Cytochrome c-Dependent Activation of Caspase-3 by Tumor Necrosis Factor Requires Induction of the Mitochondrial Permeability Transition

Marco Tafani, Timothy G. Schneider,
John G. Pastorino, and John L. Farber

*From the Department of Pathology, Anatomy and Cell Biology,
Thomas Jefferson University, Philadelphia, Pennsylvania*

The killing of L929 mouse fibroblasts by tumor necrosis factor- α (TNF- α) in the presence of 0.5 μ g/ml actinomycin D (Act D) is prevented by inhibition of the mitochondrial permeability transition (MPT) with cyclosporin A (CyA) in combination with the phospholipase A₂ inhibitor aristolochic acid (ArA). The MPT is accompanied by the release of cytochrome *c* from the mitochondria, caspase-8 and caspase-3 activation in the cytosol, cleavage of the nuclear enzyme poly(ADP-ribose)polymerase (PARP), and DNA fragmentation, all of which were inhibited by CyA plus ArA. The caspase-3 inhibitor z-Asp-Glu-Val-aspartic acid fluoromethylketone (Z-DEVD-FMK) did not prevent the loss of viability or the redistribution of cytochrome *c*, but it did prevent caspase-3 activation, PARP cleavage, and DNA fragmentation. Inhibition of the MPT reduced the activation of caspase-8 to the level occurring with TNF- α alone (no ActD). The caspase-8 inhibitor z-Ile-Glu(OMe)-Thr-Asp(OMe) fluoromethylketone (Z-IETD-FMK) did not prevent the cell killing and decreased only slightly the translocation of Bid to the mitochondria. These data indicate that induction of the MPT by TNF- α causes a release of cytochrome *c*, caspase-3 activation with PARP cleavage and DNA fragmentation. The loss of viability is dependent on the MPT but independent of the activation of caspase-3. The activation of caspase-8 is not dependent on the MPT. There is no evidence linking this enzyme to the loss of viability. Thus, the killing of L929 fibroblasts by TNF- α can occur in the absence of either caspase-3 or caspase-8 activity. Alternatively, cell death can be prevented despite an activation of caspase-8. (*Am J Pathol* 2000, 156:2111–2121)

The observation that cytochrome *c* can function as an activator of caspases¹ brought together two important aspects of the current interest in the mechanisms mediating programmed cell death (apoptosis). Considerable evidence implicates the caspases in this process.² The caspases are cysteine proteases related to interleukin 1 β -converting enzyme (ICE/caspase-1). At the same time, altered mitochondrial function is increasingly recognized as playing a critical role in apoptosis.^{3,4}

The mitochondrial permeability transition (MPT) is a well known alteration implicated as a mechanism of cell injury. The MPT refers to the regulated opening of a large, nonspecific pore in the inner mitochondrial membrane.^{5–7} Although the molecular elements that form this pore have not been definitively established, they are presumed to derive from well known membrane constituents, including the adenine nucleotide translocator, porin molecules, and the complex forming the peripheral benzodiazepine receptor.^{8,9} The MPT is a critical event in the killing of cells that follows an inhibition of mitochondrial electron transport by anoxia.¹⁰ The MPT has also been implicated as a mechanism of mitochondrial dysfunction in apoptosis.¹¹

We have shown that the overexpression of Bax in Jurkat lymphocytes results in the killing of cells by a process that displays many of the features associated with apoptosis.¹² In particular, the overexpression of Bax in the Jurkat cells induces the MPT, an event that is responsible for the loss of viability. The MPT is accompanied by the release of cytochrome *c* and, in turn, caspase-3 activation with the proteolytic cleavage of poly(ADP-ribose)polymerase (PARP) and the fragmentation of DNA. Inhibition of the MPT by cyclosporin A (CyA) prevented all manifestations of apoptosis, whereas caspase-3 inhibition prevented PARP cleavage and DNA fragmentation. The caspase-3 inhibitor was without effect on induction of the MPT and its functional consequences, namely cell death and the release of cytochrome *c*.

Several important concerns were left unresolved by this earlier study. In particular, one needs to know how relevant the findings that occurred with Bax overexpression are to cell death occurring in other models. The killing of L929 mouse fibroblasts by tumor necrosis factor- α (TNF- α) also depends on induction of the MPT.¹³ In this model, both the MPT and the cell killing induced by TNF- α are inhibited by CyA. Thus, the killing of L929 fibroblasts by TNF- α provides a very convenient system with which to further explore the nature and consequences of mitochondrial dysfunction in lethal cell injury. In the present study, we demonstrate that TNF- α causes

Accepted for publication February 15, 2000.

Address reprint requests to John L. Farber, M.D., Room 208, Jefferson Alumni Hall, Thomas Jefferson University, Philadelphia, PA 19107. E-mail: John.Farber@mail.tju.edu.

a redistribution of cytochrome *c* from the mitochondria to the cytosol that is dependent on the induction of the MPT. In addition, the functional consequences of this redistribution of cytochrome *c* and their relationship to the loss of cell viability are defined.

Materials and Methods

Cell Line

The L929 line of mouse fibroblasts (ATCC-CCL-1, American Type Culture Collection, Manassas, VA) was maintained in 25-cm² polystyrene flasks (Corning Costar Corp., Oneonta, NY) with 5 ml of Dulbecco's modified Eagle's medium (DMEM; high glucose, without pyruvate; MediaTech), containing 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (complete DMEM) and incubated under an atmosphere of 95% air and 5% CO₂. For all experiments, cells were plated at a density of 200,000/cm² in complete DMEM. After overnight incubation the cells were washed twice with phosphate-buffered saline (PBS) and placed in DMEM without serum.

Treatment Protocols

In all experiments TNF- α (Sigma, St. Louis, MO) was added to a final concentration of 2 ng/ml (22 units/ng). TNF- α was dissolved in PBS and added to the cells in 0.2% volume. Act D (Sigma) was dissolved in dimethyl sulfoxide (DMSO), further diluted in PBS, and added in 0.2% volume to a final concentration of 0.5 μ g/ml. Where indicated the cells were pretreated for 30 minutes with the following reagents before addition of TNF- α and Act D. Cyclosporin A (Biomol, Plymouth Meeting, PA) was dissolved in DMSO and added in a 0.2% volume to the cell culture media to give a final concentration of 5 μ mol/L. Aristolochic acid (Biomol) was dissolved in PBS and added in a 0.2% volume to give a final concentration of 50 μ mol/L. The cell permeable caspase-3 inhibitor (Z-Asp-Glu-Val-aspartic acid fluoromethylketone, Z-DEVD-FMK) and the cell permeable caspase-8 inhibitor (Z-Ile-Glu(OMe)-Thr-Asp(OMe) fluoromethylketone, Z-IETD-FMK; Kamyia Biomedical Co., Seattle, WA) were dissolved in DMSO and added in a 0.2% volume to give the concentrations indicated in the text. In all cases the vehicles used to prepare stock solutions of the reagents had no effect on the cells or the parameters measured at the concentrations used.

Measurement of Cell Viability

Cell viability was determined by trypan blue exclusion. After treatment the cells were trypsinized and 10 μ l of a 0.5% solution of trypan blue was added to 100 μ l of treated cells. The suspension was then applied to a hemocytometer. Both viable and nonviable cells were counted. A minimum of 200 cells was counted for each data point in a total of eight microscopic fields.

Measurement of Caspase-3

The assay is based on the ability of the active enzyme to cleave the chromophore pNA from the enzyme substrate DEVD-pNA. Extracts from treated cells were diluted 1:1 with 2 $\times$ reaction buffer (10 mmol/L Tris, pH 7.4, 1 mmol/L dithiothreitol, 2 mmol/L EDTA, 0.1% (3-[3-cholamido-propyl]dimethylammonio)-1-propanesulfonate, 1 mmol/L PMSF, 10 μ g/ml pepstatin, 10 μ g/ml leupeptin). DEVD-pNA was added to a final concentration of 50 μ mol/L, and the reaction was incubated for 1 hour at 37°C. The samples were then transferred to a 96-well plate, and the absorbance measurements were made with a 96-well plate reader at 405 nm.

Measurement of Caspase-8

The activity of caspase-8 was measured with the Apo-Alert Caspase-8 Colorimetric Assay Kit (Clontech Laboratories Inc., Palo Alto, CA) according to the manufacturer's instructions. Absorbance measurements were made with a 96-well plate reader at 405 nm.

Isolation of Cytosol and Mitochondrial Fractions and Determination of Bid or Cytochrome *c* Content

Cells were harvested by trypsinization after treatment. Soybean trypsin inhibitor was used to neutralize trypsin after harvest. Cells were centrifuged at 750 $\times g$ for 10 minutes at 4°C. The pellets were washed with SHE-PI (10 mmol/L HEPES-KOH (pH 7.4), 1 mmol/L EDTA, protease inhibitor cocktail (PI), and 250 mmol/L sucrose). The cell pellets were resuspended in SHE-PI. The cell suspension was transferred to a dounce homogenizer and the cells broken open with 20 strokes of the pestle. The homogenate was transferred to a high speed centrifuge tube containing SHE-PI and 0.5% phenol red. HE-PI (10 mmol/L HEPES-KOH, pH 7.4, 1 mmol/L EDTA, protease inhibitor cocktail (PI) containing 750 mmol/L sucrose) was laid below the homogenate. Centrifugation was conducted at 10,000 $\times g$ for 30 minutes at 4°C. The resulting mitochondrial pellet was resuspended and centrifuged a second time into 750 mmol/L sucrose in HE-PI. The mitochondria were further concentrated in a Centricon (MW cutoff of 10,000) microconcentrator and the resulting retentate analyzed for cytochrome *c*. The supernatant from the 10,000 $\times g$ spin was centrifuged at 100,000 $\times g$ for preparation of cytosol. For the detection of cytochrome *c*, mitochondrial and cytosolic fractions were separated on 12% SDS-polyacrylamide electrophoresis gels with an equal amount of protein loaded onto each lane as determined by the bicinchoninic acid assay. Kaleidoscope prestained standards (Bio-Rad) were used to determine molecular weight. The gels were then electroblotted onto nitrocellulose membranes. Cytochrome *c* was detected by a monoclonal antibody (Pharmingen, San Diego, CA) at a dilution of 1:5000. Secondary goat-anti-mouse horseradish peroxidase-labeled antibody (1:2000) was detected by enhanced chemiluminescence.

To detect Bid, the mitochondrial fraction was electrophoresed on 15% SDS-polyacrylamide gels. The gel was then electroblotted onto nitrocellulose membranes. Bid was detected with a polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500. The secondary anti-goat horseradish peroxidase-labeled antibody (1:20,000) was visualized by enhanced chemiluminescence.

Detection of DNA Fragmentation

Cells (1.0×10^6) were trypsinized and collected by centrifugation at $750 \times g$ for 10 minutes. The cell pellet was washed in PBS and then lysed in 200 μ l of 10 mmol/L Tris, pH 8.0, 10 mmol/L EDTA, 0.5% Triton X-100. The lysate was centrifuged at $13,000 \times g$ for 20 minutes at 4°C. RNase (0.2 mg/ml) was added, and the lysate was incubated for 30 minutes at 37°C. Proteinase K (0.1 mg/ml) and SDS (final concentration 1%) were added, followed by incubation at 50°C for 16 hours. DNA was extracted with phenol/chloroform and then with chloroform, precipitated with ethanol and sodium acetate, and electrophoresed on 1.2% agarose gels.

Determination of PARP Content

Cells (1.0×10^5) were trypsinized and then pelleted at $750 \times g$, resuspended in 20 μ l of SDS sample buffer, and boiled for 10 minutes. The samples were run on an 8% SDS-polyacrylamide electrophoresis gel. Protein content was determined by BCA, and molecular weight by external standards. The gels were electroblotted onto nitrocellulose membranes and probed with PARP rabbit antiserum (PA3-950, Affinity Bioreagents, Golden, CO) at 1:500 dilution. A secondary horseradish peroxidase-labeled goat-anti-rabbit antibody at 1:10,000 was detected using enhanced chemiluminescence.

Electron Microscopy

After treatment the cells were trypsinized and pelleted. The cells were then suspended and fixed in 2% glutaraldehyde with 1% tannic acid in 0.1 mol/L sodium cacodylate at pH 7.3 overnight at 4°C. The cells were rinsed three times in the sodium cacodylate buffer and then incubated in 2% osmium tetroxide in the same buffer for 2 hours at room temperature. The cells were rinsed three times in distilled water and then exposed to 1% uranyl acetate in water for 15 minutes at room temperature. The cells were then rinsed twice in distilled water, after which they were spun down into 3% agarose at 45°C and cooled to form blocks. The agarose blocks were dehydrated in graded steps of acetone and embedded in Spurr's low viscosity media. After polymerization overnight at 65°C, 80-nm sections were cut on a Reigert-Jung Ultra Cut E ultramicrotome and picked up on copper grids. The grids were poststained in uranyl acetate and bismuth subnitrate. The sections were observed in a Hitachi 7000 STEM and micrographs recorded on Kodak 4489-sheet film.

Measurement of Malate Dehydrogenase (MDH)

MDH activity was measured in L929 cytosolic extracts according to the method of Ochoa.¹⁴ Briefly, cytosolic extracts (2 mg of protein) were added to 300 μ l of MDH buffer (259 mmol/L glycylglycine, pH 7.4, 1.5 mmol/L NADH). To this, 100 μ l of 7.6 mmol/L oxalacetate was added to initiate the reaction. Readings were taken in a spectrophotometer at 340 nm and made against a blank containing all components except NADH. The decrease in the optical density between 30 and 45 seconds after the start of the reaction was used to calculate enzyme activity. The concentration of MDH in the cytosolic extracts was calculated by comparison to a standard curve. MDH and NADH were both from Sigma.

Results

Activation of Caspase-3 and Caspase-8 by TNF- α in L929 Fibroblasts

TNF- α induces the mitochondrial permeability transition (MPT) in L929 fibroblasts and, in turn, the death of virtually all of the cells.¹³ On treatment with TNF- α in the presence of either actinomycin D (ActD) or cycloheximide (CHX), the MPT (as measured by a loss of mitochondrial energization) is evident within 4 hours.¹³ As shown in Figure 1, TNF- α produced over a similar time course an increase in caspase-3 activity in the cytosol of L929 fibroblasts. Within 4 hours caspase-3 activity increased by eightfold and 16-fold by 6 hours, a result that was entirely dependent on induction of the MPT. Cyclosporin A (CyA) together with the phospholipase A₂ inhibitor aristolochic acid (ArA) prevents the development of the MPT in L929 cells treated with TNF- α .¹³ Figure 2 shows that CyA + ArA inhibited the increase in caspase-3 activity induced by TNF- α . CyA + ArA lowered caspase-3 activity in TNF- α -treated cells to that in the control cells. Importantly, direct addition of CyA + ArA to cellular extracts of TNF- α -treated cells did not inhibit caspase-3 activity (data not shown), a result demonstrating that CyA + ArA does not directly inhibit caspase-3.

The caspase family of proteases differ with respect to the recognition sequence at which they cleave a given substrate. Caspase-3 cleaves at the amino acid sequence DEVDG, which in the case of the nuclear enzyme poly(ADP-ribose)polymerase (PARP) lies between Asp216 and Gly217 in the DNA binding domain of this caspase-3 substrate.¹⁵ Z-DEVD-FMK is a specific, cell permeable and irreversible, tetrapeptide inhibitor of caspase-3.¹⁵ L929 fibroblasts were pretreated with Z-DEVD-FMK for 30 minutes prior to the addition of TNF- α . As shown in Figure 2, Z-DEVD-FMK produced a dose-dependent inhibition of the increase in caspase-3 activity, with 100 μ mol/L reducing caspase-3 activity to that of controls.

Whereas the data in Figure 2 indicate that activation of caspase-3 is entirely a consequence of the MPT, there are potential, alternative mechanisms for caspase activa-

Figure 1. TNF- α induces the activity of caspase-3. L929 cells (5.0×10^6 cells/ml) were treated with TNF- α and 0.5 μ g/ml ActD for the indicated times. Extracts of treated cells were then prepared and assayed for caspase-3 activity as described in Materials and Methods.

tion in response to TNF- α . In particular, caspase-8 can be activated as a direct consequence of the binding of TNF- α to the 55-kD receptor (TNFR-1) with recruitment of TRADD (TNFR-1-associated death domain protein) and, in turn, FADD (Fas-associated death domain protein).¹⁶ Caspase-8 can then activate downstream caspases, such as caspase-3.^{17,18} Figure 3 details the activation of caspase-8 in L929 fibroblasts treated with TNF- α . Within 2 hours, TNF- α alone (no ActD) produced a threefold increase in caspase-8 activity. It deserves emphasis that there is no loss of viability with TNF- α alone.¹³ In the presence of TNF- α and ActD, a 10-fold activation of caspase-8 occurred (Figure 3). CyA + ArA reduced the activation of caspase-8 by TNF- α and ActD to that with TNF- α alone (Figure 3). Thus, the additional activation of caspase-8 above that with TNF- α alone that occurred with TNF- α and ActD is a consequence of the MPT. Importantly, Z-IETD-FMK, a specific inhibitor of caspase-8,¹⁹ prevented both components (that with TNF- α alone plus that with TNF- α and ActD) of the activation of this enzyme, thereby reducing its activity to the control level (Figure 3).

Figure 2. DEVD-FMK or CyA + ArA inhibit caspase-3 activation by TNF- α . L929 cells (5.0×10^6 cells/ml) were pretreated for 30 minutes with either 5 μ mol/L CyA and 50 μ mol/L ArA or the indicated concentrations of DEVD-FMK. TNF- α 0.5 μ g/ml ActD were then added. After 6 hours, cell extracts were prepared and caspase-3 activity assayed as described in Materials and Methods.

Figure 3. TNF- α induces the activity of caspase-8. L929 cells (5.0×10^6 cells/ml) were treated with TNF- α alone or TNF- α and 0.5 μ g/ml ActD for 2 hours. Where indicated the cells were pretreated for 30 minutes with 5 μ mol/L CyA and 50 μ mol/L ArA or for 1 hour with 20 μ mol/L of IETD-FMK. Extracts were prepared and assayed for caspase-8 activity as described in Materials and Methods.

mitochondria

Figure 4. TNF- α causes translocation of Bid. L929 fibroblasts (5×10^6 cells) were pretreated with IETD-FMK and then with TNF- α and 0.5 μ g/ml ActD for 3 hours. Mitochondrial fraction was assayed for Bid by Western blotting as described in Materials and Methods.

TNF- α -Induced Translocation of Bid

Activation of Bid and its translocation to the mitochondria have been attributed to activation of caspase-8.^{20,21} Treatment of L929 fibroblasts with TNF- α and ActD caused the translocation of Bid to the mitochondria (Figure 4). Interestingly, despite the complete inhibition of caspase-8 activation by Z-IETD-FMK (Figure 3), the translocation of Bid is only partially prevented by this specific caspase inhibitor (Figure 4). This result is consistent with the previous report that, under conditions where the cell killing is not entirely dependent on caspase activation, Bid translocation is similarly not prevented by caspase inhibitors.²²

TNF- α -Induced PARP Depletion Is Inhibited by Z-DEVD-FMK and CyA

Cleavage of PARP is a prominent manifestation of the activation of caspase-3 in many models of apoptosis.²³ Similarly, the treatment of L929 fibroblasts (in the presence of ActD) causes a loss of the 116-kD PARP (Figure 5, lane 2). This loss of PARP protein was not apparent with TNF- α in the absence of ActD (data not shown). Despite the fact that the antibody used here (PA3-590) recognizes the carboxyl terminal of mouse PARP, it was not possible to detect the 85-kD carboxyl terminal cleavage fragment of PARP in the L929 cell extracts. However, it could be demonstrated that the loss of the native PARP protein produced by TNF- α depended on caspase-3 activity. The TNF- α -induced PARP depletion was inhibited by pretreatment of the cells with Z-DEVD-FMK (Figure 5,

lane 3). Importantly, the loss of PARP also depended on induction of the MPT as shown by the similar ability of CyA (in the presence of ArA) to maintain the control content of this enzyme (Figure 5, lane 4).

TNF- α -Induced DNA Fragmentation Is Inhibited by Z-DEVD-FMK and CyA

The cleavage of DNA to oligonucleosome size fragments is another prominent feature of apoptosis. Treatment of L929 fibroblasts with TNF- α results in DNA fragmentation within 6 hours (Figure 6a). DNA fragmentation was not detected at the same times with TNF- α in the absence of

Figure 5. DEVD-FMK or CyA + ArA inhibit PARP cleavage. L929 cells (1.0×10^5 cells) were either left untreated or pretreated for 30 minutes with either CyA + ArA or DEVD-FMK. TNF- α and 0.5 μ g/ml ActD were then added. After 6 hours the cells were harvested and PARP levels determined by Western blotting as described in Materials and Methods.

Figure 6. TNF- α produces DNA fragmentation that is inhibited by either CyA + ArA or DEVD-FMK. L929 cells (5.0×10^6 cells) were either left untreated (**a**) or pretreated for 30 minutes with either CyA + ArA or DEVD-FMK (**b**). Cells were then treated with TNF- α and 0.5 μ g/ml ActD for the times indicated (**a**) or for 6 hours (**b**). The cells were harvested and DNA extracted as described in Materials and Methods.

ActD (data not shown). This DNA fragmentation induced by TNF- α is caspase-3-dependent. As shown in Figure 6b, the caspase-3 inhibitor Z-DEVD-FMK completely prevented the evidence of DNA fragmentation at 6 hours produced by TNF- α . As with the increase in caspase-3 activity itself and the depletion of PARP protein, DNA fragmentation was also dependent on the MPT. CyA + ArA completely prevented the DNA fragmentation evident 6 hours after treatment with TNF- α .

Cytochrome c Release from Mitochondria Is Induced by TNF- α

The activation of caspases in some models of apoptosis has been attributed to the release and redistribution of cytochrome c from the mitochondria to the cytosol.^{24,25} To determine the role of cytochrome c release in the activation of caspase-3 by TNF- α , cytosolic and mitochondrial fractions were derived from homogenates of TNF- α -treated L929 cells. Figure 7a shows that TNF- α (in the presence of ActD) produced a decrease in the mitochondrial content of cytochrome c that was evident after 4 hours and more prominent at 6 hours. This depletion of cytochrome c in the mitochondrial fraction was mirrored by a concomitant increase of the protein in the cytosolic fraction. There was no change in the cellular distribution

Figure 7. TNF- α causes release of cytochrome c. L929 cells (5.0×10^6) were either treated with TNF- α and 0.5 μ g/ml ActD for the indicated time periods (**a**) or pretreated with either CyA + ArA or DEVD-FMK and then treated with TNF- α and 0.5 μ g/ml ActD for 6 hours (**b**). Cytosolic and mitochondrial fractions were assayed for cytochrome c by Western blotting as described in Materials and Methods.

of cytochrome c in cells treated with TNF- α in the absence of ActD (data not shown). The small amount of cytosolic cytochrome c evident at time zero was owing presumably to the trauma incurred by the mitochondria on homogenization and isolation of the respective sub-cellular fractions.

The release of cytochrome c produced by TNF- α is dependent on induction of the MPT. CyA + ArA prevented the release and redistribution of cytochrome c from mitochondria to the cytosol at 6 hours (Figure 7b). By contrast, Z-DEVD-FMK had no effect on either the mitochondrial loss or cytosolic accumulation of cytochrome c in L929 cells treated TNF- α (Figure 7b). Similarly, Z-DEVD-FMK did not prevent induction of the MPT as measured by loss of mitochondrial membrane potential (data not shown). In keeping with the conclusion that the release of cytochrome c is a consequence of the MPT, Figure 8 shows that 20 μ mol/L Z-IETD-FMK, a dose that prevents caspase-8 activation (Figure 3), is without effect on either the loss of cytochrome c from the mitochondria (Figure 8, top) or its accumulation in the cytosol (Figure 8, bottom).

TNF- α -Induced Release of MDH from the Mitochondrial Matrix

It has been hypothesized that the release of cytochrome c during apoptosis is the specific consequence of an as yet poorly characterized mechanism that disrupts the outer mitochondrial membrane without any loss of the mitochondrial membrane potential.²⁵ By contrast, induction of the MPT in isolated mitochondria results in the release of several proteins in addition to cytochrome c.²⁶ Similarly, we show here that cytochrome c is not the only protein whose redistribution is affected on induction of the MPT by TNF- α in intact L929 cells. MDH is a soluble protein present in the mitochondrial matrix.²⁶ Table 1 shows that cytosolic

Figure 8. TNF- α -dependent cytochrome *c* release is not blocked by IETD-FMK. L929 cells (5.0×10^6) were pretreated with IETD-FMK and then with TNF- α and 0.5 $\mu\text{g/ml}$ ActD for 4 hours. Cytosolic (**bottom**) and mitochondrial (**top**) fractions were assayed for cytochrome *c* by Western blotting as described in Materials and Methods.

extracts from control L929 cells exhibited some detectable MDH. This baseline activity is attributable to the MDH normally present in the cytosol as part of the acetyl CoA cycle and used to transport acetyl CoA across the mitochondrial membrane for utilization in fatty acid synthesis. However, the MDH activity more than doubled within 4 hours and was threefold greater than the control level after 6 hours in cytosolic extracts from cells treated with TNF- α (in the presence of ActD). This increase in the activity of MDH in the cytosol represents a change from 15% to 65% of the total

cellular enzyme (Table 1), a result that would suggest that, at least, half of the mitochondria have undergone the MPT. There was no increase in MDH in cytosolic extracts from cells treated with TNF- α alone (data not shown). Importantly, pretreatment of the cells with CyA + ArA prevented the accumulation of MDH in the cytosol produced by TNF- α (Table 1).

Induction of the MPT by TNF- α Produces Mitochondria Swelling That Is Inhibited by CyA

It has also been argued that cytochrome *c* is released from mitochondria by a mechanism distinct from the MPT that is inhibited by CyA but not accompanied by swelling of these organelles.²⁷ Examination of intact L929 cells by electron microscopy 4 hours after treatment with TNF- α (in the presence of ActD) indicated prominent mitochondrial swelling (Figure 9a). Clearing of the matrix space with loss of cristae was accompanied by increases in the size and configuration of the mitochondria. It deserves emphasis that these mitochondrial alterations occurred at a time when the nuclear membrane was still intact and the chromatin was not condensed or clumped (Figure 9a). At this time (4 hours), the plasma membrane was also intact (Figure

Table 1. MDH Accumulation in Cytosolic Extracts of TNF-Treated Cells and its Prevention by CyA + ArA

Treatment	MDH activity milliunits/ μg protein (% of total)
Control (6 hours)	78 ± 8 (16 ± 2)
TNF and ActD (4 hours)	230 ± 25 (47 ± 5)
TNF and ActD (6 hours)	320 ± 45 (65 ± 9)
TNF and ActD + CyA + ArA (6 hours)	75 ± 9 (15 ± 2)

The cells were pretreated with CyA + ArA for 30 minutes and then with 2 ng/ml TNF and 0.5 $\mu\text{g/ml}$ ActD. Cytosolic extracts were prepared and MDH activity measured after 4 or 6 hours. The data are the mean $\pm$ SD from three independent experiments.

Figure 9. TNF- α induces swelling of mitochondria that is inhibited by CyA + ArA. L929 cells were either left untreated (**left**) or pretreated for 30 minutes with CyA + ArA (**right**). TNF- α and 0.5 μ g/ml ActD were then added for 6 hours. The cells were then processed for electron microscopy as described in Materials and Methods.

9a). Mitochondrial swelling was prevented by pretreatment with CyA + ArA (Figure 9b).

The MPT but not Caspase-3 or Caspase-8 Activation Is Necessary for Cell Killing

Figure 10 demonstrates that inhibition of the activation of caspase-3 by Z-DEVD-FMK afforded no protection against the cytotoxicity of TNF- α . TNF- α (in the presence of ActD) caused a progressive loss of cell viability with almost 70% of the cells dead within 6 hours. Despite its ability to inhibit activation of caspase-3 (Figure 2), the depletion of PARP (Figure 5), and the fragmentation of DNA (Figure 6), 100 μ mol/L Z-DEVD-FMK had no effect on the rate or extent of

cell killing by TNF- α (Figure 10). By contrast, inhibition of the MPT by CyA + ArA totally prevented the loss of cell viability induced by TNF- α (Figure 10).

Figure 10 also considers the role of the activation of caspase-8 in the cell killing by TNF- α . Whereas 20 μ mol/L Z-IETD-FMK completely prevented the activation of caspase-8 (Figure 3), no effect of this specific inhibitor on the extent of cell killing was evident.

Discussion

This paper has addressed two important and controversial issues that have arisen in the course of recent studies

Figure 10. TNF- α cytotoxicity is prevented by CyA + ArA but not by DEVD-FMK or IETD-FMK. L929 cells were treated with TNF- α and 0.5 μ g/ml ActD (◆) for the indicated times. Alternatively, cells were pretreated with CyA + ArA (■), DEVD-FMK (▲) or IETD-FMK (●) followed by TNF- α and 0.5 μ g/ml ActD treatment for the indicated times. Cell viability was assessed by trypan blue as described in Materials and Methods.

of the biochemistry of cell death, namely the mechanism of mitochondrial injury that releases cytochrome *c* and the role, in turn, that this release plays in the loss of viability. The data presented above document that induction of the MPT by TNF- α is accompanied by a redistribution of cytochrome *c* from mitochondria to the cytosol. In turn, cytochrome *c* release is accompanied by activation of caspase-3, PARP degradation, and DNA fragmentation. Inhibition of the MPT by cyclosporin A (in the presence of aristolochic acid) prevented in parallel with the prevention of cell killing 1) the release of cytochrome *c*; 2) the activation of caspase-3; 3) the degradation of PARP, and 4) the fragmentation of DNA. By contrast, Z-DEVD-FMK, a cell permeable, irreversible inhibitor of caspase-3, prevented only the DNA fragmentation and PARP degradation produced by TNF- α . Z-DEVD-FMK did not prevent the redistribution of cytochrome *c* or the loss of cell viability. Thus, the present report documents for the first time that cytochrome *c* release with caspase activation and action is a consequence of the MPT induced by a physiological stimulus (TNF- α in this case) under conditions that kill cells. The cell death, however, is clearly the consequence of the MPT and not of the release of cytochrome *c* nor the activation of caspase-3. Similar events and conclusions were reported recently on the overexpression of Bax in Jurkat cells.¹² Accordingly, it deserves emphasis that the MPT has now been shown

to mediate both cell killing and cytochrome *c* release in, at least, two distinct models of cell killing in which loss of viability is accompanied by other typical manifestations of apoptosis, namely PARP cleavage and DNA fragmentation.

A role for cytochrome *c* in, at least, the nuclear events of apoptosis was first suggested in studies with cell free extracts by the requirement for an organelle fraction enriched in mitochondria.²⁸ The mitochondrial agent responsible for the degradation of DNA *in vitro* was subsequently identified as cytochrome *c*.¹ Subsequently, cytochrome *c* was shown to bind to the cytosolic protein Apaf-1, the human homologue of the *C. elegans* protein, CED-4. *In vitro* reconstitution experiments detailed that cytochrome *c* forms a complex consisting of procaspase-9 and Apaf-1.²⁹ On the addition of dATP, the complex activates procaspase-3.

More controversial has been the mechanism mediating cytochrome *c* release. Previous studies have both implicated and denied a role for the MPT in cytochrome *c* release. With isolated mitochondria, induction of the MPT released cytochrome *c*, along with other mitochondrial proteins.^{26,30,31} In intact thymocytes, the development of the MPT was inhibited by Bcl-2.³² Moreover, mitochondria isolated from cells that overexpress Bcl-2 were resistant to agents that induce the MPT. We have shown that the overexpression of Bax in Jurkat cells caused the release of cytochrome *c* as a consequence of the induction of the MPT.¹²

By contrast, in other studies the data were interpreted to indicate that the release of cytochrome *c* was not dependent on an induction of the MPT. Recombinant Bax protein induced isolated mitochondria to release cytochrome *c*, a result that was completely inhibited by CyA.³¹ Nevertheless, it was concluded that Bax-induced cytochrome *c* release occurred by a mechanism other than the MPT, because the mitochondria did not swell. In HL-60 cells treated with staurosporine, there was a redistribution of cytochrome *c* from the mitochondria to the cytosol, a change that preceded a loss of the mitochondrial membrane potential.²⁴ Jurkat cells undergoing Fas-induced apoptosis were reported to exhibit swollen mitochondria and cytochrome *c* release.³³ In this instance, rupture of the outer mitochondrial membrane was said to occur and to account for the cytochrome *c* release. Interestingly, the mitochondria still maintained a membrane potential, despite the almost complete loss of cytochrome *c* and gross deformation of their structure. The maintenance of the membrane potential was cited as evidence that the MPT was not the mechanism mediating cytochrome *c* release.

The electron micrographs presented here (Figure 9) demonstrate that CyA-sensitive mitochondrial swelling is a prominent feature in L929 cells treated with TNF- α . This change in the morphology of the mitochondria and its prevention by CyA is further evidence for induction of the MPT. As evidence for the MPT, we have previously reported that TNF- α causes CyA-sensitive mitochondrial depolarization.¹³ An additional finding that indicates the induction of the MPT is the release of MDH (Table 1). The release of MDH is interpreted as a specific consequence

of induction of the MPT, as its was prevented by CyA (Table 1).

It can be argued that during the initial phases of the evolution of the cell injury in apoptosis, normal mitochondria may coexist with those that have developed the MPT. Under such circumstances, fluorescent cationic dyes may redistribute from depolarized mitochondria to mitochondria that are still energized, an effect that may explain the inability to detect a decrease in the mitochondrial membrane potential at early time points in some models of apoptosis.⁵ Furthermore, early in the process of apoptosis, the release of cytochrome *c* from a small fraction of mitochondria that have undergone the MPT may be sufficient to activate procaspase-3. In turn, caspase-3 can further cleave procaspase-3 molecules, thereby initiating an autocatalytic cycle.

The events that follow on cytochrome *c* release are generally quite reproducible. In the TNF- α -treated fibroblasts studied here, cytochrome *c* release was accompanied by activation of caspase-3 activity (Figure 1) with degradation of PARP (Figure 5) and fragmentation of DNA (Figure 6). Caspase-3 degrades PARP directly.¹⁵ By contrast, caspase-3 results in DNA fragmentation by first activating an endonuclease (CAD) and inactivating its inhibitor, ICAD.³⁴ The increment in caspase-8 activation that depended on the MPT (Figure 3) can also be attributed to the action of caspase-3.³⁵

Similar to the killing of Jurkat cells on the overexpression of Bax,¹² caspase-3 activity, as opposed to the MPT, was not critical for the loss of viability of L929 fibroblasts (measured by disruption of plasma membrane integrity) in response to TNF- α (Figure 9). Interestingly, MCF-7 cells that lack functional caspase-3 failed to manifest many of the typical morphological manifestations associated with apoptosis, but they were still sensitive to the cytotoxicity of TNF- α .³⁶ Moreover, MCF-7 cells treated with TNF- α displayed a redistribution of cytochrome *c* from mitochondria to cytosol.²⁹ The overexpression of Bcl-X_L in MCF-7 cells inhibited the cytotoxicity and cytochrome *c* redistribution produced by TNF- α .²⁹ The microinjection of cytochrome *c* into MCF-7 cells treated with TNF- α did not overcome the protective effects of Bcl-X_L expression.³⁷ These results can be readily explained by hypothesizing that in MCF-7 cells Bcl-X_L prevents the cytotoxicity of TNF- α by inhibiting induction of the MPT. As noted above, Bcl-2, a protein closely related to Bcl-X_L, inhibited induction of the MPT in both isolated mitochondria and intact cells.

An alternative mechanism for caspase activation in response to TNF- α has been proposed. Activation of the 55-kD TNF- α receptor (TNFR-1) results in receptor aggregation and the recruitment of TRADD (TNFR-1 associated death domain protein). In turn, TRADD binds FADD (Fas-associated death domain protein). Interaction with FADD unmasks the amino-terminal death effector domain of FADD, an event that allows it to recruit caspase-8/FLICE (FADD-like interleukin-1 β -converting enzyme).¹⁶ In turn, caspase-8 activates downstream caspases, such as caspase-3.^{17,18} Such a scheme does not demand the participation of cytochrome *c* and, thus, should not depend on changes in mitochondrial structure and function.

Despite the activation of caspase-8 in response to TNF- α in L929 fibroblasts (Figure 3), a mechanism with which caspase-8 activates caspase-3 would not seem to be the predominant pathway of caspase-3 activation in TNF- α -treated L929 cells. In our study, inhibition of the MPT completely prevented the activation of caspase-3, by preventing the release of cytochrome *c*. Our data also document that the activation of caspase-8 is not necessary for the killing of L929 fibroblasts by TNF- α . Inhibition of caspase-8 activation (Figure 3) had no effect on the extent of cell killing by TNF- α (Figure 10). Similarly, caspase 8 was not necessary for the cytotoxicity of TNF- α in MCF-7 cells, which lack caspase-3. As discussed above, Bcl-X_L inhibited the cytotoxicity of TNF- α , an effect that occurred despite the fact that caspase-8 was still processed proteolytically to its active form.²⁹

The methodology used for assessing apoptosis can clearly affect the conclusions reached. Janike et al³⁸ found MCF-7 cells sensitive to the cytotoxicity of TNF- α as measured by the uptake of crystal violet. This assay is similar to the exclusion of trypan blue, in that only cells with an intact plasma membrane will be scored as viable. However, in the same study, the typical morphological features of apoptosis were not found, such as DNA laddering, cell blebbing, and the appearance of a subG1 peak. Similarly, Oberhammer et al³⁹ observed that MCF-7 cells treated with TNF- α detached from their substrate but did not manifest the typical apoptotic morphology or DNA fragmentation. As demonstrated here, in L929 fibroblasts treated with TNF- α , inhibition of caspase-3 prevented DNA fragmentation but not cell killing. Nevertheless, caspase-3 is an important determinant in apoptosis, in that it is necessary for the development of the distinguishing features (PARP cleavage and DNA fragmentation) of this form of cell death.

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