Loss of Function of Cytochrome c in Jurkat Cells Undergoing Fas-mediated Apoptosis*

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Mitochondrial function was examined in Jurkat cells undergoing Fas-mediated apoptosis. With succinate or ascorbate/tetramethylphenylenediamine as substrate, oxygen uptake by digitonin-permeabilized apoptotic mitochondria was greatly decreased as compared with control. Assessment of the function of the cytochrome c-cytochrome oxidase segment of the electron transport chain of apoptotic mitochondria showed that the activity of cytochrome oxidase appeared to be normal, but that of cytochrome c was greatly diminished. A death protease was found to participate in the events leading to the loss of cytochrome c activity, but the cytochrome did not seem to be extensively degraded during the course of apoptosis. Our results suggest that a rapid loss in mitochondrial function due at least in part to the inhibition or inactivation of cytochrome c is a potentially fatal component of the apoptosis program of Jurkat cells.

Most cells are equipped with a program whose activation results in a stereotyped series of events that culminate in the death and fragmentation of the cell. In aggregate, these events refer to as apoptosis, and their effect is to destroy the cell without releasing its contents into the external environment. During apoptosis, the surface of the dying cell is altered so as to mark the cell and its fragments as targets for macrophages, which ingest and degrade the remains.

During apoptosis, much of the cell’s contents, including many of its organelles, its cytoskeleton and its plasma membrane undergo far-reaching changes. In the nucleus, the chromatin is disrupted, lamin B (1) is degraded and the DNA is digested into fragments whose length is an integral multiple of ~200 base pairs, the length of the DNA in a nucleosome. A number of proteins, including among others poly(ADP-ribose) polymerase (2), the 70-kDa protein of the U1 small nuclear ribonucleoprotein (3), α-fodrin (4), topoisomerase I, histone H1, and phospholipase A2 (5), are cleaved into defined fragments, presumably with an alteration in their activity. Other proteins are cross-linked by transglutamination, with eventual condensation of the cell and its fragments (6). Cytoskeletal changes result in nuclear fragmentation and blebbing at the cell periphery, substance being lost when a bleb detaches from the main body of the cell, sometimes taking with it a fragment of nucleus. Alterations in cell lipids also occur, with a large increase in levels of ceramide (7), a molecule that may activate the cell death program, and the transfer of phosphatidylserine from the inner to the outer leaflet of the plasma membrane (8).

Mitochondria are also affected by the cell death program. Many studies have shown a partial depolarization of the mitochondrial membrane potential in apoptotic cells (9–15), and most (9–11, 16, 17) but not all (18) investigators believe that the oxidants thought to be important in apoptosis induced by tumor necrosis factor-α are generated by the mitochondria. Using specific substrates and tumor necrosis factor-α-treated L929 cells, Schulze-Osthoff et al. (17) demonstrated that electron flow through all four mitochondrial electron transport complexes fell steadily, declining at a rate that was approximately equal among the complexes as the cells progressed through apoptosis. We have studied mitochondrial function in Jurkat cells sent into apoptosis with an anti-Fas IgM, and report that the defect in mitochondrial electron transport observed by ourselves and others in apoptotic cells is due at least in part to the inactivation of cytochrome c.

**MATERIALS AND METHODS**

Cell Culture—Jurkat cells, a lymphoblastoid T-cell line, were the kind gift of D. Green, La Jolla Institute for Allergy and Immunology. They were cultured at 10^6 cells/ml in RPMI 1640 with 5% fetal calf serum, 2 mM L-glutamine, and penicillin-streptomycin. Individual cultures were maintained for no more than 2 months. Apoptosis was induced by treatment with anti-Fas IgM (clone CH-11, Kamiya Biomedical Co., Thousand Oaks, CA) at the concentration indicated in the figure legends.

Morphological Assessment of Apoptosis—Cells were applied to glass slides (Superfrost/Plus, Fisher), fixed for 5 min in phosphate-buffered saline containing 4% formalin solution, rinsed in methanol, and air-dried. A drop of acridine orange solution (4 g/ml) was placed over the cells, a coverslip was added, and the cells were viewed by fluorescence microscopy. Apoptotic cells were scored based on characteristic changes of chromatin condensation and nuclear fragmentation. On each slide, a minimum of 200 cells were evaluated.

Flow Cytometry—DNA content and mitochondrial membrane potential were analyzed on a Coulter Elite flow cytometer. For measurements of DNA content, cells were fixed in 50% ethanol and stored at 4 °C until analysis, when they were stained with propidium iodide (50 μg/ml), treated with RNase (10 μg/ml), and washed in Dulbecco’s phosphate-buffered saline. DNA content was calculated from the flow cytometry data using Multicycle® software (Phoenix Flow Systems, San Diego) (19). To determine the mitochondrial membrane potential during apoptosis, the cells were treated with anti-Fas as antibody (100 ng/ml) for 2 h in serum-free RPMI. During the last 30 min of treatment, the cells were loaded with di-OH(3) (0.1 μM) (14). At the conclusion of the incubation, the cells were isolated by centrifugation, resuspended in Hanks’ balanced salt solution and analyzed immediately. The zero-time value and the value for mitochondria whose transmembrane potential was discharged with carbonyl cyanide m-chlorophenylhydrazine (CCCP) were obtained from cells that had been incubated for 30 min with di-OH(3) without exposure to anti-Fas antibody.

1 The abbreviations used are: di-OH(3), 3,3′-dihexyloxycarbocyanine iodide; CCCP, carbonyl cyanide m-chlorophenylhydrazine; MOPS, 4-morpholinopropanesulfonic acid; TMPD, tetramethyl-p-phenylenediamine; Z-VAD-fluoromethyl ketone, carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone.

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**Oxygen Electrode Measurements**—A Clarke oxygen electrode (Yellow Springs Instruments, Yellow Springs, OH) with a thermomicrotector chamber was used. To induce apoptosis, cells were treated in culture (serum-free RPMI) with anti-Fas antibody (100 ng/ml) at the times indicated; control cells were incubated in a similar manner either with an irrelevant IgM antibody (an anti-cytochrome P450 monoclonal antibody generously provided by Dr. Eric Johnson) or without antibody. The cells were then centrifuged and resuspended in respiration buffer (0.25 mm sucrose, 0.1% bovine serum albumin, 10 mm KCl, 10 mm Hepes, 5 mm KH2PO4, pH 7.2) at a final concentration of 6 × 10^6 cells/ml. One-half ml of the suspension was injected into a chamber containing 2.5 ml of air-saturated respiration buffer, 1 mM ADP that had been prewarmed to 37°C. The cells were permeabilized with digitonin (final concentration 0.005%), and substrates and inhibitors were added in the following order and final concentrations: malate, 5 mm pyruvate, 5 mm; rotenone, 100 mm; succinate, 5 mm; antimycin A, 50 mm; ascorbate, 1 mm; (2 mM in experiments using horse heart cytochrome c) with TMPD (tetrathylammonium tetrafluoroborate) 0.4 mm; NaN3, 5 mm. Where noted, the uncoupling agent CCCP was added at a final concentration of 5 μM. Oxygen consumption was calibrated with air-saturated buffer, assuming 390 ng-atoms of oxygen/ml of buffer (17). Transient downward deflections indicate the points at which new reagents were added to the samples in the oxygen electrodes. Rates of azide-sensitive oxygen consumption are expressed as ng-atoms of oxygen/min/3 × 10^7 cells.

Preparation of Mitochondria from Jurkat Cells—Jurkat cells (10^8) were pelleted and resuspended in 1 ml of buffer B (buffer A plus 10 mM Hepes, 10 mM KCl, 5 mm MgCl2, 10 mM KCl, 5 mm Hepes, 5 mm KH2PO4, pH 7.2) at a final concentration of 6 × 10^6 cells/ml. One-half ml of the suspension was injected into a chamber containing 2.5 ml of air-saturated respiration buffer, 1 mM ADP that had been prewarmed to 37°C. The cells were permeabilized with digitonin (final concentration 0.005%), and substrates and inhibitors were added in the following order and final concentrations: malate, 5 mm pyruvate, 5 mm; rotenone, 100 mm; succinate, 5 mm; antimycin A, 50 mm; ascorbate, 1 mm; (2 mM in experiments using horse heart cytochrome c) with TMPD (tetrathylammonium tetrafluoroborate) 0.4 mm; NaN3, 5 mm. Where noted, the uncoupling agent CCCP was added at a final concentration of 5 μM. Oxygen consumption was calibrated with air-saturated buffer, assuming 390 ng-atoms of oxygen/ml of buffer (17). Transient downward deflections indicate the points at which new reagents were added to the samples in the oxygen electrodes. Rates of azide-sensitive oxygen consumption are expressed as ng-atoms of oxygen/min/3 × 10^7 cells.

Preparation of Mitochondria from Jurkat Cells—Jurkat cells (10^8) were incubated for 2.5–3.5 h at 37°C with or without anti-Fas antibody (100 ng/ml). Cells were pelleted and resuspended in 1 ml of buffer B (buffer A plus 10 mM Hepes, 10 mM KCl, 5 mm MgCl2, 10 mM KCl, 5 mm Hepes, 5 mm KH2PO4, pH 7.2) at a final concentration of 6 × 10^6 cells/ml. One-half ml of the suspension was injected into a chamber containing 2.5 ml of air-saturated respiration buffer, 1 mM ADP that had been prewarmed to 37°C. The cells were permeabilized with digitonin (final concentration 0.005%), and substrates and inhibitors were added in the following order and final concentrations: malate, 5 mm pyruvate, 5 mm; rotenone, 100 mm; succinate, 5 mm; antimycin A, 50 mm; ascorbate, 1 mm; (2 mM in experiments using horse heart cytochrome c) with TMPD (tetrathylammonium tetrafluoroborate) 0.4 mm; NaN3, 5 mm. Where noted, the uncoupling agent CCCP was added at a final concentration of 5 μM. Oxygen consumption was calibrated with air-saturated buffer, assuming 390 ng-atoms of oxygen/ml of buffer (17). Transient downward deflections indicate the points at which new reagents were added to the samples in the oxygen electrodes. Rates of azide-sensitive oxygen consumption are expressed as ng-atoms of oxygen/min/3 × 10^7 cells.

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**Electron Microscopy**—Control Jurkat cells and cells treated with anti-Fas antibody were collected by centrifugation (2000 rpm for 3 min at room temperature in a Beckman GPR centrifuge). The cell pellet was fixed in Karnovsky's fixative, embedded in epon resin (T. Pella Eponate 812) and cut into 100-nm sections with an LKB Ultratome V. The sections were stained with Reynolds's lead citrate and examined under a Hitachi HU 12A transmission electron microscope at 75 kV.

Replicates—Each oxygen electrode tracing shown in this paper is representative of at least two experiments.

**RESULTS**

Inhibition of Mitochondrial Electron Transport during Fas-mediated Apoptosis—Mitochondrial electron transport is mediated by four multisubunit complexes, designated complexes I–IV, that reside in the inner mitochondrial membrane (Fig. 1). Complexes I and II accept electrons from NADH and succinate, respectively, then pass these electrons on to Complex III via ubiquinone, a low molecular weight redox carrier. Complex III transfers the electrons to cytochrome c, which in turn donates them to Complex IV (cytochrome oxidase). From there the electrons are transferred 4 at a time to molecular oxygen, producing 2 molecules of water.
The function of these complexes in mitochondria from control cells and cells treated with anti-Fas antibody was assayed by the sequential addition of substrates specific for various segments of the electron transport path after permeabilization of the mitochondrial outer membrane with digitonin (Fig. 2). The malate-pyruvate combination, which transfers electrons to oxygen via NADH → I → III → IV, produced little oxygen consumption in either control or apoptotic cells. Succinate, however, passing electrons to oxygen via II → III → IV, generated oxygen uptake that was readily apparent in control cells but not detectable in apoptotic cells. Most striking were the ascorbate/TMPD results, which reflect only electron transport through cytochrome oxidase. With ascorbate/TMPD as substrate, oxygen uptake by apoptotic cells was only a small fraction of that seen with the control cells. The marked inhibition of oxygen uptake in control cells by N3 indicated that cytochrome oxidase was responsible for almost all the oxygen taken up by these cells. These results indicate that electron transport by cytochrome oxidase was greatly inhibited in apoptotic cells, a finding that could also explain the reduction in oxygen uptake in response to succinate, because succinate electrons have to pass through cytochrome oxidase on their way to oxygen.

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Fig. 3 shows the time course of cytochrome oxidase inhibition. For this experiment, a suspension of Jurkat cells was incubated with anti-Fas antibody (100 ng/ml) or a control IgM for the times noted (times include 15 min of handling at room temperature until substrate addition), then placed in the oxygen electrode cuvette. Oxygen consumption was measured using ascorbate/TMPD as the electron donor. Numerical values represent azide-sensitive oxygen consumption (ng-atmO2/min/3 x 107 cells).

Loss of Function of Cytochrome c in Apoptotic Jurkat Cells—One possible explanation for our findings would be a generalized disruption of mitochondrial structure. To investigate this possibility, we treated Jurkat cells with anti-Fas antibody for 90 min, a time point at which oxygen consumption was inhibited by more than 95%, then examined the cells by electron microscopy (Fig. 4). The nuclei of the control cells were normal in appearance, large and containing well dispersed chromatin. In contrast, the nuclei of most of the anti-Fas-treated cells showed chromatin that was condensed and of a monotonous
The inhibition of oxygen uptake in anti-Fas-treated cells (measured as % of control) was calculated from the rates of oxygen uptake shown in Fig. 3. Apoptotic nuclei were quantified as described under "Materials and Methods."

| Time (min) | Inhibition of oxygen uptake | Apoptotic nuclei |
|-----------|-----------------------------|-----------------|
| 30        | 4.0                         | 1               |
| 45        | 18.8                        | 9               |
| 60        | 71.2                        | 25              |
| 75        | 83.0                        | > 50            |

Cytochrome oxidase activity as measured by following the reoxidation of reduced cytochrome c

| Measurement | Anti-Fas | Control |
|-------------|----------|---------|
| Exp. 1      | -7.50    | -5.57   |
| Exp. 2      | -3.20    | -2.11   |
| Exp. 3      | 2.99     | -1.29   |
| $K_m$ (M)   | 10.2     | 8.2     |

We therefore focused our investigations on this redox complex.

Ascorbate/TMPD does not reduce cytochrome oxidase directly, however, but instead reduces cytochrome c, which then acts as the reducing agent for cytochrome oxidase. To measure the activity of cytochrome oxidase more directly, we employed a spectrophotometric assay based on the oxidation of exogenously added cytochrome c (21). We found to our surprise that the activities of cytochrome oxidase were similar in control and anti-Fas-treated cells (Table II). Furthermore, the interaction between cytochrome oxidase and cytochrome c did not appear to be affected by apoptosis, because the apparent $K_m$ for cytochrome c was the same for control mitochondria and mitochondria isolated from cells treated with anti-Fas antibody (Table II).

The foregoing results suggested that the cytochrome oxidase in anti-Fas-treated cells was intact, and that the failure of electron transport through Complex IV in mitochondria from apoptotic cells was the result of cytochrome c inactivation. This formulation predicts that exogenous cytochrome c would normalize oxygen consumption in apoptotic cells. To test this prediction, cytochrome c (5–45 ng in a sequential additions) was added along with ascorbate/TMPD to permeabilized cells that had been treated either with anti-Fas antibody or with no antibody for 90 min before permeabilization. Assays of oxygen uptake showed that in anti-Fas-treated cells, the very low rate of cytochrome oxidase-mediated oxygen consumption (3.5 ng-atoms/min) was largely corrected by the addition of cytochrome c (Fig. 5). At the highest concentration of cytochrome c, oxygen consumption was 80% of normal. Furthermore, the $K_m$ for cytochrome c calculated from these oxygen uptake measurements was 20 M, in reasonable agreement with the $K_m$ values determined from measurements of cytochrome c oxidation. In contrast, exogenous cytochrome c did not enhance oxygen consumption in control cells. These findings suggest that cytochrome c is specifically inactivated during Fas-mediated apoptosis.

Cytochrome c is located in the intermembrane space of mitochondria. One possible explanation for the inactivation of cytochrome c observed in our experiments was that digitonin permeabilization of the cells would render the intermembrane space accessible to cytosolic components that are normally excluded from that space. If cytochrome c inactivation were due solely to the invasion of the intermembrane space by such cytosolic components after digitonin permeabilization, then measurements of oxygen uptake obtained immediately after digitonin treatment should be similar for anti-Fas-treated and control cells, since the cytosolic components responsible for the inactivation of the cytochrome would not have had time to work. If, however, cytochrome c inactivation had occurred in anti-Fas-treated cells before digitonin permeabilization, then inactivation of cytochrome c and the consequent decrease in electron transport through cytochrome oxidase would be apparent immediately upon digitonin permeabilization. To examine these possibilities, we first treated cells for 90 min with anti-Fas antibody or no antibody, then introduced the cells into the oxygen electrode in the absence of digitonin and finally

![Fig. 4. Morphologic characteristics of mitochondria in control and apoptotic cells.](image-url)

Cells were treated with anti-Fas antibody (100 ng/ml) or an irrelevant IgM antibody, then fixed, embedded, and sectioned for electron microscopy as described in the text. A and B, control cells C and D, anti-Fas-treated cells. Regions outlined in panels A and C are shown at higher magnification in panels B and D, respectively. Original magnification for panels A and C was ×3000, and for panels B and D was ×30,000.
added digitonin together with ascorbate/TMPD. We found that oxygen uptake was already inhibited when measured immediately after digitonin permeabilization, and that it could be restored by the addition of cytochrome c (Fig. 6). These findings suggest that Fas-mediated inactivation of mitochondrial electron transport occurs in intact cells and is not merely a consequence of digitonin permeabilization. These findings also suggest that cytochrome c is a specific target of inactivation during Fas-mediated apoptosis.

Cytochrome c possesses a covalently linked heme group through which electrons are transferred. To determine if cytochrome c or its prosthetic heme group was degraded during Fas-mediated apoptosis, difference spectra (oxidized minus reduced) of the mitochondrial cytochromes were obtained using whole mitochondria obtained from control or anti-Fas-treated cells, and cytochrome c in the cell lysates was analyzed by nondenaturing gel electrophoresis, visualizing with a heme stain (22). Alterations of cytochrome c could be reflected by a change in $A_{550}/A_{563}$ in the absorption spectrum (i.e., a change in the apparent ratio of cytochrome c to cytochrome b), a change in the electrophoretic mobility of the cytochrome, or both. The spectra of control and apoptotic mitochondria were found to be similar, however, and on gel electrophoresis the cytochrome obtained from apoptotic cells was similar in quantity and mobility to the cytochrome from control cells (Fig. 7). The lack of a detectable change in the $A_{550}/A_{563}$ ratio or in the electrophoretic mobility of the cytochrome at a time when mitochondrial electron transport was $\sim 90\%$ inhibited suggests that the inactivation of cytochrome c did not involve extensive degradation of the protein or loss of the heme group. Limited proteolysis or other minor modifications of the cytochrome, however, might not have been detected in these experiments.

Studies on the Mechanism of Inactivation of Cytochrome c—Fas-mediated apoptosis is thought to involve the activation of one or more of a large group of death proteases that participate in apoptosis by cleaving a well-defined group of proteins carboxyl to an aspartate residue (23, 24). To determine if Fas-mediated inactivation of cytochrome c required protease activity, cells were preincubated for 90 min with Z-VAD fluoromethyl ketone (Kamiya Biomedical), a tripeptide inhibitor of ICE and related proteases (25), then treated with 100 ng/ml anti-Fas antibody as described. Measurements of cytochrome oxidase-dependent oxygen uptake after an additional 90 min
showed that the ICE inhibitor protected cells against Fas-mediated inactivation of cytochrome c (Fig. 8). These results indicate that an antecedent proteolytic event was needed for inactivation of electron transport through cytochrome oxidase. To ascertain whether a component in the cytosol of the apoptotic cells was responsible for the effect of apoptosis on cytochrome c, cytosol from Jurkat cells treated for 60–120 min with anti-Fas antibody was added to digitonin-permeabilized control cells (Fig. 9). Cytosol from Fas-treated cells effected an inhibition of cytochrome oxidase-dependent oxygen consumption that was apparent after a preincubation of 5–20 min; cytosol from control cells had no effect on oxygen consumption. The inactivation of electron transport by the cytosolic factor could be reversed by the addition of cytochrome c, indicating that, as in the previous experiments, cytochrome c was inactivated and cytochrome oxidase was spared.

Effect of Fas Ligation on Mitochondrial Membrane Potential—We evaluated the mitochondrial membrane potential using the fluorescent probe di-OC₆(3), which is retained in mitochondria with a normal membrane potential. CCCP was used to fully discharge the membrane potential before dye loading in the negative control. Untreated cells demonstrated a mean value (in arbitrary fluorescence units) of 24.6. Fas ligation led to a slow decline in fluorescence (Fig. 10) consistent with partial loss of mitochondrial membrane potential during apoptosis.

DISCUSSION

The decline in mitochondrial function observed in our studies and reported from other laboratories is one of several potentially fatal functional alterations that take place in apoptotic cells. An issue often raised in connection with apoptosis has to do with which one of these lethal changes is the change that is actually responsible for the death of the cell. The role of executioner is usually assigned to the earliest of the lethal changes observed to take place in the dying cell. To the extent that any of several events that take place during apoptosis has the potential to kill the cell, however, this issue is difficult to settle.
Cytochrome c in Fas-mediated Apoptosis

![Diagram of Cytochrome c in Fas-mediated Apoptosis](http://www.jbc.org/)

**Fig. 9.** Effect of Fas-treated cytosol on oxygen consumption in digitonin-permeabilized control cells. Untreated whole Jurkat cells in oxygen electrode cuvettes were permeabilized with digitonin and incubated for 15 min at 37 °C with cytosol from untreated Jurkat cells or Jurkat cells treated for 120 min with anti-Fas antibody (100 ng/ml) as described in the text. Ascorbate/ TMPD was then added and oxygen uptake was measured to assess electron transport through cytochrome oxidase. In the experiments in which the effect of cytochrome c was examined, the cytochrome (100 μM final concentration) was added to the reaction mixture in the oxygen electrode after measuring initial rate. Numerical values represent azide-sensitive oxygen consumption (ng-atoms O₂/min/3 × 10⁶ cells).

The mitochondrial membrane potential should transiently collapse (28).

Cytochrome c is located in the intermembrane space of mitochondria, raising the question as to how a cytosolic cytochrome c antagonist could reach its target. The antagonist may be generated directly in the intermembrane space during the course of apoptosis, appearing in the cytosol as a result of leakage through the outer mitochondrial membrane. Alternatively, it may be formed in the cytosol, then move to the intermembrane space to exert its effect. In either case, these results suggest that the outer mitochondrial membrane, although appearing intact by electron microscopy, allows the cytochrome c antagonist to pass through, either via a specific carrier or through an alteration in its lipid composition.

The anti-apoptosis protein Bcl-2 is located in the outer membrane of mitochondria, with a portion extending into the intermembrane space. The mechanism by which members of the Bcl-2 family affect apoptosis is not known, and it has been a particular puzzle why Bcl-2 is located in the outer mitochondrial membrane, rather than in the inner mitochondrial membrane where the electron transport complexes and the ATP-synthesizing enzyme are found. Our observation that the outer mitochondrial membrane space extends into the intermembrane space (35) would therefore be expected to collapse with the cessation of electron transport as the excess protons flow back into the matrix through the mitochondrial ATPase (Complex V). The finding that mitochondria in apoptotic cells retain a portion of their transmembrane potential raises the possibility that, like cytochrome c, the mitochondrial ATPase is also defunctionalized in some way as cells undergo apoptosis. The function of other mitochondrial elements may also be abrogated during apoptosis. Complex III, as a hypothetical example, could be inactivated through an effect on the Rieske protein, which extends into the intermembrane space (35).

Electron transport abnormalities yet to be demonstrated may account for the discrepancy in time between the early fall in oxygen uptake and the somewhat later decline in cytochrome c function in cells undergoing apoptosis.

**REFERENCES**

1. Neamaty, N., Fernandez, A., Wright, S., Kiefer, J., and McConkey, D. J. (1995) J. Immunol. 154, 3788–3795
2. Laeznik, Y. A., Kaufmann, S. H., Desnoyers, S., Poirier, G. G., and Earnshaw, W. C. (1994) Nature 371, 346–347
3. Casciola-Rosen, L. A., Miller, D. K., Anhalt, G. J., and Rosen, A. (1994) J. Biol. Chem. 269, 30757–30760
4. Martín, S. J., O'Brien, G. A., Nishikawa, W. K., McGahan, A. J., Mahboubi, A., Saido, T. C., and Green, D. R. (1995) J. Biol. Chem. 270, 6425–6428
5. Vojvodic, J., Ofner, P. A., and Walz, G. W. M. (1995) J. Immunol. 154, 1707–1716
6. Piccinetti, M. (1995) Curr. Top. Microbiol. Immunol. 200, 163–175
7. Obeid, L. M., Linares, C. M., Karolak, L. A., and Hannun, Y. A. (1993) J. Biol. Chem. 259, 1769–1771
8. Sambrook, J. G., and Steinberg, D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1390–1400
9. Macho, A., Castedo, M., Marchetti, P., Aguilar, J., J., Decaudin, D., Zamzami, N., Girard, P. M., Uriel, J., and Kroemer, G. (1995) Blood 86, 2481–2487
10. Cossarizza, A., Franceschi, C., Monti, D., Salvatori, S., Bellesis, E., Rivabene, R., Biondo, L., Rainaldi, G., Tinari, A., and Malorni, W. (1995) Exp. Cell Res. 220, 232–240
11. Zamzami, N., Castedo, M., Decaudin, D., Macho, A., Hirsh, T., Susin, S. A., Petit, P. X., Mignotte, B., and Kroemer, G. (1995) J. Exp. Med. 182, 377–377
12. Petit, P. X., LeCouet, H., Zorn, E., Dauguet, C., Mignotte, B., and Gougeon, M. L. (1995) J. Cell Biol. 130, 157-167
13. Zamzami, N., Marchetti, P., Castedo, M., Zanin, C., Vayssiere, J.-L., Petit, P. X., and Kroemer, G. (1995) J. Exp. Med. 181, 1661–1672
14. Cossarizza, A., Kalashnikova, G., Grasselli, E., Chiappelli, F., Salvadini, S., Capri, M., Barbieri, D., Trionio, L., Manti, D., and Franceschi, C. (1994) Exp. Cell Res. 214, 323–330
15. Schulze-Osthoff, K., Kramer, P. H., and Droge, W. (1994) EMBO J. 13, 4587–4596

![Graph of Fluorescence against Time](http://www.jbc.org/)

**Fig. 10.** Effect of Fas ligation on mitochondrial membrane potential as a function of time. Jurkat cells were incubated with anti-Fas antibody (100 ng/ml) for various intervals of time. During the last 30 min of the incubation, di-OC₆(3) was added. At the end of the incubation, fluorescence was analyzed by flow cytometry. 

- ●: fluorescence of cells treated with anti-Fas antibody for the times indicated; 
- △: fluorescence of cells incubated for 30 min with di-OC₆(3) after treatment with CCCP to discharge the mitochondrial transmembrane potential.
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17. Schulze-Osthoff, K., Bakker, A. C., Vanhaesebroek, B., Beyaert, R., Jacob, W. A., and Fiers, W. (1992) J. Biol. Chem. 267, 5317–5323
18. O'Donnell, V. B., Spycher, S., and Azzi, A. (1995) Biochem. J. 310, 133–141
19. Telford, W. G., King, L. E., and Fraker, P. J. (1994) J. Immunol. Methods 172, 1–16
20. Bourgeron, T., Chretien, D., Rotig, A., Munnich, A., and Rustin, P. (1992) Biochem. Biophys. Res. Commun. 186, 16–23
21. Errede, B., Kamen, M. D., and Hatefi, Y. (1978) Methods Enzymol. LIII 40–47
22. Bass, W. T., and Bricker, T. M. (1988) Anal. Biochem. 171, 330–338
23. Los, M., Van de Craen, M., Pennings, L. C., Schenk, H., Westendorp, M., Baueuerle, P. A., Dröge, W., Kramer, P. H., Fiers, W., and Schulze-Osthoff, K. (1995) Nature 375, 81–83
24. Enari, M., Hug, H., and Nagata, S. (1995) Nature 375, 78–81
25. Fearnhead, H. O., Dinsdale, D., and Cohen, G. M. (1995) FEBS Lett. 375, 283–288
26. Tan, A. (1988) The Joy Luck Club, p. 102, G. P. Putnam's Sons, New York
27. Hashimoto, T., Yoshida, Y., and Tagawa, K. (1990) J. Bioenerg. Biomembr. 22, 27–38
28. Mimura, H., Hashimoto, T., Yoshida, Y., Ichikawa, N., and Tagawa, K. (1993) J. Biochem. (Tokyo) 113, 350–354
29. Lopez-Medravilla, C., Vigny, H., and Godinot, C. (1993) Eur. J. Biochem. 215, 487–496
30. Chernyak, B. V., Dukhovich, V. F., and Khodjaev, E. Y. (1991) Arch. Biochem. Biophys. 286, 604–609
31. Ichikawa, N., Yoshida, Y., Hashimoto, T., Ogasawara, N., Yoshikawa, H., Imamoto, F., and Tagawa, K. (1990) J. Biol. Chem. 265, 6274–6278
32. Yamada, E. W., and Huzel, N. J. (1983) Biosci. Rep. 3, 947–954
33. Tunal de Gomez-Puyou, M., Muller, U., Dreyfus, G., Ayala, G., and Gomez-Puyou, A. (1983) J. Biol. Chem. 258, 13680–13684
34. Hashimoto, T., Yoshida, Y., and Tagawa, K. (1983) J. Biochem. (Tokyo) 94, 715–720
35. Hatefi, Y. (1985) Annu. Rev. Biochem. 54, 1015–1071
