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The interleukin 1β-converting enzyme inhibitor *CrmA* prevents Apo1/Fas- but not glucocorticoid-induced poly(ADP-ribose) polymerase cleavage and apoptosis in lymphoblastic leukemia cells

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Abstract Glucocorticoids (GC) induce programmed cell death (apoptosis) in immature lymphocytes and are an essential component in the therapy of acute lymphatic leukemia. The mechanism underlying GC-induced apoptosis particularly in leukemia cells is, however, not well understood. Most forms of apoptosis seem to employ a common final effector pathway characterized by specific proteolytic events mediated by interleukin 1β-converting enzyme (ICE) and/or other ICE-like cysteine proteases. These events may result in the morphologic changes characteristic of apoptosis. To determine whether a similar proteolytic pathway is activated during GC-induced leukemia cell apoptosis, we investigated poly(ADP-ribose) polymerase (PARP), a typical target of ICE-like proteases, during GC-induced apoptosis of the human acute T-cell leukemic cell line CEM-C7H2. Our studies showed proteolytic PARP cleavage suggestive of activation of ICE-like proteases that preceeded morphologic signs of apoptosis. We further established stably transfected CEM-C7H2 sublines expressing the cowpox virus protein CrmA that inhibits some, but not all, ICE-like proteases. GC-induced PARP cleavage and apoptosis were neither inhibited nor delayed in *crmA*-expressing cell lines. In contrast, *crmA* expression rendered the same lines resistant to Apo1/Fas-induced PARP cleavage and apoptosis. Thus, different proteases might be activated during the effector phases of GC- and Apo1/Fas-induced apoptosis in human leukemia cells.

Key words: Apoptosis; Glucocorticoid; Protease; ICE; Apo1/Fas; Human; Leukemia; T-cell; Poly(ADP-ribose) polymerase

1. Introduction

Genetic dissection of programmed cell death in the nematode *Caenorhabditis elegans* (*C. elegans*) led to the discovery of two essential cell death effector genes, *ced-3* and *ced-4* [1]. While the function of *ced-4* remains to be elucidated, *ced-3* has been identified as a *C. elegans* homolog of the human interleukin 1β-converting enzyme (ICE), a cysteine protease that generates the cytokine IL-1β and plays a key role in initiation of immune responses [2]. Subsequent analyses have shown that ICE, when overexpressed in appropriate cell types, can induce cell death with the morphologic characteristics of apoptosis [3]. However, ICE itself may not be the only protease important in apoptosis, since ICE-deficient mice are defective in Apo1/Fas-induced apoptosis while other forms of programmed cell death proceed unimpaired [4,5]. Meanwhile, several ICE-homologous cysteine proteases were cloned including mouse Nedd2 [6] and its human homolog Ich-1 [7], TX/Ich-2/ICErelII [8–10], ICErelIII/TY [9,11], CPP32/Apopain/Yama/Sca1 [12–16], Mch2 [17], Mch3/Sca2/ICE-LAP3 [18–20], and Mch4 and 5 [21]. Structural and functional analysis of ICE suggested that these proteases are tetramers of two small heterodimers, require an aspartic acid at the P1 position of the substrate's cleavage site and, although differing in substrate specificities, induce apoptosis when expressed in their active form in eukaryotic cells. Some of these proteases, like ICE, are activated by autocatalysis, while others, such as CPP32 and Mch-2, might require additional (ICE-like) proteases for activation [22]. Active ICE proteases cleave a variety of cellular proteins, which may then lead to the characteristic features of apoptosis [23].

One of the targets of ICE-proteases during apoptosis is poly(ADP-ribose) polymerase (PARP), also termed poly(ADP) ribosyl transferase (ADPRT), a nuclear enzyme that is activated by DNA strand breaks [24,25]. In addition to its protective action on the genome (predominantly in response to environmental stress [26]), it influences polyADP-ribosylation of nucleases implicated in internucleosomal DNA degradation [27] and consumes NAD and its precursor ATP [28], an energy source required for apoptosis [29]. Due to these various effects, PARP and its degradation may both contribute to, or interfere with, the apoptotic process [30,31]. Apoptotic inactivation of PARP involves a single cleavage of the polypeptide after aspartate 216 [12], which separates the N-terminal Zn-finger DNA-binding domain from the C-terminal automodification and polyADP-ribosylating domains. This results in liberation of an 85 kDa fragment from the 113 kDa full-length enzyme. PARP cleavage is mediated by CPP32 [12,14,15], Mch-2 [17], and perhaps other members of this family, and can be regarded a typical indicator for activation of ICE-like proteases.

The study of ICE-like proteases has been greatly facilitated by the discovery of virus proteins that specifically inactivate such enzymes, thereby preventing apoptosis of the host cell (reviewed in [32]). One of them, the cowpox virus serpin CrmA, inactivates some members of the ICE family, like ICE itself [33], but not, or to a lesser extent, others, e.g.,

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Abbreviations: FACS, fluorescence-activated cell sorter; GC, glucocorticoid(s); ICE, interleukin 1β-converting enzyme; PARP, poly(ADP-ribose) polymerase

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CPP32/apopain [15]. Hence, it can be used conveniently to distinguish ICE-like proteases in different forms of apoptosis. In agreement with data from mice made deficient for ICE by homologous recombination, *crmA* overexpression led to inhibition of Apo1/Fas-induced apoptosis [34]. Moreover, in different in vitro cell culture systems, *crmA* overexpression prevented apoptosis induced by neuronal growth factor withdrawal [35], tumor necrosis factor [36,37] and granzyme B [38].

Glucocorticoids (GC) induce programmed cell death (apoptosis) in various cells of the lymphoid lineage, including thymocytes and lymphatic leukemic cells [39,40]. The mechanisms underlying GC-induced apoptosis are poorly understood and may differ in various cell types and species. Thus, in rodent thymocytes, GC apoptosis is macromolecule neosynthesis-dependent [40,41], employs a Ca^{2+}-activated endonuclease [42], and occurs in the absence of functional ICE, as shown in corresponding knock-out mice [4]. In human leukemic cells, in contrast, GC-induced cell death may occur via transrepression rather than transactivation [43], and DNA fragmentation is mediated by a Ca^{2+}-independent endonuclease [44]. A possible requirement for ICE or other related proteases in GC-induced leukemic cell death has not been investigated.

In this study, we report that GC treatment of CEM-C7H2 acute T-cell leukemic cells, like treatment with antibodies to the death inducer membrane protein Apo1/Fas [45,46], results in the cleavage of PARP in a pattern reminiscent of ICE-like proteases followed by extensive apoptosis. However, in contrast to Apo1/Fas-induced PARP cleavage and apoptosis, which were almost completely inhibited by overexpression of the cowpox virus protein CrmA, these phenomena essentially proceeded unaltered in GC-treated and *crmA*-overexpressing CEM-C7H2 sublines. Our data suggest that GC-induced apoptosis in human leukemic cells is associated with, and perhaps mediated by, the activation of ICE-like proteases that are, however, distinct from ICE itself or other CrmA-inhibitable ICE family members.

2. Materials and methods

2.1. Cell culture

The GC-sensitive cell line CEM-C7H2 [47], a subclone of the CEM-C7 [48], and the GC-resistant cell line CEM-C1 [49] have been described previously. All cell lines were grown in 5% CO_2, saturated humidity, at 37°C in RPMI-1640 supplemented with 10% bovine calf serum (HyClone, Logan, UT), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine.

2.2. Stable transfections

Logarithmically growing CEM-C7H2 cells were washed in PBS, pelleted at $300\times g$ and resuspended at a density of 1×10^7 cells/400 μl PBS. Cells were mixed with 20 μg of plasmid pHD1.2 (chicken β-actin promoter driven *crmA* expression vector, kindly provided by V. Gagliardini) [35], incubated for 10 min on ice, and electroporated with the eletroporator (Biorad Lab., Vienna, Austria) set at 960 μF, and 300 V. After electroporation, cells were again placed on ice for 10 min, diluted in 20 ml of growth medium and seeded on 96-well flat-bottom plates. Seletion of stably transfected cells was initiated 48 h after electroporation using 1 mg/ml G418 (bioactivity 70%). G418-resistant clones were cultured and frozen in liquid nitrogen for further analyses.

2.3. Proliferation and apoptosis assay

Proliferation and apoptosis were determined as previously detailed [47]. Briefly, 2.5×10^5 cells/ml were cultured in the presence or absence of 1×10^{-7} M dexamethasone in 24-well flat-bottom plates for 24, 48, and 72 h. For determination of proliferation, cell aliquots were incubated in triplicate with [^{3}H]thymidine for a 6 h pulse, and the uptake counted by liquid scintillation. To estimate the degree of apoptosis, cells were lysed with Triton X-100, stained with propidium iodide, and fluorescence determined by flow cytometry. To study signaling through Apo1/Fas, 2×10^5 cells/ml were cultured with 0.5 μg/ml mouse monoclonal IgM antibody to Apo1/Fas (Immunotech, Marseille, France; clone CH11) or an isotype-matched control monoclonal for 6 h, and the cells processed as described above for GC-treated cells.

2.4. PARP assay

Cell pellets containing 5×10^5 cells were resuspended in 50 μl of gel loading buffer and subjected to ultrasonic treatment with a microtip 3 times for 10 s to break down high molecular weight DNA. Aliquots (20 μl) were separated in the presence of SDS in 0.75-mm-thick polyacrylamide gels containing 10% (w/v) acrylamide. Samples were electrophoretically transferred to nitrocellulose at 4°C at 40 V/120 mA for 6–12 h in a buffer containing 20% methanol, 17 mM Tris-HCl, 170 mM glycine and 0.02% (w/v) SDS. All further steps were carried out at room temperature. Remaining protein binding sites were saturated by 5% (w/v) powdered nonfat milk in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1 h. The sheets were incubated with polyclonal affinity-purified goat antibodies against human PARP for 6–12 h in TBS, washed 3 times with TBS/5% milk and incubated with alkaline phosphatase-labeled donkey antibodies against goat IgG. The filters were washed 3 times with TBS/5% milk, once with TBS, and the alkaline phosphatase activity was detecetd by the color reaction with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate.

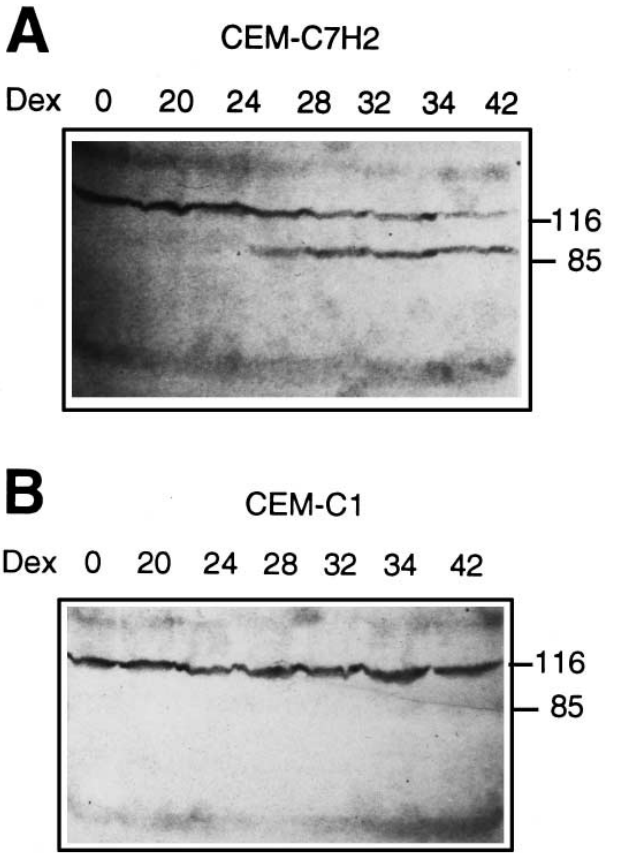

Fig. 1. PARP is cleaved during GC-induced apoptosis. GC-sensitive CEM-C7H2 (A) and GC-resistant CEM-C1 (B) cells were treated with 0.1 μM dexamethasone for the indicated time and cellular extracts subjected to Western analysis with a specific rabbit antiserum to PARP. An 85 kDa PARP-fragment, typical for cleavage by ICE-like proteases, is produced in GC-sensitive CEM-C7H2, but not in GC-resistant GR expressing, CEM-C1 cells.

3. Results

To determine whether ICE-like proteases might be activated in, and perhaps contribute to, the effector phase of GC-induced apoptosis in leukemic cells, we analyzed cleavage of PARP, a typical target of such proteases, during GC treatment. As shown in Fig. 1A, incubation of GC-sensitive CEM-C7H2 cells with 0.1 μM dexamethasone caused significant digestion of the 113 kDa PARP starting at about 28 h after treatment initiation. Hence, PARP cleavage preceeded apoptosis which generally occurred, after 48–72 h of incubation (examplified in Fig. 3A). In contrast, in CEM-C1 cells, that express low levels of functional GR but are resistant to GC-induced apoptosis [50], PARP remained intact over the entire observation period (Fig. 1B) suggesting that PARP digestion may be specifically associated with apoptosis rather than rep-

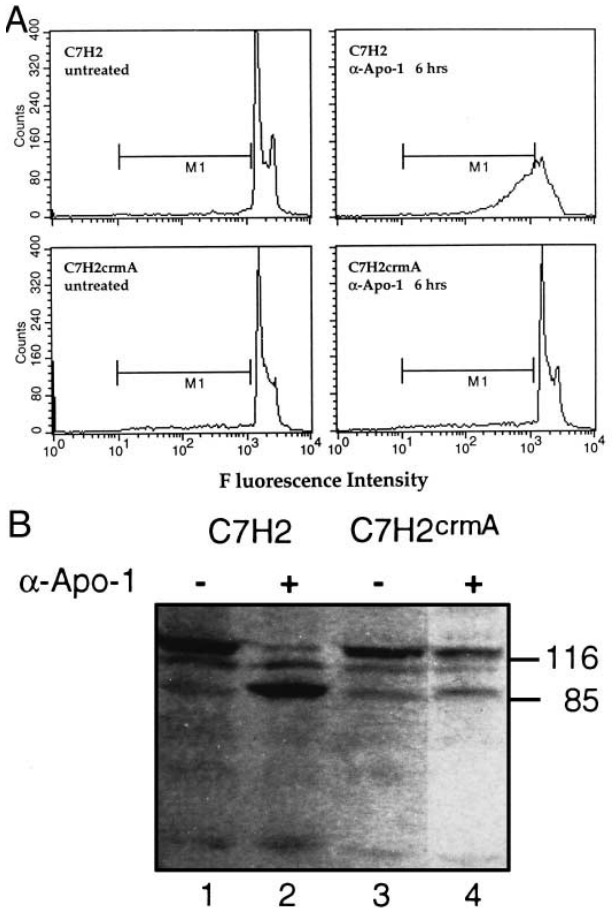

Fig. 2. Apo1/Fas-induced PARP cleavage and apoptosis are prevented by *crmA* overexpression. Parental CEM-C7H2 and *crmA*-transfected CEM-C7H2 sublines 2E8, 2G10, and 2H10 were treated with a monoclonal antibody to Apo1/Fas for 6 h and subjected to apoptosis determination by FACS (A) or Western blotting analysis with an antiserum to PARP (B). Anti-Apo1/Fas treatment induced apoptosis (hypodiploidic cells within the marker window M1) in about 60% of the parental CEM-C7H2 cells that was reduced almost to background levels by *crmA* overexpression (A). Apoptosis in CEM-C7H2 was associated with PARP cleavage (B, lane 2) that was markedly suppressed by *crmA* overexpression (B, lane 4). Shown are the results obtained with CEM-C7H2.2E8 cells, the other 2 cell lines gave essentially identical results. The percentages of apoptotic cells were 12% (untreated C7H2), 61% (anti-fas-treated C7H2), 11% (untreated C7H2.2E8), and 21% (anti-fas-treated C7H2.2E8) in the experiment shown.

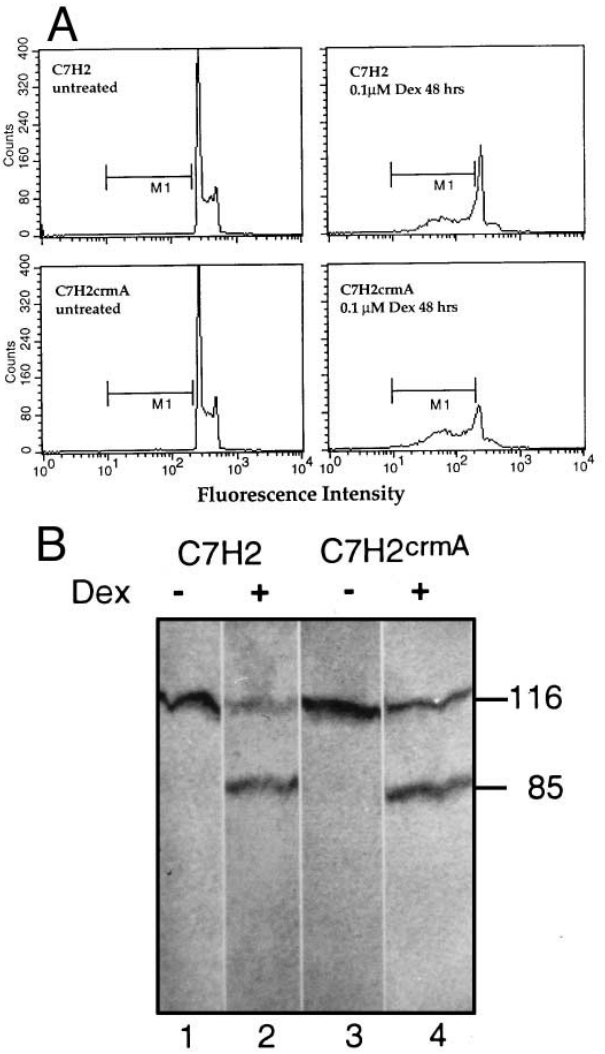

Fig. 3. CrmA does not prevent GC-induced PARP cleavage and apoptosis. Parental CEM-C7H2 and *crmA*-transfected CEM-C7H2 sublines 2E8, 2G10, and 2H10 were treated with 0.1 μM dexamethasone for 48 h and subjected to apoptosis determination by FACS (A) or Western blotting analysis with an antiserum to PARP (B). Dexamethasone treatment induced significant apoptosis (hypodiploidic cells within the marker window M1 after 48 h) both in the parental CEM-C7H2 and *crmA*-transfected cells (A). In both cell types, apoptosis was associated with PARP cleavage (B). Shown are the results obtained with CEM-C7H2.2E8 cells, the other 2 cell lines gave essentially identical results. The percentages of apoptotic cells were 4% (untreated C7H2), 60% (dex-treated C7H2), 4% (untreated C7H2.2E8), and 75% (dex-treated C7H2.2E8) in the experiment shown.

resenting an epiphenomenon of GC treatment. The cleavage pattern of PARP observed in CEM-C7H2 was indistinguishable from that seen after PARP digestion with prICE/CPP32 in other systems [12,14] strongly suggesting activation of CPP32 or another ICE-like protease in the course of GC-induced apoptosis.

Since ICE and some presumably ICE-dependent forms of apoptosis can be inhibited by overexpression of the cowpox virus protein CrmA [33], we generated CEM-C7H2 cell lines stably transfected with the *crmA* expression vector pHD1.2. Several G418-resistant cell lines were established that expressed *crmA* as judged by RNA dot-blot analysis. To ascertain that functional relevant levels of CrmA protein were ex-

pressed, we treated parental CEM-C7H2 and the *crmA* expressing CEM-C7H2 sublines 2E8, 2G10, and 2H10 with a monoclonal antibody to Apo1/Fas. As exemplified in Fig. 2, this treatment induced rapid apoptosis and PARP-cleavage in the parental CEM-C7H2 line, however, the sublines 2E8, 2G10, and 2H10 were protected by the overexpression of *crmA*. This confirmed and extended previous reports that Apo1/Fas induced apoptosis is inhibitable by CrmA [34,36,51], and clearly showed that the stably transfected cell lines expressed functional CrmA in amounts sufficient to interfere with the cell death pathway.

CEM-C7H2 and the *crmA*-expressing cell lines 2E8, 2G10 and 2H10 were then treated with 0.1 μM dexamethasone and assayed for apoptosis and PARP cleavage (Fig. 3). Neither GC sensitivity, as determined by flowcytometric DNA content analysis, nor degree of PARP cleavage, assayed by Western analyses, were reduced in the *crmA*-expressing cell lines compared to the parental CEM-C7H2. Thus, the data strongly suggested that GC-induced PARP cleavage and apoptosis occurred independent of expression of CrmA-inhibitable proteases.

4. Discussion

The induction of apoptosis in immature lymphocytes by GC, which underlies their successful application in the treatment of acute lymphoblastic leukemia, is not well understood. After binding to, and activation of, their cytoplasmic receptors, GC seem to induce apoptosis in leukemia cells mainly by down-regulation of gene expression as suggested by the observations that a transactivation-compromised, but transrepression-intact, GR mutant (LS7) still mediates GC-induced apoptosis [43]. Little, however, is known about the nature of the repressed molecules that may be either survival proteins or repressors of death-inducers.

Since ICE and ICE-like proteases have been implicated in the effector phase of various forms of apoptosis, including that triggered by antibodies to the Apo1/Fas membrane protein (see Section 1), we decided to investigate the role of such enzymes in GC-induced leukemia cell death by analyzing PARP, a typical substrate for some members (e.g., CPP32 and Mch-2) of the ICE family [12,14,17]. This study showed that, like in GC-induced rat thymocyte apoptosis [28], PARP is digested at the onset of GC- as well as Apo1/Fas-induced apoptosis in our cell system. However, while PARP cleavage and apoptosis following anti-Apo1/Fas treatment was abrogated by overexpression of the cowpox virus ICE inhibitor CrmA, these phenomena, when evoked in the same cells by GC, were neither prevented nor even delayed by CrmA. This suggested that different proteases might be activated during Apo1/Fas and GC-induced apoptosis.

While our data clearly documented that different proteases operate during Apo1/Fas- and GC-mediated apoptosis, they could not exactly define the proteases activated because the specificity of CrmA is not precisely known. CrmA inhibits ICE, and ICE deficient mice are unable to mount Apo1/Fas-induced apoptosis [5], making this enzyme the likely CrmA target responsible for Apo1/Fas apoptosis in our system as well. However, ICE itself does not cleave PARP (except at very high concentrations [52]) but may rather activate downstream ICE-like proteases (e.g., CPP32) that, in turn, pass the signal further on in a cascade-like fashion [22,23]. The GC-induced death signal apparently bypasses ICE and either directly activates the largely CrmA-resistant, PARP-cleaving CPP32/apopain [15] or another CrmA-resistant protease that might digest PARP or act upstream thereof. This conclusion is in agreement with the observation that GC-induced thymocyte apoptosis proceeds essentially unaltered in ICE knockout mice [4,5]. The exact proteases activated in the effector phase of GC-induced leukemia cell apoptosis and the mechanism leading to their activation remain to be elucidated.

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