Analysis of apoptotic cells using Beadlyte® suspension arrays

Paul W. Rhyne, Jason D. Scull, Lynn M. Stiles, and Dominic P. Eisinger

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Here we describe a new approach to study apoptosis pathways using multiplex suspension arrays. Apoptosis was induced in Jurkat T cells using the protein synthesis inhibitor, anisomycin. Cells grown in 96-well plates were treated with anisomycin for up to 7 h, washed, and lysed in their respective wells. Samples of each lysate were analyzed using Beadlyte® suspension arrays consisting of total Akt/PKB, phosphorylated Akt/PKB, active caspase-3, and single-stranded DNA (ssDNA)-specific Beadmate™ microspheres and quantified with the X-MAP™ system. We found that phosphorylated Akt levels dropped dramatically with 2 h or more of anisomycin treatment, whereas active caspase-3 levels rose sharply with 2 h of treatment, signifying the onset of apoptosis. Longer incubation with anisomycin showed increases in ssDNA, which is consistent with the characteristic degradation of DNA that occurs in late-stage apoptosis. This approach demonstrates how apoptosis pathways can be studied from a small amount of sample without the use of more lengthy techniques such as immunoprecipitation or Western blotting. The suspension array is being expanded to measure many other intracellular proteins including posttranslational modifications and should prove to be extremely useful for studying apoptosis and other important cellular pathways.

INTRODUCTION

The process of programmed cell death is initiated through at least two pathways, extrinsic and intrinsic. The extrinsic pathway is initiated by the binding of ligands to death receptors such as Fas or tumor necrosis factor receptor 1 (TNF-R1) and leads to the activation of caspase-3 and caspase-8. The intrinsic pathway is started from events inside the cell, such as damage to mitochondria. Cytochrome c is released from the mitochondria and binds to Apaf1 and procaspase-9 to form the apoptosome. The apoptosome cleaves procaspase-9 into its active form, which in turn activates caspase-3. Both of these pathways result in the activation of caspase-3, which in turn activates downstream death substrates, ultimately leading to the breakup of DNA and apoptosis (1–3).

The serine threonine kinase Akt/PKB (Akt) plays several roles, including survival, proliferation, and growth of cells. Akt is broadly expressed and is activated by recruitment to the plasma membrane and by dual phosphorylation on Thr³⁰⁸ and Ser⁴⁷³ (4,5). Akt prevents apoptosis by inhibiting Bad from sequestering the anti-apoptotic factor Bcl-XL (5,6), inhibiting the activation of caspase-3 and caspase-9 (7–9), and preventing the release of cytochrome c from the mitochondria. Akt also promotes cell survival through other mechanisms, including the phosphorylation of Mdm2, resulting in the destabilization of p53 (10).

There are many methods used to monitor apoptosis, including DNA fragmentation (11,12) and reduction of hypodiploid DNA fluorescence using cell cycle profile analysis (13). The majority of approaches used to study molecular apoptotic

events are limited to a single analyte at a time, thus requiring more sample and labor for additional analytes. The ability to measure multiple analytes (multiplex) simultaneously from a single sample would expedite the analysis of apoptotic events. We have developed a multiplex assay to monitor changes in multiple apoptotic markers using the X-MAP™ system. The multiplex suspension array utilizes capture antibodies covalently coupled to color-coded latex microspheres and biotin-labeled reporter antibodies in conjunction with streptavidin-phycoerythrin. Each analyte capture antibody is assigned a unique color-coded set of microspheres, and the amount of bound analyte is quantified using streptavidin-phycoerythrin fluorescence. The X-MAP system distinguishes between color-coded bead sets, allowing for the measurement of multiple analytes at the same time. The suspension array offers sensitivity comparable to other antibody-based immunoassays such as ELISA, only with shorter incubation times (14). The X-MAP system allows the suspension array to include up to 100 different microsphere sets to be analyzed at the same time. We have used this assay to detect changes in Akt protein levels, Akt phosphorylation, caspase-3 activation, and single-stranded DNA (ssDNA) in cells progressing through apoptosis.

MATERIALS AND METHODS

Materials and Cell Culture

The anti-caspase-3 antibodies, anti-phospho Akt antibodies, anti-total Akt antibodies, anti-ssDNA antibodies, purified

phosphorylated Akt, nonphosphorylated Akt, active caspase-3, and streptavidin-phycoerythrin were obtained from Upstate USA (Lake Placid, NY, USA). MultiScreen® 96-well filter microplates were obtained from Millipore (Bedford, MA, USA). Beadlyte® Cell Signaling Lysis buffers (containing Tris- or HEPES-buffered salts, phosphatase inhibitors, protease inhibitors, and proprietary detergents) and Cell Signaling Assay buffers (containing Tris-buffered salts, animal protein, 0.05% azide) were obtained from Upstate USA. Single-stranded calf thymus DNA was obtained from Sigma (St. Louis, MO, USA). Jurkat T cells (TIB 152, clone E6-1) were obtained from ATCC (Manassas, VA, USA) and were grown in RPMI 1640 (Fisher Scientific International, Pittsburgh, PA, USA) supplemented with 2% fetal bovine serum.

Treatment of T Cells with Anisomycin

Jurkat T cells were grown to 1×10^6 cells/mL and greater than or equal to 95% viability, as assessed by trypan blue

exclusion. The cells were collected, resuspended in medium at 1×10^7 cells/mL, and plated into two 96-well plates at 0.5×10^6 cells/well. Fifty microliters of medium containing 40 μ M anisomycin (Sigma) or medium alone were added to four replicate wells (both plates) every 60 min over a 7-h period. At the end of the assay, one of the plates was centrifuged in a 96-well plate carrier (IEC, Needham Heights, MA, USA) at 1000 rpm using an HN-SII centrifuge. The supernatants were removed, and 100 μ L Beadlyte Cell Signaling Lysis buffer A were added to each well to lyse the pelleted cells. The cells were incubated on ice for 15 min, snap-frozen, and stored at -70°C until further analysis. The second duplicate plate was used to measure cell viability as described below.

Cell Viability

ATP levels in treated cells were measured using the ATPLite-M™ Assay Kit (Packard Instrument, Meriden, CT, USA) as a measure of viability. This assay is based on

Figure 1. Detection sensitivity of Beadmates for measuring Akt protein levels, Akt phosphorylation, caspase-3 activation, and single-stranded DNA (ssDNA). Decreasing amounts of purified proteins or DNA were incubated with Beadlyte suspension arrays for the detection of (A) phosphorylated Akt, (B) total Akt, (C) active caspase-3, and (D) ssDNA. The levels of each were quantified using the X-MAP system. The figure shows the mean ($\bar{x} \pm \text{SD}$) of three replicate wells and represents data from one of three experiments.

the observation that the intracellular concentration of ATP declines rapidly when cells die or undergo apoptosis (15). The assay was carried out according to the manufacturer's instructions. Briefly, 50 μ L cell lysis solution were added to each well containing 100 μ L treated cells (see earlier section for description of cell treatment) and incubated for 5 min at 25°C on an orbital microplate shaker. Next, 50 μ L substrate solution were added to each well and incubated for 5 min at 25°C on an orbital microplate shaker. The plate was placed in the dark for 10 min and then assayed for luminescence using a Wallac Victor2™ 1420 multilabel counter (PerkinElmer Life Sciences, Shelton, CT, USA).

Suspension Array Detection of Akt Protein Levels, Akt Phosphorylation, Caspase-3, and ssDNA

The suspension array was used to detect changes in Akt protein levels, Akt phosphorylation (Ser 473), caspase-3 activation, and ssDNA levels. Twenty-five microliters of pure proteins, DNA, or cell lysates (described earlier) were serially diluted in Beadlyte Cell Signaling Assay buffer 1 and mixed with 25 μ L mixture of relevant capture beads, giving 2500 beads/set/well in a 96-well filter microplate. The mixture was incubated for 1 h at 25°C in a microplate shaker protected from light (to avoid photobleaching of the beads). The beads were washed to remove unbound sample material by placing the filter plate over a vacuum (MultiScreen vacuum manifold). One hundred microliters of Beadlyte Cell Signaling Assay buffer 1 were added to each well and then removed via vacuum. For analysis of Akt phosphorylation, caspase-3 activation, or ssDNA, the beads were resuspended

in 25 μ L reporter mixture consisting of biotin-labeled anti-phospho Akt, anti-active caspase-3, or anti-ssDNA antibodies for 1 h at 25°C (protected from light). Akt protein levels (or total Akt) were measured in separate wells using a reporter antibody that binds Akt independently of its phosphorylation status. Measuring Akt protein levels and Akt phosphorylation cannot be done in the same well because the total Akt reporter antibody binds to Akt regardless of phosphorylation status. After the reporter step, the beads were washed, resuspended in 25 μ L/well streptavidin-phycoerythrin, and incubated for 30 min at 25°C (protected from light). After the streptavidin-phycoerythrin step, the beads were washed and resuspended in 100 μ L/well of Beadlyte Cell Signaling Assay buffer 1 and analyzed with the X-MAP system (Luminex, Austin, TX, USA) where the median fluorescence intensity of at least a 50-bead set was measured.

RESULTS AND DISCUSSION

The suspension arrays for Akt protein levels and for Akt phosphorylation were validated using purified Akt proteins. The data in Figure 1A show that the anti-phospho Akt array had excellent specificity for the phosphorylated form and could detect as little as 100 pg phosphorylated Akt/well. The data in Figure 1B demonstrate that the total Akt array recognized both phosphorylated and nonphosphorylated forms of Akt when at least 100 pg/well were present, similar to the sensitivity of the phosphorylated Akt array in Figure 1A. Although the total Akt array had a slightly higher preference for the nonphosphorylated form of Akt, both forms were detected with comparable sensitivity and dynamic range.

The suspension array was also tested for the ability to detect active caspase-3 and ssDNA. The data in Figure 1C show that as little as 50 pg purified active caspase-3 and 800 pg ssDNA (Figure 1D) were detected by the assay. Although the fluorescence intensity for each portion of the suspension array varied in magnitude, the linear range of detection and assay sensitivity of each individual immunoassay was comparable. We also found no significant cross-reactivity of the various reporter antibodies with each other (data not shown).

The suspension array was then used to monitor changes in T cells as they progressed into apoptosis. Anisomycin is a protein synthesis inhibitor that has previously been shown to induce apoptosis (16–18). Exposure of cells to short durations (30 min) of anisomycin has been shown to increase phosphorylated Akt levels (19). However, longer

Figure 2. Measurement of cell viability in anisomycin-treated T cells. Jurkat T cells were incubated in the presence of anisomycin for various times. The ATP content in cells at the end of the incubation period was measured with a luminescence-based ATP assay to assess cell viability. The data are the mean ($\bar{x} \pm$ SD) of four replicate wells and represent the results from one of three experiments.

exposures (2 h) promote apoptosis, as measured by caspase-3 activation and DNA degradation (16–18). Jurkat cells were grown in 96-well plates and treated for different times with anisomycin as described in the Materials and Methods section. We first confirmed the apoptotic effects of anisomycin in our system by monitoring cell viability in treated cells via changes in ATP. The results in Figure 2 show that Jurkat cells lose viability between 2 and 3 h anisomycin treatment with maximal effects after 5 h. These results are consistent with previous findings showing that Jurkat cells undergo cell death and apoptosis after several hours of anisomycin treatment (18).

Cell lysates taken from each individual well were tested with the suspension array for phosphorylated Akt, active caspase-3, and ssDNA. A second suspension array was used to measure total Akt protein levels, since both total Akt and phosphorylated Akt could not be measured in the same well. The median fluorescence intensity measured in four replicate wells were averaged and used to create the data shown in Figure 3. Although the active caspase-3, ssDNA, and phosphorylated Akt were analyzed simultaneously from the same sample, the results of each are shown individually for

simplicity. As shown in Figure 3A, there was a small increase in phosphorylated Akt with short incubations of anisomycin (30–60 min), which diminished drastically after 2 h. This is consistent with previously published data (18,19). The data in Figure 3B show that Akt protein levels also decreased in treated Jurkat cells, but at a much later time point than the observed drop in Akt phosphorylation. These findings suggest that anisomycin (directly or indirectly) alters the phosphorylated state of Akt before causing an overall reduction of both the phosphorylated and nonphosphorylated forms of Akt.

The data shown in Figure 3C show increased levels of active caspase-3, reaching a maximum level at 3 h anisomycin treatment. These data are consistent with the findings of Shifrin and Anderson (8,18), showing increased activation levels of caspase-3 activity in cells treated with anisomycin. Note that the suspension array measures the levels of active caspase-3 directly in lysates, not the presence of caspase-3-like activity as often measured by fluorescence enzymatic assays. These data suggest that the phosphorylation of Akt and caspase-3 are inversely proportional as cells progress into apoptosis. This is not surprising, since Akt indirectly prevents

Figure 3. Suspension array detection of Akt protein, Akt phosphorylation, caspase-3 activation, and single-stranded DNA (ssDNA) in apoptotic T cells. Jurkat T cells were incubated with either anisomycin or culture medium for different lengths of time. The level of (A) phosphorylated Akt, (B) total Akt, (C) active caspase-3, or (D) ssDNA is shown as quantified by the X-MAP system. The figure shows the mean ($\bar{x} \pm \text{SD}$) of four replicate wells and is representative of one of three experiments.

the activation of caspase-3 by promoting cell survival (8,9).

The data presented in Figure 3D show that the levels of ssDNA increase in anisomycin-treated cells, reaching a maximal level after 6 h exposure. A hallmark of apoptosis is the degradation of DNA that occurs in the late stages of apoptosis, traditionally detected with agarose electrophoresis and ethidium bromide staining (17,18). However, the suspension array in this study demonstrated that DNA degradation can be detected and quantitated using an immunoassay. In a temporal order, our findings show a drop in phosphorylated Akt levels, followed by increase activation of caspase-3, and finally increased amounts of ssDNA. Thus, these findings demonstrate that the suspension array can simultaneously measure multiple apoptosis intermediates from a single sample in less than 3 h, without the need for electrophoresis, blotting, or enzymatic assays.

The Beadlyte suspension array system can be used to study the effects of other compounds on cellular pathways from a small amount of sample. The same conclusions could be reached using more traditional methods such as Western blotting, immunoprecipitation, or ELISA. However, more labor, sample, and time would be required to achieve the same results. The X-MAP system provides excellent quantitative results comparable to other methods such as ELISA. A unique advantage of the Luminex suspension array technology is that the numerical fluorescent output is automatically reported in real time as a median of at least 50 beads for each analyte result. Such a robust statistical analysis has inherent accuracy and reproducibility benefits over 2-D chip arrays that are notoriously variable in spot-to-spot reproducibility. Typically, 2–5 spots/analyte are averaged in a 2-D chip array, not the 50–100 of a Luminex assay. Other traditional immunoassays such as ELISA provide a single absorbance value as measured for the entire well. Thus, the suspension array provides an extremely reliable statistical analysis than other traditional antibody based methods (14). The assay also demonstrates how multiple aspects of cellular pathways can be studied simultaneously, in this case the phosphorylation of Akt, caspase-3, and DNA degradation. The suspension array is capable of analyzing up to 100 different analytes in a single well, thus allowing the array to include many other intracellular targets. This technology should prove extremely useful to investigators studying intracellular signaling events.

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Address correspondence to Paul W. Rhyne, Upstate USA, 10 Old Barn Rd., Lake Placid, NY 12946, USA. e-mail: prhyne@upstate.com