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# Mitochondrial DNA deletions sensitize cells to apoptosis at low heteroplasmy levels

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**Abstract**

A heterogeneous group of multisystem disorders affecting various tissues and often including neuromuscular symptoms is caused by mutations of the mitochondrial genome, which codes 13 polypeptides of oxidative phosphorylation (OXPHOS) complexes and 22 tRNA genes needed for their translation. Since the link between OXPHOS dysfunction and clinical phenotype remains enigmatic in many diseases, a possible role of enhanced apoptosis is discussed besides bioenergetic crisis of affected cells. We analyzed the pro-apoptotic impact of the mitochondrial 5 kb common deletion (CD), affecting five tRNA genes, in transmitochondrial cybrid cell lines and found a slightly enhanced sensitivity to exogenous oxidative stress ($H_2O_2$) and a pronounced sensitization against death receptor stimulation (TRAIL) at a rather low CD heteroplasmy level of 22%. Mitochondrial deletions confer enhanced susceptibility against proapoptotic signals to proliferating cells, which might explain the elimination of deletions from hematopoietic stem cells.


*Keywords:* Common deletion; mtDNA; Cybrid; Apoptosis; $H_2O_2$; TRAIL

In the last two decades, an increasing number of human disorders have been attributed to mutations of the mitochondrial DNA (mtDNA), causing dysfunction of either a single mutated electron transport chain (ETC) complex [1] or ATPase subunit [2], or leading to a disturbed mitochondrial protein biosynthesis and a more generalized ETC dysfunction. The latter biochemical phenotype is observed in point mutations of tRNAs [3,4] or large scale mtDNA deletions [5], which usually include several tRNA genes. Single heteroplasmic deletions typically cause three sporadic mitochondrial diseases, two of which occur as chronic progressive disorders of muscle and CNS in adults with an accumulation of deleted mtDNA in the affected postmitotic tissues. Chronic progressive external ophthalmoplegia [6] is a myopathy characterized mainly by ptosis, ophthalmoplegia, and progressive skeletal muscle weakness of arms and legs, while Kearns–Sayre Syndrome [7] includes heart block, retinitis pigmentosa, and cognitive impairment or dementia in addition. The third deletion disorder mainly affects blood stem cells in young children [8] leading to a severe sideroblastic anemia, which the patients survive only occasionally. Survivors of this rare childhood disease may develop the CPEO/KSS phenotype with the same deletion accumulating in muscle and CNS tissue [9–11].

Although the links between primary genetic lesions and OXPHOS defects have become increasingly clear,

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the links to the clinical phenotypes remain largely enigmatic. One fundamental issue may be the question, whether functional impairment of affected cells by lowered energy charge is the only relevant pathogenetic process or whether cell loss, probably by reactive oxygen species (ROS) induced apoptosis, plays a significant role.

For other types of mtDNA mutations, neuronal loss is well known as major constituent of the phenotype. A distinct loss of retinal ganglion cells is a typical feature of certain amino acid exchanges of the NADH:ubiquinone-oxidoreductase in Leber's Hereditary Optic Neuropathy (LHON), and massive brain necrosis can be caused by an aminoacid exchange of the mitochondrial ATPase in Leigh's Disease [1,2]. However, the impact of cellular loss on the pathogenesis of the deletion disorders is unclear at present.

At least some hints for a role of apoptosis in disorders with tRNA mutations or deletions, i.e., with a disturbed mitochondrial protein biosynthesis, have been reported in vivo. In skeletal muscle sections, Mirabella et al. [12] found an increase number of TUNEL-positive myonuclei, as well as increased caspase-3 and FAS staining in patients with both mutation types. DNA-fragmentation, as visualized by the TUNEL method, was quantitatively correlated with the heteroplasmy level of mutant mtDNA and was more prominent in COX negative muscle fibers, thus suggesting an intimate relation between ETC dysfunction and apoptosis. In addition, cytochrome *c* release from the mitochondrial intermembrane cleft into cytoplasm, a hallmark of the intrinsic apoptotic pathway, was confirmed in CPEO muscle by Western blotting [13]. While brain atrophy, white matter lesions, and basal ganglia calcifications are known to occur in encephalomyopathies associated with deletions and tRNA mutations [14–16], distinct neuronal loss was only rarely verified by precise neuropathological examination. Prominent neuronal degeneration of basal ganglia and bilateral areas of softening and total neuronal loss in the lenticular nuclei were reported in a KSS case [17] and a moderate loss of cerebellar Purkinje cells was observed in another [18]. Neuronal loss in dentate nucleus and inferior olivary nucleus [19] was found in a case with MERRF syndrome (myoclonic epilepsy with ragged red fibers).

Taken together, nerve cell loss has not been extensively studied in mitochondrial encephalomyopathies and apoptosis of muscle fibers is only a discrete phenomenon in CPEO and KSS. On the other hand, it seems reasonable to assume a selective pressure for an elimination of hematopoietic stem cells carrying deleted mtDNA from bone marrow. Decrease of the deletions was observed during improvement of Pearson's syndrome and is further underlined by the fact that deletions are usually undetectable in blood of CPEO/KSS patients. Therefore, the apoptotic impact may be higher in proliferating cells than in postmitotic ones.

In the present study, we analyzed whether single large scale deletions enhance the sensitivity of proliferating cell cultures against oxidative stress ($H_2O_2$) and against the death receptor ligand TRAIL, as triggers of the internal and external apoptotic pathways. TRAIL served as a highly specific and physiological proapoptotic compound with less pleiotropic effects as compared to $H_2O_2$ or the commonly used strong inductor staurosporine.

## Methods

*Cell culture.* Transmitochondrial cybrid cells, based on the human osteosarcoma cell line 143B and containing either the mitochondrial 5 kb common deletion ($CD_{mut}$) or a wild-type mitochondrial genome ($CD_{wt}$), were a gift from Dr. Y.H. Wei, Taipei. All cell culture reagents were purchased from PAA (Linz, Ausria) and uridine was from Sigma–Aldrich (Munich, Germany). Cybrid cells were grown at 37 °C and 5% $CO_2$ in 75 $cm^2$ cell culture flasks in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 50 μg/ml uridine, and 100 μg/ml antibiotics (penicillin/streptomycin mix). Cells were replated approximately twice a week and with medium changes every 2–3 days.

*Genotyping and BID-PCR.* Cells for DNA preparation were collected by trypsin treatment and PBS washing. DNA was isolated from cell pellets using the Spin Tissue Mini Kit (Invitek, Berlin, Germany) according to the manufacturer's instructions. To verify identical nuclear background of both cybrid lines, the forensic nonaplex-III PCR kit (Bio Type, Dresden, Germany) was used according to the manufacturer's instructions on a PTC100 thermal cycler (Biozym, Hessisch Oldendorf, Germany). PCR fragments were separated on an ABI310C capillary sequencer (Applied Biosystems, Foster City, USA) and fragments were analyzed using the GeneScan software (ABI). The determination of heteroplasmy level of the CD in the mutant cybrids was performed by Sybr Green Real-Time PCR on an ABI-Prism-7000SDS (ABI), using the Quantitect Kit (Bio-Rad, Hercules, USA). The CD breakpoint was amplified as a 350 bp fragment by means of primer shift PCR with primers CCCCTCTAGAGCCCACTGTA (forward) and GAGTGCTATAGGCGCTTGTC (reverse), while the reference (mitochondrial HVR2 region) was amplified as a 419 bp fragment with primers CTATCACCCTATTAACCACT (forward) and GTTAAAAGTGCATACCGCCA (reverse). After initial incubation at 50 °C for 2 min, hot start polymerase was activated at 95 °C for 15 min. For both amplicons, 40 PCR cycles followed with denaturation, annealing, and polymerization at 95, 55, and 72 °C for 30 s each. Afterwards the standard melting protocol was used to confirm product identity and purity by comparison to plasmids, created by cloning the CD breakpoint from a CPEO muscle and the HVR2 region into PCR-Script Amp (SK+) vectors (Stratagene, La Jolla, USA). Use of 0.01, 1, and 100 ng of cellular DNA for both systems allowed the determination of actual amplification efficacies of the cellular DNA templates by automatic slope calculation. Since slopes of both amplicons differed by less than 5%, a mean amplification rate ($A$) could be calculated according to: $A = 10^{(-1/\text{slope})}$. The relative amounts of CD and total mtDNA (HVR2) could be calculated directly from the mean $C_t$ (crossing point) difference of CD and HVR2 ($d$) according to the formula: ratio HVR2/CD $= A^d$. Heteroplasmy level of CD could be calculated according to: $H = 1/A^d \times 100\%$.

RNA from cell pellets was isolated by Trifast (Peqlab, Erlangen, Germany) and RT-PCR for detection of BID-mRNA was performed on the ABI-Prism-7000SDS under identical thermal conditions, but with 58 °C annealing temperature and the following primers:

GTTCTGACAACAGCTTCCGC (forward) and ATGCTACGGTCC ATGCTGTC (reverse). Melting curves were examined for the expected single peak at 86 °C to test product purity.

*MTT assay.* MTT assays to test the cytotoxic effects of $H_2O_2$ and TRAIL on both cybrid lines were performed in 96-well microtiter plates using 3000 cells in 100 μl culture medium per well and eight repetitions per treatment. All treatments of one experiment were represented on the same microtiter plate and cells were allowed to grow overnight. Afterwards the desired concentration (5, 10, 25, 50, and 100 ng/ml) of human recombinant TRAIL (R&D systems, Minneapolis, USA) or $H_2O_2$ (150, 300, 450, 600, and 750 μM) was added and the cells were further incubated for 24 h at 37 °C and 5% $CO_2$. Prior to the enzymatic MTT assay, the plates were inspected under an inverse microscope to verify a dose-dependent morphological effect of the compounds and micrographs were taken from untreated controls and the wells treated with the highest concentration. Then one hundred microliters of 1.5 mg/ml MTT salt (Sigma–Aldrich, Munich, Germany) in medium was applied for 2 h at 37 °C. After addition of 100 μl dimethyl sulfoxide (DMSO), the optical density (OD) was measured in an Anthos2010 ELISA reader (Anthos, Salzburg, Austria) at a wavelength of 562 nm (reference 620 nm). The mean OD of untreated cells was set to 100% and used to normalize the OD of all treated wells. The percentage of OD relative to untreated controls was taken as the percentage of surviving cells. A probable protective influence of cyclosporine A was tested by comparing 24 wells treated with 450 μM $H_2O_2$ under the above conditions with 24 wells pretreated in addition with 1 μM cyclosporine A (Sigma) for 30 min. The significance of differences between both cybrid lines for a given treatment was analyzed by a *t* test for unpaired samples.

In parallel experiments, some cells were cultured under the same conditions in eight-well culture slides to allow a nuclear DAPI staining to detect apoptotic changes by fluorescence microscopy.

*Caspase-3-assay.* $CD_{wt}$ and $CD_{mut}$ cells were plated at a density of $7 \times 10^5/25$ cm$^2$ in T25 cell culture flasks, allowed to attach, and grow overnight. A total of five flasks per cell line was then treated with 450 μM $H_2O_2$, while five flasks were used as untreated controls. After a further culture period of 24 h, protein was isolated for caspase-3-assay. Cells were harvested by trypsin detachment and PBS washing. Cells were lysed by addition of one volume share lysis buffer, supplied with the CCP32/caspase-3 fluorimetric protease assay kit (Chemicon, Temecula, USA), to the pellets, and stored on ice for 10 min. Protein content was quantified using the Bio-Rad Dc protein assay (Bio-Rad, Hercules, USA). Fluorimetric caspase-3-reaction was performed with the caspase-3 kit according to the manufacturer's instructions using 27.6 μg of cellular protein. Fluorescence was measured in a Safire fluorescence reader (Tecan, Crailsheim, Germany) at an excitation wavelength of 400 nm and emission of 505 nm. Significance of differences between unstimulated controls and $H_2O_2$ induced caspase-3 activity was tested by a *t* test for unpaired samples.

## Results

The cybrid model, introduced in 1989 by King and Attardi [20], enabled us to compare the influence of mutant and normal mtDNA in an identical nuclear background of 143B osteosarcoma cells. To ensure the identity of nuclear background, the cell lines $CD_{wt}$ and $CD_{mut}$ were analyzed by multiplex PCR with a gender marker and eight polymorphic markers (Table 1). Both lines were proven to be derived from a female individual and to have identical alleles in all eight polymorphic loci tested. Differences in apoptotic behavior should therefore be attributable to the differences between mutant and wild-type mitochondrial genomes.

Next we quantified the heteroplasmy level of CD in the mutant line. A Sybr Green real-time-PCR approach (Table 2) revealed that the mean crossing point of the CD amplicon was 2.356 cycles above the mean crossing point of the HVR2 amplicon, which represented the total copy number of all mtDNA molecules. The crossing point difference was basically the same over the entire range of template amounts between 0.01 and 100 ng per reaction. A difference above 2 cycles suggested a CD copy number below 25% of total mtDNA. A precise calculation of heteroplasmy, taking into account the actual amplification rate per PCR cycle, yielded a result of 21.7%. This mutational load was reported to be far

Table 1
Genetic identity of $CD_{wt}$ and $CD_{mut}$

| | Marker | Amelo (gm) | D3S1358 (pm) | vWA (pm) | D8S1179 (pm) | SE33 (pm) | TH01 (pm) | FIBRA (pm) | D21S11 (pm) | D18S51 (pm) |
|---|---|---|---|---|---|---|---|---|---|---|
| Cell line | $CD_{wt}$ | X/X | 15 | 17/18 | 14 | 21 | 6 | 24 | 31.2 | 17 |
| Cell line | $CD_{mut}$ | X/X | 15 | 17/18 | 14 | 21 | 6 | 24 | 31.2 | 17 |

Verification of identical nuclear background of both cybrid lines $CD_{wt}$ and $CD_{mut}$. The gender marker (gm) Amelo and the eight indicated polymorphic markers (pm) showed the same alleles for both lines.

Table 2
Common deletion (CD) heteroplasmy level in $CD_{mut}$-cells

| Parameter | d*F*/d*T* (CD) | d*F*/d*T* (HVR 2) | Slope (CD) | Slope (HVR 2) | *A* | $\Delta C_t$ | HVR2/CD | *H* (%) |
|---|---|---|---|---|---|---|---|---|
| $CD_{mut}$ | 77 °C [77 °C] | 82 °C [82 °C] | −3.451 | −3.629 | 1.914 | 2.356 | 4.615 | **21.7** |

Verification of CD and quantification of heteroplasmy in the mutant cybrids by real-time PCR. The velocity of fluorescence change (d*F*/d*T*) plotted versus the temperature during melting of PCR products revealed a single peak of 77 or 82 °C for both PCR systems, which was identical to the peak obtained from reference plasmids (in brackets). Slope, slope of plots $C_t$ (crossing point) versus relative template amount (0.01, 1, and 100 ng). *A*, calculated mean amplification rate, $\Delta C_t = C_{tCD} - C_{tHVR2}$ (mean value for all template amounts), HVR2/CD, calculated ratio for HVR2 and CD copy numbers. *H*, level of CD heteroplasmy in %.

below the level needed for a significant bioenergetic decline in this cell culture model [21], although in CPEO muscle a well-defined bioenergetic threshold for large scale deletions may not exist [22]. The wild-type cybrids contained no CD amounts detectable with the method.

Now we analyzed the reaction of both cybrid lines to $H_2O_2$ and TRAIL. Irrespective of their mtDNA status, untreated cybrids grew as completely adherent flat cells of variable shape. Pronounced morphological changes, especially rounding and detachment, could be observed generally under both compounds. High concentrations of TRAIL resulted in a high degree of complete cell lysis, especially in the mutant (Fig. 1C), whereas wild-type seemed to be less affected. While some morphologically intact, flat cells and many round, non-lysed cells were observed in the wild-type cultures treated with 100 ng/ml TRAIL, most mutant cells appeared to be lysed under these conditions (Figs. 1E and F). However, a clear-cut difference could not be verified by microscopical inspection, due to the heterogeneous distribution of morphological changes between and even within wells. For both compounds the morphological effects were clearly dose dependent and reached a maximum for the highest concentrations (Figs. 1A–C). It was recognizable that the nuclei of treated cells pointed out morphological signs of apoptosis, including nuclear fragmentation (Fig. 1D).

To quantify cell death, an enzymatic dehydrogenase assay (MTT assay) was accomplished. The treatment with $H_2O_2$ yielded only minor differences between both cell lines, which did not reach significance, except for 450 μM. The difference between both lines was more pronounced with respect to death receptor stimulation. The cybrids carrying the deletion were significantly more

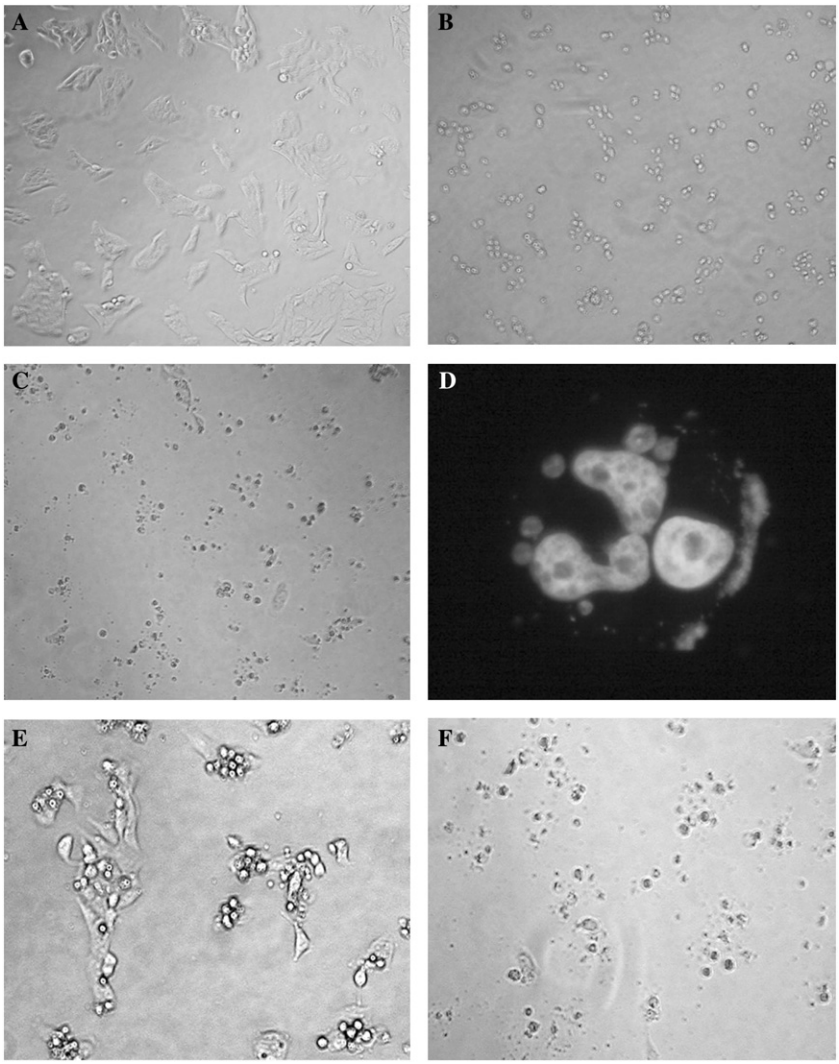


Fig. 1. (A–C) Micrographs (100×) showing mutant cybrid cells either untreated (A) or treated with 750 μM $H_2O_2$ (B) or 100 ng/ml human recombinant TRAIL (C). (D) Nuclear DAPI stain (400×) of mutant cells treated with 450 μM $H_2O_2$. (E,F) Detailed pictures of TRAIL treated wild-type (E) and mutant (F) cybrid cells.

sensitive to cell death induction ($p < 0.05$) over the whole range of TRAIL concentrations between 10 and 100 ng/ml (Fig. 2). Since the Bcl-2-family protein BID is required to couple TRAIL signalling to mitochondria, comparable BID expression in both cybrid lines was verified by RT-PCR (crossing point difference 0.11, not significant). Melting profiles of the products showed only a single peak at the expected temperature, which identified the transcripts as BID mRNAs (data not shown).

While TRAIL generally elicits apoptosis by stimulation of plasma membrane death receptors, a participation of apoptosis in $H_2O_2$ mediated cell death was confirmed by fluorimetric measurements of the proteolytic activity of caspase-3, an effector caspase which participates in the final steps of the apoptotic cascade, downstream of mitochondrial involvement. A significant 2.6-fold increase of caspase-3 activity above the endogenous level was observed by stimulation of $CD_{wt}$ cells with 450 μM $H_2O_2$ and a significant 3.1-fold induction was found in $CD_{mut}$ cells ($p < 0.05$), as shown in Fig. 3. In both cybrid lines, no significant protective effect of 1 μM cyclosporine A against 450 μM $H_2O_2$ was observed in MTT assays.

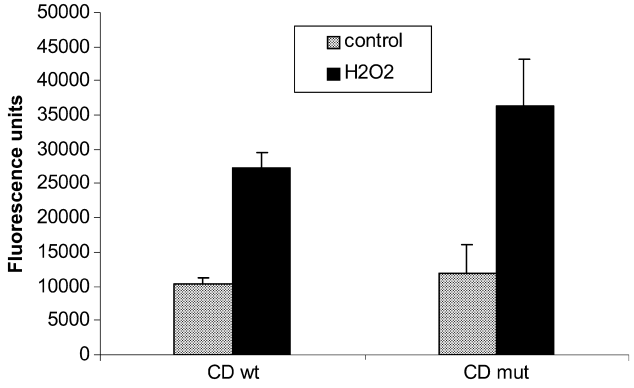


Fig. 3. Relative fluorescence after cleavage of fluorigenic caspase-3 substrate with cell lysates obtained from mutant ($CD_{mut}$) or wild type cybrids ($CD_{wt}$) without stimulation or after treatment with 450 μM $H_2O_2$.

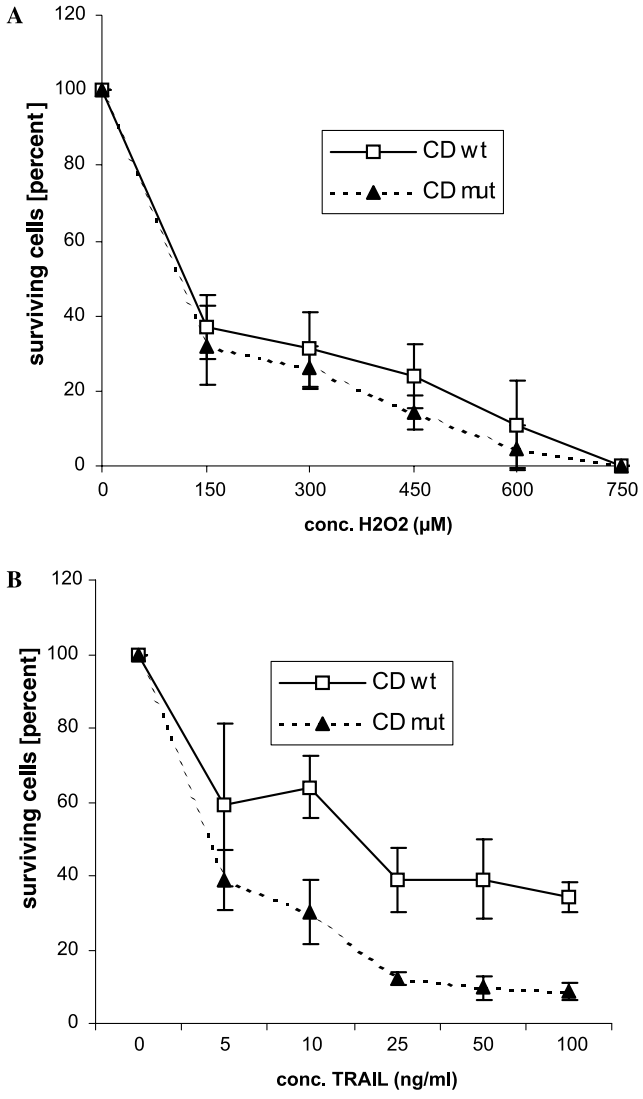


Fig. 2. Dose–response curves showing the number of surviving cells (percent), as determined by MTT assay, plotted against the concentration of the applied apoptotic stimulus $H_2O_2$ (A) or TRAIL (B).

## Discussion

In the present study, we found a significant sensitization of 143B cybrid cells carrying the mitochondrial 5 kb common deletion against apoptotic stimuli, especially against the death receptor ligand TRAIL. In an earlier report, a sensitization of 143B and Da1B based cybrids against $H_2O_2$ was found to be caused by the two most common tRNA mutations A3243G (MELAS) and A8344G (MERRF), and by the most common LHON mutation, the G11778A transition in complex I [23]. Although no direct evidence was reported, an at least partial apoptotic nature of $H_2O_2$ cytotoxicity in those cybrids was likely, since toxicity was $Ca^{2+}$ dependent and could be partially suppressed by the known inhibitor of the permeability transition pore (mtPTP), cyclosporine A. Several direct proofs of higher susceptibility of 143B based MERRF, MELAS, and CD cybrids against staurosporine and ultraviolet light (UV) induced apoptosis have been reported recently [24], including enhanced caspase-3 activation in all cybrid lines. However, the commonly used apoptotic inductor staurosporine and UV light have pleiotropic effects in the cells and UV-reaction may strongly depend on p53-status. Moreover, these treatments did not model naturally occurring apoptotic stimuli in mitochondrial disorders.

Nevertheless, the combined results of Wong and Cortopassi [23], of Liu et al. [24], and of the present study suggest that all mtDNA mutations causing a generalized OXPHOS disturbance by inhibition of mitochondrial

protein biosynthesis sensitize proliferating cells against apoptotic stimuli. A common biochemical basis of this proapoptotic effect can be the enhanced superoxide generation by complexes I and III. Such enhanced ROS production can not only be expected in LHON cybrids with selective complex I defects, but also under conditions of a partially inhibited ETC. Antioxidative defense mechanisms, such as mitochondrial glutathione, may be saturated in mutant cells to a higher degree as in wild-type cells. This explains why the capacity to scavenge externally applied oxidative stress could be reduced.

In the present study, we found that the CD sensitized 143B cells even stronger against the highly specific proapoptotic compound TRAIL as compared to $H_2O_2$, which may only partially elicit an apoptotic mode of cell death, when applied externally. The enhanced efficacy of death receptor ligands in mutant cells can be explained by the ability of mitochondria to integrate the proapoptotic effects of enhanced mitochondrial ROS production with any other signal affecting mitochondrial membrane integrity. Via caspase-8 cleavage of the proapoptotic protein BID, which was expressed in both cybrid lines, death receptor activation can be coupled to the intrinsic apoptotic pathway. While ROS may directly attack the mitochondrial membranes or increase the probability of mtPTP opening, activated BID destabilizes the outer mitochondrial membrane by integration. In line with our TRAIL data, Danielson et al. [25] reported an enhanced sensitivity of 143B based LHON cybrids against apoptosis induction via the Fas death receptor pathway.

At present, the extent of mtPTP involvement remains unclear. A closer inspection of earlier cyclosporine experiments in parental 143B cells and corresponding cybrids [23] reveals that generally only a minor fraction of $H_2O_2$ treated cells could be rescued from death by pre-treatment with 1 μM cyclosporine A, although protective effects in a MERRF mutant were highly significant. No protective effect at all was observed for the CD mutant in the present study. A general problem may be that only a fraction of cells die by apoptosis, if $H_2O_2$ is applied externally. This may explain why a highly specific stimulus (TRAIL) leads to a more pronounced difference between wild-type and mutant cells.

The bioenergetic consequences of the 5 kb CD in the 143B nuclear background and of a similar 5.2 kb deletion in a HeLa background were previously analyzed in detail. Both deletions affect exactly the same five tRNA genes (L,S,H,R,G) and the same protein genes (ND5, ND4, ND4L, ND3, COIII, and APTase 6). A threshold for a partially inhibited mitochondrial protein biosynthesis and reduced COX activity was reported to occur at 60% deleted mtDNA for the 5.2 kb deletion [26], and a decrease in mitochondrial membrane potential and ATP synthesis was measured only, if CD heteroplasmy exceeded 50–55% [21]. However, in skeletal muscle it has been shown that no thresholds occurred in citrate synthase normalized data, while adaptive mitochondrial proliferation generates such effects artificially in all tissue related data sets [22]. Measurements of the actually used cell samples of our study by Real-Time-PCR revealed a heteroplasmy of around 22% in the $CD_{mut}$ cybrid line. Whether or not a threshold effect exists in the cybrid system, the CD mediates enhanced apoptotic sensitivity under conditions of only mild bioenergetic decline. This further extends the observations of Liu et al. [24], who investigated only CD cybrids with 80% heteroplasmy for their staurosporine and UV sensitivity.

Whether apoptotic sensitization plays a role in vivo for degeneration of postmitotic cells remains unclear. Few neuropathological data are available and brain neuronal loss may in some disorders only be a response to focal ischemic insults, as in MELAS [27], or to prolonged epileptic periods, as may be argued to explain neurodegeneration in MERRF [19]. Although an enhanced load of point mutations in heart mtDNA stimulated apoptosis in a mouse model with dilated cardiomyopathy [28], the pathogenetic impact of apoptosis even in this model was questionable.

However, it seems reasonable to assume a selective loss of deletion-bearing bone marrow precursors in humans, since deletions are usually not found in the blood. In the rare condition that they are stably transmitted to bone marrow during embryogenesis, they lead to a severe hematopoietic disease, Pearson's syndrome. A gradual improvement can be associated with an elimination of the most severely affected stem cells, as can be concluded from the decreasing number of peripheral blood lymphocytes with high mutant heteroplasmy levels [29] and from the decrease of deleted mtDNA in lymphoblastoid cells [30], although some conflicting results were reported [31].

The TRAIL pathway may be of interest, since TRAIL is expressed in the bone marrow and was recently recognized as a negative regulator of normal [32,33] and pathologic erythropoiesis [34]. Among blood cell precursors, erythroblasts are especially prone to TRAIL mediated apoptosis [32] and enhanced apoptosis of deletion bearing erythroblasts may be suggested to play a role for pathogenesis and recovery in Pearson's syndrome.

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