Differential Induction of Secondary DNA Fragmentation by Topoisomerase II Inhibitors in Human Tumor Cell Lines with Amplified c-myc Expression

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ABSTRACT

In order to understand the cellular events associated with cell death after the formation of topoisomerase II-DNA cleavable complexes, we compared the induction of endonucleolytic DNA fragmentation by etoposide and its more potent analog, teniposide (VM-26) in the human cell lines HT-29 and HL-60. A new filter-binding assay is described, which allows rapid quantification of nonprotein-linked DNA fragmentation involved in apoptosis. Both cell lines showed similar loss of colony formation ability following 30 min of treatment with various VM-26 concentrations even though the initial topoisomerase II-mediated DNA single-strand break frequency was higher in HL-60 cells. DNA repair studies following drug removal indicated that VM-26-induced DNA breaks reversed rapidly and completely in HT-29 cells, while in HL-60 cells, the initial lesions persisted at and above 5 µM VM-26. In both cell lines, topoisomerase II cleavage complexes, as measured by DNA-protein cross-links by alkaline elution, reversed rapidly and completely within 2–3 h. Secondary DNA fragmentation resembling chromatin endonucleolytic cleavage by apoptosis could be detected in HL-60 cells 3 h after VM-26 or etoposide treatment but not in HT-29 cells. Secondary DNA fragmentation was also induced in the human colon cancer cell lines COLO 320, which have c-myc amplification. Since HL-60 cells also have c-myc amplification and HT-29 do not, it is possible that c-myc overexpression may be involved in secondary DNA fragmentation. Finally, our results indicate heterogeneity of cell death mechanisms after exposure to topoisomerase II inhibitors among human cancer cell lines.

INTRODUCTION

The mechanisms of cell killing by mammalian topoisomerase II inhibitors such as VP-16 and its more potent analog, VM-26, are still not understood. These drugs stabilize transient intermediates of topoisomerase II reactions which can be detected in mammalian cells as protein-linked DNA single- and double-strand breaks (1, 2). Studies of drug-resistant cell lines strongly suggest that the formation of topoisomerase II-mediated DNA breaks is essential for cell killing (1, 3–5). However, the reversibility of this DNA damage soon after drug removal (6, 7) implies that cell death must be mediated by other cellular lesions.

Recently, studies with embryonic RBCs (8), concanavalin A-stimulated splenocytes (9), and promyelocytic leukemia HL-60 cells (10–12) have suggested that topoisomerase II poisons could trigger cell killing by inducing DNA fragmentation into oligonucleosome-like fragments by apoptosis (13–16).

In the present study, we examined the importance of such a process in the case of VP-16 and VM-26 treatment. Kinetics of drug-induced DNA damage and secondary DNA fragmentation were studied at various times following drug treatment in the human promyelocytic leukemia cell line HL-60 and the human colon adenocarcinomas HT-29 and COLO 320. The occurrence of these DNA lesions was correlated with drug-induced cytotoxicity, as measured by colony formation assays. A new filter-binding assay is also described, which allows rapid quantification of nonprotein-linked DNA fragmentation involved in apoptosis.

MATERIALS AND METHODS

Chemicals. VM-26 and VP-16 (generously provided by Bristol Laboratories, Syracuse, NY) were dissolved in dimethyl sulfoxide at 10 mM and further diluted in water prior to each experiment. Radiolabeled precursors, [methyl-3H]thymidine (80.9 Ci/mmol) and [2-14C]thymidine (53.6 mCi/mmol), were purchased from New England Nuclear (Boston, MA). All other chemicals were of reagent grade and purchased either from Sigma Chemical Co. or from other local sources.

Cell Culture, Drug Treatment, and DNA Labeling. Human colon adenocarcinomas HT-29, COLO 320-HSR, and COLO 320-DMC were grown at 37°C in the presence of 5% CO2 in Eagle’s minimum essential medium (ABI, Columbia, MD), supplemented with 10% heat-inactivated fetal bovine serum (GIBCO, Grand Island, NY), 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM Eagle’s minimum essential medium nonessential amino acids, 100 units penicillin/ml, and 100 µg streptomycin/ml (ABI). The human promyelocytic leukemia HL-60 cells and mouse leukemia L1210 cells were grown in suspension culture at 37°C in the presence of 5% CO2 in RPMI 1640 medium (ABI) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), 2 mM glutamine, 100 units penicillin/ml, and 100 µg streptomycin/ml (ABI).

VM-26 and VP-16 treatments were for 30 min at the indicated concentrations. γ-Irradiation was performed on ice using a 100 µCi source at a dose rate of approximately 6 Gy/min. For measurement of DNA breaks, cells were prelabeled with [14C]thymidine (830 µCi/ml) for 48 h and then chased in isotope-free medium overnight. After VM-26 treatment, HT-29 and COLO 320 cells were collected, centrifuged, and mixed in 10 ml of ice-cold Hanks’ balanced salt solution containing drug. For DNA repair studies, cells were washed twice in 10 ml of complete medium without drug immediately following the 30 min treatment and further incubated in complete medium at 37°C for the specified times. Internal standard cells (L1210 cells) for alkaline elution were labeled with [3H]thymidine (0.2 µCi/ml) for 16 h and chased for at least 4 h in isotope-free medium prior to alkaline elution.

Colonies Formation Assays. After drug treatment, HT-29 cells were washed twice with 10 ml of Hanks’ balanced salt solution (37°C), trypsinized, and plated (in triplicate) at a density of 105, 104, and 103 cells/25-cm2 flask in 5 ml of fresh medium. Colonies were stained with methylene blue (17) and counted 14–15 days later (doubling times, ±5 h). HL-60 cells were spun down and washed twice by centrifugation/resuspension in 10 ml of drug-free Hanks’ balanced solution (37°C). Cells (105, 104, and 103) were seeded (in triplicate) into 12- x 75-mm tubes (Falcon 2058) in 4 ml of complete RPMI 1640 medium containing 0.1% of Difco Noble agar (37°C). Tubes were chilled on ice for 5 min and then transferred to the incubator (37°C, 5% CO2). Colonies were counted directly after 14 days (doubling times, ±8 ± 4 h). Results were expressed as the log of the survival fraction which was calculated from the plating efficiency of treated cells divided by the plating efficiency of untreated cells. Plating efficiencies were 50–60% and 20–40% for HT-29 and HL-60 cells, respectively.

Measurements of DNA Single-Strand Breaks by Alkaline Elution. DNA SSBs were assayed by alkaline elution as described previously (17, 18).
18). Briefly, labeled cells were loaded onto polycarbonate filters (2 μm pore size, 25 mm diameter; Nucleopore Corp., Pleasanton, CA.) and lysed with 5 ml of 2% SDS (w/v) and 0.5 mg/ml protease K at pH 10. Filters were then washed with 10 ml of 0.02 M EDTA (pH 10), and the DNA was eluted with a tetrnapropylammoniumhydroxide/EDTA solution, pH 12.1, containing 0.1% (w/v) SDS (flow rate: 0.08–0.12 ml/min; 5-min fractions). SSB frequencies were expressed in rad-equivalents using L1210 cells that had been irradiated (2000 rads) as internal standards. Alkaline elution was calibrated by using HL-60 or HT-29 cells that had been irradiated with 2000 rads. Approximately one break/106 nucleotides corresponds to 1000 rad-equivalents SSB (19).

Measurements of DNA-Protein Cross-Links by Alkaline Elution. For quantification of DPC, 14C-labeled control and drug-treated cells and 3H-labeled L1210 internal standard cells were irradiated on ice with 3000 rads and then immediately loaded onto prewashed ice-cold vinyl/ acrylic copolymer filters (Metricel membrane, 0.8 μm pore size, 25 mm diameter; Gelman Sciences Inc., Ann Arbor, MI). Cells were lysed with 5 ml of a solution containing 0.2% sodium sarkosyl-2 M NaCl-0.04 M EDTA (pH 10.0) which was subsequently washed from the filters with 5 ml of 0.02 M EDTA (pH 10.0). DNA was eluted from the filters overnight with tetrnapropylammoniumhydroxide/EDTA, pH 12.1, without SDS, at a flow rate of 0.02–0.03 ml/min. DPC were then quantitated using the “bound to one terminus” model (19).

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis. At specified times following drug removal, cellular DNA was extracted by a salting-out procedure as described previously (20). Electrophoresis was performed in 1.2% agarose gel in Tris-borate buffer (pH 8.0) containing 0.1% SDS (w/v) at 50 V for 14 h. After electrophoresis, DNA was visualized by ethidium bromide staining.

Quantification of DNA Fragmentation by Intact Chromatin Precipitation Assay. This assay was performed as described previously by Wyllie (14). Briefly, at indicated times after drug removal, 14C-thymidine-prelabeled cells were washed twice with ice-cold phosphate-buffered saline, collected by centrifugation, and disrupted in ice-cold phosphate-buffered saline containing 0.5% (v/v) Triton X-100 and 5 mM EDTA for 30 min at 4°C. The cellular lysates were then centrifuged at 12,000 ×g for 30 min in order to separate the low molecular weight DNA fragments (supernatant) from intact chromatin (pellet). Radioactivity was measured, and the amount of 14C-labeled DNA fragments released in the supernatant was expressed as a percentage of total 14C-labeled DNA.

Quantification of DNA Fragmentation by Filter-binding Assay. Usually DNA fragmentation was assayed under nondeproteinizing conditions. Approximately 0.5 × 106 prelabeled cells with 14C-thymidine were suspended in 10 ml of ice-cold Hanks' balanced salt solution and then loaded onto protein-adsorbing filters (vinyl/ acrylic copolymers filters, Metricel membrane, 0.8 μm pore size, 25 mm diameter; Gelman Sciences). Cells were then washed with an additional 10 ml of ice-cold Hanks' balanced salt solution. As soon as the washing solution had dripped through by gravity, lysis was performed with 5 ml of solution containing 0.2% sodium sarkosyl-2 M NaCl-0.04 M EDTA (pH 10.0). After the lysis had dripped through by gravity, it was washed from the filter with 10 ml of 0.02 M EDTA (pH 10.0). Filters were then processed as in the case of alkaline elution (19). Radioactivity was counted by liquid scintillation spectrometry in each fraction (loading, wash, lysis + EDTA wash, filter). DNA fragmentation was determined as the percentage of 14C-labeled DNA in the lysis fraction divided by total intracellular 14C-labeled DNA.

A variation of this filter-binding assay was also performed under deproteinizing conditions using nonprotein-adsorbing filters (polycarbonate filters), the strong ionic detergent SDS, and proteinase K in the lysis buffer in order to distinguish between protein-linked and nonprotein-linked DNA fragments. As described above, approximately 0.5 × 106 cells were suspended in 10 ml of ice-cold Hanks' balanced salt solution and then loaded onto a nonprotein-adsorbing filter (polycarbonate filter, 2 μm pore size, 25 mm diameter; Nucleopore). Cells were lysed with 5 ml of 2% (w/v) SDS and 0.5 mg/ml proteinase K at pH 10. Filters were then washed with 10 ml of 0.02 M EDTA (pH 10). DNA fragmentation was then quantified as described above.

RESULTS

VM-26-induced Cytotoxicity in HT-29 and HL-60 Cells. Exponentially growing cells were incubated for 30 min in the presence of various drug concentrations. At the end of drug treatment, cell survival was measured by colony formation assays. As shown in Fig. 1, survival of VM-26-treated cells decreased almost linearly as a function of the logarithm of drug concentration. VM-26-induced cytotoxicity was similar for both cell lines with approximately 95% cell killing at 10 μM.

Comparison of DNA Strand Breaks Induced by VM-26 in HT-29 and HL-60 Cells. Alkaline elution was used to examine the initial DNA SSB induced by 30 min of drug exposure in both cell lines. As illustrated in Fig. 2 (top), VM-26 induced more SSB in HL-60 than in HT-29 cells. A significant fraction of the DNA was found in the lysis fraction (Fig. 2, bottom). It increased similarly in both cell lines with drug concentration. The presence of DNA in the lysis fraction was probably due to DNA DSB since the pH of the lysis solution (pH 10) was too low to denature double-stranded DNA (19). The higher frequency of SSB at equitoxic VM-26 concentrations in HL-60 than in HT-29 cells is consistent with a lack of quantitative correlation between SSB and cytotoxicity between different cell lines (21–27).

Reversal of VM-26-induced DNA Break in HT-29 and HL-60 Cells. In HT-29 and HL-60 cells that had been treated for 30 min with 1 or 5 μM VM-26, SSB and DSB (Fig. 3) reversed rapidly after drug removal. At 10 μM teniposide, however, DNA breaks reversed more slowly, and at 100 μM they reversed even more slowly and not completely (approximately 50% at 6 h). DSB (DNA in lysis fraction) reversed more rapidly (Fig. 3, bottom) and more completely than SSB (Fig. 3, top).

In contrast, in HL-60 cells, DNA damage measured either as SSB (Fig. 4, top) or as the fraction of DNA in pH 10 lysis solution (DSB, Fig. 4, bottom) seems to be poorly repaired after drug removal. With the exception of 1 μM teniposide, at all other drug concentrations, repair of DNA damage was incomplete and lesions persisted after several hours following drug removal. Furthermore, at 5 and 10 μM VM-26, curves became biphasic: during the first 2 h after VM-26 removal, SSB and DSB reversed partially and thereafter persisted or even increased, suggesting the occurrence of secondary DNA fragmentation. This possibility was directly tested by measuring both
Fig. 2. Production of DNA strand breaks by VM-26 in HT-29 and HL-60 cells. Following drug treatment (30 min), DNA breaks were assayed by alkaline elution. Results are expressed in SSB rad-equivalents (top) or as the fraction of DNA in the pH 10 lysis fraction (DSB) (bottom). Points, means (bars, SD) of 3 independent experiments; O, HT-29; •, HL-60.

Fig. 3. Repair of VM-26-induced DNA damage in HT-29 cells. HT-29 cells were treated for 30 min with 1 (•), 5 (A), 10 (M), or 100 (*) µM VM-26. Drug was removed by washing cultures twice in drug-free medium, and cells were incubated further in drug-free medium. At the indicated times, DNA breaks were assayed by alkaline elution. Results are expressed in SSB rad-equivalents (top) or as the fraction of DNA in the lysis fraction of untreated cells was between 0.2 and 0.3.

Fig. 4. Repair of VM-26-induced DNA damage in HL-60 cells. HL-60 cells were treated for 30 min with 1 (•), 5 (A), 10 (M), or 100 (*) µM VM-26. Drug was removed by centrifugation in drug-free medium, and cells were incubated further in drug-free medium. At specified times, DNA breaks were assayed by alkaline elution. Results are expressed in SSB rad-equivalents (top) or as the fraction of DNA in the pH 10 lysis fraction (DSB) (bottom). Points, means (bars, SD) of 3 independent experiments. Fraction of DNA in the lysis fraction of untreated cells was between 0.2 and 0.3.

Fig. 5. Formation and reversal of DNA-protein cross-links and DNA single-strand breaks induced by VM-26 in HL-60 cells. Cells were treated for 30 min with 1 (•, O), 5 (A, A), or 10 (M, D) µM VM-26. At specified times after drug removal, SSB (closed symbols) and DPC (open symbols) were assayed by alkaline elution. Results are expressed in rad-equivalents. Points, means (bars, SD) of 3 independent experiments. Fraction of DNA SSB or the values of a representative experiment (DPC).
and HT-29 cells. Exponentially growing cells were treated with 10 μM VM-26 for 30 min. Drug was then removed and cells were incubated further in drug-free medium for the indicated times (h) before DNA extraction (number above each lane). A, agarose gel electrophoresis of DNA extracted from HL-60 cells. B, agarose gel electrophoresis of DNA extracted from HT-29 cells. Molecular weight markers (m) are λ-DNA restricted with HindIII obtained from BRL. Arrows, 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kilobases from top to bottom.

Fig. 6. Secondary DNA fragmentation following VM-26 treatment in HL-60 and HT-29 cells. Exponentially growing cells were treated with 10 μM VM-26 for 30 min. Drug was then removed and cells were incubated further in drug-free medium for the indicated times (h) before DNA extraction (number above each lane). A, agarose gel electrophoresis of DNA extracted from HL-60 cells. B, agarose gel electrophoresis of DNA extracted from HT-29 cells. Molecular weight markers (m) are λ-DNA restricted with HindIII obtained from BRL. Arrows, 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.6 kilobases from top to bottom.

Fig. 7. Quantification of secondary DNA fragmentation following VM-26 treatment in HL-60 and HT-29 cells. Cells were treated with 10 μM VM-26 for 30 min and then incubated in fresh drug-free medium for an additional 24 h after drug removal. Secondary DNA fragmentation was determined by the filter-binding assay under nonproteinizing conditions. Results are expressed as the fraction of fragmented DNA (relative to total DNA) and values are means ± SE (n indicates the number of independent determinations).

Table 1 Secondary DNA fragmentation following VM-26 treatment in human cancer cell lines overexpressing the c-myc oncogene

Cells were treated with 10 μM VM-26 for 30 min and then incubated in drug-free medium for an additional 24 h after drug removal. Secondary DNA fragmentation was determined by the filter-binding assay under nonproteinizing conditions. Results are expressed as the fraction of fragmented DNA (relative to total DNA) and values are means ± SE (n indicates the number of independent determination).

DISCUSSION

The present report indicates that at equitoxic doses of VM-26, two human tumor cell lines, HL-60 and HT-29, exhibit different DNA fragmentation following 30 min of drug exposure. Although the initial DNA damage induced by VM-26 was slightly different between the cells lines, both showed similar drug sensitivity. In HT-29 cells, topoisomerase II-mediated DNA breaks were reversed rapidly and completely. This observation is consistent with the one made in human lung adenocarcinoma (A549) and in Chinese hamster lung fibroblast...
(DC3F) cells, in which the initial DNA breaks were reversed within 1–2 h after drug removal (7, 27). In contrast, in HL-60 cells, DNA breaks failed to reverse after drug removal. Lack of reversal was not due to persistence of topoisomerase II cleavage complexes since DPC reversed completely within 3 h after drug removal. Rather, the DNA breaks observed 3 h after VM-26 removal were due to a secondary DNA fragmentation, consistent with endonucleolytic cleavage and programed cell death (apoptosis). This secondary DNA fragmentation was not detected even 48 h after drug removal in HT-29 cells. Therefore, not all cell lines fragment their DNA following treatment with topoisomerase II inhibitors. For example, HT-29 and DC3F cells show no fragmentation (present study; Ref. 27), while it is evident for HL-60 and COLO 320 cells, as well as for mitogen-stimulated lymphocytes (9–12). Similarly, a recent study with actinomycin D showed that some cell lines undergo apoptosis and others do not (32). Taken together these observations support the concept of heterogeneity in cell death pathways among different human tumor cell lines treated with the same cytotoxic agent.

Programed cell death or apoptosis has been described as occurring in response to various cytotoxic agents including a variety of chemotherapeutic drugs (9–12, 33–36). Although the biochemical events preceding apoptosis are not yet fully known, previous studies attempted to correlate the activation of programed cell death with expression of specific oncogenes, mainly c-ha-ras, c-myc, c-bcl-2, or P53 (16, 37-39). Our finding that the c-myc-amplified HL-60, COLO 320-DMC, and COLO 320-HSR cells fragmented their DNA following VM-26 treatment is consistent with the possibility that c-myc overexpression might be related to programed cell death. More experiments are under way in order to establish more unequivocally a relationship between c-myc overexpression and apoptosis.

In this report, we described a new filter-binding assay to measure endonucleolytic chromatin fragmentation. By using this assay, we have been able to demonstrate that the DNA fragments observed in agarose as a DNA ladder (Fig. 5) were not linked to proteins, since the DNA fragments were detected under nondeproteinizing conditions. Such a distinction is important when apoptosis is induced by topoisomerase inhibition since this inhibition by itself produces protein-linked DNA breaks (1, 2). When compared with the current methodologies considered indicative of apoptosis (electrophoresis of DNA fragments or Coulter sizing), the filter assay, using prelabeled breaks (1, 2), When compared with the current methodologies considered indicative of apoptosis (electrophoresis of DNA fragments or Coulter sizing), the filter assay, using prelabeled breaks (1, 2).

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