Chapter

3

A highly sensitive flow cytometric method for functional analysis of the apoptosis pathways in isolated lymphoma cells and related hematopoietic cells

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Abstract

Inhibition of apoptosis is important in hematological malignancies and specific defects in the apoptosis cascade are strongly related to the response to chemotherapy and eventual clinical outcome. Many new treatment modalities aim to induce apoptosis in leukemia and lymphoma cells. However, functional analysis of apoptosis in isolated tumor cells is hampered by high levels of spontaneous cell death in these cells and presently available methods are not sensitive enough. In this manuscript we describe an easy, highly reproducible method for detection of apoptosis in primary lymphoma cells and related cell lines. This 7AAD-beads method is based on the detection of apoptotic cells using 7AAD staining in combination with fluorescent beads. Apoptosis is distinguished from necroses by addition of a pan-caspase inhibitor. The 7AAD-beads method required few cells and was more sensitive than conventional used standard flowcytometric methods for functional analysis of apoptosis pathways in B-cell lines and isolated lymphoma cells.

We conclude that the 7AAD-beads method is highly sensitive and reproducible for detection of apoptosis in isolated lymphoma cells and related hematopoietic cells and cell lines thereby providing new insights into the pathological defects in apoptosis regulation in these cells.

Introduction

Inhibition of the apoptosis signaling pathways is important in the pathogenesis of hematological malignancies.[1,2,3] Aberrant expression of proteins involved in regulation and/or execution of apoptosis is strongly related to the patient's response to chemotherapy and clinical outcome.[4,5,6,7] Many new treatment modalities aim to induce apoptosis or restore sensitivity to apoptosis, including small-molecule antagonists against Bcl-2 and XIAP.[8,9] However, detection of the apoptosis inducing effect of these agents in primary lymphoma and leukemia cells remains unreliable and inefficient and is frequently hampered by high levels of spontaneous apoptosis. Therefore, functional analysis of the apoptosis cascade has been restricted so far to cell line models. As cell lines are generated following rigorous *in vitro* selection of immortalized cells, it is possible that cell line models do not reflect their original counterpart concerning apoptosis sensitivity.

Apoptosis is a process of strictly regulated events resulting into cell death. Upon induction of apoptosis a family of intracellular cysteine proteases, called caspases are activated.[10] Two major pathways for caspase activation have been elucidated: the intrinsic or stress-induced apoptosis pathway and the extrinsic or death receptor mediated apoptosis pathway. The intrinsic apoptosis pathway is induced after DNA damage and causes mitochondrial release of cytochrome c and activation of caspase 9 in the apoptosome.[11] The extrinsic apoptosis pathway is triggered by ligation of specific death receptors by TRAIL, TNF-α, or Fas ligand.[12] Upon ligand binding, the adaptor molecule FADD is recruited and binds to caspase 8 resulting in formation of the death inducing signaling complex (DISC) and activation of caspase 8 or 10.[13] Both caspase activation pathways induce apoptosis by the cleavage and activation of effector caspases 3 ,6 and 7, which are capable of processing many cellular substrates.[14]

Cells undergoing apoptosis display disruption of transmembrane potential in mitochondria, mitochondrial release of intermembrane proteins, activation of specific caspases, membrane blebbing, condensation of nuclear material and finally DNA cleavage and fragmentation of the cell into apoptotic bodies.[15,16,17] Many flow cytometric methods have been developed to detect different features of apoptosis, including externalization of phosphatidylserine (PS) detected by Annexin V binding,[18,19] changes in cell morphology visualized by membrane impermeable dyes such as 7-amino-actino-mycin-D (7AAD)[20] or non-vital dye propidium iodide (PI).[21] Combinations of these

methods: Annexin V/PI and Annexin V/7AAD have been widely used to discriminate apoptotic and necrotic cells from viable cells.[22,23] A major disadvantage of these methods is that they detect (pre)apoptotic cells but fail to detect fully degraded cells. This results in underestimated numbers of dead cells especially in cases with high levels of cell death. Activated caspase specific substrates are also used for detection of apoptosis.[24,25] Fluorescent detection of caspase activity as well can result in underestimating levels of apoptosis as effector caspases are only briefly activated in (pre)apoptotic cells.

In this study we described an easy, highly reproducible method for detection of apoptosis in isolated lymphoma and leukemia cells, lymphocytes and cell lines and compared it with conventional used standard flow cytometry methods: Annexin V/7AAD staining, Annexin V/PI staining and fluorescent caspase activity analysis. Using this method we were able to test the functional status of the two major apoptosis pathways. The method requires few cells and is based on quantitative detection of apoptotic cells using 7AAD in combination with fluorescent beads. Apoptosis is distinguished from necrosis by addition of a pan-caspase inhibitor in simultaneous performed experiments. The technique can be combined with other cell surface markers and can be very useful for functional assessment of the apoptosis pathways in primary lymphoma and leukemia cells, lymphocytes and cell lines.

Materials and methods

Cell lines

The following cell lines were used: Nalm6, SMS-SB (both B-ALL), Ramos (Burkitt lymphoma), 8226 (multiple myeloma cell line) and the diffuse large B-cell lymphoma cell lines SUDHL4 and HT. 8226, Nalm6, SUDHL4, and HT were cultured in RPMI 1640 medium (BioWhittaker, Cambrex, Belgium) containing 10% fetal calf serum (Hyclone, Perbio, Sweden) and 100 IU penicillin/100 μg/ml streptomycin (Gibco, Invitrogen, Grand Island, NY, USA) in a humidified 5% CO_2 atmosphere. Ramos and SMS-SB were cultured in IMDM medium (BioWhittaker) supplemented with 10% fetal calf serum and penicillin (100 IU)/streptomycin (100 μg/ml).

Isolation and selection of lymphoma cells

Lymphoma samples were obtained from our bank of isolated tumor cells. All samples tested were diagnosed between 1997 and 2005 in the Comprehensive Cancer Center of Amsterdam, according to the World Health Organization (WHO) criteria.[26] Lymphoma cells were isolated and selected from tissue biopsies as described previously.[27] In all cases tested this procedure resulted in <5% contaminating non-neoplastic cells, as tested by immuno-cytochemistry (CD20 and CD3 staining) on cytospin preparations.

Induction of apoptosis

Apoptosis was induced in 10^6 cells/ml with 500 nM etoposide (VP16, Sigma, St.Louis, MO, USA) to induce stress-induced apoptosis or 100 ng/ml hsTRAIL/Apo2L (Genentech, CA, USA) to induce death receptor induced apoptosis. Dose response curves showed that for etoposide- and hsTRAIL/Apo2L-induced cell death, 500 nM etoposide and 100 ng/ml hsTRAIL/Apo2L were the optimal cell death inducing concentrations in B-cell lines, respectively (data not shown). Cell lines and lymphoma cells were incubated for increasing periods of time at 37°C in a humidified 5% CO_2 atmosphere.

Flow cytometry

Levels of apoptosis were detected using four different FACS based methods. Fluorescence was detected by the FACS Calibur flowcytometer and analyzed using CELL-Quest software (both Becton Dickinson, San Jose, CA, USA). All tests were performed in triplicate.

1. *Annexin V-7AAD method:* Phosphatidyl serine (PS) externalization was detected with Annexin V-FITC (250 μg/ml, VPS Diagnostics, Netherlands) and cell viability with 7AAD (Via-probeTM, 2 μg/ml, Pharmingen, USA), according to the manufacturer's recommendation. After washing of the cells in phosphate buffered saline, the cell pellet was resuspended in binding buffer (10^6 cells/ml) Annexin V-FITC and 7AAD were added to the cell suspension and cells were incubated for 15 minutes.
2. *Annexin V-Propidium Iodide (PI) method:* PS externalization was determined in combination with PI (250 μg/ml, Sigma, St Louis, MO, USA), according to instructions of the manufacturer. The procedure was performed as mentioned above in #1.
3. *Caspase 3-7AAD method:* Caspase 3 activity was detected using a fluorescent FAM-DEVD-FMK substrate (CaspaTag Caspase 3-DEVD, Intergen company, Oxford, UK) in combination with 7AAD. Cells (10^6 cells/ml, $3x10^5$ cells) were incubated with 30x FAM-DEVD-FMK and incubated for one hour at 37°C under 5% CO_2 conditions. After washing of the cells in phosphate buffered saline, the cells were incubated with 7AAD for 15 minutes and fluorescence was measured.
4. *Novel 7AAD-beads method:* Cells ($5x10^4$ cells) were incubated with a standard number of fluorescent beads (Fluorospheres, Becton Dickinson, USA) and 7AAD (2 μg/ml, for 15 minutes) that was added for detection of viable (7AAD negative) cells. No washing steps were performed. Subsequently, the number of viable cells was determined by counting the number of 7AAD negative cells per 2500 beads for each individual experiment using flowcytometry. To distinguish apoptosis (i.e. caspase dependent cell death) from necrosis, cells were pre-incubated in simultaneous experiments with 25 μM of the caspase blocker Z-Val-Ala-DL-Asp-fluoromethylketone (z-VAD-fmk, Alexis biochemicals, Switzerland) one hour prior to exposure to etoposide/hsTRAIL/Apo2L. Apoptosis was determined as follows: (A/B-C/D) * 100%.
   A = the number of viable cells after etoposide/TRAIL/Apo2L treatment with z-VAD
   B = the number of viable cells in non treated samples with z-VAD
   C = the number of viable cells after etoposide/TRAIL/Apo2L treatment without z-VAD
   D = the number of viable cells in non treated samples without z-VAD

Detection of caspase 3/7 activity and caspase 9 activity

Caspase 9 and caspase 3/7 activities were determined using a luminescent assay (Caspase-Glo™ 9 assay, Promega Benelux, Leiden, Netherlands) and a fluorescent assay (Roche, Mannheim, Germany), respectively, according to the manufacturer's instructions. In brief, cells were lysed and incubated with the LEHD-containing substrate or DEVD-rhodamine 110 substrate for 1 h at 37 °C. Subsequently, luminescence was measured using a microplate luminometer (Victor2TM, Wallac, Perkin Elmer, Boston, MA, USA). The amount of free rhodamine was measured with a 492 nm excitation filter and a 535 nm emission filter using a microplate fluorescence reader (TECAN spectrafluor, Männedorf, Switzerland). Caspase activity was determined as caspase activity levels of treated samples minus caspase activity levels of untreated samples. Experiments were performed in triplicate.

May-Grünwald Giemsa staining on cytospin preparations

Cytospin preparations of cell lines were prepared and fixated for 5 minutes with methanol. Subsequently cells were stained with May-Grünwald solution for 5 minutes. After washing, cells were stained with Giemsa solution for 20 minutes and staining was analyzed microscopically.

Western blot analysis

Protein extracts of B-cell lines were prepared for western blot analysis. Cells were washed with PBS and resuspended in lysisbuffer (50 mM Tris/HCl, pH 8.0, 0.5% NP-40, 5 mM EDTA) containing protease inhibitors. An antibody against the cleaved form of Parp-1, P89 (Promega Benelux, Leiden, Netherlands) was used. The protein visualized with the enhanced chemo luminescence technique (Amersham Pharmacia Biotech, USA). The expression levels were correlated to cellular β-actin (AB-1 kit, 6.25 ng/ml, Oncogene research products, Germany) levels, measured as a reference in each sample on the blots.

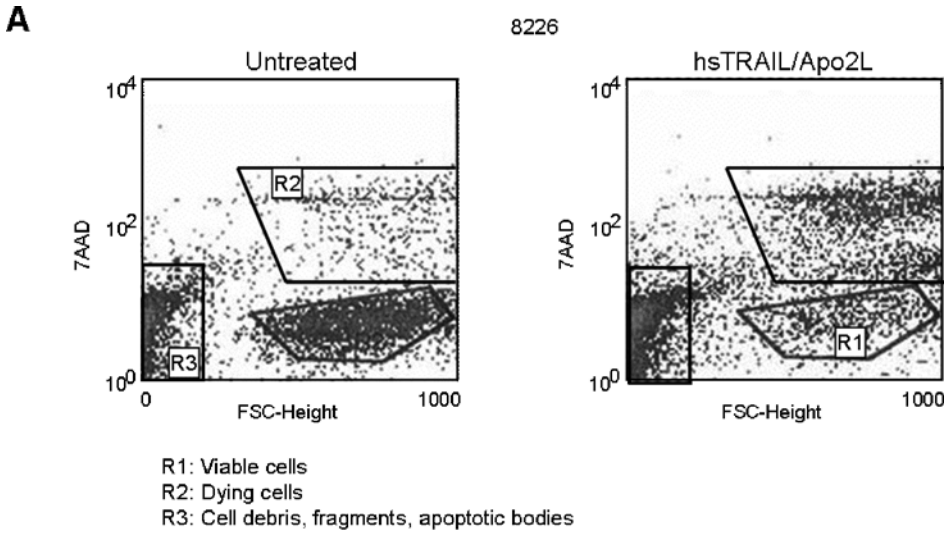
Results

Sensitive detection of apoptosis using the 7AAD-beads method in B-cell lines

We first investigated whether the 7AAD-beads method was actually a sensitive method to detect apoptosis in B-cell lines. Therefore, six B-cell lines were exposed to the apoptosis inducers hsTRAIL/Apo2L or etoposide and stained with 7AAD in combination with fluorescent beads. Figure 1A shows a representative apoptosis analysis of 8226 cells treated for 24 hours in the presence of hsTRAIL/Apo2L using the 7AAD-beads method. Combined analysis of cell size (FSC) and membrane integrity (7AAD) fluorescence revealed three populations: 1) viable cells with a normal morphology and normal membrane integrity (7AAD negative, gate R1), 2) apoptotic cells showing a complete loss of membrane integrity (7AAD high, gate R2) and 3) cell debris including cell fragments and apoptotic bodies, characterized by a extreme small size and a weak 7AAD staining. After treatment with hsTRAIL/Apo2L the percentage of apoptotic cells increased and the number of viable 8226 cells (gate 1) decreased (Figure 1A, right). In the other B-cell lines, also levels of apoptosis could be detected using the 7AAD-beads method (Figure 1B).

Validation of the 7AAD-beads method to detect apoptosis

The validity of the 7AAD-beads method was subsequently evaluated on Nalm6 and 8226 cells to examine if the cell death we observed is apoptosis. As the 7AAD-beads method involves detection of cell death in the presence of zVAD-FMK, we hypothesized that the observed cell death would be apoptotic cell death. Recent studies, using a fluorescent assay have shown that etoposide and hsTRAIL/Apo2L induced caspase 3/7 activity preceded cell death between 4 and 8 hours in B-cell lines. Therefore, we analyzed caspase 3/7 activity and the percentage apoptosis after increasing hours of incubation with etoposide or hsTRAIL/Apo2L. In addition, we detected caspase 8 or caspase 9 activity and the cleaved product of PARP-1, p89 in untreated and treated cells using a luminescence assay and western blot analysis, respectively. As expected, cell lines sensitive to etoposide or hsTRAIL/Apo2L using the 7AAD-beads method showed a clear increase in caspase 3 activity. Furthermore, Nalm6 demonstrated an increase in caspase 9 activity and 8226 in caspase 8 activity after induction with etoposide or hsTRAIL/Apo2L, respectively (Figure 2A-2B).



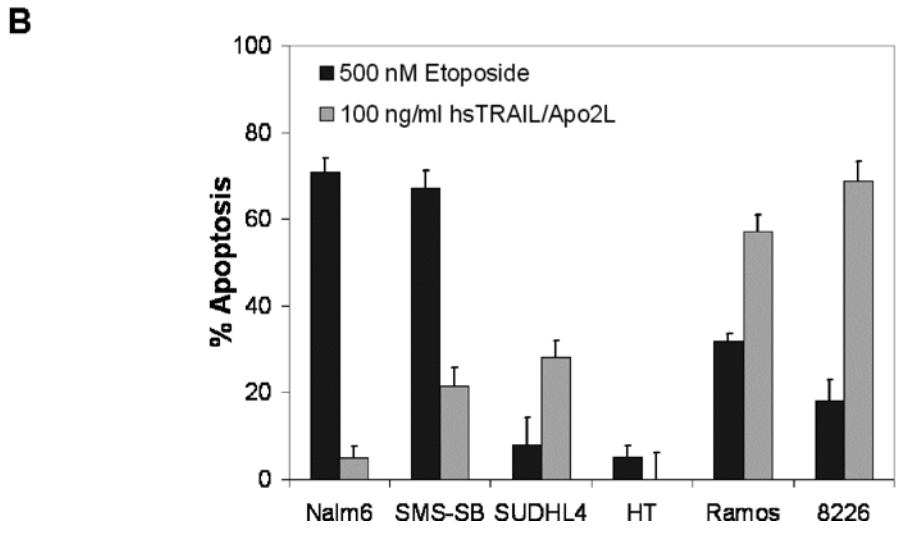
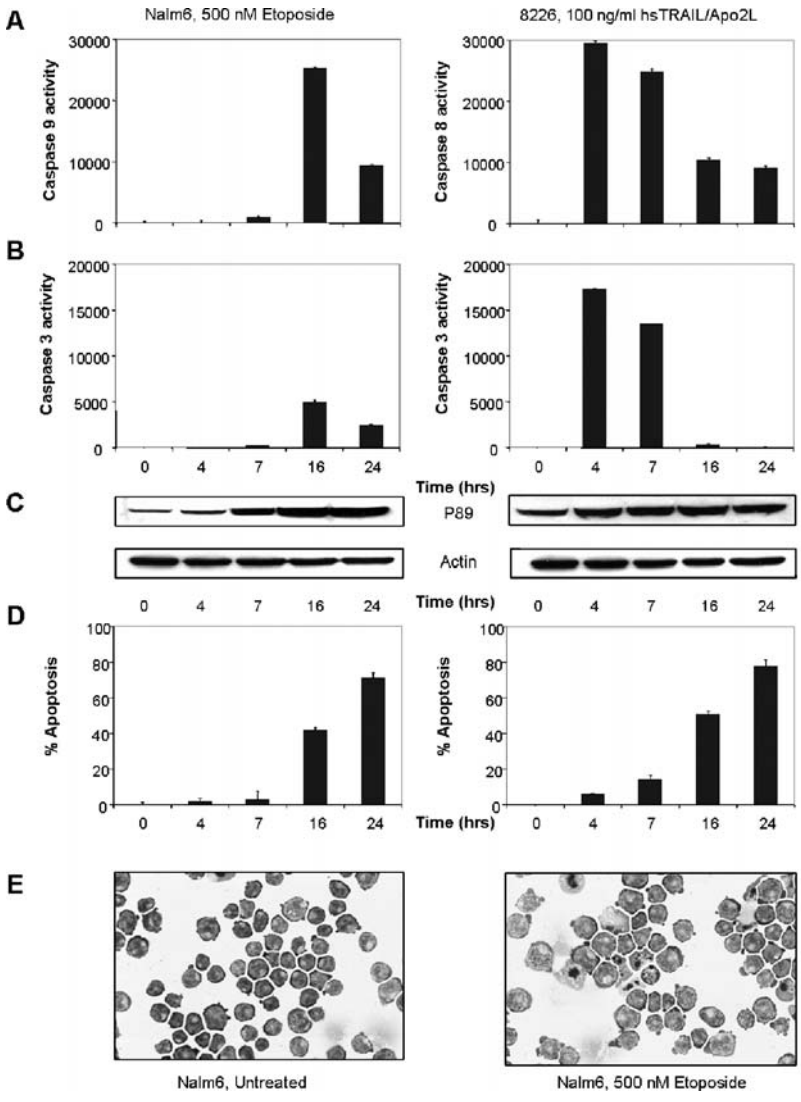


Figure 1. Detection of apoptosis induction in 8226 cells using the 7AAD-beads method.
(A) 8226 cells were treated with 100 ng/ml hsTRAIL/Apo2L for 24 hours. Subsequently, cells were incubated with fluorescent beads in combination with 7AAD for 15 minutes followed by detection of the percentage hsTRAIL/Apo2L induced apoptosis using flowcytometry. Combined analysis of cell size (FSC) and 7AAD fluorescence demonstrated three populations: gate R1: viable cells with a normal morphology and normal membrane integrity (7AAD negative), gate R2: apoptotic cells showing a complete loss of membrane integrity (7AAD high) and gate R3: cell debris fragments and apoptotic bodies (extreme small size and a weak 7AAD staining). After treatment with hsTRAIL/Apo2L a clear increase in apoptotic cells and decrease in viable 8226 cells is observed.
(B) Apoptosis detection in B-cell lines after 24 hours of treatment with 500 nM etoposide or 100 ng/ml hsTRAIL/Apo2L using the 7AAD-beads method.

Figure 2. Validation of the 7AAD-beads method for detection of apoptosis
(A) Caspase 8 and 9 activity in Nalm6 and 8226 cells treated with 100 ng/ml hsTRAIL/Apo2L or 500 nM etoposide.
(B) Caspase 3/7 activity in Nalm6 and 8226 cells treated with 100 ng/ml hsTRAIL/Apo2L or 500 nM etoposide.
(C) P89 expression was determined by western blot analysis in protein extracts of Nalm6 and 8226 cells from a time course treatment of the cells with 500 nM etoposide or 100 ng/ml hsTRAIL/Apo2L.
(D) The percentage apoptosis using the 7AAD-beads method in Nalm6 and 8226 cells treated with 100 ng/ml hsTRAIL/Apo2L or 500 nM etoposide.
(E) May-Grünwald Giemsa staining of cytospin preparations of Nalm6 cells treated with 500 nM etoposide for 16 hours. Levels of apoptosis were comparable with the obtained results using the 7AAD-beads method.



These data were also confirmed on western blot analysis. After treatment of Nalm6 cells with etoposide, the occurrence of p89 was observed (Figure 2C). Similar results were observed in 8226 cells treated with hsTRAIL/Apo2L, showing an increase in p89 expression.
Next, we investigated if the percentage apoptotic cells observed using the 7AAD-beads method actually corresponded with the presence of apoptotic cells on cytospin preparations. Nalm6 and 8226 cells were treated with etoposide and hsTRAIL/Apo2L for 24 hours and morphologic features characteristic of apoptosis were investigated with a standard May Grünwald Giemsa staining on cytospin preparations. In the non-induced samples, only sporadic apoptotic cells were detected, in contrast with the treated samples that demonstrated numbers of apoptotic cells comparable with the percentages of apoptosis observed using the 7AAD-beads method (Figure 2E).

Comparative analysis of the 7AAD-beads method with other flowcytometric methods: Annexin-7AAD staining, Annexin-PI staining and fluorescent caspase substrates

We compared the sensitivity of the 7AAD-beads method with two conventional fluorescent methods (Annexin V-7AAD and Annexin V-PI staining) in B-cell lines treated with etoposide or hsTRAIL/Apo2L. Figure 3 shows a representative experiment in 8226 cells using the three cytofluorimetric methods of apoptosis quantification in one of the B-cell lines 8226. Dual staining with Annexin V/7AAD or Annexin V/PI gave similar rates of apoptosis. Using the 7AAD-beads method we found higher levels of apoptosis in the treated cells compared to the results observed using the other two flowcytometric methods. Besides an increase in the 7AAD positive cell population also an increase in cell fragments and apoptotic bodies was found (Figure 3A). These data suggest that the 7AAD-bead method is a more sensitive quantitative apoptosis detection method than the two conventional flowcytometric methods.
One of the major features of apoptotic cell death is the activation of specific caspases, including the effector caspases (3, 6 and 7) that lead to cell disassembly. In cells that are undergoing apoptosis, active caspase 3 can be detected by flowcytometry using a fluorescent substrate, FAM-DEVD-FMK. The sensitivity of the 7AAD-beads method for detecting apoptosis was also investigated in 8226 cells treated with etoposide and stained using either the 7AAD-beads method or the fluorescent FAM-DEVD-FMK substrate. Comparison of apoptosis by 7AAD-beads staining and active caspase 3 detection was shown in Figure 3A and 3D. After 24 hours, a clear increase in apoptotic cells was observed in the treated cells using the 7AAD-beads method. However, similar to the fluorescent caspase 3 assay, no increase in active caspase 3 expression was found after treatment with etoposide using the FAM-DEVD-FMK substrate. These data suggest that detection of caspase activities as a measurement for apoptosis is only reliable after short incubation periods because caspase 3 activation proceeds early, after 4-8 hours of incubation.
Comparative analysis of all flowcytometric methods in the six B-cell lines tested was shown in table I. Similar as for 8226 cells the other sensitive B-cell lines also showed higher levels of apoptosis using the 7AAD-beads method.

Table I: Comparison of the 7AAD-beads method, Annexin V-7AAD staining and Annexin V-PI staining method in all B-cell lines tested.

B-cell line	**7AAD-beads method**		**Annexin V-7AAD**		**Annexin V-PI**	
Treatment	Etoposide	hsTRAIL/ Apo2L	Etoposide	hsTRAIL/ Apo2L	Etoposide	hsTRAIL/ Apo2L
Nalm6	70	5	64	1	61	0
SMS-SB	67	22	59	18	58	17
SUDHL4	8	28	6	22	5	24
HT	5	0	1	0	2	0
Ramos	32	57	31	48	29	51
8226	18	69	8	59	6	56

Cells were incubated with 500 nM Etoposide or 100 ng/ml hsTRAIL/Apo2L. After 24 hours of incubation, the % apoptosis was determined using the different flow cytometry methods.

Sensitive detection of apoptosis in isolated lymphoma cells of B-cell lymphoma samples

Next, we investigated whether the 7AAD-beads method is appropriate for detection of apoptosis in isolated lymphoma cells of B-cell lymphomas. Detection of apoptosis in primary lymphoma cells appeared to be quite difficult, due to high levels of spontaneous apoptosis.
Similar to the results obtained in the cell lines, the 7AAD-beads method appeared to be more sensitive than the conventional flowcytometric analysis Annexin V-7AAD in isolated lymphoma cells of DLBCL (Figure 4A). Dual staining with Annexin V-7AAD gave similar rates of apoptosis in nontreated and treated lymphoma cells. Using the 7AAD-beads method we found an increase in the 7AAD positive cell population but also in the cell debris, containing the apoptotic bodies and cell fragments.

We also investigated if it was possible to detect caspase independent cell death in isolated lymphoma cells. Therefore, we performed the 7AAD-beads method in the absence and presence of zVAD-FMK. Figure 4B represents two of the lymphoma samples tested, showing higher levels of caspase-independent cell death than caspase-dependent apoptosis. Thus using the 7AAD-beads method it was possible to detect both caspase-dependent as caspase-independent cell death in primary lymphoma cells (Figure 4B).

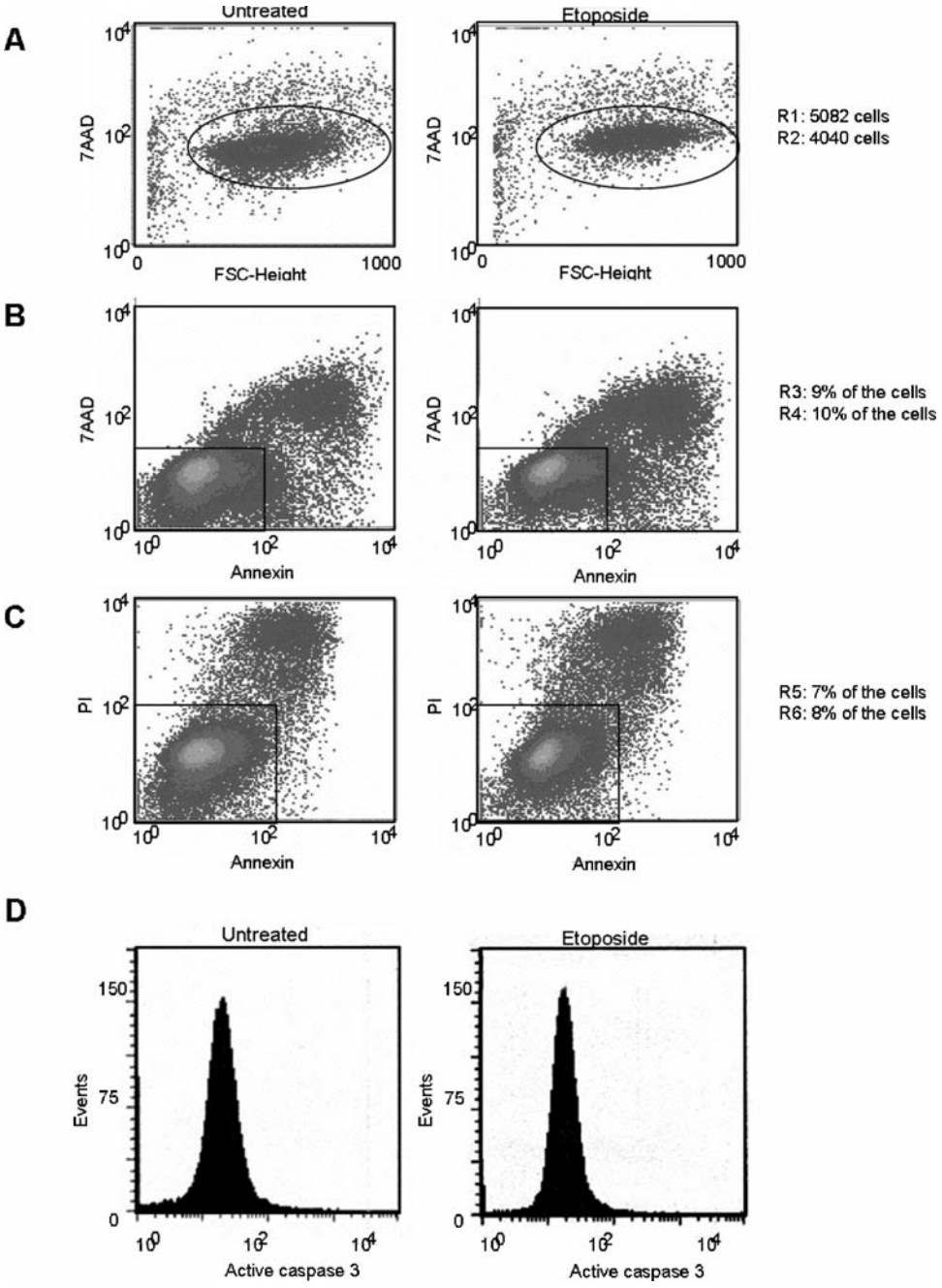
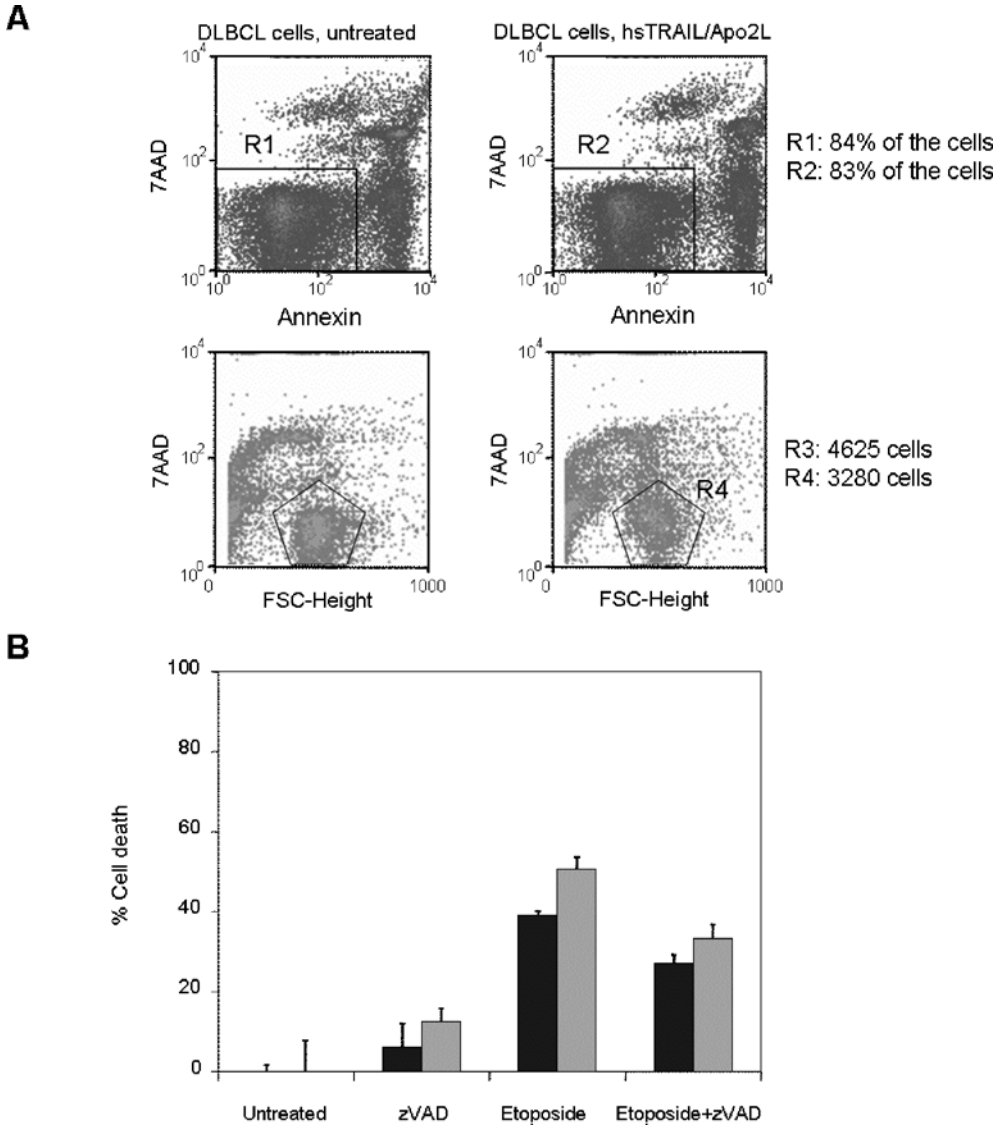


Figure 3. Comparative analysis of the 7AAD-beads method with Annexin V-7AAD staining, Annexin V-PI staining and fluorescent caspase 3 substrate.
8226 cells were incubated with 500 nm etoposide for 24 hours. Subsequently, cells were incubated with fluorescent beads in combination with 7AAD (A), Annexin V-7AAD (B), Annexin V-PI (C), or fluorescent caspase 3 substrate (D). Apoptosis was detected using flow cytometry.

Figure 4. Sensitive detection of apoptosis in isolated lymphoma cells using the 7AAD-beads method. (A) Isolated lymphoma cells of DLBCL were treated with 100 ng/ml hsTRAIL/Apo2L for 4 hours. Subsequently, cells were incubated with fluorescent beads in combination with 7AAD (bottom) or with Annexin V-7AAD (top) for 15 minutes followed by detection of the percentage apoptosis using flowcytometry. No decrease in Annexin V negative, 7AAD negative cells was observed, however the number of 7AAD-negative cells per constant number of beads showed a decrease of cells. (B) Detection of caspase dependent and caspase independent cell death in isolated lymphoma cells using the 7AAD-beads method. Isolated lymphoma cells were treated with 500 nM etoposide in the absence and presence of 25 μM zVAD-FMK and cell death was measured using the 7AAD-beads method after 4 hours of incubation.



Discussion

In this study we have demonstrated that it is possible to detect reproducible levels of apoptosis in isolated lymphoma cells and related types of cell lines using the 7AAD-beads method. Furthermore, we have shown that this technique requires few cells and can be used for detection of caspase-dependent as well as caspase-independent cell death in primary lymphoma cells.

The 7AAD-beads method was more sensitive than the standard conventional flowcytometric techniques as Annexin V-7AAD and Annexin-PI staining in primary cells as the B-cell lines tested. An explanation for the increased sensitivity of the 7AAD-beads method compared to the conventional flowcytometric methods might be that these techniques do not accurately measure the dissolved dead cells and fragments because they are too small to be recognized and therefore are not counted. In addition, both Annexin V-7AAD and Annexin-V-PI staining protocols include a wash and centrifugation step with PBS/0.03%BSA, thereby losing cell fragments and dissolved dead cells. Taken together, using the conventional flowcytometric methods for detection of apoptosis would result in inadequate low numbers of dead cells. Determination of the number of apoptotic cells in the presence of a standard number of fluorescent beads prohibits these unwanted effects.

Detection of caspase 3/7 activity is an alternative for detection of apoptosis but has the disadvantage that apoptosis can not be expressed as percentage cell death. Moreover, we have observed that caspase 3/7 activity precedes cell death with 4-8 hours in the different tested B-cell lines. Caspase 3/7 activity measurement is only sensitive as marker for apoptosis after short incubation treatments

We also demonstrated that it was possible to perform reproducible and accurate detection of apoptosis in freshly isolated lymphoma cells. Using the 7AAD-beads method, we could functionally analyze the apoptosis pathways in cases with both high and low levels of spontaneous apoptosis.[27]

Cell lines might not reflect their original counterpart concerning apoptosis sensitivity due to rigorous *in vitro* selection. Therefore, the 7AAD-beads method is an important technique for future testing of new apoptosis inducing agents and for functional analysis of the apoptosis pathways in isolated hematological tumor cells.

We conclude that the 7AAD-beads method is a highly sensitive and reproducible method for functional analysis of apoptosis in isolated lymphoma cells and related hematopoietic cell lines. This approach can provide new insights into the pathological defects in apoptosis regulation in malignant hematopoietic cells.

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