Monitor Caspase 3/7 Activity without Cell Fixation: A Novel Apoptosis Reagent from Molecular Probes

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Introduction

Apoptosis, or programmed cell death, is characterized by cell shrinkage, membrane "blebbing", and genomic fragmentation (1-3). Apoptosis is morphologically and functionally distinct from other mechanisms of cell death such as necrosis and autophagy (5-7). Apoptosis plays an important role in various biological processes including cell turnover, embryonic development and negative selection of cells during immune system development. Dysregulation of apoptosis is implicated in various human pathologies including neurodegenerative diseases, autoimmune disorders and cancer (8-9). Activation of enzymes known as caspases is an early event in the process of apoptosis and results in the cleavage of protein substrates and subsequent disassembly of the cell (10) The CellEvent® Caspase-3/7 Green Detection Reagent is a novel fluorogenic substrate designed for the detection of activated caspases 3 and 7. This cell-permeant reagent consists of a four amino acid peptide (DEVD) conjugated to a nucleic acid binding dye. During apoptosis, caspase-3 and caspase-7 proteins are activated and are able to cleave the caspase 3/7 recognition sequence encoded in the DEVD peptide. Cleavage of the recognition sequence and binding of DNA by the reagent labels apoptotic cells with a bright, fluorogenic signal, without the need for fixation and permeabilization. When used together with the SYTOX® AADvanced™ dead cell stain, apoptotic cells can easily be discriminated from live and necrotic cells.

Methods

In this study the CellEvent® Caspase 3/7 Green Flow Cytometry Assay Kit was used to monitor apoptosis in multiple cell models using a flow cytometry. Use of the reagent was compared with caspase antibody labeling, used with several apoptotic models, and multiplexed with other common apoptosis reagents.

Results

The CellEvent® Caspase 3/7 Green Flow Cytometry Assay Kit detected an increase in caspase 3/7 activity during apoptosis as confirmed by staining using an anti-active caspase 3/7 antibody. When treated with a small peptide inhibitor (Ac-DEVD-CHO), staining with CellEvent® Caspase 3/7 Green Detection reagent was reduced as was staining with the anti-active caspase 3/7 antibody. Detection of apoptotic cells using the CellEvent® Caspase 3/7 Green detection reagent was similar to staining with an Annexin V antibody conjugate.

Conclusions

The CellEvent® Caspase 3/7 Green Flow Cytometry Assay kit represents a dramatic improvement over existing reagents for caspase detection. It is compatible with live cells and does not require fixation and permeabilization. The reagent labels cells 30 minutes, permitting fast and easy multicolor labeling.

References

1. Leukemia 14, 1502 (2000); **2**. Nat Rev Mol Cell Biol. 2, (2001); **3**. Cell Cycle 3, 64 (2004); **4.** News Physiol Sci, 19, 124 (2004); **5.** Cytometry 27, 1 (1997); **6.** Br J Cancer 26, 239 (1972); **7.** Nat. Rev. Mol. Cell. Biol, 2, 545 (2001); **8.** Brown and Attardi, 2005; **9.** Cell 116, 205(2004); **10.** Cell Death and Diff. 6, 1067 (1999).

Mechanism of action

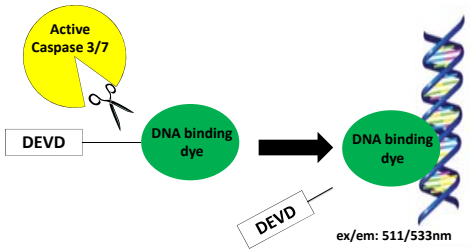

Figure 1. Mechanism of action of CellEvent® Caspase 3/7 Green Detection Reagent. The CellEvent® Caspase 3/7 Green Detection reagent is composed of a DNA binding dye conjugated to the Caspase 3 and Caspase 7 recognition sequence (DEVD) by a short peptide linker. During apoptosis, Caspase enzymes are activated as part of a signaling cascade that ultimately leads to disassembly of the cell. When applied to cell samples, the cell-permeant CellEvent® Caspase 3/7 Green Detection reagent freely enters the cytosol of cells and is cleaved by activated Caspase 3 and Caspase 7 enzymes. The released DNA binding dye binds DNA, producing a bright and stable fluorogenic signal.

Simplified workflow with the CellEvent® Caspase 3/7 Green Detection Flow Cytometry Assay Kit

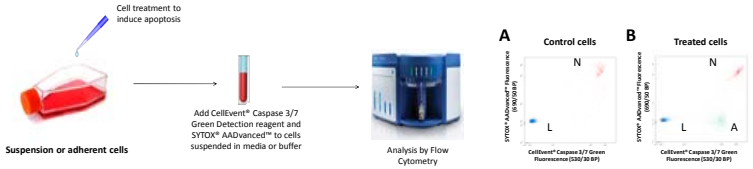

Figure 2. Staining workflow using the CellEvent® Caspase 3/7 Green Flow Cytometry Assay Kit. Adherent or suspension cells can be used with CellEvent® Caspase 3/7 Green Flow Cytometry Assay kit. Suspension cells and adherent cell samples can be stained in complete medium supplemented with 0-20% fetal bovine serum (FBS) or phosphate buffered saline (PBS) with 0-2% bovine serum albumin (BSA). Samples may be incubated at room temperature for 45-60minutes or at 37ºC for 30 minutes prior to analysis using a flow cytometer equipped with a 488nm laser and a 530/30 BP filter and a 690/50BP filter for fluorescence excitation. In this example, Jurkat cells (human T-cell leukemia) were treated with 10uM camptothecin to induce apoptosis. Samples were analyzed on an Attune® Acoustic Focusing Cytometer. The induced culture (panel B) has a greater number of apoptotic cells compared to the control culture (panel A). L=live cells (blue); A= apoptotic cells (green); N=necrotic cells (red).

Fine specificity of the CellEvent® Caspase 3/7 Green Detection Reagent

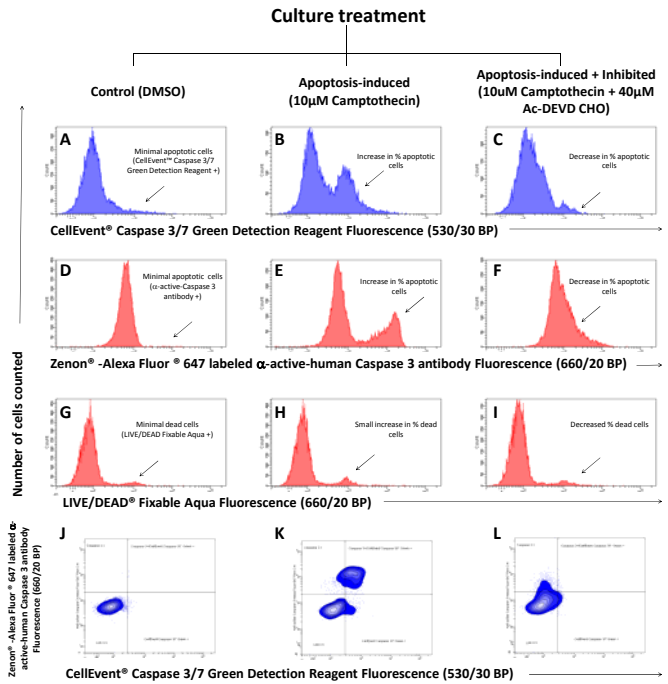

Figure 3. CellEvent® Caspase 3/7 Green Detection Reagent and an α-active caspase 3 antibody detect similar populations of apoptotic cells. Jurkat cells were treated with 10uM camptothecin alone or with the synthetic tetra peptide inhibitor for Caspase-3 (Ac-DEVD –CHO, 40µM) to inhibit caspase 3 activity. Control cells were treated with a DMSO carrier. After 3.5 hr incubation at 37ºC, cell samples were stained alone or in combination with the CellEvent® Caspase 3/7 Green Detection Reagent and the LIVE/DEAD® Fixable Aqua dead cell stain before treatment of samples with fixative. Following treatment with fixative, cells were washed, permeabilized and stained with a Zenon® Alexa Fluor® 647 labeled α-active Caspase 3 antibody. In a single-stain application, few apoptotic cells are detected by either the CellEvent® Caspase 3/7 Green Detection Reagent or the α-active Caspase 3 antibody in control cultures (panels A and D). In contrast, treatment with camptothecin results in an increase in the number of apoptotic cells detected using either the CellEvent® Caspase 3/7 Green Detection Reagent or the α-active Caspase 3 antibody (panels B, E). Co-treatment with Ac-DEVD-CHO decreases the number of apoptotic cells detected using either method (panels C and F). A small increase in the number of dead cells is detected in the culture treated with camptothecin, as expected. Multi-color staining of cell cultures indicates that most apoptotic cells are co-positive for both CellEvent® Caspase 3/7 Green Detection Reagent and the α-active Caspase 3 antibody (panel K) and staining is decreased with treatment with the Caspase 3 inhibitor. Together the data indicate that both CellEvent® Caspase 3/7 Green Detection Reagent and the α-active Caspase 3 antibody reagents are detecting similar populations of cells.

The CellEvent® Caspase 3/7 Green Flow Cytometry Assay Kit is compatible with multiple cell types and models of apoptosis

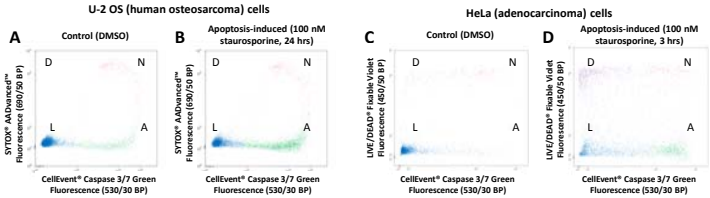

Figure 4. The CellEvent® Caspase 3/7 Green Flow Cytometry Assay Kit can be used to detect apoptotic cells in an adherent cell culture. Control cells treated with DMSO (panels A and C) and apoptosis-induced cells (panels B, C) treated with staurosporine for 3- 24 hrs were resuspended in PBS before staining with the CellEvent® Caspase 3/7 Green Detection Reagent and either SYTOX® AADvanced (U-2 OS cells) or the LIVE/DEAD® Fixable Violet dead cell stain (HeLa cells). Cells were analyzed on an Attune® Acoustic Focusing Cytometer equipped with 405nm and 488nm lasers for excitation of the CellEvent® Caspase 3/7 Green Detection Reagent, SYTOX® AADvanced™ Dead cell stain, and the LIVE/DEAD® Fixable Violet Dead Cell stain. For both U-2 OS and HeLa cell lines the induced culture has a greater number of apoptotic cells (colored green) as compared to the control culture. L=live cells (blue); A= apoptotic cells (green); N=necrotic cells (red).

The CellEvent® Caspase 3/7 Green Detection Reagent can be used in multicolor applications

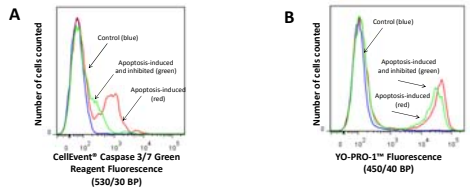

Figure 5. Multicolor staining of Jurkat cells indicates that the Caspase 3 inhibitor Ac-DEVD-CHO inhibits staining of apoptotic cells by CellEvent® Caspase 3/7 Green Detection Reagent but not staining by PO-PRO-I. Apoptosis was induced in Jurkat cells by treatment with 10uM camptothecin and inhibited in similarly induced cultures by addition of 30uM Ac-DEVD-CHO. Following incubation at 37oC for 3.5hrs, samples were analyzed for changes in Caspase 3 activity, plasma membrane permeability, and plasma membrane integrity. Samples were stained using the CellEvent® Caspase 3/7 Green Detection reagent, PO-PRO-1™ and SYTOX® Red DNA binding dyes, prior to flow cytometric analysis using a Becton Dickinson LSRII flow cytometer equipped with 405nm, 488nm, and 639nm lasers. Dead cells with compromised plasma membranes that stained positive with the cell-impermeant SYTOX® Red Dead Cell Stain and were excluded from analysis. (A) Cultures treated with 10uM camptothecin have increased staining with CellEvent® Caspase 3/7 Green Reagent (panel A, red) and increased staining with PO-PRO-1 (panel B, red) as compared to control cultures (panels A and B, blue). Cultures treated with both camptothecin and Ac-DEVD-CHO have minimal staining with the CellEvent Caspase 3/7 Green Detection Reagent (panel A, green), whereas treatment with Ac-DEVD-CHO does not decrease staining by PO-PRO-1. Together the data indicate that the CellEvent® Caspase 3/7 Green Detection reagent specifically detects changes in caspase 3 activity and can be used with multiplexing.

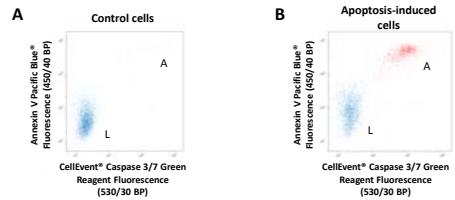

Figure 6. Multicolor staining of Jurkat cells with an Annexin V Pacific Blue™ conjugate and CellEvent® Caspase 3/7 Green Detection Reagent. Jurkat cells were treated with 10 µM camptothecin for four hours prior to staining with the CellEvent® Caspase 3/7 Green Detection Reagent, SYTOX® AADvanced™ Dead Cell Stain and an Annexin V Pacific Blue™ conjugate. Following staining, samples were acquired using an Attune® Acoustic Cytometer equipped with a 488 nm laser for excitation of CellEvent® Caspase 3/7 Green Detection Reagent and SYTOX® AADvanced™ stain, and a 405nm laser for excitation of the Annexin V Pacific Blue™ conjugate. Dead cells with compromised plasma membranes that stained positive with the cell-impermeant SYTOX® AADvanced™ dead cell stain were excluded from analysis for activation of Caspase 3/7 and outer membrane location of Annexin V. The induced culture has increased numbers of apoptotic cells (indicated in red) as compared to the control culture (B). Note that most apoptotic cells in the induced culture are positive for staining with both Annexin V Pacific Blue™ conjugate and the CellEvent® Caspase 3/7 Green Detection Reagent. L=live cells (blue); A= apoptotic cells (red).

Acknowledgements

Michelle Yan, Scott Clarke and Mike Janes, are thanked for their assistance and technical expertise.