Video Article

Identification of Intracellular Signaling Events Induced in Viable Cells by Interaction with Neighboring Cells Undergoing Apoptotic Cell Death

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Abstract

Cells dying by apoptosis, also referred to as regulated cell death, acquire multiple new activities that enable them to influence the function of adjacent live cells. Vital activities, such as survival, proliferation, growth, and differentiation, are among the many cellular functions modulated by apoptotic cells. The ability to recognize and respond to apoptotic cells appears to be a universal feature of all cells, regardless of lineage or organ of origination. However, the diversity and complexity of the response to apoptotic cells mandates that great care be taken in dissecting the signaling events and pathways responsible for any particular outcome. In particular, one must distinguish among the multiple mechanisms by which apoptotic cells can influence intracellular signaling pathways within viable responder cells, including: receptor-mediated recognition of the apoptotic cell, release of soluble mediators by the apoptotic cell, and/or engagement of the phagocytic machinery. Here, we provide a protocol for identifying intracellular signaling events that are induced in viable responder cells following their exposure to apoptotic cells. A major advantage of the protocol lies in the attention it pays to dissection of the mechanism by which apoptotic cells modulate signaling events within responding cells. While the protocol is specific for a conditionally immortalized mouse kidney proximal tubular cell line (BU.MPT cells), it is easily adapted to cell lines that are non-epithelial in origin and/or derived from organs other than the kidney. The use of dead cells as a stimulus introduces several unique factors that can hinder the detection of intracellular signaling events. These problems, as well as strategies to minimize or circumvent them, are discussed within the protocol. Application of this protocol should aid our expanding knowledge of the broad influence that dead or dying cells exert on their live neighbors, both in health and in disease.

Video Link

The video component of this article can be found at https://www.jove.com/video/54980/

Introduction

Apoptosis, or regulated cell death[1], contributes in an essential manner to the maintenance and development of tissues. Viewed most simply, apoptosis permits aged, damaged, or excess cells to be eliminated without harm to surrounding tissues[2,3]. The contribution of apoptosis to tissue homeostasis, however, is considerably more dynamic and varied. Cells dying by apoptosis acquire multiple new activities, both secreted and cell-associated, which enable them to influence the function of adjacent live cells[4-10]. Earlier studies focused on the ability of apoptotic cells to suppress inflammation[11-16], but apoptotic cells also modulate a broad range of cellular functions, including such vital activities as survival[4,9,10], proliferation[4,9,10], differentiation[17], migration[18], and growth[19]. Moreover, these effects are not restricted to professional phagocytes, like macrophages, but extend to virtually all cell types and lineages, including traditionally non-phagocytic cells, such as epithelial and endothelial cells[7,9,10,18-21].

The specific response of adjacent live cells following exposure to apoptotic cells depends on multiple factors that relate to both the viable responding cells and the apoptotic cells themselves. For example, although exposure to apoptotic cells inhibits the proliferation of both murine macrophages and kidney proximal tubular epithelial cells (PTECs), these two cell types differ dramatically in their survival response to apoptotic cells[4,6,9,10]. Apoptotic cells promote the survival of macrophages, but induce the apoptotic death of PTECs[4,6,9,10]. Notably, the response to apoptotic cells may differ even among responding cells of the same lineage, depending on the responding cell's organ of origin[10] (*e.g.*, kidney PTECs *versus* mammary epithelial cells) or state of activation[22] (*e.g.*, neutrophils). Conversely, apoptotic cells may evoke different responses in the very same cell depending on the nature of the apoptotic stimulus[10,23] or the stage of apoptosis[10,14].

Given the diverseness and complexity of the response to apoptotic cells, great care must be taken in dissecting the signaling events and pathways responsible for any particular outcome. First, responses requiring direct physical interaction between viable and apoptotic cells must be differentiated from those elicited by soluble mediators released or generated by the apoptotic cell[3,6-10]. If physical interaction is required, then a further differentiation should be performed. Signaling events may depend on receptor-mediated recognition of the apoptotic cell, independent of subsequent engulfment, or on phagocytic uptake, independent of the specific receptor that binds the apoptotic cell[3-7,9,10,19]. In the latter instance, the response is not specific to apoptotic cells, and can be triggered by any phagocytic material[4,6].

The importance of these distinctions can again be appreciated by contrasting the responses of macrophages and kidney PTECs. For both cell types, exposure to apoptotic cells alters the activity of the pro-survival kinase Akt. Modulation is dependent on physical interaction, since separation of responding and apoptotic cells by a 0.4 µm polycarbonate membrane abolishes the response[4,9,10,19]. However, the response of PTECs is receptor-mediated and independent of phagocytosis, whereas the response of macrophages is driven by phagocytosis[4,9,10,19]. This conclusion is reinforced by the fact that exposure to latex beads, a neutral phagocytic stimulus, has no effect on Akt activity in PTECs, but mimics the effect of apoptotic cells in macrophages[4,9].

Although less well studied, cells dying by necrosis, or accidental cell death[1], also modulate the function of nearby viable cells[3-10,19]. Like apoptotic cells, necrotic cells exert their effects through a variety of mechanisms, particularly the leakage of intracellular contents through their ruptured cell membrane[3,5,6,9,24]. Many cells, including PTECs and macrophages, possess distinct non-competing receptors for cells dying by necrosis[5,9]. Engagement of these receptors induces signaling events that are often opposite to those induced by engagement of the receptors for apoptotic cells[4-10,19]. For example, in PTECs, necrotic cells increase phosphorylation of Akt, whereas apoptotic cells decrease phosphorylation[9,10,19].

Here, we describe a protocol for identifying intracellular signaling events induced in viable kidney PTECs through receptor-mediated recognition of adjacent apoptotic PTECs[9,10,19]. While the protocol is specific for a conditionally immortalized PTEC cell line known as BU.MPT cells[9,10,19,25,26], it is easily adapted to cell lines that are non-epithelial in origin and/or derived from organs other than the kidney. Importantly, the use of apoptotic cells as a cell stimulus poses certain inherent experimental difficulties not present with soluble ligands. The most important of these is that apoptotic cells must be added as a suspension rather than as a solution. These difficulties, as well as strategies to minimize or circumvent them, are discussed within the protocol. Nearly all of the techniques described in the protocol are straightforward and standard to cell culture. The advantage of this protocol lies in its attention to the multiple mechanisms by which apoptotic cells modulate signal transduction within responding cells. These mechanisms include the binding via surface determinants or bridging molecules to specific receptors on the responding cell, the release of soluble mediators, and/or the engagement of the phagocytic machinery. Necrotic cells are included in all experiments to ensure that results are specific to the mode of cell death, and not a generalized response to dead cells. A careful approach, as recommended in this protocol, is critical to our understanding of the ever expanding influence that dying cells exert on their live neighbors, in both health and disease.

Protocol

1. Preparations

Note: In this protocol, two or more cell lines, or primary cell cultures, are independently prepared under specific conditions. These preparations include apoptotic cells (Protocol 1), necrotic cells (Protocol 2), and healthy responder cells (Protocol 3). After independent preparation, apoptotic or necrotic cells are added to responder cells and the resulting signal transduction is monitored (Protocols 4, 5, and 6).

1. Prepare culture media and reagents
    1. Prepare 500 mL of culture medium A (for use when growing cells under permissive conditions, see section 1.2) by combining the following: 1x Dulbecco's Modified Eagle's Medium (DMEM) containing 4.5 g/L glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate, 100 units/mL penicillin-streptomycin, 10% (v/v) fetal bovine serum (FBS), and 10 units/mL of interferon-γ (IFN-γ).
    2. Prepare 500 mL of culture medium B (for use when serum-starving cells under non-permissive conditions, see section 1.2) by omitting FBS and IFN-γ from the recipe of culture medium A. To grow cells under non-permissive conditions (prior to serum-starvation), add 10% v/v FBS to culture medium B.
    3. Prepare 0.4% (v/v) paraformaldehyde, pH 7.2, in 1x Dulbecco's phosphate buffered saline (DPBS) from 4% stock paraformaldehyde for fixation of dead cells.
    Note: Paraformaldehyde is toxic, and appropriate caution should be exercised in its use.

2. Culture BU.MPT cells
    1. Thaw from liquid nitrogen one vial of BU.MPT cells.
    Note: BU.MPT is a mouse kidney proximal tubular epithelial cell (PTEC) line derived from a transgenic mouse bearing a temperature-sensitive (ts) mutation (tsA58) of the SV40 large tumor antigen (TAg) under the control of the mouse major histocompatibility complex (MHC) H-2K^b class I promoter[25,26].
    2. Plate cells on sterile polystyrene vacuum-gas plasma treated tissue culture dishes (~ 5 x 10^6 cells per 100 mm diameter dish).
    3. Grow cells under permissive conditions in a humidified 5% (v/v) CO_2 atmosphere.
    Note: Permissive conditions, which are defined as culture at 33 - 37 ºC in the presence of IFN-γ, enable stable expression of the tsA58-mutated TAg transgene. Unlike primary cultures of mouse kidney PTEC, which do not survive more than a single passage or two, BU.MPT cultured under permissive conditions can be passaged indefinitely.
        1. Passage cells at least three times after thawing before using them in an experiment.
            1. To passage cells, add 1 mL of 0.05% trypsin per 100 mm dish, and incubate in a humidified 5% (v/v) CO_2 atmosphere at 37 ºC for 5 min. Neutralize the trypsin by adding 10 mL of medium A. Aspirate the detached cells, and split 1:3 to 1:5 into new 100 mm sterile polystyrene vacuum-gas plasma treated tissue culture dishes. Add medium A to bring the volume up to a total of 10 mL per 100 mm dish.

4. In preparation for experimental use as responding cells, grow cells to confluence in a humidified 5% (v/v) CO_2 atmosphere under non-permissive conditions (*i.e.* at 39 ºC in the absence of IFN-γ [medium B containing 10% v/v FBS]).
   Note: Under non-permissive conditions, expression of the tsA58-mutated TAg transgene is inhibited by >95%, and BU.MPT cells behave like primary cultures of mouse kidney PTEC.

2. Preparation of Apoptotic BU.MPT Cells (Protocol 1)

Note: This protocol is specific for BU.MPT cells as the source of apoptotic cells, and staurosporine as the method of apoptosis induction. Alternatively, another cell line, or primary cell culture, may be used as a source of apoptotic cells, with apoptosis induced by standardized protocols for that particular cell type and method of apoptosis induction.

1. After passage onto 100 mm diameter sterile polystyrene vacuum-gas plasma treated tissue culture dishes, grow BU.MPT cells to confluence in a humidified 5% (v/v) CO_2 atmosphere under permissive conditions (*i.e.* at 37 ºC in culture medium A at 10 mL per 100 mm dish), as described in section 1.2.3.
2. Rinse the adherent cell monolayer three times with culture medium B, using 10 mL per rinse.
3. Induce apoptosis by incubating the cells in culture medium B containing staurosporine, a nonselective protein kinase inhibitor, at 1 µg/mL for 3 h in a humidified 5% (v/v) CO_2 atmosphere at 37 ºC.
4. Aspirate the staurosporine-containing medium containing "floating" apoptotic cells that have detached from the monolayer. Centrifuge this medium for 10 min at 500 x g, discard the supernatant, wash the pellet three times with culture medium B, and add the pellet back to cells collected in the next step, 2.5.
5. Detach the remaining adherent cells from steps 2.3 and 2.4 by addition of 5 mM (ethylenediaminetetraacetic acid) EDTA in 1x Ca^{2+}- and Mg^{2+}-free DPBS at 1 mL per dish for 5 min. Aspirate the EDTA-containing medium containing detached apoptotic cells, and pool the detached cells with the "floating" apoptotic cells from step 2.4 in a sterile 15 mL polystyrene centrifuge tube.
6. Wash the apoptotic cells three times by centrifugation for 10 min at 500 x g and resuspension in 10 mL of 1x Ca^{+2}- and Mg^{+2}-free DPBS per wash.
7. After the last wash, suspend the apoptotic cells in fresh culture medium B at ~ 5 x 10^6 cells per mL before use to stimulate responder cells. Alternatively, fix washed apoptotic cells in 0.4% (v/v) paraformaldehyde in 1x DPBS for 30 min, and then wash three times with culture medium B, before suspension in culture medium B.
8. As appropriate, confirm induction of apoptosis by flow cytometry using a separate preparation of apoptotic cells (or by reserving some cells for this purpose), as previously described[6,9,10,15].
   Note: Early apoptotic cells have intact cell membranes, and appear as propidium iodide (PI)-negative and annexin V-positive cells of decreased cell size (relative to viable cells). Late apoptotic cells have non-intact cell membranes, and appear as PI-positive and annexin V-positive cells of decreased cell size. Using the current protocol, typical preparations contain ~ 85% early apoptotic and ~ 15% late apoptotic cells.
9. Add apoptotic cells to BU.MPT responder cells (Protocol 3) at an apoptotic-to-responder cell ratio of 1:1, either directly or after fixation of apoptotic cells for 30 min with 0.4% (v/v) paraformaldehyde in 1x DPBS.
   Note: Apoptotic cell fixation prior to stimulation of responders should not affect results, unless the observed signaling events are due to release of a soluble mediator (**Table 1**).

3. Preparation of Necrotic BU.MPT Cells (Protocol 2)

Note: This protocol is specific for BU.MPT cells as the source of necrotic cells, and heating as the method of necrosis induction. Alternatively, another cell line, or primary cell culture, may be used as a source of necrotic cells, with necrosis induced by standardized protocols for that particular cell type and method of necrosis induction.

1. After passage onto 100 mm diameter sterile polystyrene vacuum-gas plasma treated tissue culture dishes, grow BU.MPT cells to confluence in a humidified 5% (v/v) CO_2 atmosphere under permissive conditions (*i.e.* at 37 ºC in culture medium A at 10 mL per dish), as described in section 1.2.3.
2. Rinse the adherent cell monolayer three times with culture medium B, using 10 mL per rinse.
3. Detach the cells by addition of 5 mM EDTA in 1x Ca^{+2}- and Mg^{+2}-free DPBS at 1 mL per dish for 5 min. Aspirate the EDTA-containing medium containing detached cells, and add the cell suspension to a sterile 15 mL polystyrene centrifuge tube.
4. Wash the cells three times by centrifugation for 10 min at 500 x g and resuspension in 10 mL of Ca^{+2}- and Mg^{+2}-free DPBS per wash.
5. After the last wash, suspend the cells in fresh culture medium B at ~ 5 x 10^6 cells per mL.
6. Induce necrosis by heating cells to 70 ºC for 45 min in a water bath, gently vortexing the cell suspension every 10 min.
7. Incubate cells for 2 h in a humidified 5% (v/v) CO_2 atmosphere at 37 °C. Gently vortex the cell suspension every 15 min.
8. As appropriate, confirm induction of necrosis by flow cytometry using a separate preparation of necrotic cells (or by reserving some cells for this purpose), as previously described[6,9,10,15].
   Note: Necrotic cells have ruptured cell membranes, and appear as PI-positive cells of increased cell size (relative to viable cells). Using the current protocol, typical preparations contain ≥ 95% necrotic cells. Alternatively, induction of necrosis and loss of membrane integrity may be confirmed by Trypan blue staining.

4. Preparation of BU.MPT Responder Cells (Protocol 3)

Note: This protocol is specific for BU.MPT cells as the source of responder cells. Alternatively, another cell line, or primary cell culture, may be used as responder cells. Prior to experimental use, quiescence may be induced overnight by standardized protocols within the laboratory.

1. Grow BU.MPT cells in sterile 100 mm tissue culture dishes under permissive conditions in culture medium A at 10 mL per dish until they achieve ~ 85% confluence.
2. Aspirate culture medium A, and rinse the cells three times with culture medium B at 10 mL per rinse. Serum-starve the cells overnight for 18 to 24 h in a humidified 5% (v/v) CO_2 atmosphere under non-permissive conditions (*i.e.* at 39 ºC in culture medium B at 10 mL per dish), as described in section 1.2.4, in order to induce quiescence. Alternatively, grow cells in culture medium B plus 10% (v/v) FBS at 39 ºC for one day before serum starving cells overnight in culture medium B without FBS.
3. Label a separate tissue culture dish of confluent BU.MPT cells for each experimental condition.
4. Include the following six conditions: stimulation of viable BU.MPT responders with either vehicle or epidermal growth factor (EGF) for 15 min, following their exposure for 30 min to either no cells, apoptotic cells, or necrotic cells.
   Note: Exposure to dead cells may either stimulate or inhibit the level of activity of a given signaling molecule. Stimulation is best detected above a low baseline (*e.g.*, absence of EGF), whereas inhibition is best detected from a high baseline (*e.g.*, presence of EGF). Since the effect of stimulation with dead cells is *a priori* unknown, it is recommended to perform all studies in both the absence and the presence of EGF.

5. Stimulation of Responder Cells with Apoptotic and Necrotic Cells (Protocol 4)

1. Aspirate culture medium B from pre-labeled dishes of quiescent (serum-starved) BU.MPT responder cells (Protocol 3).
   Note: After overnight culture under non-permissive conditions, BU.MPT cells should behave like primary cultures of mouse kidney PTEC.
2. Per 100 mm dish, add 2 mL of one of the following: culture medium B (no cells); apoptotic cell suspension at ~ 5 x 10^6 cells per mL in culture medium B (Protocol 1); or necrotic cell suspension at ~ 5 x 10^6 cells per mL in culture medium B (Protocol 2).
   1. Distribute the 2 mL suspension of dead cells evenly over the 100 mm dish, and keep the time for them to settle to a minimum. To achieve this, distribute the dead cell suspension drop by drop with a wide tip pipette over the entire surface of the responder cell dishes. Use a volume of 2 mL as a compromise between minimizing the settling time and avoiding the clumping of dead cells.
      Note: The use of a suspension of dead cells as a stimulus entails a number of special considerations, which are described below in greater detail (**Table 2** and Discussion).
   2. Achieve a dead-to-responder cell ratio of ~ 1:1 by adding 2 mL of dead cells at ~ 5 x 10^6 cells per mL to a confluent 100 mm dish, which contains ~ 10 x 10^6 cells. Alternatively, in order to establish the dose-dependency of any observed signaling events, vary the ratio of dead to responder cells continuously by progressive dilution in culture medium B of the dead cell suspensions prepared in Protocols 1 and 2.
3. Gently swirl the dishes, then incubate at 37 ºC in a humidified 5% (v/v) CO_2 atmosphere.
4. After 30 min, stimulate the responder cells with either vehicle or 50 nM EGF, according to the pre-labeling of the culture dish.
5. Incubate for 15 min at 37 °C in a humidified 5% (v/v) CO_2 atmosphere.
6. Wash away the dead cells by adding 5 mL of ice-cold 1x DPBS, swirling the dish, and aspirating the wash fluid. Repeat three times.
7. Immediately place the responder cell dishes on ice for further processing.
8. Prepare cell lysates
   1. Prepare cell lysis buffer containing the following: 1× Tris buffered saline (TBS), 10 mM sodium pyrophosphate, 0.5% w/v deoxycholate, 0.1% w/v SDS, 10% glycerol, and 25 mM sodium fluoride.
      1. Immediately prior to cell lysis, add the following in the exact order given at the indicated concentrations: mini EDTA-free protease inhibitor cocktail (1 tablet per 10 mL of lysis buffer), 10% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 1 mM phenylmethanesulfonyl fluoride (PMSF), and 10 mM sodium orthovanadate.
   2. Add 700 µL of ice-cold cell lysis buffer per 100 mm dish of freshly stimulated responder cells on ice from step 5.7. Scrape cells with a cell scraper, and transfer the lysate to pre-chilled 1.5 mL microtubes. Maintain the tubes on ice for 15 min.
   3. Sonicate lysates on ice with 10 pulses of 20 Hz, less than one second each in duration. Avoid the creation of foam at the gas/liquid interface, as this can overheat the samples. Fully immerse the sonicator probe into the microtubes containing lysates before switching the sonicator on, and switch the sonicator off before removing the probe from the lysates.
   4. Centrifuge at 20,000 x g for 10 min at 4 °C, transfer the supernatants into fresh pre-chilled microtubes, and store at -70 ºC.
   5. Perform gel electrophoresis and immunoblotting on supernatants of lysates, as previously described[6,9,10,15].

6. Preventing Direct Physical Contact between Targets and Responders Using a Semipermeable Polycarbonate Membrane (Protocol 5)

1. Prepare BU.MPT responder cells according to Protocol 3, with one exception; grow cells in sterile polystyrene tissue culture treated 12-well clusters.
2. In selected wells, place a permeable support system containing a 0.4 µm polycarbonate membrane to prevent physical interaction between dead and responder cells. Include control wells without a membrane in the same experiment.
3. Aspirate culture medium B from experimental wells of serum-starved BU.MPT responder cells.
4. Follow Protocol 4 for stimulation of responder cells with apoptotic or necrotic cells, with the following modifications:
   1. Per well of the 12-well cluster, add 0.1 mL of one of the following: culture medium B (no cells); apoptotic cell suspension at ~5 x 10^6 cells per mL in culture medium B (Protocol 1); or necrotic cell suspension at ~5 x 10^6 cells per mL in culture medium B (Protocol 2).
      Note: As a confluent well of a 12-well cluster contains ~ 0.5 x 10^6 cells, the addition of 0.1 mL of dead cells at ~ 5 x 10^6 cells per mL yields a dead-to-responder cell ratio of ~ 1:1.
   2. To wells containing the permeable support system, add the dead cells on top of the 0.4 µm polycarbonate membrane within the permeable support system, so there is no direct physical interaction between dead and responder cells.

7. Inhibition of Phagocytosis with Cytochalasin D (Protocol 6)

1. Prepare BU.MPT responder cells according to Protocol 3.
2. Prepare the cytoskeletal inhibitor cytochalasin D in dimethyl sulfoxide (DMSO) at 4 mg/mL (1,000×). Add to pre-labeled dishes of BU.MPT responder cells either 10 µL of vehicle (DMSO) or 10 µL of cytochalasin D to achieve a final concentration of 4 µg/mL in 10 mL of culture medium B.
   Note: At this concentration of cytochalasin D, phagocytosis by BU.MPT cells and a macrophage cell line was inhibited by ≥90%[9,10].
3. Incubate for 2 h at 37 ºC in a humidified 5% (v/v) CO_2 atmosphere.
4. Follow Protocol 4 for stimulation of responder cells with apoptotic or necrotic cells.
5. As appropriate, confirm inhibition of phagocytosis of dead cells by flow cytometry using a separate preparation of dead and responder cells (or cells reserved for this purpose), as previously described[9,10].

Representative Results

In **Figure 1**, we provide a schematic depicting the timing and critical steps involved in the preparation of apoptotic cells (Protocol 1) and necrotic cells (Protocol 2) and the stimulation of responder cells with suspensions of apoptotic and necrotic cells (Protocol 4).

In **Figure 2**, we provide representative results showing the effect of apoptotic cells on phosphorylation of the two isoforms of the serine-threonine kinase glycogen synthase kinase-3 (GSK-3), GSK-3α and GSK-3β. GSK-3α/β has a prominent role in the regulation of cellular proliferation and survival, as well as its important role in glucose metabolism. Phosphorylation of GSK-3α at serine-21 and GSK-3β at serine-9 inhibits kinase activity and, in turn, leads to decreased phosphorylation and increased stabilization of β-catenin. β-Catenin then translocates to the nucleus, where it promotes the transcription of genes known to stimulate cell proliferation and inhibit apoptosis. Therefore, increased phosphorylation of GSK-3α/β is associated with increased proliferation and survival, whereas decreased phosphorylation of GSK-3α/β correlates with decreased proliferation and survival.

Exposure of quiescent BU.MPT responders to apoptotic cells for 30 min strongly inhibited basal phosphorylation of GSK-3α/β (**Figure 2A**). Similarly, prior exposure to apoptotic targets for 30 min strongly inhibited subsequent epidermal growth factor (EGF)-induced phosphorylation of GSK-3α/β (**Figure 2A**). In contrast, exposure of BU.MPT responders to necrotic cells had no effect on either basal or EGF-induced phosphorylation of GSK-3α/β.

To prevent physical interaction between BU.MPT responders and apoptotic cells, BU.MPT responders were grown in a permeable support system and separated from apoptotic targets by a 0.4 µm polycarbonate membrane. Prevention of physical interaction between BU.MPT responders and apoptotic cells abolished the ability of apoptotic targets to inhibit phosphorylation of GSK-3α/β (**Figure 2B**). To evaluate the role of phagocytosis, we used the cytoskeletal inhibitor cytochalasin D to prevent phagocytic uptake of dead cells. Inhibition of phosphorylation of GSK-3α/β in response to apoptotic cells occurred in the absence of phagocytosis (**Figure 2C**). We have previously shown that cytochalasin D, at the concentration used, inhibits PTEC and macrophage phagocytosis by ≥90%[9,10]. Taken together, our results show that apoptotic cell-mediated inhibition of phosphorylation of GSK3α/β requires direct physical interaction between targets and responder cells, but is independent of phagocytosis.

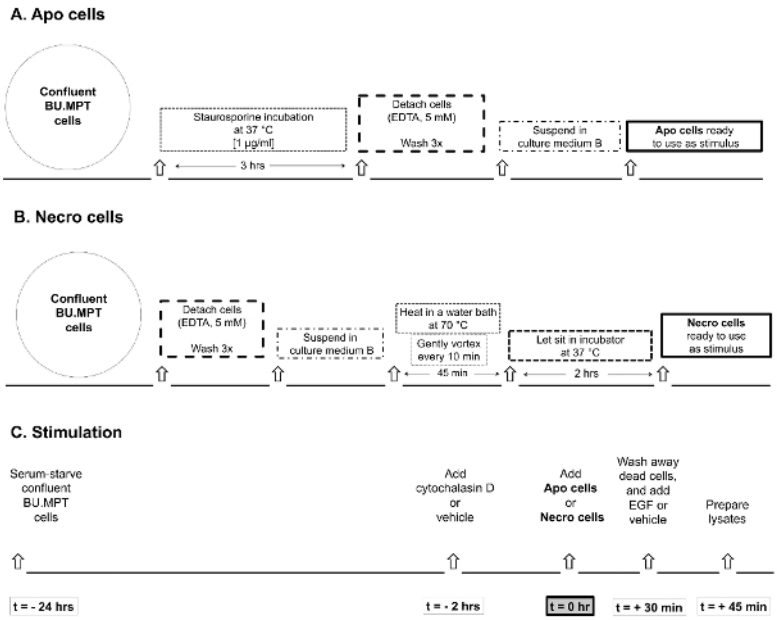

Figure 1. Schema for Induction of Intracellular Signaling Events in Viable Responder Cells by Physical Interaction with Neighboring Cells undergoing Cell Death. The line graphs depict the timing and critical events in the preparation of **(A)** apoptotic (*Apo*) cell and **(B)** necrotic (*Necro*) cell suspensions of BU.MPT cells, and in the **(C)** stimulation of viable BU.MPT responder cells with suspensions of apoptotic or necrotic cells. Please click here to view a larger version of this figure.

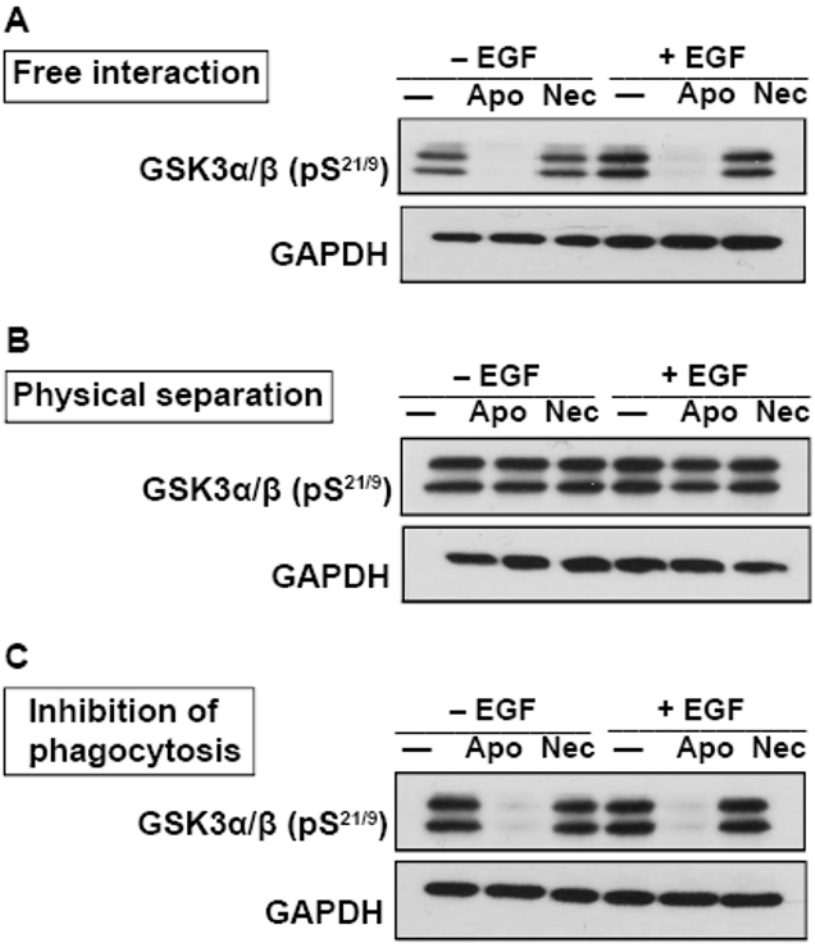

Figure 2. Inhibition of Phosphorylation of GSK3α/β in BU.MPT Responder Cells Following Exposure to Apoptotic Cells Requires Direct Cell-Cell Interaction but Is Independent of Phagocytosis. Serum-starved BU.MPT responder cells were pretreated for 2 h with **(A, B)** vehicle or **(C)** cytochalasin D (4 µg/mL), and then stimulated for 30 min with no cells (-), apoptotic cells (*Apo*), or necrotic cells (*Nec*) at a dead cell to responder cell ratio of 1:1. The responder cells were then washed, and incubated for a further 15 min in the absence or presence of EGF (50 nM), before harvesting. The source of apoptotic cells was staurosporine-treated BU.MPT cells. The source of necrotic cells was heat-treated BU.MPT cells. Interaction between dead cells and BU.MPT responders was either (**A, C**) unimpeded, enabling direct dead cell-responder physical interaction, or (**B**) with separation of dead cells and responders by a 0.4 µm polycarbonate membrane in a permeable support system. Dead cells and non-adherent responder cells were removed by washing, and BU.MPT responder cell lysates were probed with anti-phosphorylated GSK3α/β antibodies as shown. Equal loading was confirmed by probing for total glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Please click here to view a larger version of this figure.

		Effect on signaling event assuming this mechanism	
Intervention	**Soluble mediator**	**Receptor-mediated recognition**	**Phagocytosis**
Fixation of apoptotic cells*	Elimination	Persistence	Persistence
Physical separation of apoptotic and responder cells†	Persistence	Elimination	Elimination
Ultracentrifuged conditioned medium from cells undergoing apoptosis as stimulus¶	Persistence	Elimination	Elimination
Necrotic cells as stimulus	Elimination	Elimination or oppositely directed response (*e.g.*, inhibition versus stimulation)	Persistence
Latex beads as stimulus	Elimination	Elimination	Persistence
Pharmacologic inhibition of phagocytosis	Persistence	Persistence	Elimination
* Predicted results assume that fixation does not alter critical surface determinants responsible for receptor-mediated recognition and/or phagocytosis.			
† Predicted results entail two assumptions: first, that the soluble mediator is small enough to pass through the pores of the membrane separating the apoptotic and responding cells, and second, that any shed vesicles or microvesicles, often termed apoptotic bodies, are too large to pass through the membrane's pores. A role for vesicles or microvesicles would be suggested by a lack of concordance in the signaling events observed with physical separation of apoptotic and responder cells versus ultracentrifuged conditioned medium.			
¶ Ultracentrifugation (20,000 × g for 30 min) is necessary to remove any apoptotic bodies or other shed insoluble material that may mimic the effects of the intact apoptotic cell.			

Table 1. Identification of the Mechanism(s) Responsible for Signaling Events in Viable Cells Following Exposure to Apoptotic Cells. Several straightforward experimental strategies are provided to distinguish among the potential mechanism(s) responsible for signaling events observed following exposure to apoptotic cells. These mechanisms include: physical interaction of the apoptotic cell with specific receptors on the responder cell, release of soluble mediators from the apoptotic cell, and engagement of the responder cell's phagocytic machinery.

Experimental issue	**Available strategies for minimization and/or circumvention**
Restricted number of apoptotic cells per responding cell (~1:1 ratio).	1. Increase the number of apoptotic cells, but the apoptotic-to-responder cell ratio should be kept <2:1.*
Need for gravity-dependent settling of apoptotic cells.	1. Minimize the volume of medium in which apoptotic cells are suspended.
	2. Centrifuge the dish or plate to drive the apoptotic cells through the suspending medium more quickly.
Uneven spatial distribution of apoptotic cells.	1. Carefully distribute the apoptotic cell suspension drop by drop with a wide tip pipette over the entire surface of the responder cell dishes.
	2. Avoid culture dishes or wells with a diameter <35 mm, in which meniscal effects can lead to uneven distributions.
Heterogeneity of the apoptotic cell population.	1. Use a strong inducer of apoptosis.
	2. After induction, fix the apoptotic cells with paraformaldehyde, and sort by flow cytometry to obtain a more uniform population.
* At apoptotic-to-responder cell ratios >2:1, the apoptotic cells will form a confluent carpet and additional apoptotic cells will no longer make direct contact with responder cells	

Table 2. Experimental Issues Associated with the Use of a Cellular Suspension as a Stimulus of Intracellular Signaling Events. Several experimental obstacles associated with the use of a cell suspension as a stimulus are listed, as well as strategies for their partial remedy.

Discussion

We provide here a protocol for characterizing the intracellular signaling events induced in viable cells following their exposure to cells undergoing apoptosis, or other forms of cell death. The protocol emphasizes the multiple mechanisms by which exposure to apoptotic cells can modulate intracellular signaling pathways within viable responder cells. These mechanisms include: the interaction (via bridging molecules or surface determinants on the dead cell or its shed vesicles and microvesicles, often termed apoptotic bodies) with specific receptors on the responder cell; the release of soluble mediators (or even their extracellular generation[27]); and the engagement of the phagocytic machinery. Under circumstances in which substantial phagocytosis occurs, an additional mechanism to be considered is delivery of mediators, such as microRNA, enriched within the apoptotic cell or its vesicles[21]. The significance of our protocol, as compared to other methodologies, lies in its careful

dissection of the multiple mechanisms by which apoptotic cells can modulate signal transduction within responding cells. A major advantage is that most of the techniques required are straightforward and standard to cell culture.

While the protocol is specific for BU.MPT cells, an immortalized mouse kidney PTEC line, as the source of both dead and responding cells, adaptation of the protocol to other cell lines or primary cell cultures is straightforward and easily implemented. Moreover, as in our previous publications, the source of dead and responding cells need not necessarily be the same[9,10,19]. While we describe the use of staurosporine and heat as inducers of apoptosis and necrosis, respectively, the mode and triggers of cell death may also vary. While the signaling events induced in BU.MPT responders following exposure to apoptotic cells are largely independent of the mode of apoptosis induction, we have observed some differences[9,10]. For this reason, we recommend replicating key results with several different triggers of apoptotic cell death. For example, if highly phagocytic cells, such as macrophages, are used as responders[4,6], or if the duration of interaction between responders and dead cells is long enough for substantial phagocytosis to occur, then one could consider a non-toxic inducer of apoptosis, such as ultraviolet- or gamma-irradiation[4,9,10], to rule out potential phagocytic delivery of the toxin. Alternate methods of induction of necrosis may also be considered. While we have obtained identical results with heating and freeze-thawing of cells, the extensive cell clumping and debris occurring with freeze-thawing make its use problematic.

Several simple strategies can help to elucidate the particular mechanism responsible for any signaling events observed following exposure to apoptotic cells (**Table 1**). These strategies include: fixation of apoptotic cells prior to their addition to responding cells; physical separation of apoptotic and responding cells by a semipermeable membrane; the use of ultracentrifuged conditioned medium collected from cells undergoing apoptosis as a stimulus; and substitution of apoptotic cells with necrotic cells or latex beads. With certain caveats, as detailed in **Table 1**, the effect on the observed signaling event can pinpoint the responsible mechanism. For example, in the case of receptor-mediated recognition of the apoptotic cell, the signaling event should persist despite fixation of the apoptotic cell, and should be eliminated with replacement of apoptotic cells by conditioned medium or latex beads, as well as with physical separation of dead and responding cells. In response to necrotic cells, the signaling event should be eliminated or become oppositely directed (*e.g.*, inhibition *versus* stimulation).

The use of a cellular suspension as a stimulus, rather than a soluble ligand, poses several inherent and largely unavoidable experimental obstacles. Awareness of these problems, as well as potential steps to limit their interference, is critical to the successful implementation of this protocol (**Table 2**). Most of these problems derive from the experimentally restricted number of apoptotic cells per responding cell. This is in contrast to soluble factors. Even when used at the extremely low femtomolar concentrations characteristic of cytokines, the number of soluble ligands far exceeds the number of responding cells. In the case of apoptotic cells, however, steric considerations preclude the addition of dead cells at a ratio much above one dead cell per viable responding cell. To a very real degree, therefore, every dead cell matters.

There are several consequences of this restricted dead-to-responder cell ratio that can lead to a reduction or obscuring of signaling events. To interact physically with a viable responder cell, an apoptotic cell must first settle through the column of suspending medium. Even with minimal volumes, the time for settling of cells can be appreciable, as long as 10 to 20 min. Intracellular signaling events will thus be uncoordinated among responding cells. Hence, the initial contact between responder and dead cells cannot be precisely timed, but rather falls within a somewhat broad range of times. For signaling events that occur on a timescale shorter than that required for settling, this lack of synchronization can lead to an obscuring of the signal such that it is no longer distinguishable from background. Even signals of extended duration, if not of sufficient magnitude, can be susceptible to this issue.

In addition to these temporal issues, spatial matters can also adversely impact results. Meniscal effects, especially in wells and dishes of small diameter (*e.g.*, polystyrene tissue culture treated 96-well clusters), or fluid currents generated by too forceful pipetting can lead to a non-uniform distribution of dead cells, thereby creating a state of feast or famine among responder cells. Since the measured signal derives from that of all responder cells in a single well or plate, variation in the capture of dead cells may obscure weaker signals.

A final issue relates to the heterogeneity of the apoptotic cell population. Even with a strong trigger of apoptosis, such as staurosporine, any preparation of dead cells will contain cells at multiple stages of the death process. This becomes relevant, if the strength of the response to the apoptotic cell depends on its stage within the death process[10,14]. Since on average each responding cell encounters just one apoptotic cell, heterogeneity in this case can lead to weakening of the overall detected signaling event. **Table 2** provides a summary of the major suspension-related issues, as well as strategies for their partial remedy.

There are several other experimental concerns uniquely associated with the use of dead cells as a stimulus. Importantly, culture conditions may affect not only the responding cells, but also the dead cells themselves. For example, serum proteins, if present, can attach to the surface of the dead cell, and enhance or inhibit its recognition by responding cells. In troubleshooting an inconsistent result, or a result differing from that reported in the literature, attention should be given to the following culture conditions (for both responder and dead cells): degree of confluence, passage number, presence of serum, heat inactivation of serum, and nature and duration of quiescence. Finally, one needs to be aware that cell death is an inexorable aspect of all cell culture, and responder cells are therefore continually exposed to a low level of dead cells. This exposure can potentially affect the experimental response to a bolus of dead cells, since the very signals under study are being continually induced. Signal modulation may ensue through normal feedback pathways, or even through selection of a subpopulation of responder cells, especially if the induced signaling events involve survival or proliferation.

In summary, we have described a protocol for the determination of intracellular signaling events induced in viable cells by their physical interaction with adjacent dead or dying cells. Although the focus is predominantly on signaling events induced by receptor-mediated recognition of dead cells, the protocol also permits the identification of signaling events induced by phagocytic uptake or the release of soluble mediators from the dead cells. The use of dead cells as a stimulus introduces several unique factors that can hinder the detection of intracellular signaling events. Awareness of these unique factors, as well as the multiple mechanisms by which dead cells modulate intracellular signaling, is critical to the design and interpretation of experiments within this growing field of study. The protocol described here should aid in understanding the mechanisms by which dead or dying cells affect their live neighbors in both health and disease.

Disclosures

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