Lack of Release of Cytochrome c from Mitochondria into Cytosol Early in the Course of Fas-mediated Apoptosis of Jurkat Cells*

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Several groups have reported that during apoptosis, cytochrome c is released from the mitochondria into the cytosol, but we have found that in apoptotic cells the cytochrome appears to remain with the mitochondria. In hopes of reconciling these findings, we compared the results obtained from cells disrupted by our method (nitrogen cavitation) with those obtained using the cell-disruption method employed by others (homogenization). We observed that at 2 h, cytochrome c levels in apoptotic cytosols from homogenized cells exceeded control levels, whereas cytochrome c levels in apoptotic cytosols from cavitated cells were similar to control. Outer membranes of homogenized mitochondria appeared damaged because the mitochondria had become permeable to cytochrome c, whereas outer membranes of cavitated mitochondria excluded the cytochrome. 4 h after Fas ligation, both cavitated and homogenized mitochondria had released small amounts of cytochrome c into the cytosol, whereas after 6 h the cytochrome had disappeared from the cell lysate. We believe that the differences between our results and those reported by others were due to 1) our examining the cells after a short (2 h) incubation with the anti-Fas antibody, and 2) our use of nitrogen cavitation instead of homogenization to disrupt the cells.

Apoptosis comprises a series of events that occur in response to the activation of a program found in most cells whose purpose is to kill the cell without releasing its contents into the extracellular environment. The events of apoptosis usually include among other things the breakdown of the genome into nucleosomal fragments (i.e. fragments that are integral multiples of ~200 base pairs); the activation of the caspases, a family of proteases that cleave a limited set of proteins (e.g. poly-(ADP)ribose polymerase, lamin B, fodrin) on the C-terminal side of an aspartate residue; the cross-linking of actin, and the appearance of phosphatidylserine on the cell surface. Ultimately the apoptotic cell sheds its substance by blebbing, and the released fragments are taken up and degraded by mononuclear phagocytes.

The involvement of mitochondria in apoptosis was first suggested by the discovery that the anti-apoptotic protein Bcl-2 is found in the mitochondrial outer membrane (1, 2). A later report attributed apoptosis to the release of apoptosis-inducing factor from the mitochondrial intermembrane space because of the opening of the mitochondrial permeability transition pore in cells undergoing apoptosis (3). We ourselves found that cytoplasm from apoptotic cells contains cytochrome c inactivating factor of apoptosis, a substance that within minutes eliminates the ability of cytochrome c to transfer electrons to cytochrome oxidase (4). Most recently it was shown that cytochrome c, in collaboration with APAF-1, the mammalian homolog of the Caenorhabditis elegans death protein Ced-4, assists in the activation of the caspases (5, 6).

There is, however, an unsettled question concerning the participation of cytochrome c in apoptosis. Several laboratories have reported that during apoptosis, some of the cytochrome is released from the mitochondria into the cytosol where it exerts its effect on the caspases (7, 8). We, however, have reported that the cytochrome remains associated with the mitochondria during apoptosis (4, 9). For our study we measured cytochrome c spectrophotometrically, but others measured the cytochrome c by immunoblotting. In hopes of reconciling these findings, we re-examined the distribution of cytochrome c in apoptotic cells using immunoblotting to quantify the cytochrome.

MATERIALS AND METHODS

Anti-Fas antibody (clone CH-11) was purchased from Kamiya Biomedical, Thousand Oaks, CA, anti-cytochrome c antibody from Pharmingen, San Diego, and F(ab’)2 anti-mouse and anti-rabbit (human-adsorbed) alkaline phosphatase-conjugated secondary antibodies from Caltag, Burlingame CA. Jurkat cells were obtained from the American Type Culture Collection. The cells were grown in RPMI 1640, 5% fetal calf serum, 2 mM glutamate to a density of 10⁶ cells/ml. For use, they were pelleted and resuspended in serum-free medium at 4 x 10⁷ cells/ml, then incubated at 37 °C with or without 0.5 μg/ml anti-Fas IgM for the indicated lengths of time. The cells were then washed in ice-cold Dulbecco’s phosphate-buffered saline, then divided into 2 groups, one for disruption by N₄ cavitation and the other for disruption by homogenization in a Potter-Elvehjem homogenizer with a Teflon pestle. All subsequent steps were carried out on ice or at 4 °C. For N₄ cavitation, the cells were washed with MA buffer (100 mM sucrose, 1 mM EGTA, 20 mM MOPS (pH 7.4), bovine serum albumin 1 g/liter) then resuspended at 2 x 10⁸ cells/ml in MB buffer (MA buffer plus 10 mM triethanolamine, 5% Ficoll, and an antiprotease mixture consisting of aprotinin, pepstatin A, and leupeptin, each at 10 μM, and 1 mM phenylmethylsulfonyl fluoride). The cells were then disrupted by N₄ cavitation as described elsewhere. For homogenization, the cells were pelleted and resuspended at 2 x 10⁶ cells/ml in homogenization buffer (250 mM sucrose, 20 mM K⁺ HEPES (pH 7.5), 10 mM KCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride), and disrupted by homogenization by the method of Yang et al. (8). Lysates prepared by either method were centrifuged twice at 2500 x g for 5 min to remove nuclei and unbroken cells then at 25,000 x g for 30 min to isolate mitochondria. The post-mitochondrial supernatant was spun at 100,000 x g for 30 min to yield cytosol, which was saved, and membrane particles, which were discarded. The mitochondria were suspended in MC buffer (identical to MA buffer except that the sucrose concentration was 300 mM and an antiprotease mixture was included that consisted of aprotinin, pepstatin A, and leupeptin, each at 10 μM) and used for measuring oxygen consumption and cytochrome c content.

In the preparation of mitochondria, all steps were carried out at 4 °C. To quantify apoptosis, cells were fixed with 4% formalin in phosphate-buffered saline, stained with acridine orange, and then evaluated
Lack of Cytochrome c Release in Fas-mediated Apoptosis

We felt that it was important not only to try to repeat our own results, but also to see if we could reproduce the results obtained in other laboratories. One difference between our work and that of other groups was the method used for preparing mitochondria. Our method involved disrupting the cells by nitrogen cavitation in a buffer free of potassium, whereas the other groups used homogenization in a potassium-containing buffer to prepare their mitochondria. To see if these methodological differences might account for the differences in results among the various laboratories, we disrupted control and apoptotic cells by both methods and examined the cytochrome c content of the particulate and cytosolic fractions by immunoblotting. The results (Fig. 1 and Table I) indicated that at 2 h, when almost half the anti-Fas-treated cells showed apoptotic nuclei, there was no significant difference between the cytochrome c content of control and apoptotic cytosols obtained by nitrogen cavitation, whereas apoptotic cytosols from homogenized cells consistently contained more cytochrome c than the controls.

Table I

| Apoptosis          | Cytosolic cytochrome c | Mitochondrial cytochrome c |
|--------------------|------------------------|---------------------------|
|                    | Cavitate | Homogenate | Cavitate | Homogenate |
| Anti-Fas treated   | 45.0 ± 5.0 | 78.4 ± 13.0 | 191.4 ± 12.6 | 97.3 ± 6.5 |
| Control            | 2.8 ± 0.3 | 100        | 100       | 75.6 ± 6.8 |
| Significance       |          |            | p < 0.002 |          |

* NS, not significant.

Results

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corresponding control cytosols.

To determine whether the release of cytochrome \( c \) from mitochondria prepared from homogenized cells was a result of the homogenization procedure or of the relatively minor differences in the composition of our buffer versus the buffers used in the other studies, we examined cytochrome \( c \) release in cells homogenized in each of the two buffers. The results (Table II) showed that the amounts of cytochrome \( c \) released into the cytosol of anti-Fas-treated cells were similar in the two buffers, suggesting that the difference in cytochrome \( c \) release between the cavitated and homogenized samples was because of the cell disruption procedure rather than the buffers.

At later times, the release of a small amount of cytochrome \( c \) into the cytosol during apoptosis was seen even in cells disrupted by nitrogen cavitation. Table III shows the amount of cytochrome \( c \) in cavitated cytosols from cells treated for 2 and 4 h with anti-Fas, as compared with cytochrome \( c \) from control cells incubated for similar lengths of time. The results showed that cytochrome \( c \) levels were similar in the 2 h cytosols, but that at 4 h the amounts of cytochrome \( c \) in apoptotic cytosols exceeded that in the control cytosols. At 6 h, however, cytochrome \( c \) had largely disappeared from the mitochondria of apoptotic cells and was undetectable in their cytosol, though the cytochrome \( c \) distribution in the control cells was unchanged from earlier times (not shown).

Finally, experiments were carried out to assess the integrity of the outer membrane in mitochondria prepared from nitrogen-cavitated or homogenized cells. This was accomplished by determining whether exogenous cytochrome \( c \) had access to cytochrome oxidase, as measured by the ability of the cytochrome to pass electrons to cytochrome oxidase. The results (Table IV) showed that the oxidation of reduced cytochrome \( c \) by mitochondria from nitrogen-cavitated cells was barely detectable in the absence of digitonin, a steroid that permeabilizes the outer mitochondrial membrane, but was brisk when digitonin was present in the assay mixture. In contrast, mitochondria from homogenized cells were able to oxidize cytochrome \( c \) in the absence of digitonin at more than 50% the rate seen in the presence of digitonin. These findings indicate that in the nitrogen-cavitated mitochondria the outer membranes were essentially intact, although they were extensively disrupted in the homogenized mitochondria.

**DISCUSSION**

The foregoing results suggest that at least in anti-Fas-treated Jurkat cells, cytochrome \( c \) remains associated with the mitochondria during the first 2 h of apoptosis, by which time nearly 50% of the cells show the nuclear changes of apoptosis. Despite these results, our earlier findings and the findings from other laboratories clearly indicate that the interaction between cytochrome \( c \) and the other mitochondrial components is altered in some fundamental way in apoptotic cells. This is shown both by the defunctionalization of the cytochrome in apoptotic mitochondria and by the greater ease with which the cytochrome is displaced from such mitochondria as compared with its behavior in control mitochondria, as indicated by the results obtained when the cells were homogenized instead of cavitated. Elucidating the basis for this alteration is likely to lead to novel insights into the mechanism of apoptosis.

It is clear that in a cell-free system under appropriate conditions, cytochrome \( c \) is able to activate caspases, implying that it can perform this function in intact cells undergoing apoptosis. The results reported here are not in conflict with this idea. Our results do suggest, however, that in our system, cytochrome \( c \)-dependent caspase activation has to take place in the mitochondrial intermembrane space. For this to occur, it is likely that an apoptosis-dependent change in the properties of the outer mitochondrial membrane must take place so that APAF-1 and the pro-caspases can gain access to the cytochrome. Consistent with this idea, we have reported an increase in the permeability of the outer membrane of mitochondria isolated from apoptotic cells, whereas Wang and co-workers found that during apoptosis, the serine-threonine kinase Raf is transferred to the outer mitochondrial membrane (13). Further studies of the outer membrane of apoptotic mitochondria are currently in progress in our laboratory.

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