Temporal relationship between cytochrome c release and mitochondrial swelling during UV-induced apoptosis in living HeLa cells

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SUMMARY

During apoptosis, cytochrome c is released from mitochondria to the cytosol to activate a caspase cascade, which commits the cell to the death process. It has been proposed that the release of cytochrome c is caused by a swelling of the mitochondrial matrix triggered by the apoptotic stimuli. To test this theory, we measured directly the dynamic re-distribution of green fluorescence protein (GFP)-tagged cytochrome c and morphological change of mitochondria within living HeLa cells during u.v.-induced apoptosis. We observed that mitochondria did not swell when cytochrome c was released from mitochondria to cytosol during apoptosis. Instead, mitochondria swelled to spherical shapes within 10 minutes of cytochrome c release. This finding strongly suggests that cytochrome c release in apoptosis was not caused by mitochondrial swelling. This conclusion was further supported in two separated experiments using an immunostaining method and carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) treatment. In addition, we found evidence that cytochrome c was also released before mitochondrial swelling in apoptosis induced by other cell death-inducing treatments, including tumor necrosis factor (TNF) and actinomycin D.

Key words: Apoptosis, Cytochrome c, Mitochondria, GFP, Programmed cell death

INTRODUCTION

Mitochondria are known to play a central role in cell death control (Susin et al., 1998). During programmed cell death, cytochrome c is released from mitochondria into the cytosol, where it binds with Apaf1 to activate a series of caspase cascades (Cai et al., 1998; Liu et al., 1996). At present, the mechanism of cytochrome c release is not yet clearly understood. One leading hypothesis is that, a megachannel traversing across the outer and inner mitochondrial membranes called 'PT (permeability transition) pore' is induced to open during apoptosis (Green and Reed, 1998). Solutes and water in the cytosol then enter mitochondrial matrix through this channel, causing the mitochondrion to swell and rupture its outer membrane (Green and Reed, 1998). As a result, cytochrome c and other proteins in the inter-membrane space are released.

This hypothesis has been widely cited and debated in the literature (Desagher and Martinou, 2000; Eskes et al., 1998; Green and Reed, 1998; Heiskanen et al., 1999; Jürgensmeier et al., 1998; Kluck et al., 1997; Kluck et al., 1999; Krohn et al., 1999; Matsuyama et al., 2000; Minamikawa et al., 1999; Narita et al., 1998; Pastorino et al., 1998; Pastorino et al., 1999; Scarletta et al., 2000; Shimizu et al., 1999; Vander Heiden et al., 1997; von Ahsen et al., 2000; Yang et al., 1997), but so far, there is still a lack of conclusive evidence to prove or disprove it. First, the supporting evidence for the mitochondrial swelling theory is based mainly on indirect observations. For example, specific inhibitors of the PT pore such as cyclosporin A or bongkrekic acid have been found to prevent apoptosis in hepatocytes and other cell models (Bradham et al., 1998; Kroemer et al., 1997), while PT pore-opening agent like attractyloside or Ca^{2+} have been observed to induce matrix swelling and apoptosis (Jürgensmeier et al., 1998; Narita et al., 1998; Pastorino et al., 1999). Conflicting data, however, have also been reported and suggest that cytochrome c release may not be related to PT pore opening. For example, Eskes et al. observed that Bax-induced cytochrome c release could not be inhibited by either cyclosporin A or bongkrekic acid in isolated mitochondria (Eskes et al., 1998).

Second, there is a controversy about whether or not the mitochondria indeed swell during apoptosis. Some studies have reported the observation of mitochondrial swelling (Narita et al., 1998; Vander Heiden et al., 1997), while others have reported that swelling never occurred (Kluck et al., 1999; von Ahsen et al., 2000). Third, most previous studies have been carried out using fixed cells or isolated mitochondria (Antonsson et al., 2000; Eskes et al., 1998; Jürgensmeier et al., 1998; Kluck et al., 1999; Narita et al., 1998; Pastorino et al., 1999; Vander Heiden et al., 1997; von Ahsen et al., 2000), which could become swelled during the fixation or isolation processes (Scheffler, 1999). Fourth, even if mitochondria are observed to swell during apoptosis, it is still not clear whether the mitochondrial swelling is the cause or the result of cytochrome c release.

In order to overcome these difficulties and directly test the mitochondrial swelling theory, one must study the events of cytochrome c release and mitochondrial swelling in an intact living cell. Thus, we have used digital imaging techniques to measure the dynamic re-distribution of GFP labeled cytochrome c during UV-induced apoptosis in living HeLa cells, and used a red color fluorescent dye, Mitotracker, to monitor the morphological change of mitochondria at the same time. The objective of our study is to answer two important questions: (1) do mitochondria swell during apoptosis?; and (2) if they do, is the mitochondrial swelling the cause or consequence of cytochrome c release?

MATERIALS AND METHODS

Chemicals and reagents

TNFα was purchased from Clontech Lab (Palo Alto, CA); Mitotraker® Red CMXRos and goat-anti-mouse IgG antibody conjugated with FITC were from Molecular Probes (Eugene, OR); Mouse-anti cytochrome c monoclonal antibody was from PharMingen. Cytochrome c-GFP plasmid (cloned in a pEGFP-N1 vector) was kindly supplied by Brian Herman (Mahajan et al., 1998). Other chemicals were mainly from Sigma (St Louis, MO).

Cell treatments and induction of apoptosis

HeLa cells were grown on glass coverslips at 37°C in a humidified atmosphere containing 5% CO_2 in modified Eagle's Medium (MEM) supplemented with 10% fetal bovine serum (FBS) plus 100 μg/ml penicillin and 100 U/ml streptomycin. To image cytochrome c, cells were transfected with the cytochrome c-GFP plasmid using an electroporation method (Chang, 1997) and were allowed to express the fusion gene for 48-60 hours. Apoptosis was induced either by exposing cells to UV irradiation (300 μW/cm^2) for 3 minutes, by adding 10 ng/ml TNFα plus 10 μg/ml cycloheximide, or by adding 1 μM Actinomycin D to the medium. In the experiment using z-VAD-fmk (100 nM) and cyclosporine A (5 μM), both chemicals were added 1 hour before UV irradiation and kept in the medium throughout the experimental process.

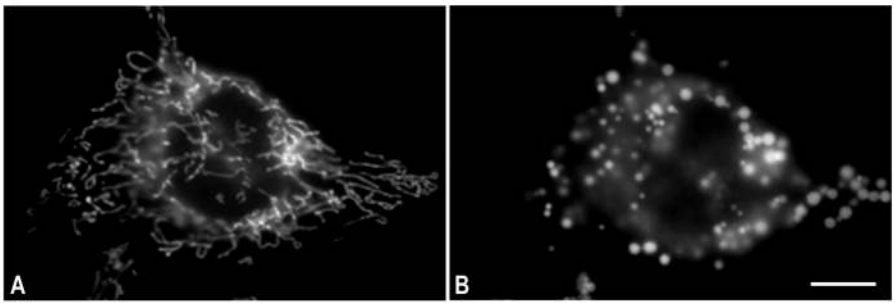
Imaging methods

For living cell measurements, we used a laser scanning confocal microscope (BioRad MRC-600) equipped with a krypton/argon laser to image cytochrome c-GFP distribution and mitochondria morphology in cells grown on a coverslip. Laser lines of 488 nm and 568 nm were used to observe cytochrome c-GFP and Mitotracker, respectively. To stain mitochondria, Mitotracker Red CMXRos was added to MEM to a final concentration of 0.5 μM and cells were incubated for 5 minutes followed by washing with MEM once. After UV irradiation, the coverslip was mounted onto a chamber containing an observation medium (Hepes-buffered Dulbecco's MEM containing 4 mM glutathione, 1 mM L-ascorbic acid, 0.5 mM DTT, pH 7.4). The cells were examined under the confocal microscope with a heating box that maintained the temperature at 37°C.

For immunostaining studies, cells were exposed to the apoptosis-inducing treatment first. After a waiting period, we washed cells and fixed them with 4% paraformaldehyde plus 0.1% glutaraldehyde for 20 minutes at room temperature. Then, cells were immunostained using the standard method (Li et al., 1999). The primary antibody used was mouse anti-native cytochrome c (1:200 dilution), while the secondary antibody was goat anti-mouse IgG conjugated with FITC (1:200 dilution). Mitochondria were stained with Mitotracker.

Quantitative analysis of cytochrome c release and morphological change in mitochondria

We used the MetaMorph 3.0 software (Universal Image, West Chester, PA) to analyze digital images recorded by the confocal microscope at different times during apoptosis. For the mitochondrial diameter measurement, we drew a line perpendicular to the mitochondrion in the magnified Mitotracker image and determined its diameter based on the line-scan profile. Several line-scan measurements were made on a single mitochondrion to obtain its average diameter. This procedure was repeated on many mitochondria within the same cell to obtain a statistical value. To quantify the distribution of cytochrome c, we examined the pixel distribution profile of the cytoc-GFP image and separated the mitochondrial signals from the cytosolic signals by choosing a proper threshold (see Fig. 3). Then, we integrated all pixels in the cell (F_{total}) and in the mitochondria (F_{mito}). The ratio of mitochondrial pixels to cytosolic pixels, $F_{mito}/(F_{total} - F_{mito})$, was used as a parameter to characterize the cytochrome c-GFP distribution between mitochondria and the cytosol.



Condition of mitochondria	No swelling	Swelling without stretching the surface
Morphology	L, d	d_2
Surface area	S_1	$S_2 = S_1$
Volume	V_1	$V_2 = 1.72V_1$
Diameter	d_1	$d_2 = 2.34d_1$

C

Fig. 1. Change of mitochondrial morphology during swelling. Mitochondria within a HeLa cell were stained by Mitotracker and imaged using a fluorescence microscope. (A) Under the normal condition, mitochondria appeared as filamentous structures. (B) After mitochondria were induced to swell by exposing the cell to a hypo-osmotic medium (5 mM potassium phosphate buffer, pH 7.4) for 1 minute, mitochondria were found to change into spherical shapes. (C) Calculated changes in volume and diameter when a mitochondrion swelled without stretching its surface. The length/diameter ratio was assumed to be five before swelling (Scheffler, 1999). Scale bar: 10 μm.

RESULTS

The swelling of mitochondria within a living cell was accompanied by a morphological change that could be monitored directly using a fluorescence imaging technique

In this study, we used Mitotracker, which is a red color fluorescent dye specifically accumulated in mitochondria, to monitor the morphology of mitochondria in living HeLa cells (Minamikawa et al., 1999). Under a fluorescence microscope, mitochondria normally appeared as thin filaments (Fig. 1A). When the mitochondria were induced to swell by various means (in this case by a brief hypo-osmotic treatment; Fig. 1), their shape changed from filamentous to spherical, and their diameters increased (Fig. 1A,B). This morphological change is due to the fact that the volume-to-surface ratio is higher in a spherical shape than in a rod shape. As water entered the mitochondrion during swelling, the organelle tended to change its shape to accommodate the extra volume while avoiding stretching its surface. This situation is analyzed using a simple mathematical model in Fig. 1C.

Cytochrome c release occurs before mitochondrial swelling in UV-induced apoptosis

In order to measure directly the event of cytochrome c release from mitochondria during apoptosis, we used GFP-labeled cytochrome c to monitor the dynamic re-distribution of cytochrome c in intact living cells. The GFP-tagged cytochrome c protein (cytoc-GFP) was produced by expressing a plasmid vector containing the cytochrome c-GFP fusion gene in HeLa cells (Mahajan et al., 1998). Using a confocal microscope operated under the two-channel measurement mode, we imaged both the distribution pattern of cytoc-GFP and that of Mitotracker simultaneously. We exposed the cells to UV light (300 $\mu W/cm^2$) for 3 minutes to induce apoptosis and then recorded the distribution of cytochrome c and Mitotracker at different times. Fig. 2 shows a sample record of this time series measurement, where two neighboring cells were caught to undergo cytochrome c release at slightly different times. Before cytochrome c was released from mitochondria, the distribution patterns of both cytochrome c and Mitotracker were the same as that of mitochondria in the normal cell, i.e., they appeared as filamentous structures (Fig. 2A). As cytochrome c was released from mitochondria of the lower cell, the cytoc-GFP in that cell became uniformly distributed over the entire cytoplasm (Fig. 2B), while the Mitotracker image shows that the morphology of mitochondria still remained filamentous (Fig. 2B). Within about 10 minutes of cytochrome c was release, mitochondria within the lower cell were found to swell and their morphology became spherical (Fig. 2C).

Similar results were observed in the upper cell in Fig. 2. Before cytochrome c was released from mitochondria, the distribution patterns of both cytoc-GFP and Mitotracker were the same and they appeared as filamentous structures (Fig. 2A-C). Cytochrome c release was found to be a highly synchronized event. At 171 minutes after the UV treatment,

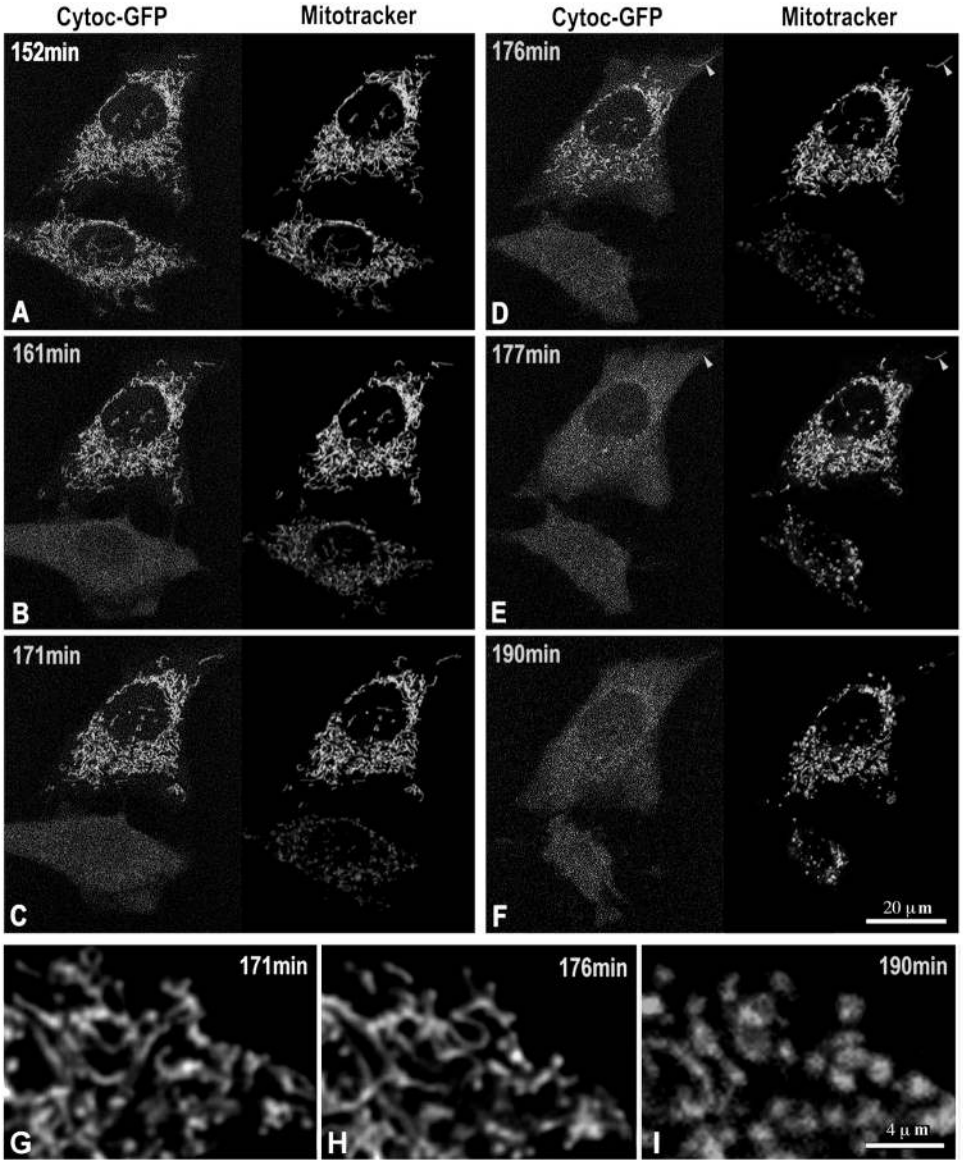


Fig. 2. (A-F) Sample records from a time-series confocal measurements of cytoc-GFP distribution (left) and Mitotracker distribution (right) on two living HeLa cells during UV-induced apoptosis. Elapsed time following the UV treatment is shown in each panel. Scale bar, 20 μm. (G-I) Magnified Mitotracker images of the upper cell shown in (C,D,F). These panels show the detailed structure of mitochondria before (G), during (H) and after (I) cytochrome c release. Scale bar: 4 μm.

none of the mitochondria in the upper cell had released their cytochrome c (Fig. 2C). Five minutes later, however, cytochrome c was found to be released from about half of the mitochondria (Fig. 2D). Another minute later, all mitochondria had finished releasing their cytochrome c (Fig. 2E). It is clear from Fig. 2E (and Fig. 2B) that after cytochrome c was released from mitochondria, the great majority of mitochondria had not swelled. The swelling did occur later. For example, 13 minutes after cytochrome c release, mitochondria in the upper cell gradually changed from filamentous shapes to spherical shapes (Fig. 2F).

In order to demonstrate the morphological change of individual mitochondrion more clearly, we magnified the Mitotracker images within Fig. 2C,D,F and showed them in Fig. 2G-I, respectively. From these magnified images, it is clearly evident that mitochondria did not swell before or during cytochrome c release (Fig. 2G,H). Instead, mitochondria became swollen only after cytochrome c was released (Fig. 2I).

Quantitative analysis of the dynamics of cytochrome c release and mitochondria swelling

To examine the temporal relationship between cytochrome c release and mitochondrial swelling, we conducted a quantitative analysis of the time-dependent change of the distribution of cytochrome c and the morphological change of mitochondria within a single living cell. The method is outlined in Fig. 3. First, we determined the diameter of the mitochondria from the magnified Mitotracker images using a line-scan method (Fig. 3A-C). The diameter of a mitochondrion was taken as the half width of its pixel profile based on the line-scan measurement (Fig. 3C). Several line-scan measurements were made on a single mitochondrion to obtain its average diameter. This procedure was repeated on many mitochondria within the same cell to obtain a statistical value. Second, to quantify the distribution of cytochrome c, we examined the pixel distribution profiles of the cytoc-GFP image in two sample regions of the cell: Region 1 contained only cytoplasm, while Region 2 contained both cytoplasm and mitochondria (Fig. 3D). By comparing the pixel distribution profiles of these two regions, we could choose a proper threshold to separate the fluorescence signal contributed by cytoc-GFP located in the mitochondria from that of the cytosolic cytoc-GFP (for more details, see Materials and Methods).

We conducted this quantitative analysis for a stack of images recorded at different time on a single HeLa cell undergoing UV-induced apoptosis. The results are shown in Fig. 4A. The release of cytochrome c from mitochondria to cytoplasm was a very rapid and highly synchronized event. We found that mitochondria started to swell only after their cytochrome c had been released. This swelling process was less synchronized than the release of cytochrome c; some mitochondria swelled almost immediately after their cytochrome c was released, while others waited for several minutes before they started to swell. In a typical cell, it took roughly 14 minutes to convert all mitochondria within the cell from a filamentous shape to a

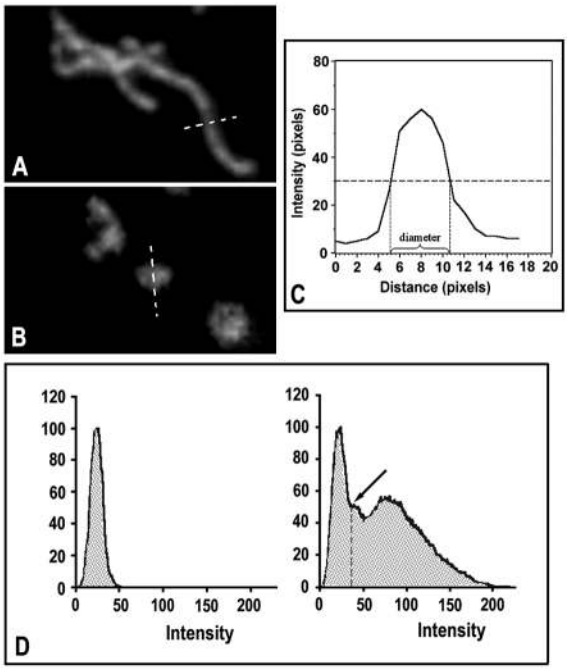


Fig. 3. Quantitative analysis of the distribution of cytochrome c and mitochondrial diameter change. (A,B) Magnified images of Mitotracker before (A) and after (B) mitochondria swelled. (C) The diameter of a mitochondrion was taken as the half width of its pixel profile based on a line-scan measurement of the Mitotracker image. (D) The distribution profiles of cytoc-GFP fluorescence intensity in a region containing only cytoplasm (left), or in a region containing both cytoplasm and mitochondria (right). The arrow marks the threshold that separates the fluorescence signal contributed by cytoc-GFP located in the mitochondria from that of the cytosolic cytoc-GFP.

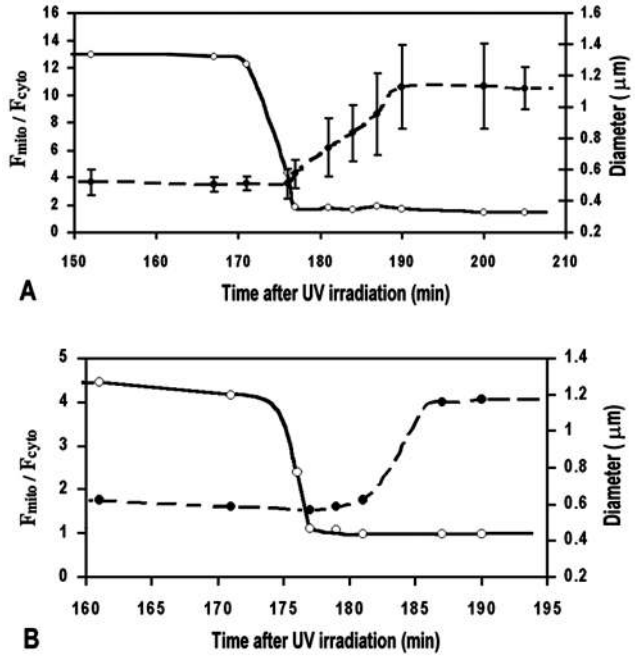


Fig. 4. (A) Dynamic change of cytoc-GFP distribution (unbroken line) and average mitochondrial diameter (broken line) in a single living cell during UV-induced apoptosis. Data are based on analysis of images obtained from the upper cell shown in Fig. 2. F_{mito}/F_{cyto} represents the fluorescence ratio of cytoc-GFP between mitochondria and cytoplasm. (B) Time-dependent changes of cytoc-GFP distribution and mitochondrial diameter as measured from a single mitochondrion (marked by arrowhead in Fig. 2D,E).

spherical shape (Fig. 4A). This relatively poor synchrony also contributed to large error bars in the measurement of the mitochondrial diameter during this swelling period.

To obtain a more detailed picture on the temporal relationship between cytochrome c release and mitochondrial swelling at a single-organelle level, we have further analyzed our images on individual mitochondria. In the periphery region of the cell where the concentration of mitochondria was less dense, one could measure both the cytochrome c distribution and morphological change in a single mitochondrion. For example, the mitochondrion marked by an arrowhead in Fig. 2D,E was shown to release its cytochrome c first without undergoing a morphological change. Results of a quantitative analysis of its cytochrome c release and change in diameter as a function of time are summarized in Fig. 4B. It is clear from this single-mitochondrion measurement that mitochondrial swelling occurred after cytochrome c was released.

Results of immunostaining studies also confirm that cytochrome c was released before mitochondria swelled during apoptosis

In the above living cell study, the distribution of cytochrome c was measured by imaging the fluorescence signal of the GFP-tagged cytochrome c. There is a remote possibility that, for some unexpected reasons, the re-distribution of endogenous cytochrome c from mitochondria might differ from that of the GFP-tagged cytochrome c. To rule out this possibility, we have conducted an immunostaining study. Here, HeLa cells (not transfected with the cytochrome c-GFP fusion gene) were fixed at different times following an UV-induced apoptotic treatment. The distribution of cytochrome c was visualized by immunostaining the cells with anti-cytochrome c antibody. The morphology of mitochondria was revealed by staining the cells with Mitotracker Red. As we compared the distribution pattern of the endogenous cytochrome c (as revealed by anti-cytochrome c) and the morphological pattern of mitochondria, we found that the HeLa cells subject to the apoptotic treatment could be classified into three different categories: (1) cells in which cytochrome c was distributed mainly in the mitochondria, which were in the normal filamentous shape (Fig. 5A); (2) cells in which cytochrome c was distributed in the cytosol, and yet, their mitochondria remained in their filamentous shape and did not swell (Fig. 5B); and (3) cells in which cytochrome c was distributed in the cytosol, while their mitochondria were in the swollen spherical shape (Fig. 5C). Category 1 cells are believed to be those that their cytochrome c had not yet been released from mitochondria to cytosol. Category 3 cells are those that their cytochrome c was released some time ago and their mitochondria had become swollen. The existence of the Category 2 cells is most interesting; it shows that when the endogenous cytochrome c was released from mitochondria to the cytosol, their mitochondria had not yet started to swell. This result confirms the finding of our living cell measurements that cytochrome c release occurred before mitochondrial swelling.

The statistical distribution of these three categories of cells is shown in Table 1. For control cells that were not treated with UV irradiation, 99.2% of cells were found to belong to Category 1. Only 0.8% of cells were in Category 3, which represented a very small population of dead cells normally found in the cell culture. No cell in Category 2 was observed.

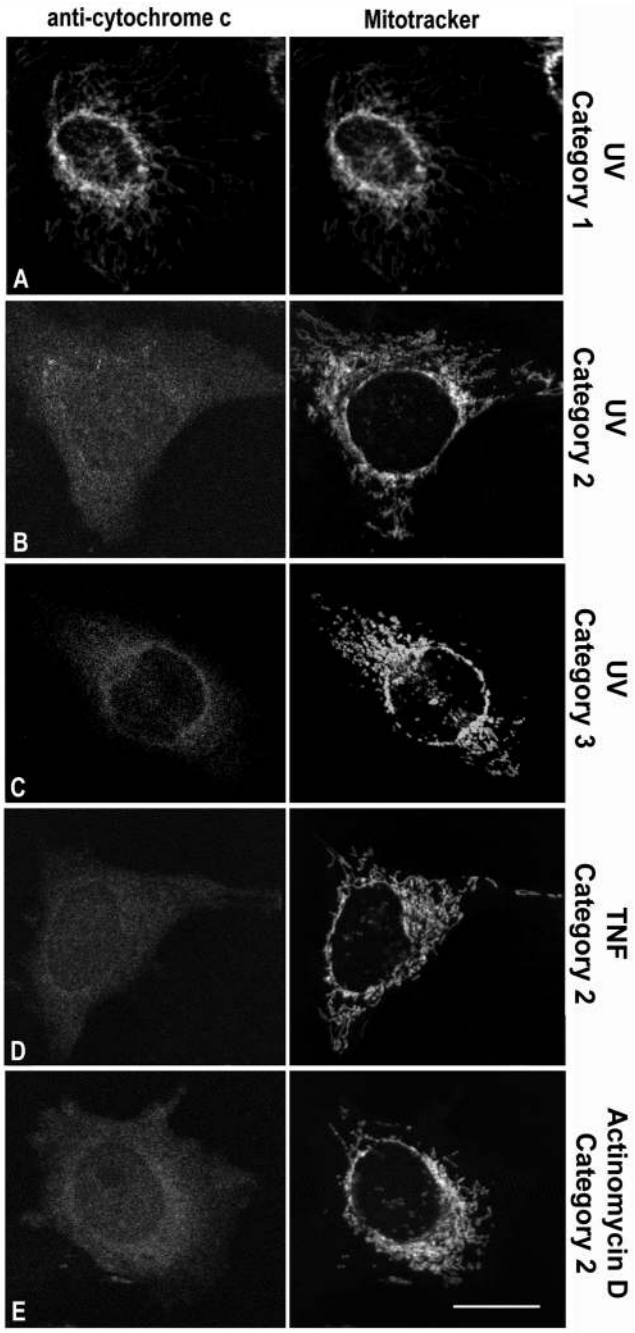


Fig. 5. Comparison of cytochrome c distribution (left) and morphology of mitochondria (right) in cells stained with anti-cytochrome c and Mitotracker Red. (A) Before the UV-treated cell entered apoptosis, cytochrome c was distributed mainly in mitochondria, which had the normal filamentous shape. (B) In a small percentage of cells undergoing UV-induced apoptosis, cytochrome c was released into the cytosol while mitochondria still remained in filamentous shape. (C) In some UV-induced apoptotic cells, cytochrome c was released into the cytosol and the mitochondria became swollen. (D,E) Results similar to those in B were observed in apoptotic cells induced by TNF (D) or actinomycin D (E) treatments. Scale bar: 20 μm.

The distribution was quite different in UV-treated cells. Three hours after UV treatment, a large number of cells were found

Table 1. Relationship between cytochrome c distribution and mitochondrial morphology in apoptotic cells

Cell treatment	Relative population of cells*			Total number of cells examined‡
	Category 1	Category 2	Category 3	
Control (without apoptotic treatment)	99.20±0.47%	0	0.80±0.47%	1978
TNF treatment for 4 hours	72.30±1.60%	2.40±0.06%	25.30±1.60%	1739
Actinomycin D treatment for 5 hours	58.30±4.50%	2.50±0.09%	39.20±4.60%	3686
3 hours after UV treatment	51.70±1.28%	2.30±0.15%	46.00±1.40%	3373
3 hours after UV treatment + z-VAD-fmk	42.40±0.35%	4.30±0.07%	53.40±0.50%	1884
3 hours after UV treatment + CsA	53.00±3.30%	3.60±1.00%	43.40±2.40%	1872

*In category 1 cells, cytochrome c was localized in mitochondria, which were filamentous; in category 2 cells, cytochrome c was distributed in cytosol; mitochondria were filamentous; and in category 3 cells, cytochrome c was distributed in cytosol; mitochondria were swollen.

‡Cells were counted from at least four independent samples.

to belong to Category 3, and the population of Category 2 cells was significantly larger than zero. The fact that the population of these Category 2 cells was much smaller than those of the other two categories indicates that the time window between the events of cytochrome c release and mitochondrial swelling must be relatively short. This finding, again, is consistent with the results of our living cell measurement.

To test whether our finding was unique in UV-induced apoptosis or a general phenomenon, we repeated the immunostaining study using various apoptotic inducing treatments, including TNF (Fig. 5D), actinomycin D (Fig. 5E) and staurosporin (data not shown). Their results were all consistent with what we found in UV-induced apoptosis. Regardless of the type of apoptotic treatment, when we examined the morphology of mitochondria and the distribution pattern of cytochrome c in a population of fixed HeLa cells that were undergoing apoptosis, we always found three categories of cells similar to those observed in UV-induced apoptosis (Table 1). In other words, there was always a small percentage of fixed cells in which cytochrome c was distributed in the cytoplasm and the mitochondria were in filamentous shape. Their existence implies that cytochrome c release must occur before mitochondrial swelling during apoptosis.

z-VAD-fmk and cyclosporin A could not inhibit cytochrome c release or mitochondrial swelling

We had applied caspase inhibitor z-VAD-fmk to UV-treated cells and found that z-VAD-fmk could block apoptosis. The application of z-VAD-fmk, however, could not inhibit cytochrome c release (Table 1). This observation is in agreement with that reported earlier by Goldstein et al. (Goldstein et al., 2000). In our experiment, we found that z-VAD-fmk could not block mitochondria swelling either. Application of a PT pore inhibitor, cyclosporin A, also failed to block UV-induced apoptosis, cytochrome c release or mitochondria swelling (Table 1). This result further argued against the hypothesis that opening of the PT pore (to cause matrix swelling) is required for cytochrome c release.

Treatment of CCCP indicated that mitochondria swelling alone could not induce cytochrome c release

To demonstrate that mitochondrial swelling alone cannot cause cytochrome c to release, we have conducted another living cell imaging experiment. After we expressed the GFP-cytochrome c fusion gene in HeLa cells and labeled them with Mitotracker (Fig. 6A), we applied carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) to induce mitochondrial swelling. Treatment of CCCP is known to cause a short circuit effect in the proton gradient of the mitochondrial inner membrane and induce mitochondria to swell (Minamikawa et al., 1999). Before application of CCCP, mitochondria were in the normal filamentous shape (Fig. 6A). Eight minutes after CCCP application, some mitochondria were seen to start to swell (Fig. 6B). After 19 minutes, most mitochondria had become swollen and changed into spherical shapes – yet cytochrome c was not released (Fig. 6C). In fact, cytochrome c was found to remain in the mitochondria even after mitochondria had been in the swollen state for more than 1 hour (Fig. 6D). Furthermore, this CCCP-induced mitochondrial swelling was reversible. Mitochondria returned to their filamentous structure after CCCP was removed and the cell was found to recover eventually. This result shows that mitochondrial swelling alone cannot cause cytochrome c to release or induce apoptosis.

DISCUSSION

According to a recent review on the functional roles of mitochondria on apoptosis (Desagher and Martinou, 2000), there are currently five major models for the mechanisms of cytochrome c release; two of which are dependent on matrix swelling. The first model is related to PT pore opening, as discussed in the Introduction here. In the second swelling model, it is thought that impairment of ATP-ADP exchange by VDAC (voltage-dependent anion channel) closure might contribute to the hyperpolarization of the inner mitochondrial membrane. Such an increase of the mitochondrial transmembrane potential could promote an osmotic matrix swelling and thus cytochrome c release (Vander Heiden and Thompson, 1999). These swelling models are not supported by the results of our study. Findings from our imaging study suggest that mitochondrial swelling was the result rather than the cause of cytochrome c release during apoptosis. Consistent with an earlier report (Goldstein et al., 2000), we found that the release of cytochrome c was a very rapid and highly synchronized event. Results of our single-cell and single-mitochondrion measurements show that mitochondria started to swell only after their cytochrome c had been released. The latency between these two events was just a few minutes.

The degree of mitochondrial swelling after cytochrome c release appeared to be relatively mild and did not necessarily cause a rupture of its outer membrane. From the simple mathematical model shown in Fig. 1C, we estimated that when

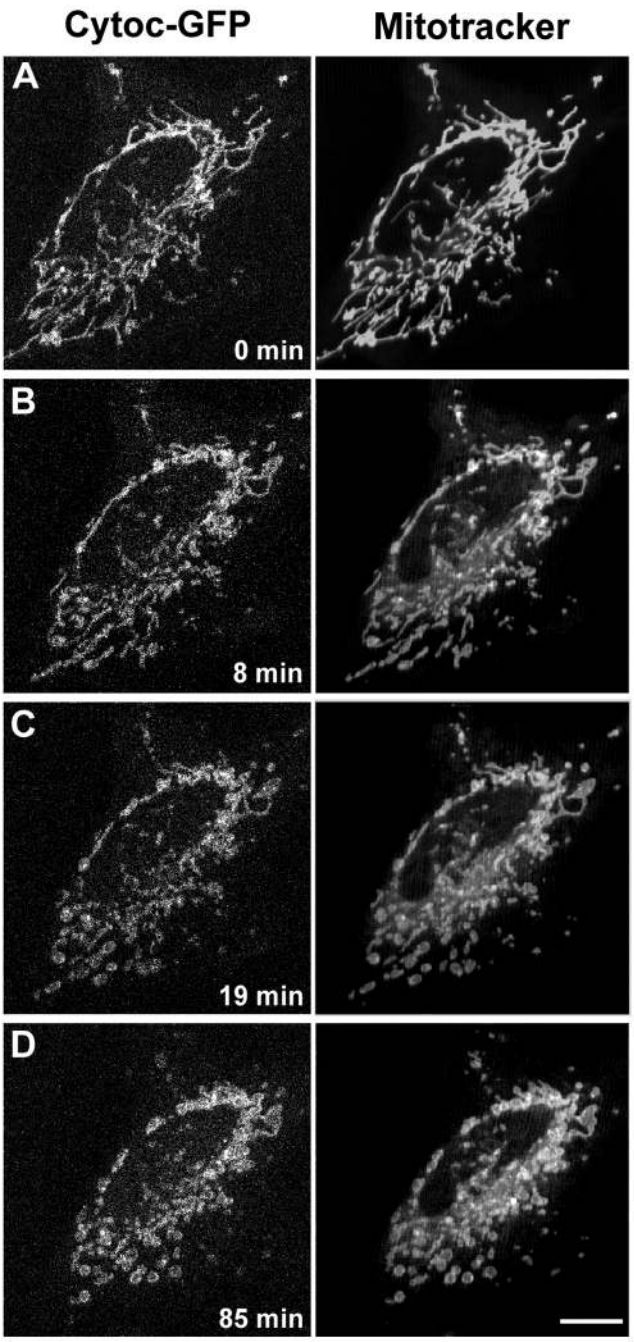


Fig. 6. Simultaneous confocal measurements of cytoc-GFP distribution (left) and Mitotracker distribution (right) in a living cell treated with CCCP (10 μM). Elapsed time following the CCCP treatment was shown in each panel. Scale bar: 20 μm.

a mitochondrion swells by changing to a spherical shape, it can increase its diameter by 2.34-fold without increasing its surface area. In the experiment shown in Fig. 2, the average diameter of mitochondria was found to increase to 2.21-fold after cytochrome c release. The extent of mitochondria swelling varied slightly from cell to cell; its average was 1.99±0.17 over six cells that we analyzed. This observed diameter change was within the theoretical limit of the non-stretching model (Fig. 1C). Thus, our data suggest that, even though mitochondria did swell after cytochrome c release, the swelling probably did not cause their outer membrane to rupture. Alternatively, this relatively small change in diameter may indicate that, after the release of cytochrome c, the filamentous mitochondria could be broken into shorter pieces before swelling.

If mitochondrial swelling was not the cause of cytochrome c release, then what would facilitate cytochrome c to redistribute during apoptosis? There are several alternative models available at present (Desagher and Martinou, 2000). Cytochrome c could be released through specific channels, such as Bax channel or Bax-VDAC channel, or through lipid pore (or protein-lipid complex) at the outer membrane of mitochondria (Martinou et al., 2000). Some recent studies have presented evidence to support these channel models (Antonsson et al., 2000; Basanez et al., 1999; Shimizu et al., 1999). For example, Shimizu et al. have reported that cytochrome c can pass through VDAC channel incorporated into lipid vesicles with the help of Bax (Shimizu et al., 1999). There is still a problem, however, with this model. The VDAC channel has a relative small opening; proteins like GST-GFP (50 kDa) could not pass through the VDAC channel (Shimizu et al., 1999). But during apoptosis, many proteins in the intermembrane space, including AIF (57 kDa), were released (Kluck et al., 1999; Susin et al., 1999). At this point, we feel that protein-lipid complex formed by Bax (or other Bcl-2 family proteins) insertion into the outer mitochondrial membrane may offer the most attractive model.

Another question is what caused mitochondria to swell after cytochrome c was released. Such mitochondrial swelling was probably not caused by a caspase feedback loop, as we found that caspase inhibitor z-VAD-fmk could not block mitochondria swelling (Table 1). As the PT pore inhibitor CsA also failed to block the swelling, one may suggest that reactive oxygen species (ROS) generated after cytochrome c release might play specific roles. It has been reported earlier that ROS can oxidize lipids and proteins in the inner membrane of mitochondria to cause membrane permeabilization and mitochondria swelling (Kowaltowski et al., 1996).

In conclusion, we have shown that during apoptosis, release of cytochrome c occurred before mitochondria swelling. The interval between the two events was relatively short (within about 10 minutes). Thus, release of cytochrome c could not be caused by rupturing the outer mitochondrial membrane due to matrix swelling. This conclusion holds true for apoptosis induced by various treatments including UV, TNF, actinomycin D and staurosporin. It is possible that, the mitochondrial swelling model might play a more appropriate role in the process of necrosis (Trump, 1981).

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REFERENCES

Antonsson, B., Montessuit, S., Lauper, S., Eskes, R. and Martinou, J. C. (2000). Bax oligomerization is required for channel-forming activity in liposomes and to trigger cytochrome c release from mitochondria. *Biochem J.* **345**, 271-278.

Basanez, G., Nechushtan, A., Drozhinin, O., Chanturiya, A., Choe, E., Tutt, S., Wood, K. A., Hsu, Y., Zimmerberg, J. and Youle, R. J. (1999).

Bax, but not Bcl-xL, decreases the lifetime of planar phospholipid bilayer membranes at subnanomolar concentrations. *Proc. Natl. Acad. Sci. USA* **96**, 5492-5497.

Bradham, C. A., Qian, T., Streetz, K., Trautwein, C., Brenner, D. A. and Lemasters, J. J. (1998). The mitochondrial permeability transition is required for tumor necrosis factor alpha-mediated apoptosis and cytochrome c release. *Mol. Cell. Biol.* **18**, 6353-6364.

Cai, J., Yang, J. and Jones, D. P. (1998). Mitochondrial control of apoptosis: the role of cytochrome c. *Biochim. Biophys. Acta* **1366**, 139-149.

Chang, D. C. (1997). Experimental strategies in efficient transfection of mammalian cells. Electroporation. *Methods Mol. Biol.* **62**, 307-318.

Desagher, S. and Martinou, J. C. (2000). Mitochondria as the central control point of apoptosis. *Trends Cell Biol.* **10**, 369-377.

Eskes, R., Antonsson, B., Osen-Sand, A., Montessuit, S., Richter, C., Sadoul, R., Mazzei, G., Nichols, A. and Martinou, J. C. (1998). Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. *J. Cell Biol.* **143**, 217-224.

Goldstein, J. C., Waterhouse, N. J., Juin, P., Evan, G. I. and Green, D. R. (2000). The coordinate release of cytochrome c during apoptosis is rapid, complete and kinetically invariant. *Nat. Cell Biol.* **2**, 156-162.

Green, D. R. and Reed, J. C. (1998). Mitochondria and apoptosis. *Science* **281**, 1309-1312.

Heiskanen, K. M., Bhat, M. B., Wang, H. W., Ma, J. and Nieminen, A. L. (1999). Mitochondrial depolarization accompanies cytochrome c release during apoptosis in PC6 cells. *J. Biol. Chem.* **274**, 5654-5658.

Jürgensmeier, J. M., Xie, Z., Deveraux, Q., Ellerby, L., Bredesen, D. and Reed, J. C. (1998). Bax directly induces release of cytochrome c from isolated mitochondria. *Proc. Natl. Acad. Sci. USA* **95**, 4997-5002.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R. and Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* **275**, 1132-1136.

Kluck, R. M., Esposti, M. D., Perkins, G., Renken, C., Kuwana, T., Bossy-Wetzel, E., Goldberg, M., Allen, T., Barber, M. J., Green, D. R. et al. (1999). The pro-apoptotic proteins, Bid and Bax, cause a limited permeabilization of the mitochondrial outer membrane that is enhanced by cytosol. *J. Cell Biol.* **147**, 809-822.

Kowaltowski, A. J., Castilho, R. F., Grijalba, M. T., Bechara, E. J. and Vercesi, A. E. (1996). Effect of inorganic phosphate concentration on the nature of inner mitochondrial membrane alterations mediated by Ca2+ ions. A proposed model for phosphate-stimulated lipid peroxidation. *J. Biol. Chem.* **271**, 2929-2934.

Kroemer, G., Zamzami, N. and Susin, S. A. (1997). Mitochondrial control of apoptosis. *Immunol. Today* **18**, 44-51.

Krohn, A. J., Wahlbrink, T. and Prehn, J. H. (1999). Mitochondrial depolarization is not required for neuronal apoptosis. *J. Neurosci.* **19**, 7394-7404.

Li, C. J., Heim, R., Lu, P., Pu, Y., Tsien, R. Y. and Chang, D. C. (1999). Dynamic redistribution of calmodulin in HeLa cells during cell division as revealed by a GFP-calmodulin fusion protein technique. *J. Cell Sci.* **112**, 1567-1577.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R. and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* **86**, 147-157.

Mahajan, N. P., Linder, K., Berry, G., Gordon, G. W., Heim, R. and Herman, B. (1998). Bcl-2 and Bax interactions in mitochondria probed with green fluorescent protein and fluorescence resonance energy transfer. *Nat. Biotechnol.* **16**, 547-552.

Martinou, J. C., Desagher, S. and Antonsson, B. (2000). Cytochrome c release from mitochondria: all or nothing. *Nat. Cell Biol.* **2**, E41-E43.

Matsuyama, S., Llopis, J., Deveraux, Q. L., Tsien, R. Y. and Reed, J. C. (2000). Changes in intramitochondrial and cytosolic pH: early events that modulate caspase activation during apoptosis. *Nat. Cell Biol.* **2**, 318-325.

Minamikawa, T., Williams, D. A., Bowser, D. N. and Nagley, P. (1999). Mitochondrial permeability transition and swelling can occur reversibly without inducing cell death in intact human cells. *Exp. Cell Res.* **246**, 26-37.

Narita, M., Shimizu, S., Ito, T., Chittenden, T., Lutz, R. J., Matsuda, H. and Tsujimoto, Y. (1998). Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. *Proc. Natl. Acad. Sci. USA* **95**, 14681-14686.

Pastorino, J. G., Chen, S. T., Tafani, M., Snyder, J. W. and Farber, J. L. (1998). The overexpression of Bax produces cell death upon induction of the mitochondrial permeability transition. *J. Biol. Chem.* **273**, 7770-7775.

Pastorino, J. G., Tafani, M., Rothman, R. J., Marcineviciute, A., Hoek, J. B. and Farber, J. L. (1999). Functional consequences of the sustained or transient activation by bax of the mitochondrial permeability transition pore. *J. Biol. Chem.* **274**, 31734-31739.

Scarletta, J. L., Sheardb, P. W., Hughesa, G., Ledgerwooda, E. C., Kuc, H. and Murphya, M. P. (2000). Changes in mitochondrial membrane potential during staurosporine-induced apoptosis in jurkat cells. *FEBS Lett.* **475**, 267-272.

Scheffler, I. E. (1999). *Mitochondria*. New York: Wiley-Liss.

Shimizu, S., Narita, M. and Tsujimoto, Y. (1999). Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* **399**, 483-487.

Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M. et al. (1999). Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* **397**, 441-446.

Susin, S. A., Zamzami, N. and Kroemer, G. (1998). Mitochondria as regulators of apoptosis: doubt no more. *Biochim. Biophys. Acta* **1366**, 151-165.

Trump, B. F., Berezesky, I. K. and Osorino-Vargas, A. R. (1981). Cell death and the disease process: the role of calcium. In *Cell Death in Biology and Pathology*. London: Chapman & Hall.

Vander Heiden, M. G., Chandel, N. S., Williamson, E. K., Schumacker, P. T. and Thompson, C. B. (1997). Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell* **91**, 627-637.

Vander Heiden, M. G. and Thompson, C. B. (1999). Bcl-2 proteins: regulators of apoptosis or of mitochondrial homeostasis? *Nat. Cell Biol.* **1**, E209-E216.

von Ahsen, O., Renken, C., Perkins, G., Kluck, R. M., Bossy-Wetzel, E. and Newmeyer, D. D. (2000). Preservation of mitochondrial structure and function after Bid- or Bax-mediated cytochrome c release. *J. Cell Biol.* **150**, 1027-1036.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P. and Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* **275**, 1129-1132.