Detection of Caspase Activation In Situ by Fluorochrome-Labeled Caspase Inhibitors

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ABSTRACT

Apoptosis is dependent on the activation of a group of proteolytic enzymes called caspases. Caspase activation can be detected by immunoblotting using caspase-specific antibodies or by caspase activity measurement employing pro-fluorescent substrates that become fluorescent upon cleavage by the caspase. Most of these methods require the preparation of cell extracts and, therefore, are not suitable for the detection of active caspases within the living cell. Using FAM-VAD-FMK, we have developed a simple and sensitive assay for the detection of caspase activity in living cells. FAM-VAD-FMK is a carboxyfluorescein (FAM) derivative of benzyloxycarbonyl-valine-alanine-aspartic acid-fluoromethyl ketone (zVAD-FMK), which is a potent broad-spectrum inhibitor of caspases. FAM-VAD-FMK enters the cell and irreversibly binds to activated caspases. Cells containing bound FAM-VAD-FMK can be analyzed by flow cytometry, fluorescence microscopy, or a fluorescence plate reader. Using FAM-VAD-FMK, we have measured caspase activation in live non-adherent and adherent cells. We show that FAM-VAD-FMK labeled Jurkat and HeLa cells that had undergone apoptosis following treatment with camptothecin or staurosporine. Non-stimulated negative control cells were not stained. Pretreatment with the general caspase inhibitor zVAD-FMK blocked caspase-specific staining in induced Jurkat and HeLa cells. Pretreatment of staurosporine-induced Jurkat cells with FAM-VAD-FMK inhibited affinity labeling of caspase-3, -6, and -7, blocked caspase-specific cell staining, and led to the inhibition of apoptosis. In contrast, the fluorescent control inhibitor FAM-FA-FMK had no effect. Measurement of caspase activation in 96-well plates showed a 3- to 5-fold increase in FAM-fluorescence in staurosporine-treated cells compared to control cells. In summary, we show that FAM-VAD-FMK is a versatile and specific tool for detecting activated caspases in living cells.

INTRODUCTION

Apoptosis is an evolutionarily conserved form of cell suicide that follows a specialized cellular process. The central component of this process is a cascade of proteolytic enzymes called caspases (14). These enzymes participate in a series of reactions that are triggered in response to pro-apoptotic signals and result in cleavage of protein substrates, causing the disassembly of the cell (20). The mammalian caspases play distinct roles in apoptosis and inflammation (12). In apoptosis, caspases are responsible for proteolytic cleavages that lead to cell disassembly (effector caspases) and are involved in upstream regulatory events (initiator caspases) (16). An active caspase consists of two large (about 20 kDa) and two small (about 10 kDa) subunits to form two heterodimers that associate in a tetramer. In common with other proteases, caspases are synthesized as precursors that undergo proteolytic maturation, either autocatalytically or in a cascade by enzymes with similar specificity (13,16,18,19). Caspase enzymes selectively recognize a four amino acid sequence with an absolute requirement for aspartate at the P₁ position.

Various approaches have been used to measure caspase activation. Among these techniques, immunoblotting and fluorogenic caspase substrates are the most widely used. Measuring caspase activation by immunoblotting is based on the detection of caspase cleavage products, which differ in molecular weight from the pro-caspases, using caspase-specific antibodies (21). Fluorogenic substrates consist of a peptide comprising the caspase recognition sequence linked to a fluorogenic reporter moiety. Upon cleavage of the fluorogenic substrate at the junction of the aspartic acid and the fluorogenic reporter molecule, the fluorescence of the reporter

becomes activated and can be detected by fluorescence spectroscopy (fluorescence plate reader). The most widely used fluorogenic caspase substrates, which are based on 7-amido-4-methylcoumarin (AMC) (7) or 7-amido-4-(trifluoromethyl)coumarin (AFC), can only be used to measure caspase activation in cell extracts and not in live cells because coumarin-labeled peptide substrates are cell impermeable and they excite at 380 and 400 nm, respectively, where autofluorescence of cells interferes with the measurement (11). Recently, new fluorogenic caspase substrates have been developed. One of these substrates is a derivative of rhodamine 110 containing the caspase-3 substrate recognition sequence DEVD covalently linked to rhodamine 110 (10). Upon enzymatic cleavage, the non-fluorescent substrate is converted into the fluorescent rhodamine 110. Although this substrate can be used to measure caspase-3 activity in a homogeneous assay either with cells or with cell extracts, it is not suitable for caspase detection in individual cells *in situ* because fluorescent rhodamine 110 diffuses out of the cell, leading to loss of

staining. A new class of fluorogenic caspase substrates has been developed by OncoImmunin, Gaithersburg, MD, USA (9). These substrates are cell permeable and become fluorescent upon cleavage by the caspase inside the cell.

We have developed a novel approach to detect active caspases in living cells. The methodology is based on carboxy-fluorescein (FAM)-labeled fluoromethyl ketone (FMK)-peptide inhibitors of caspases (4,6). These inhibitors are cell permeable and non-cytotoxic. Once inside the cell, the inhibitor binds irreversibly to the active centers of the caspases ensuring that only cells with activated caspases become labeled. Because of irreversible binding of FAM-VAD-FMK to caspases, caspase activation in each individual cell can be detected. In contrast, the fluorescent moieties of fluorogenic substrates diffuse out of the cell, limiting the sensitivity of caspase detection in single cells. Here, we show that FAM-VAD-FMK fluorescently labeled apoptotic Jurkat or HeLa cells but did not label control cells. Using FAM-VAD-FMK, caspase activation in living cells was measured by flow

Figure 1. In situ caspase detection by flow cytometry. Jurkat cells were DMSO treated (A) or treated with 2 $\mu\text{g/mL}$ camptothecin with (C) and without (B) 50 μM zVAD-FMK for 4 h. Cells were labeled with FAM-VAD-FMK for 1 h, followed by washing and analysis by flow cytometry. HeLa cells were treated with DMSO (D) or 1 μM staurosporine with (F) and without (E) 50 μM zVAD-FMK for 4 h. Cells were harvested by trypsinization, labeled with FAM-VAD-FMK for 1 h, washed, and analyzed on a Coulter Epics flow cytometer. The flow cytometry frequency histograms of the number of events (Y-axis) versus the FAM fluorescence intensity (X-axis) show two peaks. Caspase-negative cells occur within the first log decade of the X-axis, whereas caspase-positive cells lay within the second and third log decade.

cytometry, fluorescence microscopy, or a fluorescence plate reader. Fluorescence labeling of apoptotic cells was dependent on binding of FAM-VAD-FMK to activated caspases inside the cell.

MATERIALS AND METHODS

Cells

The human T cell leukemia cell line Jurkat was obtained from ATCC (Manassas, VA, USA). Cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine. Cultures with fewer than 5×10^5 cells/mL were used for experiments. The human adenocarcinoma cell line, HeLa, was obtained from ATCC, and cells were cultured in EMEM medium supplemented with 10% FCS and 2 mM L-glutamine. All cells were maintained in 5% CO₂ at 37°C.

Induction of Apoptosis

Exponentially growing Jurkat cells adjusted to 10⁶ cells/mL were stimulated with either 2 µg/mL topoisomerase I inhibitor camptothecin (Sigma, St. Louis, MO, USA) for 4 h or 1 µM concentrations of the protein kinase inhibitor, staurosporine (STS) (Sigma) for 4 h. HeLa cells were plated onto 35-mm petri dishes at 2×10^5 cells/dish and grown for 16 h. The following day, cells were treated with 1 µM staurosporine for 4 h. Where indicated, cells were simultaneously co-exposed to 50 µM caspase inhibitor zVAD-FMK (Enzyme Systems Products, Livermore, CA, USA).

Caspase Detection by FAM-VAD-FMK

FAM-VAD-FMK is commercially available as a component of the CaspaTag™ Fluorescein Caspase Activity Kit (Intergen Discovery Products, Gaithersburg, MD, USA). As specified in the kit, the FAM-VAD-FMK inhibitor was dissolved in dimethyl sulfoxide (DMSO) to obtain a 150× stock solution. Aliquots of this solution were stored at -20°C in the dark. Before use, a 30× working solution of FAM-VAD-FMK was prepared by diluting the stock solution 1:5 in PBS. For labeling, a 300-µL aliquot of cells was transferred to a fresh tube. Ten microliters of 30× FAM-VAD-FMK were added directly to the cell suspension. The cells were gently mixed and incubated for 1 h at 37°C under 5% CO₂ with tubes protected from light. Cells were washed twice with 1× Wash Buffer (PBS containing 0.5% bovine serum albumin and 0.05% sodium azide). Finally, the cell pellet was resuspended in 400 µL 1× Wash Buffer. Cells were kept on ice and were analyzed by flow cytometry within 1 h. Flow cytometry analysis was done on an EPICS™ flow cytometer (Beckman Coulter, Fullerton, CA, USA) using a 15-mW argon ion laser at 488 nm. FAM fluorescence was measured with a 530 ± 30 nm bandpass filter.

For measuring apoptosis, Jurkat cells were treated with 1 µM staurosporine for 3 h in the presence and absence of 10, 50, or 100 µM FAM-VAD-FMK. The nuclei were stained with 1 µg/mL Hoechst 33342 (Sigma) for 5 min, followed by washing and resuspension of cells in Wash Buffer. To quantify apoptosis, the percentage of cells with changes in nuclear morphology (nuclei with condensed chromatin and apoptotic bodies) was determined.

To measure caspase staining in HeLa cells, the supernatants of treated and control cells, grown in 35-mm dishes, were collected and transferred to sterile centrifuge tubes. The remaining attached cells were trypsinized and combined with the supernatants. Cells were centrifuged at 400× g, washed twice with complete medium, and resuspended in 300 µL complete medium. For labeling, 10 µL 30× FAM-VAD-FMK was added and incubated for 1 h at 37°C under 5% CO₂. Cells were washed twice with 1× Wash Buffer, and the cell pellet was resuspended in 400 µL 1× Wash Buffer, followed by flow cytometry analysis, as described above.

Affinity Labeling of Caspases

Jurkat cells (200 µL) at 2×10^6 cells/mL were treated with 1 µM staurosporine in the presence or absence of the caspase inhibitors zVAD-FMK and FAM-VAD-FMK or the control peptide FAM-FA-FMK for 4 h at 37°C and 5% CO₂. Cells were affinity labeled with 10 µM affinity label Ac-Tyr-Val-Lys(biotinyl)-Asp-2,6-dimethylbenzoyloxymethylketone (AcYVK(bio)D-aomk; Calbiochem) (8,17) for an additional hour at 37°C and 5% CO₂. Affinity labeled cells were collected by centrifugation at 400× g, washed once in ice-cold PBS, and lysed on ice for 10 min in Nonidet® P-40 buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, containing 0.5 mM phenylmethylsulfonyl fluoride, 5 µg/mL aprotinin, 5 µg/mL pepstatin, and 5 µg/mL leupeptin). Samples were vortex mixed and centrifuged for 5 min at 19000× g at 4°C. An equal volume of 2× gel loading buffer was added to 15 µL cleared extract. The samples were heated at 95°C for 5 min. Proteins were resolved on a 15% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Nonspecific binding was blocked with TBS-T (50 mM Tris-HCl, pH 7.5,

Figure 2. Inhibition of caspase staining by co-treatment with zVAD-FMK. Jurkat cells were DMSO treated and treated with 2 µg/mL camptothecin for 6 h. Then, cells were labeled with 10 µM FAM-VAD-FMK in the presence and absence of 50 µM zVAD-FMK for 1 h. Cells were washed three times with wash buffer and resuspended in 300 µL PBS. Aliquots (100 µL) were transferred to wells of a black 96-well plate. FAM-fluorescence was read in a Victor 1420 fluorescence plate reader at 485 nm excitation and 535 nm emission wavelengths. Error bars, SD of three wells.

150 mM NaCl, 0.1% Tween[®] 20) containing 5% non-fat dry milk for 1 h at room temperature. After washing two times with TBS-T, the membranes were probed with horseradish peroxidase-linked streptavidin (1:2000 dilution in TBS-T with 1% bovine serum albumin) for 1 h. The blots were washed four times with TBS-T. Labeled proteins were visualized by enhanced chemiluminescence (ECL[®]) according to the manufacturer's instructions (SuperSignal[®]; Pierce Chemical, Rockford, IL, USA).

Caspase Detection in Non-Adherent and Adherent Cells Using 96-Well Plates

Non-adherent Jurkat cells (150 μ L) at 2×10^6 cells/mL were plated per well of a black 96-well plate (Cliniplate[™]; Labsystems, Franklin, MA, USA). Cells were treated with solvent control (DMSO) or with 1 μ M staurosporine with and without 50 μ M zVAD-FMK for 4 h at 37°C/5% CO₂. To label, 5 μ L 30 $\times$ FAM-VAD-FMK were added per well, and incubation was continued for 1 h at 37°C/5% CO₂. The 96-well plate was centrifuged for 5 min at 600 $\times$ g at room temperature, and the supernatant was removed by vacuum aspiration. The wells were washed three times with 300 μ L 1 $\times$ Wash Buffer followed by centrifugation at 600 $\times$ g after each wash. PBS (100 μ L) was added per well, and fluorescence was read in a Victor 1420 fluorescence plate reader (Applied Biosystems, Foster City, CA, USA) at 485 nm excitation and 535 nm emission wavelengths.

HeLa cells were trypsinized and seeded onto wells of a black 96-well plate with clear bottom (Applied Biosystems) at a cell density of 5×10^4 cells/well and grown to confluence for 16 h at 37°C/5% CO₂. In a total volume of 100 μ L, cells were treated with solvent control (DMSO) or 1 μ M staurosporine with and without 50 μ M zVAD-FMK for 4 h at 37°C/5% CO₂. Labeling of cells was performed by the addition of 3.3 μ L 30 $\times$ FAM-VAD-FMK per well, and incubation was continued at 37°C/5% CO₂ for 1 h. Two-hundred microliters of 1 $\times$ Wash Buffer were added, followed by centrifugation at 600 $\times$ g for 5 min at room temperature. The supernatant was removed by vacuum aspiration. The wells were washed twice with 300 μ L 1 $\times$ Wash Buffer, followed by centrifugation after each wash. PBS (100 μ L) was added per well, and fluorescence was read in a Victor 1420 fluorescence plate reader at 485 nm excitation and 535 nm emission wavelengths.

RESULTS AND DISCUSSION

In Situ Caspase Detection by FAM-VAD-FMK

We have developed the fluorescein-labeled general caspase inhibitor FAM-VAD-FMK as a reagent to detect active caspases in living cells using flow cytometry, fluorescence microscopy, or a fluorescence plate reader. The flow cytometry histogram in Figure 1A shows one peak within the first log decade of the X-axis corresponding to unstained control cells. The histogram of camptothecin-induced Jurkat cells displays two peaks (Figure 1B). The peak within the first log decade corresponds to unstained non-apoptotic cells, whereas the peak appearing within the second and third log decade corresponds to FAM-VAD-FMK-stained apoptotic cells. The increase in caspase-specific fluorescence in apoptotic cells

compared to non-apoptotic cells was 30-fold. The fluorescence intensity of labeled non-apoptotic cells was approximately twice that of the autofluorescence generated by unstained cells (data not shown), indicating low background staining of non-apoptotic cells by FAM-VAD-FMK. Pretreatment with the pan-caspase inhibitor zVAD-FMK (Figure 1C) completely blocked caspase-specific fluorescence cell staining, indicating that FAM-VAD-FMK specifically labels cells containing active caspases.

To extend our findings with non-adherent Jurkat cells to other cell types, we examined caspase-specific cell staining in apoptotic and control HeLa cells. The flow cytometry histogram of staurosporine-treated HeLa cells (Figure 1E) shows a single peak within the second log decade of the X-axis with a median fluorescence 10-fold higher than with control cells, which show a peak within the first log decade of the X-axis (Figure 1D). Similar to Jurkat cells, pretreatment with zVAD-FMK (Figure 1F) blocked caspase-specific fluorescence cell staining in staurosporine-treated HeLa cells.

We have proposed that the observed labeling of apoptotic cells with FAM-VAD-FMK is due to the specific binding of FAM-VAD-FMK to caspases, which thereby inhibits the enzyme, and is not a reflection of nonspecific binding to intracellular targets as a consequence of an apoptosis-related increase in cell permeability, nor is it due to cleavage of non-caspase cellular proteins by caspase enzymes (1). To prove this claim, we measured caspase labeling in Jurkat cells treated with camptothecin for 6 h, followed by incubation with FAM-VAD-FMK with and without zVAD-FMK for 1 h. As shown in Figure 2, co-treatment with zVAD-FMK blocked staining with FAM-VAD-FMK, indicating that both zVAD-FMK and FAM-VAD-FMK compete for the same intracellular target, namely activated caspases.

Figure 3. Caspase detection by affinity labeling. Jurkat cells were treated with 1 μ M staurosporine (STS) in the presence or absence of the caspase inhibitor zVAD-FMK, FAM-VAD-FMK, or the control peptide FAM-FA-FMK for 4 h. A 10 μ M concentration of the affinity label AcYVVK(bio)D-aomk was added, and incubation was continued for 1 h. As controls, DMSO- and staurosporine-treated cells were incubated for 1 h without affinity label. Cells were washed, and cell lysates were prepared. Proteins were separated on a SDS-polyacrylamide gel, followed by transfer to nitrocellulose membranes. Labeled proteins were revealed by streptavidin-horseradish peroxidase combined with ECL detection.

Since zVAD-FMK has been shown to prevent apoptosis by inhibiting caspases (2), it is reasonable to propose that FAM-VAD-FMK would also irreversibly bind to and inhibit active caspases, thus preventing induction of apoptosis. To examine this, we measured apoptosis in staurosporine-treated cells with and without FAM-VAD-FMK. Apoptosis was quantified by counting cells with apoptotic nuclear morphology using Hoechst stain. As shown in Table 1, co-treatment of staurosporine-stimulated cells with FAM-VAD-FMK led to a decrease in the percentage of apoptotic cells. These results demonstrate that FAM-VAD-FMK acts as an inhibitor of apoptosis by inhibiting the caspase cascade. We have previously shown that there exists a strong correlation between the apoptotic index as estimated by DNA strand break labeling (TUNEL assay) and the activation of caspases in cells treated with camptothecin (1). This clearly indicates that caspase activation can be used as a definite and reliable indicator for apoptosis.

FAM-VAD-FMK Inhibits Effector Caspases

Figures 1 and 2 show that cell staining with FAM-VAD-FMK is inhibited by the general caspase inhibitor zVAD-FMK. This suggests that FAM-VAD-FMK binds to and inhibits activated caspases. To examine the specificity of FAM-VAD-FMK and zVAD-FMK for inhibiting caspases, caspase activation was measured in staurosporine-treated cells in the presence and absence of FAM-VAD-FMK or zVAD-FMK by affinity labeling. As shown in Figure 3, the pattern of

affinity-labeled bands is identical to that published by Takahashi et al. (15), who found that the four bands corresponded to caspase-3 fragments p17 and p20, to caspase-6 fragment p19, and to caspase-7 fragment p22. Fragments p19 and p20 appear as one band on a 15% SDS-polyacrylamide gel. We confirmed the published band identification (15) using specific inhibitors for caspase-3, caspase-3 and -7, and caspase-6 (data not shown). The affinity label AcYVK(bio)D-aomk only detects effector caspases, but not initiator caspases, because the latter are much less abundant (5). Staurosporine treatment of Jurkat cells led to activation of caspase-3, -6, and -7. DMSO-treated affinity labeled cells did not show any bands corresponding to activated caspases, nor did unlabeled DMSO-treated or unlabeled staurosporine-treated cells. Both zVAD-FMK and FAM-VAD-FMK inhibited affinity labeling of all the caspase fragments. As a control, the FAM-labeled cathepsin B inhibitor FAM-FA-FMK (3) did not inhibit caspase labeling. These results show that FAM-VAD-FMK and zVAD-FMK inhibit caspase labeling because of the inhibition of caspase activation, resulting in inhibition of apoptosis.

Caspase Detection in Microplates

Human disease pathogenesis often reveals an apoptotic component that either contributes to disease progression or is the direct cause. To discover drugs specific for apoptosis-related diseases, new screening technologies using living cells are required. Because of their pivotal role in cell death, caspases are the most obvious therapeutic targets for modulating

Figure 4. Caspase detection in 96-well plates. Jurkat cells (A) or HeLa cells (B) were plated into wells of a black 96-well plate. Cells were control treated (DMSO), treated with 1 μ M staurosporine (STS), or co-treated with 1 μ M staurosporine plus 50 μ M zVAD-FMK for 4 h. After labeling with FAM-VAD-FMK, cells were washed and FAM-fluorescence was read in a Victor 1420 fluorescence plate reader at 485 nm excitation and 535 nm emission wavelengths. Error bars, SD of two wells.

Table 1. Inhibition of Apoptosis by FAM-VAD-FMK

	% Apoptosis
DMSO	2.5
STS	74
STS plus	56
10 μ M FAM-VAD-FMK	
STS plus	12
50 μ M FAM-VAD-FMK	
STS plus	13
100 μ M FAM-VAD-FMK	
<p>Jurkat cells were control treated, treated with 1 μM staurosporine (STS), or co-treated with 1 μM STS plus increasing concentrations of FAM-VAD-FMK for 3 h. Cells were subsequently stained with Hoechst to detect apoptotic nuclei. After washing, an aliquot of cells was placed on a microscope slide, and nuclear staining was observed using a UV filter (excitation 395 nm, emission 450 nm). The percentage of apoptosis was determined from apoptotic nuclei cell counts of at least 100 cells.</p>	

apoptosis. To determine whether FAM-VAD-FMK could be used in an apoptotic drug screen, we measured caspase activation in staurosporine-treated Jurkat cells or HeLa cells by fluorescence spectroscopy using 96-well plates. As shown in Figure 4A, staurosporine treatment of Jurkat cells led to a 3-fold increase in FAM-fluorescence compared to control cells. FAM-fluorescence in staurosporine-treated HeLa cells was also increased 3-fold compared to control cells (Figure 4B). Pretreatment with zVAD-FMK completely abolished the staurosporine induced increase in fluorescence in Jurkat and HeLa cells. These results show that FAM-VAD-FMK can be used to measure caspase activation in living cells following drug treatment using a 96-well plate format.

In conclusion, FAM-VAD-FMK is a sensitive and specific reagent for the detection of activated caspases in living cells.

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