Involvement of CED-3/ICE Proteases in the Apoptosis of B-Chronic Lymphocytic Leukemia Cells

By Beatriz Bellosillo, Mireia Dalmau, Dolors Colomer, and Joan Gil

B-chronic lymphocytic leukemia (B-CLL) is characterized by the accumulation of long-lived B lymphocytes that express high levels of Bcl-2. We examined the involvement of CED-3/ICE-like proteases in the apoptosis of B-CLL cells. One of the substrates of these proteases is poly(ADP-ribose) polymerase (PARP). The effect of different factors that induce the apoptosis of B-CLL cells on the proteolytic cleavage of PARP has been studied. Treatment of B-CLL cells with different concentrations of dexamethasone (1 to 1,000 μmol/L) induced in a dose-dependent manner the cleavage of PARP. Dexamethasone-induced PARP cleavage after 12 hours of incubation, which was almost complete at 48 hours. PARP cleavage during apoptosis of B-CLL cells was studied in cells from eight patients and a correlation was found between cell viability and the degree of PARP cleavage. Incubation in vitro of B-CLL cells with fludarabine for 48 hours induced PARP cleavage in all the cases studied. Protein kinase C (PKC) activation with 100 nmol/L TPA (12-O-tetradecanoylphorbol 13-acetate) or incubation with interleukin-4 (10 ng/mL) prevented either dexamethasone- or fludarabine-induced proteolysis of PARP. Incubation of B-CLL cells with the CED-3/ICE-like protease inhibitor Z-VAD.fmk inhibited spontaneous and dexamethasone-induced PARP cleavage and DNA fragmentation in a dose-dependent manner. Furthermore, Z-VAD.fmk prevented the cytotoxic effect of dexamethasone. These results indicate that CED-3/ICE-like proteases play an important role in the apoptosis of B-CLL cells.

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B-CHRONIC LYMPHOCYTIC leukemia (B-CLL) is characterized by the accumulation of monoclonal CD5+ B lymphocytes. The majority of circulating cells appear to be nondividing and it has been suggested that the clonal excess of B cells results from decreased cell death rather than increased proliferation.

Apoptosis is the physiological process whereby most cells, including B lymphocytes, are eliminated, which leads to homeostasis. Apoptosis is characterized by morphological and biochemical changes that include cell shrinkage, membrane blebbing, chromatin condensation, and endogenous endonuclease activation. Apoptosis of B-CLL lymphocytes can be regulated by different cytokines. When B-CLL cells are placed in culture medium, they undergo apoptosis. This spontaneous apoptosis in cultured B-CLL cells is probably triggered by the absence of survival factors present in vivo. Candidate survival factors that prevent apoptosis of B-CLL cells in vitro are: interleukin-4 (IL-4), interferon-γ (IFN-γ), IFN-α, IL-2, IL-6, IL-8, and IL-13. On the other hand, IL-10 and IL-5 induce apoptosis in these cells. Glucocorticoids and other chemotherapeutic agents used clinically, like chlorambucil, 2-chloro-2-deoxyadenosine and fludarabine, induce apoptosis in CLL lymphocytes, suggesting that apoptosis is one of the mechanisms of their therapeutic action.

The proto-oncogene product Bcl-2 has been shown to inhibit cell death induced by a variety of apoptotic stimuli and in numerous cell types. The Bcl-2 protein is overexpressed in B-CLL lymphocytes, which may inhibit the apoptosis of these cells. The mechanism of action of Bcl-2 is still unknown. It has been proposed that Bcl-2 has either an antioxidant activity or the capacity to interfere with intracellular calcium signaling. Protein kinase C (PKC) activation and also incubation with different cytokines, including IL-4, IL-8, and IFN-α, increase Bcl-2 levels in B-CLL cells and consequently block apoptosis. These findings suggest that a common step, inhibitable by Bcl-2, is activated during B-CLL apoptosis.

It is becoming evident that cysteine-proteases of the CED-3/ICE family play an important role in apoptosis. Inhibition of these proteases can block apoptosis triggered by different stimuli. They are synthesized as proenzymes, which are proteolytically processed to form active heterodimeric enzymes, but the mechanisms of control are largely unknown. Several substrates of these proteases have been described. Some of these proteases can cleave and inactivate poly(ADP-ribose) polymerase (PARP), an enzyme that participates in DNA repair and genome maintenance. Although the significance of PARP cleavage during apoptosis is not clear, it can be used as a marker of activation of CED-3/ICE-like proteases and apoptosis.

CED-3/ICE-like proteases are involved in the apoptosis of different cell lines. Recently, involvement of CED-3/ICE-like proteases in the apoptosis of Ramos-Burkitt lymphoma cells has been reported. However, the implication of these proteases in the apoptosis of primary tumor cells has not been studied. Here we attempt to determine whether CED-3/ICE-like proteases are involved in the apoptosis of B-CLL cells. The knowledge of the mechanisms underlying B-CLL apoptosis could contribute to the design of new treatments for B-CLL.

MATERIALS AND METHODS

Patients. Eleven patients (six men and five women) with B-CLL, who had not received treatment, median age 64 years (range,
and Fludarabine on B-CLL Lymphocytes

Biologically and clinically, B-CLL cells show a high degree of aberrant differentiation, which could result in their resistance to standard chemotherapy. The median peripheral blood

47 to 75 years) were studied. B-CLL was diagnosed according to standard clinical and laboratory criteria. The median peripheral blood leukemia was 104 × 10⁹ leukocytes per liter (range, 19 to 425 × 10⁹). Leukemic cells were phenotyped for cell surface markers by flow cytometry and were positive in all cases for CD5 and CD19. According to Binet’s classification,⁴⁸ at the time of inclusion four patients were at stage A, three patients were at stage B, and four patients were at stage C.

Reagents. 12-O-tetradecanoylphorbol 13-acetate (TPA) and 3,4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co (St Louis, MO). Dexamethasone was obtained from Merck KGaA (Darmstadt, Germany). Fludarabine was obtained from Schering AG (Berlin, Germany). Recombinant human IL-4 was obtained from Genzyme (Cambridge, MA). N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD.fmk) and N-benzyloxycarbonyl-Phe-Ala-fluoromethyl ketone (Z-FA.fmk) were obtained from Enzyme Systems Products (Dublin, CA). Anti-PARP polyclonal antibody (Vi.5) raised against the recombinant human PARP overproduced in SF9/baculovirus was kindly provided by Dr Gilbert de Murcia (Strasbourg, France).

Isolation of B-CLL cells. Peripheral blood lymphocytes from B-CLL patients were obtained from the Hematology Laboratory at Hospital Clinic, Barcelona, Spain. Mononuclear cells from peripheral blood samples were isolated by centrifugation on a Ficoll/Hypeaque (Seromed, Berlin, Germany) gradient and cryopreserved in liquid nitrogen in the presence of 10% dimethyl sulfoxide (DMSO).

Cell culture. B-CLL lymphocytes were cultured immediately after the thawing of the cells at a concentration of 2 to 5 × 10⁹ cells/mL in RPMI 1640 culture medium (GIBCO-BRL, Paisley, Scotland) supplemented with 10% heat inactivated fetal calf serum (FCS), 2 mmol/L glutamine and gentamicin 0.04 mg/mL at 37°C in a humidified atmosphere containing 5% carbon dioxide.⁴ factor was added at the beginning of the culture.

Cell viability assay. Cell viability was determined by the MTT assay.⁴ B lymphocytes (5 × 10⁶ cells/well) were incubated in 96-well plates in the absence or presence of factors in a final volume of 100 μL. After 48 hours, 10 μL of MTT (5 mg/mL in phosphate-buffered saline) was added to each well for an additional 6 hours. The blue MTT formazan precipitate was dissolved in 100 μL of isopropanol: 1 mol/L HCl (24:1). After 1 hour of incubation at 37°C, the absorbance values at 540 nm were determined on a multiwell plate reader. Cell viability is expressed in percentage with respect to cells incubated with 100 mmol/L TPA, which maintains cell viability.⁴³,⁴⁵ Western blot analysis of PARP cleavage. Cells were lysed with Laemmli sample buffer⁴⁴ and equal amounts of protein estimated by Bradford analysis of fragmented DNA (results not shown). The blue MTT formazan precipitate was dissolved in 100 μL of isopropanol: 1 mol/L HCl (24:1). After 1 hour of incubation at 37°C, the absorbance values at 540 nm were determined on a multiwell plate reader. Cell viability is expressed in percentage with respect to cells incubated with 100 mmol/L TPA, which maintains cell viability.⁴³,⁴⁵ Western blot analysis of PARP cleavage. Cells were lysed with Laemmli sample buffer⁴⁴ and equal amounts of protein estimated by the BCA Protein Assay (Pierce, Rockford, IL) were separated by electrophoresis on 10% or 12% polyacrylamide gel and transferred to Immobilon-P (Millipore, Bedford, MA) membranes. After blocking for 1 hour with 5% dried skimmed milk in TBST (50 mmol/L Tris HCl pH 8.0, 150 mmol/L NaCl, 0.5% Tween-20), the filters were incubated with Vi.5 PARP antibody diluted 1:1,000 in 5% dried skimmed milk in TBST. Antibody binding was detected using a secondary antibody (swine antirabbit immunoglobulin, DAKO, Glostrup, Denmark) conjugated to horseradish peroxidase diluted 1:500 in 5% dried skimmed milk in TBST and an enhanced chemiluminescence (ECL) detection kit (Amersham, Buckinghamshire, UK).

Analysis of DNA fragmentation. Analysis of DNA fragmentation by agarose gel electrophoresis was performed essentially as previously described.⁴⁵ Five million cells were pelleted and lysed in 10 mmol/L Tris-HCl pH 7.4, 0.2% Triton X-100, 1 mmol/L EDTA. After incubating for 20 minutes at 4°C, cell lysates were centrifuged at 14,000g for 15 minutes to separate low molecular weight DNA from intact chromatin. The supernatant was treated with 0.2 mg/mL of proteinase K in a buffer containing 150 mmol/L NaCl, 10 mmol/L Tris HCl pH 8.0, 40 mmol/L EDTA and 1% sodium dodecyl sulfate (SDS) for 6 hours at 37°C. The DNA preparations were extracted twice with phenol baking soda to remove proteins. DNA was precipitated with 140 mmol/L NaCl and two volumes of ethanol at −20°C overnight. DNA precipitates were recovered by centrifugation at 14,000g for 15 minutes, washed twice in 70% ethanol, and air dried. DNA pellets were dissolved in 5 μL of double-distilled water and treated for 1 hour at 37°C with RNase (Boehringer Mannheim, Mannheim, Germany). A total of 5 μL of loading buffer was added to each tube and the DNA preparations were electrophoresed in 1% agarose gels. Gels were stained with ethidium bromide and visualized under ultraviolet (UV) light.

RESULTS

PARP cleavage during glucocorticoid-induced apoptosis of B-CLL cells. First, the effect of the incubation in the absence of any factor (control) or with 10 μmol/L dexamethasone or fludarabine (5 μg/mL). Cell viability was determined by the MTT assay described in Materials and Methods. Data are shown as the mean ± SD. Abbreviation: ND, not determined.

Table 1. Cytotoxic Effect of Dexamethasone and Fludarabine on B-CLL Lymphocytes

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Control</th>
<th>Dexamethasone</th>
<th>Fludarabine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>99 ± 9</td>
<td>ND</td>
<td>54 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>102 ± 18</td>
<td>31 ± 2</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>3</td>
<td>65 ± 1.6</td>
<td>52 ± 1</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>4</td>
<td>29 ± 1</td>
<td>13 ± 0.2</td>
<td>7 ± 0.1</td>
</tr>
<tr>
<td>5</td>
<td>55 ± 3</td>
<td>25 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>6</td>
<td>25 ± 1</td>
<td>18 ± 1</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>7</td>
<td>83 ± 13</td>
<td>52 ± 12</td>
<td>37 ± 19</td>
</tr>
<tr>
<td>8</td>
<td>69 ± 0.3</td>
<td>22 ± 0.3</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>9</td>
<td>65 ± 4</td>
<td>27 ± 0.4</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>10</td>
<td>100 ± 2.5</td>
<td>ND</td>
<td>32 ± 4</td>
</tr>
<tr>
<td>11</td>
<td>68 ± 2</td>
<td>50 ± 0.3</td>
<td>25 ± 5</td>
</tr>
</tbody>
</table>

B-CLL lymphocytes were incubated at 37°C for 48 hours in the absence of any factor (control) or with 10 μmol/L dexamethasone or fludarabine (5 μg/mL). Cell viability was determined by the MTT assay as described in Materials and Methods. Data are shown as the mean ± SD. Abbreviation: ND, not determined.

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Effect of fludarabine on the cleavage of PARP. The effect of another drug that also induces apoptosis but has a different mechanism of action was next studied. Fludarabine, a purine analogue commonly used in B-CLL treatment, induces apoptosis in these cells. The effect of 5 μg/mL fludarabine on the viability of lymphocytes from B-CLL patients after 48 hours of incubation is shown in Table 1. All patients were sensitive to fludarabine and this compound induced apoptosis as shown by DNA fragmentation analysis (results not shown). We next analyzed whether fludarabine induced PARP cleavage. Incubation of B-CLL cells from six patients with fludarabine for 48 hours in vitro induced PARP cleavage in all cases (results not shown). The degree of proteolysis depended on the patient studied and ranged from 57% to 95% (mean value, 83.4 ± 14).

PKC activation and IL-4 inhibit PARP proteolysis. Factors such as the phorbol ester TPA, an activator of PKC, and IL-4 have been described as inhibitors of apoptosis in asone-induced PARP cleavage. B-CLL lymphocytes were incubated with or without 10 μmol/L dexamethasone for the indicated times. Incubation of B-CLL cells from three patients with 100 nmol/L TPA inhibited glucocorticoid-induced PARP cleavage. Incubation of cells from patient 6 (which had a high degree of spontaneous apoptosis) with 100 nmol/L TPA blocked PARP cleavage (Fig 3B). This effect was also observed when cells were incubated with 10 ng/mL IL-4. Finally, we tested if TPA blocked fludarabine-induced apoptosis. TPA inhibited both DNA degradation (results not shown) and PARP cleavage (Fig 3C). The dose-response study after 48 hours of incubation with dexamethasone (Fig 1B) showed that PARP cleavage was dose-dependent and detectable in the presence of 1 μmol/L dexamethasone. The proteolysis of PARP was complete when a high dose of dexamethasone (1 mmol/L) was used.

PARP cleavage was further studied in lymphocytes from eight additional B-CLL patients. Cells were incubated with two doses of dexamethasone, for 24 hours. DNA and protein extracts were obtained following incubation of cells for 24 hours in 1 or 10 μmol/L dexamethasone. Agarose gel electrophoresis of DNA had the characteristic ladder-like pattern of apoptosis (results not shown). Analysis of the protein extracts by Western blot showed that in all cases dexamethasone treatment increased the proteolysis of PARP, which was shown by an increase in the cleavage product and/or a decrease in the quantity of native enzyme. A correlation was found between viability and the degree of PARP cleavage (Fig 2). Thus, the cells most sensitive to dexamethasone (patients no. 2 and 8) showed a high dexamethasone-induced proteolysis of PARP, and the least sensitive cells (patients no. 3 and 7) had a very low degree of PARP cleavage. The spontaneous cleavage of PARP also depended on the patient studied, so cells from patients 4 and 6 had a very high degree of spontaneous cleavage of PARP, which correlated with a high spontaneous loss of viability.

Inhibition of CED-3/ICE–like proteases blocks apoptosis of B-CLL cells. To confirm the involvement of CED-3/ICE–like proteases in the apoptosis of B-CLL cells, we used a specific inhibitor of these proteases, Z-VAD.fmk. The dose response study of Z-VAD.fmk on the inhibition of spontaneous and dexamethasone-induced apoptosis was performed with cells from patients 6 and 9, respectively. As seen in Fig 4A, 20 to 50 μmol/L Z-VAD.fmk decreased the proteolysis of PARP and 200 μmol/L completely blocked it.
apoptosis (patients no. 5 and 6), Z-VAD.fmk reduced the spontaneous loss of viability.

DISCUSSION

This report describes the involvement of CED-3/ICE–like proteases in the apoptosis of B-CLL cells. To our knowledge, this is the first report of a role for CED-3/ICE–like proteases in the apoptosis of primary cancer cells. The results presented herein show that activation of CED-3/ICE–like proteases in B-CLL cells is a common step in spontaneous, glucocorticoid- and fludarabine-induced apoptosis. Furthermore, apoptosis of B-CLL cells is blocked by a specific

Incubation of B-CLL cells from four patients with 200 μmol/L Z-VAD.fmk for 48 hours in the presence or absence of dexamethasone showed that PARP proteolysis was inhibited in all cases studied (Fig 4B). Z-FA.fmk (200 μmol/L), a compound similar to Z-VAD.fmk, which lacks the aspartic acid residue that is necessary to inhibit CED-3/ICE–like proteases, did not block PARP degradation (data not shown). Furthermore, analysis by agarose gel electrophoresis showed that 200 μmol/L Z-VAD.fmk also inhibited both spontaneous and dexamethasone-induced DNA fragmentation almost completely (Fig 5).

Finally, we studied the effect of this inhibitor on the viability of B-CLL cells. Z-VAD.fmk (200 μmol/L) maintained B-CLL lymphocytes viability for 4 days, although incubation for longer periods of time decreased it (64% ± 8% after 7 days of incubation). The effect of Z-VAD.fmk on the viability of B-CLL lymphocytes incubated for 48 hours in the absence or presence of dexamethasone was studied. As shown in Fig 6, Z-VAD.fmk inhibited the cytotoxic effect of dexamethasone in all the patients studied. Although Z-VAD.fmk had no significant effects on B lymphocytes from patients 7, 8, and 9, in those patients with high spontaneous
inhibitor of CED-3/ICE proteases, suggesting that these proteases are essential for the apoptosis of these cells.

At present, the mechanisms that control CED-3/ICE-like proteases are largely unknown. There seems to be a cascade of proteases similar to the one involved in the activation of the complement, but the signal that starts this apoptotic cascade is not known. Recently, it has been reported that CD95/FasR uses the adaptor protein FADD/MORT1 physically to engage FLICE/MACH, the apical component of a proteolytic cascade of CED-3/ICE-like proteases.

Our results also show that PKC activation inhibits spontaneous, glucocorticoid- and fludarabine-induced apoptosis and blocks PARP cleavage. Furthermore, PARP cleavage is blocked by IL-4. These findings suggest that both PKC and IL-4 signal transduction pathways cross-talk with the different apoptotic pathways in a common step upstream of the activation of the proteases that cleave PARP. This common step could be Bcl-2, as both PKC activation and IL-4 increase the levels of Bcl-2 protein. Recently, biochemical evidence that Bcl-2 functions upstream of the CED-3/ICE family proteases has been reported, as Bcl-2 prevents the processing of these proteases and the cleavage of poly(ADP-ribose) polymerase.

Although it has been used as a marker of apoptosis, the significance of PARP cleavage during this process is unknown. Mice with a disrupted PARP gene do not show altered apoptosis, however, a dominant negative of PARP induces apoptosis of transfected cells. PARP has been implicated in different processes including DNA repair, DNA replication, and transcription. Interestingly, in B-CLL cells PARP is the most abundant protein to bind to damaged DNA. It has been proposed that a central function of CED-3/ICE proteases in apoptosis is the cleavage of PARP and other nuclear repair proteins, thereby abolishing their critical homeostatic functions.

The CED-3/ICE-like proteases present in B-CLL cells have not been identified, but some of these proteases are expressed in B lymphocytes. The identification of these proteases in B-CLL cells and the knowledge of their mechanisms of control could be very important to design new therapies for this disease. In this respect, direct activation of these proteases to induce apoptosis may be achieved.

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