Effect of IGF-1 on the balance between autophagy of dysfunctional mitochondria and apoptosis

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Abstract Mutations in mitochondrial DNA (mtDNA) cause excessive production of mitochondrial reactive oxygen species (ROS) and shorten animal life span. We examined the mechanisms responsible for removal of mitochondria with deleterious mtDNA mutations by autophagy. Incubation of primary cells and cell lines in the absence of serum promotes autophagy of mitochondria with deleterious mtDNA mutations but spares their normal counterparts. The effect of serum withdrawal on the autophagy of dysfunctional mitochondria is prevented by the addition of IGF-1. As a result of the elimination of mitochondria with deleterious mutations, excessive ROS production, characteristic of dysfunctional mitochondria, is greatly reduced. Mitochondrial autophagy shares a common mechanism with mitochondrial-induced cell apoptosis, including mitochondrial transition pore formation and increased ROS production.

Keywords: Mitochondria; Autophagy; Apoptosis; IGF-1

1. Introduction

Mutations in mitochondrial DNA (mtDNA) accelerate aging-related diseases and decrease animal life span [1]. Deleterious mtDNA mutations cause incomplete electron transport chain with increased reactive oxygen species (ROS) production, thus causing a vicious loop to produce further mtDNA mutations [2]. DNA damage as a result of ROS production accelerates cellular aging [3]. MtDNA is more sensitive to DNA damage as a result of oxidative stress than nuclear DNA [4,5].

Elimination of dysfunctional mitochondria will reduce ROS-induced cellular damage and is expected to contribute to increased longevity [2]. The process of mitochondrial turnover is carried out by lysosomal autophagy [6]. We hypothesized that the elimination of dysfunctional mitochondria by autophagy is responsible for the effect of autophagy genes on longevity. Consistent with this hypothesis are recent findings that genes that control lysosomal autophagy are essential for life span extension in *Caenorhabditis elegans* [7].

To study the role of autophagy in the maintenance of mtDNA integrity and reduction of ROS production, we used cell lines and primary cells with deleterious mtDNA mutations and deletions as compared to their WT counterparts. Since mutations in IGF-1 pathway have been shown to affect both animal life span and sensitivity to oxidative stress [8–10], we examined the effect of serum withdrawal and its replacement by IGF-1 on the autophagy of mitochondria with mtDNA mutations or deletions. We found that the autophagy of mitochondria with deleterious mtDNA mutations is activated following serum withdrawal, thus reducing excessive production of ROS by dysfunctional mitochondria. The effect of serum withdrawal on mitochondrial autophagy is prevented by the addition of IGF-1.

2. Materials and methods

2.1. Cell cultures

The human osteocarcinoma cell lines 143B (WT) and its mtDNA-less derivative Rho^0 (ATCC, Manssas, VA) were kept in continuous logarithmic growth in Dulbecco's modified Eagle's medium – high glucose supplemented with 10% FCS, 1 mM sodium pyruvate, and 50 μg/ml uridine – supplemented with sodium pyruvate (20 mM) and 10% FCS by subculturing them twice weekly at a concentration of 2×10^4 cells/cm^2 with standard trypsinization. Primary fibroblast cultures from MELAS patient containing heteroplasmic mitochondrial $tRNA^{Leu(UUR)}$ gene mutation [23] and normal control were cultured in RPMI1640 medium supplemented with 10% FCS. IGF-1 and high affinity phosphorylated IGFBP-1 were purchased from Sigma (Oakville, Ontario, Canada).

2.2. Bcl-2 transfection

The full-length Bcl-2 cDNA was cloned into pcDNA3 with *neo* cassette (Invitrogen, Burlington, ON). Osteocarcinoma cells 143B (WT) and its mtDNA-less derivative Rho^0 were transfected using the cationic lipid LipoAMINE PLUS (Life Technologies, Burlington, ON). After 48 h, the cells were selected in medium containing 1 mg/ml Geneticin (G418; Life Technologies) and stable transfectants were isolated after 14 days under selection. Overexpression of Bcl-2 was comparable in WT and Rho^0 transfectants (<10-fold as revealed by Western blotting). The resistance to apoptosis of Bcl-2 transfectants was confirmed by growth following γ-irradiation.

2.3. Flow cytometry

Following incubation of cultured cells as described in figure legends, cells were stained for 60 min in complete medium at 37 °C with the indicated fluorescent dye: green-fluorescing MitoTracker Green (MTG, 0.5 μM, Molecular Probes, Eugene, OR) for specific staining of mitochondria; LysoTracker Red or Green for staining lysosomes; and

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Abbreviations: mtDNA, mitochondrial DNA; ROS, reactive oxygen species; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes; MPT, controlled mitochondrial permeability transition; IGFBP-1, IGF-1 binding protein; IAPs, inhibitors of apoptosis; CM-H_2-DCFDA, 5,6-carboxymethyl-2′,7′-dichlorofluorescein diacetate; MTG, MitoTracker Green; LTR, LysoTracker Red

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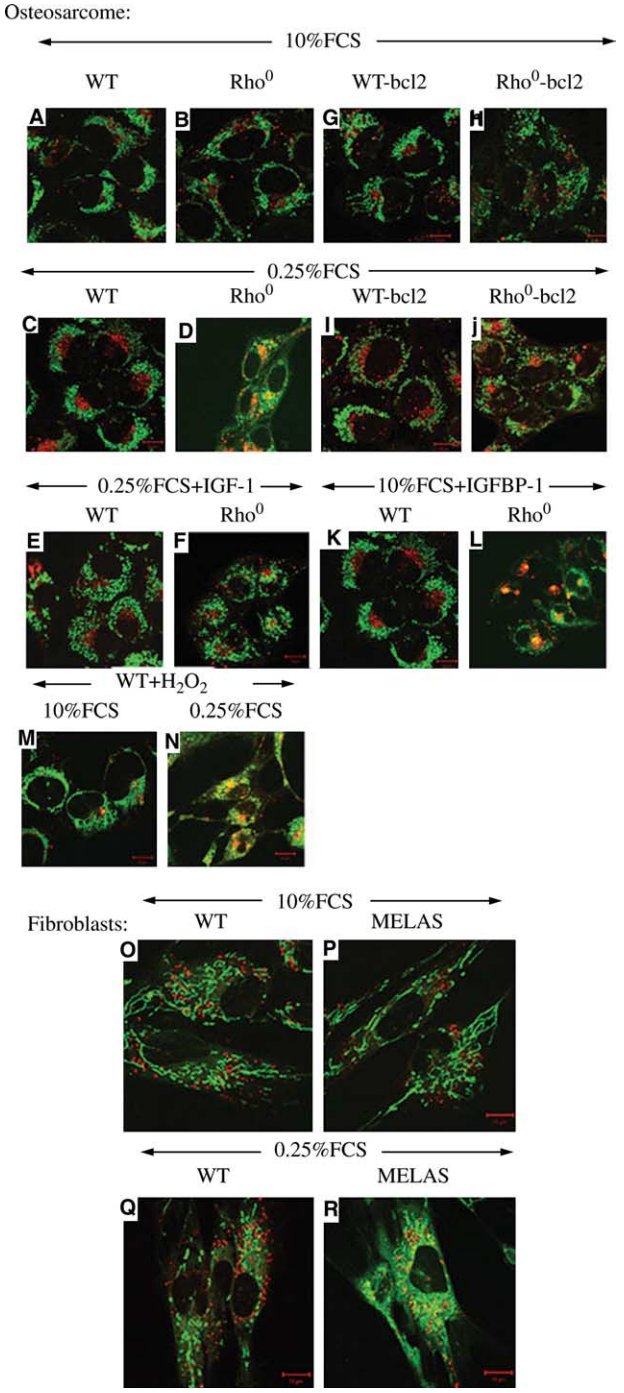
Annexin V FITC (PharMingen, San Diego, CA) and propidium iodide for labeling apoptotic cells. ROS production was assessed by flow cytometer using oxidation-sensitive fluorescent probe 5,6-carboxymethyl-2′,7′-dichlorofluorescein diacetate (10 μM, CM-H_2-DCFDA, Molecular Probes, Eugene, OR) stained for 30 min in serum-free medium. Cell fluorescence was analyzed in a FACScan flow cytometer (Becton Dickenson, Franklin Lakes, NJ).

2.4. Confocal microscopy

Cells were incubated in culture media with various supplements as indicated. After 16 h, cells were loaded with green-fluorescing mitochondrial marker MitoTracker Green (MTG) and after 60 min lysosomes were labeled with LysoTracker Red (LTR) for additional 60 min. After loading of fluorescent dyes, cells were incubated at 37 °C for 1 h in the presence of one-third of the initial concentration to maintain steady state loading. Fluorescence was imaged with laser confocal microscope.



3. Results

To determine the role of serum in the elimination of mitochondria with deleterious mtDNA mutations, we studied the effect of serum withdrawal on lysosomal autophagy of mitochondria. We compared mitochondrial autophagy in cells with normal mtDNA, to osteosarcoma cells with deleted mtDNA (Rho^0), or to fibroblasts from a patient with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) caused by point mutation in the leucine mitochondrial tRNA [11] (Fig. 1). Both WT osteosarcoma cells with normal mtDNA and Rho^0 osteosarcoma cells with deleted mtDNA exhibit minimal mitochondrial autophagy in the presence of 10% FCS (Fig. 1A and B). However, serum withdrawal results in autophagy of numerous mitochondria in Rho^0 cells but not in WT cells. The effect of serum withdrawal is prevented by the addition of IGF-1 (Fig. 1C–F). Likewise, addition of high affinity phosphorylated IGF-1 binding protein (IGFBP-1) to sequester serum IGF-1 [12] causes an increase in mitochondrial autophagy in Rho^0 cells incubated in the presence of 10% FCS but not in WT cells (Fig. 1K and L). Overexpression of Bcl-2 transgene prevents the autophagy of dysfunctional mitochondria in Rho^0Bcl-2 cells incubated in the presence of 10% serum (Fig. 1G–J). Thus, Bcl-2-controlled mitochondrial permeability transition (MPT) formation is a common trigger for both cell apoptosis and mitochondrial autophagy.

ROS production by hydrogen peroxide preferentially causes mtDNA mutations in cultured cells [13]. To further confirm the role of mtDNA mutations induced by ROS on the autophagy of mitochondria, we incubated WT osteosarcoma cells in the presence of hydrogen peroxide for 24 h and monitored mitochondrial autophagy. Mitochondria of WT cells undergo autophagy in the presence of hydrogen peroxide especially at low serum concentration, reflecting increased lysosome activation at low serum (Fig. 1N).

For the process of elimination of dysfunctional mitochondria to be effective, mitochondria with deleterious point mutations must also undergo autophagy. To determine whether this is the case, we used primary fibroblast cultures from a patient with the mitochondrial disease MELAS, caused by point mutation in mitochondrial tRNA. Indeed, mitochondria of fibroblasts from MELAS patient, but not from a normal donor, undergo ex-

Fig. 1. Effect of IGF-1 on autophagy of mitochondria by lysosomes in WT cells with normal mtDNA or in cells with mtDNA mutations or deletions. Cultured human osteosarcoma cells (A–N) or human fibroblasts (O–R), incubated in complete DMEM. Cells were incubated in the presence of either 10% or 0.25% FCS with IGF-1 (50 nM) or IGFBP-1 (100 nM), as indicated. Green dots represent free mitochondria labeled with MTG alone; red dots represent free lysosomes labeled with LTR alone; and yellow dots are sites of overlap of MTG and LTR fluorescent and represent autophagy of mitochondria by lysosomes [24]. Dots were counted in 20 cell scans in four experiments. The average number of mitochondria undergoing autophagy (yellow dots) in Rho^0 cells incubated in the presence of 0.25% FCS (e.g., D) is 18.5(±2.1)/cell ($P < 0.02$ compared to WT cells); in Rho^0 cells incubated in the presence of 10% FCS+IGFBP-1 (e.g., L) is 13.7(±3.6)/cell ($P < 0.05$ compared to WT cells); in WT cells incubated in the presence of 10% FCS+H_2O_2 (e.g., N) is 9.6(±2.9)/cell ($P < 0.05$ compared to WT cells without H_2O_2); in MELAS cells incubated in the presence of 0.25% FCS (e.g., R) is 11.2(±4.0)/cell ($P < 0.05$ compared to WT cells); cells incubated under all other conditions described above (e.g., A, B, C, E, F, G, H, J, I, K, M, O, P and Q) contained an average of <3 yellow dots/cell.

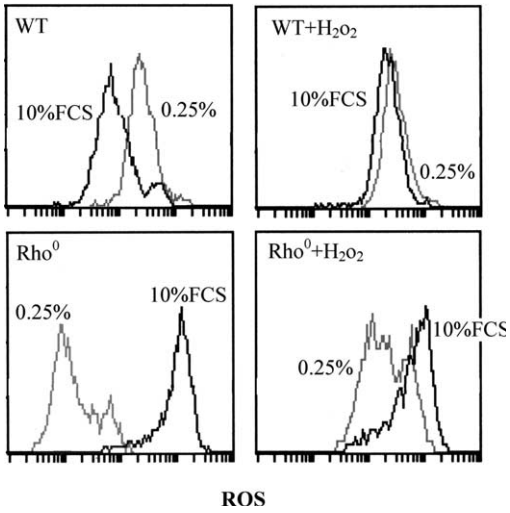


Fig. 2. Effect of serum and hydrogen peroxide on ROS production and lysosome activation in osteosarcoma cells. ROS production was measured following 8 h incubation of osteosarcoma cells in the presence of the indicated serum concentrations and/or 100 μM hydrogen peroxide (C and D). Cells were incubated in complete medium in the presence of 0.25% or 10% FCS as indicated. After 8 h, cells were labeled with the oxidation-sensitive fluorescent probe (10 μM, CM-H_2-DCFDA, Molecular Probes, Eugene, OR) stained for 30 min in serum-free medium and analyzed by flow cytometry. WT cells (A and B) and Rho^0 (C and D) were incubated in the presence (B) or absence (A and C) of hydrogen peroxide. Similar results were obtained in four individual experiments.

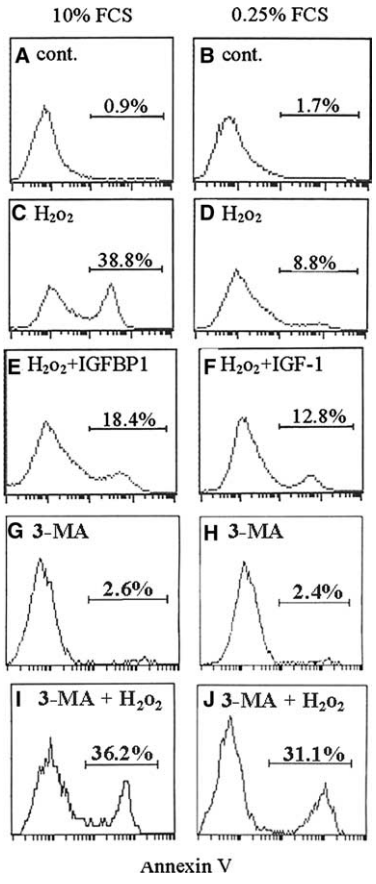


Fig. 3. Roles of IGF-1 and mitochondrial autophagy on the sensitivity of fibroblasts to oxidative stress-induced apoptosis. Fibroblasts were incubated in complete medium in the presence of 10% or 0.25% FCS with or without IGF-1 (50 nM), IGFBP-1 (100 nM) or the inhibitor of autophagy 3-methyladenine (5 mM) as indicated for 24 h. Apoptotic cells were monitored by Flow cytometry following annexin V staining. Bars represent apoptotic cells with their percentage indicated. In four individual experiments, average percentage of apoptotic cells incubated at 10% serum in the presence of H_2O_2 (36.0 ± 2.6, panel C) is significantly different ($P < 0.01$) from both cells incubated at 10% serum and H_2O_2 (9.6 ± 1.3, panel D) or at 10% serum in the presence of IGFBP-1and H_2O_2 (16.2 ± 2.8, panel E). Likewise, average ROS production by cells incubated at 0.25% serum in the presence of H_2O_2 (panel D) is significantly different ($P < 0.05$) from cells incubated at 0.25% serum in the presence of IGF-1and H_2O_2 (13.1 ± 1.3, panel F) or from cells incubated at 0.25% serum in the presence of H_2O_2 and 3-MA (32.2 ± 4.1, panel J). Testing for significance of differences was assessed by Student's t test (numbers represent means ± S.D. of four experiments, con = control).

tensive autophagy in response to serum withdrawal (Fig. 1O–R). Similar to osteosarcoma cells, addition of IGFBP-1 to MELAS cells incubated in the presence of 10% serum promoted an increase in autophagy (data not shown).

To determine the effect of autophagy of dysfunctional mitochondria on ROS production, we monitored ROS production in cultured osteosarcoma cells by flow cytometry (Fig. 2). Rho^0 cells with incomplete electron transport chain produce significantly more ROS than WT cells at 10% serum concentration (Fig. 2C). Incubation of Rho^0 cells at low serum concentration resulted in marked reduction of ROS production (Fig. 2A) consistent with the extensive elimination of ROS-producing dysfunctional mitochondria by autophagy observed at low serum (as illustrated in Fig. 1). In contrast, the absence of reduction in ROS production by cells with normal mtDNA, at low serum concentration, is evidence for the selectivity of lysosomal autophagy towards dysfunctional mitochondria. Incubation of WT cells in the presence of hydrogen peroxide causes smaller increase in ROS production at low serum concentration as compared to that of cells incubated in the presence of high serum concentration (Fig. 2B and D), consistent with increased autophagy of mitochondria with hydrogen peroxide-induced mtDNA mutations [13].

There is no significant cellular apoptosis of either WT, MELAS fibroblasts or of WT and Rho^0 osteosarcoma cells in the absence of serum despite the increase in mitochondrial autophagy under these conditions (data not shown). To explain the absence of cell apoptosis despite significant increase in ROS production, we determined the effect of serum withdrawal on the sensitivity of fibroblast cultures to oxidative stress-induced apoptosis by H_2O_2 (Fig. 3). Fibroblasts are more resistant to cell apoptosis induced by oxidative stress at low serum concentration than at 10% FCS. Addition of IGF-1 to fibroblasts cultured in low serum restores the sensitivity of fibroblasts to oxidative stress. Conversely, sequestration of IGF-1 by the specific IGFBP-1 resulted in increased resistance to oxidative stress-induced apoptosis at 10% FCS (the effect of IGFBP-1 is reversed by excess IGF-1). Inhibition of autophagy by 3-methyladenine results in an increase in H_2O_2-induced apoptosis in cells incubated at low serum concentration to levels comparable to that of H_2O_2-induced apoptosis in the presence of 10% serum (Fig. 3C and J). These results are consistent with the elimination of ROS-producing dysfunctional mitochondria of cells treated with hydrogen peroxide at low serum concentration (Fig. 1M and N). These data are in agreement with a recent report of increased resistance to

oxidative stress in mice with heterozygous inactivation of the IGF-1 receptor gene [10].

4. Discussion

We hypothesize that mitochondrial turnover by autophagy (half life time of 14 days [14]) maintains the integrity of mtDNA and thus links mitochondrial function to its survival. For such a mechanism to operate, it should be able to effectively detect and eliminate non-functional mitochondria with deleterious mutations or deletions in mtDNA. Deleterious mutations in mtDNA genes result in incomplete electron transport chain and uncoupling of oxidative phosphorylation and result in increased ROS production and low ATP production [15]. Excessive ROS production combined with low ATP levels of dysfunctional mitochondria trigger MTP formation [16] and promote their autophagy. Mitochondria with deleterious mtDNA mutations have much higher basal ROS production and thus are more susceptible to lysosomal autophagy. The elimination of dysfunctional mitochondria by autophagy at low IGF-1 levels results in significant reduction in ROS production (Fig. 2). The observations made using this cell culture model outline a trade-off between mitochondrial autophagy that maintains the integrity of mtDNA and cell apoptosis controlled by IGF-1 pathway.

There is abundant evidence that the genes coding for individual steps of this signaling pathway control life span in various animal species [8–10,25]. Based on the observations in this manuscript, we propose that this pathway provides a mechanism of life span extension by caloric restriction. According to this hypothesis, during caloric restriction increased efficiency of energy utilization is achieved by increasing aerobic respiration by the elimination of inefficient dysfunctional mitochondria. In this situation, low IGF-1 levels result in lysosomal autophagy of dysfunctional mitochondria by MPT-dependent mechanism sparing functional mitochondria and thus result in reduced ROS production that contributes to increased life-span [17]. However, since MPT formation may also initiate cell apoptosis, a necessary trade-off is the inhibition of ROS-mediated apoptosis. Indeed, withdrawal of IGF-1 induces cell resistance to apoptosis by oxidative stress (Fig. 3) consistent with observation made in IGF-1 receptor deficient mice [10].

Mitochondrial transition pore (MTP) plays a critical role in the initiation of apoptosis [18]. The observation that mitochondrial autophagy is sensitive to inhibition by Bcl-2 suggests a role for MTP in the initiation of autophagy of dysfunctional mitochondria. Since eukaryotic cells contain hundreds of mitochondria with a high turnover rate, in most cases opening of the mitochondrial MTP in a single mitochondrion may result in the autophagy of dysfunctional mitochondria without cell apoptosis. Thus, in cases of autophagy of only a few dysfunctional mitochondria, cell apoptosis is avoided since the limited cytochrome *c* released is neutralized by the cytoplasmic inhibitors of apoptosis (IAPs) [19]. In cases where many dysfunctional mitochondria accumulate, significant release of cytochrome *c* overcomes the threshold for apoptosis inhibition by IAPs.

Comparative sequence analysis suggests that the metazoan mtDNA originated from parasitic eubacteria about 850 million years ago [20]. During a long co-evolutionary process the promitochondrion lost over 90% of its original genes, the majority of which now reside in the metazoan cell nucleus [21]. Functionally similar sets of mtDNA genes were retained independently in both Fungi and Holozoa concomitant with the emergence of a multicellular body plan [22]. The observation that a similar selection of genes was retained by the mtDNA over several hundred million years of evolution suggests a stable evolutionary solution for successful endosymbiosis of mitochondrial and nuclear genomes. Since mtDNA replicates independently of nuclear DNA, for endosymbiosis to succeed, a mechanism must exist to link the replication of an individual mtDNA molecule to its oxidative phosphorylation function. We propose that autophagy of mitochondria with deleterious mtDNA mutations provides an evolutionary conserved mechanism that links respiratory function of individual mitochondria to their survival during cellular life span.

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