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Marked mitochondrial alterations upon starvation without cell death, caspases or Bcl-2 family members

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ABSTRACT

Dictyostelium HMX44A cells can withstand starvation under monolayer conditions for a few days without dying. They die only when the differentiation factor DIF-1 is exogenously added. Still, when HMX44A were subjected to starvation without addition of DIF-1 they showed, by electron microscopy and electron tomography, gross mitochondrial lesions including marked cristae alterations with frequent "holes" probably originating from dilated cristae. Since these cells did not die as shown for instance by FACS analysis, these results showed unexpected resilience of cells bearing markedly altered mitochondria, and thus showed that apparently destructive mitochondrial alterations may not lead to cell death. Also, these marked mitochondrial lesions could not be caused by caspases or bcl-2 family members, which these cells do not encode.

1. Introduction

Mitochondria can be causally involved in caspase-dependent apoptotic cell death, in particular through fission [1] possibly involved in cytochrome *c* release [2]. Some mitochondrial lesions may also be a consequence rather than a cause of apoptosis, for instance through mitochondria-detrimental effects of activated caspases [3]. More generally, the relationships between mitochondrial structure and function, on the one hand, and apoptosis and other types of cell death on the other hand [4], are only partially understood. Still, it is often believed that marked mitochondrial alterations lead to cell death. Do they?

To answer this question, we took advantage of the two-step induction of cell death in *Dictyostelium discoideum*. In this organism, in vitro monolayer approaches [5] revealed and permitted the study of an autophagic vacuolar type of cell death [6–8], and, when the atg1 autophagy gene was mutated, a necrotic type of cell death [9,10]. Importantly, starvation by itself did not lead to either type of cell death, as repeatedly shown [6–10], but was required for subsequent induction of cell death. This induction was achieved through exogenous addition of differentiation-inducing factor (DIF-1), a small dichlorinated molecule that is a main natural morphogen in *Dictyostelium* [5]. This required addition dissects out starvation (which sensitizes to death) from addition of DIF-1 (that triggers death), which constitutes a significant analytical advantage of this experimental system.

Another question bears on what inflicts mitochondrial lesions in a stressed or dying cell. Caspases and bcl-2 family members have been implicated [3,11,12]. Of note, in *Dictyostelium* cells there are no caspase-family members (except one paracaspase gene which is not involved in autophagic or necrotic cell death), no Bcl-2 family member and no BH3 (Bcl-2 family domain)-bearing molecule [13,14], which therefore cannot take part in mitochondrial alterations.

We report here on marked mitochondrial lesions including apparent "holes" likely due to extreme dilation of cristae, revealed by electron microscopy (EM) and subsequently electron tomography (ET), in *Dictyostelium* cells subjected to starvation only, without addition of DIF thus with no induction of cell death, and in the absence of caspases or bcl-2 family members.

2. Materials and methods

2.1. Cells and cell culture

Dictyostelium discoideum axenic strain HMX44A.atg1-1 described earlier [9] was routinely grown at 22 °C in HL5 [15] containing Blasticidin (10 μg/ml). Starvation conditions and monolayer experiments were as described [10]. When added, DIF-1 was at a final concentration of 100 nM.

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2.2. Electron microscopy

Cells were processed as described earlier [15]. Briefly, cells were prefixed at 22 °C by adding an equal volume of 2% glutaraldehyde in PBS buffer pH 7.2 (grade I; Sigma-Aldrich) to the culture medium. After 20 min the medium was replaced by 1% glutaraldehyde in PBS for 1 h at 4 °C. Cells washed in PBS, concentrated in 2% agarose (LMP Agarose, Sigma A9414), washed again in PBS and postfixed in 1% Osmium Tetroxide (EMS 19150) for 1–2 h at 4 °C. Samples washed again in distilled water and treated with 1% uranyl acetate (EMS 22400) for 1 h at 4 °C in the dark. Samples washed again with distilled water, dehydrated in a graded series of acetone and embedded in Epon (Polysciences 8792). Ultrathin sections (60–90 nm) were collected, stained with uranyl acetate and lead citrate and examined using a Zeiss EM 912 electron microscope.

2.3. Morphometric analysis of mitochondrial alterations

Electron micrographs of randomly selected cells of each stage (with or without DIF-1) were collected and the statistical analysis was done using ImageJ (http://rsb.info.nih.gov/ij/). Surface area is in μm^2. The number of cells and the total number of mitochondria analyzed were, for starved cells without DIF-1 26 and 428 respectively, and for starved cells with DIF-1 19 and 318 respectively.

2.4. Electron tomography

Before recording electron microscopy projections of semi-thin sections (250–500 nm), 10 nm colloidal gold particles were applied on both surfaces of the sections to function as fiducial markers for subsequent image alignment. The specimens were placed in a high-tilt specimen holder (Fischione type 2020; Fischione Instruments, Pittsburgh, PA, U.S.A.) and datasets were recorded at 200 kV (Tecnai 20 LaB6; FEI Company, Eindhoven, The Netherlands). Angular tilt range was from −65° to +65° with an increment of 1°. Images (1024×1024 square pixels) were recorded using a CCD (charge-coupled-device) camera (Temcam F214; TVIPS GmbH, Germany). The sections were pre-irradiated to avoid shrinking effects during recording [16]. Automated data acquisition of the tilt series was carried out using Xplore 3D (FEI Company, Eindhoven, The Netherlands). For dual axis tomography [17], the grids were manually rotated over 90°, and a second tilt series was acquired over the same tilting range. For image alignment, the colloidal gold particles were used as fiducial markers. Tomograms were computed for each tilt axis using the R-weighted back-projection algorithm and combined into one double-tilt tomogram with the IMOD software package [18]. We recorded and reconstructed in total more than 35 single and double-tilt series of mitochondria.

2.5. Modelling and analysis of tomographic data

Double-tilt tomograms were analyzed and modelled using the IMOD software package [18].

3. Results

3.1. Starvation results in abnormal mitochondrial morphology irrespective of addition of DIF-1

We used *Dictyostelium* HMX44A cells, which produce little or no DIF-1 but are sensitive to exogenous DIF-1. The mitochondrial EM lesions we saw upon starvation were similar whether these cells had been further mutated for the atg1 autophagy gene or not. Since HMX44A.atg1-1 cells formed the basis of our necrotic cell death model [9,10], only results obtained with these cells ("cells" throughout this paper) are presented in detail here. Fig. 1 shows a control cell in rich medium with typical vegetative cell morphology, large vacuoles, dense endoplasmic reticulum and a small Golgi system (inset b). The latter indicated that the methodology used allowed good preservation of these organelles. The mitochondria were round, compact with preserved cristae (inset a).

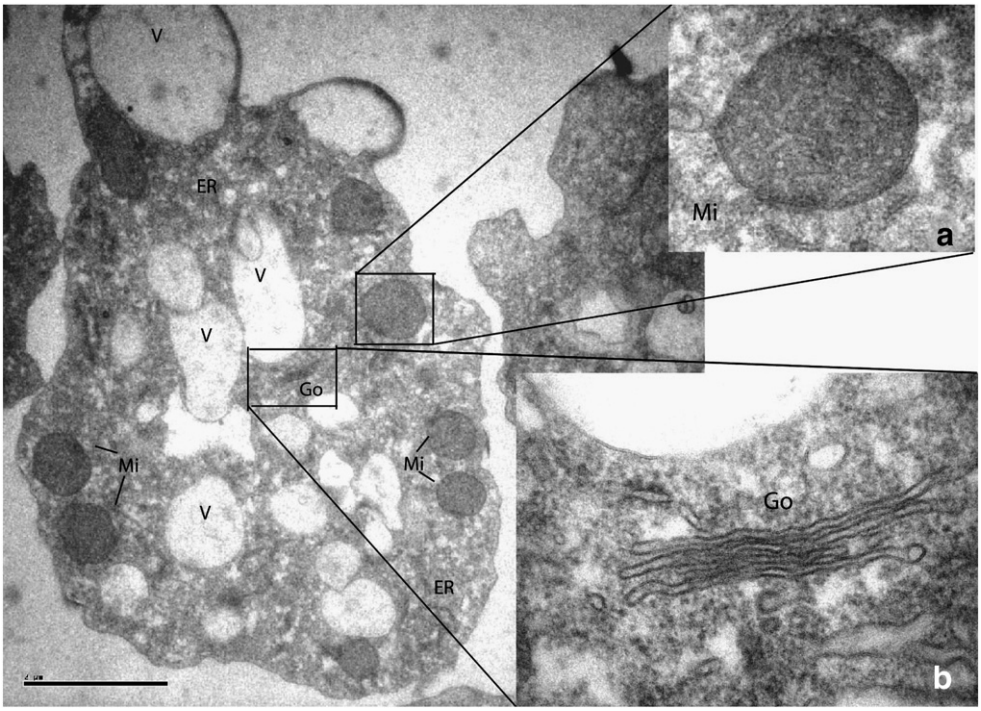

Fig. 1. Ultrastructural morphology of a representative vegetative cell. HMX44A.atg1 cells in rich medium, showing large vacuoles (V), dense endoplasmic reticulum (ER), small Golgi (Go). The mitochondria (Mi) occupy a substantial portion of the cytoplasmic volume and their diameter ranges from 0.5 to 1.0 μm. They are round, with a "compact" morphology and they display a dense matrix, with normal cristae. Bar, 2 μm. (Inset a) Higher magnification of a vegetative mitochondrion showing compact morphology, with no obvious alterations. (Inset b) Higher magnification showing a Golgi apparatus with its usual morphology, indicating good preservation of the cell organelles.