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Robust autophagy/mitophagy persists during mitosis

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Abstract

From microscopic observations of autophagosome content it has been argued that autophagy is shut down during mitosis to protect the relative short-lived organelles spindle and chromosomes from the process while they are contiguous with cytosol. However, without autophagy, buildup of dysfunctional mitochondria arising from the intense energy demands of mitosis potentially poses a hazard to accurate partition of chromosomes. Here we show using biochemical markers of autophagosomes and mitophagosomes and a blockade at the lysosomal clearance step that autophagy/mitophagy persists during mitosis at robust levels equal to interphase. This suggests a mechanism that insulates normal spindle and chromosomes from autophagy and potentially recognition of defects in spindle and chromosomes by the autophagic process.

Keywords

aneuploidy; cell synchronization; citrate synthase; LC3; lysosomal inhibitor; mitochondrial mass; mitophagosome; mitotic cell death; mitotic spindle; nocodazole

Introduction

Eukaryotic cells utilize the ubiquitin-proteasome pathway to degrade short-lived protein substrates conjugated with a polyubiquitin chain and the autophagy-lysosome pathway to isolate larger subcellular organelles and macromolecular particles with a membrane that fuses with lysosomes.[1] The ubiquitin-proteasome pathway has been clearly implicated in regulation of genomic activity in the interphase nucleus,[2] and in mitosis including mitotic spindle checkpoint mechanisms that insure accurate partition of chromosomes.[3] The autophagy-lysosome pathway is thought to be largely cytosolic based on distribution of autophagosomes in interphase cells.[1] The status of the autophagy-lysosome pathway during mitosis is unclear.

After nuclear membrane breakdown, the mitotic spindle and condensed chromosomes constitute short-lived organelles continuous with the cytosol that are potentially subject to autophagy in addition to normal cytosolic organelles as mitochondria. Based on electron microscopic observations, it has been argued that autophagy is cell cycle regulated and shut down during mitosis to protect spindle and chromosomes until nuclear membrane closure subsequent to cytokinesis.[4] A similar trend of autophagic inhibition in G_2/M phase was also reported.[5] Mitochondria, one of the largest and most abundant types of cytosolic organelles, are contiguous with the mitotic machinery and particularly active during the energy intensive processes driving spindle formation and chromosomal separation. Mitochondrial

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morphology is determined by a balance of fission and fusion events. Fusion is a brief event of about 100 seconds in mammalian cells followed by fission and the average frequency of fusion events in mammalian cells is about once every 5–20 minute per mitochondrion. Mitochondria fission produces fragmented daughter units with unevenly membrane potentials.[6] The depolarized mitochondria are sequestered by isolation membranes and targeted to lysosomes and eliminated in as short as 20 minutes.[7] The autophagy-lysosomal pathway is the primary pathway for maintenance of mitochondrial homeostasis and removal of potentially lethal dysfunctional mitochondria by a specialized autophagic sub-pathway called mitophagy.[8] The reported high percentage of fragmented mitochondria in mitotic cells[9,10] likely reflects the high energy demands of mitosis and consequently the need for sufficient rates of mitophagy to maintain homerostasis and prevent mitochondria-associated damage. Thus it could equally be argued that the shutdown of autophagy and more specifically mitophagy would potentially subject the cell to buildup of worn out defective mitochondria and the accompanying destructive forces including cell death associated with mitochondria at this particularly labile period in the cell cycle.

Based on this reasoning, we examined autophagy and more specifically mitophagy in mitotic cells using GFP-LC3 as marker and lysosomal inhibitors to block clearance of autophagosomes by lysosomes.

Results and Discussion

Mitotic cells cultured under normal conditions accumulated few autophagosomes as indicated by a reduction of GFP-LC3 punctate foci and LC3-II levels

To examine autophagic activity during mitosis, we generated a stable Hela cell line expressing the autophagosome marker GFP-LC3 and examined its distribution in interphase cells and cells at different substages of mitosis (Fig. 1A, control). LC3 was distributed uniformly across the cytosol with less than 10% of the interphase cells carrying several punctate foci indicative of autophagosomes (Fig. 1B). Consistent with the previous report that a paucity of autophagosomes may indicate a deficiency in the autophagy-lysosomal pathway in mitotic cells,[4] the GFP-LC3 signal was distributed across the cell (except the area of condensed chromosomes) at all mitotic substages. No punctate foci of GFP-LC3 indicative of autophagosomes were apparent in majority of mitotic cells (Fig. 1A and B, control). The conversion of LC3-I to LC3-II is generally considered to be coincident with initial formation of autophagic isolation membranes.[11] Therefore, its appearance reports activity of the autophagic pathway prior to fusion of autophagosomes with lysosomes. To assess isoforms of LC3, mitotic cells were enriched by synchronization by a thymidine blockade and capture of loosely attached cells in mitosis by shakeoff,[12] LC3 isoforms in the resultant fractions was analyzed by immunoblot. DNA staining (not shown) indicated that more than 95% of the cells in the shakeoff fraction were in mitosis. Intensity of the mitotic marker phosphorylated histone H3 (p-H3) confirmed that the shakeoff fraction was enriched in mitotic cells relative to the mixture of interphase and mitotic cells left attached to culture dishes (Fig. 1C, lane 1 vs. 2). Again the direct immunoblot analysis, particularly the reduced levels of LC3-II in untransfected cells (Fig. 1C, lane 2 vs. 1), was consistent with the reported reduction in autophagosomes during mitosis.[4]

Both interphase and mitotic cells dramatically accumulated autophagosomes when lysosomal activity was inhibited

To test whether the reduction in autophagosomes in mitotic cells was due to inhibition of the autophagy-lysosomal pathway or rapid clearance of autophagosomes, we treated cells with NH_4Cl or Bafilomycin A1 to block lysosome acidification,[13,14] thus preventing the clearance of autophagosomes. A large number of punctate foci of GFP-LC3 were observed

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in majority of mitotic cells at all substages (Fig. 1A and B, NH_4Cl and Bafilomycin A1 panels) equal to that observed in cells in interphase. A dramatic increase of GFP-LC3-II intensity was observed in the total lysate of treated cells (Fig. 1D). Endogenous LC3-II levels were dramatically increased in mitotic cells captured by shakeoff equal to the mixture of attached cells in interphase and mitosis when wild-type Hela cells were treated with NH_4Cl (Fig. 1C). Therefore, the majority of punctate foci that appeared in mitotic cells were LC3-II-associated autophagosomes rather than LC3-I aggregates. Autophagosomes were constantly formed and degraded during interphase or mitosis, and the total number of autophagosomes actually formed drastically exceeded the number of autophagosomes we can observe when cells were cultured in media without lysosomal inhibitor.

The accumulated autophagosomes were newly formed during mitosis

To distinguish the possibility that the accumulated autophagosomes were formed in mitosis but not inherited from interphase, mitotic cells were collected from thymidine-nocodazole double synchronized cells and treated with NH_4Cl for different lengths of times. LC3-II levels were increased immediately after treatments (Fig. 1E). The results suggested that high levels of LC3-II in mitotic cells was obviously accumulated after lysosomal blockade and unlikely caused by upregulation of LC3 expression.

The accumulated autophagosomes in the presence of lysosomal inhibitors mainly contained mitochondria

The colocalization of punctate foci of GFP-LC3 and mitochondria labeled with MitoTracker Red CMXRos dye in the presence of NH_4Cl indicated the significant presence of mitophagosomes (Fig. 2A). The mitochondria within mitophagosomes were mainly round and small and accumulated among the other mitochondrial populations in the presence of either NH_4Cl or Bafilomycin A1 (Fig. 2B). Citrate synthase activity was considered as an exclusive biomarker of mitochondria mass.[15] Treatment with cycloheximide blocked protein synthesis and total proteins in cells are expected to decrease because of other protease activities. A long-term blockade of autophagy with NH_4Cl in mitotic cells that were maintained in nocodazole-containing medium resulted in an elevation of citrate synthase activity relative to total proteins (Fig. 2C). These results indicated that the formation of autophagosomes particularly mitophagosomes and clearance through the autophagy-lysosomal pathway is robust during mitosis.

Materials and Methods

Expression of GFP-LC3 by stable transfection

Hela cells were transfected with a plasmid carrying the EGFP-LC3 fusion[16] and subjected to 200 μg/mL of G418 selection for two weeks. A stable cell line that exhibited the same response profile to untransfected Hela cells to starvation in Hanks' medium was selected for study.

Immunofluorescence analysis

Spread monolayered interphase cells and round mitotic cells were visualized with a Zeiss LSM510 laser confocal system. GFP-LC3 was used for labeling of autophagosomes and MitoTracker Red CMXRos (MitoTracker) for mitochondria.[16,17] Stages of the cell cycle were identified by DNA stain TO-PRO-3 iodide or 4′,6-diamidino-2-phenylindole (DAPI). Interphase cells were about 1–2 μm thick while spherical mitotic cells were about 10 μm diameter. The images were scanned through a plane with reference at the middle part of a sphere. The acquired images were exported to Adobe Photoshop, processed and then

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imported into ImageJ for RGB split and colocalization analysis with a ColocalizeRGB Plugin.

Cell cycle fractionations and immunoblot analysis

Mitotic cells were enriched by synchronization with 2 mM thymidine for 20 hr followed by release from the thymidine blockade for 20 hr in the absence or presence of 10 μM nocodazole as described.[12] Loosely attached mitotic cells (Shakeoff) were harvested by vigorous shake off from the remaining mixture (Attached) of interphase cells and mitotic cells attached to the dish. The stages of cell cycle were confirmed by DAPI staining as described above and majority of shakeoff cells were in mitosis. Cell lysates were prepared in lysis buffer and protein concentrations were determined as described.[17] The same amount of total protein for each fraction was loaded on SDS-PAGE with three replicas. The intensity of LC3-I and LC-II were detected by an anti-LC3 polyclonal antibody from Nuvus Biological. The mitotic status of each fraction was monitored with an antibody against phosphorylated histone H3 (p-H3) from Santa Cruz Biotechnology, Inc. Loading was further controlled by β-actin.

Citrate synthase assay in mitotic cells

Mitotic cells harvested from thymidine and nocodazole double blocked Hela cells by shake off as described above were transferred to the same culture medium containing nocodazole and untreated or treated with NH_4Cl and/or cycloheximide for different times. The collected cells were frozen for citrate synthase assay using kit purchased from Sigma (CS0720). Cell lysates were prepared by CelLytic MT Cell Lysis Reagent and citrate synthase activity was measured according to the procedure in the technical bulletin supplied by the manufacture. Total protein content in the lysates was assayed by the BCA method. Briefly, samples were mixed with assay buffer (100 mM Tris, pH 8.1) containing 300 μM acetyl CoA, 100 μM 5,5-dithio-bis-(2-nitrobenzoic) acid (DTNB). Reaction was initiated by adding 0.5 mM oxaloacetic acid and the enzyme kinetic was recorded spectrophotometerically at 412 nm at 30°C. Citrate synthase activity was expressed as nmol/min per mg of protein. The accumulation of citrate synthase activity was calculated based on the variation of the activity between different time points with the activity at time zero as the basis.

Concluding Remarks

Our results using a blockade of autophagosome clearance via lysosomal activity indicate that the basal levels of autophagy in both interphase and mitotic cells is much higher than anticipated by observance of steady-state levels of autophagosomes. A similar situation was observed in neuronal cells where autophagy was also initially believed to be relatively inactive because of the small number of autophagosomes accumulated in neurons of healthy brains. Suppression of autophagy-regulatory genes Atg5, Atg7 or inhibition of lysosomal proteases in primary neurons resulted in accumulation of ubiquitylated proteins and autophagosomes suggesting basal levels of autophagy in neurons was quite active. It was concluded that autophagic intermediates are normally scarce because of exceptionally rapid clearance in healthy neurons.[18] The apparent reduction in steady-state levels of autophagosomes in mitosis relative to interphase may indicate a more efficient formation of autophagosomes in interphase cells or clearance in mitotic cells.

In summary, our results suggest that the autophagy-lysosomal pathway in mitotic cells is sufficiently active to protect mitotic cells from the destructive effects of a buildup of defective mitochondria and other normally cytosolic organelles during mitosis. Chromosomal material has been observed in autophagosomes under oxidative stress.[19] How the normal mitotic spindle and chromosomes that are transiently foreign organelles in the

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cytosol relative to interphase, are protected from the persistently robust autophagy during mitosis is an intriguing question. Such mechanisms may be important in protection of accurate chromosomal partitioning, but also in recognition of spindle and chromosome defects.

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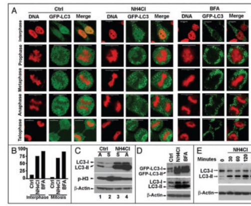

Figure 1.

The autophagic pathway occurs in mitotic cells similar to interphase cells. (A) Punctate foci of GFP-LC3-containing autophagosomes at all mitotic substages revealed by overnight blockade of autophagosome clearance with lysosomal inhibitor NH_4Cl (20 mM) or Bafilomycin A1 (BFA, 10 nM). The cell cycle stages for Hela cells stably expressing GFP-LC3 were identified by staining with DNA dye TO-PRO-3 iodide (1 μM) after cells were fixed and permealized as described.[20] Total GFP-LC3 signal thus appears more intense in rounded mitotic cells. Scale bar, 10 μm in all panels. (B) Percentages of interphase or mitotic cells exhibiting GFP-LC3 punctate foci after treatment overnight with lysosomal inhibitors NH_4Cl or Bafilomycin A1 (BFA). (C) Autophagosome accumulation revealed by LC3-II accumulation in mitotic cells. Wild type mitotic cells were enriched by synchronization with 2 mM thymidine for 20 hr followed by release from the thymidine blockade for 20 hr as described previously.[12] NH_4Cl was added to medium 12 hrs before cells were harvested in order to collect sufficient mitotic cells for analysis. Loosely attached mitotic cells (Shakeoff) were harvested by vigorous shake off from the remaining mixture (Attached) of interphase cells and mitotic cells attached to the dish. Equal amounts of protein from each sample were analyzed by immunoblot with the indicated antiserum. p-H3, phosphorylated histone H3 indicative of mitotic cells. (D) Specific accumulation of GFP-LC3-II and LC3-II in Hela cells stably expressing GFP-LC3 in response to NH_4Cl or Bafilomycin A1 as in (A). Cell lysates were collected from treated cells without synchronization and fractionation. (E) Rapid accumulation of LC3-II in mitotic cells treated with NH_4Cl. Mitotic cells were collected by shakeoff from wild type Hela cells that were released from thymidine blockade but further blocked in mitosis with 5 μM nocodazole for 20 hr as described previously[12] and maintained in fresh medium containing the same concentration of nocodazole. The cells were treated with 20 mM of NH_4Cl for the indicated times and used to prepared lysates for immunoblot. The intensity of β-actin served as the loading control. Ctrl, control.

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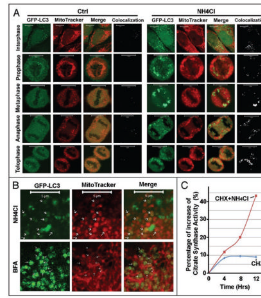

Figure 2.

Accumulation of mitophagosomes in mitotic cells in the presence of NH_4Cl. (A) The colocalization of GFP-LC3 punctate foci (green) with mitochondria labeled with MitoTracker Red CMXRos dye (red) as previously described.[17] Mitotic stages were identified with DAPI staining. The punctate foci of bright green GFP-LC3 in stably transfected Hela cells overlapping with weak red punctate foci of dysfunctional mitochondria was indicative of mitophagosomes. Colocalized punctate foci in white were revealed by analysis with an ImageJ ColocalizeRGB Plugin. Scale bar, 10 μm in all panels. (B) The colocalization of GFP-LC3 punctate foci with fragmented mitochondria in interphase cells stably expressing GFP-LC3 treated with lysosomal inhibitor NH_4Cl and Bafilomycin A1. A small portion of a single interphase cell was enlarged and shown. White arrows point to individual mitochondria contained in autophagosomes (mitophagosomes). Scale bar: 5 μm. (C) Mitochondria accumulation as indicated by the citrate synthase activity. Mitotic cells collected and maintained as described in Figure 1E were treated with protein translation inhibitor cycloheximide (CHX, 1 μg/ml) and NH_4Cl individually or in combination but for extended periods of time. The percentages of increase of citrate synthase activities at different times from the initial activity at time zero were plotted.