

TIMING YOUR APOPTOSIS ASSAYS

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The term apoptosis refers to a series of specific morphological changes associated with cell death. We present a number of factors that can affect the timeline of biochemical events associated with apoptosis to emphasize the importance of monitoring these biochemical events over an appropriate time period.

Introduction

Cell death can occur by a spectrum of morphologically and biochemically distinct pathways, including apoptosis, necrosis and autophagy (1). The term "apoptosis", coined by Kerr *et al.* (2), describes cell death characterized by defined morphological changes, including cell shrinkage, chromatin condensation, loss of nuclear membrane integrity, plasma membrane blebbing and eventually apoptotic body formation. Although apoptosis refers to a purely morphological change, biochemical events accompany these morphological changes. These events include initiator and effector caspase activation, release of cytochrome c from mitochondria, externalization of phosphatidylserine on the plasma membrane, poly (ADP-ribose) polymerase (PARP) cleavage and internucleosomal DNA fragmentation.

Not all of these biochemical events are specific to apoptosis, and not all events occur in all apoptotic cells, in all stages of apoptosis or in response to all apoptosis-inducing stimuli. The time course of these biochemical events often differs, depending upon a wide variety of factors, such as the cell line or tissue, apoptosis-inducing agent, drug concentration or stimulus intensity, and exposure time. Therefore it is critical to choose wisely the biochemical events that will be measured to show that cell death occurs by apoptosis and to understand the kinetics of cell death. In this article, we discuss the importance of monitoring these biochemical events over an appropriate time period to ensure that the period of activity is not missed. We also present some of the factors that can influence the timeline of detection of the associated biochemical events.

Timeline of Biochemical Events During Apoptosis

Apoptosis typically progresses through the extrinsic or intrinsic pathways. The extrinsic (receptor-mediated) pathway involves receptor binding, followed by activation of the initiator caspase, caspase-8, which in turn activates caspase-3 or amplifies caspase-3 activation by cleaving the Bcl-2 family member Bid. Bid cleavage leads to cytochrome c leakage, the formation of a protein complex called the apoptosome and activation of caspase-9 (3). The intrinsic (mitochondrial) pathway involves members of the Bcl-2 family that regulate cytochrome c release from the mitochondria. Once released from the mitochondria, cytochrome c can interact with Apaf-1, dATP

and procaspase-9 to form the apoptosome. As part of the apoptosome, caspase-9 is processed and activated, and proceeds to activate caspase-3. In both pathways, the late apoptotic events take place after activation of the effector caspases and include exposure of phosphatidylserine on the external surface of the plasma membrane (which can be measured by annexin V binding), cleavage of poly (ADP-ribose) polymerase (PARP) and internucleosomal DNA fragmentation.

Exactly which biochemical events occur will depend on the apoptotic pathway. In addition, the time required to detect different events or activities after apoptosis induction often varies. For example, treating Jurkat cells with anti-Fas monoclonal antibody (mAb) activates the extrinsic pathway of apoptosis by causing trimerization of the Fas receptor and inducing a conformational change that allows assembly of the death-inducing signaling complex (DISC). In anti-Fas mAb-treated Jurkat cells, caspase-8 activity peaked after only 3

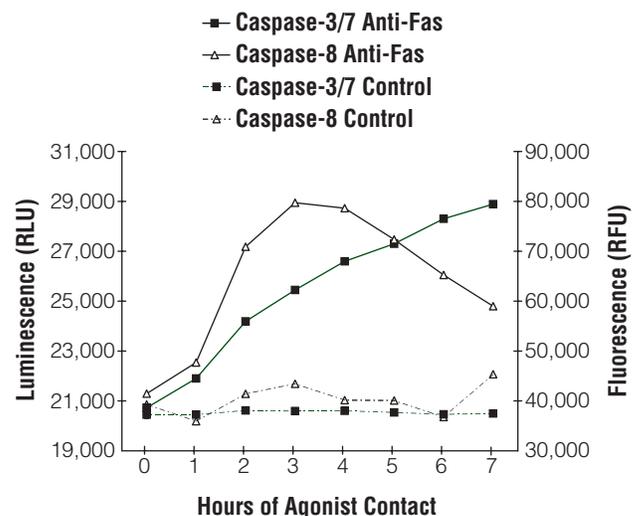


Figure 1. Time dependence of caspase-8 and caspase-3/7 activities. Jurkat cells were seeded at a density of 25,000 cells per well, and anti-Fas mAb or vehicle was added every hour for 7 hours. Caspase-8 was detected using the luminescent Caspase-Glo® 8 Assay with Z-LETD-aminoluciferin as the substrate. Caspase-3/7 was detected using a modification of the fluorescent Apo-ONE® Homogeneous Caspase-3/7 Assay, which contains (Z-DEVD)₂-R110 as the substrate. After a 1-hour incubation at room temperature, luminescence and fluorescence were recorded in the same well (4).

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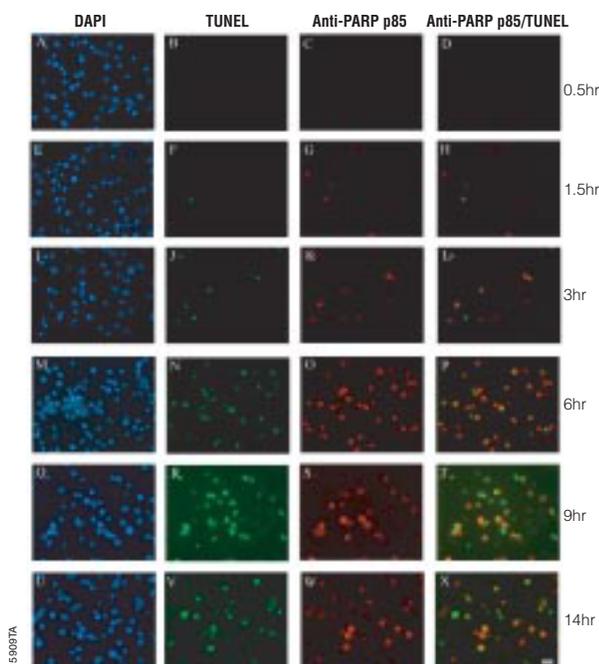


Figure 2. Time course of TUNEL and PARP cleavage. Jurkat cells were treated with anti-Fas mAb for the times noted. Cells were double labeled using the DeadEnd™ Fluorometric TUNEL System (Cat.# G3250) to assess DNA fragmentation and with Anti-PARP p85 Fragment pAb (Cat.# G7341), which specifically recognizes the cleaved form of PARP. Each row shows the same field of cells at the noted time point. Each column shows a different staining method as noted. For each time point, the DAPI staining shows the number of nuclei in each field. Scale bar = 20µm.

hours of treatment, whereas caspase-3/7 activity increased over the 7 hours measured (Figure 1). PARP cleavage peaked at 6 hours, while DNA fragmentation, a late-stage event, did not peak until after 9 hours of treatment (Figure 2). Monitoring these activities before or after the optimal time period could have resulted in little to no signal above background (compare caspase-8 activity in vehicle control and anti-Fas mAb-treated cells at 1 hour and at 7 hours, Figure 1), leading to the erroneous conclusion that the treatment did not induce apoptosis. Thus, it is critical to empirically determine the time at which the activity of interest peaks. Perform initial studies to determine the optimal time, or be sure to examine the activity over a sufficiently broad time period with appropriate time increments to capture peak activity.

Many factors can affect the timeline of apoptotic events, including the cell line or tissue examined, apoptosis-inducing agent, drug concentration or stimulus intensity, and exposure time. We discuss a number of these factors here to emphasize the importance of monitoring these biochemical events over an appropriate time period.

Effects of Cell Line or Tissue

Different cell lines or tissues may respond differently to identical conditions. The same agent may induce apoptosis in one cell line but not in other cells. In some cases, the result of the treatment is the same (cell death), but the mechanism is quite different. Treating HL60 and K562 cells with similar concentrations of doxorubicin produced an apoptotic morphology and DNA laddering in HL60 cells but a necrotic morphology and no DNA fragmentation in K562 cells (5).

Even with an identical apoptosis-inducing stimulus, timelines for the progression of different cells through apoptosis vary. The time required for an activity to peak in one cell line will likely differ from that for another cell line. For example, DNA fragmentation in response to staurosporine treatment peaked at 5–6 hours in EL4 cells (6) but at 24 hours in HCE cells (7). When consulting published protocols to identify apoptosis-inducing conditions, do not assume that the effect on your cell line will be the same as that on other cells.

In general, cultured cells induced to undergo apoptosis exhibit signs of apoptotic events much sooner (within hours; 5–10) than cells within tissues (within days; 11–14). When studying an in vivo model, some late apoptotic events may never be detected, as apoptotic cells in tissues are often phagocytosed before late events can be detected.

Some events may be underestimated because the cell line or tissue of interest does not express a necessary protein. The breast cancer cell line MCF-7 lacks a functional caspase-3 gene product, so apoptosis may be underestimated by assays that use DEVD substrates to detect caspase-3/7 activity. Likewise, a cell that does not express a necessary receptor cannot be induced to undergo apoptosis by the extrinsic pathway. A classic example is anti-Fas mAb treatment to induce the extrinsic pathway in Fas receptor-bearing Jurkat cells. Cells that lack the Fas receptor will not undergo apoptosis in response to anti-Fas mAb treatment.

Because of heterogeneity and differences in phases of the cell cycle, individual cells within the same population can undergo apoptotic events at different times. For example, in HeLa cell cultures exposed to ultraviolet light, some cells lost plasma membrane integrity, a late event in apoptosis, before other cells in the same population underwent cytochrome c release from the mitochondria, a relatively early event in apoptosis (10). Both the time required after UV light treatment to detect the initial apoptotic event and the length of time intervals between apoptotic events varied. In this example, the release of cytochrome c from a single cell occurred during a 5-minute time period, but cytochrome c release from the entire cell population was detected over the course of many hours. Thus, population heterogeneity can reduce the need to monitor the activity of interest over a narrow time period. However, to obtain an accurate temporal resolution of events, single cells must be analyzed (10,15).

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Effects of Apoptosis-Inducing Agent

Different drugs or stimuli can have different effects on the same cell line or tissue. Some agents have no effect on a cell line, whereas other agents might induce necrosis, rather than apoptosis, or even induce different apoptosis pathways. For example, treatment with staurosporine, a protein kinase C inhibitor, but not with anti-Fas mAb induces apoptosis in HeLa cells, which are Fas receptor-deficient. Some methods such as treatment with kinase inhibitors, exposure to ultraviolet (UV) light or X-ray irradiation are more effective at inducing apoptosis in a majority of cells or tissues. However, we recommend choosing an appropriate apoptosis-inducing agent carefully. Consult published literature or perform preliminary experiments to be sure that the agent induces apoptosis in your cell line of interest. The Promega Apoptosis Assistant, which is available at: www.promega.com/apoasst/ also can be a useful resource.

Effects of Drug Concentration or Stimulus Intensity and Exposure Time

The concentration of a drug or intensity of the stimulus can also affect the cells' response. A lower drug concentration may have no effect or even an unintended effect. For example, treating HeLa cells with low concentrations of the cardiac glycosides ouabain, strophanthidin, digoxin and digoxigenin stimulated, rather than inhibited, cell growth as measured by a change in absorbance following crystal violet staining. At higher drug concentrations, the same drugs induced apoptosis (16). At an inappropriately high concentration or intensity, an apoptosis-inducing stimulus can induce necrosis rather than apoptosis.

Exposure time also has an effect. In general, induction of apoptosis using lower drug concentrations or a less intense stimulus requires longer exposure times. Thus, EC_{50} values decrease as exposure time increases (9,16). Whereas exposure of HeLa cells to low cardiac glycoside concentrations for 48 hours stimulated cell growth, growth after 72 or 96 hours was neither stimulated nor inhibited. At higher drug concentrations, the inhibitory effect on cell growth was more pronounced with increasing exposure times. Thus, the time that a cell is subjected to a toxin or injury can also greatly affect experimental results and conclusions. Treatments that seem to have little or no short term effect can have dramatically different effects over the long term.

Even under conditions that induce apoptosis, the times at which specific apoptotic events occur can vary with exposure time. The time at which caspase-3 activity peaks in tamoxifen-treated HepG2 cells depends upon the drug concentration and also the exposure time (Figure 3; 17). At the higher tamoxifen concentrations, caspase-3 activity peaked after only 1–2 hours, but at lower tamoxifen concentrations, caspase-3 activity did not peak until after a

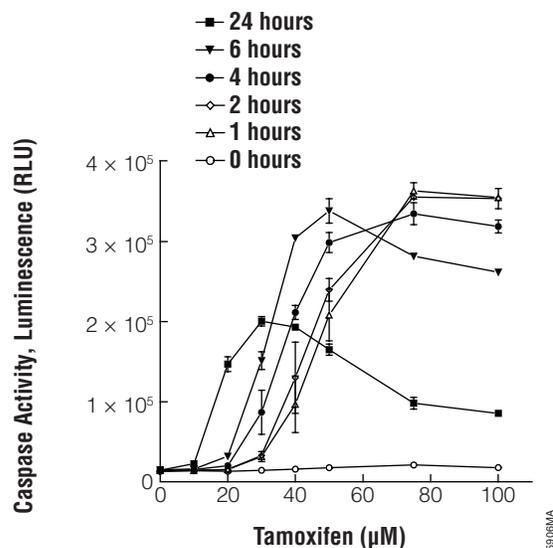


Figure 3. Caspase-3 activity measured from HepG2 cells treated with 0–100µM tamoxifen for 0–24 hours. HepG2 cells were seeded at 25,000 cells/well in a 96-well plate and cultured overnight. Tamoxifen stock solution in DMSO was further diluted into RPMI medium and added at staggered times. All wells without tamoxifen contained a final concentration of 0.2% DMSO. Caspase-3/7 activity was measured using the luminescent Caspase-Glo® 3/7 Assay. The Caspase-Glo® 3/7 Reagent (100µl/well) was added at the appropriate time point after tamoxifen addition, and the assay plates were incubated at ambient temperature for 1 hour prior to recording luminescence. The luminescence value for the control of culture medium without cells was 708 ± 68 (data not shown). Data shown represent the mean ± SD (n = 3). Reprinted with permission from Moravec, R. and Riss, T. (2004) *ASSAY Drug Dev. Tech.* 2, 1–12.

much longer exposure (24 hours). We recommend performing an initial dose response curve to identify an appropriate drug concentration or stimulus intensity to induce apoptosis in the cell or tissue of interest. Be sure to examine the activity over a sufficiently broad time period with appropriate time increments to capture peak activity.

Effect of Cell Culture Conditions

As described above, the cell line used to examine apoptosis can have a dramatic effect on experimental results. However, a perhaps lesser known fact is that the culture conditions can also have an effect (9). Factors such as cell density, passage number and the density to which the parental stock culture was grown can affect cell physiology, and subsequently, may affect the ability to respond to a stimulus. Relatively high cell numbers seem to have a protective effect; perhaps the drug is binding nonspecifically to the cell surface or cellular proteins in the medium to reduce the effective drug concentration. When HepG2 cells at different densities were treated with increasing concentrations of tamoxifen, drug potency increased (lower EC_{50} value) as cell number decreased (Figure 4). Because the exposure time required for peak caspase-3/7 activity increases

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with decreasing tamoxifen concentration (Figure 3), this suggests that a longer exposure time is required to detect the specific apoptotic event when higher numbers of cells are used. When preparing cell cultures to study apoptosis, be sure to use well established culture procedures and consistent cell numbers to minimize these variables.

Summary

Apoptosis can occur by a variety of biochemically distinct mechanisms, and the timeline of apoptosis-related biochemical events depends on a number of factors. This discussion is not meant to be an exhaustive list but an introduction to the factors that can affect the timeline of apoptosis. When designing an apoptosis study, be sure to take these factors into account. Perform preliminary experiments to identify appropriate assay conditions. Exercise care when choosing a biochemical marker of apoptosis to be sure that the marker is relevant in your experimental system. Perform an initial dose response curve to identify the appropriate drug concentration or stimulus intensity to induce apoptosis in the cell or tissue of interest. Examine the biochemical event over a sufficiently broad time period with appropriate time increments to capture peak activity and minimize erroneous conclusions based on ill-timed assay points. ■

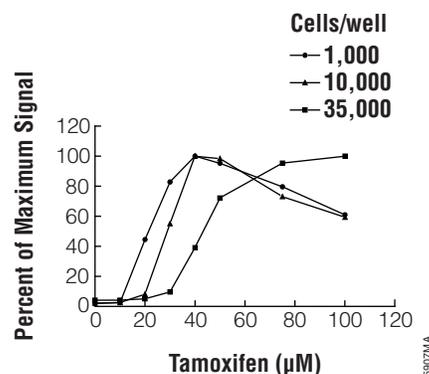


Figure 4. Effect of cell number on the timing of caspase activation. HepG2 cells were seeded at 1,000, 10,000 or 35,000 cells per well in 96-well plates and exposed to various concentrations of tamoxifen for 4 hours. Caspase-3/7 activity was measured using the luminescent Caspase-Glo® 3/7 Assay, and the results were expressed as percentages of maximum signal for each cell density. All data points represent the mean ± SD (n = 3). Reprinted with permission from Moravec, R. and Riss, T. (2004) *ASSAY Drug Dev. Tech.* **2**, 1–12.

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Additional Information and Protocols

Protocols and Applications Guide, Apoptosis, Chapter 3 (www.promega.com/paguide/chap3.html)

Related Products

Product	Size	Cat. #
Caspase-Glo® 3/7 Assay*	2.5ml	G8090
Caspase-Glo® 8 Assay*	2.5ml	G8200
Caspase-Glo® 9 Assay*	2.5ml	G8210
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml	G7791
CaspACE™ FITC-VAD-FMK In Situ Marker	50µl	G7461
DeadEnd™ Colorimetric TUNEL System	20 reactions	G7360
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250
Caspase Inhibitor Z-VAD-FMK	50µl	G7231
Caspase Inhibitor Ac-DEVD-CHO	100µl	G5961
Anti-PARP p85 Fragment pAb	50µl	G7341
Anti-ACTIVE® Caspase-3 pAb	50µl	G7481
Anti-Cytochrome c mAb	100µg	G7421

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