Studies on the Mechanisms and Kinetics of Apoptosis Induced by Microinjection of Cytochrome c in Rat Kidney Tubule Epithelial Cells (NRK-52E)

Seung H. Chang, Patricia C. Phelps,
Irene K. Berezsky, M. Lee Ebersberger, Jr.,
and Benjamin F. Trump

*From the Department of Pathology, University of Maryland
School of Medicine, Baltimore, Maryland*

Recent reports substantiating the role of cytochrome c in the induction of apoptosis led us to examine the kinetics and mechanisms involved in this process as an extension of our ongoing studies of cell injury and cell death. Microinjection of cytochrome c into NRK-52E kidney cells produced rapid apoptosis, which usually began within 30 minutes and reached a maximum of 60–70% by 3 hours. The changes that occurred included four phases: an initial shrinkage phase, an active phase, a spherical phase, and a necrotic phase. For morphological purposes, the progressive changes were followed by phase-contrast and fluorescence microscopy, transmission and scanning electron microscopy, and time-lapse video microscopy. Cells first showed shrinkage, then displayed multiple pseudopods, which rapidly extended and retracted, giving the cells a bosselated appearance. During this active phase there was chromatin condensation, mitochondria were swollen but retained membrane potential, and the endoplasmic reticulum was dilated. Within 2–4 hours, active-phase cells became spherical and smooth-surfaced but were still alive, the nuclei showed chromatin clumping, the mitochondria underwent high-amplitude swelling but retained membrane potential, the endoplasmic reticulum was highly dilated, and many large apical vacuoles were present. Elevation of $[Ca^{2+}]_i$ was seen at the late spherical phase, shortly before cell death. Pretreatment with the caspase 3 inhibitor (Ac-DEVD-CHO) prevented apoptosis, whereas overexpression of *Bcl-2* did not. Depletion of cellular ATP by cyanide inhibition of energy metabolism prevented cytochrome c from inducing the active and later phases of apoptosis. The results clearly indicate that cytochrome c-induced apoptosis is a dynamic and energy-requiring process that has a distinct active and spherical phase before cell death. (*Am J Pathol* 2000, 156:637–649)

Apoptosis is an important mechanism of cell death in the kidney during development and after many and diverse injuries.¹ As part of our ongoing studies of cell injury and cell death in the kidney, we are exploring the detailed stages and mechanisms of apoptosis and comparing them with those of oncosis. As the prelethal changes of oncosis have been well characterized, we wished to carefully examine the changes of apoptosis before and after cell death.^{2,3}

Cytochrome c release into the cytosol has been implicated as an intermediate event in the initiation of apoptosis after a variety of toxic and other stimuli and injury in several cell types^{4–9}; it also serves as a useful model, inasmuch as we found that after microinjection, the early phase of apoptosis usually begins within 30 minutes. This means that the progression of changes in single injected cells can be followed by many important techniques. These include the use of time-lapse phase-contrast video microscopy, digital imaging fluorescence, and confocal microscopy to analyze mitochondrial membrane potential, mitochondrial permeability transition, cytosolic calcium concentration $[Ca^{2+}]_i$, and cell death, as well as phase-contrast and electron microscopy for morphological characterization.

The results clearly defined three principal prelethal stages or phases of apoptosis and characterized the morphology at the light and electron microscope levels. We also showed that ATP is required for the initiation of apoptosis, that the loss of mitochondrial membrane potential and the precipitation of the mitochondrial permeability transition occur immediately before cell death, and that increased $[Ca^{2+}]_i$ occurs in the prelethal phase just before cell death. The results also revealed that after apoptotic cell death the necrosis phase is essentially identical to the necrotic phase that is seen after the induction of oncosis, eg, by chemical anoxia or nephrotoxins such as mercuric chloride.

Materials and Methods

Cell Culture

All media and additives were obtained from Gibco-BRL. The NRK-52E normal kidney cell line (CRL 1571) was

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obtained from the American Type Culture Collection. This cell line is derived from the proximal tubule epithelium (PTE) of the rat kidney, is nontumorigenic, and retains many morphological and physiological characteristics of PTE *in vivo*.^{10,11} Cells were grown in low-glucose Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and antibiotics (100 U/ml penicillin, 100 μ g/ml streptomycin) and maintained at 37°C in 5% CO₂, 95% air. Cells were plated onto 0.8 × 1.4 cm segments of letter-identified, grid-embossed glass slides (Cel-Line) and grown to about 40% confluency for microinjection.

Microinjection and Examination of Cells

Before microinjection, a grid slide with cells was transferred to a special culture dish that was constructed by predrilling a hole in the bottom of the dish and gluing a cover glass over the hole with aquarium cement. The dish was placed on the stage of an Olympus IMT-2 inverted microscope equipped with a Narishige microinjection system. The cytoplasm of the cells, which averaged 4 μ l in volume, was microinjected with approximately 1 fl of 500 μ mol/L (6.2 mg/ml) rat heart cytochrome *c* (Sigma) containing 5 μ g/ml Texas Red dextran (Molecular Probes). For each slide usually four to five grid windows of cells were injected, to achieve a total of 200–300 injected cells. The cells were followed by phase-contrast and fluorescence microscopy, using a Texas Red, UV, or rhodamine filter, for up to 24 hours. For time-lapse video microscopy, a video camera was interfaced between the microscope and the tape recorder. Images were recorded at 1/60 of normal time.

Examination and Evaluation of Apoptotic Cells

Apoptotic cells were evaluated by phase-contrast and fluorescence microscopy; the presence of Texas Red fluorescence was used to confirm that the cell had been microinjected. In addition, the DNA-binding fluorophore dye Hoechst 33342 (1 μ mol/ml) was used to further evaluate chromatin morphology in living cells. The percentage of Texas Red positive cells that showed morphological features of apoptosis was calculated every 30 minutes for 5 hours in 18 separate experiments. Propidium iodide was used to identify dead cells. In addition, cells were fixed for 10 minutes in 2% phosphate-buffered paraformaldehyde, followed by 20 minutes in absolute methanol, and then mounted with Vectashield mounting medium (Vector Labs) containing 0.5 μ g/ml diamidino phenylindole (DAPI) for DNA staining. Microinjected cells fixed in buffered paraformaldehyde were stained with an ApopTag plus fluorescein assay kit from Intergen Co. (Purchase, NY) (formerly a product of Oncor, Gaithersburg, MD) to detect DNA fragmentation in morphologically identified apoptotic cells. ApopTag uses the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling assay (TUNEL assay) to specifically stain single- and double-stranded DNA breaks associated with apoptosis. Cells were stained according to the instructions of the manufacturer. The percentage of the fluorescein-pos-

itive cells was calculated over a 4-hour period in several separate experiments. The Texas Red remained visible after fixation and again was used to identify microinjected cells.

Cytosolic Calcium [Ca²⁺]_i Analysis

Cells grown on glass coverslips mounted over holes in 60-mm dishes were incubated with 5 μ mol/L Fura 2/AM (Molecular Probes, Eugene, OR) for 90 minutes at 22°C after microinjection of cytochrome *c*. Analysis was performed by digital imaging fluorescence microscopy (DIFM), using a Nikon inverted microscope and a dual-excitation chopper-based Tracor Northern FluoroPlex III and image analysis system (Tracor Northern, Madison, WI) as previously described.¹² After loading, three to five fields of Fura-2-loaded uninjected control cells were selected, and image pairs were collected at 340/380-nm excitation. Two hours after microinjection of cytochrome *c* and Fura-2/AM loading, image pairs were collected at 20-minute intervals for up to 6 hours in fields having apoptotic cells. [Ca²⁺]_i calibrations were performed as described previously.¹² The ratioed image pairs were displayed using a pseudocolor or gray scale to indicate levels of [Ca²⁺]_i.

Mitochondrial Membrane Potential Analysis

The mitochondrial membrane potential (MMP) (terminology defining the integrity of the inner mitochondrial membrane and associated respiratory chain complexes) was measured indirectly by use of the vital fluorochrome dye rhodamine 123. Loss of membrane integrity is indicated when the dye leaves the mitochondria. The time of opening of the mitochondria permeability pore(s) (high-conductance pore(s)), which precipitates the mitochondrial permeability transition, is also often indirectly established by the loss of rhodamine 123 from mitochondria. Cytochrome *c*-microinjected cells were incubated with 5 μ g/ml rhodamine 123 (Sigma) for 20–40 minutes at 37°C. Mitochondrial staining in apoptotic cells was monitored and photographed for 4 hours by both fluorescence and phase microscopy with the inverted Olympus microscope. Bright fluorescence was maintained by reincubating the grids periodically in medium with rhodamine 123.

Electron Microscopy

For transmission electron microscopy (TEM), cells were plated onto grid slides precoated with a thin carbon film, microinjected with cytochrome *c*, and then fixed after 0.5, 1, 1.5, 2, and 4 hours with 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.3) for 2–16 hours, followed by 1% osmium tetroxide fixation (1 hour at 22°C). Before fixation, apoptotic cells were located on grid slide windows and accurately mapped on a clear acetate sheet for future reference and for final epoxy block trimming. Cells on grid slides were gently dehydrated and infiltrated with Polybed 812 epoxy resin (Polysciences), mounted on a blank epoxy block, and cured. Blocks were

trimmed using the prefixation acetate maps to pinpoint exact cell location, and thin sections were cut, stained, examined, and photographed with a JEOL EX1200 transmission electron microscope. For scanning electron microscopy (SEM) samples were fixed, dehydrated, critical-point dried, gold coated, examined, and photographed with an AMR-1000 scanning electron microscope.

Treatment of Cells with Protease and Enzyme Inhibitors

Cells were incubated with either the caspase-3 inhibitor Ac-DEVD-CHO (Ac-Asp-Glu-Val-L-aspartic acid aldehyde) or the caspase 1-inhibitor YVAF-CHO (Ac-Tyr-Val-Ala-ASP aldehyde) (Bioscience, King of Prussia, PA) for 1.5 hours before microinjection of cytochrome *c*. Both inhibitors were used at various concentrations ranging from 5 to 200 $\mu\text{mol/L}$ and were kept in the media throughout the course of the experiment.

Microinjection of Bcl-2

Cytochrome *c* was microinjected into NRK-52E cells that had been stably transfected with the *Bcl-2* gene (NRK-Bcl-2) and which overproduced Bcl-2 protein.¹³ In separate experiments, the vector *pD5-neo/Bcl-2* was microinjected into the nuclei of NRK-52E cells 24 hours before the coinjection of cytochrome *c* and Texas Red dextran. The vector was kindly supplied by Dr. Paul Amstad.¹⁴

Actin Staining

Injected cells on grid slides were sampled at 30-minute intervals up to 4 hours, fixed in 1.5% formaldehyde (10–30 minutes), and then stored in PBS. Cells were first permeabilized with Triton X-100 (0.5%) for 3 minutes before staining for 30 minutes with a 1:10 dilution of fluorescein phalloidin (Molecular Probes). Grids were then mounted on coverslips with Vectashield medium and photographed by fluorescence microscopy, using either a fluorescein filter or a dual Texas Red/fluorescein filter.

ATP Depletion

Cells grown on grids were preincubated in DMEM without serum for 30 minutes and then treated with freshly prepared potassium cyanide to inhibit mitochondrial respiration and sodium iodoacetate to block glycolysis (KCN + IAA), in equal concentrations ranging from 0.25 to 1 mmol/L for different times (0.5, 1, 1.5, 2–4 hours) before microinjection with cytochrome *c*. The percentage of dead cells was calculated for each time point, and a final optimum concentration of 0.25 mmol/L was selected. Cells were also grown in flasks for adenosine triphosphate (ATP) evaluation and treated with 0.25 mmol/L KCN and 0.25 mmol/L IAA for time periods similar to those mentioned above but were not microinjected with cytochrome *c*. At zero time and at each time point, a flask

of cells was exposed to 1 ml trypsin-EDTA for 10 minutes, followed by the addition of 2 ml DMEM without serum. Aliquots of cells were analyzed by a luciferase method,¹⁵ using the Sigma Assay Kit and a luminometer (model TD-20-20; Turner Designs). ATP concentrations were expressed in fmol/cell.

Results

Microinjection of cytochrome *c* resulted in a very rapid initiation of the apoptotic pathway in NRK-52E cells. After microinjection, the number of injected cells that became apoptotic over a period of 5 hours was counted at 30- or 60-minute time intervals. The progressive morphological changes were analyzed and recorded. The cell changes that occurred during apoptosis after microinjection were divided into an initial shrinkage phase, an active phase, a quiescent spherical phase, and a final necrosis phase at cell death. Results are presented in correlation with these phases.

Control Cells

The untreated NRK-52E cells in culture formed colonies of epithelial cells that were polyhedral in shape and contained eccentric nuclei that bulged upward and had multiple nucleoli. At 100% confluency, the cells exhibited a compact cobblestone appearance, as reported previously.^{10,11} The surfaces of the cells were covered with short microvilli (Figure 1A), and the apical region contained vesicles, tubules, and vacuoles similar to those of PTE *in vivo*. The mitochondria were elongated and had a moderately dense matrix (Figure 2A). A network of actin was situated immediately below the apical cell surface, and a well-developed network of actin bundles was seen in the cytosol with phalloidin staining. In apical regions of the cells, microtubules were associated with the actin filaments and were seen elsewhere in the cytoplasm, where they formed a characteristic network. The Golgi apparatus was well developed and surrounded by multivesicular bodies, secondary lysosomes, and occasional triglyceride droplets.

Shrinkage and Active Phase

Within 30 minutes after microinjection of cytochrome *c*, some cells began to exhibit apoptotic changes, but others changed less rapidly. Within 2–3 hours approximately 60–70% of the microinjected cells became apoptotic, as evaluated by morphological criteria (Figure 3). In the experiments where cells were stained with ApopTag, about 70–80% of the microinjected cells became fluorescent (or ApopTag-positive) within 2–3 hours (Figure 4). Initially, the cells exhibited a very rapid shrinkage phase, becoming roundish and leaving behind cytoplasmic strands (Figures 1B and 5B). The active phase immediately followed this. In this phase the cells exhibited rapid extension and retraction of buds or pseudopods (not to be confused with bleb formations seen in oncosis)

Figure 1. Scanning EM of a control cell and cells microinjected with cytochrome *c*. **A:** A typical untreated epithelial cell with many short microvilli and thin peripheral processes (original magnification, $\times 2400$). **B:** A contracted cell in the early (1 hour) active phase of apoptosis, showing multiple pseudopods on the cell body and on remnant cytoplasmic strands (**arrows**) (original magnification, $\times 2800$). **C:** Cells in late active phase (2 hours) that have formed aggregates and show large pseudopods. The upper group includes three or four active-phase cells (original magnification, $\times 3100$). **D:** Two spherical-phase cells (4 hours), identified by shape and lack of pseudopods. Occasional pitted areas are seen at the cell surface (**arrows**) (original magnification, $\times 3500$). These areas are presumed to correspond to vacuoles, as seen in Figure 2D.

over the entire cell surface (Figures 1B, 1C, 5C, 6E, 7A, and 7B). Many of these pseudopods had secondary pseudopods (Figure 1B, arrows) that also extended and retracted. This active phase lasted for a period of approx-

imately 30 minutes to 4 hours, although occasionally a few cells remained active for as long as 6 hours. In the early active phase there is positive cell staining after use of the TUNEL ApopTag kit (Figure 8, A and B).

Figure 2. **A:** Transmission EM of a normal untreated cell, showing a nucleus and elongated mitochondria (original magnification, $\times 3750$). **B and C:** Active-phase cells after microinjection, showing multiple pseudopods, often containing annular patterns of RER profiles, dense free ribosomes, and nuclear fragments or processes (**arrows**). Cellular organelles are concentrated in one area, mitochondria (M) are mostly condensed or slightly swollen, and the RER is dilated (original magnification, $\times 4500$). In **B** at lower left, note a process of a necrotic cell (**arrowhead**). Also note at middle right a pseudopod with a nuclear process showing dense chromatin (**arrow**). In **C** at lower left, there is a narrow band region of chromatin with nuclear pores (**arrowheads**); also note two pseudopods with a nuclear process (**arrows**). **D:** A spherical phase cell with an eccentric nucleus having peripheral patches of dense chromatin, nuclear budding (**arrow**), highly dilated RER, swollen mitochondria (M), and large apical vacuoles (V) (original magnification, $\times 3000$). An outlined area of nuclear filaments is enlarged (**inset**) (original magnification, $\times 8250$).

In this active phase most of the organelles were aggregated in the perinuclear regions, while the cell periphery was relatively organelle-free (Figure 2, B and C). The nuclei showed chromatin condensation, which was mild at first and then extreme. At the same time, multiple patches of chromatin were seen with the use of DAPI on fixed cells (Figure 7D) or Hoechst 33342 dye on living cells (Figure 5D), giving the appearance of nuclear fragmentation. These nuclear "fragments" were often observed in the extending and retracting pseudopods (Figure 2, B and C). However, in many EM sections it was

seen that these fragments were often connected to the main body of the nucleus by a very narrow stem and therefore represented nuclear processes (see Figure 12). Occasionally, portions of pseudopods pinched off and were observed floating in the medium. During this phase, an almost immediate disorganization of the normal actin network occurred, which was replaced by patchy homogeneous areas of actin staining throughout the cell. The actin staining did not extend into the pseudopods. DIFM revealed a slight increase of $[Ca^{2+}]_i$ (Figures 9B, a–c). SEM of this phase clearly revealed the details of the

Figure 3. Graph of percentage of apoptotic cells (in media with serum) seen over a 5-hour time period in four experiments after microinjection with cytochrome *c* and in one experiment where cells were microinjected with Bcl-2 plasmid (pD5-neo-Bcl-2) 24 hours before cytochrome *c* microinjection. Also shown is a curve representing cells that were incubated with 50 mmol/L Ac-DEVD-CHO for 1.5 hours before, during, and after microinjection with cytochrome *c*, which shows minimal apoptosis similar to that of control cells.

pseudopods and the retraction processes and showed that groups of adjacent apoptotic cells tended to form aggregates (Figure 1C).

TEM revealed that the apoptotic cells were shrunken with a denser cytosol than the controls (Figure 2, B and C). The pseudopods typically contained free ribosomes and sometimes profiles of rough endoplasmic reticulum (RER) (Figure 10), which often assumed an annular pattern (Figure 2C). Mitochondria and/or nuclear fragments or long and irregular nuclear processes were also observed in the pseudopods (Figures 2B, 2C, and 10). Aggregates of ~20-nm filaments were observed in the cytosol (Figure 10, inset). In the apical regions of the cell, enlarged vesicles and vacuoles were seen. The RER

Figure 4. Graph of percentage of cytochrome *c* microinjected cells that were positive for ApopTag staining over a period of 4 hours, indicating DNA strand breaks characteristic of apoptosis.

cisternae were often dilated, as were those of the Golgi apparatus.

The mitochondria differed from the controls in that the profiles were rounded instead of elongated (Figures 2B, 2C, and 10). On occasion, mitochondrial profiles showed matrical condensation. Studies using rhodamine 123 revealed persistence of the mitochondrial membrane potential (MMP) (Figure 6D), but there was no mitochondrial swelling.

Spherical Phase

Beginning approximately 2–4 hours after microinjection, the cells became spherical and quiescent (Figures 1D, 2D, 7C, 8C, 8D, and 9B, d–f). SEM confirmed the spherical shape and showed some remnants of the retraction processes (Figure 1D). The surface of these cells did not show pseudopods, but instead often revealed a pitted appearance (Figure 1D, arrows) corresponding to the vacuoles seen by TEM. Spherical cells showed positive staining after the use of the TUNEL ApopTag assay kit, indicating DNA fragmentation (Figure 8 C,D). TEM typically showed a polarized appearance with the eccentric nucleus at one pole and a series of variably sized membrane-bound vacuoles at the other pole (Figure 2D). Many of these apical vacuoles were highly dilated, and some could be seen to communicate with the cell surface.

The cytosol was relatively electron-dense, with closely packed ribosomes (Figure 11). The cytosol also contained numerous phagolysosomes (Figure 11, arrowheads) and frequently contained bundles of filaments similar to those seen in the active phase. At this stage the nuclei showed intense chromatin clumping (Figure 12) and marked shape changes, sometimes resulting in multiple nuclear profiles in any one thin-section plane. In most cases the nuclear processes, which were often quite small, could be identified by the recognition of a nuclear envelope and the fact that many distant nuclear protrusions could be seen to connect with the main part of the nucleus (Figure 12, inset). However, nuclear chromatin could sometimes be seen in the pseudopods of active-phase cells without any identifiable nuclear envelope (Figure 2, B and C, arrows). Most nuclei were markedly irregular and frequently contained nuclear inclusions composed of aggregates of filaments (Figure 2D, inset). Nucleoli often showed marked segregation. The RER, on the other hand, was even more dilated than in the active phase (Figures 11 and 12). In general, the mitochondria were seen as rounded profiles that showed high-amplitude swelling of the inner compartments (Figure 11, arrows). The MMP, however, remained unchanged in virtually all of the cells observed (Figure 6F). At the late stages of the spherical phase, but before cell death, $[Ca^{2+}]_i$ was markedly increased (Figure 9B, d–f).

Necrotic Phase

Fragments of cells in the necrotic phase were seen adjacent to cells in the active and spherical phases (Figure 2B, lower left, arrowhead). In these cells, the cytosol was

Figure 5. Series of phase-contrast images of the same group of live cells after microinjection of cytochrome *c* (original magnification, $\times 785$). **A:** Just after microinjection. **B:** Thirty minutes after microinjection, showing a cell beginning to shrink (arrow). **C:** Thirty-two minutes after microinjection, showing the start of active-phase apoptosis (arrow). **D:** A fluorescent image (UV filter) of the cells stained with Hoechst 33342 at 35 minutes, demonstrating early chromatin condensation in the apoptotic cell (arrow).

swollen, as was the RER and the mitochondria, which often showed flocculent intramatrix densities. These changes were identical to those seen after many other injuries that do not cause apoptosis, such as ischemia or mercuric chloride toxicity.

Cytosolic Calcium Analysis

DIFM studies showed that normal cells had a $[Ca^{2+}]_i$ range of 50–100 nmol/L, and cells in the active phase showed a slight increase to about 200 nmol/L (Figure 9B, a–c). There was an increase to approximately 900 nmol/L in the late stages of the spherical phase, just before cell death (Figure 9B, d–f).

Caspase-3 Inhibition

Pretreatment of cells with Ac-DEVD-CHO before and after microinjection of cytochrome *c* prevented all of the changes of the active phase, whereas pretreatment with YVAD-CHO apparently had no effect (Figure 3).

Excess Bcl-2

Microinjection of cells with cytochrome *c* after overexpression of *Bcl-2*, resulting from either transfection by *NRK-Bcl-2* or microinjection with the *pD5-neo/Bcl-2* vector, resulted in no inhibition of the apoptotic pathway (Figure 3).

ATP Analysis

Preincubation of cells with 0.25 mmol/L KCN + 0.25 mmol/L IAA for 1.5 hours before cytochrome *c* microinjection showed a maximum of about 30% apoptosis in microinjected cells by 4 hours. However, preincubation for 2 hours or longer with 0.25 mM KCN + 0.25 mM IAA followed by microinjection of cytochrome *c* resulted in the complete absence of apoptosis (Figure 13). (Note: Control cells in DMEM without serum, microinjected with cytochrome *c*, resulted in almost 100% of the cells becoming apoptotic by 1–2 hours, whereas microinjected cells in DMEM with serum had a maximum of about 60–70% apoptosis by 2–3 hours (Figure 3).)

Control cells and cells pretreated with 0.25 mmol/L KCN + 0.25 mmol/L IAA for from 0.5 to 4 hours, but not postmicroinjected with cytochrome *c*, were assayed for ATP. Control cells contained approximately 20 fmol ATP/cell; by 2 hours of treatment, cells had approximately 4 fmol/cell, and by 4 hours they had approximately 2 fmol/cell.

Discussion

Summary of Kinetics

These studies clearly show that microinjection of cytochrome *c* in NRK-52E cells resulted in apoptosis, followed by cell death and necrosis. The changes proceeded rapidly and were particularly dramatic when

Figure 6. Sets of phase-contrast images (left) and their corresponding fluorescent images (right) of control cells and cells microinjected with cytochrome *c*, followed by incubation with rhodamine 123 (original magnification, $\times 430$). **A** and **B**: Control cells. **B**: The fluorescent staining of thread-like mitochondria. **C** and **D**: Two groups of active-phase cells. **D**: Staining of condensed mitochondria. **E** and **F**: Two groups of spherical-phase cells. **F**: Shows staining of mitochondria, indicating maintenance of mitochondrial membrane potential. (Arrows in **D** and **F** indicate apoptotic cells.)

viewed by time-lapse video microscopy. The apoptotic phase began as early as 30 minutes after microinjection, and within 2–3 hours 60–70% of the microinjected cells (as evaluated morphologically) and 70–80% by the ApopTag assay were apoptotic. Cell death, as indicated

by nuclear propidium iodide staining, occurred at the late spherical phase, frequently when the cells were detaching from the substrate.

Examination of time-lapse video tapes (accelerated 60 times) revealed that the process of cytochrome *c*-induced apoptosis was extremely dynamic. (The excellent review on cell death by G. Majno and I. Joris² provides earlier descriptions of this phenomenon.) In our most graphic video sequences, the changes occurred explosively once they began. The initial shrinkage phase of the cell was followed by active pseudopod formation around the periphery of the cell. Although this active phase typically lasted for 1–1.5 hours, some cells continued active pseudopod formation for as long as 6 hours. This prolonged active and prelethal phase of apoptosis strongly suggests that ATP is required for this violent activity, as has been suggested by other models. Reports by Tsujimoto¹⁶ and Lieberthal et al¹⁷ support the hypothesis that both cytochrome *c* and a sizable pool of ATP are necessary to induce apoptosis. Our preliminary experiments using KCN + IAA as a model of inhibition of energy metabolism showed that cells treated with 0.25 mmol/L concentrations of both for 2 hours before microinjection with cytochrome *c* resulted in a reduction of ATP and complete prevention of apoptosis. The ATP assay data showed a correlation between the loss of ATP and the absence of apoptosis, which also supports the hypothesis that apoptosis is an ATP-requiring phenomenon. The maintenance of ATP in the active phase provides an explanation for the relatively normal-appearing mitochondria and normal range of cytosolic calcium seen in this phase. After the active phase, the cells again became quiescent. They changed shape, rounded up, and became more or less spherical. The cells remained in this conformation until they began to die (as shown by uptake of PI into nuclei) and detach from the coverslip.

Morphological Alterations

Shape Changes

The initial shrinkage and retraction of the cells in the first phase as observed by phase-contrast microscopy is most likely related to loss of water from the cytosol. Bortner and co-workers¹⁸ have hypothesized that shrinkage is secondary to loss of K^+ and Na^+ from the cytosol in that K^+ at normal concentrations (when efflux is prevented) inhibited DNA fragmentation and caspase-3 activity, suggesting an important early role for K^+ loss in apoptosis.

The subsequent active phase of pseudopod formation and the ultimate rounding up and retraction of the cells imply marked dynamic alterations in the cytoskeleton, probably involving actin and associated proteins. During this phase, actin staining patterns were markedly abnormal, with loss of the normal F-actin patterns and replacement by patchy, homogeneously staining areas throughout the cell. Actin staining appeared to be concentrated at the base of the pseudopods, whereas the pseudopods *per se* showed almost no staining. Similar bands of actin

Figure 7. Sets of phase-contrast and companion fluorescent images of cells after comicroinjection of cytochrome *c* and fixable Texas Red dextran (original magnification, $\times 540$). **A** (phase) and **B** (fluorescent): Cells 2 hours after microinjection. Groups of active apoptotic cells with pseudopods (**A**, **arrows**) also show Texas Red fluorescence (**B**, **arrows**), verifying positive microinjection (four cells are not positive for microinjection; **A**, **arrowheads**). **C** (phase) and **D** (fluorescent): Cells 4 hours after microinjection. Some cells are in the spherical phase (**C**, **arrows**) and show condensed chromatin after fixation and DAPI staining (**D**, **arrows**).

Figure 8. Phase and fluorescent images of cells after comicroinjection with cytochrome *c* and Texas Red dextran, fixed and stained with the ApopTag assay kit to determine DNA fragmentation (original magnification, $\times 500$). **A**: Texas Red fluorescent image of cells 30 minutes after microinjection and in early active phase. **B**: A fluorescein fluorescent image of the identical area, showing ApopTag-positive staining. **C**: Phase-contrast image of cells in the spherical phase 3 hours after microinjection. **D**: A fluorescein fluorescent image of the identical area, showing ApopTag-positive staining.

Figure 9. A: Phase-contrast images of Fura-2-loaded cells after microinjection with cytochrome *c*. **A: a, b, and c:** Active-phase cells at 2 hours, 2 hours, and 3 hours, respectively. **A: d, e, and f:** Spherical-phase cells at 5 hours, 5.25 hours, and 6 hours, respectively. **B:** Ratioed and normalized images of the identical cells in **A**. Cells were analyzed for $[Ca^{2+}]$, by digital imaging fluorescence microscopy. Images are displayed in gray tone, ranging from ~ 200 nmol/L (darkest) to 900 nmol/L calcium (whitest). The calcium range for the active phase cells was from 150 to 200 nmol/L (**B, b** and **c**), and for the spherical cells the maximum range was 900 nmol/L (**B, d**, cell 2), 500 nmol/L (**B, e**, cells 1 and 2), and 500 nmol/L (**B, f**, cell 3). **Scale bar: A and B, 22.3 μ m.**

Figure 10. Transmission EM of an active-phase cell, showing portions of several pseudopods and part of the central organelle area. Mitochondria appear to be slightly swollen and rounded, and the RER is dilated. Pseudopods contain a few mitochondria, free ribosomes, RER, and an occasional nuclear process with condensed chromatin (left pseudopod, **arrowhead**). Within the organelle region are phagolysosomes (**arrows**) and areas of filaments measuring ~ 200 nm (original magnification, $\times 16,500$). The **inset** shows a prominent area of filaments from another active-phase cell (original magnification, $\times 16,500$).

Figure 11. Transmission EM of the perinuclear area of a spherical phase cell. A dense chromatin area in the nucleus is just visible at the bottom of the micrograph. Mitochondria are rounded (**arrows**) and show high-amplitude swelling of the inner compartment, the cytoplasm is densely packed with free ribosomes, the RER is dilated, and phagolysosomes are present (**arrowheads**) (original magnification, $\times 15,600$).

staining were also observed at the base of cytoplasmic blebs in rat PTE cells after treatment with HgCl_2 .¹⁹

The fact that caspase-3 inhibition eliminates all of these shape changes and changes in actin staining leads us to presume that caspase-3 is directly involved. Actin is known to be a major cellular substrate for caspase-3, and Williams's group²⁰ has reported that caspase-3 cuts gelsolin, which is a Ca^{2+} -dependent actin-severing and -capping protein. The cleaved gelsolin fragments can, in turn, sever actin polymers. Microinjection of cells with NH_2 -terminal gelsolin fragments was shown to result in actin depolymerization and cellular shape changes, whereas gelsolin-null cells were shown to be resistant to the apoptotic shape changes induced by tumor necrosis factor (TNF) plus cycloheximide.²⁰ Because our data showed no significant $[\text{Ca}^{2+}]_i$ elevation during the active phase, we presume that normal concentrations of $[\text{Ca}^{2+}]_i$ are sufficient for these putative gelsolin-induced changes.

Mitochondrial Changes

The mitochondria also underwent dynamic changes in conformation, which related to the phases of apoptosis. In the untreated NRK cells, the mitochondria appeared as long, thread-like bodies that were in continual movement, as seen in time-lapse video microscopy. By TEM, they appeared as elongated profiles with moderately dense matrices and narrow intercrystal spaces. During the active phase, where there is dramatic extension and retraction of pseudopods, mitochondria remained in their orthodox conformation. Some, however, show matrical condensation. The latter can be seen during periods of rapid ATP utilization, such as state 3 respiration,²¹ and is

completely compatible with the presumed ATP requirement for cells in the active phase, as mentioned above.

When the cells entered the quiescent spherical phase, the mitochondria began to show varying degrees of matrical swelling, similar to that which occurs in oncosis in renal tubules after other types of prelethal injury, such as total ischemia *in vivo*,²² or after exposure to many nephrotoxins, including mercuric chloride.¹² This swelling is known to be reversible and is usually associated with ATP depletion.³ In the spherical phase the MMP initially remains intact, as measured by rhodamine 123 retention, demonstrating that the mitochondrial permeability transition had not occurred. The progression to cell death followed by necrosis then takes place and the mitochondria show flocculent densities. The identical changes leading up to cell death and necrosis are shared by apoptosis and oncosis, as recently pointed out by LeMasters.²³

Because treatment with Ac-DEVD-CHO prevented all of the mitochondrial changes, it is presumed that caspase-3 is either directly or indirectly involved. On the other hand, overexpression of *Bcl-2* did not prevent these mitochondrial changes, which implies that, at least in this system, *Bcl-2* overexpression did not prevent changes in mitochondrial inner membrane permeability. Furthermore, the observation that *Bcl-2* overexpression did not protect against apoptosis may render it less likely that caspase-3 produces mitochondrial swelling by attacking the mitochondrial permeability transition pore complex directly.²⁴ In our experiments, *Bcl-2* protection was not observed downstream of cytochrome c, as suggested by Rosse et al.²⁵ However, they used Bax-induced apoptosis, which they reported invokes a different type of cell death pathway.²⁵ At the same time it has been reported

Figure 12. Transmission EM of a spherical-phase cell, showing areas of intense chromatin clumping and a large, dramatic nuclear-chromatin bud with a bilobular configuration (original magnification, $\times 15,600$). The **inset** shows a smaller nuclear chromatin process, which can be identified by the presence of a nuclear envelope and small strands that connect the process with the main body of the nucleus (**arrows**) (original magnification, $\times 13,000$).

that Bcl-2 can be cleaved, at least in some circumstances, by caspases. Furthermore, cleavage of Bcl-2 may not only inactivate these proteins, but may also produce fragments that promote apoptosis.²⁶

The results of previous work in our laboratory have indicated that Bcl-2 protection resulting from oxidant injury is due to mitochondrial buffering of increased $[Ca^{2+}]_i$.¹³ This buffering has also been reported to occur in neural cells.²⁷ Another possibility for this protection is that the change in inner membrane permeability may be secondary to caspase-induced activation of a phospholipase A2, as suggested by Atsumi et al²⁸ and Wissing et al.²⁹

This study has shown that microinjection of cytochrome *c* into NRK-52E cells results in rapid apoptosis that can be separated into four distinct morphological phases: shrinkage, active, spherical, and necrotic. It was also found that the caspase-3 inhibitor Ac-DEVD-CHO totally prevented apoptosis, whereas excess Bcl-2 did not. In addition, preliminary data showed that ATP is a requirement for the active phase of apoptosis.

Figure 13. Graph of percentage of apoptotic cells in media without serum over a 5-hour period after different pretreatment times with 0.25 mmol/L KCN + 0.25 mmol/L IAA, followed by microinjection of cytochrome *c*. Zero time represents the time of microinjection. Control cells (no chemical treatment) had almost 100% apoptosis by 1–2 hours. Cells pretreated for up to 1.5 hours before microinjection showed a reduction to about 30% apoptosis by 4 hours, and cells pretreated for 2 hours or longer before microinjection had no apoptotic cells.

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