A novel 96-well scintillation proximity assay for the measurement of apoptosis

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Abstract

The translocation of phospholipids across the plasma membrane has been widely documented as one of the earliest measurable biochemical events of apoptosis. Using fluorescently labelled annexin V, which preferentially binds phosphatidylserine (PS) in the presence of Ca²⁺, the externalization of PS can be measured and apoptosis quantified using flow cytometry. Conventional detection methods utilizing annexin V, while faster than *in situ* DNA end-labelling or DNA laddering, require extensive sample preparation which may compromise samples and makes rapid, high volume screening prohibitive. This paper describes a novel assay for the measurement of apoptosis based upon binding of radiolabelled annexin V to apoptotic cells attached to the growth surface of a 96-well scintillating microplate (Cytostar-T[®]). We compared measurements of apoptosis made by flow cytometry to those obtained with the scintillating microplate in three model systems, treatment of: mouse connective tissue (L-M) cells with lymphotoxin (LT), human lung carcinoma (H460) cells with Apo-2 ligand and human umbilical vein endothelial (HUVE) cells with staurosporine. In this assay, we compare both direct and indirect labelling methods by utilizing either iodinated annexin V or biotinylated annexin V/[³⁵S] streptavidin to radiolabel apoptotic cells. The signal detected is a direct consequence of the binding of annexin V to externalized PS on apoptotic cells and the proximity of the label to the base of the plate. Using this method, separation of bound and unbound radiolabel signal occurs directly within the well resulting in a sensitive assay that requires minimal manipulation and can accommodate a large number of samples.

Abbreviations: Apo-2L, Apo-2 ligand; BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; LT, lymphotoxin; PI, propidium iodide; PS, phosphatidylserine

Introduction

Apoptosis, or programmed cell death, is the physiological counterpoint to proliferation in higher organisms. While removal of damaged cells via apoptosis is critical to preserving homeostasis in healthy tissue, dysregulation of apoptosis can lead to uncontrolled growth, invasion and/or destruction of normal tissue (Wyllie et al., 1992). Understanding the basic control

mechanisms for apoptosis may lead to new therapies to combat disease progression.

One well established system for demonstrating cell death is cytokine-induced apoptosis. Activation of the receptors for cytokines such as tumor necrosis factor (TNF α), Lymphotoxin (LT or TNF β), or Apo2 ligand (Apo-2L) triggers an intracellular cascade of proteases which propagate an apoptotic signal (Ashkenazi et al., 1998). Once apoptosis has been initiated, the

Figure 1. Crosssection of a single well from a Cytostar-T[®] scintillating microplate.

cells undergo distinct morphological and biochemical changes such as membrane blebbing, membrane flipping, nuclear condensation, DNA fragmentation, and ultimately cell dissolution into discrete apoptotic bodies. Phosphatidylserine (PS) externalization, considered to be one of the first and most consistent cellular phenomenon associated with apoptosis, is readily measured by the specific binding of annexin V, a Ca^{2+} -dependant phospholipid binding protein (Huber et al., 1991). The pancellular nature of PS translocation makes annexin V binding useful for screening apoptosis in a wide variety of cell types. Quantitative measurement of apoptosis with flow cytometry, utilizing annexin V, while faster than *in situ* DNA end-labelling or DNA laddering, requires extensive sample preparation which may compromise samples and makes rapid, high volume screening prohibitive (Vermees et al., 1995). This paper describes a novel assay for the measurement of apoptosis based upon annexin V binding to apoptotic cells attached to the growth surface of a 96-well scintillating microplate (Cytostar-T[®]).

To evaluate the utility of this assay for the measurement of apoptosis we examined two members of the TNF cytokine family, LT which binds to TNFR1

and TNFR2 (Wong et al., 1996) and Apo-2L (Pitti et al., 1996) which binds death receptors DR4 and DR5 (Sheridan et al., 1997). Both are potent inducers of apoptosis in a wide variety of tumor cell types. We also examined the ability of staurosporine, a broad spectrum inhibitor of protein kinases, (Martin et al., 1995) to induce apoptosis in normal human umbilical vein endothelial cells (HUVEC). Thus, using various agents to induce an apoptotic response on a number of different suspension and adherent cell types, we demonstrate the versatility of detection in Cytostar-T[®] plates using annexin V.

Materials and Method

Materials

Annexin V-FITC (APOPTEST-FITC), annexin V-biotin (APOPTEST-BIOTIN), unconjugated annexin V and $10\times \text{Ca}^{2+}$ binding buffer were purchased from NeXins Research BV (Hoeven, the Netherlands). Propidium iodide (PI) was purchased from Molecular Probes (Eugene, OR) and diluted in dH_2O to a working stock of $4 \mu\text{g/ml}$. FITC-labelled streptavidin

was obtained from DAKO (DAKO Corporation, Carpinteria, CA). Crystallized BSA was from ICN Biomedicals (Aurora, IL). Staurosporine, obtained from CLONTECH (Palo Alto, CA) was reconstituted in DMSO to a stock concentration of 100 $\mu\text{g/ml}$. Actinomycin D was purchased from Sigma (St. Louis, MO) and dissolved in ethyl alcohol (100%) to a working stock of 5 mg/ml. Apo-2L and rTNF- β (lymphotoxin) were obtained from Genentech, Inc. (South San Francisco, CA). The [^{35}S]streptavidin, [^{125}I]annexin V and the Cytostar-T scintillating microplates were purchased from Amersham Life Science (Arlington Heights, IL).

Cell lines and culture medium

Murine connective tissue cells (L-M), human lung carcinoma cells (H460, A549 and SK-MES-1), human cervical carcinoma cells (ME-180), human breast carcinoma cells (BT474 and SK-BR-3), and human lung fibroblast cells (WI-38) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Human colon carcinoma cells (HCT116) were also obtained from the American Type Culture Collection (ATCC) and were suspension adapted at Genentech. Human mammary epithelial (HME) cells were purchased from Clonetics (San Diego, CA). Human umbilical vein endothelial (HUVE) cells were purchased from Cell Systems (Kirkland, WA). Human B (9D) cells were obtained from Genentech, Inc. The L-M cells were cultured in phenol-red free medium 199 (M199, GIBCO/BRL, Grand Island, NY) supplemented with 0.5% Bacto-peptone, 24 mM Hepes, 2 mM L-glutamine, 5 $\mu\text{g/ml}$ insulin, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin (GIBCO/BRL). HUVE cells were cultured in CS-C complete medium containing 10% FBS (Cell Systems) and supplemented with 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. The HME cells were cultured in serum-free mammary epithelial cell basal medium (Clonetics). The 9D cells were cultured in RPMI 1640 medium containing 10% FBS and 2 mM L-glutamine. All remaining cell lines were cultured in phenol-red free Dulbecco modified Eagle medium: Ham's F-12 (50:50) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin. Cells were routinely kept in a humidified incubator at 37 °C with 5% CO_2 . The cells were tested and found free of *Mycoplasma* infection prior to initiation of experiments.

Induction of apoptosis by lymphotoxin, Apo-2L or staurosporine

To measure apoptosis in L-M cells induced by LT, the cells were treated with 1 $\mu\text{g/ml}$ Actinomycin D (as described by Kramer et al., 1986) in combination with varying concentrations of lymphotoxin (0.5–62 pg/ml) for 24 h. Apoptosis was induced in A549, HCT116, SK-MES-1, ME-180 and H460 cells by treatment with Apo-2L (1.0 ng/ml–2.5 $\mu\text{g/ml}$) for 24 h. WI-38, SK-BR-3, BT474, HME and HUVE cells were treated with Apo-2L at doses ranging from 1.5 ng/ml–10 $\mu\text{g/ml}$ for 24 h. For the suspension cell lines (HCT116 and 9D) Apo-2L (1.0 ng/ml–2.5 $\mu\text{g/ml}$) was diluted down the plate in a 0.05ml volume prior to cell addition. HUVE cells were incubated for 48 h in the presence of 17.0 pg/ml–0.33 $\mu\text{g/ml}$ staurosporine. Cell lysis was induced in L-M, H460, HCT116 and HUVE cell lines by repeat freeze/thawing at –20 °C.

Flow cytometry

Two different approaches were taken to analyze annexin V binding by flow cytometry. The cells were either labelled directly with annexin V-FITC (as described by Moore et al., 1998) or indirectly using annexin V-biotin followed by streptavidin-FITC. For both procedures, cells were seeded in 100 $\times$ 20 mm dishes at a cell density of $1.5 \times 10^6/\text{dish}$ and allowed to attach overnight at 37 °C. After induction of apoptosis (as described above), the nonadherent cells were collected into individual tubes and the adherent cells detached using 1 ml of 2.5X trypsin-EDTA (GIBCO/BRL) in phosphate buffered saline (PBS). The detached cells were combined with the corresponding floating cells and pelleted. The assay medium was decanted, the pellet resuspended in Ca^{2+} binding buffer (NeXins B.V.) and the cells stained by one of the following methods:

Direct Labelling Procedure

The cell suspension was divided evenly into 3 tubes which received either annexinV-FITC only (1.0 $\mu\text{g/ml}$), propidium iodide (PI) only or both annexin V-FITC and PI. The PI was added in order to distinguish cells which had lost membrane integrity, and was added to a final concentration of 40 ng/ml. The cells were allowed to sit on ice for 15 min prior to analysis. In the experiments comparing direct vs. indirect labelling, PI staining was omitted.

Figure 2. Phase (a, d), fluorescent (e, f) or a combination phase/fluorescent (b, e) micrograph of L-M cells with no treatment (a, b, c) or treated with 15.6 pg/ml LT (d, e, f). Untreated cells show little annexin V-FITC binding (c) while treatment with LT results in significant binding of annexin V-FITC (f).

Figure 3. Histograms displaying dose-dependent apoptotic responses for L-M cells treated with LT (left), H460 cells with Apo-2L (center) and HUVEC cells treated with staurosporine (right) measured by binding of annexin V-FITC. Untreated cells show low levels of fluorescence (a), while treatment with 62 pg/ml LT (b, left panel), 0.83 μ g/ml Apo-2L (b, center panel) or 0.33 μ g/ml staurosporine (b, right panel) results in a shift in fluorescence intensity. The frequency of annexin V-FITC positive cells diminishes as the drug concentrations are decreased (2-fold dilutions for LT, 3-fold dilutions of drug for Apo-2L and staurosporine, c-g on each panel). The percent of annexin V-FITC positive cells is displayed within each histogram. Two-color quadrant plots are displayed for control and high dose treatments showing the shift in percent of apoptotic cells after treatment from an annexin V-FITC negative/PI negative to an annexin V-FITC positive/PI positive cell population. The percent of cells detected is displayed in each quadrant.

Figure 4. Comparison of annexin V-FITC (■) binding to annexin V-biotin/streptavidin-FITC (□) on L-M cells treated with LT (A) for 24 h, H460 cells with Apo-2L (B) for 24 h, and HUVEC cells treated with staurosporine (C) for 48 h. No detectable signal resulted with an irrelevant biotinylated anti-sheep IgG control (○), or with the streptavidin-FITC alone (●).

Indirect Labelling Procedure

Annexin V-biotin was added to a final concentration of 1.0 μ g/ml and allowed to incubate for 20 min on

ice. The cells were pelleted and the supernatant decanted to remove unbound annexin V-biotin. The cells were then incubated with streptavidin-FITC (diluted

1:250 in binding buffer) for 20 min on ice before being analyzed. An irrelevant biotinylated anti-sheep IgG (Vector, Burlingame, CA) was run in parallel with the annexin V-biotin to verify binding specificity of annexin V for apoptotic cells.

Flow cytometric analysis was performed on a FAC-Scan (Becton Dickinson, San Jose, CA) equipped with an Argon ion laser emitting 488 nm. Annexin V-FITC fluorescence emission was detected utilizing a 560 nm short-pass dichroic filter followed by a 530 nm band-pass filter with a band width of 30 nm. PI fluorescence was detected utilizing a 640 nm long-pass dichroic filter followed by a 650 nm long-pass filter. Compensation was determined from single-color samples using a positive apoptotic control. A total of 10,000 events was collected per sample and the percentages of cells of each phenotype determined.

All experiments measuring apoptosis by flow cytometry were performed twice with duplicate dishes run in each experiment. The data shown are those of representative experiments.

Fluorescent staining with annexin V-biotin/streptavidin-FITC

L-M cells were seeded in a 6-well plate at a density of 5×10^5 /well. After treatment with lymphotoxin, 1 μ g/ml annexin V-biotin in $2 \times \text{Ca}^{2+}$ binding buffer was added to the cells. The plate was allowed to incubate for 15 min with gentle agitation to facilitate binding. The plate was centrifuged for 5 min and the media removed. Streptavidin-FITC was added to a final concentration of 1 μ g/ml in $2 \times \text{Ca}^{2+}$ binding buffer and the plate was incubated for 30 min with gentle agitation. Phase and fluorescent images were obtained using a Nikon Axiovert Microscope with a 20 $\times$ objective.

Measurement of apoptosis in a 96-well scintillating microplate

The utility of Cytostar-T[®] scintillating microplates for measuring apoptosis was evaluated using a direct labelling procedure with iodinated annexin V and a two-step (indirect) procedure using annexin V-biotin/[³⁵S]streptavidin. For both procedures, cells were seeded in a volume of 0.1 ml at a density of 4.0×10^4 /well for L-M, SK-BR-3, SK-MES-1, BT474, HCT116 and ME-180 cells; 2.3×10^4 /well for H460, WI-38 and HME cells; 6×10^4 /well for 9D cells and 2.0×10^4 /well for HUVE cells and allowed to attach overnight at 37 °C. After cell attachment, the

media was changed to remove damaged cells resulting from plating conditions and the cells were treated with LT, Apo-2L or staurosporine as previously described. For the suspension cell lines (HCT116 and 9D) cells were added directly to the dose conditions in a 0.05 ml volume. Blank wells were used to determine nonspecific binding of either the radiolabelled annexin or radiolabelled streptavidin.

Direct Labelling Procedure

After induction of apoptosis, 0.5 μ Curie (5.0×10^5 cpm/well) of [¹²⁵I]annexin V in 0.05 ml of $3 \times \text{Ca}^{2+}$ binding buffer containing 2.5% BSA (w/v) was added to all wells. After a 30 min incubation at room temperature, the plates were centrifuged for 7 min at 1100 rpm to bring floating cells in contact with the base of the plate. To minimize radioactive crosstalk between wells, the medium was removed from each well using a multichannel pipet with care taken not to disturb the cell layer. The plates were then heat-sealed and counted.

Indirect Labelling Procedure

Following induction of apoptosis, 4 μ g/ml annexin V-biotin in 0.05 ml of $2 \times \text{Ca}^{2+}$ binding buffer containing 2.5% BSA (w/v) was added to the appropriate wells and the plates incubated for 30 min at room temperature. After allowing the annexin V-biotin to bind, 0.05 ml of $2 \times \text{Ca}^{2+}$ binding buffer (with BSA) containing 0.46 μ Curie (3.0×10^4 cpm/well) [³⁵S]streptavidin was added to all wells. To control for nonspecific binding, certain wells on each plate received [³⁵S]streptavidin in the absence of annexin V-biotin. The plates were incubated for two hours at room temperature with gentle agitation. Apoptotic cells were brought in contact with the base of the plate by centrifugation as with the direct method. However, no media removal was required before the plates were sealed and counted.

Cytostar-T[®] plates were counted using a 1450 MicroBeta Trilux scintillation counter (EG&G Wallac). Since the phenol-red was removed from the medium it was not necessary to optimize count conditions with regard to color quench. However, substitution with phenol containing medium did not alter the counting efficiency with these isotopes (data not shown). For both the direct and indirect labelling methods the counter was set using CPM mode with normal coincidence counting parameters. The default window settings were used, 5–1020 for [¹²⁵I] annexin V and 150–650 for annexin V-biotin/[³⁵S]streptavidin. The

Figure 5. Apoptosis detected by binding of [¹²⁵I] annexin V (■) or annexin V-biotin/[³⁵S] streptavidin (□) to L-M cells treated with LT (A) for 24 h, H460 cells treated with Apo-2L (B) for 24 h, and HUVE cells treated with staurosporine (C) for 48 h.

Figure 6. Dose response curves for L-M cells after 24 h treatment with LT (A), H460 cells after 24 h treatment with Apo-2L (B), and HUVE cells after 48 h treatment with staurosporine (C) comparing increasing annexin V-biotin/[³⁵S]streptavidin binding (CPM, ■) to a corresponding decrease in cell viability as measured by crystal violet staining (OD, □).

Figure 7. Comparison of rates of cell death using indirect binding of annexin V-biotin/[^{35}S]streptavidin to three different cell lines: H460 cells (■) and suspension adapted HCT116 cells (●) after treatment with 0.092 $\mu\text{g/ml}$ Apo-2L or HUVE cells (□) after treatment with 0.037 $\mu\text{g/ml}$ staurosporine. H460 cells were assayed at 4, 12, 24 and 32 h; HCT116 cells at 4, 8, 10, and 24 h and HUVE cells at 12, 24, 48 and 72 h to determine maximal annexin V binding.

counter was normalized for both isotopes based upon the manufacturer's recommendations and crosstalk correction was performed with all experiments using iodinated annexin V.

All experiments measuring apoptosis in Cytostar-T[®] scintillating microplates were repeated three times with each point run in triplicate. The data shown are those of representative experiments.

Measurement of cell viability by crystal violet staining

Following analysis of annexin V binding on adherent cell lines in the Cytostar-T[®] plates, the monolayers were washed once with PBS to remove all non-adherent cells and the wells were stained for 15 min with a 0.5% solution of crystal violet in methanol. The relative cell number was determined by measuring optical density of the dry plate at 540 nm using an SLT 340 ATC platereader (Salzburg, Austria). The data

shown are those of representative experiments ($n=3$ for each treatment group).

Results and Discussion

The Cytostar-T[®] scintillating microplates are sterile 96-well, tissue culture treated microplates containing a mixture of scintillant and polystyrene in the base which have been used to measure a spectrum of cell associated events using the principles of scintillation proximity (Picardo et al., 1997). Using these plates we have developed an assay for the measurement of apoptosis based upon the binding of radiolabelled annexin V to externalized PS as diagrammed in Figure 1.

In order to validate the use of this 96-well scintillating microplate for quantitative analysis of apoptosis, we compared measurements of apoptosis made by flow cytometry to those obtained with the scintillating plate system using model systems in which the induction of apoptosis was previously established us-

Table 1. Detection of apoptosis in multiple cell lines using Cytostar-T[®] scintillating microplates

Cell line	Cell type	Growth	ED ₅₀ (ug/ml) ^a
A549	lung carcinoma	adherent	0.311±0.023
NCI-H460	lung carcinoma, large-cell	adherent	0.017±0.004
ME-180	cervical carcinoma	adherent	0.233 ±0.025
SK-MES-1	lung carcinoma, squamous	adherent	0.066±0.009
SK-BR-3	breast adenocarcinoma	adherent	0.048±0.003
BT474	breast ductal carcinoma	adherent	0.947±0.031
HCT116	colon carcinoma	adherent	0.112±0.017
HCT116	colon carcinoma	suspension	0.077±0.012
9D	B cell	suspension	0.033±0.009
HUVEC	umbilical vein endothelial	adherent	2.56±0.145
HMEC	mammary epithelial	adherent	negative
WI-38	lung fibroblast	adherent	negative

^a ED₅₀ values were obtained after overnight treatment with Apo-2L as measured by annexin V-biotin/[³⁵S]streptavidin binding in a Cytostar-T[®] scintillating microplate.

Values are mean ± standard error except for A549, ME-180, 9D and HCT116 suspension where ED₅₀ values are represented as ranges.

ing DNA laddering (Wong et al., 1994 and Sheridan et al., 1997). In one of the model systems, we show that annexin V does indeed bind to LT treated L-M cells by visualizing cells exposed first to annexin V coupled to biotin, followed by streptavidin-FITC. Figure 2f shows the presence of a large proportion of FITC-labelled apoptotic cells, compared to untreated cells (Figure 2c). The fluorescently labelled cells also appear rounded up in the phase contrast micrographs (Figures 2d, e) in contrast to the untreated cells which remain flattened and healthy (Figures 2a, b).

For quantitative measurement by flow cytometry, three cell types (L-M, H460 and HUVE cells) were labelled directly with annexin V-FITC after treatment with the apoptosis inducing agents. Flow cytometric methods for measuring apoptosis have been reported by several groups (Vermes et al., 1995; Martin et al., 1995; Van Engeland et al., 1996; Siegel et al., 1998; Moore et al., 1998). Figure 3 shows a dose dependent induction of apoptosis as measured by annexin V-FITC binding upon treatment of L-M cells with LT (left panel), H460 cells with Apo-2L (center panel) and HUVE cells with staurosporine (right panel). Treatment results in a two-log shift in fluorescence intensity (Figure 3b) which diminishes upon serial dilution of each drug (c-g on each panel). By the double staining procedure (shown in each panel as

quadrant plots), measuring both PI uptake and annexin V-FITC binding to cells with externalized PS, the total percentage of apoptosis detected was the same as detected using annexin V-FITC binding alone (Figure 3). The hypodiploid DNA has been gated out of the dual analysis in Figure 3, however this population appears as described by Moore et al., 1998. We further verified that similar quantitation by flow cytometry could be obtained with indirect labelling. Separate dose-effect experiments using annexin V-biotin/streptavidin-FITC demonstrated similar killing curves to those observed by direct labelling, as indicated in Figure 4.

Figure 5 shows that the 96-well scintillation proximity assay, using directly labelled annexin or annexin V-biotin/[³⁵S]streptavidin, duplicated the results observed by flow cytometry for all three cell types. To further examine the correlation between the percentage of annexin positive cells detected by flow cytometry and the total annexin binding detected in the microplate, linear correlation analysis was performed (data not shown). Comparing the indirect methods of annexin V binding detected by either streptavidin-FITC or [³⁵S]streptavidin, correlation coefficients were obtained for each cell line: H460 cells treated with Apo-2L, $r = 0.9562$; L-M cells treated with LT, $r = 0.9337$ and for HUVE cells treated with staurosporine, $r = 0.9089$. However, the microplate-based assay

measures the total apoptosis occurring within the well instead of apoptosis as a percent of a total cell population as in flow cytometry. Because we don't have the ability to stain with PI and resolve necrotic cells, and since apoptosis and necrosis can differ in the timing with which the cell loses membrane integrity, the ability of scintillation proximity to resolve these two types of cell death was a concern. To this end, cells were subjected to repeat freeze/thawing which resulted in cell lysis similar to what one would observe with necrosis. No apoptotic signal was detected after total cell lysis was induced (data not shown). To confirm that PS-containing cell fragments resulting from cell lysis would be too light to settle and therefore too distant to signal, we centrifuged the plates to sediment the cell debris but were still unable to detect annexin V binding.

While separation of bound and unbound material in the wells eliminated the concern of false positives resulting from necrosis it was still necessary to monitor false negatives resulting from loss of all the cells. Since apoptosis is a dynamic event, using the correct window for measuring optimal annexin V binding was critical. While HUVE cells treated with staurosporine required a minimum of 48 h to achieve maximal annexin V binding, the H460 cells, which are very sensitive to Apo-2L, show maximal annexin V binding after only 4 h (Figure 7). The suspension adapted HCT116 cells by comparison have an intermediate response rate to induction with Apo-2L with a narrow window for detection of apoptosis relative to either H460 or HUVE cells (Figure 7). With such variation in rates of cell death it was preferable to choose a longer incubation time and run extensive dose curves in order to detect false negatives for each agent tested. As expected, a decrease in signal was observed under all conditions evaluated when a sufficiently high dose of LT, Apo-2L or staurosporine was used. To confirm that this loss in signal was due to a diminished viable cell density, the cells remaining attached to the plate were stained with crystal violet and a measure of cell density obtained. This confirmed that the decrease in signal (seen in Figure 6B) correlated with a significant loss of apoptotic cells capable of binding annexin V. By combining measurement of annexin V binding with crystal violet staining, we were able to decrease the dose range, maximizing sample number per plate without missing potent inducers of apoptosis because of varying rates of cell death. However, crystal violet staining measures only the cells which remain attached to the growth surface and represents

a subset of the total cells measured when obtaining an annexin V binding signal. This is shown in Figures 6a and 6c where high annexin V binding corresponds to a low viable cell number. Staining the cells with alamar Blue[®] instead of crystal violet enables measurement of cell viability on the same population analyzed by annexin V binding without extensive manipulation. However, in experiments where we have compared alamar Blue[®] to crystal violet staining we have seen equivalent results (data not shown).

While the two-step labelling process is a good way to measure induction of apoptosis in many cell types there were some primary cells tested which did not survive the two hour incubation required for the binding of [³⁵S] streptavidin to annexin V-biotin. In our system, the binding of annexin V to PS is faster than the binding of streptavidin to biotin. Therefore, it was important to evaluate the utility of a one-step method. Using directly labelled annexin V we measured similar dose response relationships for the induction of apoptosis, (Figure 5) while significantly decreasing the amount of time required for detection of a maximal signal (30 min). However, due to the crosstalk associated with the use of ¹²⁵I, it was necessary to remove most of the media from the wells.

The utilization of fluorescently labelled annexin V to measure apoptosis by flow cytometry has, until now, been the quickest means of evaluation. However, this method requires cell detachment which can damage cells and increase PS exposure (Van Engeland et al., 1996). In addition, trypsinization and wash steps can decrease manageable sample numbers and add to sample preparation time. Methods which enable quantitation of apoptosis on undetached cells rely on manual counts of labelled cells. Higher throughput assays for apoptosis measuring ICE protease (CPP32) activity or PARP cleavage address internal signalling events but not the cellular end result. This may allow compounds which induce apoptosis by unknown signalling mechanisms to evade detection. Likewise, demonstration of DNA fragmentation by gel electrophoresis, although sensitive and reliable, is laborious, not quantitative and is limited by small sample capacity. The pancellular nature of PS externalization allows screening in a wide variety of cell types (Table 1) and detection of apoptosis induced by diverse compounds irrespective of signalling mechanisms. Further, the in-well separation unique to scintillation proximity technology allows the immediate measurement of apoptosis without further manipulation which may damage or remove apoptotic cells. By

combining the specificity of annexin V binding and the sensitivity of a radioactive readout we have developed a real-time, quantitative alternative to more conventional methods of measuring apoptotic cell death, with the added advantages of rapid data acquisition, minimal manipulation and accommodation of large sample numbers.

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