Fas-dependent and -independent Mechanisms of Cell Death following DNA Damage in Human Colon Carcinoma Cells

Istvan Petak, David M. Tillman, Franklin G. Harwood, Rudolf Mihalik, and Janet A. Houghton

Department of Molecular Pharmacology, St. Jude Children’s Research Hospital, Memphis, Tennessee 38105

ABSTRACT

In thymidylate synthase-deficient (TS−) colon carcinoma cells, thymineless death is mediated via Fas/Fas ligand (FasL) interactions after thymidine deprivation and inhibited by the Fas-inhibitory monoclonal antibody NOK-1. The objective of the study was to elucidate whether other modes of DNA damage induced by doxorubicin, topotecan, and etoposide (VP-16) could elicit a similar cytotoxic response in TS− cells by signaling via the Fas death receptor. After a 72-h drug exposure, a loss in clonogenic survival was not prevented by NOK-1. TS− cells were resistant to the induction of thymineless death after thymidine deprivation but were not cross-resistant to doxorubicin, topotecan, or VP-16. A close correlation was found between acute induction of apoptosis (24 h) and up-regulated expression of FasL at high concentrations of each of the three agents (0.3–3 μM doxorubicin, 0.3–3 μM topotecan, and 10–90 μM VP-16), which was caspase dependent but Fas independent. At all drug concentrations, cell cycle distribution analyses demonstrated marked accumulation of cells in the G2-M phase. At nanomolar drug concentrations, prolonged arrest of TS− cells in G2-M phase resulted in the up-regulation of FasL expression and the delayed appearance of apoptotic cells (6 days), which could also be inhibited by the general caspase inhibitor Z-VAD-FMK, but not by NOK-1 or Fas-Fc. In clonogenic assays, Z-VAD-FMK did not rescue cells treated with VP-16 in contrast to treatment with CH-11 or thymineless stress, suggesting an irreversible commitment to cell death in G2-M phase. Expression of FasL at all drug concentrations appeared to be unrelated to the mechanism of drug-induced apoptosis. This was in contrast to the Fas-dependent regulation of thymineless death, which could be inhibited by blocking Fas/FasL interactions.

INTRODUCTION

We have demonstrated previously that TS− cells selected from the wild-type GC/c7 human colon carcinoma cell line undergo thymineless death after dThd withdrawal, which is regulated by Fas/FasL interactions and signaling via the Fas death receptor (1). TS− cells undergo acute apoptosis after dThd deprivation and simulate the thymineless state induced by treatment of parental GC/c1 cells with FUra/LV combinations (2). Thymineless stress induced by dThd withdrawal in TS− cells or FUra/LV treatment of GC/c1 cells initiated an imbalance in the dATP/dTTP pools, followed by inhibition of DNA synthesis (2). Subsequent DNA damage comprised the formation of single- and double-stranded DNA breaks (3), nucleosomal DNA ladders (2, 4), and morphological features of apoptosis (5). The response of cells to cytotoxic stress and DNA damage may depend on the cell type, together with the type and extent of DNA damage. Some reports have indicated that certain DNA-damaging agents may induce apoptosis via Fas signaling (6–9), whereas others suggest that stress-induced apoptosis caused by drug treatment may be independent of Fas (10–12). The objective of this study was to elucidate whether, in addition to DNA damage induced by dThd starvation, other modes of DNA damage induced in TS− cells by chemotherapeutic agents could also induce cell death by signaling via the Fas death receptor. Data demonstrated a close correlation between loss in clonogenic survival, decrease in numbers of cells, the acute induction of apoptosis, and up-regulated expression of FasL in TS− cells in response to thymineless stress and that blocking Fas/FasL interactions by the NOK-1 inhibitory MoAb completely protected cells from loss in clonogenic survival over a period of 5 days. In contrast, other types of DNA damage induced by doxorubicin (intercalator and dual topoisomerase I and II inhibitor), VP-16 (inhibitor of topoisomerase II), and topotecan (inhibitor of topoisomerase I) induced loss in clonogenic survival at considerably lower drug concentrations (50–100-fold) than those that initiated acute apoptosis. Under these conditions, no protection from loss in clonogenic potential or the induction of apoptosis was obtained by blocking Fas/FasL interactions. The loss in ability of cells to clone at low drug concentrations was due to the induction of a prolonged G2-M-phase arrest that ultimately led to cell death and apoptosis after 6 days. This delayed form of apoptosis could be inhibited by caspase inhibitors, but loss in clonogenic survival could not be prevented by inhibition of caspase activation. At high drug concentrations, an acute form of cell death was apparent at 24 h, where a close correlation existed between the induction of apoptosis and loss in numbers of cells. However, despite drug-induced expression of FasL, this also appeared to be independent of Fas signaling because blocking Fas/FasL interactions did not prevent the drug-induced acute apoptotic response. Data indicated that at low concentrations of DNA-damaging agents, cells accumulated in the G2-M phase of the cell cycle in the absence of acute apoptosis, which resulted in delayed apoptosis and loss in clonogenic survival after drug removal, independent of signaling via Fas but dependent on caspase activation. In contrast, the regulation of thymineless death in TS− cells was dependent on signaling via the Fas death receptor, indicating that that not all forms of DNA damage induce subsequent apoptosis via the Fas signaling pathway in colon carcinoma cells.

MATERIALS AND METHODS

Cell Lines. A TS− human colon carcinoma cell line selected from parental GC/c1 cells, which is deficient in thymidylate synthase mRNA and protein and auxotrophic for dThd, has been well characterized (4). TS− cells were maintained in the presence of folate-free RPMI 1640 containing 10% dialyzed fetal bovine serum (dPBS), 80 nM 6′-methylpentahydrololate, 712 μM Ca2+, and 20 μM dThd.

Clonogenic Assays. TS− cells were plated at a density of 3000 cells/well in 6-well plates (Falcon). Cells were also treated with the NOK-1 MoAb (PharMingen; 100 ng/ml) or a mouse IgG1 isotype-matched control MoAb (PharMingen; 100 ng/ml) at the time of plating. After overnight attachment,
cells were washed with 2 ml of HBSS (37°C) and subsequently deprived of dThd by refeeding with dThd-free medium containing the respective Abs. At various times for up to 7 days, cells were rescued by adding dThd (20 μM) to individual wells, and clonogenic survival was determined 11 days after dThd restoration (1). Alternatively, cells were plated in the presence of NOK-1 and subsequently treated for 72 h with doxorubicin (0.01–100 nM), topotecan (0.01–100 nM), or VP-16 (0.1–1 μM) in the presence of dThd and in either the absence or presence of Abs. Clonogenic survival was determined 11 days after removal of the drugs (1). To determine the influence of caspase activation on loss in clonogenic survival after the induction of thymineless stress or treatment with anti-Fas (CH-11; 3–30 ng/ml; MBL International Corp.) or VP-16 (0.1–1 μM; Bristol-Myers-Squibb), additional clonogenic survival assays were conducted in the presence of the general caspase inhibitor Z-VA-D-FMK (100 μM; Enzyme Systems Products) during 72-h exposures.

Analysis of Cell Numbers. Cells were plated in 6-well plates at a density of 400,000 cells/well. After overnight attachment, cells were treated with doxorubicin (0.3–3 μM), topotecan (0.3–3 μM), and VP-16 (10–30 μM) for periods of up to 96 h. Cells were subsequently enumerated using a Coulter Particle Counter (5).

Apoptosis and Cell Cycle Analyses. TS− cells were plated at a density of 400,000 cells/well in 6-well plates. After overnight attachment, cells were deprived of dThd or treated with varied concentrations of doxorubicin, topotecan, or VP-16 for periods of up to 96 h before harvest. Both the floating cells and attached cells were pooled after trypsinization, fixed in 70% ethanol, and stored at −20°C before analysis. Apoptotic cells were detected as a sub-G1 fraction after propidium iodide staining and analysis using a Becton Dickinson FACSscan (13, 14). The influence of cotreatment with Z-VA-D-FMK (100 μM) for 72 h on the induction of acute apoptosis (72-h exposure to 10–100 μM VP-16) or delayed apoptosis (72 h after a 72-h coincubation with 0.1–1 μM VP-16) was also examined. Furthermore, the effect of NOK-1 (50 μg/ml) or Fas-Fe (50 μg/ml; a chimeric fusion protein generously provided by Dr. Carl Ware; the La Jolla Institute for Allergy and Immunology, San Diego, CA), which prevent Fas/FasL interactions, was examined on the induction of rapid apoptosis (72-h exposure to 30 μM VP-16) or delayed apoptosis (72 h after a 72-h exposure to 1 μM VP-16). For evaluation of the cell cycle distribution, samples were collected as described and immediately centrifuged and resuspended in 0.005% propidium iodide containing 0.1% sodium citrate and 0.1% Triton X-100, filtered, and analyzed by FACSscan.

Expression of FasL. FasL expression was determined by semiquantitative RT-PCR in TS− cells at various times during dThd deprivation or during treatment with doxorubicin (0.3–3 μM), topotecan (0.3–3 μM), or VP-16 (10–90 μM) for up to 96 h, as described previously (Ref. 5; acute apoptosis). FasL expression was also determined at 72 h after exposure to 1 μM VP-16 and at 72 h after the end of drug exposure (delayed apoptosis). β-Actin was used as a control to monitor RT-PCR amplification efficiency and the quality of the cDNA from 2 μl of the template at 25 cycles, as reported previously (5). PCR products were separated by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining and UV light illumination. Quantitation was by optical densitometry of the reverse gel image using a Hewlett Packard Scan Jet IIIC. Intensity of the signal was determined by comparison with a calibrated photographic gray scale (Kodak) in the linear range of detection.

Selection of CH-11-resistant Clones. TS− cells were selected for resistance to Fas-mediated apoptosis by treatment with the cytolytic anti-Fas MoAb CH-11 (MBL International Corp.). Cells (100 × 105) were mutagenized with EMS (300 μg/ml) in T-175 flasks for 22 h. Cells were cultured for an additional 48 h in the absence of EMS and subsequently selected for 72 h in 200 ng/ml CH-11. After growth for an additional 7 days in the absence of CH-11, surviving cells were mutagenized with EMS and selected in CH-11 (200 ng/ml) for a second time. Surviving colonies were allowed to grow for 2 weeks and were subsequently ring-cloned and placed in T-12.5 flasks in 2 ml of dFBS-containing medium with dThd (20 μM), and the colonies were expanded. Three colonies were selected for further evaluation.

RESULTS

Fas Dependency of Thymineless Death in TS− Cells. We demonstrated previously that autocrine signaling via Fas was involved in the regulation of thymineless stress-induced cell death after dThd withdrawal in TS− cells (1). Fig. 1A demonstrates that after dThd withdrawal from asynchronously growing TS− cells, complete protection from thymineless death and loss in clonogenic survival oc-

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Fig. 1. Clonogenic survival of TS− cells after the induction of thymineless stress (A) or after a 72-h exposure to varied concentrations of topotecan (B), doxorubicin (C), or VP-16 (D), as described in “Materials and Methods.” Results are the mean ± SD of triplicate determinations at each drug concentration.
curred when cells were incubated simultaneously with NOK-1. Cells incubated with the IgG1 isotype-matched control in the absence of dThd demonstrated the same kinetics of cell kill as cells exposed to dThd deprivation alone: cells lost 50% of clonogenic potential in 72 h; and only 5% of cells formed colonies at 5 days after dThd deprivation. In contrast, cells coincubated with NOK-1 in the absence of dThd were completely protected from thymineless death at this time.

**Fas Independence of the Sensitivity to DNA-damaging Agents.**

To determine the influence of the Fas signaling pathway on the regulation of cell death in TS<sup>−</sup> cells in response to different modes of DNA damage, clonogenic survival of cells after a 72-h exposure to varied concentrations of topotecan (Fig. 1B), doxorubicin (Fig. 1C), and VP-16 (Fig. 1D) was determined in the absence or presence of NOK-1. Thus, loss in clonogenic survival was induced by all three agents, with less than 10% clonogenicity remaining at topotecan, doxorubicin, and VP-16 concentrations of 30 nm, 30 nm, and 1 μM, respectively, and IC<sub>50</sub> values of 10, 5, and 150 nm, respectively. In contrast to the protection of TS<sup>−</sup> cells from thymineless death by simultaneous incubation with the NOK-1 MoAb, no protection from the cytotoxic effects of these three agents was provided during coincubation with the inhibitory Ab for 72 h. Furthermore, no protection was provided by NOK-1 when cells were coincubated with the MoAb additionally after drug treatment (data not shown).

**Sensitivity of Fas-resistant TS<sup>−</sup> Clones to DNA-damaging Agents.**

Characterization of the Fas resistance phenotype demonstrated wild-type sequence for Fas in all clones after DNA sequencing of the death domain (and extracellular binding domain). However, Fas-resistant clones were found to express lower levels of Fas (reduced by 40%) as determined by RNase protection and FACS analyses (data not shown). To confirm the lack of involvement of the Fas death receptor in regulation of the cellular sensitivity of TS<sup>−</sup> cells to doxorubicin, topotecan, and VP-16, studies were conducted in five clones. When asynchronous populations of Fas-resistant clones were exposed to thymineless stress, >80% survival was obtained at 5 days after dThd withdrawal, in contrast to only 5% in the case of TS<sup>−</sup> cells (Fig. 2A), demonstrating that TS<sup>−</sup> cells selected for resistance to CH-11 and hence for resistance to signaling via Fas were also cross-resistant to the induction of thymineless death after dThd deprivation.

The sensitivities of CH-11-resistant TS<sup>−</sup> clone 2 and clone 5 to doxorubicin, topotecan, and VP-16 in a clonogenic survival assay after 72-h drug exposures are shown in Fig. 2, B–D, in comparison to the sensitivities of TS<sup>−</sup> cells to the three agents. No change in the sensitivities to the three DNA-damaging agents was obtained in CH-11-resistant clones, confirming the lack of influence of signaling via Fas on the sensitivity of TS<sup>−</sup> cells to these agents.

**Induction of Apoptosis and Expression of FasL.**

The influence of thymineless stress or drug exposure on the induction of apoptosis in TS<sup>−</sup> cells, on the numbers of cells surviving after 72 h, and on the expression of FasL was determined. After dThd withdrawal (Fig. 3), apoptosis determined by FACS analysis was detected by 24 h and increased to almost 45% at 5 days (Fig. 3). This correlated temporally with the loss in numbers of surviving cells, which decreased to <50% at 5 days, and to the progressive induction of the expression of FasL (Fig. 3).

After 72-h drug exposures, a concentration-dependent increase in apoptotic cells was detected at concentrations of 0.3–3 μM doxorubicin and topotecan and 10–90 μM VP-16 (Fig. 4A), but not at lower drug concentrations. Analysis of the drug concentration-dependent loss in the number of surviving cells after treatment for 72 h correlated with the percentage of cells undergoing apoptosis at this time (Fig. 4). FasL expression, as determined by RT-PCR, was induced at the same concentrations of doxorubicin, topotecan, and VP-16 after 72-h exposures that induced apoptosis and loss of cells after drug exposure. Furthermore, expression of FasL also increased with increasing drug concentrations (Fig. 4C). There was also a close correlation between the three parameters during drug treatment and incubation time at 1–30 μM drug concentrations (Fig. 5). However, the concentrations of drugs that induced apoptosis and up-regulated expression of FasL were considerably higher (50-fold) than those that effectively reduced clonogenic survival.
Apoptosis was thus determined to be acute under these conditions. The fate of cells treated with doxorubicin (0.1 μM), topotecan (0.1 μM), and VP-16 (1 μM) at low drug concentrations that reduced clonogenic survival by 90% (IC₉₀) was also examined by FACS analysis (Fig. 6). At the end of drug exposure (72 h), only a small fraction of cells were undergoing apoptosis (15%). However, after removal of drugs and further incubation in drug-free medium, 40–50% of treated cells demonstrated evidence of apoptosis after an additional 3 days, and apoptosis continued to increase with increasing time of incubation. On examination of the expression of FasL, a low level of FasL expression was detected after a 72-h exposure to VP-16.

Fig. 3. Relationship between the induction of apoptosis (A), decrease in numbers of surviving cells (B), and up-regulated expression of FasL (C) in TS⁻ cells after dThd withdrawal. The experiments were conducted and quantitated as described in “Materials and Methods.”

Fig. 4. The percentage of TS⁻ cells undergoing apoptosis (A), loss in numbers of surviving cells (B), and up-regulated expression of FasL (C) increased with increasing concentrations of doxorubicin, VP-16, and topotecan 72 h after drug exposure. Experiments were conducted as described in “Materials and Methods.” Results for apoptosis and survival assays represent the mean ± SD of three determinations/point.
Expression of FasL (determined by RT-PCR) during incubation with doxorubicin (1 μM) and survival assays represent the mean ± SD of triplicate determinations at each time point. Drug-treated cells had accumulated in G2-M phase at the end of drug exposure, and survival enter a prolonged and irreversible G2-M-phase arrest that ultimately leads to apoptosis.

Influence of Drug Treatment on Cell Cycle Distribution. The distribution of TS- cells in G0-G1, S phase, and G2-M phase after treatment with concentrations of doxorubicin (0.3–3 μM), topotecan (0.3–3 μM), and VP-16 (10–90 μM) that induced acute apoptosis are shown in Fig. 7A. In response to doxorubicin and topotecan, there was an increase in G2-M-phase cells from 20% to between 60% and 75% after drug treatment, and in response to VP-16, there was an increase in G2-M-phase cells up to 44%. As the concentrations of drugs were increased in cells undergoing acute apoptosis, the percentage of cells in G2-M phase decreased, correlating with the increase in percentage of apoptotic cells.

At low concentrations of drugs that reduced clonogenic survival (0.1 μM doxorubicin; 0.1 μM topotecan; 1 μM VP-16), 70%–80% of drug-treated cells had accumulated in G2-M phase at the end of drug exposure (72 h; Fig. 7B). Furthermore, there was a decline in the fraction of TS- cells in G2-M phase with increasing time of incubation. Data suggested that TS- cells arrested in G2-M phase at low concentrations of DNA-damaging agents that reduce clonogenic survival enter a prolonged and irreversible G2-M-phase arrest that ultimately leads to apoptosis.

Effect of Inhibition of Caspase Activation on Apoptosis and Clonogenic Survival. The effect of the general caspase inhibitor Z-VAD-FMK (100 μM) on the induction of both acute and delayed apoptosis during a 72-h exposure to VP-16 was determined and compared with the effect on apoptosis induced by thymineless stress and after treatment with anti-Fas (CH-11, 3–50 ng/ml). During incubation with VP-16 (10–30 μM) or CH-11 (50 ng/ml) and under dThd-free conditions, Z-VAD-FMK inhibited the induction of acute apoptosis (Fig. 8A). In addition, 72-h exposure to low concentrations of VP-16 (0.1–1 μM) in the presence of the caspase inhibitor also resulted in a reduction in the degree of apoptosis induced (Fig. 8B).

Although Z-VAD-FMK protected TS- cells from loss in clonogenic survival after the induction of thymineless stress and ligation of Fas after treatment with CH-11, no protection was demonstrated after treatment with 0.1 μM VP-16 (Fig. 9).

Effect of Blocking Fas/FasL Interactions on the Induction of Acute and Delayed Apoptosis. TS- cells were treated with VP-16 at concentrations of 30 μM (acute apoptosis) or 1 μM (delayed apoptosis) for 72 h in the absence or presence of NOK-1 (50 μg/ml) or Fas-Fc (50 μg/ml) to prevent the ligation of FasL to its receptor. Apoptosis was subsequently evaluated at the end of drug exposure (acute apoptosis) or after an additional 72 h under VP-16-free conditions (delayed apoptosis) and in the presence or absence of inhibitors of Fas/FasL interactions (Fig. 10). Blocking ligation of Fas under both conditions did not prevent either acute or delayed apoptosis induced by VP-16.

DISCUSSION

Fas is a cell surface receptor comprising a type I integral membrane protein that expresses a cytoplasmic death domain and belongs to the tumor necrosis factor receptor superfamily (15). After up-regulated expression and ligation of the natural ligand, FasL, in response to stimuli including thymineless stress (1, 16) and cytotoxic agents (6–9), apoptosis is initiated after rapid signaling from the receptor. Human colonic epithelial cells constitutively express Fas (17), and an intact Fas signaling pathway has been shown to be capable of regulating apoptosis in human colon carcinoma cell lines (5, 18, 19).

The cytotoxic activity of anticancer agents has been described to be both dependent (6–9) and independent (10–12) of signaling via Fas. In Jurkat T leukemia cells, apoptosis induced by doxorubicin appeared to proceed via activation of FasL, analogous to the dependence of activation-induced apoptosis in T lymphocytes on transcription and expression of FasL (6). Furthermore, competitive binding of either FasL (using a Fas-Fc chimeric protein; Ref. 6) or Fas (using a

Fig. 5. Induction of acute apoptosis. Time course for the appearance of apoptotic TS- cells (A; determined by FACS analysis), loss in numbers of surviving cells (B), and up-regulated expression of FasL (C; determined by RT-PCR) during incubation with doxorubicin (1 μM), topotecan (1 μM), or VP-16 (30 μM), as described in “Materials and Methods.” Apoptosis and survival represent the mean ± SD of triplicate determinations at each time point.

Fig. 6. Induction of delayed apoptosis. Incubation of TS- cells in drug-free medium after 72-h exposure to IC50 concentrations of doxorubicin (0.1 μM), topotecan (0.1 μM), or VP-16 (1 μM). Percentages of apoptotic cells were determined in triplicate. Expression of FasL, as determined by RT-PCR, is described in “Materials and Methods.”
neutralizing anti-Fas Ab) effectively inhibited death induced by VP-16 and VM-26 (7), suggesting that apoptosis induced by these agents was mediated via Fas signaling. Of interest was that interference with Fas/FasL interactions inhibited anisomycin- but not radiation-induced apoptosis in Jurkat cells, suggesting that not all stress stimuli involving DNA damage use the same cell death pathway (10).

In this study, we demonstrated that Fas-dependent loss in clonogenic survival of TS \(^2\) cells after dThd withdrawal correlated closely with the loss in cell numbers over a period of 5 days, the onset and induction of apoptosis determined by FACS analysis, and the up-regulated expression of FasL. The importance of thymineless death in the mechanism of cell killing by FUrLV combinations in human colon carcinoma cell lines (16), xenografted tumors in mice (20, 21), and colon carcinomas in humans (22, 23) has been well established. Furthermore, up-regulation of the expression of Fas by IFN-\(\gamma\) in colon carcinoma cell lines has been shown to be important in increasing the cytotoxic activity of FUrLV, which is dependent on thymineless stress-induced DNA damage (16). Therefore, taken together, data suggest that Fas is an important determinant of the extent to which colon carcinomas are sensitive to the induction of thymineless death.

In contrast, the cytotoxic response to both nanomolar and micromolar concentrations of doxorubicin, topotecan, and VP-16 was independent of regulation via Fas. At low drug concentrations, drug-induced loss in clonogenic survival was not inhibited by the anti-Fas inhibitory MoAb NOK-1. Of interest was that FasL expression was induced under these conditions. However, this appeared to be an associated stress response and was not causative of the induction of apoptosis because the delayed apoptotic response after VP-16-induced G\(_2\)-M-phase arrest at a low drug concentration could not be inhibited by blocking Fas/FasL interactions with either NOK-1 or Fas-Fc. A close correlation between the loss in numbers of surviving cells, induction of apoptosis, and up-regulated expression of FasL was obtained in response to treatment with doxorubicin, topotecan, and VP-16, but at considerably higher drug concentrations (50-fold) than required to induce loss in clonogenic survival. Whereas evidence suggests that TS \(^-\) cells undergo apoptosis and cell loss after dThd deprivation as cells enter S phase, resulting in no apparent accumulation of cells in S phase during this time, the cellular response to DNA-damaging agents was different. It was apparent that at all drug concentrations, TS \(^-\) cells were arrested in the G\(_2\)-M phase of the cell cycle and that at the higher concentrations (micromolar concentrations), this was associated with acute induction of apoptosis and up-regulated expression of FasL. At lower drug concentrations (nanomolar concentrations), the initial arrest of cells in G\(_2\)-M occurred in the absence of apoptosis. However, it is evident that these cells were already committed to cell death because apoptosis ultimately took place after the equivalent of several cell doublings. Furthermore, the caspase inhibitor Z-VAD-FMK did prevent the induction of delayed apoptosis induced by VP-16, indicating that this late form of cell death was a caspase-dependent apoptosis, after commitment of TS \(^-\) cells to cell death in the G\(_2\)-M phase of the cell cycle. Thus, studies conducted at drug concentrations that induce apoptosis in the acute phase may not necessarily be indicative of the actual mechanism by which cells ultimately commit to cell death. It is also evident that the induction of apoptosis because the delayed apoptotic response after VP-16-induced G\(_2\)-M-phase arrest at a low drug concentration could not be inhibited by blocking Fas/FasL interactions with either NOK-1 or Fas-Fc. A close correlation between the loss in numbers of surviving cells, induction of apoptosis, and up-regulated expression of FasL was obtained in response to treatment with doxorubicin, topotecan, and VP-16, but at considerably higher drug concentrations (50-fold) than required to induce loss in clonogenic survival. Whereas evidence suggests that TS \(^-\) cells undergo apoptosis and cell loss after dThd deprivation as cells enter S phase, resulting in no apparent accumulation of cells in S phase during this time, the cellular response to DNA-damaging agents was different. It was apparent that at all drug concentrations, TS \(^-\) cells were arrested in the G\(_2\)-M phase of the cell cycle and that at the higher concentrations (micromolar concentrations), this was associated with acute induction of apoptosis and up-regulated expression of FasL. At lower drug concentrations (nanomolar concentrations), the initial arrest of cells in G\(_2\)-M occurred in the absence of apoptosis. However, it is evident that these cells were already committed to cell death because apoptosis ultimately took place after the equivalent of several cell doublings. Furthermore, the caspase inhibitor Z-VAD-FMK did prevent the induction of delayed apoptosis induced by VP-16, indicating that this late form of cell death was a caspase-dependent apoptosis, after commitment of TS \(^-\) cells to cell death in the G\(_2\)-M phase of the cell cycle. Thus, studies conducted at drug concentrations that induce apoptosis in the acute phase may not necessarily be indicative of the actual mechanism by which cells ultimately commit to cell death. It is also evident that the induction of

![Fig. 7. A, cell cycle phase distribution of TS \(^-\) cells after 72-h drug exposures to increasing concentrations of doxorubicin, VP-16, or topotecan that cause the acute induction of apoptosis. B, cell cycle distribution of TS \(^-\) cells was examined at the end of drug exposure (72 h) and at intervals after drug removal. Data were analyzed by FACS analysis as described in “Materials and Methods.”](image-url)
acute apoptosis was not dependent on Fas/FasL interactions because simultaneous incubation of VP-16 with NOK-1 or Fas-Fc failed to prevent induction of apoptosis. This suggested that, like the delayed apoptotic response, the induction of acute apoptosis was independent of Fas. Both acute and delayed apoptosis required caspase activation, as demonstrated by an inhibitory response in the presence of Z-VAD-FMK.

There have been several reports of dual mechanisms of action demonstrated by DNA-damaging agents including doxorubicin (24), daunomycin (24), VP-16 (25, 26), and bleomycin (27), dependent on the concentrations of the agents used. For doxorubicin and daunomycin, EC_{50} concentrations led to G_{2}-M-phase arrest and cytostasis in HeLa cells in the absence of inhibition of DNA synthesis, ultimately resulting in delayed apoptosis and cell death. In contrast, at 10× EC_{50} concentrations, apoptosis was detected as early as 3 h after drug treatment (24). Different cellular responses to VP-16 have been reported in thymocytes at different drug concentrations (25). In addition, Lock and Strubinskiene (26) reported dual modes of death in HeLa cells induced by VP-16. One mode was prevented by Bcl-2, which inhibited loss in cell viability and induction of apoptosis, whereas the second, which induced loss in clonogenic survival and comprised the formation of giant, multinucleated cells characteristic of mitotic catastrophe, was not affected by Bcl-2. Similarly, at low concentrations of bleomycin exposure, DC-3F Chinese hamster lung fibroblasts arrested in G_{2}-M phase and became enlarged and polynucleated before dying. This response paralleled the mitotic death observed after ionizing radiation (27) and also after cisplatin-induced cellular cytotoxicity (28, 29). In contrast, apoptosis and rapid DNA fragmentation were observed at high bleomycin concentrations.

It is therefore evident that the acute induction of apoptosis after treatment of cells with DNA-damaging agents is not a prerequisite for the induction of cellular sensitivity to these agents. The data of Smith et al. (30) in a small cell lung carcinoma cell line suggested that traverse of G_{1}-S phase and early S phase in the presence of VP-16 led to trapping and enhanced availability of topoisomerase IIα and subsequent irreversible arrest of cells in G_{2}-M phase in the presence of limited DNA fragmentation. After G_{2}-M phase arrest of DC-3F cells at low bleomycin concentrations, cells died after a time period equivalent to three doubling times (27). In the current study, we demonstrated a similar G_{2}-M-phase arrest in the TS- human colon carci-
nomal cell line at low, nanomolar concentrations of doxorubicin, topotecan, and VP-16, resulting in loss of clonogenic survival. In contrast to the induction of thymineless death, this was independent of regulation via Fas. However, similar to the studies described above, the induction of G2/M-phase arrest in TS cells at nanomolar drug concentrations ultimately led to the induction of apoptosis. Both acute and delayed forms of apoptosis are caspase dependent; however, inhibition of apoptosis by caspase inhibitors did not restore clonogenic survival.

It is not clear which form of cell death may be responsible for the antitumor activity of these drugs in vivo. However, inhibitors of both topoisomerase I and II have demonstrated marked schedule dependence. Against human tumor xenograft models, topotecan and irinotecan have demonstrated superior therapeutic efficacy when administered at low doses in protracted schedules in comparison to more intense shorter-term schedules of administration (31, 32). In clinical trials, prolonged administration of VP-16 demonstrated superior therapeutic efficacy when administered at low topoisomerases I and II have demonstrated marked schedule dependence.

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