Mitochondrial DNA depletion causes morphological changes in the mitochondrial reticulum of cultured human cells

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Abstract Depletion of mitochondrial DNA (mtDNA) causes defects in respiratory activity and energy production. Recent studies have shown mitochondria to exist primarily as reticular networks, having tubular cristae. Using fluorescence microscopy and transmission electron microscopy, we have examined mitochondrial morphology and interior structure in wildtype and mtDNA-depleted ρ0 human fibroblasts and 143B osteosarcoma cell lines. MtDNA depletion results in compromise of the mitochondrial continuum and causes a reduction in amount of cristal membranes, often prompting the remaining cristae to adopt a circular appearance in the mitochondrial interior. These changes emphasize the tight relationship between mitochondrial structure and function.

Key words: Mitochondrion; ρ0; Mitochondrial DNA; Ultrastructure

1. Introduction

Mitochondria provide cellular energy via oxidative phosphorylation, utilizing the multisubunit complexes of the respiratory chain to create a transmembrane potential, which drives ATP synthesis by the F_1F_0 ATP synthase. Mitochondria also have a number of important roles in cellular homeostasis, participating in calcium regulation, redox regulation, and production of second messengers [1,2]. Additionally, there is now extensive evidence that mitochondria play a critical role in apoptosis, or programmed cell death [3,4]. The majority of mitochondrial proteins are synthesized in the nucleus and imported into the mitochondrion. However, this organelle has its own genome (mtDNA) which encodes 13 proteins and 22 tRNAs. Despite its small size (16.6 kb) relative to the nuclear genome, mtDNA is critically important for effective mitochondrial function and ATP production. Point mutations of mitochondrial-encoded tRNAs and proteins, as well as deletions of segments of mtDNA, have been diagnosed in a wide variety of clinical bioenergetic disorders [5,6].

One particularly interesting mitochondrial disorder is mtDNA depletion syndrome. First described in 1991, this syndrome is characterized by a tissue-specific loss of mtDNA until mtDNA is fully depleted [7,8]. MtDNA depletion can be achieved in cultured cell lines by treating with ethidium bromide, resulting in a ρ0 phenotype. Such ρ0 cells show a characteristic lack of mitochondrial-encoded protein subunits, resulting in a disruption of respiratory function and prompting cellular energy production by glycolysis [9,10]. ρ0 cells are increasingly being studied to characterize the role that mitochondria play in apoptosis. By using cells devoid of mtDNA, the role that enzymes of oxidative phosphorylation play in this process can be evaluated. Recent studies have shown that ρ0 cells retain the ability for mitochondrially regulated apoptosis [11,12]. Here, we examine the effects of mtDNA depletion on both the overall morphology and internal structure of human mitochondria. This is important because it is becoming increasingly clear that the morphological features of mitochondria control and amplify many of the functions of this organelle.

2. Materials and methods

2.1. Fluorescence microscopy

Lung fibroblast (MRC-5) and osteosarcoma (143 B) cells, both wildtype (WT) and ρ0, were grown on glass coverslips using high glucose Dulbecco's modified Eagle's medium (HG-DMEM) supplemented with 10% fetal calf serum and uridine in 5% CO_2 atmosphere. MRC-5 cells (WT and ρ0) were stained with 0.25 μM JC-1 for 1 h and then mounted on a slide. Photographs of living cells were acquired on slide film using a 35 mm Canon camera within 20 min of mounting. Osteosarcoma cells were grown to 30% confluence and transfected with green fluorescent protein (GFP) targeted to mitochondria. GFP was fused to the leader sequence of the E1α subunit of pyruvate dehydrogenase and transfected using Fugene6 reagent (Boehringer Mannheim). Twenty-four hours after transfection cells were washed with phosphate buffered saline and fixed in two steps (4% cold paraformaldehyde for 5 min followed by 8% paraformaldehyde at room temperature for 30 min). Images were acquired using an image intensified CCD camera (Pentamax, gen IV) mounted on an Axioskop 2 Zeiss microscope.

2.2. Transmission electron microscopy (TEM)

MRC5 fibroblasts were seeded onto 60 mm Permanox culture dishes and incubated at 37°C in HG-DMEM medium until confluent (2 days for WT, 1 week for ρ0 cells). Fixation for Spurr's resin embedment was in the Permanox dishes in either (a) 1% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.2, followed by 2% osmium tetroxide in the same buffer, or (b) 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodylate, pH 7.4 with 8 mM $CaCl_2$ [13] followed by 1% osmium tetroxide, pH 7.4. Dehydration was in ethanol, with or without a 1 h step in 2% uranyl acetate in 50% ethanol. Cells to be embedded in LR White resin were fixed with 2.5% glutaraldehyde, 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, postfixed with 1% osmium tetroxide in the same buffer, and dehydrated in ethanol. Ultrathin sections were counterstained with uranyl acetate and lead citrate, and viewed on a Philips 300 or CM12 electron microscope. Serial sections used to follow the interconnection of the mitochondrial reticulum were aligned using features

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Abbreviations: mtDNA, mitochondrial DNA; TEM, transmission electron microscopy; WT, wildtype; HG-DMEM, high glucose Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; OM, outer membrane; IM, inner membrane; CM, cristal membrane

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on the cell surface which were continuous through more sections than the portion of reticulum being followed.

2.3. Image analysis

Measurements of mitochondrial internal structure were conducted by scanning TEM negatives into Adobe Photoshop 4.0. These images were then opened in NIH Image 6.2 for analysis. Mitochondria were measured by tracing the outer (OM), inner (IM), and cristal (CM) membranes and calculating the area of the profile measured. Ideally, dual membranes opposing one another in the interior of the profile defined the cristae, although the electron density of the mitochondrial matrix and the orientation of cristae to the sectioning plane sometimes made it feasible to trace only one side of a crista. Data were compiled to achieve a total mitochondrial survey area of more than 1 μm^2 for each data set, comparing ratios of OM/IM, CM/IM, and CM/total membrane. Data sets were compiled for WT fibroblast fixations in Spurr's preparations A and B, as well as LR White. Data for ρ0 fibroblasts were taken in Spurr's preparations A and B.

3. Results

3.1. ρ0 cells have altered mitochondrial morphology as visualized by fluorescence microscopy

The overall morphology of mitochondria was studied in two cell lines, using different approaches to identify the organelle. Fibroblasts (MRC5) were incubated with JC-1, a fluorescent dye which accumulates exclusively in mitochondria as a result of the membrane potential generated by the organelle. Fig. 1A shows the reticular organization of mitochondria in normal fibroblasts using JC-1. The use of fluorescence microscopy reveals that the mitochondria of mammalian cells typically exist as a reticulum [14], rather than a wide dispersion of discrete cigar-shaped organelles within the cell. The reticular nature of mitochondria is equally well viewed in the 143B osteosarcoma cell shown in Fig. 1B. In this case, the arrangement of mitochondria was revealed by transfecting cells with GFP targeted to the mitochondrial matrix, using the mitochondrial targeting leader sequence of the E1α subunit of pyruvate dehydrogenase. Studies, largely in yeast, have indicated that the reticular nature of the mitochondrion is dynamic with the overall mitochondrial morphology the result of frequent fusion and fission events [15,16]. Fig. 1D,E shows the organization of the mitochondrial mass in ρ0 cells of JC1-labeled fibroblasts and GFP-labeled osteosarcoma cells, respectively. In the ρ0 form of these cells, the reticulum appears disrupted, yielding a distribution of small individual organelles; this fragmentation is highlighted in the higher magnification images in Fig. 1C,F. Similar results have been obtained for myoblasts, utilizing polyclonal antisera [17]. It should be noted that ρ0 mitochondria still take up JC-1, and they retain a significant, although lowered, membrane potential in the absence of a functional system of oxidative phosphorylation, consistent with measurements of membrane potential in ρ0 mitochondria [18]. While no quantitative analysis is attempted, the total amount of mitochondrial volume does not appear altered between the normal and ρ0 cells; only the morphology is altered.

3.2. The morphology and internal structure of mitochondria of ρ0 cells is altered based on TEM studies

Fig. 2 details the features of mitochondria of WT fibroblasts when examined by TEM. The flattened nature of these cells means that much of the cell volume is contained within thin sections of monolayers of cells. As a result, the reticular nature of the mitochondria in these cells can often be observed. The appearance of both elongate and circular mitochondrial profiles (Fig. 2A) is consistent with a mitochondrial reticulum as it intersects the sectioning plane at various angles. Note that the mitochondrial profiles have a constant diameter.

A cross-section through the mitochondrial reticulum in normal fibroblasts shows a distinct outer and inner membrane running roughly parallel along the organelle, with the cristal membrane spaced regularly along the reticulum. Fig. 2B demonstrates the cristal arrangement of WT mitochondria in higher magnification. There are numerous cristae, generally extending across the mitochondrial profile. Fig. 2C illustrates the connection of the cristal and inner membranes at a cristal junction. Recent electron tomography studies with brain and muscle tissue have shown that the cristal membranes are not irregular invaginations of the inner membrane as once thought; they are structurally distinct compartments, with

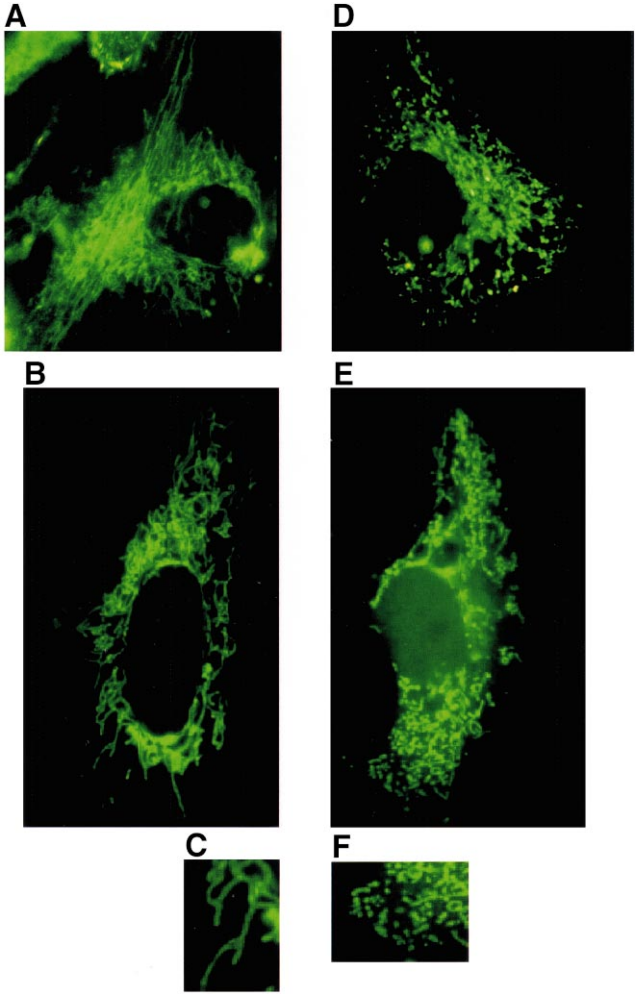

Fig. 1. Mitochondrial organization in WT and ρ0 cultured human cell lines. A: WT MRC5 fibroblast stained with JC-1. B: WT 143B osteosarcoma cell transfected with mitochondrially targeted GFP. C: Higher magnification image of B. A–C demonstrate the predominantly reticular organization of mitochondria in WT cell lines. D: ρ0 MRC5 fibroblast stained with JC-1. E: ρ0 143B osteosarcoma cell transfected with mitochondrially targeted GFP. F: Higher magnification image of E. D–F show the disruption of the mitochondrial reticulum in ρ0 cells, appearing as a population of vesicular organelles.

each crista linked to the intermembrane space between the outer and inner membranes by a few relatively narrow connections [19,20].

TEM images of ρ0 cells show clearly altered morphology of their mitochondria. Many regions are swollen such that the diameter of the mitochondrial tube is much more variable than in WT cells. Where the reticulum is retained, the fatter regions are linked by narrower regions that become more obvious in serial thin sectioning of cells (Fig. 3A–D). Such serial sections also show that the reticulum is degraded in ρ0 cells. At the far left is an isolated mitochondrial profile which fails to connect with the nearby profiles. Similar fragments separated from the main reticulum are common.

Significantly, the mitochondria in ρ0 cells still retain the distinct outer and inner membranes seen in normal cells; this is true even in the narrowest parts of the modified reticulum, as well as in regions of fragmented organelles. Based on a quantitation described in detail in Section 2, the ratio of cristal membranes to inner membranes in ρ0 cells was often close to 1, while the same ratio in WT cells is greater than 2. The agreement of ratios for WT mitochondria in several fixation/embedment protocols indicates that these calculated ratios accurately reflect the membrane state in living cells. Most of the cristae of appreciable length in ρ0 mitochondria appear as curved or circular cristae, as displayed in the higher magnification image of Fig. 3A. These often fail to extend across the profile. The cristal membranes present in mitochondria of ρ0 cells are not distributed evenly in reticular regions. They are concentrated to swollen regions and are essentially absent from the narrow portions, as evident in Fig. 3B,C.

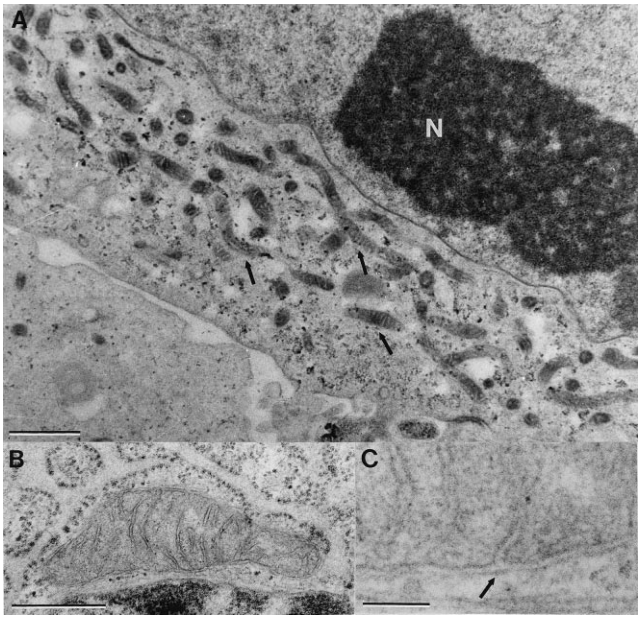

Fig. 2. A–C: Mitochondrial structure in wildtype MRC5 fibroblasts. A: Mitochondrial reticulum as seen in a single thin section. Numerous long, wavy mitochondrial profiles (arrows) are seen between the nucleus (N) and the edge of the cell. 13 700×; scale bar 1.0 μm. B: Mitochondrial profile showing numerous parallel cristae which extend most of the way across the profile. 35 500×; scale bar 0.5 μm. C: Cristal junction, showing cristal membranes connected to the inner membrane (arrow). 125 000×; scale bar 0.1 μm.

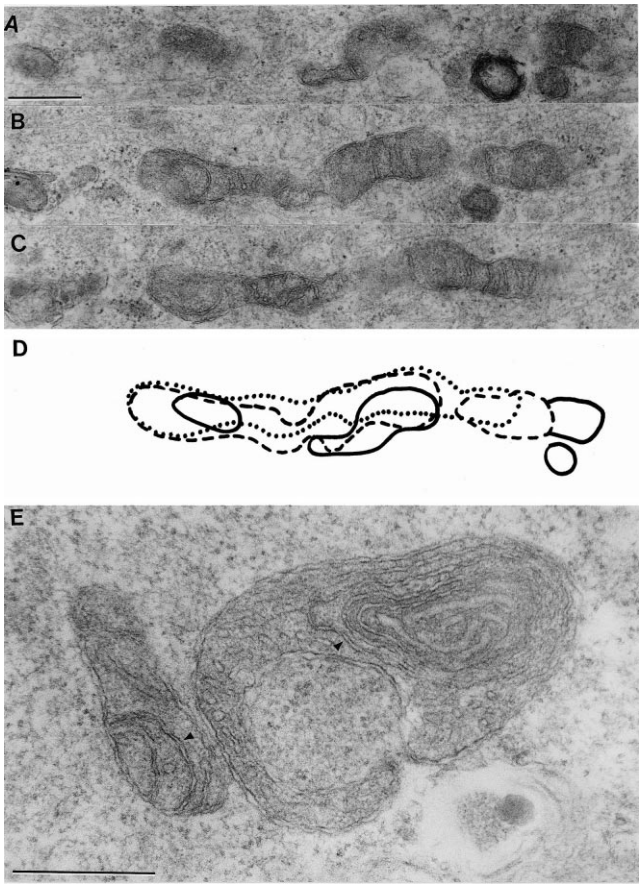

Fig. 3. ρ0 mitochondria viewed in TEM. All scale bars 0.5 μm. A–C: Serial sections revealing the limited interconnection of swollen mitochondria. Note isolated profile at far left which fails to connect with other profiles. These mitochondria show the irregular bulging and constriction typical of ρ0 mitochondrial morphology. D: Tracings of the profiles in A–C based on alignment with reference points external to the mitochondria. E: Representative profiles of ρ0 mitochondria, displaying circular and curved cristal motifs (arrowheads).

4. Discussion

MtDNA encodes 13 different polypeptides, all components of electron transfer complexes or the ATP synthase. In ρ0 cells, these complexes of the oxidative phosphorylation machinery are not fully assembled. Our ongoing studies to assess the protein composition of normal and ρ0 cells, as well as previous limited data, indicate that the synthesis and assembly of enzymes involved in other functions of mitochondria are not reduced, and for some proteins synthesis may be enhanced. Nevertheless, the selective loss of only a few of the total number of proteins within the mitochondrion leads to a dramatic reduction in the amount of cristal membrane and disorganization of the cristae, yet there is no observable alteration of the inner membrane. Such an effect may be explained if the cristal membrane is functionally distinct from the inner membrane, housing the respiratory chain complexes within a regulated mitochondrial subcompartment, while substrate and ion transporters, as well as protein translocators, are localized to the inner membrane. Perotti and co-workers found that cytochrome oxidase activity is localized almost exclusively within the cristal spaces and not in the intermembrane space of mi-

tochondria [21], supporting a functional distinction between the cristal and inner membranes. ρ0 cells, with their enrichment of inner membrane relative to cristal membranes, appear to be an ideal system in which to examine protein localization between inner and cristal membranes.

In addition to the depletion of cristal membranes, the mitochondrial reticulum is clearly altered in ρ0 cells. Instead of a reticulum with a uniform profile diameter, the reticulum seen in ρ0 mitochondria appears much like a necklace of beads with swollen regions linked by narrow connections. The swollen regions have distorted cristal membranes, while the linker regions do not appear to contain cristae. TEM of serial thin sections of cells confirms the results from fluorescence microscopy that fragmented mitochondria co-exist with the mitochondrial reticulum in ρ0 cells. It is as though a reticulum is constantly being generated in new cell growth, but this becomes fragmented in ρ0 cells, possibly by pinching off in the narrower regions, to give a population of disrupted mitochondria. Thus mtDNA depletion results in a compromised mitochondrial reticulum with reduced cristal content, consistent with a need for maintenance of respiratory function to retain an energized mitochondrial network.

The idea that mitochondria exist predominantly as reticula is not new, but has gained additional credence with newer labeling approaches to examining organelle morphology, such as those used here. ATP synthase mutants deficient in ATP production continue to maintain a hyperpolarized reticulum [22], suggesting that it is the loss of transmembrane potential which is responsible for alteration of the mitochondrial reticulum in ρ0 cells. The significance of the reticular nature of mitochondria is only now becoming appreciated. One possibility is that the mitochondrial continuum allows for ATP production and ATP use to occur simultaneously in different parts of the cell [23]. Calcium release from the endoplasmic reticulum may cause waves of mitochondrial redox potential, with the end result of fast, efficient regulation of energy production; this idea is supported by findings of close association of mitochondria with the endoplasmic reticulum [14,24], and reports of cell-wide waves of redox potential in cardiomyocytes [25]. Such a bioenergetic signaling function would likely be obsolete in ρ0 mitochondria, where no oxidative phosphorylation is occurring.

Our findings demonstrate the structural changes of which mammalian mitochondria are capable, in terms of overall morphology and internal structure. Mitochondrial morphology and ultrastructure similar to that in ρ0 cells have been observed in apoptotic cells [26,27]. Clearly, further work is needed to reconcile the morphological changes that can occur in mitochondria with changes in function of this organelle. It will be necessary to account for the variations in structure of mitochondria and to understand the dynamics of the structural changes that are a part of the bioenergetics of living cells.

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