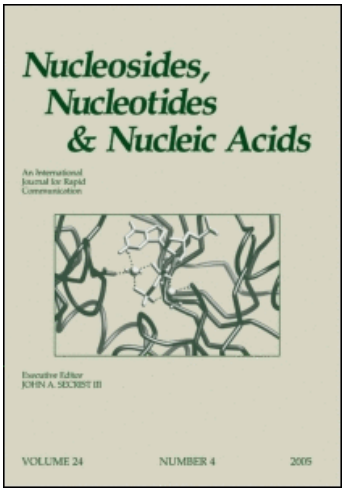

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MOLECULAR MECHANISMS OF MITOCHONDRIAL DNA DEPLETION DISEASES CAUSED BY DEFICIENCIES IN ENZYMES IN PURINE AND PYRIMIDINE METABOLISM

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□ *Mitochondrial DNA depletion syndrome (MDS), a reduction of mitochondrial DNA copy number, often affects muscle or liver. Mutations in enzymes of deoxyribonucleotide metabolism give MDS, for example, the mitochondrial thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) genes. Sixteen TK2 and 22 dGK alterations are known. Their characteristics and symptoms are described. Levels of five key deoxynucleotide metabolizing enzymes in mouse tissues were measured. TK2 and dGK levels in muscles were 5- to 10-fold lower than other nonproliferating tissues and 100-fold lower compared to spleen. Each type of tissue apparently relies on de novo and salvage synthesis of DNA precursors to varying degrees.*

Keywords Mitochondrial DNA depletion syndrome; thymidine kinase2; deoxyguanosine kinase; mtDNA synthesis

Mitochondria in mammalian cells contain multiple copies of a circular DNA molecule of 16,569 bp coding for 13 polypeptides, which are components of the oxidative phosphorylation complex. Mitochondrial DNA (mtDNA) proliferation occurs in all cells in the body through out life. The proteins responsible for mtDNA replication and maintenance are encoded by nuclear genes and are imported into mitochondria. There are more than 200 nuclear gene products involved in this process and the roles of several of these in human disease have in recent years been elucidated.[1–4]

The first mitochondrial diseases described were caused by point mutations, deletions and insertions in mtDNA and now more than 100 mutations of this type are known and often affect tissues with high energy demands

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such as nerve cells, for example, Leber's hereditary optic neuropathy or muscles such as ocular myophaties as seen in Pearson syndrome.[2]

A hallmark of mitochondrial diseases is the large interindividual and tissue variability in onset and severity of symptoms. These occur when a critical level is reached, usually 5–20% of normal mt function. One reason for this heterogeneity is that cells in tissues have multiple mt genomes, and therefore usually varying mixtures of mutant and wildtype mtDNA (heteroplasmy). The variable repair and recombination capacity of mtDNA in different tissues and situations, as well as the complex mechanisms involved mt induced apoptosis, are also contributing factors.[1,2]

This review is focused on the molecular mechanisms of mitochondrial disorders where nuclear gene products engaged in mtDNA precursor metabolism are involved. Several diseases of this type have been described during the last eight years.[2–5] The most prevalent molecular defect is a general reduction of mtDNA copies (below 30%). This type of disease is named mitochondrial DNA depletion syndrome (MDS) and affect mainly muscles, liver, and brain.[1–4]

The best described MDS is mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), which is caused by mutations in the thymidine phosphorylase (TP) gene.[5] TP catalyzes the reversible phosphorolysis of thymidine (dThd) to thymine and 2-deoxyribose 1-phosphate and is primarily a catabolic enzyme. The clinical features of MNGIE are gastrointestinal dysmotitity, cachexia, ptosis and progressive external opthalomoplegia, peripheral neuropathy and biochemical signs of mitochondrial dysfunction. MNGIE was the first clear link between pyrimidine metabolism and mitochondrial disease. The patho-physiological mechanism behind MNGIE is that a lack of TP results in high blood dThd levels, which lead to increased mitochondrial dTTP pools. This unbalanced mtDNA precursor level causes defective mtDNA replication.[6]

CELLULAR AND mtDNA PRECURSOR METABOLISM

A summary of the major steps in DNA precursor synthesis is shown in Figure 1. The salvage of extra-cellular nucleosides (NdR e.g dThd) via membrane nucleoside transporters is followed by 5′-phosphorylation in the cytosol catalyzed by thymidine kinase 1 (TK1) or deoxycytidine kinase (dCK) using nucleoside triphosphates as phosphate donors. Thereby, the deoxynucleoside monophosphate (dNMP) products are trapped inside the cells and this activation step is usually rate limiting. Subsequently dNMPs are further phosphorylated by nucleoside monophosphate kinases (NMPKs), of which there are four enzymes, specific for each base. The deoxynucleoside diphosphates produced are then phosphorylated by nucleoside diphosphate kinase (NDPK) to the deoxynucleoside triphosphates (dNTPs) needed for

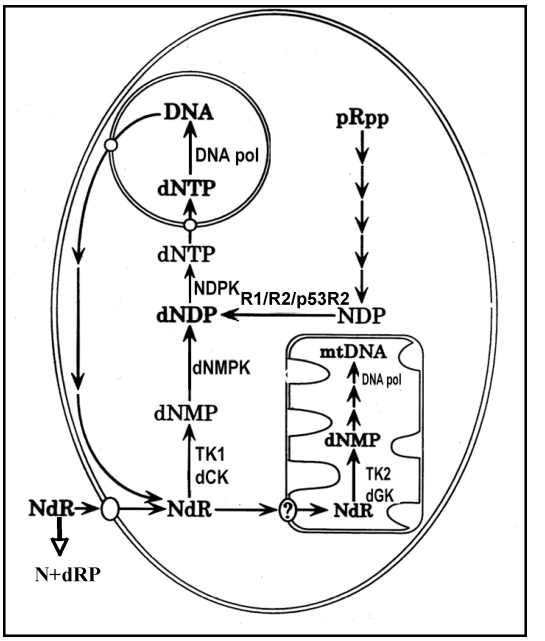

FIGURE 1 Schematic representation of the major routes of DNA precursor metabolism. Abbreviations are defined in the text.

the DNA polymerases in the nucleus. 5′-nucleotidases (5′-NTs) are responsible for the hydrolysis of (d)NMPs,[7,8] thereby opposing the action of the nucleoside kinases.

The major route of dNTP synthesis is via the *de novo* pathway where ribonucleotide reductase (R1 and R2 [alternatively p53R2] subunits) reduces ribonucleoside diphosphates (NDPs) to form deoxyribonucleoside diphosphates (dNDPs),[9,10] which then are phosphorylated by NDPK to the dNTPs (Figure 1). In mitochondria there is no clear cut evidence of de novo synthesis of DNA precursors but there are two deoxynucleoside kinases, thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK), that can phosphorylate dThd and dCyd, and dGuo and dAdo, respectively. The dNMPK products are then supposedly activated further in analogy with the cytosolic reactions leading to dNTPs that can be utilized by mtDNA polymerase (POLG) to synthesize mtDNA. A mitochondrial deoxynucleotide carrier has been described but the nature of this protein is still not defined and although there is evidence for nucleotide transport across the mitochondrial membrane, its molecular characterization remains to be determined.[11]

MECHANISMS OF MDS AND mtDNA METABOLISM

In addition to mtDNA depletion, large scale mtDNA deletions and point mutations are associated with some mutations in the TP gene.[12] Another

disorder of this complex type is linked to mutations in the POLG gene, which cause Alpers syndrome. This disease is one of the oldest phenotypes associated with mitochondrial dysfunction and it is characterized by epileptic seizures, psychomotor regression and liver disease.[13] A further example of the variable and complex phenotypes associated with mutations in mtDNA metabolizing enzymes, are some mutations in the POLG gene which have been linked to MDS with no point mutations or deletions but with a multitude of symptoms both from nerves, muscles, liver and other tissues.[14] It is estimated that about 10% of the MDS patients are due to POLG mutations.[4]

Mutations in the Succinyl CoA Synthase (SUCLA2) gene and the inner membrane protein MPV gene have recently been linked to MDS[15,16] and constitute about 1% of the cases. Patients with SUCLA 2 mutation show variable onset encephalomyopathy. It was suggested that the association observed between SUCLA 2 and the mitochondrial NDPK enzyme may lead to defects in mtDNA precursor synthesis when the former is mutated.[15] In case of MPV17 mutations, the clinical symptoms are liver failure and the pathophysiological mechanism is still unknown.[16]

P53R2 Mutations and MDS

Very recently mutations in the small subunit of the key de novo enzyme ribonucleotide reductase e.g. the p53-controlled R2 (p53R2), was shown to be linked to MDS.[9] The unexpected finding was that these mutations were found in functionally conserved amino acids in p53R2 gene. The symptoms were early onset hypotonia, myopathy, lactic acidosis, and kidney disease. There are two genes for R2 in mammalian cells; one expressed only in S phase cells and the other one (p53R2), induced by tumor suppressor protein p53, is expressed constitutively in post mitotic tissues such as muscle. The large subunit R1 is also found in this type of tissue but at reduced levels (approximately 5%) compared to proliferating cells, and together with p53R2 it can catalyze production of the DNA precursors needed for mtDNA synthesis.[9,10] The mutations in p53R2 contribute to approximately 2% of the MDS cases.[4]

TK2 and dGK Mutations and MDS

The two mitochondrial deoxynucleoside kinases, TK2 and dGK are dimeric enzymes, which show about 30% sequence homology. They belong to the same enzyme family as dCK and several other broadly specific deoxynucleoside kinases.[7] The three-dimensional structure of dGK is known but that of TK2 is still not determined.[7,17] Exon 1 of both TK2 and dGK contains a mitochondrial targeting signal sequence but there are different forms of TK2 N-terminal sequences reported.[8,13] Here the shorter version of mitochondrial signal sequence (33 amino acids) is used in the numbering

of the TK2 residues, since it has been shown to be sufficient for mitochondrial import in vitro.[20]

The substrate specificities of the two mitochondrial kinases are such that TK2 can most efficiently phosphorylate dThd but dCyd is also well accepted. dGK is most effective with dGuo but it can also phosphorylate dAdo. Thus, TK2 and dGK can together provide all four dNMPs that after further phosphorylation give dNTPs for mtDNA replication. dTTP serves as an efficient feed back inhibitor for TK2 and dGTP for dGK.[7] The essential role of both TK2 and dGK were clearly demonstrated in 2001, where almost simultaneously mutations in the TK2 and dGK genes were shown to lead to MDS.[23,24] In the case of TK2, this was associated with skeletal muscle defect, while with dGK deficiency liver failure often combined with encephalopathy was observed. About 1% of the MDS cases were caused by TK2 mutations and 10% with dGK mutations.[4] Later studies revealed that in both cases some mutations and combinations of mutations gave multiple organ symptoms but one basic question that will be addressed here is the molecular basis for the tissue specificity of the symptoms associated with defects in purine and pyrimidine metabolism.

Since the initial studies of the Israeli families a number of new cases have been reported and at present 16 point mutations, deletions and insertions have been reported in the TK2 gene (Figure 2A), leading to in 20 patients predominantly muscle defects but also cases with various forms of neuropathies.[21,22] The onset of symptoms was at birth or at 2 years of age and most patients died of respiratory failure at 1–3 years of age but some survived more then 10 years. Ten patients were found to be compound heterozygotes and the mutations were fairly evenly distributed from exon 3 to exon 10 (Figure 2A).

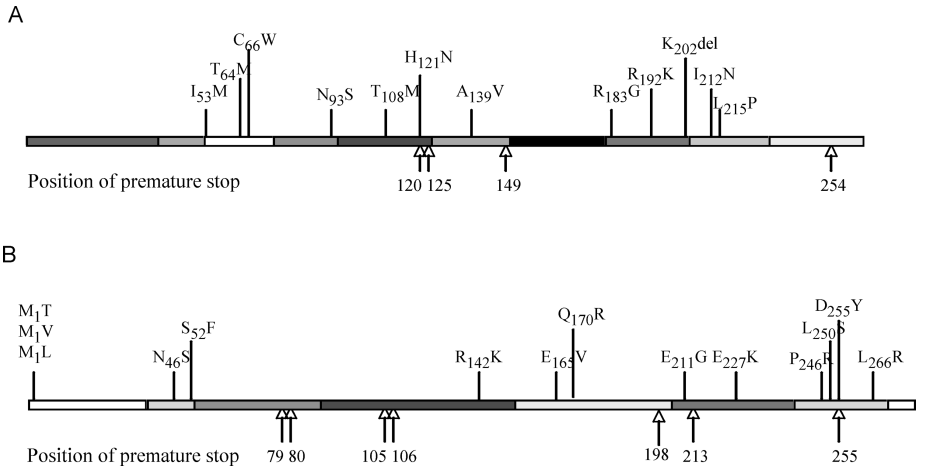

FIGURE 2 Distribution of the known mutations in the TK2 (A) and dGK (B) genes which caused MDS (from references[21–29] and reference therein).

Several of the point mutations in TK2 were reconstructed in vitro by site-directed mutagenesis, expressed and characterized with regard to their enzyme functions.[25,27] The I53M substitution is in the highly conserved phosphate binding sequence and the corresponding recombinant protein showed no detectable activity with dThd or dCyd. One patient with this mutation showed onset at 1 month and died at 2 years of age, while another showed onset at 15 month and was alive at the age of 2. The T108M mutant TK2 showed 1.1% and 0.7% activity with dThd and dCyd, respectively, as compared to wtTK2 and the three patients with this single mutation showed onset at 12–16 months and died after 3–4 years. H121N mutant TK2 had normal dThd activity and 70% reduced dCyd activity. One patient had this single mutation while two patients were compound heterozygotes containing also the T108M substitution. These patients showed relative long survival (approximately 4 years).

The R192K mutant TK2 showed 2% dThd and 4% dCyd activity compared to wtTK2 and one heterozygote patient with R192K and T108M mutations was alive at the age of 12 years. I212N mutant TK2 had a residual dThd and dCyd activity of 0.7% and 0.01 %. These patients showed relative shorter survival. Overall there was a tendency that if the reconstructed TK2 mutants showed some enzyme activity the disease developed slower.[21,27]

Figure 2B shows the presently known 22 point mutations, deletions, and insertions in the dGK gene. Of 12 patients characterized, 8 were compound heterozygotes.[24,28] The mutations were distributed in all exons but there appeared to be an over-representation of point mutation and insertions in exon 6 (Figure 2B). Residues S52 and E211 are involved in the binding of phosphate donor and R142 interacts with the 5′-OH group of the deoxynucleoside substrates, while the rest of the mutations do not represent active site residues.[17] Reconstructed dGK mutant enzymes demonstrated that the C-terminal region of dGK was essential for activity, that is, truncations of 11, 18, and 23 amino acids from the C-terminal L277, representing part or the entire α-helix 9, all showed less than 0.01% of wtdGK activity.

The R142K mutant dGK had <1% activity with dGuo and no detectable activity with dAdo, L250S mutant dGK had <1% activity with both dGuo and dAdo as compared with wt dGK, while E227K mutant dGK had about 3% and 5% dGuo and dAdo activities compared to wtdGK, respectively.[26,27] In most of these cases early onset and severe multiorgan disease was seen with less than one year survival, however, in one case with compound heterozygote R142K and E227K mutations the patient was subjected to liver transplantation and was alive at 11 years of age.[26–28] A recent case of a reversible dGK deficiency causing MDS was observed in a child who was a compound heterozygote for N46S and L266R point mutations.[29] Mitochondria from this patient's fibroblasts were analyzed and showed 25% and 9% residual activity with dAdo and dGuo, respectively, and it was suggested that this could play a crucial role in the phenotype reversal.

Expression of TK2 AND dGK in Different Tissues and Tissue Specific Variation in DNA Precursor Metabolism

The cytosolic and mitochondrial deoxynucleoside kinases (TK1, dCK, TK2, and dGK) as well as 5′-(deoxy)nucleotidase (5′-(d)NT) activities were determined in whole cell extracts from mouse tissues, including different skeletal muscles, testis, brain, adipose tissue, liver, spleen, kidney, lung, and heart using selective enzyme assays (Table 1).[30] TK2 activity was very low in all tissues, particularly in heart and skeletal muscle. Expression of TK1 activity was also low in all tissues, except spleen where it was several orders of magnitude higher. There was a significant correlation (rho 0.98 $P = 0.01$) between the TK1 and dCK activities. dGK activities were higher than any of the other dNK activities in all tissues, except spleen and testis. The variations in 5′-dNT activities, measured with dUMP as substrate, were about 10-fold and there was a correlation between these levels and those of TK1 and dCK (rho = 0.73, 0.78 and $p = 0.03$, 0.04, respectively). When the ratio of the dNK and 5′-dNT activities was used to estimate the capacity of the tissues to salvage deoxynucleosides, it was evident that muscle samples showed 5- to 10-fold lower levels compared to the other nonproliferating tissues and 100-fold lower compared to spleen. These results help explain the high susceptibility of muscle tissues to perturbations in mtDNA precursor synthesis, but they also demonstrate that each major type of tissue has a different capacity and rate of dNTP metabolism both with regard to the de novo and salvage pathways as well as the levels of catabolic and anabolic enzymes.

TABLE 1 Activity of cytosolic and mitochondrial deoxynucleoside kinases and 5′-nucleotidase in extracts from mouse tissues

	TK1	dCK	TK2	dGK	5′dNT
Liver	1.9	3.4	2.6	37.1	49
Lung	1.4	3.2	1.9	23.1	147
Kidney	2.1	3.1	3.1	36.6	160
Testis	6.4	10.7	4.6	8.1	42
Spleen	218	54.9	nd	22.2	504
Brain	4.8	9.2	8.2	15.9	160
Pancreas	1.3	2.9	1.3	7.8	157
Heart	0.8	1.0	1.0	20.2	70
Biceps	0.2	0.3	0.2	5.8	13
Vastus	0.1	0.1	0.5	5.1	10
Brown fat	1.9	3.5	3.9	23.4	145

Activities of deoxynucleoside kinases and 5′-nucleotidase in total tissue extracts from mice were measured and the results are from Rylova et al.[30] and are expressed in pmoles monophosphates formed per mg wet tissue per minute and and for 5′-dNT the results are in nmoles dUMP hydrolysed per min and mg wet tissue. Values are the mean of duplicate measurements of tissue extracts from three separate mice and the SD was <20%. nd, not determined.

GENERAL CONCLUSIONS

The enzymology of DNA precursor metabolism is now relatively well established but only in recent years has this basic knowledge been connected with metabolic diseases and in particular with mitochondrial diseases such as MDS. Characteristics for this type of disease are the large variations in time of onset and severity of symptoms. The results summarized in this review exemplified by many mutations in eight different DNA metabolic enzymes demonstrate that both the salvage and de novo pathways for deoxynucleotide synthesis are required for mtDNA precursor synthesis in all cells, including post mitotic tissues. The balance between the two anabolic pathways as well as the catabolic pathways varies in different tissues and metabolic situations as well as in individual patients. This may explain the different symptoms, severity and time of onset of MDS caused by defects in purine and pyrimidine enzymes.

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