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Comparison of methods based on annexin-V binding, DNA content or TUNEL for evaluating cell death in HL-60 and adherent MCF-7 cells

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Abstract. HL-60 and MCF-7 cells were treated with 0 15 μM camptothecin (CPT) or with the solvent dimethylsulfoxide (DMSO) for the controls, for 2, 3 and 4 h or for 24, 48 and 72 h, respectively The apoptotic index (AI) was then evaluated in parallel by the following flow cytometric methods (1) double staining of unfixed cells with fluoresceinated annexin V and propidium iodide (PI), this after detachment by trypsinization in the case of MCF-7 cultures, (2) prefixation in 70% ethanol, extraction of degraded, low molecular weight DNA with 0 2 M phosphatecitrate buffer and analysis of the DNA content stained with PI, (3) TUNEL, i e labelling of DNA strand breaks with biotin-dUTP, followed by staining with streptavidin–fluorescein and counterstaining with PI In HL-60 cells, the three methods gave similar results for the AI (3–4% in the controls and at 2 h of CPT treatment, and 35–43% at 3 and 4 h after CPT) This indicates that CPT-induced membrane alteration and DNA fragmentation occurred concomitantly in those cells For MCF-7 cells, CPT-induced apoptosis developed more slowly, the AI, whether based on annexin V or on DNA content, remained unchanged at 24 h, then was increasing to 8% at 48 h and to 25% at 72 h of treatment In these cells, the TUNEL index did not increase prior to 72 h, and the increase was minor (up to 9% vs 2–3% in the controls) at 72 h of the treatment This indicates that in MCF-7 cells DNA strand breaks cannot be effectively labelled, which may be due to inaccessibility of 3′-OH ends in the breaks to exogenous terminal deoxynucleotidyl transferase The mechanism of endonucleolytic DNA fragmentation thus may be different, depending on the cell type

INTRODUCTION

Previous studies showed that inhibition of topoisomerase I by camptothecin (CPT) arrests progression of the DNA replication forks and induces cell death (Hsiang, Lihou & Liu 1989,

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D'Arpa, Beardmore & Liu 1990). The mechanism of induction of cell death was attributed to the collision of the replication fork with the drug-stabilized topoisomerase I-DNA complexes (Hsiang *et al.* 1989; D'Arpa *et al.* 1990). Cells of leukaemic lineage such as HL-60 or KG-1 die by apoptosis shortly after administration of CPT. Apoptosis of these cells is characterized by typical changes in their morphology that involve cytoplasm and chromatin condensation followed by nuclear fragmentation and formation of apoptotic bodies. Endonucleolytic DNA fragmentation, preferential to internucleosomal sections, parallels the morphological changes (Del Bino, Skierski & Darzynkiewicz 1990, Del Bino & Darzynkiewicz 1991). However, in certain cell types, primarily of epithelial origin, inhibition of S-phase progression by CPT leads to growth imbalance, secondary changes and atypical apoptosis (Kung *et al.* 1990). These cells lack the classical apoptotic features but manifest other characteristics that are secondary to prolonged perturbation of cell cycle progression. While in most cell types topoisomerases inhibitors act directly on S-phase cells to induce apoptosis, in some, such as human colon carcinoma, Chinese hamster ovary cells or lung cells, they induce prolonged G_2 arrest which subsequently leads to apoptosis (Solary *et al.* 1993). The mechanism of induction of apoptosis by CPT, and the apoptotic process itself, thus appears to be different, depending on the cell type (Kung *et al.* 1990, Darzynkiewicz *et al.* 1996).

The present study was aimed at comparing the mode of death of the promyelocytic leukaemic HL-60 cells and MCF-7 breast carcinoma cells. These cell lines differ in expression of the tumour suppressor gene *p53*, lacking in HL-60 (Collins 1987) and present (wt) in MCF-7 cells (Watson *et al.* 1997) and appear to exhibit different sensitivity towards induction of apoptosis. Three distinct features of apoptotic mode of cell death were measured (1) the exposure of phosphatidylserine at the outer leaflet of the plasma membrane, as detected by FITC-conjugated annexin V; (2) the presence of sub-G_1 diploid peak on cellular DNA content frequency histograms; and (3) the presence of DNA strand breaks *in situ*, in fixed cells by labelling them with fluorochrome conjugated nucleotides using exogenous terminal deoxynucleotidyl transferase (TdT).

The data indicate that both the kinetics of induction of apoptosis and mechanism of endonucleolytic DNA cleavage are different in MCF-7 and in HL-60 cells, and that the diverse aspects of the execution of the apoptotic 'programme' can be dissociated as separately induced events.

MATERIALS AND METHODS

Cell culture

The human promyelocytic leukaemia cell line HL-60 was obtained from Dr Z. Darzynkiewicz (Cancer Research Institute, NY Medical College, Elmsford, NY). The cells were grown in suspension and propagated in RPMI-1640 medium supplemented with 10% heat-inactivated bovine serum, 100 μg/ml streptomycin and 2 mM L-glutamine, all obtained from Gibco (Paisley, UK). To maintain exponential growth, the cultures were divided every third day by dilution to a concentration of 2×10^5 cells/ml, as described (Del Bino *et al.* 1990). The human breast adenocarcinoma cell line MCF-7 was obtained from Dr G. Leclercq (Bordet Institute, Bruxelles, Belgium). Cells were maintained at 37°C in a 5% CO_2 humidified atmosphere, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, glutamine (3.2 nM), penicillin, streptomycin (Gibco, Paisley, UK). The cells from cultures growing attached to the surface of the flasks were collected by trypsinization and reseeded at low density to maintain continuous,

exponential growth The cultures were periodically tested for mycoplasma infection by PCR ELISA (Boehringer Mannheim Biochemicals, Bruxelles, Belgium) test

Cell treatment

Exponentially growing HL-60 and MCF-7 cells were exposed, respectively, to short (2–3 4 h) and long (24–48–72 h) treatment with camptothecin (0 15 μM CPT), prior assays having shown that the latter cell line responded much more slowly Stock solution was prepared by dissolving camptothecin (Sigma-Aldrich, Bornem, Belgium) in dimethylsulfoxide (DMSO) at a concentration of 1 mg/ml and was kept frozen at −20°C Dilution of the drug to final concentration was accomplished with culture medium Control cultures were treated with the equivalent concentrations of DMSO which had not detectable effect on any of the measured parameters

Measurement of annexin V binding

It has been shown that loss of phospholipid asymmetry of the plasma membrane is an early event of apoptosis (Fadok *et al* 1992, Koopman *et al* 1994) The annexin V binds to negatively charged phospholipids, like phosphatidylserine During apoptosis the cells react to annexin V as soon as chromatin condenses but before the plasma membrane loses its ability to exclude PI Hence by staining cells with a combination of fluoresceinated annexin V and PI it is possible to detect nonapoptotic live cells, early apoptotic cells and late apoptotic or necrotic cells (Koopman *et al* 1994, Vermes *et al* 1995)

Cells from exponentially growing and CPT-treated cultures were collected at the time indicated and for MCF-7 cells by adding the floating cells to the trypsinized ones and analysing them together

Aliquots of $0\,5 \times 10^6$ cells were centrifuged (1000 rpm) for 5 min and washed with PBS The cell pellet was resuspended in 100 μl of labelling solution (ANNEXIN-V-FLUOS, Boehringer Mannheim) containing 2 μl annexin V labelling reagent and 0 1 μg propidium iodide (Calbiochem, La Jolla, CA) and incubated for 10–15 min, as per manufacturer's instructions Immediately after adding 0 4 ml of incubation buffer (10 mM HEPES/NaOH, 140 mm NaCl, 5 mM $CaCl_2$) analysis of green (annexin V) and red (PI uptake) fluorescence of individual cells was measured with a FACScan flow cytometer (Becton Dickinson, Erembodegem, Belgium) using the standard optics and Lysys II software Electronic compensation was required to exclude overlapping of the two emission spectra At least 10^4 cells were collected and stored for each sample

Smears were obtained by cytocentrifugation, using a Shandon Cytofuge (Life Sciences International, Zellik, Belgium) and the 5×10^4 cells were directly fixed in 2% paraformaldehyde in PBS (pH 7 2) and stained with haematoxylin and eosin (0 25% in 80% alcohol) After dehydration in alcohol the smears were mounted with DPX Gurr Merck, Belgolabo, Overigse, Belgium The preparations were examined under a Zeiss photomicroscope using a 40 × oil immersion objective (photos at final magnification ×650)

DNA content

Following fixation in 70% ethanol, the cells were rinsed in HBSS, centrifuged and resuspended in 40 μl of phosphate–citric acid buffer containing 192 parts of 0 2 M Na_2HPO_4 and 8 parts of 0 1 M citric acid (pH 7 8) at room temperature for 30 min to facilitate removal of low molecular weight DNA from apoptotic cells The cells were then stained with 10 μg/ml propidium iodide (Calbiochem, La Jolla, CA) plus 0 1% RNAse A (Sigma) and incubated for 30 min in the dark at room temperature The red (PI) DNA fluorescence of individual cells

was measured with a FACScan flow cytometer (Becton Dickinson, Erembodegem, Belgium) utilizing the standard filter combination. Data were acquired and processed using Lysys II and analysed with Cell Fit softwares (Becton Dickinson). Cells in M- and G_2-phases were discriminated from cell doublets, based on the difference in electronic pulse shape. All experiments were repeated at least three times with essentially identical results.

TdT assay for DNA strand breaks

Following drug treatment cells were fixed in 1% formaldehyde in PBS for 15 min on ice. After washing once in PBS, the cells were resuspended in cold 70% ethanol and stored at −20°C. Cell aliquots were then rehydrated in PBS and resuspended in 50 μl of a solution containing: 0.1 M sodium cacodylate (pH 7.0; Sigma), 0.1 mM 1,4-dithiothreitol (Sigma), 0.05 mg/ml BSA, 5 units of TdT and 0.5 nmol biotin-16-dUTP (both from Boehringer Mannheim Biochemicals, Bruxelles, Belgium). The cells were incubated in 100 μl of the staining buffer which contained 3 μg/ml of streptavidin-fluorescein (Boehringer Mannheim), 4 concentrated saline-sodium citrate buffer (Sigma), 0.1% Triton X-100 and 5% (w/v) nonfat dry milk (Gloria, Nestlé, Bruxelles, Belgium). Cells were incubated in the staining buffer at room temperature for 30 min in the dark, rinsed with PBS containing 0.1% Triton X-100, and resuspended in 1 ml of PBS containing 5 μg/ml PI and 0.1% RNase A, as described (Gorczyca *et al.* 1992; Li *et al.* 1996). The percentage of apoptotic cells were determined on a FACScan flow cytometer (Becton Dickinson), where the green fluorescence (b-dUTP) was proportional to the number of DNA strand breaks and the red PI fluorescence to DNA content. The data from 10^4 cells were collected and stored using Lysis II software.

Table 1. Determination of cell cycle distribution (%) and AI* apoptotic cells in HL-60 cells treated with 0.1 g/ml camptothecin (CPT)

Time (h)	Treatment	AI* (% *total* population)	Distribution of *nonapoptotic* cells in the cycle phases (%)		
			G_1	S	G_2 + M2
2	CONTROL	5	35	46	19
	CPT	3	37	47	16
3	CONTROL	2	28	54	18
	CPT	37	61	13	26
4	CONTROL	7	36	44	20
	CPT	49	76	9	15

*The apoptotic cells (AI) are recognized following staining of cellular DNA as cells with DNA content lower than that of G_1 cells (sub-G_1 peak).

Table 2. Comparison of the incidence of apoptosis as concurrently detected by three methods in HL-60 cells treated with 0.1 μg/ml of camptothecin (CPT) for up to 4 h

Time (h)	Treatment	% apoptosis		
		Annexin	sub-G_1 DNA	TUNEL
2	CONTROL	2	5	*n*.d.
	CPT	3	3	4
3	CONTROL	2	2	*n*.d.
	CPT	29	37	39
4	CONTROL	4	7	4
	CPT	38	49	42

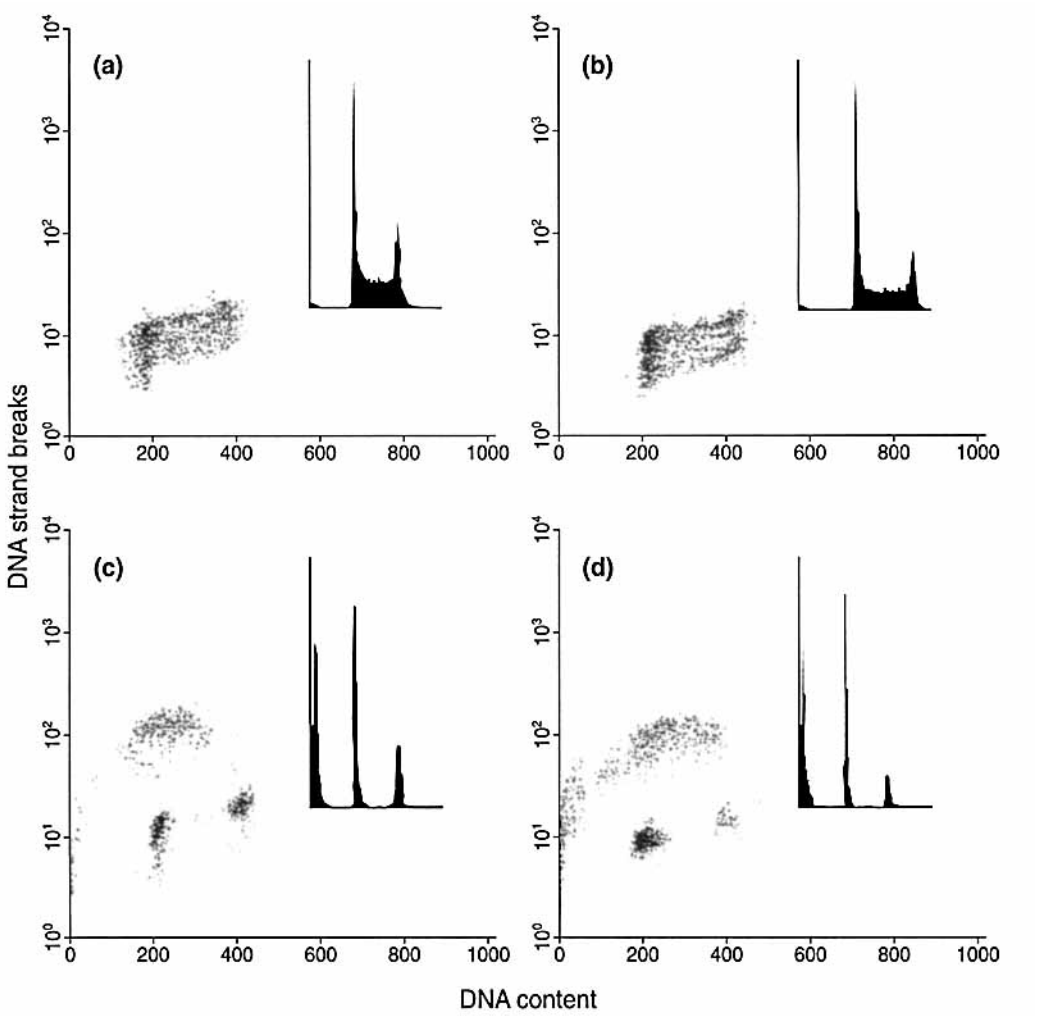


Figure 1. Effect of camptothecin on the appearance of DNA strand breaks in HL-60 cells treated for 0, 2, 3 and 4 h. The bivariate dot plots represent the distribution of cells with respect to their DNA content, abscissa (linear scale) and biotinylated dUTP incorporation, ordinate (log scale). The insets represent the DNA content frequency distributions after extraction with phosphatecitric acid buffer: this shows the presence of cells with sub-G_1 diploid peak in camptothecin-treated cells at 3 h and 4 h and the concomitant detection of DNA breaks in cells with an S-phase DNA content.

RESULTS

Exposure of exponentially growing HL-60 cells to CPT induced apoptosis of S-phase cells. Apoptosis was observed early during the treatment (3–4 h) and manifested by the loss of cells with an S-phase DNA content and appearance of cells with fractional DNA content, identified as cells with DNA values below that of G_1 cells, on the DNA content frequency histograms (Figure 1, insets). Thus, for example, after 4 h of treatment with CPT the proportion of S-phase cells was decreased by 80% (Table 1) while there were 49% apoptotic cells, identified as the cells with fractional DNA content.

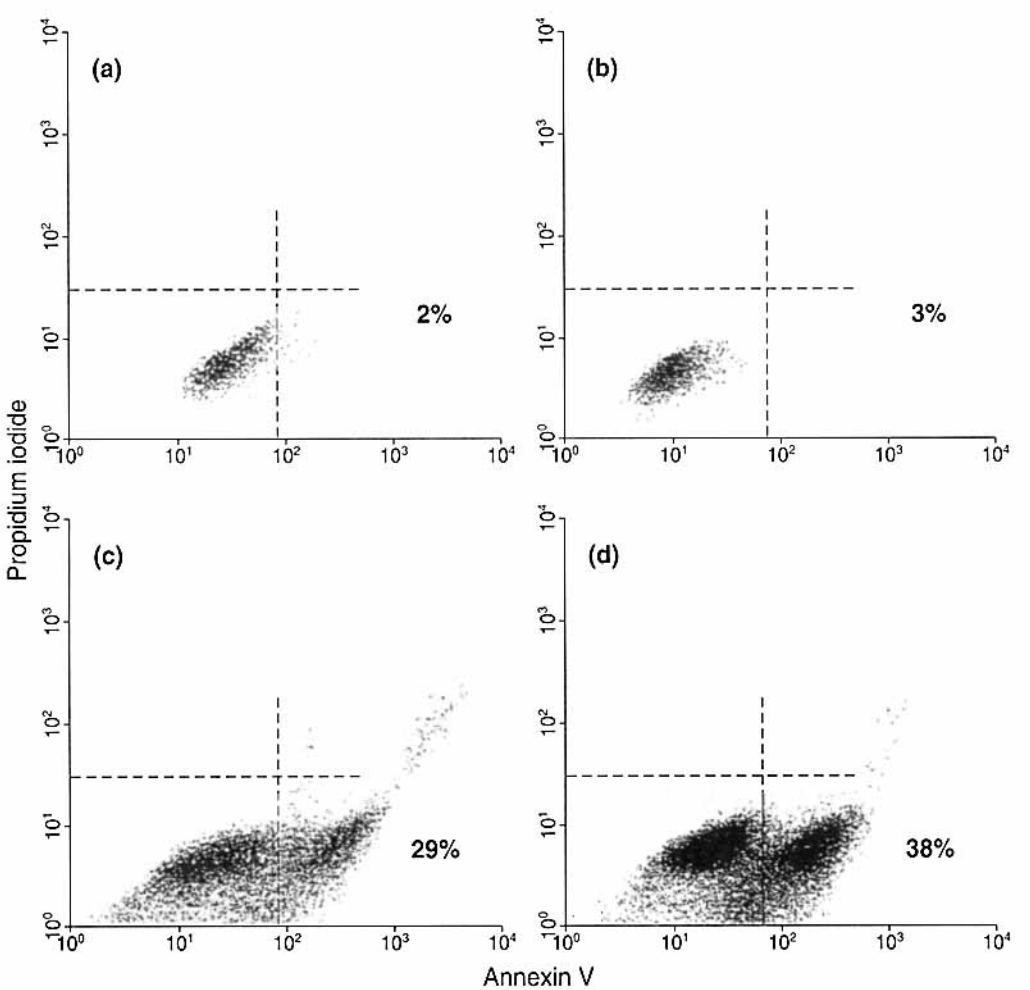


Figure 2. Cytograms of annexin V binding (abscissa) vs. PI uptake (ordinate): live (annexin V−/PI−), apoptotic (annexin V+/PI−) and necrotic or necrotic-like (annexin V+/PI+) cells are shown in (c), (d) and (a) and (b), respectively. Cytograms of annexin V binding (abscissa) vs. PI uptake (ordinate) in HL-60 cells treated with 0.1 g/ml of camptothecin for 0, 2, 3 and 4 h, respectively, showing progressive increase in the incidence of cells in the annexin+/PI^- compartment.

Similar frequency of apoptosis was observed based on DNA strand break labelling assay (TUNEL), in which biotinylated dUTP is incorporated into DNA strand breaks. Figure 1 shows bivariate distributions of DNA content vs. DNA strand break labelling of exponentially growing HL-60 cells incubated in the absence or presence of 0.1 μg/ml of CPT for 2, 3 or 4 h. This assay, like DNA content analysis, also indicates that apoptosis was selectively affected S-phase cells.

The proportion of cells reactive with the annexin V-FITC conjugate (Figure 2) was somewhat lower than that with fractional DNA content. Thus, after 3 h of treatment of HL-60 cells with 0.1 μg/ml of CPT the incidence of annexin V positive and PI negative cells—i.e. apoptotic cells was 29% vs. 2% in the control; it increased to 38% after 4 h (Table 2).

Table 3. Comparison of the incidence of apoptosis as concurrently detected by three methods in MCF-7 cells treated with 0.1 μg/ml camptothecin (CPT)

Time (h)	Treatment	% Apoptosis		
		Annexin	sub-G_1 DNA	TUNEL
24	CONTROL	2	5	1
	CPT	5	4	2
48	CONTROL	2	n.d.	1
	CPT	6	11	2
72	CONTROL	5	5	1
	CPT	26	21	9

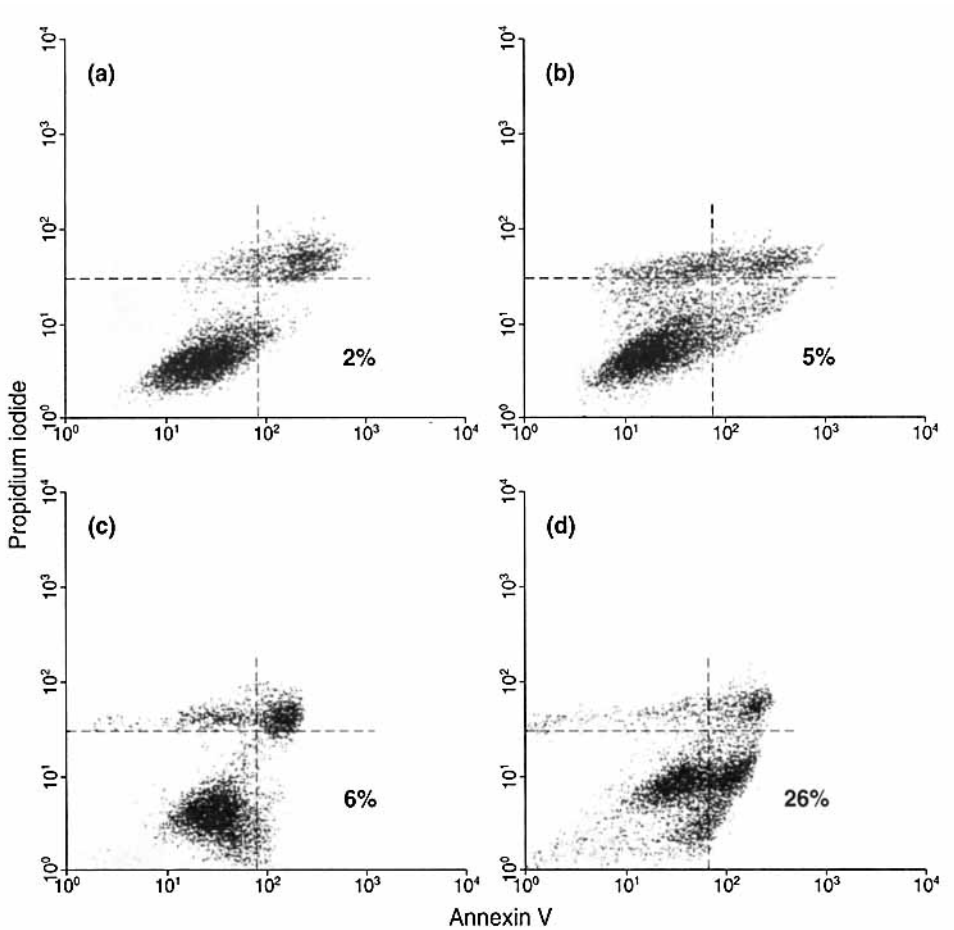


Figure 3. Cytograms of annexin V binding (abscissa) vs. PI uptake (ordinate): live (annexin V−/PI−), apoptotic (annexin V+/PI+) and necrotic or necrotic-like (annexin V+/PI+) cells are shown in (c), (d) and (a) and (b), respectively. (The latter being present at the same incidence—about 20%—in all conditions, including the controls, most likely reflecting artefactual membrane damage due to EDTA treatment). Cytograms of annexin V binding (abscissa) vs. PI uptake (ordinate) in MCF-7 cells treated with 0.1 μg/ml of camptothecin for 0–24–48 or 72 h, respectively. The compartment annexin+/PI− after a 72-h treatment is 26%.

Table 4. Determination of the cell cycle distribution (%) and AI* apoptotic cells in MCF-7 cells treated with 0.1 μg/ml camptothecin (CPT)

Time (h)	Treatment	AI* (% *total* population)	Distribution of *nonapoptotic* cells in the cycle phases (%)		
			G_1	S	$G_2 + M$
24	CONTROL	5	33	46	21
	CPT	4	8	84	8
48	CONTROL	n.d.	n.d.	n.d.	n.d.
	CPT	11	7	70	23
72	CONTROL	5	58	27	15
	CPT	21	9	61	30

*The percentage of apoptotic cells represents the fraction of the cells in the sub-G_1 peak on DNA frequency distribution histograms (see Figure 4).

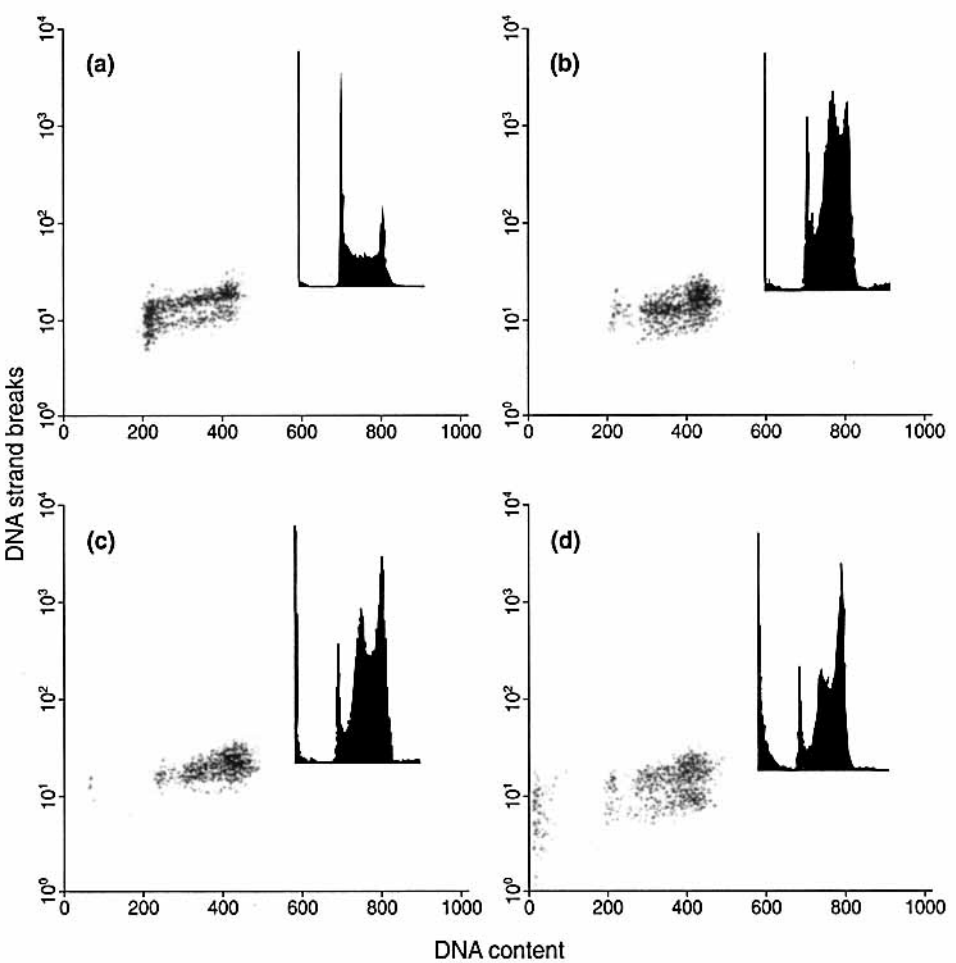
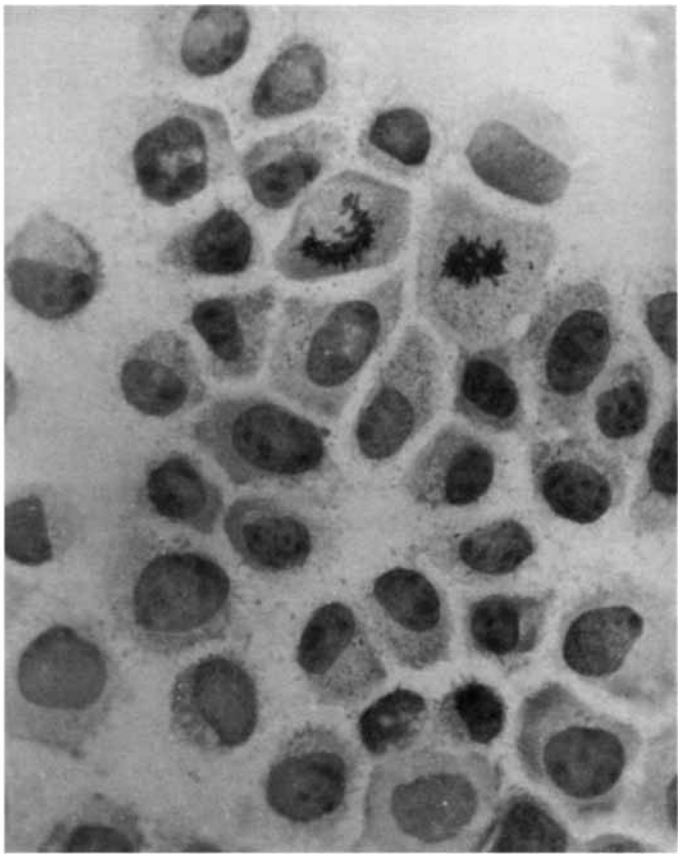


Figure 4. Effect of camptothecin on DNA degradation and on the appearance of DNA strand breaks in MCF-7 cells treated for 0–24–48 and 72 h. Data show the presence of sub-G_1 compartment with low DNA content (insets), but no increase in the DNA strand break associated fluorescence.

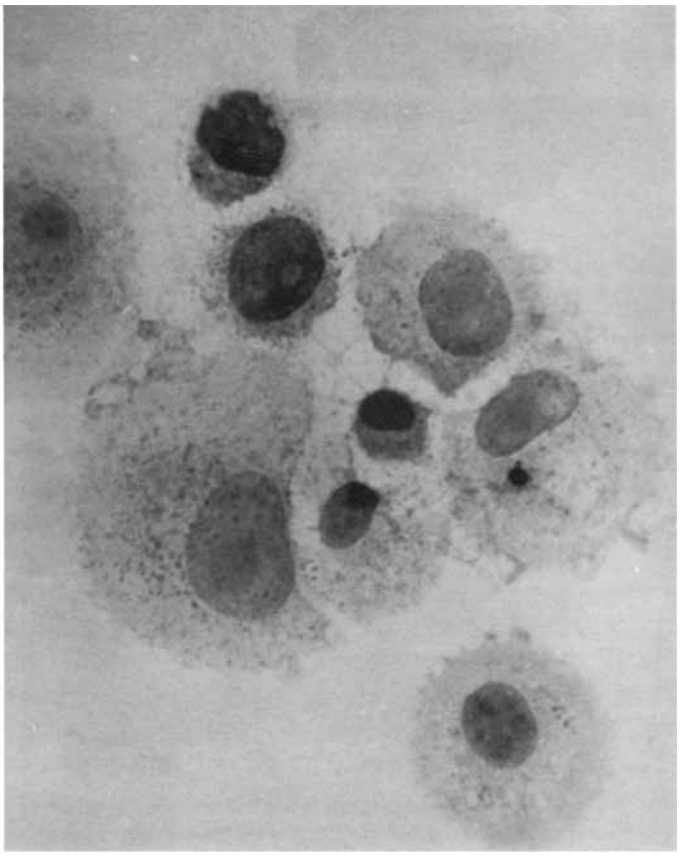


a

Figure 5 Changes in the nuclear morphology of MCF 7 cells untreated (a) or treated (b c) with 0 1 μg/ml of CPT for 24 h (b) or 72 h (c) The cells were stained with haematoxylin and eosin and observed under a Zeiss photomicroscope (final magnification ×650) CPT treatment induced cellular enlargement and growth arrest in S and G_2/M phases (b) associated with chromatin condensation at periphery of the nucleus with a horseshoe shape (c)

The response of MCF-7 cells to CPT differed from that of HL-60 cells in that there was no evidence of significantly increased AI during the first 24 h of treatment (Table 3) The presence of cells with fractional DNA content, as that of annexin V+/PI−, was detected only after 48 and 72 h Their percentage, however, still remained below that observed in HL-60 cultures during the initial 4 h Thus there were only 26% of annexin V+/PI− cells (Figure 3) and 21% cells with fractional DNA content (Table 3) in cultures of MCF-7 cells incubated with CPT for 72 h However, the proportion of cells with DNA strand breaks was minor both after 48 h (2%) and after 72 h (9%) of treatment with CPT (Table 3)

The effects of CPT on the cell cycle distribution was also different in MCF-7 and in HL-60 cells In contrast to HL-60 cells that showed diminished number of S-phase cells, the CPT treatment resulted in the accumulation of MCF-7 cells in S and $G_2 + M$ and only a few cells remained in G_1-phase after 48 or 72 h incubation with the inhibitor (Table 4, Figure 4) Many cells showed morphological features of apoptosis, namely the presence of condensed chromatin at the periphery of the nucleus with a horseshoe shape (Figure 5) However, these cells did not show the presence of DNA strand breaks that would be detected by the TUNEL assay (Figure 4)



b

Figure 5. Continued.

DISCUSSION

Previous studies on cells of the haematopoietic lineage growing in suspensions indicated that loss of membrane asymmetry occurs very early during apoptosis being initiated at a time of activation of the caspase cascade and preceding nuclear condensation, cell shrinkage and breakdown of nuclear matrix constituents (Koopman *et al* 1994, Vermes *et al* 1995, O Brien *et al* 1997) In this study direct comparison of the annexin V affinity assay fractional DNA content and the TUNEL assay indicates that in CPT-treated HL-60 cells the externalization of phosphatidylserine at the outer leaflet of the plasma membrane as well as the appearance of a sub-G_1 diploid peak on DNA frequency histograms can be detected at approximately the same time as the presence of DNA strand breaks by the TUNEL assay However the cells reactive with annexin V-FITC (e g 29% after 3 h) were less numerous than those with DNA strand breaks or with fractional DNA content (37 or 39%, respectively, Table 2) This might mean that, in those cells, the 'time window' through which apoptotic cells are recognized by the annexin V-FITC assay is narrower than in the assays based on the detection of DNA fragmentation Alternatively, it may indicate that membrane asymmetry is not necessarily associated to DNA fragmentation

CPT-induced apoptosis of HL-60 cells was quite selective to S-phase cells, confirming our earlier results (Del Bino *et al* 1991, Darzynkiewicz *et al* 1992) This was apparent from the

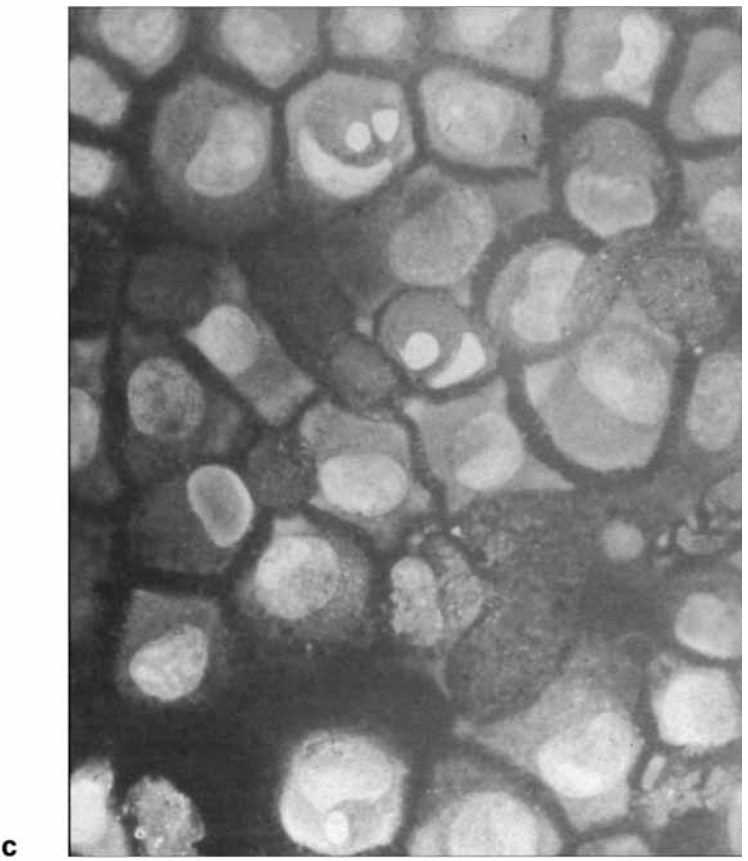


Figure 5. Continued.

decrease of the percentage of cells with an S-phase DNA content (Figure 1) within the nonapoptotic cell population and, conversely, from the fact that the cells with DNA strand breaks had an S-phase DNA content.

Exposure of MCF-7 cells to CPT led to apoptosis, which become apparent after a delay of 48–72 h. At that time, most cells in these cultures were arrested in S and G_2/M, many being enlarged and exhibiting signs of growth imbalance. Growth imbalance invariably occurs as a result of the prolonged cell arrest, when RNA and protein synthesis continue (Traganos *et al.* 1982). The present work shows that the assay based on annexin V binding can be adapted for evaluating apoptosis in cell growing adherent. Apoptosis of MCF-7 cells was manifested by exposure of phosphatidylserine on cell surface (as detected by the annexin V-FITC conjugate assay) and by the appearance of cells with a fractional (sub-G_1) DNA content. Inspection of CPT-treated MCF-7 cells by microscopy indicated the presence of the typical areas of condensed chromatin at the nuclear periphery, with a characteristic horseshoe or sickle shape (Figure 5).

DNA fragmentation in MCF-7 cells was detected by extraction of low MW DNA with phosphate-citric acid buffer (Gong *et al.* 1994), apoptotic cells then being recognized by their DNA content being lower than the G_1 peak. However, the incidence was higher than that of the cells in which DNA strand breaks could be detected by the TUNEL assay. This apparent

discrepancy might be explained by the fact that in MCF-7 cells 3′-OH termini in DNA strand breaks were inaccessible to the reagents used in the TUNEL assay. This would indicate that the apoptosis-associated endonuclease in MCF-7 cells has different specificity, compared to that of HL-60 cells. It is also possible that in MCF-7 cells, like in some other epithelial cells (Oberhammer *et al.* 1993, Otto *et al.* 1996), DNA cleavage does not progress into internucleosomal sections, but stops after generation of 50–300 kb fragments. While the latter may still be extracted from the cell, the number of DNA strand breaks would remain too small to be detected by the TUNEL assay. Indeed, evidence was provided that, whereas MCF-7 cells do transcribe a DNase-I-like gene, the resulting product is inactive, neither internucleosomal DNA fragmentation, nor Ca^{2+}/Mg^{2+}—dependent nuclease being detectable in the drug-treated cells undergoing apoptosis (Otto *et al.* 1996).

In HL-60 cells, apoptosis occurred rapidly (3–4 h after administration of CPT) and was limited to S-phase cells, which agrees with previous observations by Gong *et al.* (1994). It is known that such rapid induction of apoptosis by CPT in HL-60 cells is prevented by aphidicolin, an inhibitor of DNA polymerase (Darzynkiewicz *et al.* 1996). It has been proposed that the collision between the progressing DNA replication fork and the DNA-topoisomerase I complex stabilized by CPT, triggers apoptosis (Hsiang *et al.* 1989, D'Arpa *et al.* 1990, Del Bino *et al.* 1990). Little is known, however, about the mechanism that detects such a lesion and triggers the apoptotic pathway. In MCF-7 cells, expression of *p53*, via activation of *p21*, would act to arrest cells in S and G_2/M and might slow down the triggering of apoptosis, thus giving time for repairing DNA lesions (Halicka *et al.* 1997, Gradzka & Szumiel 1996, Whitacre & Berger 1997). Accumulation of secondary changes, including growth imbalance and/or defective transcription of damaged genes that are essential for cell survival, would then ultimately lead to cell death in the next cell cycle.

It has been observed (Cohen *et al.* 1992, Pandey *et al.* 1994, Sun *et al.* 1994, Pandey *et al.* 1997) that endonucleolytic DNA fragmentation is not necessary for inducing nuclear chromatin condensation during apoptosis, indicating that DNA fragmentation and other nuclear changes might be caused by separate mediators. Our present results with MCF-7 cells fit with this observation, by showing that chromatin condensation took place in the absence of DNA breakage detectable by the TUNEL assay.

This underlines the necessity of using different methods, aimed at detecting the different aspects of the apoptotic process for a complete evaluation of apoptosis.

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