High Molecular Weight DNA Fragmentation: A Critical Event in Nucleoside Analogue-induced Apoptosis in Leukemia Cells

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ABSTRACT

Cleavage of DNA into internucleosomal fragments is one of the characteristics of apoptosis. However, searches for in vivo evidence of nucleosomal DNA fragmentation in leukemia cells freshly obtained from patients during chemotherapy frequently failed to reveal nucleosomal multimers (DNA ladders). It is not clear whether this type of DNA cleavage is an essential event in drug-induced apoptosis and thus a denominator of cell killing, or whether the internucleosomal DNA fragments are merely the by-products of the apoptotic process. Here, we report our investigation into the role of DNA fragmentation in apoptotic cell death induced by anticancer nucleoside analogues, both in cell culture and in leukemia patients undergoing chemotherapy. Using a 5'-end DNA-labeling technique and pulsed field gel electrophoresis, we detected fragmentation of DNA in two distinct size classes, internucleosomal and high molecular weight (predominantly 50 kb) DNA fragments, in a human leukemia cell line exposed to the nucleoside analogues fludarabine and gemcitabine. We further demonstrated that the two types of DNA fragmentation were separate events, distinguishable by their requirements for Ca2+ and responses to phorbol ester treatment. The drug-treated cells underwent morphological changes of apoptosis even after internucleosomal DNA fragmentation was selectively inhibited by intracellular Ca2+ chelation, or by treatment with phorbol ester. In contrast, neither apoptotic morphology nor internucleosomal DNA fragmentation was observed when the high molecular weight DNA fragmentation was blocked by inhibition of nucleoside analogue incorporation into DNA. These results suggest that cleavage of DNA into large fragments may be an initial event that is critical for drug-induced apoptosis, whereas activation of a Ca2+-dependent endonuclease to cleave DNA at internucleosomal sites is not an absolute requirement for the execution of the apoptotic cell death program. Further studies of leukemic lymphocytes obtained from 9 patients with chronic lymphocytic leukemia during therapy with fludarabine revealed high molecular weight DNA fragmentation, which was correlated with a decrease of peripheral lymphocytes in 6 patients, whereas only 1 of the 15 patients evaluated for nucleosomal DNA fragments showed the DNA ladders. These results indicate that high molecular weight DNA fragmentation occurs in vivo, and may be correlated with the cytotoxic action of the anticancer drugs. Further study of the association of high molecular weight DNA fragmentation with clinical response to chemotherapy is warranted.

INTRODUCTION

Apoptosis or programmed cell death is a fundamental biological process in embryogenesis and tissue development (1–3). A variety of stimuli (4–7) or depletion of growth factors (8, 9) induces apoptosis in different experimental systems. Morphological changes typical of apoptosis include cell shrinkage, nuclear condensation, cell surface blebbing, fragmentation of nuclei, and clearance of the apoptotic cells by phagocytosis (1). Cleavage of cellular DNA at the internucleosomal sites has been observed in cells undergoing apoptosis induced by many agents and physiological conditions (1–4, 6). This cleavage produces DNA fragments the size of integer multiples of a nucleosome length (180–200 bp). Because of their characteristic patterns revealed by agarose gel electrophoresis, the nucleosomal “DNA ladders” are widely used as markers of apoptosis. It remains unclear, however, whether the nucleosomal DNA cleavage is an essential event required by the apoptotic cell death program and thus is critical for apoptosis, or whether the internucleosomal DNA fragments are merely the products of apoptosis.

Recent studies suggest that the apoptotic process is involved in the killing of cancer cells by irradiation (10, 11) and anticancer drugs (12–16). The nucleoside analogues fludarabine (F-ara-A3) and gemcitabine (dFdC) are two drugs with clinically proven anticancer activity (17–20). Our previous studies demonstrated that both compounds induce apoptosis in human leukemia CEM cells in vitro (12, 13). F-ara-A and dFdC are each incorporated into DNA and terminate DNA strand elongation (21, 22). F-ara-A-terminated DNA fragments cannot be ligated with an adjacent DNA fragment by human DNA ligase I (23). The incorporated analogue residues in DNA resist excision by the 3'→5' exonuclease of DNA polymerase ε (22, 24). The

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3 The abbreviations used are: F-ara-A, 9-β-D-arabinofuranosyl-2-fluoroadenine (the more soluble formulation of this compound, F-ara-A3 monophosphate, for clinical usage is known as fludarabine); dFdC, 2',2'-difluorodeoxycytidine (gemcitabine); CEM, human T lymphoblastoid CCRF-CEM cells; BAPTA-AM, bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetra(acetoxymethyl)ester; HMW, high molecular weight; PMA, phorbol 12-myristate 13-acetate; CLL, chronic lymphocytic leukemia; dThd, thymidine.
incorporation of each analogue into DNA is strongly correlated with the loss of cell clonogenicity (21, 22). Deletion of large DNA fragments was observed in cells that survived a short-term F-ara-A incubation (25). Thus, damage to cellular DNA by the terminal incorporation of these drugs appears to be the key mechanism of their cytotoxicity.

When human T lymphoblastoid CEM cells are exposed to fludarabine and gemcitabine in culture, the cells undergo morphological changes typical of apoptosis and generate nucleosomal DNA fragments (12, 13). Incubation of lymphocytes from patients with CLL with fludarabine and 2-chlorodeoxyadenosine in vitro also induced apoptosis, as demonstrated both morphologically by light and electron microscopy and biochemically by nucleosomal-sized DNA fragmentation in CLL cells (26, 27). However, quantitation of the in vitro drug-induced nucleosomal DNA fragmentation was heterogeneous among patients. Furthermore, attempts to detect this type of DNA fragmentation in fresh peripheral blood samples obtained from leukemia patients undergoing chemotherapy failed to reveal nucleosomal ladders, despite a rapid decrease of peripheral leukemia cells (28). Additionally, classic apoptotic morphology has been shown in Molt-4 cells despite a demonstrable lack of oligonucleosomal cleavage (29), whereas this type of DNA fragments were detected in cells showing the ultrastuctural features of necrosis (30). This paradox led us to investigate the role of DNA fragmentation during apoptosis in leukemia cells both in vitro and in vivo. The present study used a 32P 5'-end DNA-labeling technique and pulsed field gel electrophoresis to investigate the change of DNA integrity in cells induced to undergo apoptosis by fludarabine and gemcitabine. Two types of DNA fragmentation, internucleosomal and HMW DNA fragments, were revealed in a cell line and in fresh leukemia cells treated with the drugs. The relationship between the apoptotic cell death process and the two types of DNA fragmentation is discussed.

MATERIALS AND METHODS

Chemicals and Reagents. The nucleoside analogues 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, cycloheximide, and PMA were obtained from Sigma Chemical Co. (St. Louis, MO). BAPTA-AM was purchased from Molecular Probes (Eugene, OR). Protease K, alkaline phosphatase (from calf intestine), and RNase (DNase free) were obtained from Boehringer Mannheim Co. (Indianapolis, IN). T4 polynucleotide kinase was obtained from United States Biochemicals Co. (Cleveland, OH). [γ-32P]ATP was obtained from ICN Radiochemicals (Irvine, CA). The Betascope 603 blot analyzer, used in the quantitation of radioactivity in dried agarose gels, was a product of Betagen (Waltham, MA).

Cell Culture and Examination of Cell Morphology. The human T-lymphoblastoid cell line, CCRF-CEM, was maintained in exponential growth in RPMI 1640 suspension culture medium supplemented with 5% fetal bovine serum. Drug exposures were carried out when cells were in the middle phase of exponential growth (approximately 5 × 10^5 cells/ml). After incubation with drugs, aliquots of cells 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. Apoptotic cells were scored and expressed as the number of cells exhibiting morphology typical of apoptosis per 200 cells counted (n = 7–14 fields).

Patients and Sample Preparation. All patients studied for in vivo HMW DNA fragmentation were diagnosed as B-cell CLL according to the National Cancer Institute criteria for the diagnosis of CLL (31), and entered the study after informed consent was obtained according to institutional guidelines. Co-expression of CD5 with B-cell antigens and isotypic light chain was demonstrated by flow cytometry in all cases. Patient ages ranged from 46 to 74 years, with a median age of 63 years. Rai stages ranged from I to IV with most being stage I. Performance status was 0–2. Eight of the nine patients received fludarabine-based therapy on study as described below. Four patients (patients 6–9), who were previously untreated, received fludarabine (30 mg/m2) for 3 days and one dose of mitoxantrone (12 mg/m2) on the third day: one patient (patient 1) was given fludarabine (30 mg/m2) for 5 days combined with continuous granulocyte-colony-stimulating factor (5 μg/kg); patient 2 received fludarabine (30 mg/m2) for 4 days with a single dose of doxorubicin (100 mg/m2) on the first day; patient 4, who had failed previous fludarabine therapy, received fludarabine combined with enolplatin; and patient 3 received single-agent fludarabine (30 mg/m2) for 5 days. Patient 5, who previously failed fludarabine therapy, was treated with 2-chlorodeoxyadenosine (0.14 mg/m2 for 5 days) at the time of DNA fragmentation study. At designated times, freshly obtained peripheral blood was subjected to an erythrocyte lysis procedure using ammonium chloride. The WBCs were immediately processed for analysis of DNA fragmentation as described below. The samples generally contained more than 90% lymphocytes.

DNA Fragmentation Assays. To detect and quantitate internucleosomal DNA fragmentation, DNA isolated from the control and drug-treated cells in vitro or from samples freshly obtained from CLL patients was labeled with 32P at the 5'-ends as described previously (12). The DNA was fractionated by electrophoresis in a 2% agarose gel, stained with ethidium bromide, and photographed. An autoradiograph was made after the gel was dried at 60°C under vacuum. The radioactivity in the DNA bands was quantitated by a Betascope 603 blot analyzer as described previously (12).

To quantitate HMW DNA fragmentation, the DNA of CEM cells was prelabeled with [14C]dTTh for 48 h (0.02 μCi/ml every 12 h, × 4), and then cells were incubated with the indicated compounds for 4 h. 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 kept at 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 TE buffer (10 mM Tris-HCl, pH 7.8, and 1 mM EDTA) for 3 h with buffer changes each hour, the plugs were analyzed by pulsed field gel electrophoresis (CHEF-DR II; Bio-Rad Laboratories, Richmond, CA) 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 HMW DNA fragments was quantitated by Betascope after the gel was dried at 60°C under vacuum. Because the DNA of CLL cells from patients was not labeled with 14C, large-size DNA fragments were quantitated by densitometry scanning (Ephortec Joyce Loebl Densitometer, Gateshead, England) of the negative films after the gels were stained with ethidium bromide.

Statistical Analysis. A computer-assisted program for linear regression analysis (32), which is based on the least-square principle, was used to evaluate the relationship between chemotherapy-induced HMW DNA fragmentation in vivo and the decrease of leukemic lymphocyte counts. Because the absolute lymphocyte counts varied greatly among patients, the change in peripheral lymphocytes was expressed as the percentage of lymphocyte counts with reference to the pretreatment sample from the same patient. Likewise, the amount of HMW DNA fragments in leukemic cells from patients during therapy was converted to relative units (percentage of DNA fragments found in the pretreatment sample) for data analysis. This data conversion allows the comparison of HMW DNA fragmentation among patients with heterogeneous lymphocyte counts.

RESULTS

Two Distinct Classes of DNA Fragmentation in Drug-induced Apoptosis in Vitro. Human T lymphoblastoid CEM cells were incubated with F-ara-A or dFdC to induce apoptosis in vitro. A radioactive DNA-labeling technique (12) was used to detect internucleosomal DNA fragmentation induced by the nucleoside analogues. Labeling of cellular DNA with 32P at the 5'-end clearly demonstrated internucleosomal DNA fragmentation after incubation with F-ara-A (Fig. 1A, Lane 2) and dFdC (Fig. 1B, Lane 2). The nucleosomal DNA bands were not detected in control cells (Fig. 1, Lanes 1). This DNA fragmentation was inhibited when cells were incubated with F-ara-A or dFdC in the presence of 2 μM aphidicolin (Fig. 1, Lanes 3), which almost completely inhibited DNA replication in CEM cells at this concentration and blocked incorporation of the nucleoside analogues into DNA (13, 21). This result suggests that incorporation of the analogues into DNA is required for the induction of internucleosomal DNA fragmentation. Inhibition of DNA synthesis by a 4-h treatment with aphidicolin alone was not sufficient to induce DNA fragmentation (data not shown).

Drug-induced DNA fragmentation was also inhibited when cells were incubated with F-ara-A or dFdC in the presence of 2 μM BAPTA-AM (Fig. 1, Lanes 4), indicating that internucleosomal DNA fragmentation is a Ca2+-dependent event. This is in agreement with the observation in other experimental systems that a Ca2+-dependent endonuclease might be responsible for the cleavage of cellular DNA at internucleosomal linkages (4, 33, 34). The inhibitory effect of BAPTA-AM on drug-induced nucleosomal DNA fragmentation was observed both in exponentially growing cells and in synchronized S-phase cells. In a separate experiment, we demonstrated that incubation of CEM cells with 50 μM BAPTA-AM for 45 min did not affect the DNA synthesis activity of the cells as determined by [3H]TdR incorporation. Prolonged incubation (4 h) with BAPTA-AM decreased DNA synthesis activity to 69% of the control. This moderate inhibitory effect on DNA synthesis might decrease the incorporation of the nucleoside analogues into DNA, and thus might contribute to the inhibition of drug-induced DNA fragmentation. However, because cells retained about 70% DNA synthesis activity in the presence of BAPTA, a significant amount of the analogues was incorporated into DNA to initiate apoptosis. This is supported by the evidence that HMW DNA fragmentation was induced by the analogues in the presence of 50 μM BAPTA-AM (see details below and Fig. 2).

Internucleosomal DNA fragmentation was observed when the protein synthesis inhibitor cycloheximide was included in the cell culture with F-ara-A or dFdC (Fig. 1, Lanes 5). The amount of DNA fragments was similar to that of samples incubated with either F-ara-A alone (Fig. 1A, Lane 2) or dFdC alone (Fig. 1B, Lane 2). In a separate experiment, we demonstrated that neither puromycin, another inhibitor of protein synthesis, nor actinomycin D, an RNA synthesis inhibitor, blocked the induction of internucleosomal DNA fragmentation induced by F-ara-A or dFdC (data not shown). Because cycloheximide, puromycin, and actinomycin D themselves were toxic to CEM cells and induced comparable amounts of DNA fragmentation when used alone (data not shown), caution should be taken in interpreting these results regarding the requirement of new RNA and protein synthesis in the nucleoside analogue-induced DNA fragmentation process. The generation of internucleosomal DNA fragments was blocked when cells were incubated with

![Fig. 1](https://clinicalcancerres.aacrjournals.org/content/full/1007/640/)

Internucleosomal DNA fragmentation induced by F-ara-A and dFdC in CEM cells. DNA that was isolated from cells incubated for 4 h with 30 μM F-ara-A or 1 μM dFdC and the indicated compounds was labeled with 32P at the 5' terminus and fractionated on 2% agarose gels as described in "Materials and Methods." Each lane, 0.6 μg DNA. A, autoradiograph of a gel containing DNA samples from cells incubated with F-ara-A and the indicated compounds for 4 h. B, autoradiograph of a gel containing DNA samples from cells treated with dFdC and the indicated metabolic modulators. Ethidium bromide staining of the gels revealed that approximately equal amounts of DNA were loaded in each lane (data not shown). APH, aphidicolin, 2 μM; BAP, BAPTA-AM; 50 μM; CHX, cycloheximide, 50 μg/ml; PMA, phorbol 12-myristate 13-acetate, 1 nM.
either nucleoside analogue plus the phorbol ester PMA (Fig. 1, Lanes 6). Because phorbol ester is an activator of protein kinase C under these conditions (35), it is possible that activation of this enzyme led to the blockage of internucleosomal DNA fragmentation. The exact signal transduction pathway involved in this process requires further study. In a separate study, we observed that incubation of CEM cells with 1 nM PMA, by a yet unknown mechanism, increased the DNA synthesis activity by 94% as measured by [3H]dThd incorporation. This nevertheless excludes the possibility that PMA might inhibit drug-induced nucleosomal DNA fragmentation by inhibiting incorporation of the analogues. Inhibition of drug-induced DNA damage by phorbol ester has also been observed in leukemic cells from CLL patients incubated with fludarabine and phenylalanine mustard (36).

Because the nucleoside analogues terminate DNA strand elongation (21, 22) and cause deletion of DNA fragments of 30 kb or greater (25), we investigated the possibility that generation of HMW DNA fragments might be involved in drug-induced apoptosis. Fig. 2 shows the pulsed field gel electrophoresis analysis of DNA from cells prelabeled with [14C]thymidine and incubated with the analogues. HMW DNA fragments, with a predominant size of 50 kb, were visualized in samples incubated with F-ara-A (Fig. 2, Lane 2) or dFdC (Fig. 2, Lane 8). Molecular size analysis of the DNA in the gel by Betascope indicated that the size range of the large fragments extended between 5 and 500 kb, with the majority of the fragments located between 35 and 70 kb (data not shown). The gel was run under non-denaturing conditions; therefore, the fragments reflected double-stranded breaks of cellular DNA. It is expected that F-ara-A and dFdC were incorporated only into the newly synthesized (daughter) DNA strand and most likely terminated its elongation, the double-strand breaks suggest that an active cellular process may have been involved in the cleavage of the parental DNA strand. The induction of HMW DNA fragments by F-ara-A and dFdC was both concentration and time dependent (data not shown). A separate HMW DNA band located at approximately 1500–2000 kb was observed in both the control and the drug-treated cells (Fig. 2, Lanes 1–12). The amount of DNA in this band in the control cells, as determined by Betascope analysis, was not different from that of the drug-treated samples. It appears, therefore, that the megabase-sized DNA fragmentation was not induced by the drugs in CEM cells under the experimental conditions.

The same metabolic modulators used to characterize nucleosomal DNA fragmentation were then used to further evaluate the HMW DNA fragmentation process. When cells were incubated with F-ara-A or dFdC in the presence of aphidicolin, no HMW DNA fragmentation was induced by the analogues (Fig. 2, Lanes 3 and 9). This result indicates that, as with the induction of internucleosomal DNA fragmentation, the generation of large DNA fragments was dependent on incorporation of the analogues into cellular DNA, and was not induced by the inhibition of DNA synthesis with aphidicolin. When cells were incubated with BAPTA-AM and F-ara-A or dFdC, the depletion of intracellular Ca2+ by BAPTA-AM did not inhibit the generation of large DNA fragments (Fig. 2, Lanes 4 and 10). These results strongly suggest that the HMW DNA fragmentation is independent of intracellular calcium. This is in direct contrast to the internucleosomal DNA fragmentation, which was a Ca2+-dependent event and was inhibited by BAPTA-AM (Fig. 1, Lanes 4). Furthermore, PMA did not block the drug-induced HMW DNA fragmentation (Fig. 2, Lanes 6 and 12), whereas the generation of internucleosomal DNA fragments was inhibited by PMA (Fig. 1, Lanes 6). Thus, the two types of DNA fragmentation appear to be separate events, distinguishable with respect to their reaction requirements (i.e., Ca2+ and signaling pathways, i.e., response to phorbol ester). Neither BAPTA-AM nor PMA alone caused HMW DNA fragmentation during a 4-h incubation (data not shown). As was the case for internucleosomal fragmentation, cycloheximide (50 μg/ml, 4 h) appeared not to affect the generation of HMW DNA fragments associated with F-ara-A and dFdC treatment (Fig. 2, Lanes 5 and 11). As noted above, however, a high concentration (50 μg/ml) of cycloheximide was toxic to CEM cells and was able to induce both HMW and nucleosomal DNA fragmentation in these cells. In a separate experiment, we demonstrated that a lower concentration (5 μg/ml) of cycloheximide alone inhibited protein synthesis by about 90% in CEM cells as determined by [3H]leucine incorporation, but did not cause a significant amount of HMW DNA fragmentation during a 4-h incubation. In contrast, a higher concentration of cycloheximide (50 μg/ml) inhibited protein synthesis by 97% and caused fragmentation of 22% cellular DNA into HMW fragments by a yet unknown mechanism. Addition of 5 μg/ml cycloheximide to CEM cells incubated with F-ara-A reduced the amount of F-ara-A-induced HMW DNA fragmentation by about 76% during a 4-h incubation (data not shown). Thus, it is likely that synthesis of new protein may be involved in the HMW DNA fragmentation process.
Correlation between DNA Fragmentation and Apoptotic Morphology. The relationship between the morphological changes of apoptosis and DNA fragmentation was investigated to evaluate the role of each type of DNA fragmentation in drug-induced apoptosis. Fig. 3A shows the morphology of control CEM cells. Morphological changes typical of apoptosis, including cell shrinkage, nuclear condensation, and fragmentation of nuclei, were observed when cells were incubated with 30 μM F-ara-A for 4 h (Fig. 3B). Quantitative evaluation revealed that there were 62 ± 2 apoptotic cells/200 cells in the F-ara-A-treated samples (Table 1). Few apoptotic cells (4 ± 1/200 cells) were observed in the sample incubated with F-ara-A and aphidicolin (Fig. 3C and Table 1), in which the formation of both large and internucleosomal DNA fragments was inhibited. The intracellular Ca²⁺ chelator BAPTA-AM, which inhibited internucleosomal DNA fragmentation without affecting HMW DNA fragmentation, did not block the generation of apoptotic bodies (Fig. 3D); 76 ± 4 apoptotic cells were counted per 200 cells in the F-ara-A/BAPTA-AM-treated samples. Furthermore, a significant number of apoptotic cells (58 ± 2/200 cells) was scored in samples incubated with F-ara-A plus PMA, in which the large DNA fragments remained, but the
analogue-induced internucleosomal DNA fragmentation was inhibited. Similar results were observed in cells treated with dFdC and the metabolic modulators (Table 1). Incubation of CEM cells with 1 nm PMA alone for 4 h did not induce apoptosis. Thus, in both F-ara-A- and dFdC-treated samples, the inhibition of internucleosomal DNA fragmentation by either BAPTA-AM or PMA failed to block the appearance of apoptotic cells. These data strongly suggest that cleavage of cellular DNA into internucleosomal DNA fragments is not an essential requirement for the execution of the cell death program. Instead, the internucleosomal DNA fragments may be a critical step in the irreversible commitment to apoptosis induced by the anticancer drugs. This hypothesis is supported by the fact that inhibition of HMW DNA fragmentation by aphidicolin blocked both internucleosomal DNA fragmentation and apoptotic morphological changes (Table 1).

**DNA Fragmentation in Vivo.** Leukemia cells from nine patients with CLL receiving fludarabine-based therapy were analyzed for nucleosomal- and large-sized DNA fragmentation. DNA from leukemic lymphocytes obtained from patients before and during therapy were immediately processed both for \(^{32}\)P 5'-end labeling and for pulsed field gel electrophoresis. As shown in Table 2, HMW DNA fragmentation was observed in the leukemia cells from six of the nine patients (patients 1, 2, 3, 6, 8, and 9) during the course of chemotherapy. These six patients also showed a decrease of peripheral blood lymphocytes at the time when large DNA fragmentation was observed (Table 2). No significant increase in HMW DNA fragments was observed in three other patients. Blood lymphocyte counts of two of these three patients (patients 4 and 5) remained unchanged during therapy. Patient 7 showed a decrease of lymphocyte counts and revealed a high level of HMW DNA fragments but no net increase in both pretreatment and posttreatment samples. In contrast, nucleosomal DNA fragmentation was not detected in leukemic lymphocytes of eight of the nine patients, including six patients who showed a decrease of peripheral lymphocytes. Only one patient (patient 2) revealed nucleosomal DNA fragments. This patient also showed HMW DNA fragmentation and a decrease of blood lymphocytes (Table 2). In a prior study, we analyzed samples from an additional six patients, and none of them showed nucleosomal DNA fragments (data not shown). These results suggest that HMW DNA

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**Table 1** Relationship between drug-induced DNA fragmentation and generation of apoptotic cells

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA fragments (% of drug-treated cells)</th>
<th>Apoptotic cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nucleosomal</td>
<td>Large</td>
</tr>
<tr>
<td>F-ara-A (30 μM)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+ aphidicolin</td>
<td>8 ± 2</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>+ BAPTA (2 μM)</td>
<td>17 ± 7</td>
<td>102 ± 6</td>
</tr>
<tr>
<td>+ CHX (50 μM)</td>
<td>159 ± 7</td>
<td>153 ± 7</td>
</tr>
<tr>
<td>(50 μg/ml) + FMA (1 nM)</td>
<td>19 ± 6</td>
<td>120 ± 17</td>
</tr>
<tr>
<td>dFdC (1 μM)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>+ aphidicolin</td>
<td>5 ± 3</td>
<td>3 ± 2</td>
</tr>
<tr>
<td>+ BAPTA (2 μM)</td>
<td>11 ± 4</td>
<td>148 ± 11</td>
</tr>
<tr>
<td>(50 μM) + CHX (50 μg/ml)</td>
<td>101 ± 4</td>
<td>174 ± 14</td>
</tr>
<tr>
<td>(50 μM) + PMA (1 nm)</td>
<td>19 ± 5</td>
<td>144 ± 17</td>
</tr>
</tbody>
</table>

"CEM cells were labeled with \([^{3}H]dTd\) for 48 h (0.02 μCi/ml every 12 h, × 4), and then incubated with the indicated compounds for 4 h. The cultures were split into three portions for the quantitative determination of (a) internucleosomal DNA fragments, (b) large DNA fragments, and (c) apoptotic cells as described in "Materials and Methods." The positive control values for F-ara-A- and dFdC-induced internucleosomal fragments were 4472 ± 716 cpm and 3856 ± 792 cpm, respectively. The value for the negative control sample (without drug treatment) was 560 ± 70 cpm. The positive control values for F-ara-A- and dFdC-induced large DNA fragments were 1205 ± 23 cpm and 1558 ± 212 cpm, respectively. The value for the negative control sample (without drug treatment) was 80 ± 2 cpm. Apoptotic cells were scored by light microscopy and expressed as the number of apoptotic cells per 200 cells counted (n = 7–14 fields). Spontaneous apoptotic cells in the control sample were 1.5 ± 1/200 cells. Incubation with 50 μg/ml cycloheximide alone for 4 h caused generation of nucleosomal DNA fragments (4589 ± 50 cpm), HMW DNA fragments (22.5%), and apoptotic cells (28 ± 4/200 cells examined).

**Table 2** Relationship between DNA fragmentation and peripheral absolute lymphocyte counts in CLL patients before and after chemotherapy

<table>
<thead>
<tr>
<th>Patient</th>
<th>Time (h)</th>
<th>DNA fragments</th>
<th>Absolute lymphocyte count/μl × 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HMW</td>
<td>Nucleosomal</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
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</tr>
<tr>
<td>4</td>
<td>0</td>
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</tr>
<tr>
<td>5</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>-</td>
<td>-</td>
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"- DNA fragmentation not different from the background of pretreatment sample; ±, DNA fragmentation increased by 50–100% over the background; +, increased by 101–200%; ++, increased by 201–300%; ++++, increased more than 300%.
The heterogeneity in the amount of HMW DNA fragments generated by fludarabine therapy among patients suggests that this activity might be correlated with the cell killing activity of anticancer drugs.

To quantitatively evaluate the relationship between the in vivo drug-induced HMW DNA fragmentation and the decrease of leukemic lymphocytes, the relative amount of HMW DNA fragments was plotted against the relative peripheral lymphocyte counts measured as described in "Materials and Methods." A computer-assisted linear regression program (32) was used to analyze the correlation between the two parameters and its statistical significance.

DISCUSSION

The present study demonstrates two types of DNA fragmentation associated with nucleoside analogue-induced apoptosis in vitro. The HMW DNA fragmentation is a Ca²⁺-independent event that is closely associated with drug-induced apoptosis. Although internucleosomal DNA fragmentation is considered to be a characteristic hallmark of apoptosis, our study demonstrates that it is not an essential element of the cell death process in the response of CEM cells to nucleoside analogues. Recently, Falcieri et al. (29) reported that morphological apoptosis apparently occurred without concomitant internucleosomal DNA fragmentation in Molt-4 cells treated with the protein kinase inhibitor staurosporine. Collins et al. (30) suggested that internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. Another interesting observation was reported by Oberhammer et al. (37) who showed cleavage of DNA to 300-kb and/or 50-kb fragments prior to or in the absence of internucleosomal fragmentation. Those studies, however, used ethidium staining which has limited sensitivity in detecting small DNA fragments. In the present study of drug-induced apoptosis, the 5'-end-labeling technique extended the limits of sensitivity for detection of DNA fragments by 1000-fold relative to ethidium staining (12), and permitted quantification of the changes in DNA fragmentation as affected by a variety of metabolic modulators. This made it possible to evaluate the role of the two types of DNA fragmentation in apoptosis at a higher level of sensitivity. When we investigated the temporal relationship between the two types of DNA fragmentation, we found that both the HMW and the internucleosomal DNA fragmentation occurred 2–3 h after drug incubation. The lack of a detectable time delay between the two types of DNA fragmentation probably reflects the rapid onset of drug-induced DNA cleavage associated with apoptosis.

It is of interest to note that the majority of the HMW DNA fragments were located at approximately 50 kb (Fig. 2). DNA fragments of similar sizes were observed in rat fibroblast H11ras-R3 cells undergoing apoptosis induced by serum depletion (37). Dexamethasone- and DNA topoisomerase II-reactive agents also induced the generation of large DNA fragments in thymocytes (38, 39). Because depletion of serum imposed no direct damage to DNA, Oberhammer et al. (37) concluded that the 50-kb DNA fragments must be the products of an active cellular process. It was suggested that the activation of endonucleolytic activity at the sites where chromatin loop domains are anchored to the nuclear scaffold releases the 50-kb DNA fragments (37, 40), but that DNA topoisomerase II may not be directly involved in this process (41). In the case of drug-induced apoptosis, the detailed mechanism by which the nucleoside analogues induce HMW DNA fragmentation is not clear at the present time. Because the nucleoside analogues are potent DNA chain terminators, their incorporation into DNA imposes an immediate damage to the elongating DNA strand. It is possible that termination of DNA replication by the incorporated analogues may serve as the signal that triggers the activation of a Ca²⁺-independent endonuclease, which cleaves DNA at or near the anchorage sites of the chromatin loop domains to the nuclear scaffold.

The present study in CLL patients demonstrated that HMW DNA fragmentation occurred in vivo during chemotherapy. Although the number of patients in this initial study is limited, statistical analysis of the data shown in Table 2 suggest that drug-induced HMW DNA fragmentation is significantly correlated with the decrease of peripheral leukemic lymphocytes during chemotherapy (Fig. 4). Because HMW DNA fragments were detectable in vivo and the levels of fragmentation were heterogeneous among patients, it is important to seek correlation between drug-induced HMW DNA fragmentation and clinical outcome, and to evaluate the possibility of using this parameter as an indicator of prognosis. In contrast, there is only a low incidence (1/15 patients) of detecting in vivo nucleosomal DNA fragmentation, which did not appear to be correlated with the decrease of peripheral lymphocytes during therapy. This is
consistent with the results of in vitro experiments (Table 1). Other studies in patients with leukemia also demonstrated the lack of detectable nucleosomal DNA fragmentation during chemotherapy, despite a rapid decrease in the peripheral leukemic cell counts (28, 42).

The reason for the low frequency of detecting in vivo nucleosomal DNA fragmentation remains unclear at the present time. One possibility is that because nucleosomal DNA fragmentation is a product of drug-induced apoptosis rather than a required event of the cell death program, leukemia cells can be killed in vivo without generating nucleosomal fragments. An alternative possibility is that nucleosomal DNA fragments may indeed be produced in vivo during drug-induced apoptosis. However, cells with nucleosomal DNA fragments may represent a more advanced stage in the apoptotic process. In vivo, multiple changes may take place which cause the cells in an advanced apoptotic stage to be readily recognized and rapidly cleared by the reticuloendothelial system, and thus decrease the possibility of detecting nucleosomal DNA fragments in the peripheral blood samples. If so, the possibility of detecting in vivo nucleosomal DNA fragments may depend on the ability of the reticuloendothelial system of the individual patients to eliminate apoptotic cells in vivo. Detection of nucleosomal DNA fragmentation in peripheral WBCs and bone marrow cells during therapy was recently reported (43). Therefore, it is likely that nucleosomal DNA fragmentation in peripheral lymphocytes may not be predictive of clinical response to chemotherapy. On the other hand, HMW DNA fragmentation may represent an early event of changes in chromosomal structure during apoptosis, which is readily detectable in patient samples. Because this appears to be correlated with the cell killing activity of the anticancer drugs, we conclude that further study to investigate the possibility of using HMW DNA fragmentation as a predictor of clinical response to chemotherapy is warranted.

Recent studies on the oncogene bcl-2 indicated that overexpression of the bcl-2 protein may confer cells a survival advantage and lead to some degree of resistance to apoptosis induced by certain antineoplastic agents (44–46). Furthermore, immunoblotting analysis of fresh samples from CLL patients demonstrated that bcl-2 protein was overexpressed in the majority of the cases, and that CLL cells with a higher level of bcl-2 protein delayed DNA fragmentation and survived longer (47). Immunoblotting analysis of fresh samples from CLL patients also demonstrated that bcl-2 protein was overexpressed in the majority of the cases, and that CLL cells with a higher level of bcl-2 protein delayed DNA fragmentation and survived longer (47). Furthermore, the expression levels of bcl-2 protein, it is possible that bcl-2 may play a role in regulating HMW DNA fragmentation in nucleoside analogue-induced apoptosis. Thus, a future study to evaluate the relationship between bcl-2 expression and drug-induced HMW DNA fragmentation will be important in both understanding the apoptotic process and developing strategies to overcome drug resistance to chemotherapy.

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