1. Introduction

Apoptosis is a form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area. It requires the activation of specific genes that lead to a series of distinctive morphological and biochemical features.

These changes include the activation of cellular proteases (caspases), chromatin condensation, mitochondrial depolarization, oligonucleosomal DNA degradation or cell fragmentation without the involvement of inflammatory response (White 1996; Salvasen and Dixit 1997; Hetts 1998). Mitochondria play a leading role in triggering and mediating the apoptotic processes. Studies in vitro have shown that the disruption of the mitochondrial transmembrane potential ($\Delta\psi$) and the release of mitochondrial proteins (cytochrome c and apoptosis inducing factor) into the cytoplasm are able to initiate and activate different apoptotic pathways (Liu et al. 1996; Kroemer et al. 1997; Kluck et al. 1997; Zhivotosky et al. 1998). Apoptosis also plays an important role in pathologic conditions, including neurological disorders. Its execution pathway is critically regulated at the mitochondrial level. Too little or too much apoptosis plays a role in a great many diseases. When programmed cell death does not work right, cells that should be eliminated may hang around and become immortal (e.g. cancer and leukemia). When apoptosis works overly well, it kills too many cells causing tissue damage (e.g. strokes and neurodegenerative disorders). Apoptotic features in human pathology are documented in neoplasms, autoimmune diseases, stroke and some neurodegenerative disorders such as familial amyotrophic lateral sclerosis, Alzheimer's, Parkinson's, and Huntington's diseases (Hetts 1998).

Evidence of apoptosis in muscle specimens was investigated in patients with genetically defined mitochondrial encephalomyopathies. Whatever the mechanism, the final common step in mitochondrial encephalomyopathies is a defect of energy production resulting from respiratory chain impairment. Cellular necrosis is rarely observed in these
disorders. Other mechanisms, such as incorrect assembly of the defective enzyme complex or other mitochondrial proteins, the production of free radicals and ways of cell death other than necrosis such as apoptosis, may be postulated to explain the phenotypic variability and the severity of mitochondrial diseases.

A previous study indicated that apoptotic cell death was dramatically increased in muscle biopsies from patients carrying mtDNA mutations in genes encoding bioenergetic proteins compared to those carrying mutations in structural genes (Mirabella et al. 2000). Furthermore, in most cases, the severity of the apoptotic phenotype correlated with the percentage of mitochondria in a given cell carrying the mutation and with the severity of clinical disease phenotype. Therefore, apoptosis may indeed play a role in the development of mitochondrial encephalomyopathies, although the exact pathway involved remains uncertain. The apoptotic cell death in mitochondrial encephalomyopathies is supported by a number of studies from last few years (Formichi et al. 2003; Ikezoe et al. 2002; Umaki et al. 2002; Aure et al. 2006). Most of the detailed studies regarding apoptosis in OXPHOS disorders have been carried out on LHON (Danielson et al. 2002; Ghelli et al. 2003; Zanna et al. 2003; Battisti et al. 2004). Apoptosis contributes to mitochondrial pathology and is tightly linked to mitochondrial proliferation (Zanna et al. 2005).

To access the consequences of OXPHOS defect upon cell survival, apoptotic cell death of six lymphoblast cell lines established from patients with complex I deficiency (as discussed in chapter 3 and 5) were analyzed and comparison of apoptosis was done with three control cell lines established from healthy individuals. The patient cell lines were named according to the unique variants identified in their mtDNAs and genotyped as G4812C, T3866C, T3394C, T11916A, C4640A and T4216C.
2. Materials and Methods

2.1. Materials
TMRM, Propidium Iodide (PI), Poly-L-lysine, Paraformaldehyde and Hoechst 33258 were from Sigma. ATP determination kit and RNAase A were purchased from Invitrogen, USA. Galactose was obtained from HiMedia laboratories, India.

2.1. Cell lines and Cell Culture
Lymphoblast cell lines from six patients (complex I deficient) and three controls as established earlier (chapters 3 and 5) were grown in glucose and glucose free-galactose supplemented medium (RPMI 1640 plus 10% FBS) for 24, 48 and 72 hours. Both patient and control cells were thawed at the same time and maintained exactly in similar culture conditions. Cell death analysis was performed after each time point. The qualitative analysis of apoptotic nuclei was performed by confocal microscopy and time dependent quantitative analysis of apoptotic cells was performed by flow cytometry.

2.3. Hoechst 33258 DNA staining
Morphological analysis of apoptotic nuclei was done by observing changes in the nuclear chromatin of cells detected by Hoechst staining. Approximately $1 \times 10^6$ cells were harvested and washed once with PBS and fixed in 4% paraformaldehyde. The cells were smeared on poly-L-lysine coated slides and stained with Hoechst 33258 for 30 minutes, followed by examination on Confocal Microscope LSM 510 (Carl Zeis, JENA, Germany). Hoechst 33258 stained cells were illuminated with an argon laser tuned for UV (353-365nm) and resulting fluorescence was detected at 480nM. The stock solution for Hoechst dye was made in glass distilled water and stored at -20°C.
2.4. Measurement of apoptosis by Propidium Iodide staining

FACS was used to measure degradation of DNA. Propidium iodide was used to detect DNA breakdown as described previously with minor modifications (Darzynkiewicz et al. 1994). Cells were harvested, washed once with PBS and fixed in 70% ethanol. The cells were kept on ice for 1-2 hours and centrifuged at 300g for 5 minutes. The ethanol was removed; cells were washed once again with PBS and resuspended in 100 µl of PBS. RNAase A to a final concentration of 50µg/ml was added and incubated at room temperature for 15 minutes. After this, 1ml propidium iodide solution (50µg/ml) made in PBS was added. The cells were incubated in dark for 10 minutes and analyzed by flow cytometry.

2.5. Mitochondrial membrane potential

TMRM was used to detect loss in mitochondrial membrane potential during apoptosis. Cells were grown either in glucose or galactose supplemented medium (24, 48 and 72 hours.), harvested and resuspended in fresh serum free medium, adjusted to concentration of 1×10^6 cells/ml. To this, TMRM was added to a final concentration of 100nM and incubated at 37°C in CO₂ incubator for 30 minutes. The cells were washed once with PBS and analyzed by FACS. The percentage of cells containing polarized or depolarized mitochondria was determined by histogram analysis of the changes in fluorescence intensities using Cell Quest Pro-software (BD Biosciences).

2.6. Measurement of Cellular ATP pool

A commercially available kit from Invitogen, which is based on bioluminescent detection using luciferin-luciferase system, was used to measure the ATP levels. Briefly, 1×10^6 cells (both patient and control group) grown either in glucose or galactose medium were boiled in 100µl buffer containing 100mM Tris, 4mM EDTA (pH 7.75) for 2 minutes. The samples were centrifuged at 1000g for 10 minutes and an ATP level in the supernatants was measured by adding luciferase agent. ATP
concentrations were calculated from a log-log plot of standard curve data. The luminescence was read in Floroskan Ascent FL system (Lab systems, USA).

2.7. Estimation of ROS levels in galactose medium
FACS using DCFDA and DHE probes measured ROS production in galactose medium as described in chapter 5.

2.8. Statistical analysis
Statistical analysis was performed as described in previous chapters; $P$ greater than 0.05 and 0.01 were considered to be statistically significant.
3. Results

3.1. Nuclear staining and Cell cycle analysis

After 24 hours of culture in galactose medium, approximately, 15-20% of the patient cells showed chromatin condensation and nuclear fragmentation under confocal microscopy using Hoechst 33258 DNA staining (Figure 6.1). Cell cycle analysis based on flow cytometry was used to quantitatively estimate apoptotic cell death. Gating of the subdiploid cell population on a linear scale excluded cells with extensive DNA degradation (typical of necrosis) to distinguish apoptotic cells from necrotic cells (Herrmann et al. 1994).

The percentage apoptotic cells in glucose medium were similar between patient and control group, no significant differences were observed even after 72 hours of culturing (Figure 6.2). Quantitative analysis of sub-G1 region of cells grown in galactose medium showed a time dependent increase in the percentage of apoptotic cells (Figure 6.3). The apoptosis in galactose medium started from 24 hours and was highest after 72 hours of culturing. Both control and patient cells showed a typical G1S/G2M distribution in galactose medium on flow cytometry. More than 35% of patient cells after 72 hours of incubation in galactose medium moved to subdiploid area, which is typical of apoptotic cells. However, only 14% of the control cells were in the subdiploid area (Figure 6.3).
3.2. Loss of mitochondrial membrane potential

Loss of mitochondrial membrane potential is the first event in apoptotic cell death. The culturing of complex I defective cell lines in galactose medium induced a significant reduction in the TMRM fluorescence. Figure 6.4 showing a clear increase in the cells with no excited TMRM signal i.e. cells with depolarized mitochondria (left panel of histograms, marker-M2) after 48 hours of culturing in galactose medium. The increase in cells with depolarized mitochondria was significantly higher in patient group than control group (P< 0.05). Two patient derived cell lines with mtDNA genotypes T3394C and T11916A showed the highest percentage of cells with depolarized mitochondria (Figure 6.4). These two patients had a complex I assembly defect as described in chapter 3 and highest ROS accumulation (described in chapter 5). Mitochondrial depolarization also started after 48 hours of culturing in galactose medium.
Figure 6.2: Analysis of percentage apoptotic cells by Flow cytometry in glucose medium. The DNA content of each phase was quantified and the percentage of apoptotic cells is shown in control and patient cell lines. (A) Histogram plots for cell cycle analysis in one representative control and complex I defective patients with mtDNA genotypes T3394C, T3866C, T11916A, G4812C, C4640A and T4216C. (B) Histograms showing mitochondrial membrane potential loss analyzed after 72 hours. No significant difference between control and OXPHOS defective cell lines was observed in glucose supplemented medium.
Figure 6.3: Analysis of percentage apoptotic cells by Flow cytometry in galactose medium. The DNA content of each phase was quantified and the percentages of apoptotic cells are shown in control and patient cell lines. A significant (2 fold) increase in apoptotic cells was observed in patient cell lines compared to controls in galactose supplemented medium. (A) Histogram plots for control and patient cell lines with mtDNA genotypes T3394C, T3866C and T4216C (B) Histogram plots for control and patient cell lines with mtDNA genotypes G4812C, C4640A and T4216C.
Figure 6.4: Loss of mitochondrial membrane potential: The cells were grown in galactose supplemented medium for 24, 48 and 72 hours and mitochondrial membrane potential was observed at each time point by TMRM fluorescence (A) Histogram plots for control and patient cell lines with mtDNA genotypes T3394C, T3866C and T4216C. (B) Histogram plots for control and patient cell lines with mtDNA genotypes G4812C, C4640A and T4216C
3.3. Cellular ATP levels

ATP levels were measured for cells grown in glucose or galactose medium. In glucose medium, control and patient cells had a similar ATP content. By replacing glucose with galactose, ATP levels decreased slightly after 10 hours in patient group. However, after longer incubation periods (16 hours) in galactose medium, cellular ATP levels decreased significantly ($P<0.05$) in patient cells compared to controls (Figure 6.5). It is therefore evident that complex I deficient cells were unable to survive when forced to rely on mitochondrial respiratory chain to synthesize ATP.

**Figure 6.5: Determination of cellular ATP levels.** Both control and complex I deficient cells were grown in glucose and galactose supplemented medium (RPMI 1640) for 10 or 12 hours. Cells were harvested and once washed with PBS. ATP levels were measured by luciferin-luciferase assay. Data are mean ±SD of two independent experiments.
3.4. ROS production in galactose medium

In glucose medium, where we observed an increased ROS production by complex I defective lymphoblasts (as described in Chapter 5), however, when the same cells were shifted to a medium with glucose being replaced by galactose, a significant decline in ROS levels were evident. This was true for both control and patient derived cell lines (Figure 6.6).

![Graph showing ROS levels](image)

**Figure 6.6: Measurement of ROS levels in galactose supplemented RPMI 1640 medium.** Cells were harvested and washed in phosphate buffered saline and resuspended in PBS containing 1% BSA, incubated with 10μM DCFDA and 1μM DHE for 20 minutes at 37°C. Fluorescence intensity was immediately analyzed by flow cytometry (FACS vantage). A minimum of 10000 events were analyzed per sample. Control here means the cells which were grown in glucose supplemented medium.

4. Discussion

Oxidative phosphorylation is the main source of ATP under aerobic conditions. A defective OXPHOS may result in declined ATP production through electron transport chain. In glucose medium, cells in culture mostly derive ATP by means of glycolysis (anaerobic respiration). However, if glucose is replaced by galactose, it is weakly recognized by glucose 6-phosphate, and slows down glycolysis. Under this condition, cells will be more dependent on mitochondrial pathway for ATP synthesis through respiratory chain rather than on glycolytic pathway. The cells which are
OXPHOS deficient will not be able to generate enough ATP through ETC compared to normal cells which will generate significant quantities of ATP through ETC, hence normal cells will be able to survive to a better extent in galactose medium compared to OXPHOS defective cells. This experimental condition has been used previously to identify impaired respiratory function (Robinson et al. 1992; Petrova-Benedict et al. 1992; Robinson 1996). We used the same strategy to detect the consequences of complex I deficiency upon cell survival in our patient derived cell lines. Both control (n=3) and patient cell lines (n=6) were grown in galactose medium for different time points and cell death was quantified after each time point by means of flow cytometry. In the glucose-medium, no apoptotic cell death was evident for patient derived cell lines (Figure 6.1). This is quite possible because, in glucose medium the OXPHOS deficient cells generate enough ATP through glycolysis and will not be solely dependent on mitochondrial pathway for ATP synthesis. However, a slow growth (in glucose medium) of two patient cell lines with mtDNA genotypes T3394C & T11916A was observed glucose medium.

The first important observation was the significant growth impairment of all lymphoblast cell lines in glucose free/galactose medium. This is likely due to nutrient shortage since it was abolished after replacing the growth medium. Secondly, the type of cell death was apoptotic in galactose medium. This was confirmed by some typical hallmarks of apoptosis such as changes in nuclear morphology and chromatin condensation (Figure 6.1).
Figure 6.7: Direction of metabolite flow when cultured lymphoblasts are grown in glucose and galactose supplemented media. When glucose is the substrate, large amounts of lactate are formed with only a small percentage of pyruvate being oxidized to CO₂. When galactose is the substrate, the bulk of pyruvate which is formed at much slower rate, is converted to CO₂ and water. Thus, ATP is generated largely by glycolysis when glucose is the substrate but from mitochondria when galactose is the substrate.

In galactose medium, significant increase in apoptotic cell death was observed in patient lymphoblasts (2-fold) compared to controls. However, some percentages of control cells do also undergo apoptotic cell death. The apoptotic cell death was highest in two patient cell lines having mtDNA genotypes T3394C and T11916A. Actually, in these two patients, a defect in complex I assembly was observed (Chapter 3). Whether the impaired complex I activity or assembly was because of these two mtDNA variants, remains to be determined. Hence, the above results suggest that an unassembled or partially assembled complex I has a greater impact on cell survival in vitro compared to an inactive but assembled complex I as seen in other patient derived cell lines with mtDNA genotypes as G4812C, T3866C, T4216C and C4640A. These cell lines have partially inactive but fully assembled complex I.
Apoptosis is an active process that requires energy for its accomplishment, but a reduction in the level of ATP makes cells more susceptible to programmed cell death (Richter et al. 1996; Leist et al. 1997). According to previous studies, the induction of selective respiratory chain deficiencies makes cells more vulnerable to Fas-mediated apoptosis (Asoh et al. 1996) and the use of respiratory chain inhibitors induce apoptosis in vitro (Wolvetang et al. 1994; Marton et al. 1997; Higuchi et al. 1998).

Complex I deficiency leads to enhanced ROS, decrease in mitochondrial membrane potential and simultaneous decrease in ATP synthesis by complex I dependent substrates (as described in chapter 3). The further consequences of these events may lead to a condition where the cell has to decide whether to enter necrosis or apoptosis. Again, there are evidences that complex I is either directly or indirectly involved in apoptosis e.g. the gene associated with GRIM-19 (retinoid-interferon-induced mortality 19) (Angell et al. 2000; Fearnley et al. 2001), that was originally isolated as cell death activator, and later shown to be a subunit of NDUFS1 bovine
complex I. This protein has been shown to be a critical mitochondrial substrate for caspase 3 (Ricci et al. 2004) pointing again towards an important role for complex I in apoptotic cell death.

The sequence of events in complex I driven apoptosis remains to be elucidated but based on the literature and present work it can be said that the loss of transmembrane potential due to diminished complex I activity (flow of protons to outer membrane) results in the generation of ROS (Zamzami et al. 1995). However, even though ROS was elevated in glucose medium in patient cells (chapter 5) but in galactose medium, there was no increase in ROS but rather a decrease was observed. This is indicating that cells are not dying due to oxidative stress in galactose medium. ATP levels are reduced in patient lymphoblasts to significant levels. However, there isn’t complete cessation of ATP synthesis, since complex II (as in chapter 3, we observed an increase in ATP synthesis by complex II dependent substrates in patient cell lines) can still channel electrons into ETC, but the ATP levels are maintained at a lower level by both complex II and defective complex I in patient lymphoblasts, that enable the cells to undergo apoptosis in galactose medium.

There are evidences that suggest apoptosis is an ATP requiring process, and the level of ATP or ATP/ADP ratio serves as a marker to differentiate between apoptosis and necrosis (Richter et al. 1996). Our study also strengthens the previously reported role of ATP in apoptosis and that ATP levels are a sensor for the cell to decide to go for apoptosis or necrosis. The ROS levels in galactose medium were lower compared to glucose medium and hence the cell death is most probably occurring by energy deficit only. But in vivo, the cell death can be either due to oxidative stress or energy deficit or by both.

In earlier studies, Battisti et al. (2004) have analyzed the oxidative stress mediated apoptosis in LHON patients using primary cultures of peripheral blood lymphocytes. Our results also strengthen previous studies (Battisti et al. 2004) that peripheral blood lymphocytes or cell lines
established from blood cells can be used to trace out the respiratory chain defect if cultured in galactose-supplemented medium. The observations from our findings could also have profound implications for therapy of mitochondrial diseases by using anti-apoptotic drugs, in addition to most current therapies, which are focused on the use of antioxidant vitamins and bioenergetic-supporting drugs. But more in vivo studies are required to support these results.