Overexpression of Bcl-2 or Bcl-xL Inhibits Ara-C-induced CPP32/Yama Protease Activity and Apoptosis of Human Acute Myelogenous Leukemia HL-60 Cells

Ana Maria Ibrado, Yue Huang, Guofu Fang, Linda Liu, and Kapil Bhatta

Division of Hematology/Oncology, Department of Medicine, Winship Cancer Center, Emory University School of Medicine, Atlanta, Georgia 30322

ABSTRACT

Ara-C has been shown to induce apoptosis of human acute myelogenous leukemia HL-60 cells. The DNA repair enzyme poly(ADP-ribose) polymerase (PARP) is known to be degraded during apoptosis. PARP as a substrate is cleaved by the Yama protease, encoded by the CPP32/Yama gene. Yama belongs to the interleukin 1β converting enzyme/ced-3 family of cysteine proteases that are activated as a cascade, producing proteolytic cleavage of specific substrates that result in the morphological and biochemical features of apoptosis. In the present studies, we determined the effect of high intracellular levels of the antiapoptosis Bcl-2 or Bcl-xL protein on Yama protease activation and PARP degradation during Ara-C-induced apoptosis. For this, we utilized HL-60/Bcl-2, HL-60/Bcl-xL, or control HL-60/neo cells, which were created by transfection of the cDNA of the bcl-2, bcl-xL, or the neomycin-resistant genes, respectively. As compared to HL-60/neo, HL-60/Bcl-2 and HL-60/Bcl-xL cells have 5-fold greater expression of Bcl-2 and Bcl-xL, respectively. However, these cell lines have similar levels of p32Yama and PARP. Treatment with 10 or 100 μM Ara-C for 4 h produced DNA fragmentation and morphological features of apoptosis in HL-60/neo cells. This was associated with the cleavage and activation of p32Yama and PARP degradation but not with the induction of Yama mRNA. In contrast, in HL-60/Bcl-2 and HL-60/Bcl-xL cells, Ara-C-induced p32Yama activation by its cleavage, PARP degradation and apoptosis were significantly inhibited. High Bcl-2 and Bcl-xL levels in these cells also inhibited Yama protease activity, PARP degradation, and apoptosis due to clinically relevant concentrations of etoposide and mitoxantrone. These results suggest that the activation of the Yama protease and PARP degradation are involved in Ara-C-, etoposide-, or mitoxantrone-induced apoptosis. In addition, they suggest that Bcl-2 and Bcl-xL antagonize drug-induced apoptosis by a mechanism that interferes in the activity of a key cysteine protease that is involved in the execution of apoptosis.

INTRODUCTION

After interaction with their intracellular targets, anticancer drugs engage the final mechanism of apoptotic cell death (1, 2). Apoptosis is an active and gene-directed form of cell death with well-characterized morphological and biochemical features (3, 4). The latter include large (5—300 kb) and internucleosomal DNA fragmentation as well as the proteolytic cleavage of the DNA repair enzyme PARP2 (4, 5). Ara-C, etoposide, and mitoxantrone are important antileukemic drugs which, at their clinically relevant concentrations, have been shown to induce apoptosis of human acute myelogenous leukemia cells (6—8). Recent evidence suggests that the activation of a cascade of cysteine proteases of the ICE/ced-3 family may exert a pivotal role in the execution of apoptosis (9, 10). Their overexpression induces, and their inhibition suppresses, apoptosis (10—12). The substrates for the mammalian cell death proteases include PARP, lamin, and β-actin, which are degraded with the onset of apoptosis (10, 12—14). The CPP32/Yama gene, a member of the ICE/ced-3 family, encodes for the Yama protease (32 kd), also called apopain, which is activated by proteolytic cleavage into p20 and p12 subunits (9, 15). In turn, Yama cleaves PARP into an 85-kd fragment generated during apoptosis (9, 15). Degradation of PARP could lead to inhibition of DNA repair, activation of an endonuclease, depletion of NAD and ATP, and ultimately cell death (5, 12, 15). During anticancer drug-induced apoptosis, although PARP degradation has been observed (5), neither the induction of the mRNA of CPP32/Yama nor its activation by cleavage has been examined.

Among the growing numbers of genes that regulate apoptosis is the bcl-2 family of genes (16). The bcl-2 gene encodes for the p26Bcl-2, which suppresses apoptosis (16). bcl-x is a member of the bcl-2-related family of genes, and it shares significant homology with bcl-2 in its BH1 and BH2 regions (16, 17). By alternate splicing, bcl-x encodes for two important protein isoforms, of which the longer, Bcl-xL, inhibits apoptosis, whereas the shorter isoform, Bcl-xS, facilitates apoptosis by acting as a dominant inhibitor of Bcl-2 and Bcl-xL (16, 17). Bcl-xL has a pattern of expression distinct from Bcl-2 and has been shown to block apoptosis where Bcl-2 is ineffectual (18, 19). High intracellular levels of Bcl-2 and Bcl-xL have also been shown to inhibit drug-induced apoptosis (20, 21). Although previous reports have suggested several diverse mechanisms that may underlie the antiapoptotic effect of Bcl-2 or Bcl-xL (16), the precise mechanism responsible for this effect would have to be operative distally and should impair the activation of the proteases involved in the execution of apoptosis. In the present studies, utilizing HL-60/Bcl-2 and HL-60/Bcl-xL cells, we demonstrated that both Bcl-2 and Bcl-xL overexpressions inhibit Ara-C-, mitoxantrone-, effects, and etoposide-induced Yama protease activation, PARP degradation, and apoptosis.

MATERIALS AND METHODS

Reagents. To determine Bcl-xL and Bax expression, the rabbit anti-Bcl-x and anti-Bax sera were utilized, respectively (22, 23), which were kindly provided by Dr. John C. Reed of the Burnham Institute (La Jolla, CA). Monoclonal anti-Bcl-2 (No. 124) was obtained from DAKO Corp. (Carpinteria, CA).

Cells and Transfection of the bcl-xL Gene. Human myeloid leukemia HL-60 cells were cultured to logarithmic growth phase in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum. Viable cells (5 x 10⁶) were washed with ice-cold PBS and electroporated with purified, linearized pSFFVneo-bcl-xL or pSFFV-neo plasmids (a gift from Dr. Gabriel Nunez; University of Michigan, Ann Arbor, MI) (17). The electroporation parameters were 500 microfarads (capacitance), 300 V, 0.75 kV/cm (field strength), and 14 ms (time constant), with a Bio-Rad Gene Pulser (Hercules, CA). Transfected cells were selected in RPMI 1640 containing 10% fetal bovine serum and 1 mg/ml G418 (Geneticin; Life Technologies, Inc.) for 2 weeks. HL-60/Bcl-2 cells were transfected with the cDNA of bcl-2 gene, as described previously (24). The clones expressing high levels of Bcl-2 or Bcl-xL were subcloned further by limiting dilution. Representative subclones of each of HL-60/neo, HL-60/Bcl-2, and HL-60/Bcl-xL cells were passaged twice per week and used for the studies described below.

Northern Blot Analysis. Total RNA was extracted from HL-60/neo, HL-60/Bcl-2, and HL-60/Bcl-xL cells by the acid-phenol- guanidium thiocyanate.
method (7). The 0.85-kb cDNA for CPP32/Yama gene was released from pcDNA-Yama (kindly provided by Dr. Vishva Dixit, University of Michigan, Ann Arbor, MI) by digestion with EcoRI and Xbal, and labeled with [32P]dATP by random priming. Northern blot analyses were performed by hybridization with this 32P-labeled cDNA probe (heat denatured) for 20 h at 42°C, as described previously (7).

Detection of Internucleosomal Fragmentation of Genomic DNA by Agarose Gel Electrophoresis. After incubations with the designated concentrations and schedules of the drugs, 1 x 10^6 cells were pelleted. The genomic DNA was extracted and purified, and its purity was determined spectrophotometrically (6). Agarose gel electrophoresis of 1.0 µg of DNA was performed as described previously (6).

Activation of Yama Protease and PARP Degradation. During and after exposure to the designated concentrations and schedules of the drugs, cells were harvested, and immunoblot analyses of p32Yama and PARP expression were determined in HL-60/neo versus HL-60/Bcl-2 and HL-60/Bcl-xL cells, utilizing a previously described method (24). Briefly, protein was extracted from cells with lysis buffer [142.5 mM KCl, 5 mM MgCl2, 10 mM HEPES (pH 7.2), 1 mM EDTA, 0.2% NP-40, and 0.2 mM phenylmethylsulfonyl fluoride] supplemented with 0.2 trypsin inhibitory units/ml aprotinin, 0.7 µg/ml pepsatin, and 1 µg/ml leupeptin. Appropriate protein amounts (20 µg) were subjected to SDS-PAGE (10% gel). After electrophoresis, proteins were transferred to nitrocellulose sheets (0.5 A at 100 V; 4°C) for 1-3 h. The blots were blocked in 5% nonfat dry milk solution for 3 h at room temperature with gentle shaking (5% nonfat milk [w/v/PBS/0.02% sodium azide (pH 7.4)]). This was followed by incubation with the respective antibody (1:1000 dilution) at room temperature and then with antirabbit or antimouse peroxidase-conjugated secondary IgG antibodies. Immune complexes were detected with an enhanced chemiluminescence detection method by immersing the blot for 1 min in a 1:1 mixture of chemiluminescence reagents A and B (Amersham UK, Little Chalfont, UK) and then exposing to Kodak XCL film for a few seconds.

RESULTS

Bcl-2, Bcl-xL, and Bax Expressions. Western blot analyses of proteins from untreated HL-60/neo, HL-60/Bcl-2, and HL-60/Bcl-xL cells were performed to compare Bcl-2, Bcl-xL, and Bax expression in the three cell types. Fig. 1 demonstrates that, as compared to HL-60/neo, HL-60/Bcl-xL cells contain approximately 5-fold higher Bcl-xL, but equivalent levels of Bcl-2 and Bax. In contrast, as compared to HL-60/neo, HL-60/Bcl-2 cells possess 5-fold higher Bcl-2 levels but equivalent levels of Bcl-xL and Bax. These results were representative of three separate experiments.

Ara-C-Induced Yama Protease Activation and PARP Degradation and Apoptosis. Previous studies demonstrated that the exposure to ≥1.0 µM Ara-C for 4 h induces apoptosis of HL-60 cells (6), whereas this is inhibited in HL-60/Bcl-2 and HL-60/Bcl-xL cells (27). To determine whether Ara-C-induced apoptosis was associated with the activation of the cysteine protease cascade, we determined the p32Yama levels and activity in HL-60/neo versus HL-60/Bcl-2 and HL-60/Bcl-xL cells that had been exposed to 1.0, 10.0, or 100 µM Ara-C for 4 h. Fig. 2A shows a dose-dependent activation of p32Yama brought about by its cleavage, represented here as a decline in its levels on the Western blot, due to treatment of HL-60/neo cells with 10 and 100 µM Ara-C. Fig. 2B shows that the exposure of HL-60/neo cells to 10 or 100 µM but not 1.0 µM Ara-C also caused the degradation of PARP into its 85-kd fragment. In HL-60/neo cells, both the activation of Yama protease and PARP degradation were coincidental with Ara-C-induced internucleosomal DNA fragmentation of apoptosis (Fig. 2C). These Ara-C-induced intracellular effects were not observed in HL-60/Bcl-2 and HL-60/Bcl-xL cells (Fig. 2, A—C). It is noteworthy that the levels of p32Yama and PARP were not significantly different in the untreated HL-60/neo versus HL-60/Bcl-2 and HL-60/Bcl-xL cells (Fig. 2, A and B). We also determined whether Ara-C-induced Yama protease activity in HL-60/neo was associated with the induction of its mRNA by Ara-C. Northern blot analyses showed that Ara-C treatment had no significant effect on CPP32/Yama mRNA levels in either HL-60/neo or HL-60/Bcl-xL cells (data not shown). The temporal relationship of Yama protease activation, PARP degradation, and internucleosomal DNA fragmentation in Ara-C-treated HL-60/neo cells was also explored. Fig. 2, D and E, shows that 3 h of exposure to 100 µM Ara-C caused a down-regulation of the p32Yama protease levels and PARP degradation. These effects of Ara-C were coincidental with Ara-C-induced internucleosomal DNA fragmentation, which is observed after 3 h of exposure to Ara-C (Fig. 2F).

To demonstrate that both the decline of p32Yama levels and the degradation of PARP into its 85-kd fragment are due to the activities of the member(s) of the ICE/ced-3 family of cysteine proteases, which are responsible for the biochemical and morphological features of apoptosis, we examined the effect of YVAD-cmk, a known inhibitor
Fig. 2. Protein (20 μg) from cell lysates was electrophoresed in 10% SDS-PAGE gels, transferred to nitrocellulose, and probed with anti-Yama and anti-PARP antibodies to determine p32Yama levels (A and D) and p116PARP degradation (B and E) in untreated and Ara-C-treated cells. C demonstrates the induction or absence of the apoptosis-associated internucleosomal DNA fragmentation ladder in HL-60/neo, HL-60/Bcl-2, or HL-60/Bcl-xL cells treated with 1.0, 10.0, or 100 μM Ara-C for 4 h; F demonstrates these effects in HL-60/neo cells treated with 100 μM Ara-C for 2, 3, or 4 h. Lane M, 123-bp marker DNA ladder.

of the cysteine protease family, on the Ara-C-mediated effects on the p32Yama and PARP levels, as well as on the induction of apoptosis. Gel scanning densitometry revealed approximately a 45% decline in p32Yama levels and a complete degradation of p116 kD PARP after treatment with 100 μM Ara-C for 4 h (Fig. 3, A and B, Lane 2). Cotreatment with YVAD-cmk reversed significantly, by approximately 50%, both Ara-C-induced decline of p32Yama (Fig. 3A) and PARP degradation (Fig. 3B) in HL-60/neo cells (Fig. 3, A and B, Lane 3 versus 2). Although treatment with YVAD-cmk alone had little effect (Fig. 3C, Lane 4), cotreatment with Ara-C and YVAD-cmk significantly inhibited Ara-C-induced internucleosomal DNA fragmentation of apoptosis (Fig. 3C, Lane 3 versus 2). After these treatments, the percentage of cells displaying the morphological features of apoptosis was evaluated according to a previously described method (6). Cotreatment with YVAD-cmk and Ara-C resulted in a lower percentage of apoptotic cells, as compared to treatment of HL-60/neo cells with Ara-C alone (18.0 versus 32.0%, mean of three experiments). To confirm that during Ara-C-induced apoptosis the decline in Yama levels (Fig. 2A) was associated with its cleavage into a 20-kd fragment and the generation of a cysteine protease activity, the extracts of Ara-C-treated cells were examined for their ability to cleave in vitro-translated PARP. Fig. 4 demonstrates that Ara-C treatment of HL-60/neo cells generated a 20-kd cleavage product of Yama (Fig. 4A, Lane 2). This produced cysteine protease activity, which degraded in vitro-translated PARP into its 85- and 31-kd cleavage products (Fig. 4B, Lane 2). Fig. 4 also demonstrates that these effects of Ara-C are inhibited by Bcl-xL overexpression in HL-60/Bcl-xL cells (Fig. 4, A and B, Lane 4). A similar inhibition was also observed in Ara-C-treated HL-60/Bcl-2 cells (data not shown). The results presented in Figs. 2–4 are representative of three separate experiments.

Etoposide- and Mitoxantrone-induced Yama Protease Activity and Apoptosis. Next, we examined whether those antileukemic drugs that engage different intracellular targets prior to inducing apoptosis also activate the cysteine protease p32 Yama resulting in PARP degradation. Fig. 5A demonstrates that clinically relevant concentrations of etoposide (1.0 μM) and mitoxantrone (1.0 μM) activate Yama protease and degrade PARP into an 85-kd fragment in HL-60/neo cells. Again, this occurred in association with etoposide- and mitoxantrone-induced apoptosis of HL-60/neo cells (Fig. 5B). In contrast, in HL-60/Bcl-2 and HL-60/Bcl-xL cells, both drugs were unable to induce Yama cleavage and PARP degradation or apoptosis (Fig. 5A) or internucleosomal DNA fragmentation of apoptosis (Fig. 5C).
that the induction of apoptosis by anticancer drugs with diverse mechanisms of action involves activation of the key cysteine protease CPP32/Yama and PARP degradation, and these events are inhibited significantly by Bcl-2 or Bcl-xL overexpression.

**DISCUSSION**

Proteolytic degradation of specific substrates is responsible for many of the morphological and biochemical features of apoptosis (9, 10, 15). Activation of the cysteine proteases belonging to the ICE/ced-3 family initiates a cascade of protease activity that results in apoptosis (9, 10). In the present report, we demonstrate that during Ara-C, mitoxantrone and etoposide-induced apoptosis of HL-60/neo cells, one key protease encoded by the CPP32/Yama gene is activated, which results in the degradation of PARP. In contrast, in HL-60/Bcl-2 and HL-60/Bcl-xL cells, which possess high Bcl-2 and Bcl-xL levels, respectively, the Yama protease activation by these drugs and PARP degradation, as well as the morphological and DNA fragmentation of apoptosis, are inhibited markedly.

In HL-60/neo cells, the proteolytic cleavage and activation of the p32Yama and resulting PARP degradation immediately preceded the DNA fragmentation during the drug-induced apoptosis. However, the precise mechanism by which Ara-C, mitoxantrone, and etoposide cause activation of the Yama protease is not clear. Besides being a substrate for Yama, PARP has also been shown to be degraded by Mch2 and Mch3, the other novel cysteine proteases related to Yama (28, 29). Mch3 in turn is cleaved and activated by Yama (28). Data presented here show clearly that Ara-C treatment results in the cleavage of p32Yama into its 20-kd fragment and the generation of PARP cleavage activity. p20Yama subunits can also form an active heteromeric enzyme complex with p12Mch3 subunits to induce apoptosis (28). Therefore, it is possible that in Ara-C-treated HL-60/neo cells, the drug-induced PARP degradation may not be solely due to Yama.

**Fig. 4.** Western analysis of the levels of the full-length p32Yama protein and its cleavage product (A), and determination of the cysteine protease activity (B) by the in vitro cleavage of 35S-labeled PARP by cellular protein extracts from untreated and Ara-C-treated (100 μM for 4 h) HL-60/neo and HL-60/Bcl-xL cells.

**Fig. 5.** A, Western blot analyses to determine p32Yama levels and p116PARP degradation into an 85-kd fragment in untreated and etoposide-treated (1.0 μM for 4 h) or mitoxantrone-treated (1.0 μM for 4 h) HL-60/neo, HL-60/Bcl-2, and HL-60/Bcl-xL cells. B, etoposide- or mitoxantrone-induced internucleosomal DNA fragmentation ladder in HL-60/neo versus HL-60/Bcl-2 or HL-60/Bcl-xL cells; M, lane containing the 123-bp marker DNA ladder.
but also to the activity of Mch3, Mch3/Yama heteromer, and/or other proteases. In addition, although not assayed, activation of multiple ICE-like cysteine proteases, including lamin proteases, e.g., Mch2α, are required for the generation of all of the features of apoptosis (13, 14, 29). These may also have been induced during Ara-C-, etoposide-, and mitoxantrone-induced apoptosis.

The results presented here demonstrate that treatment with Ara-C, mitoxantrone, or etoposide does not up-regulate either the mRNA of CPP32/Yama or the p32Yama levels before inducing apoptosis of HL-60/neo cells. A previous report suggested that drug-induced serine phosphorylation of Bcl-2 inhibits its ability to interfere with apoptosis (30). However, in other reports, during drug-induced apoptosis of HL-60/neo cells, Western analyses did not show any significant change in the p26Bcl-2 or p29Bcl-xL protein bands (27), because the migration of the bands on the Western blot would have been modified had their size been affected by phosphorylation (31, 32). This has been noted in some but not all cell lines (31). As speculated elsewhere (31), it is possible that the putative kinase responsible for the Bcl-2 phosphorylation is not activated by Ara-C, mitoxantrone, or etoposide treatment in HL-60/neo or HL-60/Bcl-xL cells (33).

Previous reports have demonstrated that in human leukemic cells, overexpression of Bcl-2 or Bcl-xL does not impair either the interaction of the drugs with their intracellular targets or the resulting cell cycle perturbations (24, 27), but the drug-induced biochemical and morphological features of apoptosis were blocked (8, 24). In the present report, our findings show that Bcl-2 and Bcl-xL interfere in the activation of a key cysteine protease that results in the degradation of PARP. Although not examined specifically, because these proteases can cleave and activate other cysteine proteases and amplify the protease cascade (10), overexpression of Bcl-2 or Bcl-xL may significantly down-regulate the activity of the protease cascade that executes apoptosis. Taken together, these findings also suggest that the antiapoptotic effect of Bcl-2 or Bcl-xL is exerted downstream to the drug-induced cell cycle perturbations or early DNA damage but is exerted proximal to or at the level of the cysteine proteases the activity of which produces the features of apoptosis. This conclusion is supported by a recent report that demonstrated that in Jurkat T cells, treatment with staurosporine (2 μM) or anti-APO-1 antibody (200 ng/ml) for 3 h activates CPP32/Yama and ICE-LAP3 (Mch 3) cysteine proteases as well as apoptosis, and this was blocked by overexpression of Bcl-2 or Bcl-xL (34). However, our findings further extend these observations by showing that CPP32/Yama activation, PARP degradation, and apoptosis are triggered by commonly used antileukemic drugs, and these events are inhibited in human acute myelogenous leukemia cells by overexpression of Bcl-2 or Bcl-xL. Previous reports have suggested several different mechanisms underlying the antiapoptotic activity of Bcl-2 or Bcl-xL. These have included: (a) antioxidant effects (35); (b) inhibition of intracellular calcium flux (36); (c) control of R-ras- or Raf-mediated signal transduction (37, 38); and (d) blocking the nuclear localization of p53 and Myc (39).

Our results suggest that, if operative, these mechanisms should result in the inhibition of the Yama protease and PARP degradation in conjunction with the other features of apoptosis. Whether Bcl-2 or Bcl-xL inhibits the activation of Yama, and/or another protease that activates Yama, by a direct physical interaction or indirectly through other regulators of the proteases remains to be established.

In summary, data presented here indicate that apoptosis that follows the interaction of the anticancer drugs with diverse intracellular targets involves CPP32/Yama protease activation and PARP degradation. Additionally, these findings suggest that the activity of the protease cascade is a potential, downstream target of the antiapoptotic activity of Bcl-2 and Bcl-xL, which may be an important mechanism of clinical drug resistance in cancer cells.


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