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Co-clustering of Golgi complex and other cytoplasmic organelles to crescentic region of half-moon nuclei during apoptosis

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Abstract

Early apoptosis is defined by stereotypic morphological changes, especially evident in the nucleus, where chromatin condenses and compacts, and assumes a globular, half-moon or crescent-shaped morphology. Accumulating evidence suggests that cytoplasmic organelles such as mitochondria and the Golgi complex are major sites of integration of pro-apoptotic signaling. In this study, cytoplasmic organelles including Golgi complex, mitochondria, endosomes, lysosomes, and peroxisomes were shown to condense at the same unique region adjacent to the crescentic nucleus during a relatively early stage of apoptosis induced by staurosporine or other agents. The co-clustering phenomenon may be caused by shrinkage of cytoplasm during apoptosis although cytoskeletal markers actin and tubulin were not condensed and appeared excluded. These data suggest the co-clustering of cytoplasmic organelles plays an interesting role during the progression of the apoptotic process. It is possible that modification of pro-apoptotic proteins may arise as a result of the interplay of these cytoplasmic organelles.

Keywords

Apoptosis; Golgi complex; Staurosporine

1. Introduction

Apoptosis is a form of highly regulated cell death that results in the orderly removal of cells that are senescent, unneeded, defective and destined to die. Apoptosis is defined by stereotypic morphological changes, especially evident in the nucleus, where the chromatin condenses and compacts, and assumes a crescent or half-moon shape prior to the typical fragmented morphology represented as nuclear bodies (Leist and Jaattela, 2001). Albeit the precise role of nuclear apoptotic changes has been questioned, there is no doubt that apoptosis is also

characterized by a variety of biochemical changes, which occur not only in the nucleus but are also associated with several cytoplasmic organelles. Established by both biochemical and genetic approaches, caspases are among the best characterized enzymes that have essential roles at various stages of the apoptotic process (Grutter, 2000). In addition, other proteases including granzyme B, cathepsins, and histone-associated proteases have been implicated in eliciting characteristic nuclear changes that occur during apoptosis (Robertson *et al.*, 2000). However, many of the biochemical events and molecular mechanisms underlying apoptotic morphological changes have been difficult to define, mostly because apoptosis often occurs in an asynchronous manner (Martelli *et al.*, 2001). It has become increasingly evident that the initiation and execution of apoptosis is a multistage process dependent upon a tightly regulated and well-defined program. Molecular participants in this program are present in different subcellular compartments including the plasma membrane, Golgi complex, mitochondria, and nucleus. The interplay among these compartments and the exchange of specific signaling molecules are critical for the systematic progression of apoptosis (Robertson *et al.*, 2000).

The Golgi complex is a cytoplasmic organelle has a central function in the secretory pathway required for the processing of complex sugar structures on many proteins and lipids, and for the sorting of these modified proteins and lipids to their correct subcellular destinations (Farquhar and Palade, 1998). Since 2002, we and others have reported fragmentation of the Golgi complex during apoptosis (Nozawa *et al.*, 2002; Mukherjee *et al.*, 2007; Walker *et al.*, 2004; Chiu *et al.*, 2002). We also reported that preceding fragmentation there was clustering of the Golgi complex in early apoptosis (Nozawa *et al.*, 2002). It has been reported that the Golgi complex may also play an important role in apoptosis (Bennett *et al.*, 1998; Jones *et al.*, 1999; Mancini *et al.*, 2000; Chiu *et al.*, 2002). Furthermore, accumulating evidence suggests that other organelles, including the endoplasmic reticulum (ER), lysosomes, and mitochondria, are also major points of integration of pro-apoptotic signaling or damage (Ferri and Kroemer, 2001). These observations prompted us to further investigate the fate of the Golgi complex as well as other cytoplasmic organelles during apoptosis using well established cell culture models.

2. Materials and methods

2.1. Antibodies

Rabbit antibodies to golgins (giantin, golgin-97, GM130/golgin-95) were produced in New Zealand White rabbits by immunization with the respective recombinant proteins as published previously (Fritzler *et al.*, 1993; Fritzler *et al.*, 1995; Griffith *et al.*, 1997; Nozawa *et al.*, 2002). Mouse anti-actin and anti- α -tubulin antibodies were obtained from Sigma (St Louis, MO). Rabbit anti-early endosome protein EEA-1 (Selak *et al.*, 1999) and rabbit anti-peroxisome marker PMP70 were obtained from Invitrogen (Carlsbad, CA). Mouse anti-lysosome protein LAMP1 was kindly provided by Drs. August and Hildreth (Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore MD). Human anti-poly-ADP-ribose polymerase (PARP) antibodies and anti-mitochondria E2 component of the pyruvate dehydrogenase complex used in this study was obtained from our serum banks as described (Nozawa *et al.*, 2002).

2.2. Induction of cell death

HEp-2, HeLa, R9ab, and NRK cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (Life Technologies, Rockville, MD), containing 10% fetal calf serum under standard conditions (Nozawa *et al.*, 2002). Induction of apoptosis was typically achieved by exposure of the cells to 2 μ M staurosporine (STS, Alexis, San Diego, CA) for 4 h or 100 μ M etoposide (Alexis) for 30 h (Nozawa *et al.*, 2002). The treated cells were used for indirect immunofluorescence (IIF) and

electron microscopy (EM). For immunoblotting analysis, HEp-2 cells were also exposed to 2 μ M STS up to 28 h, or etoposide ranging from 10 μ M to 100 μ M up to 30 h. Spontaneous cell death prior to the experiments was minimized by maintaining cells in exponential growth. Cell viability was quantified by trypan blue exclusion at the beginning of every experiment to ensure that cell cultures used in the experiments maintained >95% cell viability.

2.3. Indirect immunofluorescence

HEp-2 cells were grown on 8 chambered tissue culture slides (Becton Dickinson, Franklin Lakes, NJ) and apoptosis was induced by one of the agents described above. After the treatment, cells were fixed and permeabilized in a mixture of 1:3 methanol:acetone (v/v) at -20°C for 2 minutes, or fixed with 4% paraformaldehyde in PBS and then permeabilized with 0.5% Triton X in PBS. IIF was performed as reported previously (Nozawa *et al.*, 2002). Primary antibodies were diluted in PBS (anti-giantin, 1/400; anti-golgin-97, 1/200; anti-LAMP1, 1/100; anti-EEA1, 1/100; anti-PMP70, 1/100; anti-actin, 1/100; anti- α -tubulin, 1/100; anti-mitochondria E2 component of the pyruvate dehydrogenase complex, 1/1600). The bound antibodies were labeled with a combination of corresponding secondary antibodies including Alexa 488 conjugated goat anti-rabbit IgG antibody, Alexa 488 conjugated goat anti-mouse IgG antibody (Invitrogen), rhodamine conjugated goat anti-mouse IgG antibody (Caltag, Burlingame, CA), Cy3 conjugated goat anti-rabbit IgG antibody (Caltag), and Alexa 568 conjugated goat anti-human IgG antibody (Invitrogen). Cells were counterstained with the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). Fluorescent images were captured with a Zeiss Axiovert 200M microscope fitted with a Zeiss AxioCam MRm camera using x10 (0.75NA), x20 (0.75NA), x40 (0.75 NA) or x63 (1.4 NA) objectives. All the exposure times and gain settings within one set of experiments were equivalent. Color images were processed using Adobe Photoshop version 7. The estimation of the percentage of cells at each morphological stage described below for Golgi staining in apoptotic cells was obtained by scoring three to five hundred cells in each experiment. For specific mitochondrial staining, medium containing 50 nM of mitochondrion-selective probe, Mitotracker Red 580 (Invitrogen), was added to HEp-2 cells grown in the tissue culture slide and incubated at 37°C for 30–45 min. After incubation, the cells were washed and fixed with paraformaldehyde and permeabilized with 0.5% Triton X as described above.

2.4. Immunoblotting analysis of cell lysates

HEp-2 cells were grown in 35mm tissue culture dishes (7×10^6 /plate). After incubation in the presence of apoptosis inducing reagents, the cells were washed in PBS and collected into lysis buffer containing 150mM NaCl, 1mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 80mM Tris-HCl, 0.1% NP-40 and Complete Protease Inhibitor cocktail (Roche, Mannheim, Germany). Lysates were passed several times sequentially through 18–27 gauge needles to shear the DNA, and protein concentrations were determined by Protein DC Assay Kit (Bio-Rad, Hercules, CA) to ensure equal loading of proteins in each SDS-PAGE lane. After determination of protein concentration, lysates were mixed with an equal volume of 2X gel sample buffer containing 6% sodium dodecyl sulfate (SDS), 20% glycerol, 10% β -mercaptoethanol, and 0.02% bromphenol blue and stored at -80°C until used. The equivalent amount of total lysate protein was loaded onto each lane of a 10% gel, separated by electrophoresis, and transferred to nitrocellulose membranes using a Semi-Dry Trans-Blot apparatus (Bio-Rad). Immunoblotting was performed as described previously (Nozawa *et al.*, 2002). Detection of bound antibodies was achieved using horseradish peroxidase (HRP)-conjugated goat anti-human or anti-rabbit IgG antibody (Caltag) at 1/5000 dilution in combination with chemiluminescence (Super Signal; Pierce, Rockford, IL).

2.5. Live cell imaging of GFP-golgin-97 undergoing apoptosis

Full-length golgin-97 cDNA (Griffith *et al.*, 1997) was subcloned into the pHRGFP-N1 vector (Stratagene, La Jolla, CA) and the in-frame fusion of GFP to golgin-97 was verified by direct nucleotide sequencing. The construct was co-transfected with red fluorescent protein encoding plasmid pDsRed2-C1 (Clontech, Palo Alto, CA) to observe the Golgi complex and whole cell morphology using live cell imaging. HeLa cells were grown in eight-chamber coverslip slides (Becton Dickinson, Franklin Lakes, NJ) under semi-confluent condition one day prior to transfection. Transfection was performed according to the manufacturer's protocol for Polyfect Transfection Reagent (Qiagen, Valencia, CA). In brief, cells were incubated with serum-free Opti-MEM (Gibco, Carlsbad, CA) containing an equal amount of the DNA constructs (0.1 µg each) and 2 µl of Polyfect. After 5 h, the medium was replaced with 10% fetal calf serum containing medium and cells were cultured at 37°C for additional 24 h prior to the exposure to 2 µM STS for 4 h to induce apoptosis and analyzed by live cell IIF microscopy.

2.6. Electron Microscopy (EM) and Immuno-EM

HeLa cells were grown at log phase in 35-mm tissue culture dishes, treated with 2 µM STS for 4 h, trypsin treated briefly to release cells which were then collected as a pellet after brief centrifugation. Standard EM was performed using 2.5% glutaraldehyde fixative in 0.1M sodium cacodylate buffer followed by cacodylate buffered 1% osmium tetroxide, dehydration in graded ethanol and embedded in resin. Immuno-EM was performed as described (Yang *et al.*, 2004). In brief, the cell pellet was fixed for 1.5 h in ice-cold 4% paraformaldehyde and 0.025% glutaraldehyde in 0.1M phosphate buffer pH 7.4. Following fixation, free aldehydes were quenched in 50 mM glycine in 0.1 M phosphate buffer. The cells were then resuspended in warm 7.5% gelatin in PBS in microfuge tubes for 10 min and then pelleted at 10,000 rpm for 1 min. The tubes were placed on ice and the solidified gelatin pellets were removed, transferred to an ice-cold 50mM glycine solution, cut into small cubes, and then transferred to 2.3M sucrose in 0.1M phosphate buffer at 4°C. Following sucrose infiltration for cryoprotection, each block of tissue was placed on a cryo pin-head, excess sucrose solution was removed and the pin was immersed and stored in liquid nitrogen. Thin sections (~100nm) were cut using glass knives and picked up in wire loops dipped in a mixture of methylcellulose and sucrose. The sections were placed directly onto parlodion-coated 200 mesh nickel grids. Each grid was processed on individual droplets of the following solutions at room temperature: 50 mM glycine, 10% fetal calf serum (FCS) in PBS, rabbit anti-GM130 antibody diluted 1/400 in 10% FCS, washed in 0.2% FCS, incubated in Protein A tagged with 10nm gold (Dr. J. Slot, University of Utrecht), washed in PBS, fixed in 1% glutaraldehyde in PBS, washed in double-distilled H₂O and contrasted in uranyl oxalate (pH 7). Individual grids were then picked up in ice-cold uranyl acetate/methyl cellulose (pH 4) mixture using copper wire loops, which were then blotted with filter paper to remove excess solution. The grids were then allowed to dry overnight before examination on a Philips CM-100 transmission electron microscope. Controls included incubation with Protein A gold alone, normal human serum and rabbit antibodies to giantin.

2.7. Detection for disruption of mitochondrial transmembrane potential ($\Delta\Psi_m$)

HEp-2 cells were grown on 8 chambered tissue culture slide and apoptosis was induced by 2 µM STS for 4 h. For the detection of mitochondrial dysfunction characterized as disruption of mitochondrial transmembrane potential ($\Delta\Psi_m$) during early apoptosis, HEp-2 cells were incubated with 50 nM Mitotracker Red CMXRos (Invitrogen) at 37°C for 1 hour, and the cells were processed for IIF.

3. Results

3.1. Morphologic changes of Golgi complex during apoptosis

We previously reported that the morphological changes of the Golgi complex depend upon the stage of apoptosis (Nozawa *et al.*, 2002). Approximately, 10 to 20% of HEP-2 cells exposed to 2 μ M STS for 4 h exhibited unique morphology and clustering of the Golgi complex (Nozawa *et al.*, 2002). Interestingly, the nuclei were half-moon or crescent shaped and the Golgi complex was usually condensed at the indented region. This clustering of the Golgi complex was considered an early stage of apoptosis that preceded complete Golgi complex and nuclear fragmentation (Figure 1a). Another 10 to 20% of HEP-2 cells exhibited fragmentation of the nuclei and disappearance of Golgi complex; accordingly these cells were defined as late apoptotic cells (Figure 1a). Apoptosis was also confirmed by immunoblotting analysis using anti-PARP antibody and the characteristic 85kDa apoptotic fragment was confirmed from the cell lysates treated with 2 μ M STS for 4h or longer (Figure 1b). Cell counting showed that 10–20% cells were at an early stage of apoptosis with half-moon nuclei, 10–20% cells were at the late stage of apoptosis with nuclear fragmentation, and 60–80% cells were still apparently unaffected at this time point. However, the majority of cells exposed to STS for 24h exhibited late stage apoptosis characterized by complete fragmentation of their nuclei and Golgi complex, and only a few cells exhibited half-moon nuclei (data not shown, (Nozawa *et al.*, 2002)). To confirm this, a similar experiment was performed using HeLa cells. An identical pattern was also observed with HeLa cells. However, HeLa cells appeared more sensitive to apoptotic induction by STS than HEP-2 cells. At 90 min of 1 μ M STS treatment, 10 to 20% HeLa cells exhibited half-moon nuclei and another 10 to 20% cells were at the late stage of apoptosis. Interestingly, when the cells underwent the same treatment following replacement of fresh medium without STS and incubated an additional 3 hours, the number of half-moon nuclei cells decreased dramatically (1 to 3%), and late apoptotic cells increased ranging from 20 to 30% (data not shown). This observation suggests that cells with clustered Golgi complex are irreversibly committed to the apoptotic process and that the apoptotic process can proceed without further apoptotic stimuli. Therefore, the characteristic half-moon nuclei with Golgi clustering could be considered an important indicator for commitment to the apoptotic process.

Another obvious difference between apoptotic cells and non-apoptotic cells was noted in the staining of nucleoli by propidium iodide (PI) (Figure 1a). In control untreated cells, the nucleoli and its components could be clearly distinguished in the nucleus. However, in both early and late apoptotic cells, the nucleoli were disassembled and their components released into the cytoplasm. Horky *et al* and other investigators have reported nucleolar RNP segregation and extrusion in apoptotic cells (Horky *et al.*, 2001; Martelli *et al.*, 2000; Biggiogera *et al.*, 1997; Biggiogera *et al.*, 1998).

To exclude the possibility that the morphological changes of the Golgi complex during apoptosis was a unique feature that followed STS treatment, we also examined apoptosis induced by etoposide. When HEP-2 cells were treated with 10 to 100 μ M etoposide for 30 hours as previous described (Wu *et al.*, 2002), comparable results were obtained from both IIF and immunoblotting analysis (Figure 1c, 1d). Furthermore, similar clustering of Golgi complex was also observed when apoptosis was induced by gamma irradiation of HEP-2 cells (Supplemental figure 1).

To explore whether the clustering of Golgi complex during apoptosis was specific to human cells, cell lines from other species were examined. Supplemental figure 2 shows similar clustering of Golgi complex in STS-induced apoptosis of a normal rabbit lung fibroblast line R9ab. The same clustering of Golgi complex during early apoptosis induced by STS was also observed in a normal rat kidney fibroblast line NRK (data not shown).

To rule out potential fixation artifacts, we also investigated morphological changes of the Golgi complex during apoptosis in live cells. GFP-golgin-97 fusion expression vector was co-transfected with red fluorescent protein encoding plasmid pDsRed2-C1 to observe the Golgi complex and whole cell morphology, respectively. The clustering of the Golgi complex adjacent to half-moon nuclei during early apoptosis was also observed in HeLa cells expressing GFP fused golgin-97 and treated with 1 μ M STS for 2 hours (Figure 2). These data suggest that the clustering of the Golgi complex in apoptotic cells is potentially a general feature of experimentally induced apoptosis and it is feasible to distinguish relatively early from late stage apoptosis by monitoring the morphological changes of the Golgi complex and nucleus.

3.2. Clustering of mitochondria and the mitochondrial membrane dysfunction in early stage apoptosis

Next we investigated whether this clustering phenomenon is also observed in other cytoplasmic organelles. It has been shown that mitochondria have an active function in apoptosis (Ferri and Kroemer, 2001) and disruption of mitochondrial transmembrane potential ($\Delta\Psi_m$) during early apoptosis induced by various stimuli in diverse cell types, represents an irreversible commitment to cell death, preceding the characteristics of late apoptosis, such as DNA condensation and degradation as well as formation of apoptotic bodies (Zamzami et al., 1996; Marchetti et al., 1996). Moreover, Vos *et al* and other investigators have reported that clustering of mitochondria precedes cell death when apoptosis was induced by TNF α or TRAIL (De Vos et al., 1998; Thomas et al., 2000). These reports prompted us to investigate the mitochondrial contribution to the apoptotic process as well as Golgi complex. HEP-2 cells were treated with 2 μ M STS for 4 hours and cells are stained with the mitochondria specific probe Mitotracker Red 580, and anti-giantin antibody. In early apoptosis with the half-moon nuclei as a marker, mitochondria were clustered in the vicinity of the Golgi complex adjacent to the nuclei. However in late stage apoptosis, mitochondria were broadly dispersed throughout the cytoplasm (Figure 3). These data suggest that clustering of mitochondria may represent an early manifestation of apoptosis. To confirm whether this phenomenon is actually one of earliest signs of apoptosis, we checked the disruption of mitochondrial transmembrane potential ($\Delta\Psi_m$), which is thought to be one of the earliest events of apoptotic process (Ly *et al.*, 2003). To detect mitochondrial transmembrane potential ($\Delta\Psi_m$), Mitotracker Red CMXros was used since it is known to accumulate to a lesser degree in mitochondria with depolarized membranes compared to intact membranes (Poot and Pierce, 1999). As expected, in contrast to control cells, early apoptotic cells were characterized by Golgi complex clustering and reduced intensity of Mitotracker Red CMXros (Figure 4). The observed $\Delta\Psi_m$ may be induced by mitochondria and Golgi complex clustering. This clustering event is associated with and may even activate pro-apoptotic molecules localized in these organelles and thereby play an important role for the subsequent apoptotic process.

3.3. Co-clustering of other cytoplasmic organelles during apoptosis

Other cytoplasmic organelles such as lysosomes have also been implicated in the integration of pro-apoptotic signaling or damage sensing (Ferri and Kroemer, 2001; Salvesen, 2001). Accordingly, we investigated the distribution of other cytoplasmic organelles during early apoptosis. Double immunostaining was performed in combination with anti-golgin antibody (giantin or golgin-97) and several antibodies for marker proteins of cytoplasmic organelles. Figure 5 shows the co-staining of Golgi complex and lysosomes, endosomes, peroxisomes or cytoskeletal proteins actin and tubulin in the half-moon nuclei containing apoptotic cells and control cells. In untreated controls, these cytoplasmic organelles and cytoskeletal proteins were distributed throughout the cytoplasm. Interestingly, these cytoplasmic organelles all condensed adjacent to the nucleus and in the same region as the clustered Golgi complex (Figure 5). This co-clustering phenomenon may be a result of simple shrinkage of cytoplasm followed by apoptosis although actin and tubulin, which are major cytoplasmic structural components, were

not enriched at the same region (Figure 5). Figure 6 shows that in the majority apoptotic cells with the half-moon nuclei, either centrosome also migrated to the concave region of the crescentic nucleus and co-clustered with the Golgi complex and other cytoplasmic organelles

3.4. EM analysis of co-clustering of Golgi complex and other cytoplasmic organelles during early apoptosis

Figure 7 shows HEP-2 cells treated with 2 μ M STS for 4 h using standard EM and immuno-EM. Cytoplasmic organelles including Golgi complex, lysosomes, mitochondria, and centrosomes could be identified at the same region adjacent to the half-moon nucleus. These data demonstrate the unique co-clustering event of many cytoplasmic organelles detectable in both IIF and EM.

4. Discussion

In this study, we have demonstrated a novel extensive co-clustering of cytoplasmic organelles such as mitochondria, early endosomes, lysosomes, peroxisomes, adjacent to the half-moon nucleus during an early stage of apoptosis in contrast to the late stage, which was defined by fragmentation of the nucleus. Whether the centrosomes or any of the other organelles play an active role in the co-clustering of cytoplasmic organelles is unclear and will need further investigation. As demonstrated here, the co-clustering of cytoplasmic organelles in early apoptosis was not restricted to STS-induced apoptosis and was also observed in apoptosis induced by etoposide or gamma irradiation. As shown in the Western blot analysis of cells treated with either STS or etoposide, caspase-dependent degradation of PARP was demonstrated and thus it is likely the co-clustering of cytoplasmic organelles is also caspase-dependent.

Increasing evidence suggests that apoptotic progression requires caspase activation and some contribution of cytoplasmic organelles. It is well known that mitochondrial release of cytochrome C can trigger activation of caspases (Mak and Yeh, 2002). Disruption of mitochondrial transmembrane potential during early apoptosis induced by various stimuli in diverse cell types represents an irreversible commitment to cell death (Marchetti *et al.*, 1996). This precedes late characteristics of apoptosis, such as DNA condensation and degradation, and formation of apoptotic bodies (Zamzami *et al.*, 1996; Marchetti *et al.*, 1996). Susin and co-workers have identified procaspase-2 and procaspase-9 in mitochondria, and both of these were released into the cytosol and became active caspases in STS-induced early apoptosis (Susin *et al.*, 1999). In addition to caspase-2 and caspase-9, Nechushtan and co-workers have demonstrated that Bax, a member of pro-apoptotic molecule of the Bcl-2 family, clustered adjacent to mitochondria during apoptosis (Nechushtan *et al.*, 2001). Interestingly, our data showed that mitochondria and other cytoplasmic organelles are co-clustered with Golgi complexes in the same region during a relatively early stage apoptosis.

It has been shown that ganglioside GD3, which is an acidic glycosphingolipid synthesized in the Golgi complex, plays a crucial role in early events in apoptotic cascades initiated by ceramides or by activation of the surface receptor CD95/Fas/APO-1 (Malisan and Testi, 2002; De Maria *et al.*, 1997). Ganglioside GD3 induces mitochondrial permeability transition resulting in cytochrome C release, which in turn triggers late stage apoptotic events (Kristal and Brown, 1999; Scorrano *et al.*, 1999). Ouasti *et al.* have recently shown membrane scrambling between the Golgi complex and mitochondria that is triggered by death receptor signaling and the resulting global change in the membrane traffic of intracellular organelles could influence cell death (Ouasti *et al.*, 2007).

Another possibility for the cytoplasmic co-clustering during early apoptosis is that many biochemical events can occur in this region with the condensation of lysosomes, peroxisomes,

and endosomes. Hydrogen peroxide, which is stored in peroxisomes, possesses a proteolytic function (Nozawa *et al.*, 2002). It is possible that modification of pro-apoptotic macromolecules may arise as a result of the close proximity to these cytoplasmic organelles, such as lysosomes and peroxisomes which are rich in enzymes including cathepsins, glycosidases, and phosphatases, which may play an important role to facilitate the later events in apoptosis. These enzymes are acid hydrolases that are strictly localized to the acidic lumen of the organelle. However, if the lysosomal membrane is partially disrupted and the pH of the cytoplasm shifts to an acidic milieu during apoptosis, these enzymes may help promote cleavage of pro-apoptotic proteins. In support of this concept, it has been reported that intracellular acidification occurs during apoptosis (Pervaiz and Clement, 2002; Wolf and Eastman, 1999) and lysosomal cathepsins cause the proteolytic activation of Bid, a member of the pro-apoptotic Bcl-2 family, which then translocates to the mitochondria and mediates release of cytochrome C (Ferri and Kroemer, 2001). Therefore, pro-apoptotic signaling proteins might be modified by the clustering event and this modification might be required for the systematic progression of apoptosis.

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Abbreviations

PARP	poly-ADP-ribose polymerase
DAPI	4',6-diamidino-2-phenylindole
IIF	indirect immunofluorescence
PI	propidium iodide
STS	staurosporine
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand

References

- Bennett M, Macdonald K, Chan SW, Luzio JP, Simari R, Weissberg P. Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science* 1998;282:290–293. [PubMed: 9765154]
- Biggiogera M, Bottone MG, Martin TE, Uchiyama T, Pellicciari. Still immunodetectable nuclear RNPs are extruded from the cytoplasm of spontaneously apoptotic thymocytes. *Exp Cell Res* 1997;234:512–520. [PubMed: 9260922]
- Biggiogera M, Bottone MG, Pellicciari C. Nuclear RNA is extruded from apoptotic cells. *J Histochem Cytochem* 1998;46:999–1005. [PubMed: 9705965]
- Chiu R, Novikov L, Mukherjee S, Shields D. A caspase cleavage fragment of p115 induces fragmentation of the Golgi apparatus and apoptosis. *J Cell Biol* 2002;159:637–648. [PubMed: 12438416]

- De Maria R, Lenti L, Malisan F, d'Agostino F, Tomassini B, Zeuner A, Rippo MR, Testi R. Requirement for GD3 ganglioside. *Science* 1997;277:1652–1655. [PubMed: 9287216]
- De Vos K, Goossens V, Boone E, Vercammen D, Vancompernelle K, Vandenabeele P, Haegeman G, Fiers W, Grooten J. The 55-kDa tumor necrosis factor receptor induces clustering of mitochondria through its membrane-proximal region. *J Biol Chem* 1998;273:9673–9680. [PubMed: 9545301]
- Farquhar MG, Palade GE. The Golgi apparatus: 100 years of progress and controversy. *Trends Cell Biol* 1998;8:2–10. [PubMed: 9695800]
- Ferri KF, Kroemer G. Organelle-specific initiation of cell death pathways. *Nat Cell Biol* 2001;3:E255–E263. [PubMed: 11715037]
- Fritzler MJ, Hamel JC, Ochs RL, Chan EKL. Molecular characterization of two human autoantigens: Uniques cDNAs encoding 95- and 160-kD proteins of a putative family in the Golgi complex. *J Exp Med* 1993;178:49–62. [PubMed: 8315394]
- Fritzler MJ, Lung CC, Hamel JC, Griffith K, Chan EKL. Molecular characterization of golgin-245: a novel Golgi complex protein containing a granin signature. *J Biol Chem* 1995;270:31262–31268. [PubMed: 8537393]
- Griffith KJ, Chan EKL, Lung CC, Hamel JC, Guo X, Miyachi K, Fritzler MJ. Molecular cloning of a novel 97-kd Golgi complex autoantigen associated with Sjögren's syndrome. *Arthritis Rheum* 1997;40:1693–702. [PubMed: 9324025]
- Grutter MG. Caspases: key players in programmed cell death. *Curr Opin Struct Biol* 2000;10:649–655. [PubMed: 11114501]
- Horky M, Wurzer G, Kotala V, Anton M, Vojtesek B, Vacha J, Wesierska-Gadek J. Segregation of nucleolar components coincides with caspase-3 activation in cisplatin-treated HeLa cells. *J Cell Sci* 2001;114:663–670. [PubMed: 11171371]
- Jones SJ, Ledgerwood EC, Prins JB, Galbraith J, Johnson DR, Pober JS, Bradley JR. TNF recruits TRADD to the plasma membrane but not the trans-Golgi network, the principal subcellular location of TNF-R1. *J Immunol* 1999;162:1042–1048. [PubMed: 9916731]
- Kristal BS, Brown AM. Apoptogenic ganglioside GD3 directly induces the mitochondrial permeability transition. *J Biol Chem* 1999;274:23169–23175. [PubMed: 10438487]
- Leist M, Jaattela M. Four deaths and a funeral: from caspases to alternative mechanisms. *Nat Rev Mol Cell Biol* 2001;2:589–598. [PubMed: 11483992]
- Ly JD, Grubb DR, Lawen A. The mitochondrial membrane potential ($\Delta\psi(m)$) in apoptosis; an update. *Apoptosis* 2003;8:115–128. [PubMed: 12766472]
- Mak TW, Yeh WC. Signaling for survival and apoptosis in the immune system. *Arthritis Res* 2002;4 (Suppl 3):S243–S252. [PubMed: 12110144]
- Malisan F, Testi R. GD3 ganglioside and apoptosis. *Biochim Biophys Acta* 2002;1585:179–187. [PubMed: 12531552]
- Mancini M, Machamer CE, Roy S, Nicholson DW, Thornberry NA, Casciola-Rosen LA, Rosen A. Caspase-2 is localized at the Golgi complex and cleaves golgin-160 during apoptosis. *J Cell Biol* 2000;149:603–612. [PubMed: 10791974]
- Marchetti P, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, Haeflner A, Hirsch F, Geuskens M, Kroemer G. Mitochondrial permeability transition is a central coordinating event of apoptosis. *J Exp Med* 1996;184:1155–1160. [PubMed: 9064332]
- Martelli AM, Robuffo I, Bortul R, Ochs RL, Luchetti F, Cocco L, Zwyer M, Bareggi R, Falcieri E. Behavior of nucleolar proteins during the course of apoptosis in camptothecin-treated HL60 cells. *J Cell Biochem* 2000;78:264–277. [PubMed: 10842321]
- Martelli AM, Zwyer M, Ochs RL, Tazzari PL, Tabellini G, Narducci P, Bortul R. Nuclear apoptotic changes: an overview. *J Cell Biochem* 2001;82:634–646. [PubMed: 11500941]
- Mukherjee S, Chiu R, Leung SM, Shields D. Fragmentation of the Golgi apparatus: an early apoptotic event independent of the cytoskeleton. *Traffic* 2007;8:369–378. [PubMed: 17394485]
- Nechushtan A, Smith CL, Lamensdorf I, Yoon SH, Youle RJ. Bax and Bak coalesce into novel mitochondria-associated clusters during apoptosis. *J Cell Biol* 2001;153:1265–1276. [PubMed: 11402069]
- Nozawa K, Casiano CA, Hamel JC, Molinaro C, Fritzler MJ, Chan EKL. Fragmentation of Golgi complex and Golgi autoantigens during apoptosis and necrosis. *Arthritis Res* 2002;4:R3. [PubMed: 12106502]

- Ouasti S, Matarrese P, Paddon R, Khosravi-Far R, Sorice M, Tinari A, Malorni W, Degli EM. Death receptor ligation triggers membrane scrambling between Golgi and mitochondria. *Cell Death Differ* 2007;14:453–461. [PubMed: 17008914]
- Pervaiz S, Clement MV. A permissive apoptotic environment: function of a decrease in intracellular superoxide anion and cytosolic acidification. *Biochem Biophys Res Commun* 2002;290:1145–1150. [PubMed: 11811982]
- Poot M, Pierce RH. Detection of changes in mitochondrial function during apoptosis by simultaneous staining with multiple fluorescent dyes and correlated multiparameter flow cytometry. *Cytometry* 1999;35:311–317. [PubMed: 10213196]
- Robertson JD, Orrenius S, Zhivotovsky B. Review: nuclear events in apoptosis. *J Struct Biol* 2000;129:346–358. [PubMed: 10806085]
- Salvesen GS. A lysosomal protease enters the death scene. *J Clin Invest* 2001;107:21–22. [PubMed: 11134173]
- Scorrano L, Petronilli V, Di Lisa F, Bernardi P. Commitment to apoptosis by GD3 ganglioside depends on opening of the mitochondrial permeability transition pore. *J Biol Chem* 1999;274:22581–22585. [PubMed: 10428836]
- Selak S, Chan EKL, Schoenroth L, Senécal JL, Fritzler MJ. Early endosome antigen. 1: An autoantigen associated with neurological diseases. *J Investig Med* 1999;47:311–318.
- Susin SA, Lorenzo HK, Zamzami N, Marzo I, Brenner C, Larochette N, Prevost MC, Alzari PM, Kroemer G. Mitochondrial release of caspase-2 and -9 during the apoptotic process. *J Exp Med* 1999;189:381–394. [PubMed: 9892620]
- Thomas WD, Zhang XD, Franco AV, Nguyen T, Hersey P. TNF-related apoptosis-inducing ligand-induced apoptosis of melanoma is associated with changes in mitochondrial membrane potential and perinuclear clustering of mitochondria. *J Immunol* 2000;165:5612–5620. [PubMed: 11067917]
- Walker A, Ward C, Sheldrake TA, Dransfield I, Rossi AG, Pryde JG, Haslett C. Golgi fragmentation during Fas-mediated apoptosis is associated with the rapid loss of GM130. *Biochem Biophys Res Commun* 2004;316:6–11. [PubMed: 15003503]
- Wolf CM, Eastman A. Intracellular acidification during apoptosis can occur in the absence of a nucleus. *Biochem Biophys Res Commun* 1999;254:821–827. [PubMed: 9920824]
- Wu D, Ingram A, Lahti JH, Mazza B, Grenet J, Kapoor A, Liu L, Kidd VJ, Tang D. Apoptotic release of histones from nucleosomes. *J Biol Chem* 2002;277:12001–12008. [PubMed: 11812781]
- Yang Z, Jakymiw A, Wood MR, Eystathioy T, Rubin RL, Fritzler MJ, Chan EKL. GW182 is critical for the stability of GW bodies expressed during the cell cycle and cell proliferation. *J Cell Sci* 2004;117:5567–5578. [PubMed: 15494374]
- Zamzami N, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer G. Mitochondrial control of nuclear apoptosis. *J Exp Med* 1996;183:1533–1544. [PubMed: 8666911]

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

Fig. 1.

Representative morphological changes and clustering of the Golgi complex in HEp-2 cells undergoing apoptosis. HEp-2 cells were treated with or without 2 μ M STS for 4 h to 28 h (**a**, **b**) or 10 to 100 μ M etoposide for 30 h (**c**, **d**). Indirect immunofluorescence was performed using rabbit anti-giantin and Alexa 488 conjugated goat anti-rabbit IgG antibody (green). Cells were counterstained by DAPI (blue) and propidium iodide (PI, red) (**a**), or DAPI alone (**c**). In cells with half-moon nuclei, clustering of Golgi complex was observed. In the late stage as defined by nuclear fragmentation, staining for Golgi complex indicated the absence of typically Golgi membrane and vesicular stacks. Scale bar, 10 μ m. Apoptosis was also monitored by immunoblotting analysis of cells exposed to STS (**b**) or etoposide (**d**) using human anti-PARP antibody. Numbers to the left of each blot represent relative molecular mass (kDa).

Fig. 2.

Apoptotic clustering of Golgi complex in live HEp-2 cells transfected with GFP-fusion golgin-97 construct. To rule out fixation artifacts in the clustering of Golgi complex during apoptosis, GFP-golgin-97 fusion expression vector was co-transfected with red fluorescent protein (RFP) encoding plasmid pDsRed2-C1 to observe the Golgi complex and nuclear morphology in live cells. Cells expressing GFP-golgin-97 and RFP were treated with or without 2 μ M STS for 2 h and examined for morphological changes of the Golgi complex using live cell imaging. In cells treated to undergo apoptosis, the Golgi complex was clustered in a juxtannuclear position in the two representative images shown with the half-moon nuclei. Outlines of the nucleus are shown in the RFP panels. Scale bar, 10 μ m.

Fig. 3.

Mitochondria co-clustering with Golgi complex at same region adjacent to half-moon nuclei during apoptosis. For further confirmation of apoptosis, we also monitored distribution of mitochondria during apoptosis. HEp-2 cells were treated with or without 2 μ M STS for 4 h and the cells were stained with anti-giantin antibody (green) and the mitochondria specific probe Mitotracker 580 (red). Nuclei were counterstained by DAPI (blue). The mitochondria revealed broad distribution throughout whole cytoplasm in normal control cells. Scale bar, 10 μ m.

Fig. 4.

Depolarization of mitochondrial transmembrane potential ($\Delta\Psi_m$) in apoptotic cells with half-moon nuclei. To confirm mitochondrial dysfunction during apoptosis, we investigate disruption of mitochondrial transmembrane potential as the marker of early apoptosis using Mitotracker Red CMXRos. HEP-2 cells were treated with or without 2 μ M STS for 4 h and the cells were stained with anti-giantin antibody (green) and Mitotracker Red CMXRos (red). The nucleus was counterstained by DAPI (blue). The intact mitochondria membrane was represented as intense staining in normal control cells. In apoptotic cells with half-moon nuclei characterized by clustering of the Golgi complex as well as late apoptotic cells, mitochondrial transmembrane exhibited reduced intensity of Mitotracker Red CMXRos. Scale bar, 10 μ m.

Fig. 5.

Co-clustering of lysosomes, peroxisomes, and endosomes and the Golgi complex in apoptotic cells with half-moon nuclei. HEp-2 cells were treated with 2 μ M STS for 4h and used for double immunofluorescence using a combination of rabbit anti-giantin or mouse monoclonal anti-golgin-97 antibodies as Golgi complex markers and costaining with (a) lysosome marker LAMP1, (b) peroxisome marker PMP70, (c) early endosome marker EEA-1, (d) actin, or (e) tubulin. Cytoplasmic organelles including lysosomes, peroxisomes, and endosomes were enriched adjacent to half-moon nuclei and in the same region as the Golgi complex.

Fig. 6. Positioning of Golgi complex relative to the centrosome during apoptosis. Panels A–C show HEP-2 cells treated with 2 μ M STS for 4h to induce apoptosis. Double immunostaining was performed using rabbit anti-giantin antibody and Alexa 488-goat anti-rabbit IgG (green, A, D, G, J) and human anti-pericentrin antibody and Alexa 568-goat anti-human IgG (red, B, E, H, K). Nuclei were counterstained by DAPI (blue). Panel C, F, I, L show merged images. In addition, three representative apoptotic cells with half-moon nuclei (D–L) are also shown. Arrows show centrosomes in relationship to the Golgi complex. Arrowhead shows a mitotic cell.

Fig. 7.

Electron microscopic images illustrating the co-clustering of Golgi complex and other cytoplasmic organelles during apoptosis. HEP-2 cells treated with 2 μ M STS for 4 h were analyzed by standard EM (**A**, **B**) or processed for immuno-EM (**C**, **D**) stained with rabbit anti-GM130 antibody and labeled by immuno-gold as described in Methods. Panel **A** and **C** show the crescentic nuclei (Nu). In panel **B**, enlarged from **A**, Golgi (G), mitochondria (M), lysosomes (arrows), lipid (L), centrosome (C), and electron dense residual bodies (R) are visible. Rabbit anti-GM130 antibody on the cryo-ultrathin sections (**C**) has labeled the Golgi membranes clearly detected in the enlargement (**D**).