Cancer Chemother Pharmacol (1995) 36: 181–188

ORIGINAL ARTICLE

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Fludarabine- and gemcitabine-induced apoptosis: incorporation of analogs into DNA is a critical event

Received 19 May 1994 / Accepted 25 October 1994

Abstract The nucleoside analogs fludarabine and gemcitabine inhibit cellular DNA synthesis by two different mechanisms: (1) direct termination of DNA strand elongation after the triphosphate of each drug is incorporated into DNA; and (2) indirect inhibition of DNA synthesis by decreasing cellular dNTPs through inhibition of ribonucleotide reductase. The present study demonstrated that incorporation of the analogs into DNA is critical for the cytotoxic action of these drugs in human T lymphoblastoid CEM cells. S phase cells, which actively incorporated the analogs into DNA, were most sensitive to the cytotoxic action of these compounds. A relatively short-term (5–24 h) cessation of cellular DNA synthesis without analog incorporation was not sufficient to cause cell death. The drug-treated cells died through apoptosis characterized by generation of internucleosomal DNA fragmentation and apoptotic morphology. Induction of high molecular mass (50–500 kb) DNA fragmentation was also observed in cells undergoing apoptosis; this type of DNA degradation was strongly correlated with the analog-induced cell death process. Inhibition of the analog incorporation into DNA by aphidicolin blocked both types of DNA fragmentation and apoptotic morphology, indicating the essential role of analog incorporation into DNA in drug-induced cell death.

Key words Apoptosis · Nucleoside analog · DNA incorporation

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This work was supported in part by grants CA28596 and CA55164 from the National Cancer Institute, Department of Health and Human Services, and by grant DHP-1 from the American Cancer Society

Introduction

Cell proliferation and apoptosis (programmed cell death) are two fundamental biological processes whose delicate balance is essential for embryogenesis, tissue development, and the maintenance of normal tissue organization [1, 6, 24, 40]. Failure to appropriately execute the cell death program when needed may be a factor in carcinogenesis [32]. Cells undergoing apoptosis show typical changes in morphology which include cell shrinkage, nuclear condensation and margination, fragmentation of nuclei, cell membrane blebbing, and rupture of the cells into debris [40]. Cleavage of cellular DNA at the internucleosomal linkages to generate nonrandom DNA fragments (or nucleosomal ladders) is a characteristic biochemical marker of apoptosis [1, 3, 6]. Recent studies indicate that generation of high molecular mass DNA fragments may also be an important event in apoptotic cell death [12, 25, 36]. In experimental systems, cells can be induced to undergo apoptosis by depletion of appropriate growth factors [5, 39] or by the addition of exogenous stimuli such as glucocorticoid [3], tumor necrosis factor [31], cell surface-specific antibodies [17, 33], irradiation [30, 38], and a variety of cytotoxic drugs [2, 4, 8, 19, 20]. Induction of apoptosis in cancer cells in experimental systems may provide a basis for the development of new therapeutic approaches.

The nucleoside analogs 9-β-D-arabinofuranosyl-2-fluoroadenine (fludarabine, F-ara-A), 1-β-D-arabinosylcytosine (ara-C), and 2′,2′-difluorodeoxycytidine (gemcitabine, dFdC) are anticancer agents with clinically proven therapeutic activity. These drugs trigger apoptotic cell death in human leukemia cells [9, 11, 27]. Each analog is incorporated into DNA and, to different extents, terminates the extension of the elongating DNA strand [15, 16, 21]. The incorporation of these nucleosides into DNA correlates with their cytotoxicity [15, 16, 21]. Thus, it appears that cells active in DNA synthesis, or S phase cells, are most susceptible to the cytotoxic action of these compounds. These observations led us to hypothesize that S phase-specific incorporation of the nucleoside analogs into DNA

is involved in the initiation of apoptosis. The present investigation was conducted to test this hypothesis. Our results demonstrate that both F-ara-A and dFdC induced apoptosis during S phase in synchronized human T lymphoblastoid cells, and that the incorporation of the analogs into DNA is required for the initiation of the cell death program.

Materials and methods

Chemicals and reagents

The nucleoside analogs F-ara-A and dFdC were kindly supplied by Dr. V. L. Narayanan (Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute) and Dr. L. W. Hertel (Lilly Research Laboratories), respectively. Aphidicolin and cycloheximide were from Sigma Chemical Co. (St. Louis, Mo.). Proteinase K, alkaline phosphatase (from calf intestine), and RNase (DNase-free) were from Boehringer Mannheim Co. (Indianapolis, Ind.). T4 polynucleotide kinase was from United States Biochemicals Co. (Cleveland, Ohio). [γ-^{32}P]ATP was from ICN Radiochemicals (Irvine, Calif.). The Betascope 603 blot analyzer, used in the quantitation of radioactivity in dried agarose gels, was manufactured by Betagen (Waltham, Mass.).

Cell culture and synchronization

Cells of the human T lymphoblastoid cell line CCRF-CEM (CEM cells) were maintained in exponential growth in RPMI-1640 suspension culture medium supplemented with 5% fetal bovine serum. Cell doubling time was approximately 24 h under these culture conditions. The cells were synchronized by double aphidicolin block [22]. CEM cells were incubated with 2 μM aphidicolin for 24 h, washed into fresh medium for 12 h, and then incubated with 2 μM aphidicolin for another 24 h. This treatment blocked the cells at the G_1/S boundary. The cells were washed and incubated in fresh medium to allow their progression through the cell cycle. At various times after release from aphidicolin block, portions of the cells were assayed for DNA synthesis activity by [^{3}H]thymidine incorporation (1 μCi/ml, 15 min) and analyzed for cell cycle phase distribution by flow cytometry after the cells were treated with pepsin and RNase and stained with propidium iodide.

Drug exposures and examination of cell morphology and membrane integrity.

At various times after release from aphidicolin block, the synchronized cells were incubated with 10 μM F-ara-A or 1 μM dFdC for 4 h. Aliquots were centrifuged onto glass slides, fixed with methanol, and stained with Wright-Giemsa stain. Cell morphology was examined by light microscopy using a Nikon HFX-II microscope. Membrane integrity was analyzed by trypan blue exclusion using a hemacytometer.

DNA fragmentation assays

To detect and quantitate internucleosomal DNA fragmentation, DNA was purified from the control and drug-treated cells and labelled with ^{32}P at the 5′-end as described previously [11]. The DNA samples were fractionated in a 2% agarose gel, stained with ethidium bromide, and photographed. An autoradiograph of the same gel was made after the gel had been dried at 60 °C under vacuum. The radioactivity in the DNA bands was quantitated using the Betascope 603 blot analyzer as described previously [11].

Pulsed-field gel electrophoresis was used to analyze high molecular mass DNA fragmentation. Exponentially growing CEM cells were prelabelled with [^{14}C]Thd for 48 h (0.02 μCi/ml every 12 h, × 4) and then either incubated with the indicated concentrations of F-ara-A or dFdC for 4 h or synchronized at S phase with double aphidicolin block before the addition of the analogs. The cells were embedded in 0.6% agarose plugs containing 75 mM NaCl, 5 mM EDTA, and 5 mM Tris-HCl, pH 7.8. The plugs were cooled to 4 °C for 20 min and then incubated in lysis buffer containing 1% sarkosyl, 50 mM EDTA, 50 mM Tris-HCl, pH 7.8, and 0.2 mg/ml proteinase K at 45 °C for 16 h. After washing with 10 mM Tris-HCl buffer (pH 7.8) containing 1 mM EDTA for 3 h with a buffer change each hour, the cellular DNA in the plugs was analyzed with a CHEF-DR II pulsed-field gel electrophoresis system (Bio-Rad Laboratories, Richmond, Calif.) at 200 V with a switch time of 50 s for 16 h at 7 °C. The electrophoresis buffer contained 50 mM Tris-borate, pH 8.2, and 1 mM EDTA. The gel was stained with ethidium bromide and photographed. The radioactivity associated with the high molecular mass DNA fragments was quantitated using the Betascope after the gel had been dried at 60 °C under vacuum.

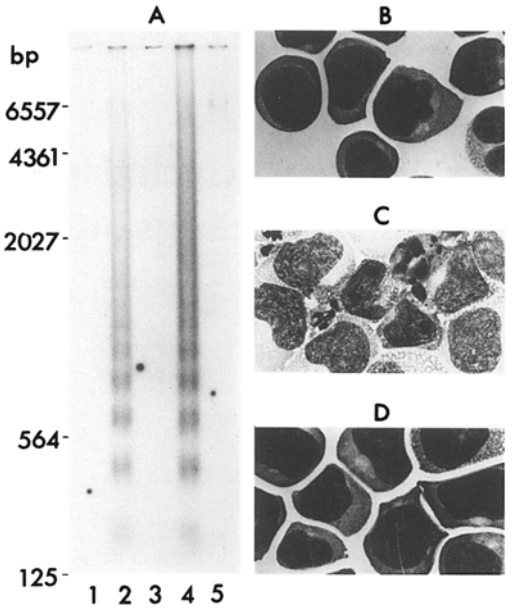

Fig. 1 A–D Induction of apoptosis in exponentially growing CEM cells by F-ara-A and dFdC. **A** Autoradiograph of a 2% agarose gel containing DNA samples from cells treated as follows: *lane 1* control, *lane 2* 10 μM F-ara-A for 4 h, *lane 3* 10 μM F-ara-A and 2 μM aphidicolin for 4 h, *lane 4* 1 μM dFdC for 4 h, *lane 5* 1 μM dFdC plus 2 μM aphidicolin for 4 h. **B** Light microscopic morphology of control CEM cells. **C** Light microscopic morphology of CEM cells treated with 10 μM F-ara-A. **D** Light microscopic morphology of CEM cells treated with 10 μM F-ara-A and 2 μM aphidicolin (×360)

Results

Induction of apoptosis by F-ara-A and dFdC was first evaluated in exponentially growing CEM cells. When the cells were incubated with 10 μM F-ara-A (Fig. 1A, lane 2) or 1 μM dFdC (lane 4) for 4 h, cellular DNA was cleaved into internucleosomal fragments with molecular masses of integer multiples of 180–200 bp, the DNA "ladders" characteristic of apoptosis. Microscopic examination of the F-ara-A-treated sample (Fig. 1C) and dFdC-treated sample (data not shown) revealed changes in cell morphol-

ogy typically seen in cells undergoing apoptosis. These include cell shrinkage, nuclear condensation, fragmentation of nuclei, and rupture of the apoptotic cells into debris. DNA fragmentation (Fig. 1A, lane 1) and the morphological changes were not observed in the control sample not treated with the analogs (Fig. 1B). When the cells were incubated with F-ara-A or dFdC in the presence of 2 μM aphidicolin, the drug-induced internucleosomal DNA fragmentation was blocked (Fig. 1A, lanes 3 and 5). No apparent apoptotic morphology was seen in cells incubated either with F-ara-A and aphidicolin (Fig. 1D) or with dFdC plus aphidicolin (data not shown). Because aphidicolin is an inhibitor of DNA polymerases and, at a concentration of 2 μM, blocks more then 95% of DNA synthesis in CEM cells [15], the data presented in Fig. 1 indicate that inhibition of analog incorporation into DNA blocked the cell death process. Thus, incorporation of the nucleoside analogs into DNA appears to be critical in drug-induced apoptosis.

Because F-ara-A and dFdC are incorporated into DNA during replication in S phase cells, we evaluated the susceptibility of CEM cells to apoptosis induced by the analogs at different stages of the cell cycle. CEM cells were synchronized by incubation with aphidicolin as described in Materials and methods. Flow cytometric analysis demonstrated that the double aphidicolin treatment blocked the cells at the G_1/S boundary (Fig. 2A), and of these cells, 83% accumulated at G_1 phase and 9% at early S phase (Fig. 2B). After washing into fresh medium, the population proceeded synchronously through the cell cycle. Between 4 and 6 h after incubation in fresh medium, the majority (73%) of the cells were in S phase, later the population shifted into G_2/M phases. The time course of DNA synthesis in the synchronized cell population as measured by [^{3}H]thymidine incorporation (Fig. 2C) was correlated with the percentage of S phase cells determined by flow cytometry.

At various times after release from aphidicolin block, corresponding to different stages of progression through the cell cycle, portions of the culture were incubated with either 10 μM F-ara-A or 1 μM dFdC for 4 h. Cells were then evaluated for damage to membrane integrity by trypan blue exclusion. As shown in Fig. 2C, exposure of the predominantly G_1 phase cells (at time 0 h with 2 μM aphidicolin in the culture medium) to either F-ara-A or dFdC for 4 h did not result in loss of dye-excluding cells. However, as the cells entered S phase, identical incubation with the compounds caused increasing numbers of cells to lose the ability to exclude trypan blue. The maximum effect was seen at 6 h when >70% of the cells were in S phase. In contrast, F-ara-A or dFdC had little effect on membrane integrity after cells had moved out of S phase. Thus, it appeared that the degree of damage to cellular membranes by the nucleoside analogs was proportional to the number of cells in S phase.

Cell samples treated with the nucleoside analogs at different stages of the cell cycle were examined by microscopy for morphologic alterations. Exposure of the S phase cells (6 h after release of the aphidicolin block) to F-ara-A

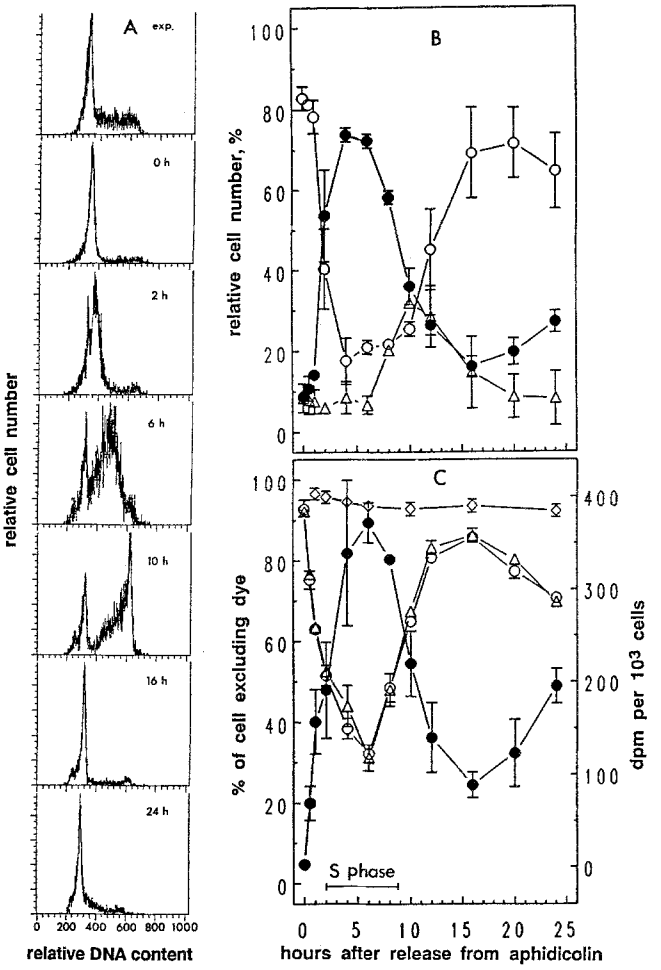

Fig. 2 A–C Cell cycle-specific damage to the cell membrane by nucleoside analogs. **A** Flow cytometric analysis of exponentially growing (*exp.*) cells and of synchronized cells at the indicated time after release from aphidicolin block. **B** Cell cycle phase distribution of the synchronized cells (● cells in S phase, ○ G_1 phase, Δ G_2/M phases). **C** Time course of DNA synthesis activity in control cells (●) and damage to membrane integrity by a 4-h incubation with 10 μM F-ara-A (○), 1 μM dFdC (Δ), or without drug (◇) at the indicated times after release from aphidicolin block. DNA synthesis was determined by [^{3}H]thymidine incorporation. Cell membrane integrity was evaluated by trypan blue exclusion assay and is expressed as the percentage of intact cells excluding the vital dye. Each point represents the mean ±SD of two experiments

caused morphologic changes typical of apoptosis in a major portion of the cells. As indicated in Fig. 3B, some cells had shrunk and exhibited nuclear condensation, and other exhibited clusters of membrane blebs and fragmented nuclei or had ruptured into debris. In contrast, when cells were incubated with F-ara-A 16 h after release from aphidicolin block (predominantly non-S phase cells at this time), most of the cells appeared morphologically intact (Fig. 3C). The single apoptotic body in Fig. 3C probably reflects a small number of S phase cells sensitive to F-ara-A at that time. These results strongly suggest that S phase cells are most susceptible to induction of apoptosis by F-ara-A. Similar results were observed in experiments with dFdC (data not shown). For yet unknown reasons,

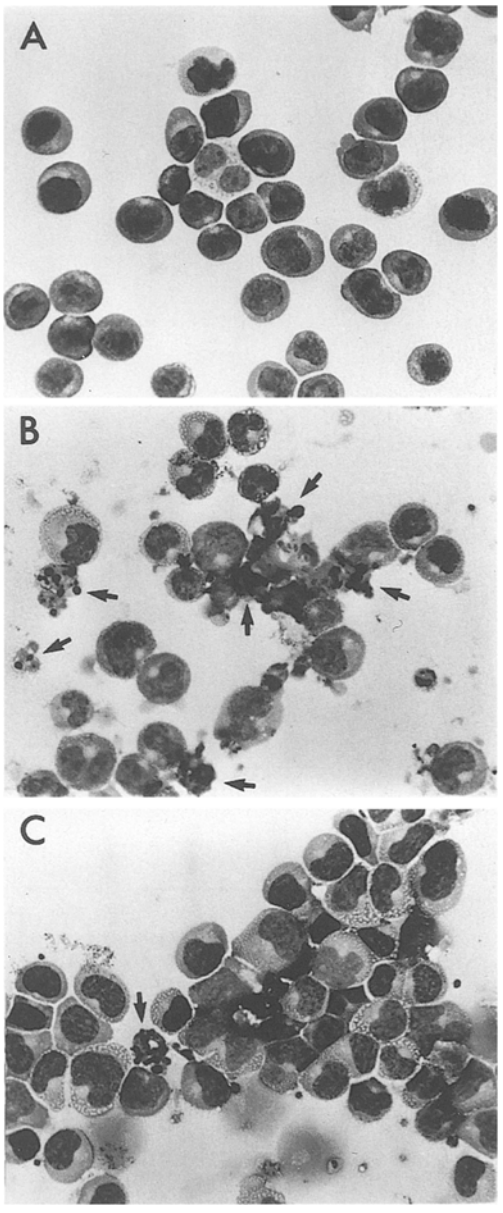

Fig. 3 A–C S phase-specific apoptosis induced by F-ara-A. **A** Microscopic morphology of exponentially growing CCRF-CEM cells. **B** Six hours after release from the aphidicolin block, cells were incubated with 10 μM F-ara-A for an additional 4 h. **C** Identical incubation with F-ara-A 16 h after release from aphidicolin block. The *arrows* indicate apoptotic cells

cytoplasmic vacuoles were observed in both S phase cells and non-S phase cells treated with F-ara-A (Fig. 3 B, C). This might reflect the disturbance of cellular metabolism by the drug. If so, the metabolic disturbance by F-ara-A was not cell cycle-specific.

To quantitate the internucleosomal DNA fragmentation in cells treated with the analogs at different phases in the cell cycle, DNA from control and F-ara-A-treated cells was labelled with ^{32}P and analyzed in a 2% agarose gel. Ethidium bromide staining of the gel (Fig. 4A) demonstrated that each lane contained approximately the same amount of DNA. DNA fragments of nucleosomal multimers, however, were barely visible under these conditions and did not allow accurate quantitation by densitometric scanning. Autoradiography of the same gel, however, clearly revealed separation of DNA fragments or nucleosomal ladders (Fig. 4B). Evaluation of individual lanes in Fig. 4B indicated that in the absence of F-ara-A treatment, nucleosomal DNA fragments were not detectable in DNA samples from exponentially growing cells (lane 2) or cells synchronized by aphidicolin (lane 3). Thus, incubation of CEM cells with aphidicolin alone in the synchronization procedure did not cause any detectable DNA fragmentation. Furthermore, when the synchronized cells were maintained in 2 μM aphidicolin and incubated with 10 μM F-ara-A for an additional 4 h, no nucleosomal ladders were generated (lane 4). These results indicate that incorporation of F-ara-A into DNA is critical for induction of apoptosis in a synchronized population, as was the case in exponentially growing cells (Fig. 1A, lane 3).

When the synchronized cells were washed free of aphidicolin and incubated in fresh medium to allow their progression into S phase, exposure to the same dose of F-ara-A (10 μM, 4 h) resulted in a cell cycle-dependent DNA fragmentation (Fig. 4, lanes 5–15). Nucleosomal ladders were readily detectable 30 min after release from aphidicolin block (Fig. 4, lane 5). The amount of fragmented DNA varied depending on the stage of the cell cycle. The amount of F-ara-A- or dFdC-induced fragmented DNA increased as the cells progressed into S phase (Fig. 5). DNA fragmentation reached its peak value at 6 h, when the cells were predominantly in S phase (Fig. 2C). However, when the S phase cells (at 6 h) were incubated with F-ara-A or dFdC in the presence of 2 μM aphidicolin, DNA fragmentation was inhibited (data not shown). Figure 5 also demonstrates that the amount of fragmented nucleosomal DNA decreased as the cells cycled out of S phase with the trough level at 16 h, when most of the cells were not in S phase. These results are in agreement with the time courses of the F-ara-A-induced cell cycle-dependent membrane damage (Fig. 2) and changes in cellular morphology (Fig. 3).

Previous studies have demonstrated that F-ara-A and dFdC are incorporated into DNA and terminate strand elongation. Most of the incorporated F-ara-AMP residues are located at the 3′-ends of DNA strands [15] whereas dFdCMP residues are located at the penultimate positions [16]. In the present study, we compared the amount of analog incorporation in exponentially growing cells and in synchronized S phase cells by incubating the respective cell populations with either 10 μM [^{3}H]F-ara-A or 1 μM [^{3}H]dFdC for 4 h and then quantitating the radioactivity associated with purified DNA. Incorporation of [^{3}H]F-ara-AMP into DNA was 2.1-fold higher in S phase cells than in the exponentially growing cells. A 1.6-fold increase in [^{3}H]dFdCMP incorporation was observed in S phase cells.

Recent studies have suggested that high molecular mass DNA fragmentation is associated with apoptosis [12, 13, 25, 35, 36]. Because F-ara-A and dFdC terminate DNA strand elongation [15, 16] and cause deletion of DNA

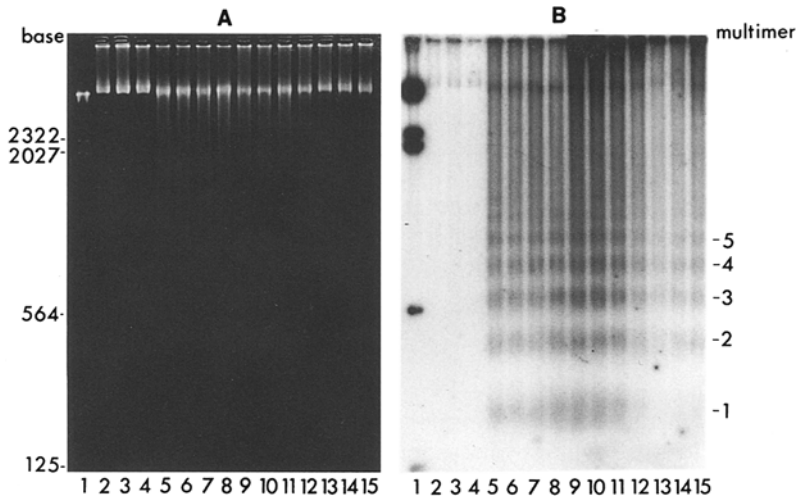

Fig. 4 A, B Internucleosomal DNA fragmentation induced by F-ara-A in synchronized CCRF-CEM cells. DNA (1 μg) from control and drug-treated cells at different stages in the cell cycle was labelled with ^{32}P and fractionated in a 2% agarose gel as described in Materials and methods. **A** Photograph of the gel stained with ethidium bromide. **B** Autoradiograph of the same gel. *Lane 1* DNA molecular mass markers II (Boehringer Mannheim), *lane 2* DNA from exponentially growing cells, *lane 3* DNA from synchronized cells without F-ara-A treatment, *lane 4* DNA from synchronized cells treated with 10 μM F-ara-A for 4 h while the cells were maintained in 2 μM aphidicolin, *lanes 5 to 15* DNA from the synchronized cells incubated with 10 μM F-ara-A at 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 20, and 24 h after release from aphidicolin, respectively. The greater intensity of radioactivity at the high molecular mass region in *lanes 9 and 10* reflects the existence of large DNA fragments which were produced as a consequence of F-ara-A incorporation [12, 13] and provided more DNA strand ends for 5′-end labeling

fragments up to 30 kb in cells that survive short term F-ara-A incubation [14], we used pulsed-field gel electrophoresis to investigate the possibility that generation of larger DNA fragments might be involved in drug-induced apoptosis. Figure 6 shows the analysis of DNA from either exponentially growing or synchronized S phase CEM cells (4 h after release from double aphidicolin block) incubated with the analogs. High molecular mass DNA fragments, with a predominant size of 50 kb, were visualized in exponentially growing cells incubated with 10 μM F-ara-A (lane 2) or 1 μM dFdC for 4 h (lane 5). The amount of drug-induced large DNA fragments increased substantially when S phase cells were incubated with identical concentrations of analogs for the same period (Fig. 6, lanes 3 and 6). Molecular size analysis of the DNA fragments using the Betascope indicated that the size range of the large fragments extended between 50 and 500 kb. In contrast, incubation of CEM cells with F-ara-A or dFdC in the presence of aphidicolin blocked the

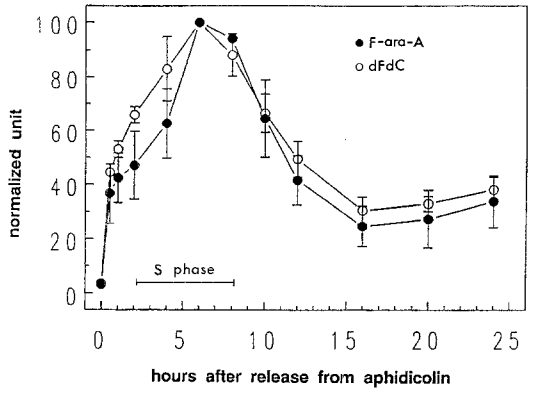

Fig. 5 Quantitation of nucleosomal DNA fragmentation induced by F-ara-A (●) or dFdC (○). Cellular DNA was labelled and analyzed as described in the legend to Fig. 4. The radioactivity in the first five nucleosomal bands was quantitated using the Betascope. The sum of the radioactivity of the five bands in the 6-h sample (2679 ± 250 cpm and 3613 ± 209 cpm for F-ara-A- and dFdC-treated samples, respectively) was assigned the value of 100 units for normalization of the other samples. Each data point is the mean ± SD of two to three determinations

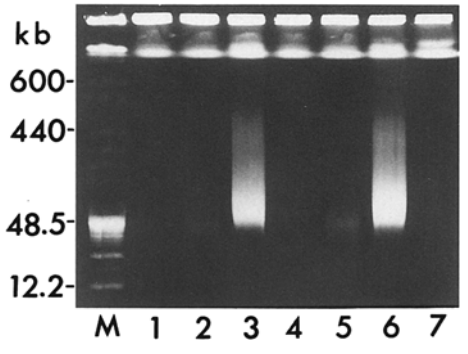

Fig. 6 High molecular mass DNA fragmentation induced by F-ara-A and dFdC in CEM cells. Cells incubated with or without drugs were embedded in agarose plugs and the cellular DNA was analyzed by pulsed-field gel electrophoresis as described in Materials and methods. *Lane M* Molecular mass markers, *lane 1* exponentially growing CEM cells without drug treatment, *lane 2* exponentially growing CEM cells incubated with 10 μM F-ara-A for 4 h, *lane 3* S phase CEM cells incubated with 10 μM F-ara-A for 4 h, *lane 4* exponentially growing CEM cells incubated with 10 μM F-ara-A plus 2 μM aphidicolin for 4 h, *lane 5* exponentially growing CEM cells incubated with 1 μM dFdC for 4 h, *lane 6* S phase CEM cells incubated with 1 μM dFdC for 4 h, *lane 7* exponentially growing CEM cells incubated with 1 μM dFdC and 2 μM aphidicolin for 4 h

generation of large DNA fragments (Fig. 6, lanes 4 and 7). Thus, incorporation of the analogs into DNA appears to be required for drug-induced high molecular mass DNA fragmentation. These results are consistent with internucleosomal DNA fragmentation and morphologic changes observed in the two cell populations exposed to the analogs, and support the conclusion that S phase cells are most sensitive to induction of apoptosis by F-ara-A and dFdC.

Discussion

The present study demonstrates that the nucleoside analogs F-ara-A and dFdC exert their cytotoxic action by induction of apoptosis. Inhibition of the analog incorporation into DNA by aphidicolin blocked drug-induced DNA fragmentation and prevented the generation of apoptotic morphology. Thus, the incorporation of the analogs into DNA appears to be a critical event in triggering the apoptotic cell death program. Because incorporation of the analogs into DNA results in termination of DNA synthesis [15, 16], it is important to determine whether the cessation of DNA replication per se or the incorporated analog in DNA is responsible for the initiation of apoptosis.

Several lines of evidence suggest that incorporation of the analogs into DNA is the key element in causing cytotoxicity. First, when CEM cells were incubated either with aphidicolin alone or with aphidicolin plus F-ara-A or dFdC, DNA synthesis was inhibited and no analog was incorporated into the DNA. Neither internucleosomal DNA fragmentation nor apoptotic morphology was observed under these conditions. High molecular mass DNA fragments were not generated when incorporation of F-ara-A or dFdC into DNA was inhibited. Second, S phase cells, which actively synthesize DNA and incorporate analogs during replication, were most sensitive to induction of apoptosis by the analogs. Inhibition of DNA synthesis by aphidicolin in the synchronized population did not trigger apoptosis. Rather, aphidicolin blocked nucleoside analog-induced apoptosis in S phase cells. Third, in a cell clonogenicity study [15], 5-h exposure of CEM cells to 10 μM F-ara-A inhibited DNA synthesis by 95% and caused a 75% loss of cell survival. Incubation of CEM cells with 1 μM aphidicolin for 5 h resulted in the same degree of DNA synthesis inhibition, but did not lead to loss of cell clonogenicity. Thus, it is clear that a short-term (5 h) interruption in DNA synthesis is not a lethal event to CEM cells, whereas incorporation of analogs into DNA appears to be critical in causing cell death.

An alternative interpretation of the inhibitory effect of aphidicolin on drug-induced apoptosis is that DNA replication might be required to convert the drug-induced DNA damage to a form necessary for initiation of apoptosis. It is possible that although aphidicolin inhibited DNA synthesis, replication with drug-induced DNA damage was prevented and the apoptotic program was not triggered. However, this is unlikely because aphidicolin inhibits the incorporation of F-ara-A and dFdC into DNA and thus eliminates the direct DNA chain termination effect of the analogs. Although DNA damage might be mediated by an indirect mechanism rather than by direct chain termination, the nature and extent of such damage is unknown. In a separate experiment, in which CEM cells were first incubated with 10 μM F-ara-A for 1 h to allow incorporation into DNA and then aphidicolin was added for an additional 3 h, DNA fragmentation occurred despite the presence of aphidicolin (data not shown). This is consistent with the conclusion that incorporation of analogs into DNA is critical in drug-induced apoptosis.

Both F-ara-A and dFdC inhibit ribonucleotide reductase and cause a decrease of intracellular dNTPs [7, 10, 26]. It is possible that the disturbance of deoxynucleotide pools by F-ara-A or dFdC may also contribute to induction of apoptosis by the analogs. Because arabinosylcytosine (ara-C), which is not an inhibitor of ribonucleotide reductase, induces apoptosis in human myeloid leukemia cells [9], it appears that inhibition of ribonucleotide reductase is not a requisite for nucleoside analogue-mediated apoptosis. In a separate study, we have demonstrated that cellular dNTP pools increase slightly (about 10%) in the presence of aphidicolin, presumably due to its inhibition of dNTP incorporation into DNA. Thus, it appears that aphidicolin does not affect the activity of ribonucleotide reductase. No significant change in the formation of cellular F-ara-ATP and dFdCTP was observed in the presence of aphidicolin (data not shown).

Two types of DNA fragmentation were observed in analog-induced apoptosis. Both F-ara-A and dFdC induced internucleosomal DNA fragmentation. Nucleosomal DNA ladders have been detected in a variety of experimental systems and have been used as a biochemical marker of apoptosis. A Ca^{2+}-dependent endonuclease is thought to be responsible for the cleavage of DNA at internucleosomal linkages [3, 23]. The second type of drug-induced DNA cleavage was associated with the generation of higher molecular mass DNA fragments, ranging between 50 and 500 kb. The pulsed-field gel electrophoresis used in this study was run under non-denaturing conditions; therefore the fragments reflected double-strand breaks of cellular DNA. Because F-ara-A and dFdC were incorporated only into the newly synthesized DNA strand and terminated its elongation, the double-strand breaks suggest that an active cellular process may have been involved in the cleavage of the template DNA strand. The high molecular mass DNA fragmentation is also observed in rat fibroblasts induced to undergo apoptosis by serum depletion [25] and in thymocytes treated with dexamethasone and DNA topoisomerase II-reactive agents [35]. It has been proposed that the cleavage of chromatin into large DNA fragments may be the initial stage of DNA fragmentation in apoptosis [36]. However, neither the enzyme responsible for high molecular mass DNA fragmentation nor the mechanism that triggers this cleavage has been identified.

In the case of nucleoside analog-induced apoptosis, our preliminary studies have demonstrated that high molecular mass DNA fragmentation is a Ca^{2+}-independent event [13]. Chelation of intracellular Ca^{2+} blocked the analog-induced

internucleosomal DNA fragmentation, but did not affect the generation of large DNA fragments. Thus, it appears that a Ca^{2+}-independent endonuclease is involved in this process.

The mechanism by which F-ara-A and dFdC induce DNA fragmentation and eventually lead to cell death is not clear at the present time. We speculate that during DNA replication there is a surveillance mechanism, or sensor, that detects DNA damage or, in the present case, DNA strand termination by the incorporated analogs. We hypothesize that the sensor may be functionally homologous to the *E. coli* RecA protein [29, 34] or the nucleotide-excision repair protein complex in yeast [37]. This sensor recognizes DNA damage and initiates DNA repair. Because DNA polymerases δ and ε have $3'->5'$ exonuclease activities that are assumed to serve a proofreading function for mismatched nucleotides, these enzymes may be considered candidates to participate in such a surveillance mechanism. When F-ara-A or dFdC is incorporated into DNA, the analogs terminate the elongating DNA strand at the incorporation sites. Although DNA damage by a terminally incorporated analog is detected by the sensor, repair of the lesions would be difficult because excision of the incorporated analogs is not efficient [16, 18] and ligation of the analog-terminated DNA is inhibited by F-ara-A monophosphate at the 3′-terminus and by free F-ara-ATP [41]. Failure to repair the terminally incorporated analogs would eventually lead the sensor mechanism to trigger apoptosis. Because enzymes such as DNA polymerases and ligases are involved in both replication and repair, the same or a similar surveillance mechanism might be associated with both processes. If so, it should be possible to introduce DNA damage in quiescent cells by agents such as UV irradiation and cisplatin, and thus evoke DNA repair. The active DNA repair synthesis in quiescent cells should allow the incorporation of nucleoside analogs into the repair patches. This incorporation would lead to inhibition of further repair synthesis due to DNA chain termination activity of the analogs. Assuming that the same or similar surveillance and response mechanisms are associated with DNA repair and replication, it should be possible to induce apoptosis in quiescent cells by combining nucleoside analogs with agents that damage DNA. This hypothesis is currently under investigation [28].

References

1. Arends MJ, Morris RG, Wyllie AH (1990) Apoptosis, the role of the endonuclease. Am J Pathol 136:593
2. Barry MA, Behnke CA, Eastman A (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol 40:2353
3. Cohen JJ, Duke RC (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J Immunol 132:38
4. Evans DL, Dive C (1993) Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. Cancer Res 53:2133
5. Fairbairn LJ, Cowling GJ, Reipert BM, Dexter TM (1993) Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. Cell 74:823
6. Fesus L, Davis PJA, Piacentini M (1991) Apoptosis: molecular mechanisms in programmed cell death. Eur J Cell Biol 56:170
7. Gandhi V, Plunkett W (1988) Modulation of arabinosylnucleoside metabolism by arabinosylnucleotides in human leukemia cells. Cancer Res 48:329
8. Gorczyca W, Gong J, Ardelt B, Traganos F, Darzynkiewicz Z (1993) The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. Cancer Res 53:3186
9. Gunji H, Kharbanda S, Kufe D (1991) Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-β-D-arabinofuranosylcytosine. Cancer Res 51:741
10. Heinemann V, Xu Y-Z, Chubb S, Sen A, Hertel LW, Grindey GB, Plunkett W (1990) Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2′,2′-difluorodeoxycytidine. Mol Pharmacol 38:567
11. Huang P, Plunkett W (1992) A quantitative assay for fragmented DNA in apoptotic cells. Anal Biochem 207:163
12. Huang P, Plunkett W (1993) Replicon-sized DNA fragmentation induced by nucleoside analogs. Proc Am Assoc Cancer Res 34:289
13. Huang P, Plunkett W (1994) Drug-induced apoptosis: relationship between DNA fragmentation and cell death. Proc Am Assoc Cancer Res 35:313
14. Huang H, Siciliano MJ, Plunkett W (1989) Gene deletion, a mechanism of induced mutation by arabinosyl nucleosides. Mutat Res 210:291
15. Huang P, Chubb S, Plunkett W (1990) Termination of DNA synthesis by 9-β-D-arabinofuranosyl-2-fluoroadenine, a mechanism for cytotoxicity. J Biol Chem 265:16617
16. Huang P, Chubb S, Hertel LW, Grindey GB, Plunkett W (1991) Action of 2′,2′-difluorodeoxycytidine on DNA synthesis. Cancer Res 51:6110
17. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S-I, Sameshima M, Hase A, Seto Y, Nagata S (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 66:233
18. Kamiya K, Huang P, Plunkett W (1993) Excision of incorporated fludarabine from DNA by human DNA polymerase ε. Proc Am Assoc Cancer Res 34:349
19. Kaufmann SH (1989) Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: a cautionary note. Cancer Res 49:5870
20. Kaufmann SH, Karp JE, Jones RJ, Miler KB, Schneider E, Zwelling LA, Cowan K, Wendel K, Burke PJ (1994) Topoisomerase II levels and drug sensitivity in adult acute myelogenous leukemia. Blood 83:517
21. Major PP, Egan EM, Beardsley GP, Minden MD, Kufe DW (1981) Lethality of human myeloblasts correlates with the incorporation of arabinofuranosylcytosine into DNA. Proc Natl Acad Sci USA 78:3235
22. Matherly LH, Schuetz JD, Westin E, Goldman ID (1989) A method for the synchronization of cultured cells with aphidicolin: application to the large-scale synchronization of L1210 cells and the study of the cell cycle regulation of thymidylate synthase and dihydrofolate reductase. Anal Biochem 182:338
23. McConkey DJ, Hartzell P, Duddy SK, Hakansson H, Orrenius S (1988) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin kills immature thymocytes by Ca^{2+}-mediated endonuclease activation. Science 242:256
24. Michaelson J (1991) The significance of cell death. In: Tomei LD, Cope FO (eds) Apoptosis, the molecular basis of cell death. Cold Spring Harbor Laboratory Press, New York, p 31
25. Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 12:3679

26. Parker WB, Bapat AR, Shen J-X, Townsend AJ, Cheng C-Y (1988) Interaction of 2-halogenated dATP analogs (F, Cl, and Br) with human DNA polymerases, DNA primase, and ribonucleotide reductase. Mol Pharmacol 34:485
27. Robertson LE, Chubb S, Meyn RE, Story M, Ford R, Hittelman WN, Plunkett W (1993) Induction of apoptotic cell death in chronic lymphocytic leukemia by 2-chloro-2′-deoxyadenosine and 9-β-D-arabinosyl-2-fluoroadenine. Blood 81:143
28. Sandoval A, Plunkett W (1994) Inhibition of UV-induced DNA repair in quiescent human peripheral blood lymphocytes by fludarabine stimulates apoptosis. Proc Am Assoc Cancer Res 35:395
29. Sassanfar M, Roberts JW (1990) Nature of the SOS-inducing signal in Escherichia coli, the involvement of DNA replication. J Mol Biol 212:79
30. Sellins KS, Cohen JJ (1987) Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. J Immunol 139:3199
31. Tartaglia LA, Ayres TM, Wong GH, Goeddel DV (1993) A novel domain within the 55 kd TNF receptor signals cell death. Cell 74:845
32. Thompson HJ, Strange R, Schedin PJ (1992) Apoptosis in the genesis and prevention of cancer. Cancer Epidemiol Biomarkers Prev 1:597
33. Trauth BC, Klas C, Peters AMJ, Matzku S, Moller P, Falk W, Debatin K-M, Krammer PH (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science 245:301
34. Walker GC (1984) Mutagenesis and inducible responses to deoxyribonucleic acid damage in Escherichia coli. Microbiol Rev 48:60
35. Walker PR, Smith C, Youdale T, Leblanc J, Whitfield JF, Sikorska M (1991) Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. Cancer Res 51:1078
36. Walker PR, Kokileva L, LeBlanc J, Sikorska M (1993) Detection of the initial stages of DNA fragmentation in apoptosis. Biotechniques 15:1032
37. Wang Z, Wu X, Friedberg EC (1993) Nucleotide-excision repair of DNA in cell-free extracts of the yeast Saccharomyces cerevisiae. Proc Natl Acad Sci USA 90:4907
38. Warters RL (1990) Radiation-induced apoptosis in a murine T-cell hybridoma. Cancer Res 52:883
39. Williams GT, Smith CA, Spooncer E, Dexter TM, Taylor TR (1990) Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 343:76
40. Wyllie AH, Kerr JFR, Currir AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251
41. Yang SW, Huang P, Plunkett W, Becker FF, Chan JYH (1992) Dual mode of inhibition of purified DNA ligase I from human cells by 9-β-D-arabinofuranosyl-2-fluoroadenine triphosphate. J Biol Chem 267:2345