

Detection of DNA Strand Breaks in Individual Apoptotic Cells by the *in Situ* Terminal Deoxynucleotidyl Transferase and Nick Translation Assays¹

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ABSTRACT

DNA strand breaks which occur in HL-60 cells as a result of activation of endonuclease during apoptosis induced by cell treatment with the DNA topoisomerase I inhibitor camptothecin and topoisomerase II inhibitors teniposide, 4'-(9-acridinylamino)-3-methanesulfon-*m*-anisidide, and fostriecin were labeled *in situ*, in individual fixed and permeabilized cells, with biotinylated dUTP (detected by fluoresceinated avidin), using the terminal deoxynucleotidyl transferase or nick translation assays. During the early stage of apoptosis, prior to nuclear fragmentation, the breaks were predominantly localized at the nuclear periphery, close to the nuclear envelope. In more advanced stages, all cellular DNA, then localized within the cell as dense, homogeneous granules of a variety of sizes, was strongly labeled, indicating extensive and more uniform distribution of breaks throughout genomic DNA. Bivariate analysis of the incorporated biotinylated dUTP and cellular DNA content by flow cytometry made it possible to estimate the kinetics of the labeling reaction and relate DNA breaks to cell position in the cycle. The kinetics of biotinylated dUTP incorporation was faster, and the distinction of cells with DNA breaks was more pronounced, using the terminal transferase rather than the nick translation assay. Camptothecin, teniposide, and 4'-(9-acridinylamino)-3-methanesulfon-*m*-anisidide induced DNA breaks preferentially in S-phase cells, having little effect on cells in the G₁ phase of the cycle. In contrast, fostriecin affected cells indiscriminately, in all phases of the cell cycle. The method of detection of DNA strand breaks (3'-hydroxyl termini) in individual cells offers several advantages and can be applied to clinical material (tumor biopsies) to study the induction of apoptosis in tumors during treatment, as a possible prognostic marker. The protein-associated DNA breaks in the "cleavable" DNA-topoisomerase complexes, which are the primary lesions induced by the inhibitors and precede apoptosis, were not detectable by the present methods.

INTRODUCTION

Apoptosis is a specific mode of cell death recognized by a characteristic pattern of morphological, biochemical, and molecular changes (1-3). Cell death by apoptosis is common during embryogenesis (4, 5), in normal tissue and organ involution (6, 7), and in cytotoxic immunological reactions (8, 9), and it occurs naturally at the end of the life span of differentiated cells (10, 11). This mode of cell death has recently become a focus of interest in oncology (12, 13). Apoptosis of tumor cells is triggered by ionizing radiation (14, 15), hyperthermia (16), and a variety of antitumor drugs (17-24), as well as following deprivation of growth factors (25, 26). There is a growing body of evidence that the efficacy of antitumor treatment may be associated with the intrinsic ability of tumor cells to respond by apoptosis (12, 13, 27). The possibility of modulating cell responsiveness by apoptosis, therefore, opens new antitumor strategies, and the mechanism by which particular agents can trigger the apoptotic response of tumor cells has become a subject of intense study. The interest in apoptosis in oncology also stems from the observation that,

at least in some tumors, malignancy may be associated with the loss of the ability of the cell to undergo spontaneous apoptosis rather than with increasing cell proliferation rates (28, 29).

There are several methods of detection of apoptosis. Morphological changes involve a characteristic pattern of condensation of chromatin and cytoplasm, which makes it possible to identify these cells by microscopy (1-3). The landmark of apoptosis is endonucleolysis, with nuclear DNA initially degraded at the linker sections to fragments equivalent to single and multiple nucleosomes (3). The latter are revealed as the typical "ladder" on agarose gels during electrophoresis. The flow cytometric methods of identifying apoptotic cells (for a review see Ref. 30) are based on measurement of cellular DNA content (31, 32), increased sensitivity of DNA to denaturation (33), or altered light scatter properties (34). In the present study, we describe and compare two methods which are based on detection of the extensive DNA breakage which characterizes apoptosis. In these methods the 3'-hydroxyl termini of DNA breaks are labeled with b-dUTP,³ either by exogenous TdT assay or *Escherichia coli* DNA polymerase (NT assay). Both methods (35) were applied to detect apoptosis of HL-60 cells triggered by DNA topoisomerase I (CAM) and topoisomerase II inhibitors (TN, *m*-AMSA, and FST). The NT assay was originally proposed by Fehsel *et al.* (36) as a method to detect apoptotic fibroblasts in histological specimens and was used by Jonker *et al.* (37) to identify apoptotic thymocytes following ionizing radiation. Since this paper was submitted for publication, TdT and NT assays were applied in studies on the effects of protease inhibitors on apoptosis induced by topoisomerase inhibitors (38) and used to monitor apoptosis during the treatment of human leukemias (39).

MATERIALS AND METHODS

Cells. All experiments were performed on HL-60 and MOLT-4 cells during their exponential phase of growth. The cells were maintained in RPMI (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, as described previously (33, 40). The cells were split every second day and were diluted 1:1 a day before each experiment. Cell densities in cultures did not exceed 5 × 10⁵ cells/ml. Peripheral blood mononuclear cells, obtained from patients diagnosed with CML, prior to and during chemotherapy, were isolated and prepared as described elsewhere (39). Breast carcinoma cells were obtained by aspiration biopsy, and the cells were dispersed mechanically by repeated syringing through an 18-gauge needle.

Drugs. CAM (Sigma Chemical Co., St. Louis, MO) dissolved in DMSO at a concentration of 3 mM was stored at -20°C. TN (VM-26; Bristol Myers Co., Wallingford, CO) dissolved in DMSO at a concentration of 5 mM was kept at 4°C. *m*-AMSA, obtained from the Investigational Drug Branch, Division of Cancer Treatment, National Cancer Institute, was dissolved in DMSO at a concentration of 0.3 mM and was kept at -20°C. FST (Phosphotrienin; Ben Venue Laboratories, Inc., Bedford, OH) was kindly provided by Dr. Werbel of Parke-Davis (Ann Arbor, MI). Fresh stock solutions of FST were prepared at a concentration of 5 mM by adding 2 ml of Hanks' balanced salt solution to the lyophilized substance. Further dilutions were achieved by addition of RPMI

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³ The abbreviations used are: b-dUTP, biotinylated dUTP; NT, *in situ* nick translation assay; TdT, terminal deoxynucleotidyl transferase assay; CAM, camptothecin; TN, teniposide; VM 26; *m*-AMSA, 4'-(9-acridinylamino)-3-methanesulfon-*m*-anisidide; FST, fostriecin; phosphotrienin; PBS, phosphate-buffered saline; DMSO, dimethyl sulfoxide; PI, propidium iodide; CML, chronic myelogenous leukemia.

1640. The cells were treated with the drugs for up to 6 h. Control cultures were treated with solvent (DMSO) alone, which at the highest dose (0.1%) had no detectable effect on any of the parameters measured.

Cell Fixation. Following incubation with the drugs, the cells were fixed in 1% formaldehyde in PBS (pH 7.4) for 15 min on ice. Then, after washing in 3 ml of PBS, cells were resuspended in 70% ice-cold ethanol and immediately transferred to the freezer. The cells were stored at -20°C for 1–3 days before being subjected to the *in situ* NT or TdT assay. To study the effect of fixation on the TdT procedure, in some experiments, cells were fixed only in 70% ice-cold ethanol. In still other experiments, to extract histones (41), the cells were fixed in 70% ice-cold ethanol (1 h) and then treated with 0.1 M HCl on ice.

DNA Gel Electrophoresis. Untreated or drug-treated cells were collected by centrifugation, washed in PBS (without Ca^{2+} and Mg^{2+}), resuspended (5×10^6 cells) in 0.5 ml TBE (45 mM Tris-borate buffer, 1 mM EDTA; pH 8.0) containing 0.25% Nonidet P-40 (Sigma) and 0.1 mg/ml RNase A (Sigma), incubated at 37°C for 30 min, treated with 1 mg/ml proteinase K (Boehringer Mannheim, Indianapolis, IN), and incubated for an additional 30 min at 37°C . After incubation, 0.1 ml of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol; Ref. 35) was added, and 25 μl of the tube content (equivalent of approximately 2×10^5 cells/well) were transferred to the gel. Horizontal 1.5% agarose gel electrophoresis was performed at 2 V/cm for 6 h, and DNA in gels was visualized under UV light after staining with 5 $\mu\text{g}/\text{ml}$ of ethidium bromide (Polysciences, Inc., Warrington, PA).

NT Assay. Following fixation, the cells were rinsed once with PBS, and 2×10^5 cells were suspended in 12.5 μl of nick translation buffer consisting of 2.5 mM MgCl_2 , 10 mM β -mercaptoethanol (Bio-Rad Laboratories, Richmond, CA), 50 mM Tris (pH 7.8), 10 $\mu\text{g}/\text{ml}$ bovine serum albumin, 1 unit *E. coli* DNA polymerase, 0.2 nmoles unlabeled dATP, dGTP, and dCTP, and 0.2 nmoles biotin-16-dUTP. The cells were incubated in nick translation buffer (35), with gentle agitation every 15 min, at 15°C , for up to 6 h, as indicated in the figures. Afterward, the cells were washed in PBS and resuspended in 100 μl of the staining buffer, which contained 2.5 $\mu\text{g}/\text{ml}$ fluoresceinated avidin, $4 \times$ saline-sodium citrate buffer (Sigma) ($1 \times \text{SSC} = 0.15 \text{ M NaCl}, 0.015 \text{ M Na-citrate}$), 0.1% Triton X-100, and 5% (w/v) nonfat dry milk. Cells were incubated with this buffer for 30 min at room temperature in the dark. Nucleotides, biotin-16-dUTP, avidin-fluorescein isothiocyanate, and DNA-polymerase were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

TdT Assay. After fixation and washing, cells were resuspended in 50 μl of a solution containing 0.1 M sodium cacodylate (pH 7.0) (Sigma), 1 mM CoCl_2 (Sigma), 0.1 mM dithiothreitol (Sigma), 0.05 mg/ml bovine serum albumin (Sigma), 10 units of terminal transferase (Boehringer Mannheim Biochemicals), and 0.5 nmoles biotin-16-dUTP (TdT buffer). In some experiments, in addition to b-dUTP, unlabeled dATP, dGTP, and dCTP were included, each at a concentration of 20 μM (35). The cells were incubated in this solution at 37°C , for time intervals between 15 min and 3 h, rinsed in PBS, and resuspended in 100 μl of the staining buffer containing fluoresceinated avidin, prepared as described above for the NT assay. The TdT assay was also performed on cytospin preparations, on slides containing untreated and drug-treated HL-60 cells. The specimens were fixed for 15 min in 1% formaldehyde in PBS (pH 7.4), rinsed in PBS, and transferred to 70% ice-cold ethanol for 1 h. After rinsing in PBS, excess PBS was removed, and 25 μl of TdT buffer were pipetted onto slides. The specimens were kept in a humidified chamber for 30 min at 37°C . The slides were then rinsed in PBS and incubated in the dark for 30 min with the staining buffer.

Flow Cytometry. Following incubation in staining buffer, the cells were rinsed in PBS with 0.1% Triton-X 100 and resuspended in 1 ml of PBS containing 5 $\mu\text{g}/\text{ml}$ of PI and 0.1% RNase A (both from Sigma). Flow cytometry was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The red (PI) and green (fluorescein) fluorescence emissions from each cell were separated and measured using the standard optics of the FAC-Scan. The data from 1×10^4 cells were collected, stored, and analyzed using LYSYS II software. The signals of green fluorescence were measured using both linear and logarithmic amplification for each sample.

RESULTS

Exposure of HL-60 cells to DNA topoisomerase I (CAM) or II (TN, *m*-AMSA, FST) inhibitors triggered apoptosis, which was recognized

by characteristically altered cell morphology and the appearance of DNA fragments equivalent to approximately 200 bp and their multiples (Figs. 1 and 2). The cell response by apoptosis was rapid. The changes in cell morphology, manifesting as condensation of chromatin and nuclear fragmentation, were observed 4 h after the addition of CAM or TN. The integrity of plasma membrane, however, was preserved for several hours after the initial chromatin changes were seen. DNA degradation was detected by electrophoresis as early as 3 h after cell exposure to CAM or TN. No apoptosis was observed in the case of MOLT-4 cells exposed to the same concentrations of the drugs, for up to 6 h (not shown).

HL-60 cells from cultures in which apoptosis induced by DNA topoisomerase inhibitors was confirmed by morphology and gel electrophoresis were fixed, permeabilized, and subjected to either TdT or NT assays. Subsequently, the cells were incubated with fluoresceinated avidin. Numerous cells (generally over 30%) became intensely labeled under these conditions, which would indicate incorporation of b-dUTP by these cells (Fig. 3). Attempts have also been made to use the directly labeled, fluoresceinated dUTP (instead of b-dUTP followed by fluoresceinated avidin), which could simplify the procedure. Although fluoresceinated dUTP was incorporated into apoptotic cells in the NT procedure, the labeling was much less intense, and thus the method was less sensitive (not shown).

No labeling was seen when exogenous DNA polymerase or terminal transferase was excluded from the incubation mixture. Also, no incorporation of b-dUTP was observed in MOLT-4 cells, untreated or treated with the same inhibitors for up to 4 h (not shown); unlike HL-60, MOLT-4 cells do not respond immediately with apoptosis to topoisomerase inhibitors (22). Fewer than 5% of the cells were labeled in the exponentially growing, untreated HL-60 cultures, consistent with the observation that approximately the same percentage of HL-60 cells undergoes spontaneous differentiation and apoptosis (22). The labeled cells withstood intensive cell washings, but the label was removed by cell incubation with 1 mg/ml of DNase I (not shown). These data provide evidence that b-dUTP was covalently incorporated by the exogenous enzymes and that DNA breaks appearing in apoptotic cells served as primers for the reaction. Both methods (TdT and NT) were thus successful in labeling DNA strand breaks in apoptotic cells.

Two patterns of cell labeling with b-dUTP, dependent on the duration of cell treatment with the inhibitors, were observed (Fig. 3).

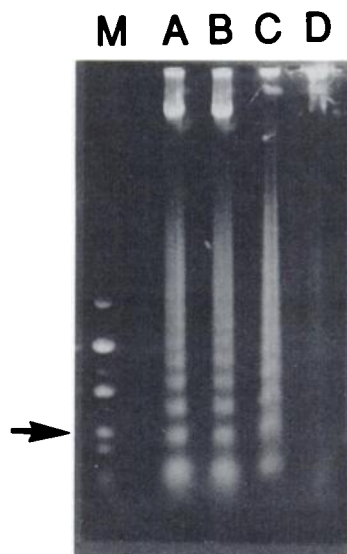


Fig. 1. Agarose gel electrophoresis of DNA from HL-60 cells treated for 4 h with 0.15 μM CAM (A–C; DNA preparations from different experiments) and from untreated cells (D). Lane M, molecular weight lane; arrow, 476-bp DNA band.

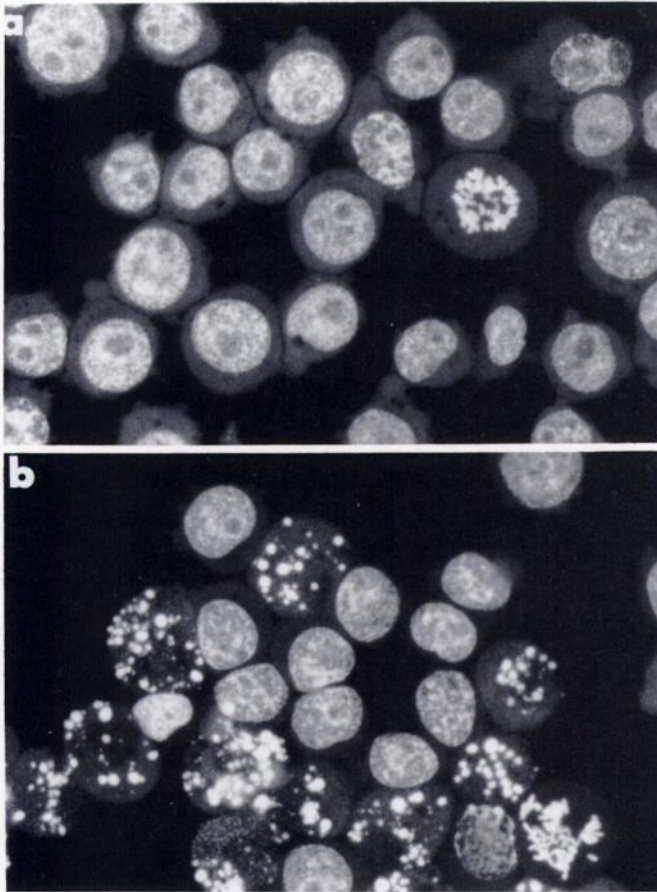


Fig. 2. Morphology of the HL-60 cells, untreated (A) and treated (B) with 0.15 μM CAM for 4 h. The cells were cytocentrifuged, stained with the DNA-specific fluorochrome diamidino-2-phenylindole, and counterstained with the protein stain sulforhodamine 101. The photographs were taken under UV light (epillumination) microscope (Nikon, 40 \times objective lens).

Short-term treatment with CAM or TN (1.5–2 h) led to labeling which was more predominant on the nuclear periphery; the nuclei were ring-shaped (green fluorescence) and had rather weak and uniform overall fluorescence. This staining pattern suggested that the first DNA breaks occurred in the regions close to the nuclear envelope. Longer exposures to the inhibitors (3–4 h) resulted in chromatin fragmentation; the chromatin granules exhibited very bright fluorescence.

Flow cytometric measurements of cells labeled in the TdT or NT assays and then counterstained with PI made it possible to quantitate the incorporation of b-dUTP and relate it to cellular DNA content, *i.e.*, to cell position in the cycle (Fig. 4). The NT reaction was much slower compared to that of TdT. Although the rate was decreased after 2 h of incubation, the b-dUTP progressively accumulated in the cells for up to 6 h in the NT reaction (Fig. 5). In contrast, maximal labeling in the TdT assay was already seen after 30 min, and actually a minor decrease in cell fluorescence was observed thereafter. The labeling of apoptotic cells in the TdT assay was approximately 60% higher in the presence of only biotinylated dUTP, compared to that observed in the presence of all four deoxynucleotides. The distinction of apoptotic cells based on the intensity of green fluorescence of labeled *versus* unlabeled cells (signal:noise ratio) was more pronounced in the TdT compared to the NT assay (Figs. 4 and 5). Thus, after 30 min of incubation in the TdT assay the green fluorescence of apoptotic S-phase cells (peak value) was equivalent to 300 channels, which represents a 25-fold increase over the 12-channel value of the S cells in the control. A comparable increase observed after 45 min of incubation in the NT assay was only 35 channels (from 15 to 50), which

represents a 2.3-fold (35:15) increase (Fig. 4B *versus* 4F; note the exponential scale of the coordinate). At incubation times that are acceptable in practice, therefore, the TdT assay was over 10-fold more sensitive compared to the NT assay.

The use of ethanol or formaldehyde in cell fixation was compared, and the effect of dissociation of histones on NT reaction was studied in subsequent experiments (Fig. 6). Apoptotic cells, when fixed in ethanol and then subjected to the NT procedure, lost a significant portion of DNA (Fig. 6D). Incorporation of b-dUTP by these cells was diminished compared to apoptotic cells fixed in formaldehyde. Their distinction, however, *vis-à-vis* cells that did not respond with apoptosis, was very pronounced, based on both decreased DNA stainability and increased b-dUTP incorporation (Fig. 6D). Pretreatment with 0.1 M HCl increased the ability of all cells (apoptotic and nonapoptotic) to incorporate the precursor, and the distinction of apoptotic cells was diminished (Fig. 6C).

Because, as mentioned, the loss of DNA from apoptotic cells fixed in formaldehyde was relatively minor, it was possible to study apoptosis with respect to the cell position in the cycle. The data indicated that apoptosis induced by CAM, TN, or *m*-AMSA (Fig. 7) was selective for S-phase cells. This was most convincing in the case of CAM-treated cells, where, regardless of whether the NT or TdT assay was used, nearly total loss of S-phase cells from the cell population that did not incorporate the precursor was evident (Fig. 7, B and F). The position on the DNA histograms of S-phase cells that incorporated b-dUTP was somewhat shifted toward lower DNA values, suggesting

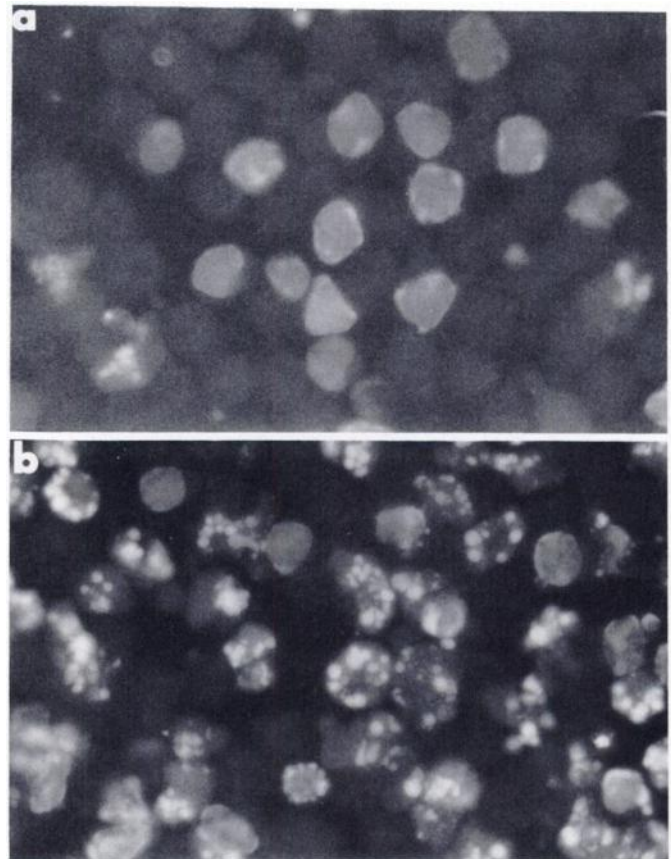


Fig. 3. TdT reaction *in situ*, in HL-60 cells treated with 0.15 μM CAM for 2 (A) or 4 (B) h. The cells were cytocentrifuged, fixed in 1% formaldehyde for 15 min, incubated in the presence of exogenous terminal transferase and b-dUTP, and counterstained with fluoresceinated avidin. At an early stage of apoptosis, prior to nuclear disintegration, the labeled DNA strand breaks were more numerous on the nuclear periphery (A). Only a few cells are labeled; outlines of the unlabeled cells are barely visible. Chromatin granules that were present in apoptotic cells after 4 h of treatment with CAM were intensely and uniformly labeled (B). Blue light (BG 12 filter) epillumination, 40 \times objective lens.

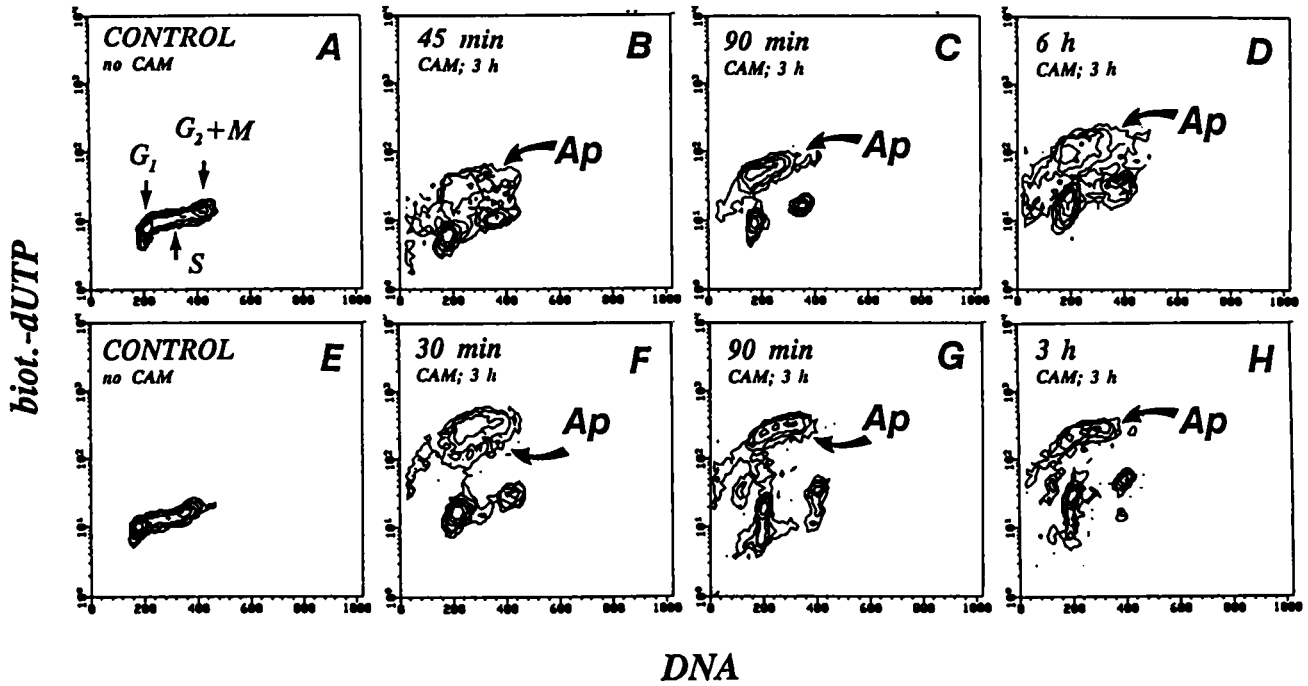


Fig. 4. Labeling of DNA breaks in apoptotic cells with b-dUTP during NT (A-D) or TdT (E-H) reaction, after different periods of incubation in these assays. The untreated cells (control) were incubated in NT assay for 90 min and in TdT assay for 30 min. Incorporation of b-dUTP peaked after 30 min in the TdT assay but continued to up to 6 h in the NT assay.

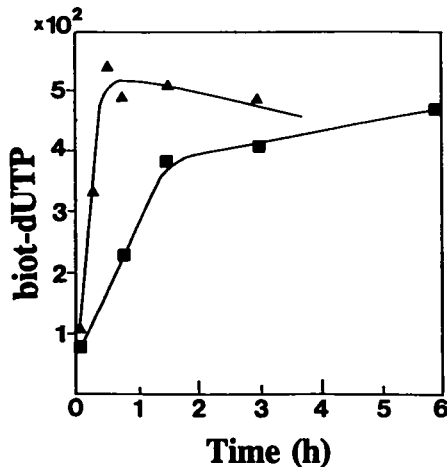


Fig. 5. Kinetics of b-dUTP incorporation into DNA of apoptotic cells in TdT (▲) and NT (■) assays. The mean green fluorescence intensity of the labeled cell population (Ap), as seen in Fig. 4, was measured, using a linear rather than logarithmic amplifier, and was plotted as a function of time of incubation in the respective assays.

that a minor fraction of DNA was still extracted from these cells during the procedure, despite fixation in formaldehyde.

In contrast to CAM, TN, or *m*-AMSA, apoptosis induced by FST was not selective for S-phase cells (Fig. 8). There were also many cells which were continuously entering apoptosis, *i.e.*, the cells were very heterogeneous with respect to the stage of apoptosis, in cultures treated with FST. This resulted in a less pronounced distinction of the apoptotic cell population due to the presence of cells characterized by intermediate values of b-dUTP incorporation in these cultures.

Fig. 9 illustrates that the TdT assay can be applicable to detecting apoptosis-associated DNA strand breaks in human tumor cells. Fig. 9 (A and B) represents the distribution of peripheral blood cells of a patient with CML (49% blasts) before treatment and 24 h after administration of VP-16. The proportion of cells with DNA strand breaks is clearly increased during the treatment. Fig. 9C shows a specimen of breast carcinoma obtained by aspiration biopsy. A large

number of aneuploid cells are characterized by DNA strand breaks labeled in the TdT assay; these cells are presumed to represent tumor cells undergoing spontaneous apoptosis. Details on the application of the TdT assay for monitoring leukemia chemotherapy are presented elsewhere (39).

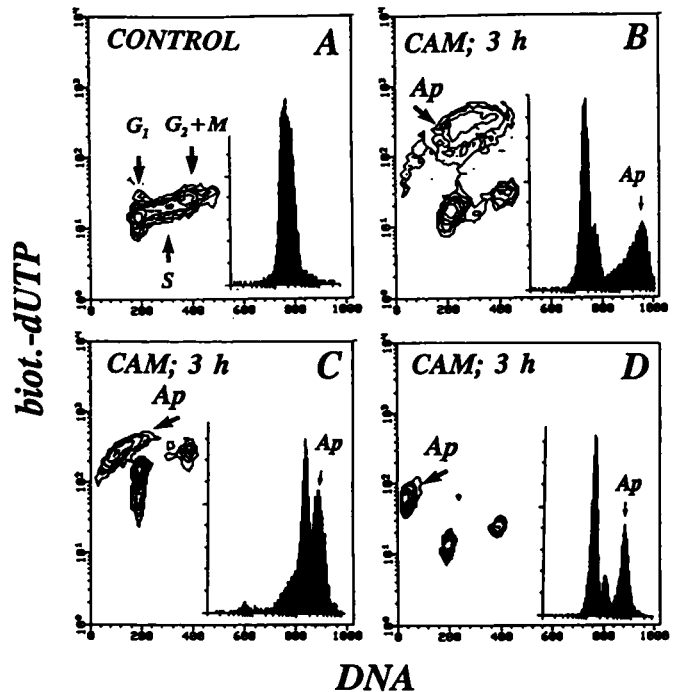


Fig. 6. Effect of cell fixation on b-dUTP incorporation in the TdT assay. A and B, cells untreated and treated, respectively, with $0.15 \mu\text{M}$ CAM for 4 h were fixed in 1% formaldehyde and incubated for 30 min in TdT assay. C, cells fixed in 70% ethanol and then extracted with 0.1 M HCl at $0-4^\circ\text{C}$ for 10 min. D, cells fixed in 70% ethanol (no formaldehyde prefixation). The increased cell labeling in C suggests that the HCl treatment induced additional DNA breaks, also in G_1 - and G_2 -phase cells. A decrease in DNA content of apoptotic cells following ethanol fixation was evident and was more pronounced when cells were not treated with HCl (D). Insets, frequency distributions of b-dUTP incorporation.

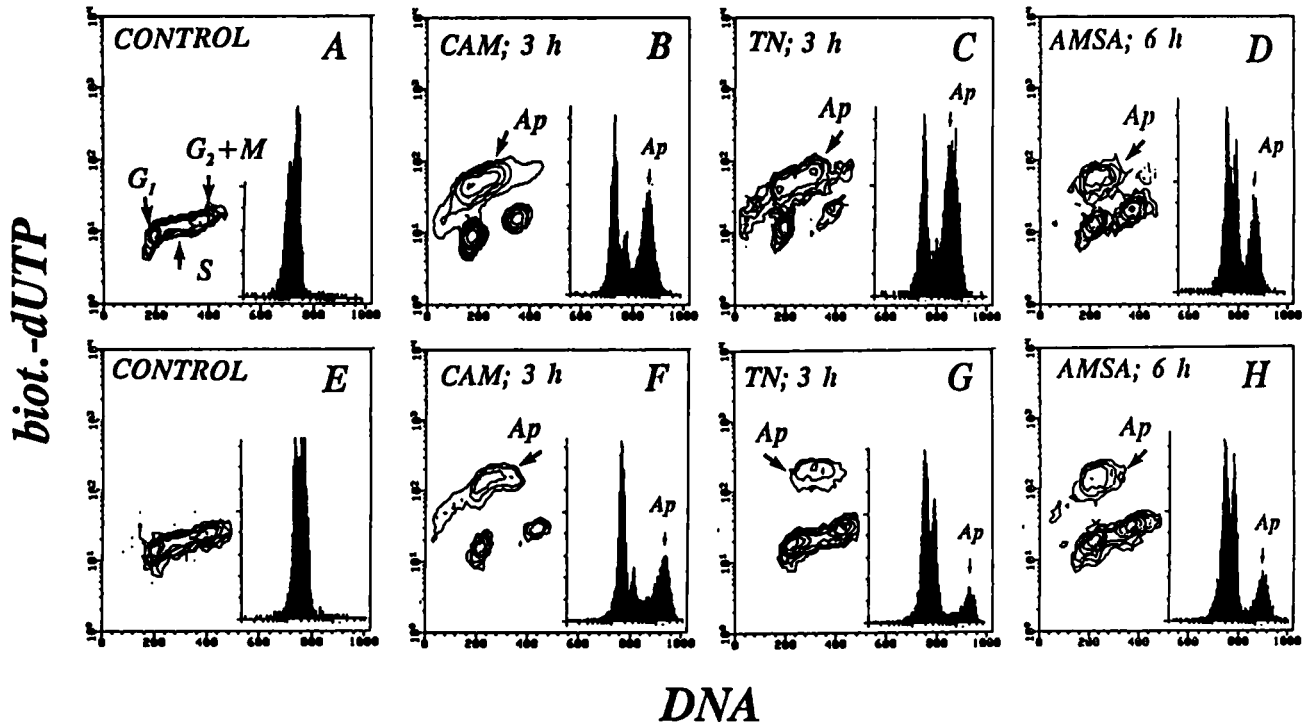


Fig. 7. Labeling of DNA strand breaks with b-dUTP in the NT (A–D) and TdT (E–H) assays, in HL-60 cells treated with different DNA topoisomerase inhibitors (0.15 μ M CAM, 1 μ M TN, and 0.25 μ M *m*-AMSA) and incubated for 3 h in the NT or 30 min in the TdT assay, respectively.

DISCUSSION

The present study shows that DNA strand breaks that occurred in cells undergoing apoptosis as a result of activation of an endogenous endonuclease were labeled *in situ* with a biotinylated nucleotide in the reaction using either exogenous DNA polymerase (NT assay) or TdT assay. The incorporated precursor was detected by fluoresceinated avidin. The labeled cells were then identified either by image analysis or by flow cytometry. In the flow cytometric analysis, simultaneous counterstaining of DNA with PI made it possible not only to identify cells with degraded DNA but also to reveal their position in the cell cycle. The labeling was very intense, and the labeled cells were highly heterogeneous, which required the use of an exponential rather than a linear scale for plotting green (fluorescein) fluorescence.

Two pools of DNA are detected in apoptotic cells (30). DNA of relatively low molecular weight (200–1000 base pairs; mono- and oligonucleosomes) is diffusely dispersed in the cell and, following cell fixation with 60 or 70% ethanol and subsequent washings in buffered saline, it can diffuse out and is lost from the cell. The remaining DNA is of higher molecular weight or is still attached to the nuclear matrix, and it cannot be removed from ethanol-prefixed cells by their rinsing

(30). Thus, apoptotic cells present themselves on the DNA frequency histograms as the cells with reduced DNA content (“sub- G_1 ” cell population) due to loss of the diffusible DNA fraction. We have fixed the cells in either formaldehyde or ethanol; in the latter case the cells were also treated with 0.1 N HCl to extract histones. Formaldehyde fixation, most likely by cross-linking the low-molecular-weight DNA to other cellular constituents, prevented any significant loss of DNA from apoptotic cells. This was advantageous for two reasons: (a) incorporation of biotinylated dUTP was more extensive in formaldehyde-fixed cells, most likely because more DNA, and thus more DNA strand breaks, were retained; and (b) apoptosis could be correlated with cell cycle position. Ethanol fixation, on the other hand, made it possible to identify apoptotic cells based on two parameters, namely the DNA loss and precursor incorporation, simultaneously (Fig. 6D). Treatment with HCl probably induced additional DNA breaks, resulting in an increase in dUTP incorporation by all (apoptotic and non-apoptotic) cells (Fig. 6C).

Both methods were quite specific for apoptotic cells. No significant incorporation of dUTP in either the TdT or NT assay was observed in the case of MOLT-4 cells which, unlike HL-60, do not immediately

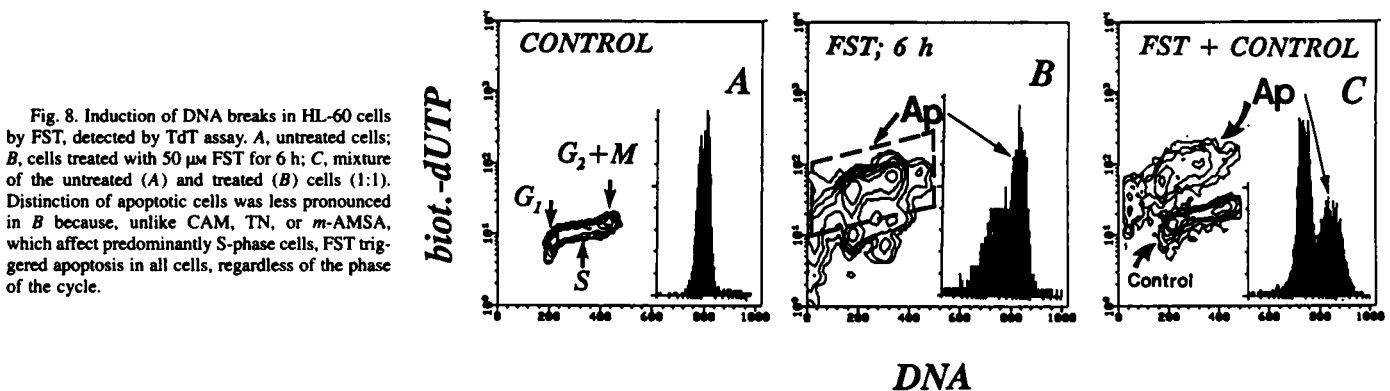


Fig. 8. Induction of DNA breaks in HL-60 cells by FST, detected by TdT assay. A, untreated cells; B, cells treated with 50 μ M FST for 6 h; C, mixture of the untreated (A) and treated (B) cells (1:1). Distinction of apoptotic cells was less pronounced in B because, unlike CAM, TN, or *m*-AMSA, which affect predominantly S-phase cells, FST triggered apoptosis in all cells, regardless of the phase of the cycle.

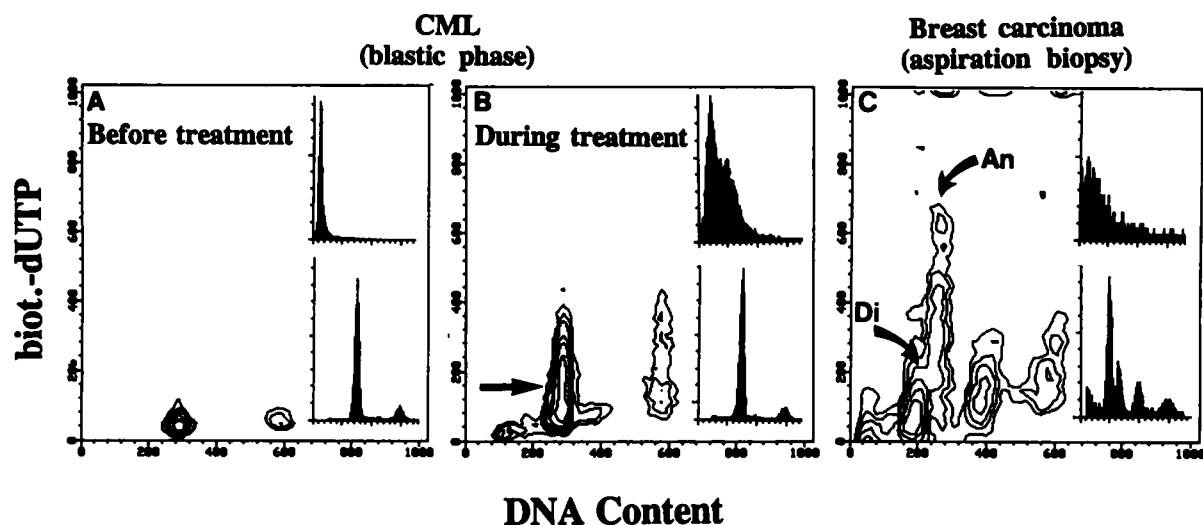


Fig. 9. DNA strand breaks detected by TdT assay in blasts from peripheral blood of the CML patient, before (A) and during (B) chemotherapy (24 h after administration of VP-16) and in breast carcinoma cells (C). A large number of cells with DNA strand breaks (50%; cells above the arrow) were detected during treatment of CML; the control cells, incubated in TdT assay but in the absence of the TdT enzyme (not shown), were located below the arrow (B). Breast carcinoma was aneuploid (An), with a DNA index (DI) of 1.3. Note that most cells with DNA strand breaks were aneuploid, whereas very few diploid cells (Di) were labeled with b-dUTP. Upper insets, univariate frequency histograms representing biot.-dUTP incorporation; lower insets, DNA frequency histograms.

respond with apoptosis to topoisomerase inhibitors (22). Likewise, no incorporation of the precursor by HL-60 cells in G₁ or G₂, following their treatment with CAM, TN, or *m*-AMSA, was noted. Thus, "cleavable complexes" formed as a result of exposure of cells to these topoisomerase inhibitors (42–44), which are presumed to exist in drug-treated MOLT-4 or in G₁ HL-60 cells, were not labeled under present conditions. This is not surprising, considering that despite the DNA breakage, the 3'-hydroxyl termini in the "cleavable complexes" (42–44) are covalently linked to DNA topoisomerase I, and thus it was unlikely that they could become labeled in the TdT or NT assays. About 5% of the cells from the untreated HL-60 cultures undergo spontaneous apoptosis (40), and the equivalent cell number has been seen to incorporate the precursor in the control cultures.

Labeling of cells which die by necrosis, in the TdT reaction, is at least one order of magnitude lower compared with labeling of apoptotic cells (39). The cells containing primary DNA strand breaks induced by γ -irradiation with doses up to 25 Gy are also labeled distinctly less than apoptotic cells (39). Thus, clearly the number of DNA strand breaks (free 3'-hydroxyl termini) present in cells which die by apoptosis is markedly higher than during necrosis or immediately (primary strand breaks) following γ -irradiation.

At the very early stages of apoptosis, prior to nuclear fragmentation, the presence of DNA breaks was detected predominantly at the periphery of the nucleus. This would indicate that initiation of DNA degradation during apoptosis starts at the chromatin regions close to the nuclear envelope.

Of both methods tested, TdT offers certain advantages over the NT assay. The maximal intensity of labeling (cell fluorescence) of apoptotic cells was clearly higher with the TdT than the NT procedure; the former assay thus was more sensitive, providing a very pronounced distinction between unaffected and apoptotic cells, with practically no overlap in fluorescence values with cells that did not undergo apoptosis. The kinetics of dUTP incorporation also favors the TdT procedure: the incorporation was rapid and a 30-min incubation time was adequate to strongly label apoptotic cells, compared with several hours required for the NT assay to maximize labeling.

Multiple b-dUTP molecules are added per single DNA strand break in the TdT assay. The stoichiometry of this reaction, thus, in terms of amount of incorporated nucleotides per DNA break, may be in conflict with the sensitivity of the method. The chain length of the synthesized

polynucleotide is determined by the ratio of the monomer (biotinylated dUTP) to the initiator (free 3'-hydroxyl termini) (45). With a constant molar ratio of the monomer per cell, the reaction will approach completion (plateau) when the monomer is exhausted. Thus, if cells with different numbers of DNA breaks are compared, the length of the synthesized polynucleotide chain per DNA break may be quite different. At the plateau, therefore, where the sensitivity of the method is the highest, the intensity of cell fluorescence may not correlate with the number of DNA breaks per cell. To ensure that incorporation of labeled nucleotide is proportional to the number of DNA breaks, the reaction should be carried out at a high monomer:initiator ratio and be terminated prior to plateau.

Preferential apoptosis of S-phase cells was observed following treatment with DNA topoisomerase inhibitors CAM, TN, or *m*-AMSA but not FST. The first three inhibitors of DNA topoisomerases induce the formation of cleavable complexes (42–44, 46). Collision of DNA replication forks with such complexes has been proposed as a mechanism inducing cell death (46). The collision, apparently, triggers apoptosis in HL-60 cells, whereas in other cells, which do not respond immediately by apoptosis (*e.g.*, MOLT-4), cell death is delayed. In contrast, FST, which does not induce the cleavable complexes (47), causes the apoptosis of all cells, regardless of the phase of the cell cycle. The mechanism of cell kill by FST thus appears to be different from that of the drugs which form cleavable complexes.

There are several advantages that TdT or NT assays offer in the study of apoptosis: (a) The reactions are based on the direct labeling of 3'-hydroxyl termini of DNA breaks, and thus the lesions measured are identifiable at the molecular level. (b) The DNA breaks occur very early in apoptosis, prior to changes in cell morphology (Fig. 3); the method thus detects apoptotic cells which cannot yet be recognized based on changes in morphology. These assays can be applied, therefore, to study the very early events of apoptosis. (c) Since DNA content is measured in addition to DNA breaks, apoptosis can be related to the cell's position in the cycle or to DNA ploidy if cells of different ploidies are present in the same sample (*e.g.*, tumor and host cells in tumor specimens). Rapid flow cytometric analysis thus yields in seconds information on several thousand cells per sample, relating the number of DNA breaks in each cell to its position in the cycle or to its DNA ploidy, (b) The reactions can be carried out on fixed cells, and the time of cell storage in ethanol can vary, with no effect on the

detection of DNA breaks. It is possible, thus, to accumulate and transport clinical samples (e.g., to assess the response of the tumor to treatment by apoptosis), which can be subsequently analyzed. Our most recent data, obtained since the submission of this paper, indicate that the response of human leukemias to various drugs, in particular DNA topoisomerase II inhibitors, can be successfully monitored with the TdT assay (Fig. 9; Ref. 39).

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