Protease Activation Is Required for Glucocorticoid-Induced Apoptosis in Chronic Lymphocytic Leukemic Lymphocytes

By Joya Chandra, Joyce Gilbreath, Emil J Freireich, Kay-Oliver Kliche, Michael Andreeff, Michael Keating, and David J. McConkey

Recent work has demonstrated that glucocorticoids, nucleoside analogues, and other cancer chemotherapeutics induce apoptosis in chronic lymphocytic leukemia (CLL) cells. In this study, we investigated the involvement of protease activation in these responses using selective peptide inhibitors of the interleukin-1β converting enzyme (ICE)/caspase family and a Ca2+-activated protease we recently implicated in thymocyte apoptosis. Apoptosis was associated with proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) and increased caspase protease activity, and cell-permeant caspase antagonists (Z-VAD(O-Me)fmk and Boc-D(Obz)cmk) blocked apoptosis in response to the glucocorticoid methylprednisolone or the nucleoside analogue fludarabine, indicating that caspase activation was required for these responses. However, a peptide-based inhibitor of the Ca2+-dependent lamin protease (Z-APFcmk) also completely suppressed DNA fragmentation and the cleavage of lamin B1. Strikingly, treatment of cells with Z-APFcmk alone led to characteristic PARP cleavage, depletion of the precursor forms of two ICE family proteases (CPP32 and ICH-1), and phosphatidylserine exposure, suggesting that blockade of the lamin protease led to activation of the ICE family. Our results implicate the lamin protease as a target for Ca2+ during chemotherapeutics-induced apoptosis in CLL lymphocytes, and they identify a novel functional interaction between the protease and members of the ICE family.

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MATERIALS AND METHODS

Materials. The esterified peptide caspase inhibitor, Z-VAD(O-Me)fmk, and the mouse anti-PARP monoclonal antibody C2-10 were purchased from Enzyme Systems Products, Inc, Dublin, CA. The peptide inhibitor of the Ca2+-dependent lamin protease, Z-APFcmk, was purchased from Bachem Bioscience (King of Prussia, PA). The serine protease inhibitors Na-p-tosyl-l-lysine chloromethyl ketone (TLCK) and TPCK were purchased from Sigma Chemical Co (St Louis, MO), and calpain inhibitors I and II were from Boehringer Mannheim Corp (Indianapolis, IN). Monoclonal antibodies for human CPP32 and ICH-1 were purchased from Transduction Laboratories, Lexington, KY. A chicken antiseraum specific for lamin B1 was generously provided by Dr Scott Kaufmann, Department of Cell Biology and Hematology, The University of Texas M.D. Anderson Cancer Center, Houston, TX.

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Table 1. Effects of Protease Inhibitors on Glucocorticoid- and Fludarabine-Induced Apoptosis

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Apoptosis was quantified by PI staining and FACS analysis after 48 hours of culture. Abbreviations: ND, not determined; MPS, methylprednisolone succinate; M/A, MPS/zAPFcmk; M/V, MPS/zVADfmk.

*10 μmol/L zAPFcmk.

²25 μmol/L zVADfmk.

³10 μmol/L methylprednisolone.

Oncology, Mayo Clinic, Rochester, MN. The rabbit antichicken antibody was from Cappel Laboratories (Durham, NC), and horseradish peroxidase-conjugated antimonoclonal and antirabbit antibodies were from Amersham Corp (Arlington Heights, IL).

Patients, cell isolation, and incubation criteria. All patients fulfilled the National Cancer Institute’s (NCI) criteria for the diagnosis of CLL. Some of the patients had received prior therapy, although none within the last 6 months before experimentation.

Immunophenotyping by dual-parameter flow cytometry showed coexpression of CD5 with B-cell antigen and isotypic light chain expression. Clinical staging was based on the system described by Rai.\textsuperscript{7} Freshly isolated peripheral blood was fractionated by Ficoll-Hypaque (Winthrop Pharmaceuticals, New York, NY) sedimentation at 4°C, and cells were analyzed by flow cytometry (FACScan, Becton Dickinson).

ICE activity assay. Protease activity measurements were conducted as described previously.\textsuperscript{8} Cells were resuspended in binding buffer containing 1 μg/ml fluorescein isothiocyanate (FITC) conjugated annexin V (Nexins Research BV, Hoeven, The Netherlands) and incubated for 30 minutes at 4°C, and cells were analyzed by flow cytometry (FACScan, Becton Dickinson).

Immunoblotting. Cells were lysed for 1 hour at 4°C in a lysis buffer A containing 25 mmol/L HEPES (pH 7.4), 5 mmol/L EDTA, 2 mmol/L dithiothreitol (DTT), and 10 μmol/L digitonin for 15 minutes on ice. The lysates were clarified by centrifugation (12,000 g), and supernatants were incubated with 50 μmol/L Asp-Glu-Val-Asp-aminomethyl coumarin DEVD-AMC (Enzyme Systems Products, Inc) at 37°C in the dark. Relative activities were then measured in a spectrofluorimeter (400 nm excitation, 505 nm emission); blanks included supernatants processed as outlined above without dye and supernatants incubated with excess BACMK (25 μmol/L).

DNA fragmentation analysis. Quantification of apoptosis by propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) analysis was performed as described previously.\textsuperscript{9} Following incubation with various agents in vitro, cells were pelleted by centrifugation and resuspended in phosphate-buffered saline containing 50 μg/mL PI, 0.1% Triton X-100, and 0.1% sodium citrate. Samples were stored at 4°C for 16 hours and vortexed prior to FACS analysis (FL-3 channel; Becton Dickinson FACScan, Mountain View, CA).

Annexin V binding. Exposure of surface phosphatidylserine was quantified by surface annexin V staining as described previously.\textsuperscript{10} Cells were resuspended in binding buffer containing 1 μg/ml fluorescein isothiocyanate (FITC) conjugated annexin V (Nexins Research BV, Hoeven, The Netherlands) and incubated for 30 minutes at 4°C, and cells were analyzed by flow cytometry (FACScan, Becton Dickinson).

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Fig 1. Induction of apoptosis in a representative CLL patient isolate. (A) Cells were incubated in the absence or presence of 10 μmol/L methylprednisolone with or without 25 μmol/L zAPFcmk for 48 hours, and apoptosis was quantified by PI staining and FACS analysis. Representative results are presented from one patient (not included in Table 1). (B) Glucocorticoid treatment induces exposure of phosphatidylserine. Cells were incubated in the absence or presence of 10 μmol/L methylprednisolone with or without 25 μmol/L zAPFcmk, and PS exposure was measured by binding of annexin V as described in Materials and Methods. Representative results from two experiments (patients 24 and 25 from Table 1).
Fig 2. ICE family protease activation in CLL apoptosis. (A) Cleavage of PARP. Cells were incubated for 16 hours in the absence or presence of 10 μmol/L methylprednisolone with or without 25 mmol/L zAPFcmk or 25 mmol/L zVADfmk, and PARP integrity was assessed by immunoblotting. Arrows indicate positions of intact PARP (p116) and the 85 kD caspase-generated PARP fragment (p85). Results from one experiment (patient 22) representative of four independent replicates with different patient isolates. (B) Measurement of caspase activity. Cells were incubated for the times indicated in the absence or presence of 10 μmol/L methylprednisolone, and hydrolysis of the ICE protease substrate DEVD-AMC was measured in a spectrofluorimeter as described in Materials and Methods. Results from two representative patient isolates (not included in Table 1) analyzed on the same day. Levels of DNA fragmentation were measured in parallel (16 hours): (h), patient A, control—2%; (●), MPS—30%; (○), patient B, control—9%; (●), MPS—16%. Similar results were observed in three other patient isolates.

experiments) or overnight (lamin B1 experiments) in a TBS-T buffer (25 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, and 0.05% Tween-20) containing 3% (wt/vol) nonfat dried milk. Blots were then probed overnight with antibodies to PARP or CPP32 or for 1 hour with a chicken antitubulin B1 antiserum, and blots were developed using species-specific secondary and/or tertiary antisera. Immunoreactive material was then visualized by enhanced chemiluminescence (ECL; Amersham Corp) according to the manufacturer’s instructions.

RESULTS

Previous work has shown that glucocorticoid hormones and nucleoside analogues induce DNA fragmentation characteristic of apoptosis in some (but not all) CLL lymphocytes in vitro and in vivo.1 We treated cells from 25 different CLL patients for 48 hours with 10 μmol/L methylprednisolone (MPS), a concentration that was found to be optimal in dose-response analyses in previous work1 and measured apoptosis by PI staining and FACS analysis (Table 1; representative results from one patient are presented in Fig 1A). Apoptotic cells can be readily distinguished from viable cells with this procedure by their subdiploid DNA content. Variable responses were observed, consistent with our previous findings.3 Surface annexin staining and FACS analysis demonstrated that glucocorticoid treatment induced surface phosphatidylserine exposure on the cells, which is another hallmark biochemical feature of apoptotic cell death (Fig 1B). An optimal concentration12 of the nucleoside analog, fludarabine, also promoted the appearance of cells with a hypodiploid DNA content, demonstrating that the effects were not limited to steroid treatment.

We next investigated the involvement of the caspases in these responses using biochemical assays for their activation. Glucocorticoid-induced cell death was associated with nearly complete cleavage of PARP to an 85-kD fragment characteristic of caspase-mediated proteolysis,8 an event that was inhibited by zVADfmk (Fig 2A). Glucocorticoids also induced time-dependent hydrolysis of a fluorogenic caspase substrate (DEVD-AMC) in apoptosis-sensitive patient isolates, but not in apoptosis-resistant ones (Fig 2B). Furthermore, the cell-permeant caspase antagonist zVAD(O-Me)fmk8 inhibited glucocorticoid-induced DNA fragmentation in a majority of patient samples (Fig 3A, Table 1) and prevented DNA fragmentation in some CLL isolates treated with the nucleoside analog, fludarabine (Fig 3A), demonstrating that the effects were not limited to glucocorticoid-induced apoptosis. Together, these results indicate that caspase activation is required for induction of apoptosis in CLL cells.

We then investigated the involvement of the nuclear scaffold protease in these responses. A peptide inhibitor of the nuclear Ca2+-dependent lamin protease (zAPFcmk)5 com-
Fig 3. Effects of protease inhibitors on apoptosis. (A) Comparison of the ICE antagonist zVADfmk with the serine protease antagonist TPCK. Cells were incubated for 48 hours in the absence or presence of 10 μmol/L methylprednisolone or 10 μmol/L fludarabine with or without 25 μmol/L zVADfmk or 25 μmol/L TPCK, and the percentage of hypodiploid cells was determined by PI staining and FACS analysis as outlined in Materials and Methods. Results from two representative experiments (patients 6 and 21 in Table 1) (△, experiment 1; ■, experiment 2). (B) Effects of the peptide antagonist zAPFcmk. Cells were incubated as described above with or without 25 μmol/L zAPFcmk, and the percentage hypodiploid cells was determined by PI staining. Results from three representative experiments (patients 8, 11, and 9 in Table 1) (□, experiment 1; ■, experiment 2; △, experiment 3). (C) Effects of TLCK. Cells were incubated for 48 hours in the absence or presence of 10 mmol/L methylprednisolone with or without 50 mmol/L TLCK, and the percentage of hypodiploid cells was determined by PI staining and FACS analysis. Results from four representative patients (5, 6, 7, and 20 in Table 1) (□, patient 5; ■, patient 6; △, patient 7; △, patient 20).
Lytic degradation of one of their key substrates, and a specific either lamin cleavage or DNA fragmentation in CLL cells. It promotes activation of at least two caspases, proteolytic degradation of one of their key substrates, and a specific surface change associated with apoptosis. Together, these results strongly suggest that the Ca\(^{2+}\)-dependent lamin protease acts downstream of the caspases in CLL lymphocytes.

**DISCUSSION**

The observation that cancer chemotherapeutics induce apoptosis in CLL cells and other tumor cell types\(^{13,15}\) provides a mechanistic framework for their actions and suggests the existence of novel mechanisms for drug resistance involving specific suppression of physiologic cell death. Detailed information about the biochemical and molecular mechanisms involved in apoptosis is now emerging, with the implication of the caspases in the effector phase of the process being one of the most notable.\(^{5}\) With this in mind, we investigated the potential involvement of the caspases in chemotherapy-induced apoptosis in CLL lymphocytes. The results show that specific cleavage of the caspase substrate, PARP, is a feature of glucocorticoid-induced cell death. Furthermore, direct measurement of caspase activity using a fluorogenic substrate demonstrated a correlation between the effects of glucocorticoid on caspase activation and DNA fragmentation. Time course analysis indicates that caspase activation slightly precedes DNA fragmentation (J. Chandra, unpublished observations), consistent with a role for caspase activation at an early stage of the response. Finally, cell-permeant peptide caspase inhibitors blocked endogenous endonuclease activation in most patient samples. Together, our results show for the first time that activation of one or more members of the ICE family participate in glucocorticoid-induced apoptosis in CLL cells.

In our previous work on CLL cells, we showed that glucocorticoid-induced apoptosis is associated with an early, sustained increase in the cytosolic Ca\(^{2+}\) concentration, and Ca\(^{2+}\)-buffering agents blocked DNA fragmentation and delayed cell death.\(^{5}\) We proposed that one target for Ca\(^{2+}\) in these cells is the endogenous nuclear endonuclease responsible for chromatin cleavage, as incubation of isolated nuclei from untreated, drug-sensitive CLL cells in the presence of Ca\(^{2+}\) resulted in oligonucleosomal DNA fragmentation characteristic of apoptosis in whole cells.\(^{1,3}\) Indeed, the level of endonuclease activity detected by this approach predicts the level of DNA fragmentation observed in whole cells exposed to glucocorticoid,\(^{3}\) indicating that it is tightly coupled to the response, and the endonuclease remains an attractive candidate target for Ca\(^{2+}\) in the cells. However, the results of the present study indicate that the nuclear lamin protease is another important target for Ca\(^{2+}\) in CLL cells. Activation of this protease is also required for endonuclease activation in whole thymocytes and isolated thymocyte nuclei,\(^{7}\) and it is therefore possible that the link between drug sensitivity and endonuclease activity identified in our previous work is related to levels of this protease. Other recent work has demonstrated that the cytosolic caspases MCH-2\(^{14,15}\) and CPP32\(^{26}\) can also degrade the lamins in other model systems. However, we can exclude a requirement for caspase-3 activation in our system, because activation of the protease in response to treatment with zAPFcmk alone did not result in either lamin cleavage or DNA fragmentation in CLL cells. Although this has not yet been cloned, preliminary efforts in
Fig 5. Effects of the Ca\(^{2+}\) protease inhibitor on processing of two different members of the ICE family. (A) Effects on CPP32. Cells were incubated for 16 hours in the absence or presence of 10 \(\mu\)mol/L methylprednisolone with or without 10 \(\mu\)mol/L zAPFcmk or 25 \(\mu\)mol/L zVADfmk, and levels of the precursor form of CPP32 were analyzed by immunoblotting. Results of one experiment (patient 22, Table 1) that were typical of three independent replicates. (B) Effects on ICH-1L. Cells were incubated as described above and levels of the precursor form of ICH-1L were determined by immunoblotting. Results of one experiment (patient 23, Table 1) that were typical of three independent replicates.

this laboratory and another suggest that the nuclear scaffold protease is structurally related to the proteosome,\(^{17}\) a multisubunit protease complex\(^{18}\) that has recently been implicated in apoptosis.\(^{19-21}\) Precisely how this protease regulates endonuclease activation will be the focus of future investigation.

Inhibitors of the Ca\(^{2+}\)-dependent lamin protease promote PARP cleavage, processing of two distinct members of the ICE family, and surface exposure of phosphatidylserine, while they completely block drug-induced lamin B\(_1\) cleavage and endonuclease activation. Although the biochemical mechanisms underlying these seemingly contradictory observations are still under investigation, our ongoing work suggests a possible explanation, outlined schematically in Fig 6. Previous work has shown that one of the inhibitors
of the nuclear scaffold (NS) protease,\textsuperscript{7} TPCK, blocks the activity of the transcription factor, NFκB, by preventing proteolytic degradation of its inhibitor, IκB.\textsuperscript{22-27} Other recent work has shown that NFκB inhibits apoptosis,\textsuperscript{23-27} and protease inhibitors that block IκB degradation can directly induce apoptosis in certain model cell lines.\textsuperscript{25} The biochemical properties of TPCK and zAPFcmk are similar (they both possess a phenylalanine residue at the critical P1 site that binds the protease active site), and in electrophoretic mobility shift (EMSA) assays, we have found that the levels of active NFκB, which are very high in most of the isolates we have tested, are rapidly lowered by both TPCK and zAPFcmk (J. Chandra, unpublished observations). Therefore, if NFκB participates in the suppression of apoptosis in CLL, its inhibition by zAPFcmk could account for the caspase activation and phosphatidylserine exposure observed in CLL cells treated with the inhibitor. However, TPCK and zAPFcmk also completely inhibit Ca\textsuperscript{2+}-mediated lamin B\textsubscript{1} cleavage and endonuclease activation in isolated nuclei, so that although upstream events of apoptosis still occur, the process is arrested upstream of the nuclear events in cells treated with these inhibitors. Importantly, our results predict that agents that inhibit the NFκB protease without blocking the NS protease would be extremely effective inducers of apoptosis in CLL cells. Our ongoing work suggests that proteasome inhibitors (calpain inhibitor I, MG-132, and lactacystin) may fall into this category: they induce apoptosis in all of the patient isolates we have tested so far, including cells that are substantially resistant to glucocorticoids or nucleosides. Hopefully, by elucidating the biochemical mechanisms involved, we can use this information to identify better treatments for CLL and other hematopoietic malignancies.

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