Induction of Apoptotic Cell Death in Chronic Lymphocytic Leukemia by 2-Chloro-2'-deoxyadenosine and 9-β-D-Arabinosyl-2-fluoroadenine

By L.E. Robertson, Sherri Chubb, Raymond E. Meyn, Michael Story, Richard Ford, Walter N. Hittelman, and William Plunkett

2-Chloro-2'-deoxyadenosine (CldAdo) and 9-β-D-arabinosyl-2-fluoroadenine (F-ara-A) have shown marked activity in the treatment of indolent lymphoid malignancies. Based on the susceptibility of various lymphocyte populations to apoptosis, we investigated whether CldAdo or F-ara-A would induce this process in lymphocytes from patients with chronic lymphocytic leukemia (CLL). In vitro exposure of leukemic lymphocytes to CldAdo or F-ara-A for 24 to 72 hours elicited features of apoptosis visible by light and electron microscopy. Analysis of DNA integrity showed DNA cleavage into nucleosomal-sized multimers. Using a quantitative assay, drug-induced DNA fragmentation was both time and dose dependent. Inhibition of active macromolecular synthesis did not prevent drug-induced fragmentation; however, both drug-induced and spontaneous DNA fragmentation were prevented by intracellular calcium chelation. In vitro culture with phorbol ester generally decreased drug-induced DNA cleavage. After prolonged incubation, CLL cells exhibited spontaneous cleavage; albeit, at significantly lower rates than drug-treated cells. Heterogeneity was observed for spontaneous and drug-induced DNA fragmentation and was significantly lower in B-lymphocytic cells obtained from patients with high-risk and refractory disease. We conclude that CldAdo and F-ara-A are potent inducers of apoptotic death in CLL and that this feature correlates with the disease status.

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MATERIALS AND METHODS

Materials. RPMI medium 1640, trypan blue, and EcoX174 RF DNA/Hae III molecular weight marker were purchased from GIBCO Biologic Research Laboratories (Grand Island, NY). 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid, tetra(acetoxy-methyl)ester (BAPTA-AM) was purchased from Molecular Probes (Eugene, OR). All other chemicals were purchased from Sigma Chemical Company (St Louis, MO).

Patients, cell isolation, and incubation conditions. All patients fulfilled the National Cancer Institute (NCI) criteria for the diagnosis of the disease. Cell isolation was performed as previously described.23 Cultivation conditions and drug exposures were performed to optimize the conditions for the detection of apoptosis. Drug sensitivities were determined in a quantitative assay, using propidium iodide and DNA/Hae III digestion profiles.

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of CLL. Immunophenotyping by dual-parameter flow cytometry showed coexpression of CD5 with B-cell antigens and isotypic light-chain expression. Clinical staging was based on the system described by Rai and response criteria were similar to the NCI-sponsored guidelines, with the designation of a nodular complete response group for patients having residual marrow lymphoid nodules as the only clinical evidence of disease.

Freshly obtained peripheral blood was fractionated by Ficoll-Hypaque (Winthrop Pharmaceuticals, New York, NY) sedimentation. Nonadherent mononuclear cells were then resuspended in complete medium (RPMI 1640 supplemented with streptomycin, penicillin, and 10% fetal bovine serum) at a concentration of 0.5 to 1 × 10⁷ cells per mL. Cells were incubated at 37°C in an atmosphere of 5% CO₂ with various drugs for the times indicated.

Cell number, size, and viability. The cell count and volume were determined by a Coulter Counter Model ZM and a Coulter Channelizer 256 (Coulter Electronics, Hialeah, FL). Cell viability was assessed by trypan blue exclusion using a hemacytometer.

DNA quantification. Cells were washed and then resuspended in hypotonic lysis buffer containing 10 mmol/L Tris, 1 mmol/L EDTA, and 0.2% Triton X-100 (pH 7.5) (Sigma). DNA of nucleosomal size, which is soluble at low salt concentrations, remains in the supernatant, whereas intact genomic-sized DNA is insoluble and is present in the pellet. The DNA content of the pellet and supernatant was determined by the diphenylamine reaction using deoxyadenosine as a standard. Results are expressed as the percentage of total DNA that resisted sedimentation.

DNA extraction and electrophoresis. Cells were washed, lysed, and centrifuged as described above. The supernatant fractions were removed and precipitated in two volumes of isopropanol and 0.3 mol/L sodium acetate. The crude DNA preparation was resuspended in 10 mmol/L Tris and 1 mmol/L EDTA containing proteinase K. DNA was extracted using equal volumes of phenol-chloroform and chloroform-isooamyl alcohol. The aqueous phase was collected and the DNA was precipitated in two volumes of ice-cold ethanol and 0.15 mol/L NaCl. After resuspension in Tris-EDTA and incubation with DNAase-free RNAse, loading buffer was added; the samples were then electrophoresed for 3.5 hours at 50 V in 1.8% agarose gels. After electrophoresis, DNA was stained with ethidium bromide and the gels photographed using Polaroid type 57 (Polaroid Corporation, Cambridge, MA) film exposed to UV light. An Hae III digest of φX174 was run on each gel to provide standards of 1353, 1078, 872, 603, 310, 271, 234, 194, 118, and 72 bp.

Light microscopy. Cells were cytocentrifuged at 600 rpm using a Shandon cytocentrifuge (Shandon Incorporated, Pittsburgh, PA). The cells were fixed in 100% methanol, stained with Wright-Giemsa, and examined for nuclear changes consistent with apoptosis.

Electron microscopy. Cells were washed and fixed overnight at 4°C with 2% glutaraldehyde in 0.2 mol/L sodium cacodylate buffer. Cells were postfixed in 1% osmium tetroxide in 0.2 mol/L sodium cacodylate buffer, dehydrated in a graded series of buffers, embedded in resin, and thinly sectioned. Sections were poststained in uranyl acetate and lead citrate and viewed using a JEOL JEM 1200 Ex electron microscope (Joel, Tokyo, Japan).

Nucleotide extraction and analysis. Nucleotides were extracted from cells with 0.4 N HClO₄, as previously described. The neutralized HClO₄-soluble extracts were analyzed by high-pressure liquid chromatography using a Paristel-10 SAX (Whatman, Clifton, NJ) anion exchange column (250 × 4 mm). Triphosphates of CldAdo and F-ara-A were separated from natural nucleotides by a linear gradient run over 60 minutes at 1 mL/min, starting at 100% buffer A (0.005 mol/L NH₄H₂PO₄, pH 2.8) and concluding at 100% buffer B (0.75 mol/L NH₄H₂PO₄, pH 3.7).

**RESULTS**

Induction of DNA fragmentation by CldAdo and F-ara-A. CLL lymphocytes were exposed to different concentrations of drug for various times to determine the dose response and kinetics. Untreated lymphocytes were also analyzed for the extent of spontaneous fragmentation. Evaluation of untreated lymphocytes derived from four CLL patients showed the amount of soluble DNA that could not be sedimented at 13,500g increased from undetectable levels to approximately 15% over 24 hours and then remained essentially unchanged over 72 hours (Fig 1). In contrast, leukemic lymphocytes treated with 3 µmol/L CldAdo showed fragmentation levels over 40% after 24 hours and increasing to approximately 70% by 72 hours. Levels of DNA fragmentation after treatments with 3 µmol/L F-ara-A also increased with time.

As shown in Fig 2, the degree of fragmentation increased with escalating concentrations of drug. The amount of DNA that resisted sedimentation increased markedly to 30 µmol/L, after which increases in concentration were less marked. CldAdo consistently induced higher fragmentation than F-ara-A at equal molar concentrations. Varying the source of serum (autologous versus fetal bovine serum) did not affect the rate of spontaneous or drug-induced DNA fragmentation.

**Induction of oligonucleosomal DNA cleavage by CldAdo and F-ara-A.** To determine whether the enhanced DNA fragmentation observed resulted from cleavage between nucleosomes, DNA was separated by agarose gel electrophoresis. Incubation of CLL cells with increasing concentrations of CldAdo and F-ara-A was undertaken; agarose gel electrophoresis of fragmented DNA obtained from an equal number of cells showed increasing amounts of the DNA fragments in an oligonucleosomal pattern over time in cells exposed to 3 µmol/L CldAdo, whereas no fragmentation was observed at zero time (Fig 3A). CLL cells incubated with increasing
concentrations (30 nmol/L to 30 μmol/L) of F-ara-A also showed a ladder pattern (Fig 3B).

CldAdo and F-ara-A induction of morphologic changes consistent with apoptosis. CLL cells incubated with CldAdo and F-ara-A were examined by light and electron microscopy to determine whether these treatments induced the morphologic features of apoptosis. Light microscopy of CLL lymphocytes incubated with 0.3 to 3 μmol/L CldAdo for up to 72 hours showed characteristic changes of apoptosis, including chromatin condensation, nuclear margination, and apoptotic body formation (Fig 4). In addition, degenerate anucleate cells lacking chromatin staining began to appear between 48 and 72 hours. Similar changes were observed in cells treated with F-ara-A.

The electron microscopic findings were also consistent with apoptosis, showing cells with decreased size and intensely osmiophilic chromatin forming crescents, toroids, and micronuclei (Fig 5). An ultrastructural feature of necrosis, mitochondrial swelling, was occasionally present in cells with apoptotic nuclear changes. Membrane dissolution appeared after prolonged drug exposure. Despite these extensive morphologic aberrations and the DNA fragmentation previously described, the loss of cell viability, as defined by membrane integrity to trypan blue uptake, lagged behind the morphologic changes by 48 to 72 hours.

Failure of RNA and protein synthesis inhibitors to prevent CldAdo and F-ara-A–induced DNA cleavage. The induction of DNA fragmentation in rat thymocytes has been shown to require nascent mRNA and protein synthesis, suggesting gene expression is necessary. To assess whether DNA fragmentation in CLL cells is under the control of active metabolism, cells were incubated with actinomycin D, cycloheximide, or puromycin at concentrations inhibitory to RNA and protein synthesis in the human lymphoblastoid cell line CCRF-CEM. Preincubation with actinomycin D or a protein synthesis inhibitor for 1 hour failed to prevent CldAdo-induced fragmentation (Table 1). Further study using drug-free cultures showed that these agents in fact enhance rather than abrogate DNA fragmentation in CLL cells (not shown).

Effect of calcium chelator on CldAdo– and F-ara-A–induced DNA cleavage. Studies of radiation- and glucocorticoid-stimulated apoptosis in rat thymocytes have documented the critical role of transient increases in cytosolic calcium. Modification of calcium homeostasis modulates this response. In CLL cells incubation with the extracellular calcium chelator, ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA), had minimal inhibitory effects on the extent of DNA fragmentation induced by CldAdo (Table 2). In contrast, CldAdo-induced fragmentation was reduced to the level of background detection by the potent intracellular calcium chelator, BAPTA-AM (50 μmol/L). The level of fragmentation in untreated cells at 48 hours was also decreased to less than 1% by incubation with 50 μmol/L BAPTA-AM (not shown).

Partial inhibition of CldAdo– and F-ara-A–induced DNA cleavage by phorbol ester. To assess if protein kinase C activation had an effect on fragmentation, CLL cells were preincubated with phorbol ester. CLL cells from patients with levels of spontaneous fragmentation ranging from 3% to 41% were preincubated with phorbol ester. CLL cells from patients with levels of spontaneous fragmentation ranging from 3% to 41% were preincubated with tetradecanoylphorbol 13-acetate (TPA) before the addition of CldAdo. This resulted in a decrease of CldAdo-induced DNA fragmentation by 48%.

Fig 3. Separation of soluble DNA from CLL cells incubated with CldAdo or F-ara-A. Low molecular weight DNA obtained from equal aliquots of cells was isolated and resolved by agarose gel electrophoresis as described in Materials and Methods. (A) DNA from CLL cells incubated with 3 μmol/L CldAdo over time: lane 1, molecular weight marker; lane 2, 24 hours; lane 3, 48 hours; lane 4, 72 hours; lane 5, 0 hours. (B) DNA from CLL cells incubated with different concentrations of F-ara-A for 48 hours: lane 1, molecular weight marker; lane 2, 0.03 μmol/L; lane 3, 0.3 μmol/L; lane 4, 3 μmol/L; lane 5, 30 μmol/L.
Table 1. Effect of RNA and Protein-Synthesis Inhibitors on DNA Fragmentation in CLL Cells Exposed to 3 μmol/L CldAdo

<table>
<thead>
<tr>
<th>DNA Fragmentation (%)</th>
<th>Actinomycin D μg/mL</th>
<th>Cycloheximide μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Inhibitor</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>65</td>
<td>72</td>
<td>63</td>
</tr>
<tr>
<td>49</td>
<td>49</td>
<td>75</td>
</tr>
<tr>
<td>48</td>
<td>67</td>
<td>84</td>
</tr>
<tr>
<td>48</td>
<td>71</td>
<td>91</td>
</tr>
</tbody>
</table>

Suspensions of cells were preincubated in the presence of actinomycin D or cycloheximide for 1 hour followed by incubation with 3 μmol/L CldAdo for 48 hours. DNA fragmentation was quantified as described in Materials and Methods. Each result is from duplicate samples. Standard deviations did not exceed 10%.

Association of CldAdo- and F-ara-A-induced cleavage and pronounced decreases in cellular nucleoside triphosphate levels. To assess the effects of the two analogues on nucleotide pools, CLL cells were incubated with both drugs over 72 hours (Table 4). This showed decreases in nucleoside triphosphate levels, whereas levels remained normal or slightly increased in untreated lymphocytes. After 48 hours of incubation with 3 μmol/L CldAdo, levels of uridine triphosphate (UTP), ATP, and guanosine triphosphate (GTP) were approximately 20% of control levels. At 30 μmol/L the decrease in nucleotide triphosphate pools was even greater. The decrease in triphosphate levels for both drugs was dose dependent (Table 4) and time dependent over 72 hours (not shown).

Clinical correlation of spontaneous and drug-induced DNA fragmentation. Freshly isolated cells from 17 CLL patients were incubated alone or with 3 μmol/L CldAdo or F-ara-A for 48 hours to assess DNA fragmentation. Interpatient variation was evident for spontaneous and drug-induced fragmentation. Spontaneous DNA fragmentation after incubation of the lymphocytes for 48 hours ranged from 3% to 64% (median 21%) (Table 5). Lymphocytes from patients with Rai stage 0 and I disease exhibited greater levels of DNA fragmentation than those of patients with more advanced...
Drugs-induced apoptosis in CLL

For the first time, swelling of the mitochondria, a feature of necrosis, was observed; however, high-amplitude swelling of the mitochondria, a feature of necrosis, was also consistent with apoptosis. At the ultrastructural level, nuclear changes characteristic of apoptosis were observed; however, high-amplitude swelling of the mitochondria, a feature of necrosis, was observed in some cells undergoing apoptosis. After prolonged exposure, membrane dissolution also occurred. This event was presumably caused by secondary necrotic degeneration, although it is possible that some cells died by means of necrosis or a mixture of both processes.

In the present study in vitro exposure of CLL lymphocytes to CldAdo and F-ara-A triggered extensive DNA fragmentation and cell death characteristic of apoptosis as defined by morphology and agarose gel electrophoresis of DNA. The time course of vital dye uptake was also consistent with apoptosis. At the ultrastructural level, nuclear changes characteristic of apoptosis were observed; however, high-amplitude swelling of the mitochondria, a feature of necrosis, was observed in some cells undergoing apoptosis. After prolonged exposure, membrane dissolution also occurred. This event was presumably caused by secondary necrotic degeneration, although it is possible that some cells died by means of necrosis or a mixture of both processes.

DNA fragmentation was observed during short-term culture in the absence of drugs in most cases. In contrast to drug-induced apoptosis, the degree of fragmentation was several fold lower and did not seem to increase dramatically on prolongation of incubation. The dose-response curve of DNA fragmentation illustrates the marked sensitivity of CLL cells to these agents. CldAdo-treated lymphocytes consistently exhibited more quantitative DNA cleavage than those treated with equal concentrations of F-ara-A; however, clinically

Table 2. Effect of Calcium Chelators on DNA Fragmentation in CLL Cells Exposed to 3 μmol/L CldAdo

<table>
<thead>
<tr>
<th>DNA Fragmentation (%) 3 μmol/L CldAdo</th>
<th>EGTA (mmol/L)</th>
<th>BAPTA-AM (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Chelator</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>59</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>80</td>
</tr>
</tbody>
</table>

Suspensions of cells were preincubated in the presence of EGTA or BAPTA-AM for 15 minutes followed by the addition of 3 μmol/L CldAdo for 48 hours. DNA fragmentation was quantified as described in Materials and Methods. Each result is from duplicate samples. Standard deviation did not exceed 10%.

Table 3. Effect of Phorobol Ester on DNA Fragmentation in CLL Cells in the Presence and Absence of 3 μmol/L CldAdo

<table>
<thead>
<tr>
<th>DNA Fragmentation (%)</th>
<th>No CldAdo</th>
<th>CldAdo</th>
</tr>
</thead>
<tbody>
<tr>
<td>No TPA/TPA</td>
<td>3.0/4.5</td>
<td>34/9</td>
</tr>
<tr>
<td>3.5/2.0</td>
<td>41/9</td>
<td></td>
</tr>
<tr>
<td>7.6/5.7</td>
<td>45/11</td>
<td></td>
</tr>
<tr>
<td>39/44</td>
<td>63/57</td>
<td></td>
</tr>
<tr>
<td>41/44</td>
<td>62/32</td>
<td></td>
</tr>
</tbody>
</table>

Suspensions of cells were preincubated in the presence of 10 nmol/L TPA for 15 minutes and incubated with 3 μmol/L CldAdo for 48 hours. DNA fragmentation was quantified as described in Materials and Methods. Each result is from duplicate samples. Standard deviations did not exceed 10%.

Table 4. Nucleotide Triphosphate Levels in CLL Cells

<table>
<thead>
<tr>
<th>Drug (concentration)</th>
<th>% of Control</th>
<th>UTP</th>
<th>ATP</th>
<th>GTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CldAdo (3 μmol/L)</td>
<td>19</td>
<td>20</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>CldAdo (30 μmol/L)</td>
<td>5</td>
<td>5</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>F-ara-A (3 μmol/L)</td>
<td>50</td>
<td>48</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>F-ara-A (30 μmol/L)</td>
<td>7</td>
<td>7</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

CLL cells were treated with various concentrations of CldAdo or F-ara-A. After incubation with the indicated drug concentrations for 48 hours, nucleotides were extracted and analyzed as outlined in Materials and Methods. Each value represents pooled data derived from three patients with testing performed in triplicate. Standard deviations did not exceed 5%.

Table 5. Effect of CldAdo and F-ara-A on DNA Fragmentation in CLL Cells

<table>
<thead>
<tr>
<th>DNA Fragmentation (%)</th>
<th>Rai Stage</th>
<th>Prior Therapy</th>
<th>Prior Response</th>
<th>Spontaneous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>–</td>
<td>–</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>+</td>
<td>PD</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>–</td>
<td>–</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>+</td>
<td>PR</td>
<td>22</td>
</tr>
</tbody>
</table>

CLL cells isolated from 17 CLL patients were incubated alone or with CldAdo or F-ara-A 3 μmol/L for 48 hours. DNA fragmentation was assessed as the percentage of DNA resisting sedimentation. Each result is from duplicate determinations. Standard deviations did not exceed 10%.

Abbreviations: CR, complete response; PR, partial response; PD, progressive disease.

DISCUSSION

In the present study in vitro exposure of CLL lymphocytes to CldAdo and F-ara-A triggered extensive DNA fragmentation and cell death characteristic of apoptosis as defined by morphology and agarose gel electrophoresis of DNA. The time course of vital dye uptake was also consistent with apoptosis. At the ultrastructural level, nuclear changes characteristic of apoptosis were observed; however, high-amplitude swelling of the mitochondria, a feature of necrosis, was observed in some cells undergoing apoptosis. After prolonged exposure, membrane dissolution also occurred. This event was presumably caused by secondary necrotic degeneration, although it is possible that some cells died by means of necrosis or a mixture of both processes.

DNA fragmentation was observed during short-term culture in the absence of drugs in most cases. In contrast to drug-induced apoptosis, the degree of fragmentation was several fold lower and did not seem to increase dramatically on prolongation of incubation. The dose-response curve of DNA fragmentation illustrates the marked sensitivity of CLL cells to these agents. CldAdo-treated lymphocytes consistently exhibited more quantitative DNA cleavage than those treated with equal concentrations of F-ara-A; however, clinically
achievable levels of CldAdo range from 30 to 300 nmol/L depending on the method of administration, whereas levels of F-ara-A reach approximately 3 μmol/L.7

CLL does not result directly from enhanced proliferation, but rather is caused by the progressive accumulation of long-lived lymphocytes with enhanced viability. Paradoxically, short-term culture renders many of these cells susceptible to apoptosis. Increasing the cell density or changing the source of serum did not affect this spontaneous rate of cell death. One assumes this arises because of the lack of an unidentified essential cytokine. Recently it was shown that allogeneic bone marrow–derived stromal cells prevent the loss of B-lineage acute lymphoblastic leukemia cells though apoptosis.38 Whether in vitro survival of CLL cells would be enhanced by optimizing cell culture conditions using a variety of media containing feeder cells and/or growth factors in these experiments remains unknown.

Much knowledge concerning apoptosis comes from studies performed in immature rat thymocytes, which readily undergo apoptosis. Several studies have shown that glucocorticoid-induced DNA fragmentation in thymocytes can be blocked by inhibition of protein and mRNA synthesis.2,3 Because glucocorticoids are able to induce a protein possessing nuclease activity, it is postulated that new mRNA and protein synthesis is required for synthesis of the endonuclease. However, these observations are controversial and some data suggest that these results were generated by the experimental conditions.40,41 Inhibition of protein synthesis by cycloheximide has been shown to stimulate fragmentation in human HL-60 cells,42,43 and T-acute lymphoblastic leukemia cells.44 Similar findings in CLL cells were obtained by Collins et al5 and confirmed by our data. Together, these results suggest constitutive expression of the endonuclease. Alternatively, cycloheximide and actinomycin D could suppress the synthesis of a repressor protein that has a shorter half-life than the endonuclease resulting in a subsequent release of apoptosis. Finally, these inhibitors could directly cause DNA fragmentation by means of additional effects on DNA synthesis and conformation.

DNA cleavage in thymocytes is associated with elevations in cytosolic calcium, which stimulates a calcium-magnesium-dependent endonuclease.37 We found depletion of extracellular calcium with EGTA failed to block fragmentation. In contrast, the intracellular chelator BAPTA-AM completely eliminated DNA fragmentation. This would suggest that intracellular calcium-storing organelles, such as mitochondria, may be important in regulating the increase in cytosolic calcium. When cytosolic calcium reaches exceedingly high levels, a low-affinity, high-capacity calcium pump located in the inner mitochondrial membrane is activated and takes up cytosolic calcium. The 25-Kd Bcl-2 protein, which localizes to the inner mitochondrial membrane, has been shown to provide a survival advantage by inhibition of apoptosis in B lymphocytes.22,23 The role of Bcl-2 in modulating cytosolic calcium levels has not yet been established.

Activation of protein kinase C–dependent phosphorylation by phorbol esters resulted in a variable inhibition of drug-induced DNA fragmentation in four of five CLL patients. The response of CLL cells to phorbol esters has been shown to be heterogeneous and is associated with increased expression of cytoplasmic IgG, surface IgG, Ia, and tartrate-resistant acid phosphatase.46,47 Our data and those of McConkey et al,48 which show that phorbol ester-driven CLL cells are less susceptible to apoptosis, suggest that the state of cellular activation in CLL is important for chemosensitivity.

Although subject to controversy,48,49 it is thought that quiescent lymphocytes contain DNA strand breaks that are continuously being formed and repaired. It is hypothesized that CldAdo treatment of normal resting lymphocytes causes an increase in the amount of DNA strand breaks and thus leads to activation of poly(ADP-ribose) polymerase, which results in a lethal depletion of cellular NAD and ATP and consequential cell death.27,28 Our investigations showed equally dramatic decreases in nucleotides other than ATP, suggesting alternative mechanisms for the decrease in ATP levels.

Pronounced interpatient variation was evident in spontaneous and drug-induced fragmentation and was significantly higher in lymphocytes obtained from patients with low-risk disease and no prior therapy. Patients who had previously untreated CLL and high levels of spontaneous DNA fragmentation after short-term culture had a high complete response rate, suggesting that the propensity of CLL cells to undergo spontaneous apoptosis correlates with a favorable response to chemotherapy. The lowest levels of spontaneous fragmentation occurred in cells from five patients who had been refractory to a variety of regimens including fludarabine. This is consistent with the hypothesis that a relatively apoptosis-resistant phenotype may develop with disease progression. The observed interpatient variation has implications for studies using CLL cells as an experimental model for apoptosis, and the underlying predisposition of the leukemic cell population to apoptosis should be considered.

The primary mechanism of action and the cellular targets of CldAdo and F-ara-A in proliferating cells are well known. The present in vitro study of CLL cells shows that these chemotherapeutic agents activate a program of cell death. This process is not dependent on nascent mRNA and protein expression; however, it is apparently dependent on elevations of cytosolic calcium and the state of cellular activation and/or protein kinase C status. Not all cell populations were equally sensitive with CLL lymphocytes from patients with advanced-stage and refractory disease being less prone to apoptosis. Processes uncovered by the investigation of CLL cells in vitro may lend insight into the signals that are involved in chemotherapy-induced cell death, and assays that measure endonucleolytic cell death may have clinical use in monitoring chemosensitivity and predicting response.

REFERENCES


Induction of apoptotic cell death in chronic lymphocytic leukemia by 2-chloro-2'-deoxyadenosine and 9-beta-D-arabinosyl-2-fluoroadenine

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