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A Role for Mitochondrial DNA and Sperm Survival

Review

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This review outlines the role of mitochondria in providing energy for cell function and discusses the relevance of mutations to various cell types within the mitochondrion's own genome. In particular, we describe some of the mutations reported in sperm mitochondrial DNA (mtDNA) and discuss the relevance of these mutations to sperm function. A strong link has been established between the effects of free radicals on the mitochondrial genome and the mitochondrial inner membrane potential ($\Delta\psi_m$). We examine whether this link is applicable to spermatozoa and suggest that those cells with higher levels of mtDNA mutation may have a weakened $\Delta\psi_m$ that may affect the function of the organelle. We then discuss the role that mitochondria have in mediating cell death and how they may regulate sperm survival.

The Importance of Mitochondria

Mitochondria are complex organelles in the cytoplasm of all aerobic animal and plant cells. They are the site of most of the chemical reactions that convert the chemical energy in food to adenosine triphosphate (ATP). The double membranes of mitochondria regulate the transport of molecules. Large molecules transit through the outer membrane through nonspecific porin channels; the inner membrane is heavily invaginated and forms cristae, the distinct, shelflike internal layers that are impermeable even to small ions and molecules. It is within the cristae that the enzymes of the electron transfer chain (ETC) are located (Figure 1). The Krebs cycle and β-oxidation also take place within the inner membrane. These processes precede oxidative phosphorylation and result in the removal of hydrogen from metabolic fuels and the release of CO_2.

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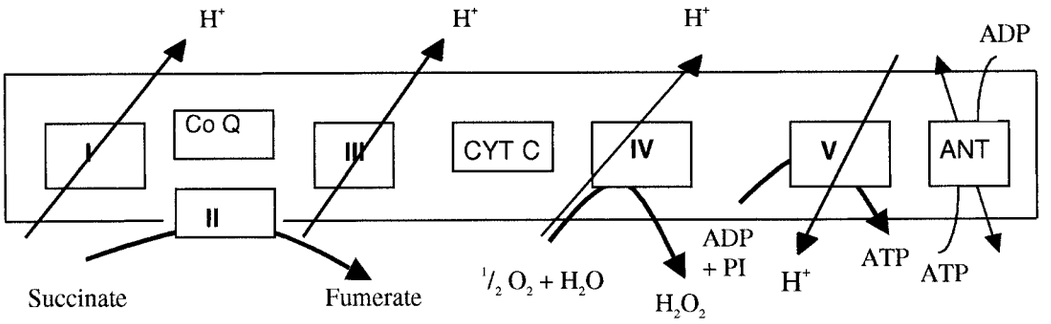
Oxidative Phosphorylation

Generation of ATP by mitochondria is vital to the survival of most cells because it is either the primary or only source of ATP and thus is essential for cellular homeostasis (Moyes et al, 1998). The major contributor to cellular ATP is oxidative phosphorylation, in which electrons are separated from hydrogen atoms and are transferred from one electron-acceptor molecule to another to oxidize the cytochromes and, as a consequence, lose much of their energy. This energy is employed to pump protons into the surrounding media to establish an electrochemical gradient that provides the energy for ATP synthesis (Figure 1). The electrons that are passed down the ETC are united with the protons and react with molecular oxygen to result in the production of H_2O. This process achieves the transduction of redox energy into a proton gradient, which then acts as the driving force for ATP synthesis. Indeed, the importance of oxidative phosphorylation to the process has been demonstrated by classical experiments using mitochondrial-specific inhibitors (Slater, 1979; Figure 1).

A cell's requirement for oxygen reflects its metabolic activity and the levels of ATP that its mitochondria produce are often indicative of the cell's function (Moyes et al, 1998). Cells with a large oxidative capacity, such as those found in liver, heart, muscle, and neural tissue, have several thousand mitochondria per cell (Brown and Wallace, 1994). The lungs have a high demand for oxygen uptake, which is reflected in an oxygen tension of 160 mm Hg, whereas spermatozoa are produced in the testis in almost anoxic conditions with a partial pressure of 4 to 16 mm Hg (Setchell, 1978). Spermatozoa have 22 to 28 mitochondria per cell, a relatively low number considering their need for motility. Furthermore, sperm mitochondria tend to be responsive to the glucose concentration of the female reproductive tract (Storey, 1980).

Mitochondrial Genome

Mitochondria possess their own genome, which is compartmentalized away from the nuclear DNA. The human mtDNA genome is extremely compact and circular, and consists of 2 strands. The complete sequence of the 16 569 base pair (bp) human mitochondrial genome has been described by Anderson et al (1981). It encodes 13 polypeptide subunits of the mitochondrial ATP-generating pathway, along with the 22 tRNAs and 2 rRNAs (12S and 16S) that are necessary for mRNA expression (Figure



Complex	I	II	III	IV	V
Inhibitor	Rotenone		Antimycin	KCN	Oligomycin
nDNA subunits	>18	4	8	10	10
mtDNA subunits	7	0	1	3	2

Figure 1. The electron transfer chain. The majority of subunits are encoded by nuclear DNA but all of the complexes except for complex II have subunits encoded by mtDNA. CoQ indicates coenzyme Q; CYTC, cytochrome C; ANT, adenine nucleotide transporter; nDNA, nuclear DNA; mtDNA, mitochondrial DNA; KCN, potassium cyanide; and H^+, proton.

2). Each of the complexes of the ETC, except for complex II, has genes encoded by the mitochondrial genome, whereas the remainder of the subunits of the ETC are encoded by the nucleus. Unlike nuclear DNA, the mitochondrial genes have none or only a few noncoding bases between them and thus lack introns (Figure 2). However, there is 1 noncoding sequence, the displacement loop (D-loop), which is vital to mtDNA replication (Figure 2).

Mutations and Deletions in mtDNA

Various mutations and deletions have been identified in mtDNA and are associated with different disease states. These mutations or deletions can be categorized into at least 4 groups.

1. Maternally inherited point mutations of mtDNA, such as neurogenic weakness, ataxia, retinitis pigmentosa (NARP; Fryer et al, 1994); myoclonus epilepsy with ragged-red fibers (MERRF; Shih et al, 1991); mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episodes (MELAS; Kobayashi et al, 1990); and Leber's hereditary optic neuropathy (LHON; Brown et al, 1992).
2. Single sporadic deletions or insertion-duplication of mtDNA, such as Kearns-Sayre syndrome (KSS; Schon et al, 1989).
3. Mutations of mtDNA inherited as Mendelian traits, such as chronic external progressive opthalmoplegia (CPEO; Zeviani et al, 1989).
4. Multiple mtDNA (ΔmtDNA) deletions that are associated with aging, such as somatic mutations that are nongermline (eg, Zhang et al, 1992).

mtDNA Mutations and Deletions in Spermatozoa

The mitochondrial genome is particularly susceptible to base substitution (Polyak et al, 1998). Although many studies have highlighted the presence of maternally inherited mtDNA point mutations, to date, few studies have investigated these mutations in spermatozoa. One such example is that of a 50-year-old male patient from a 4-generation family that suffers from mitochondrial encephalomyopathy. This condition was caused by a maternally inherited point mutation in the tRNA leucine (UUR) gene, which a pedigree study of this family indicated reflected a heteroplasmic trend in the segregation of the mitochondria (Folgero et al, 1993). Heteroplasmy is characterized by both wild type and deleted molecules being present in a mitochondrion, cell, or tissue, whereas in homoplasmy, only wild type or deleted molecules are present. Although an mtDNA analysis was not performed on that patient's spermatozoa, biochemical studies indicated that the mutation affected sperm motility. Specifically, the authors showed that the addition to the media of complex II substrates pyruvate and succinate, which bypassed the complex I mutation, resulted in a threefold increase in motility.

Various large-scale deletions have been found at extremely low levels within the mitochondial genome that are both somatic and multiple in nature. Typical markers of overall ΔmtDNA are in the 4977-bp (Figure 2) and 7.4-kb ''common'' deletions and their incidence in the diseases of the brain, heart, liver, and skeletal muscle has been well documented (Ikebe et al, 1990; Zhang et al, 1992, 1995, 1999; Pallotti et al, 1996; Ferlin et al, 1997).

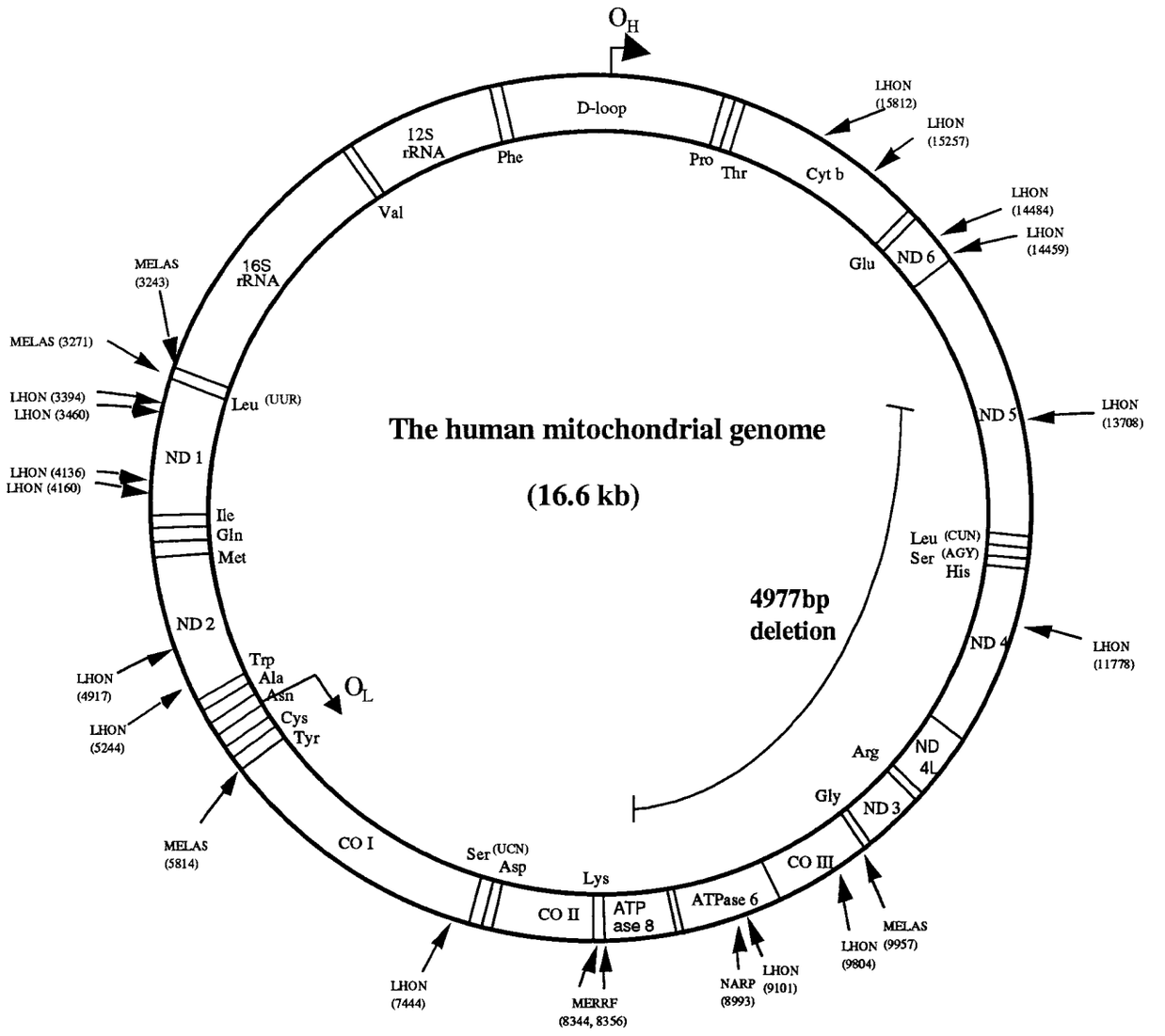


Figure 2. The mitochondrial genome. The D-loop comprises the H-strand origin of replication (O_H), the H- and L-strand promoters, and conserved sequence boxes. The 2 promoters are located approximately 150-bp apart, do not overlap, and function independently. The L-strand origin of replication (O_L) is located at ⅔ of the molecule length and is surrounded by genes encoding 5 tRNAs. The information content of each strand is dissymmetrical because the heavy (H) strand encodes 12 proteins, 14 transfer RNAs, and the 2 ribosomal RNAs, whereas the light (L) strand encodes only 1 protein (ND6) and 8 tRNAs (Clayton, 1982, 1992). Replication requires initiation of O_H and proceeds around to O_L. Once O_L has been reached, replication of the L-strand can then take place. The genes encoded by the mitochondrial genome are complex I (NADH dehydrogenase; ND1, 2, 3, 4, 4L, 5, and 6); complex III (ubiquinol:cytochrome C oxidoreductase) cytochrome B complex IV; (cytochrome C oxidase; CO I, II, and III); complex V (ATP synthase; ATPase 6 and 8). tRNAs are identified by their respective 3-letter codes.

However, a clearer picture is emerging that the common deletions are not closely related to the overall rate of somatic deletion. Indeed, the diagnostic value of the 4977-bp common deletion is limited and controversial, especially for spermatozoa. Kao et al (1995) employed a semiquantitative polymerase chain reaction (PCR) method and demonstrated the ratio of 4977-bp deletion to wild type mtDNA to be in the range of 0.0032% to 0.0708% for the 80% and less than 50% Percoll-fractionated spermatozoa, respectively. Furthermore, they concluded that there was a negative correlation between the 4977-bp deletion and sperm motility. However, other data suggest that this particular deletion alone is of little value and is not correlated to semen quality (Cummins et al, 1998) or sperm parameters (St. John et al, 1997b). Indeed, the variance in the results of these studies suggests that an analysis should be conducted on single sperm in which more discrete anomalies may be identified.

In contrast to conventional PCR, long PCR allows amplification of the whole or a large part of the mitochon-

drial genome (Cheng et al, 1994a,b). This technique combines normal polymerase activity with that of a 3′/5′ exonuclease to enhance the length of the resulting product and has been extensively exploited in the assessment of multiple deletions associated with CPEO (Fromenty et al, 1996, 1997), skeletal muscle (Melov et al, 1995), polymeglia rhumatica (Reynier and Malthiery, 1995), and the testis (St. John et al, 1997a). Its use is justified by the presence of multiple deletions and the subsequent formation of minicircles of mtDNA observed from the total detection system (Katsumata et al, 1994; Hayakawa et al, 1996). However, unlike the total detection system, long PCR requires one reaction instead of many. Amplification of either the entire genome or the most heavily deleted region with long PCR, incorporating the 4977-bp deleted region, indicated multiple deletions in patients with oligoasthenozoospermia (Kao et al, 1998; Reynier et al, 1998). One of these reports identified the presence of 2 types of 7.4-kb deletions and, through quantitative conventional PCR, observed that these deletions had a higher incidence in those patients with primary infertility and oligoasthenozoospermia (Kao et al, 1998). Our studies to date suggest that considerably more deletions are present in samples from men with oligoasthenoteratozoospermia (OATS; St. John et al, 1999), which implies that mtDNA depletion is perhaps also indicative of poor morphology.

In terms of the testis, abnormal mitochondria were also reported in the spermatids and Sertoli cells of the patient with encephalomyopathy described by Folgero et al (1993). This raises 2 fundamental issues associated with mitochondrial disease and genetics. First, spermatozoa originate from the primordial germ cells but are produced in the testis. The somatic cells associated with the testis are all derived from a different embryonic ridge, the genital ridge (Larsen, 1993), suggesting that heteroplasmic segregation is evident within the testis. As a result, depletion of ATP could arise through 2 independent embryonic sources. This then could lead to a cyclic loss in energy production with the support cells, for example, the Sertoli cells, being depleted of energy and thus unable to maintain efficient spermatogenesis (Cummins et al, 1994). Second, it has been hypothesized that loss of testicular function could be age-specific and, more important, indicative of premature aging. To this extent, one study has analyzed the proportion of deleted mtDNA in a range of human tissues with the 4977-bp deletion first appearing in the second and third decades of life in human muscle and liver tissues, though the deletion was not detectable in the testis until the age of 60 (Lee et al, 1994b). However, another study showed that, for men who were diagnosed with azoospermia or severe oligozoospermia (men aged less than 45 years old), a correlation existed between the number of testicular spermatozoa retrieved following testicular biopsy and the level of multiple ΔmtDNA observed in the biopsied tissue as detected by long PCR (St. John et al, 1997a). Even though this study did not employ age-matched controls, the presence of the deletions suggests that spermatogenic failure may arise through premature aging of the testis.

How Do Multiple ΔmtDNA Deletions Arise?

The large-scaled ΔmtDNA are characteristically often flanked by direct repeats, which are regarded as intrinsic to their formation. In the 4977-bp deletion, a repeated sequence of 13-bp flanks either side of the region marking the deletion, and in the deleted molecule, the 5′ repeat persists, whereas the 3′ is lost (Zhang et al, 1992). Other deletions, such as the 7.4-kb ''common'' deletion and the 15-bp microdeletion in the COX III subunit are also flanked by repeats, albeit of fewer bases. For the ''common'' deletions, the 5′ repeat is typically located on the H-strand and the 3′ repeat in situated on the L-strand, which suggests a process of strand jumping during polymerase activity (Ozawa, 1995; Figure 3a). To this extent, 2 molecular models have been proposed to account for the formation of large-scale deletions: slipped-mismatching (Shoffner et al, 1989; Figure 3b), and illegitimate elongation of the D-loop strand during replication (Buroker et al, 1990; Figure 3c). The former involves an ''illegitimate'' realignment of direct repeats between the H strand and the complementary L-strand during replication, which produces a DNA loop that is then excised and results in the formation a shortened template for the subsequent elongation of the newly formed L-strand. The second, illegitimate elongation of the D-loop strand during replication necessitates the elongation of the D-loop strand and exposure of the first repeats followed by extension of the H-strand. This results in displacement of the elongated D-loop strand, which then misaligns with a downstream repeat on the L-strand. During the next round of replication, the region between the repeats is bypassed by the elongating D-loop strand and results in a smaller genome.

These 2 hypotheses are far from complete, and although they may account for the deletions in which either the 5′ or 3′ repeat is retained (Larsson and Holme, 1992), they do not account for those deletions that are not surrounded by repeats but nevertheless have breakpoints (Lee et al, 1994a; Moslemi et al, 1996). Other, more complex mechanisms include a nuclear mutational–driven event (Mansouri et al, 1997; Schapira et al, 1998) or the incorporation of free radicals and the clonal expansion of existing deletions in the stem cells. This review now concentrates on the latter of these hypotheses.

Mitochondrial DNA and Aging

Regions of the mitochondrial genome are subject to attack by free radicals with some regions more susceptible to

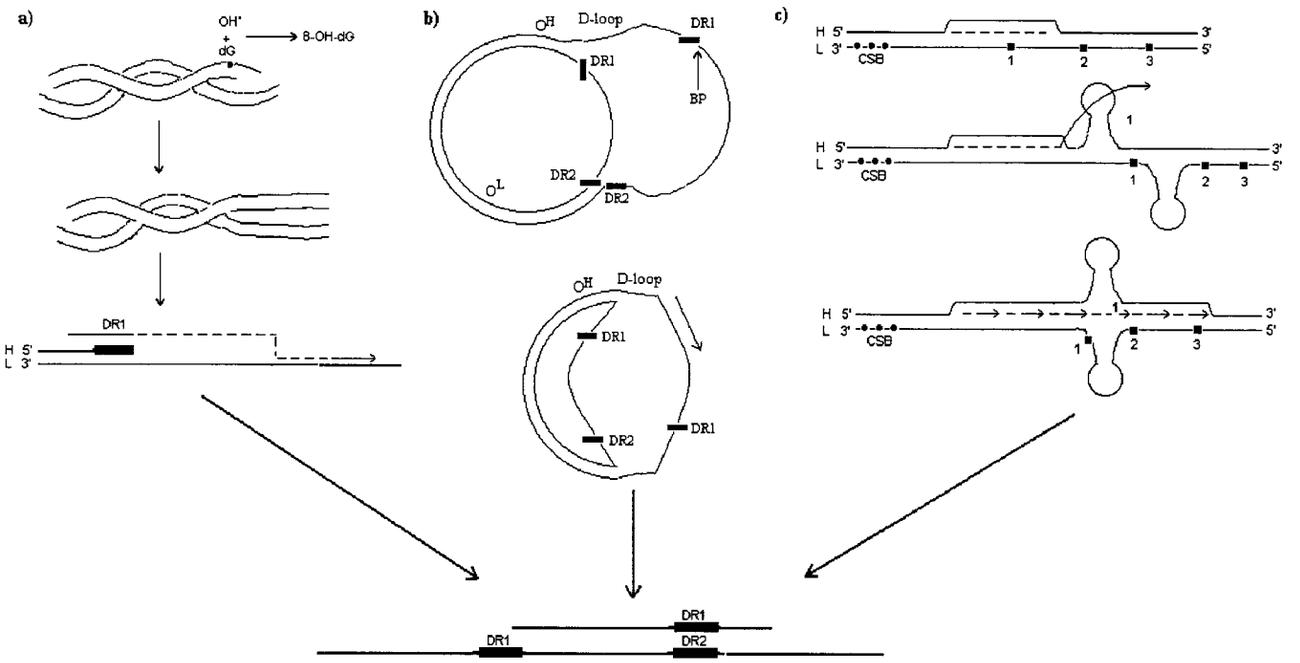


Figure 3. Three mechanisms proposed to be mediators of mtDNA deletions. **(a)** Free radicals such as $OH^{\cdot}$ can complex with deoxyguanosine (dG), causing a conformational change in the DNA. Polymerase misreading ensues with direct repeat 1 (DR1) being retained and DR2 lost, the sequence between these 2 repeats also being eradicated. This results in the encoding of a hybrid protein (Ozawa, 1999). **(b)** The slip-replication model. The D-loop proceeds around to the L-strand origin of replication (O_L) and displaces the H-strand, allowing DR1 to base pair with DR2 on the L-strand caused by a breakpoint (BP) occurring just after DR1. The remainder of the H-strand is degraded while the 3′-hydroxyl of the displaced DR1 is ligated to the 5′-phosphate (Shoffner et al, 1989). **(c)** The illegitimate elongation model. A short D-loop strand is partially displaced by the H-strand, and the 3′ end of the D-loop reinvades at a downstream copy, forcing the L-strand into a hairpin loop. Extension of the D-loop into a nascent H-strand results in a copy being lost (Buroker et al, 1990). CSB indicates conserved sequence box; O_H, H-strand origin of replication; O_L, L-strand origin of replication; and $OH^{\cdot}$, hydroxyl radical. Each mechanism appears to occur randomly and results in both wild type and deleted molecules being identified in the investigated samples.

deletion than others. Indeed, regions that are more susceptible to mutation are up to 100 times more susceptible than nuclear DNA, which is protected by histones (Pesole et al, 1999). Previously, it was understood that mtDNA had little or no repair mechanisms; however, more recent data suggest that some repair mechanisms appear to be present, although their effectiveness is controversial (Bohr and Anson, 1995; Thyagarajan et al, 1996; Lunt and Hyman, 1997; Yakes and Van Houten, 1997; Bohr and Dianov, 1999). These factors, coupled with the free radical theory of aging (Harman, 1960), which argues that free radicals cause nonspecific damage to macromolecules such as DNA, lipids, and proteins, has led to the mitochondrial theory of aging (Linnane et al, 1989). This theory proposes that somatic (ie, nongermline) accumulations of mtDNA mutations during life are a major cause of human aging and degenerative diseases.

Cytochrome c, a vital transporter of electrons in the ETC, has also been strongly implicated in the mitochondrial theory of aging. This complex is highly effective at transforming hydrogen peroxide (H_2O_2) to the hydroxyl radical ($OH^{\cdot}$), the most reactive of the oxygen free-radical species, through the Fenton reaction (Hayakawa et al, 1989). The Fenton reaction is a normal biochemical event, the generation of free radicals is regulated by scavenging enzymes such as manganese superoxide dismutase (SOD), catalase, and glutathione peroxidase. However, the newly formed $OH^{\cdot}$ will accumulate and, at some stage, outnumber SOD and glutathione peroxidase, the enzymes that can neutralize its actions. The consequence of this imbalance is that nonscavenged $OH^{\cdot}$ complexes with mtDNA, producing 8-hydroxydeoxyguanosine (8-OH-dG; Hayakawa et al, 1989; Giulivi et al, 1995; Kowaltowski and Vercesi, 1999). Ozawa's hypothesis (1995) of a ''vicious cycle'' proposes that double-stranded separation of the mtDNA molecule ensues with the subsequent deletion encoding for a defective ETC, which propagates a further increase in $OH^{\cdot}$ formation and damage (Figure 3a). Many of the large-scale deletions observed occur at hot regions (ie, those within the genome that have bent DNA structures). These regions are identifiable through 2-dimensional gel electrophoresis because they show abnormal mobility, whereas regions that rarely exhibit deletions do not (Hou and Wei, 1996). Specifically, the 2 AT-rich sequences flanking the 5′-end breakpoint of the 4977-bp deletion confer this bent structure to the

DNA and thus render it more susceptible to free radical attack (Hou and Wei, 1998). To support these findings, Mn-SOD has been shown to increase with age in human skin before age 60 but subsequently decreases, whereas 8-OH-dG in total DNA and the 4977-bp deletion showed an age-dependent increase (Lu et al, 1999). Furthermore, 8-OH-dG increases exponentially with age in heart and liver mtDNA and can be correlated with the incidence of the 7.4-kb deletion (Hayakawa et al, 1991, 1992, 1995). A greater accumulation of 8-OH-dG has also been observed in fragmented mtDNA in comparison with intact mtDNA (Suter and Richter, 1999).

In cardiomyopathy, the heart mitochondria generate oxygen free radicals through cyclic ischemia and reperfusion, which can result in an 8- to 2200-fold increase in somatic mtDNA mutations (Corral-Debrinski et al, 1991, 1992). Patients with Alzheimer's disease have an increased mtDNA somatic mutation rate and accumulation of deletion in neurons, which results in associated dementia. Patients with late-onset Alzheimer's disease who have been analyzed before age 75 have a 15-fold higher cortical somatic deletion level than age-matched controls, whereas those who were analyzed after age 75 harbor fivefold less cortical deletion levels (Corral-Debrinski et al, 1994). Patients with Huntington's disease show similar symptoms with 5- to 11-fold increases in cortical deletion against age-matched controls (Horton et al, 1995). Furthermore, in vitro, long exposure (60 minutes) of Simian virus 40–transformed fibroblasts to H_2O_2, a precursor to OH• formation, demonstrated extensive ROS-induced mtDNA damage (Yakes and Van Houten, 1997).

Although a strong correlation between free radical levels and mtDNA deletions has been made, the accuracy of techniques for measuring $OH^{\cdot}$ and its conversion to 8-OH-dG, for example, is controversial. Indeed, because much of these data are correlative, they must be viewed with caution. Specifically, the levels of 8-OH-dG that can be detected through the use of high-performance liquid chromatography/mass spectrometry are 10- to 100-fold higher than those measured with high-performance liquid chromatography/electrochemical detection (Beckman and Ames, 1996). A more recent report suggests that quantification of oxidative DNA adducts is biased by artifactual oxidation, to the extent that it is not clear whether mtDNA is more or less prone to oxidative damage than nuclear DNA (Beckman and Ames, 1999).

Implication of Reactive Oxygen Species

Several studies have detected a clonal expansion of specific deletions that are present early in life (Johnston et al, 1995; Oldfors et al, 1995; Tengan et al, 1997; Zhang et al, 1999) that are not associated with the ''vicious cycle.'' In one case, a positive correlation was observed between age and common deletion levels in controls ($r = 0.80$) and those patients with skeletal muscle disorders ($r = 0.69$). In this instance, the rate of accumulation of the age-related common deletion was the same in each group (Tengan et al, 1997). This clonal hypothesis complements the concept of the founder molecule theory (Marchington et al, 1997) and the exponential increase in deletion with age. Furthermore, it has been demonstrated in other cellular systems that the nuclear background of the cell can determine whether wild type or deleted molecules would predominate (Dunbar et al, 1995). We hypothesize that in the testis, where the nuclear background of spermatogonia would bias replication of deleted molecules, coupled with a decrease in the number of sperm mitochondria occurring at the spermatocyte/spermatid stage (Hecht et al, 1984; Larsson et al, 1996), there would be preferential accumulation of deleted molecules. Perhaps this mechanism would also contribute to the exclusion of the paternal mitochondrial genome from being transmitted to the offspring because the embryo would favor transmission of intact mtDNA. However, the effect of these deletions is only important when a clear loss of mitochondrial function is identified.

Do ΔmtDNA Influence $\Delta\psi_m$ and Mitochondrial Function?

$\Delta\psi_m$ is important to a functional mitochondrion. This potential is achieved through protons being pumped across the inner mitochondrial membrane as a result of oxidative phosphorylation and is used as a marker for assessing overall mitochondrial function (Cortopassi and Wong, 1999). When any mutation or deletion is detected in the mitochondrial genome, it is important to determine whether this gene defect has an adverse effect on cellular function. Indeed, many of the mitochondrial genome-driven diseases have debilitating effects and can be fatal (Wallace, 1993), and it is the degree of heteroplasmy that prevails in one particular tissue type that dictates the onset of a particular phenotype. One technique for determining such a phenotype is the rho-0 cell model. These cell lines are mtDNA depleted but can still synthesize mitochondria because of the transcription of the nuclear-encoded genes responsible for synthesis of proteins associated with the mitochondrion and those of the ETC. Rho-0 cells are essentially maintained in an anaerobic medium containing pyruvate and uridine (King and Attardi, 1988). Foreign mtDNA can be incorporated in these cell lines through transfection techniques, thus creating functional cybrids. Viable cybrids are selected by transferring the cybrid from the anaerobic media to an aerobic environment (King and Attardi, 1988, 1996). Those cybrids that have sufficient oxidative phosphorylation capacity survive.

However, the evidence in favor of using rho-0 cell model systems is conflicting. The use of rho-0 cell lines to undertake such analysis has been criticized because they maintain $\Delta\psi_m$. It is interesting, however, that the $\Delta\psi_m$

is lower in rho-0 cells than it is in the cells they were created from (Appleby et al, 1999). Furthermore, cybrids of Alzheimer's disease exhibit a significantly decreased uptake of H^3-TPP^+, a marker of overall $\Delta\psi_m$, compared with controls and $\Delta\psi_m$ can be recovered following treatment with cyclosporin A (CsA), an agent that inhibits opening of the mitochondrial transition pore (MTP; Cassarino et al, 1998). Indeed, the permeability transition (PT) arises as a result of the formation of MTP in the inner mitochondrial membrane, which depolarizes and allows low molecular weight molecules such as Ca^{2+} and ROS to permeate (Zoratti and Szabo, 1995), resulting in the loss of $\Delta\psi_m$. Interestingly however, a study employing cybrids that were transfected with the mitochondrial genomes from enucleated skin fibroblasts from a patient with CPEO provided clear evidence of a critical threshold. In cybrids containing less than 50 to 55% ΔmtDNA, no difference from controls was recognized, but when the proportion of ΔmtDNA exceeded this threshold, the $\Delta\psi_m$, rate of ATP synthesis, and cellular ATP/ADP ratio decreased (Porteous et al, 1998), which are all good indicators of mitochondial function (Cortopassi and Wong, 1999). Indeed, MELAS and MERRF fibroblasts are particularly susceptible to increased ATP demand (James et al, 1999; see also Attardi et al, 1995). Added to this, in rho-0 cells that carry the MERRF mutation, a decrease in oxygen consumption is observed and is accompanied by an increase in mean mitochondrial superoxide production (Cortopassi and Wong, 1999). Administration of H_2O_2 to an rho-0–transfected cell line containing one of the MERFF, MELAS, or LHON genotypes demonstrated significant sensitivity to oxidant stress against controls, and as before, these transformants were protected by CsA (Cortopassi and Wong, 1999).

We hypothesize that sperm mitochondria, which harbor a high proportion of mutated mtDNA, would also be particularly susceptible to H_2O_2 and other free radical activity, and result in a decrease in $\Delta\psi_m$. Indeed, a positive correlation exists between $\Delta\psi_m$ and sperm motility (Troiano et al, 1998). To date, one study has attempted to transfect sperm mitochondria into a rho-0 cell line, although the recovery of viable cybrids postselection was less than 1% (Manfredi et al, 1997). Failure to achieve higher uptake is probably the result of complex disulphide bonding in the sperm midpiece, which also results in our inability to isolate a pure population of sperm mitochondria (Sutovsky et al, 1996). It would thus appear that a full analysis of the role of mtDNA deletions and their ability to affect $\Delta\psi_m$ and sperm viability needs to be undertaken.

Effect of $\Delta\psi_m$ Loss

The generation of free radicals, which results in the degradation of $\Delta\psi_m$, is particularly well documented (Zamzami et al, 1995, 1996). In particular, H_2O_2 and *tert*-butylhydroperoxide (*t*-BOOH), an inorganic compound, are adept at this. *t*-BOOH modulates the mitochondrial calcium-dependent formation of H_2O_2 through an up-regulation in oxygen consumption that increases $\Delta\psi_m$ to allow the accumulation of sufficient calcium. This then results in the release of H_2O_2 to the nucleus, which reacts with the iron bound to chromatin and generates the hydroxyl radical through the Fenton reaction (Guidarelli et al, 1997). The loss of $\Delta\psi_m$ can also result in the release of specific apoptotic factors, for example cytochrome C (Scarlett and Murphy, 1997) and apoptotic inducing factor (AIF; Susin et al, 1999) from the mitochondria, which initiate the process of cell degradation (Zamzami et al, 1996). It therefore seems possible that mitochondria can regulate the cell's response to local environmental factors through their sensitivity. Apoptosis is a form of cell death characterized by the catabolic actions of proteases and nucleases and can be triggered by ligation of certain receptors—for example, Fas. Necrosis, however, is induced by nonphysiological effectors such as toxins and physical and chemical changes (Zamzami et al, 1997).

A strong link has been established between apoptosis and mitochondria and specifically a mitochondrial-initiated cascade of downstream events (see Wilson, 1998, for a review). This complex process can be partially regulated by 2 members of the Bcl-2 family. One member of the family, Bcl-2, the antiapoptotic factor, is involved in regulating the mitochondrial process of apoptosis through calcium flux into and from the mitochondrial matrix (Baffy et al, 1993; Murphy et al, 1996). This process is regulated by the MTP, which is particularly sensitive to agents such as *t*-BOOH, H_2O_2, protonophore, or atractyloride (Zamzami et al, 1996). Recent data from a cell line that overexpresses Bcl-2 have demonstrated that this particular antiapoptotic factor was vital for maintaining $\Delta\psi_m$ by regulating ion transport in the presence of the protonophore SF6847 (Shimizu et al, 1998). However, overexpression of Bax, the proapoptotic member of the Bcl-2 family, induces the disruption of $\Delta\psi_m$ (Xiang et al, 1996). Furthermore, normal cellular function requires the heterodimerization of Bcl-2 and Bax to maintain a balance between cell death and cell proliferation (for a review, see Kroemer, 1997). It is interesting that the testis appears to be finely regulated by Bax and Bcl-2 to the extent that, in mice, overexpression of Bcl-2 (Knudson et al, 1995) and deficiency of Bax (Furuchi et al, 1996) leads to spermatogenesis that fails to complete to the mature spermatozoa stage.

Apoptosis has been proposed to be a regulator of the overproliferation of the clonal progeny. One particular factor implicated in spermatogenesis is the cell surface protein receptor, Fas (Lee et al, 1997). We have shown in humans that poor-quality samples containing mature spermatozoa have proportionally higher levels of Fas expres-

sion than normal samples (Sakkas et al, 1999). To this extent, we have argued that these cells have been rescued in the testis through an abortive apoptotic mechanism, even though they had been targeted for cell death. We envisage that such a mechanism could also exist for other apoptotic markers such as Bax, especially as the up-regulation of Bcl-2 and Bax expression takes place during the early stages of spermatogenesis (Rodriguez et al, 1997). Up-regulation of Bax or one of the Bcl-2 proapoptotic family members in the testis would normally ensure that the developing spermatozoa would undergo cell death. However, this could be countered by up-regulation of Bcl-2 or one of the Bcl-2 antiapoptotic factors in an attempt to rescue the cell. Indeed, this may appear to be a normal mechanism because Bcl-2 family members possess both homologous and heterologous binding sites and maintenance of normal cell function would require equal levels of expression of proapoptotic and antiapoptotic factors (for a review, see Kroemer, 1997). These cells would again be labeled throughout spermatogenesis, as is the case for Fas, and this expression would be detected in mature spermatozoa. Consequently, we would predict that those spermatozoa with high levels of ΔmtDNA and poor $\Delta\psi_m$ might have an imbalance in their expression of Bcl-2 and Bax.

Conclusion

It is now pertinent for new lines of investigation to be conducted into the role of mitochondrial-mediated sperm survival. First, it is apparent that spermatozoa are sensitive to H_2O_2. Low levels of H_2O_2 are associated with hyperactivation, capacitation, and the acrosome reaction in vivo (de Lamirande et al, 1997). Higher concentrations of H_2O_2 lead to sperm immobilization through depletion of intracellular ATP (de Lamirande et al, 1997; Armstrong et al, 1999), lipid peroxidation, and cell death (de Lamirande et al, 1997). To this extent, we need to determine whether poor-quality spermatozoa are more likely to undergo loss of $\Delta\psi_m$ through a free radical–mediated event followed by the subsequent loss of cell function. Indeed, our preliminary studies have demonstrated that sperm mitochondria labeled with a mitochondrial-specific dye are susceptible to free radical attack mediated through both H_2O_2 and *t*-BOOH, which triggers the loss of $\Delta\psi_m$ and renders the cell nonviable (St. John et al, unpublished observations).

Second, because poor-quality sperm samples have high levels of ΔmtDNA, physiological and biochemical studies are required to determine whether these deletions can truly effect both survival and function of the sperm cell. However, a priority is to develop techniques to isolate human sperm mitochondria. We then need to transfect these isolated sperm mitochondria into rho-0 cells to create cybrids to determine the relevant $\Delta\psi_m$ threshold characteristics that are appropriate to spermatozoa and their ability to survive.

Third, because apoptotic factors can be linked to a mitochondrial-initiated cacsade of downstream events, analysis of these factors in terms of sperm survival requires investigation. These results could indicate definitive markers of defective spermatogenesis and eventually have clinical implications relevant to an andrologist's report to the physician and patient.

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