
Original Articles

p21WAF1 Prevents Down-modulation of the Apoptotic Inhibitor Protein c-IAP1 and Inhibits Leukemic Apoptosis

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Abstract

Background: Prior studies indicate that leukemias expressing high levels of the p21WAF1 cell cycle inhibitor have a poorer prognosis than p21WAF1-negative leukemias. Although p21WAF1 is up-regulated by p53 in the setting of DNA damage, the prognostic significance of p21WAF1 is independent of p53 status. The molecular basis of the negative prognostic effect of p21WAF1 remains obscure, but it is believed to result from decreased apoptosis of p21WAF1-expressing leukemias.

Materials and Methods: We studied the effects of p21WAF1 on apoptosis of K562 leukemic cells, which lack wild-type p53 and do not express endogenous p21WAF1. An inducible p21WAF1 system was used and the effect of p21WAF1 induction on susceptibility to etoposide-mediated apoptosis was measured.

Results: p21WAF1 decreased apoptotic death of K562 leukemic cells in response to etoposide. Analysis of intermediaries in the apoptotic pathway indicated that p21WAF1 had no effect on cytochrome c release or cleavage of procaspase-3. In contrast,

p21WAF1 was protective against cleavage of caspase targets poly(ADP-ribose)polymerase (PARP), retinoblastoma protein (Rb), and lamin. The expression of the inhibitor of apoptosis protein c-IAP1, which inhibited the function of executioner caspases 3 and 7, was studied. c-IAP1 protein expression was found to be present in a majority of leukemic blasts from untreated patients, but absent in normal differentiating myeloid progenitor cells. In K562 cells, treatment with etoposide in the absence of p21WAF1 induction resulted in post-transcriptional down-modulation of c-IAP1 levels. c-IAP1 loss involved proteasomal, rather than caspase, degradation pathways. Expression of p21WAF1 sustained c-IAP1 protein levels in the presence of etoposide.

Conclusions: Etoposide-mediated apoptosis involves down-modulation of the anti-apoptotic protein c-IAP1. Our findings support the hypothesis that p21WAF1 contributes to leukemic chemoresistance by stabilizing c-IAP1 levels in the presence of chemotherapy.

Introduction

The cell cycle inhibitor p21WAF1, is reported to be a negative prognostic factor in leukemia, associated with leukemic chemoresistance (1). In vitro, p21WAF1 is noted to have anti-apoptotic or pro-apoptotic effects in different settings. Exogenous p21WAF1 is reported to decrease differentiation-associated apoptosis of myocytes (2), as well as apoptosis in response to radiation (3), p53 overexpression (4), chemotherapeutic agents (3,5,6), Fas activation (7), prostaglandin

A2 (8), and growth factor deprivation (9). The ability of p21WAF1 to inhibit DLD-1 colon carcinoma cell apoptosis by adriamycin is associated with its ability to bind to cyclin-dependent kinase 2(cdk2) (3), although a truncated p21-WAF1 protein retaining cdk2 binding does not protect irradiated leukemic cells from apoptosis (10). C-terminal cleavage of p21-WAF1 by a caspase-3-like activity is reported in early apoptosis in myeloid leukemic cell (10) and endothelial cell (9) models. p21WAF1 cleavage may disrupt the ability of p21WAF1 to protect against apoptosis.

In contrast, induced endogenous p21WAF1 appears to contribute to apoptosis of serum-

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deprived fibroblasts (11), and exogenous p21WAF1 is reported to promote apoptosis of breast cancer cell lines (12), irradiated glioma cells (13) and *cis*-platinum-exposed retinoblastoma cells (14). Recently, p21WAF1 was shown to promote receptor-mediated apoptosis of lymphoma cells (15). Moreover, several reports indicate that exogenous or induced p21WAF1 neither protects nor sensitizes cells to apoptosis (16–19). Clearly, the interaction of p21WAF1 with apoptotic pathways is complex.

A better understanding of the mechanisms involved with p21WAF1 effects on apoptosis clearly is needed to reconcile these conflicting observations. Differing effects of p21WAF1 on apoptosis may clarify why p21WAF1-transfected cells are not tumorigenic in xenograft models (20) and yet, cancers expressing high levels of p21WAF1 are radio- or chemoresistant (1,21) and have a poorer prognosis (1). Conceivably, anti-apoptotic effects of p21WAF1 could derive from its ability to inhibit cdk2, causing cells to arrest in a G1 cell cycle phase, in which they are relatively insensitive to the effects of pro-apoptotic agents that target S-phase mechanisms. However, pro-apoptotic effects of p21WAF1 also occur in settings of growth arrest (11), so resistance to apoptosis may not be simply an epiphenomenon of cell cycle arrest (8,22). p21WAF1 also interacts with many targets other than cdk-cyclin complexes, including interactions with signal transduction machinery such as casein kinase II (23) and SAPK (24), or binding complexes with partners such as E2F (24), gadd45 (25) and Myd 118 (26). Direct interaction of p21WAF1 with apoptotic machinery also has been reported. In Fas-mediated apoptosis of (HepG2) cells, p21WAF1 was shown to form an inhibitory complex with procaspase-3 (7,27). p21WAF1 also was shown to bind (ask1) (28), which may lead to inhibition of (JNK/SAPK)-mediated apoptosis. The recruitment of p21WAF1 into pro- or anti-apoptotic pathways may depend upon the availability of specific p21-WAF1-interacting proteins.

We studied apoptosis in the erythroleukemic cell line K562. These cells are p53-negative and do not exhibit basal expression of p21-WAF1. K562 cells are relatively resistant to apoptosis. Early steps in the apoptotic pathway, such as release of mitochondrial cytochrome c, occur in K562 cells 1–2 days (29), rather than hours (30), after etoposide addition. Once initiated, execution of the apoptotic program proceeds similarly to that in sensitive cells

(29). The slower kinetics of cell death in K562 cells facilitated the study of cell cycle changes coincident with etoposide-mediated apoptosis in the presence and absence of p21WAF1.

Our investigations of the anti-apoptotic effects of p21WAF1 measured a number of molecular constituents of apoptotic pathways to determine whether p21WAF1 induction altered the ability to sense damage or altered the ability to complete apoptosis in response to sensed damage. We found that etoposide overcame the p21WAF1-induced G1 arrest without cleaving p21WAF1 and that p21WAF1 partially protected cells from apoptosis. Whereas, sensation of damage was not altered by p21WAF1, execution of the apoptotic program was diminished distal to the point of procaspase-3 cleavage. p21WAF1 decreased cleavage of caspase targets PARP, (Rb), and lamin. The induction of p21WAF1 stabilized levels of (c-IAP1), an inhibitor of distal apoptotic mediators, which was otherwise down-modulated by etoposide.

Materials and Methods

Cells and Culture

K562 cells were transfected with inducible murine p21WAF1 cloned into the LacSwitch system (Stratagene, Inc., La Jolla, CA) or with vector control and were double-selected in neomycin (500 μ g/ml) and hygromycin (300 μ g/ml) as described. Single clones were grown and analyzed for p21WAF1 inducibility and cell cycle effects. Two clones designated IWAF-1; Neo-1 were used in this study. In this paper, IWAF cells refer to K562 cells stably transfected with the inducible p21WAF1 plasmid, and Neo cells are K562 cells stably transfected with vector control plasmid. Cells were maintained at a density of $<10^6$ /ml in RPMI medium containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD). IPTG (Boehringer Mannheim, Indianapolis, IN) was added to 2 mM concentration to induce p21WAF1 expression. Etoposide (Boehringer Mannheim) was added at 1:1000 from a 100 mM frozen stock in 0.1% dimethylsulfoxide (DMSO); control flasks received 0.1% DMSO alone. Leukemic cells were received, in most cases, direct from plasmapheresed blast specimens obtained from newly presenting patients as part of routine care, in accordance with Institutional review board (IRB) guidelines. Extracts from leukemic cells were prepared immediately upon receipt.

Reagents

The cdk2 inhibitor, roscovitine, and proteasome inhibitors, (MG132) and Lactocystin, were obtained from Calbiochem, Inc. (La Jolla, CA). z-DVED-FMK (DEVD) and z-VAD-FMK (zVAD) were purchased from Kamiya Biomedical Company (Seattle, WA). Mimosine was obtained from ICN Biomedicals (Costa Mesa, CA). All inhibitors were dissolved at 400-1000X concentration in DMSO and aliquots maintained at -70°C prior to use. DMSO was added as a vehicle control in all experiments.

Western Blots and Antibodies

At times indicated in figure legends, cells were harvested and prepared for immunoblotting analysis. Cells were prepared in two ways, with similar results. Two million cells were either suspended in protein gel loading buffer and flash frozen, or suspended in high-salt buffer as previously described (31). Equal microgram amounts were loaded on SDS-PAGE and blotted onto Protran (Schleicher and Schuell, Keene, NH) membrane using standard techniques. Antibodies used consisted of rabbit polyclonal anti-caspase-3 (Pharmingen, Torrey Pines, CA), goat polyclonal anti-c-IAP1 and anti-c-IAP2 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-c-IAP1 (Pharmingen; yielding similar results to polyclonal antibody), mouse monoclonal anti-Lamin, anti-(Rb), rabbit polyclonal anti-PARP (Upstate Biotechnology, Lake Placid, NY), and rabbit polyclonal anti-p21WAF1 (Santa Cruz). Appropriate secondary antibodies were used and blots were developed using SuperSignal kit (Pierce, Rockford, IL).

Cell Cycle

Samples for cell-cycle analysis were prepared from freshly harvested cells as previously described (32). In brief, K562 cells (0.5 million/sample) were washed in wash buffer [phosphate-buffered saline (PBS) containing 2% FBS and 0.1% sodium azide], fixed in 1% paraformaldehyde for 15 min in ice, washed, and then resuspended in cold 75% ethanol and kept at -20°C for 20 min. After 20 min, cells were washed in wash buffer and resuspended in 0.25% Triton X-100 in wash buffer. After a 5 min incubation on ice, cells were washed, pelleted, and resuspended in wash buffer containing RNase 0.2 mg/ml and propidium iodide (Sigma, St. Louis, MO) 20 $\mu\text{g}/\text{ml}$. After 20 min,

the cells were analyzed for DNA content by flow cytometry.

Cytochrome C Release

To assay release of cytochrome C from mitochondria, cells were collected by centrifugation, washed in ice-cold PBS, then centrifuged again. The cell pellets were resuspended in 400 μl of resuspension buffer [20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 10 mM KCl, 1.5 mM MgCl_2 , 1.0 mM EDTA, 250 mM sucrose, 1 mM DTT, 1.5 mM phenylmethylsulfonyl fluoride (PMSF), 3 $\mu\text{g}/\text{ml}$ leupeptin, 20 $\mu\text{g}/\text{ml}$ aprotinin], then lysed by 20 strokes in a type B dounce homogenizer (Wheaton, Millvale, NJ). The homogenates were subjected to microcentrifugation for 20 min at 4°C , and the resulting pellets, containing the mitochondria, were resuspended in 100 μl of resuspension buffer. The supernatants, containing cytosol and released cytochrome C, were further clarified by microcentrifugation for an additional 20 min. Protein aliquots (10 $\mu\text{g}/\text{lane}$), representing cytosolic or mitochondrial fractions, were resolved on a 14% SDS-PAGE gel, transferred to nitrocellulose, then probed with monoclonal antibody directed against cytochrome C (Pharmingen).

Preparation of Genomic DNA

Cells were collected by centrifugation, washed in PBS, then centrifuged again. The cell pellets were lysed in 10 mM Tris (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5% SDS, 200 $\mu\text{g}/\text{ml}$ proteinase K, then incubated at 50°C for 4 hr. Following incubation, the lysates were subjected to sequential extraction with phenol/ T_{10}E_1 , phenol/ CHCl_3 , and CHCl_3 , then precipitated overnight. The dried DNA pellets were resuspended in T_{10}E_1 containing 50 $\mu\text{g}/\text{ml}$ RNase A, and incubated for 2 hr at 37°C . Following sequential extractions and ethanol precipitation, the DNA pellets were resuspended in 40 μl of T_{10}E_1 buffer. DNA samples (5 $\mu\text{g}/\text{lane}$) were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

Northern Blots and Probe Generation

RNA was prepared using RNEasy columns (Qiagen, Inc., Valencia, CA) from K562 IWAF transfectants exposed to medium or etoposide (100 μM) for 48 hr, following overnight prein-

cubation in isopropyl B-Z-thiogalactopyranoside (IPTG; 2mM). Northern blots analysis was performed as described previously (33), and membranes were probed with radiolabelled c-IAP1 probe. The probe encompassed bp 185-2437 of c-IAP1 and was generated by reverse transcription polymerase chain reaction (RT-PCR) amplification of K562 RNA using primers S: ATTG ATC ACC TAC TCA TGC ACA AAA CTG C and AS: TCG GCA CCA TAA CTC TGA TGA AC for 30 cycles of amplification.

Results

p21WAF1-induced G1 Arrest is Disrupted by Etoposide Without Cleavage of p21WAF1

To study modulation of etoposide-induced apoptosis by p21WAF1, cells were transfected with p21WAF1 expressed from the inducible LacSwitch promoter (Stratagene, La Jolla, CA). In this system, p21WAF1 expression was regulated negatively by a cotransfected Lac repressor, and was induced by addition of IPTG to the medium. The level of p21WAF1 expression achieved in this system was similar to the level expressed in HL60 cells induced to differentiate by chemical agents (data not shown). p21WAF1 gene expression was induced stably within 2 hr of IPTG addition, leading to growth arrest (34). K562 cells stably transfected with the inducible p21WAF1 construct (IWAF cells) and cells selected after transfection with control plasmid (Neo) were preincubated for 6 hr with IPTG or with no inducer, and then exposed to etoposide or to carrier (0.1% DMSO) for 48 hr. Preincubation of cells with IPTG for 24 hr gave the same results as preincubation for 6 hr. Figure 1 demonstrates that the levels of induced p21WAF1 are equivalent in the presence or absence of etoposide. There was no detectable cleavage of p21WAF1, as was reported in apoptosis induced by serum-deprivation or irradiation (9,10). It was also notable that in K562 cells, in contrast to 3T3 cells (35), etoposide did not induce endogenous p21WAF1 on its own. Induction of p21WAF1 led to G1 arrest within 24 hr (Fig. 2, IWAF column, +IPTG). Interestingly, subsequent addition of etoposide to p21WAF1-expressing cells undermined the G1 checkpoint established by p21WAF1, causing a significant number of cells to proceed to the S and G2 phases and to undergo apoptosis (Fig. 2,

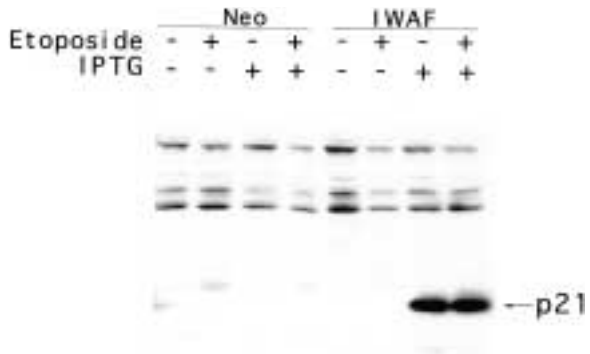


Fig. 1. Expression of p21WAF1 is not altered by etoposide. K562 cells stably transfected with vector control (Neo) or inducible p21WAF1-vector (IWAF) were exposed to medium or to IPTG for 6 hr as indicated; etoposide (100 μ M) or carrier (0.1% DMSO) were then added. Protein extracts were prepared 48 hours after etoposide addition and blotted for p21WAF1 expression. IPTG, p21WAF1 inducer; DMSO, dimethylsulfoxide.

IWAF+ Etoposide column, +IPTG, and Fig. 5). Similar effects were seen after a 24-hr preincubation with IPTG. The partial abrogation of the p21WAF1-mediated G1 block by etoposide was not a result of p21WAF1 cleavage or p21WAF1 protein instability (Fig. 1) and might be derived from Rb cleavage in the presence of etoposide (see below).

By 24 hr after etoposide addition, a shift to S and G2/M phases DNA contents was apparent in etoposide-treated cells (35). Substantial cell fragmentation was manifested by the appearance of subnuclear DNA peaks on cell cycle analysis. This shift was more evident after 48 hr (Fig. 2 and Table 1). A subpopulation of cells in the G0/G1 phase remained prominent in p21WAF1-expressing cells, although less than in the absence of etoposide (33% vs. 68%).

Partial Protection from Apoptosis by p21WAF1

Induction of p21WAF1 prior to and during etoposide exposure clearly did not provide total protection against apoptosis. However, a partially protective effect was evident. Figure 3 shows cell viability and DNA laddering analysis of cells exposed to etoposide in the presence or absence of induced p21WAF1. At 48 hr after etoposide addition, 71% of cells expressing p21WAF1 were viable in terms of trypan blue exclusion, compared with 43% of p21WAF1-nonexpressing cells.

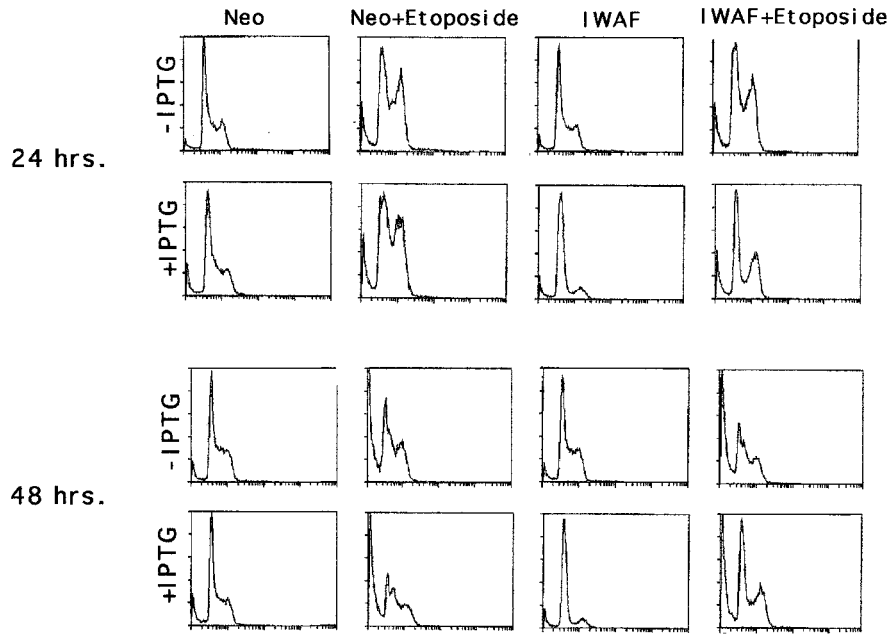


Fig. 2. Cell cycle profiles in the presence or absence of p21WAF1 and/or etoposide. Vector control (Neo) or inducible p21WAF1-vector (IWAF) cells were preincubated in the presence or absence of

p21WAF1 inducer (IPTG) for 6 hr, after which etoposide was added as indicated. Cells were harvested 24 or 48 hr later and DNA content determined by propidium iodide staining and flow cytometry.

Etoposide-induction of Cytochrome c Release and Procaspase-3 Cleavage was Unaltered by p21WAF1

The release of cytochrome c from mitochondria into the cytosol is considered an early response to chemotherapy agents triggering a proteolytic cascade mediated by caspase proteins. This consists of the activation of caspase-9, which cleaves procaspase-3, yielding activated caspase-3. Like Martins et al. (29), we

found that the addition of high-dose etoposide to K562 cells resulted in cytosolic relocation of cytochrome c and cleavage of caspase-3. Interestingly, the induction of p21WAF1 in IWAF cells did not affect either of these processes, as shown in Figure 4. This indicated that transduction of damage signals initiating the apoptotic pathway were intact in the presence of p21WAF1.

Table 1. Cell cycle profiles of Neo and IWAF cells exposed to medium or etoposide (E) after pre-incubation in medium or in IPTG (I)

	DAY ONE				DAY TWO			
	Frag.	G1	S	G2/M	Frag.	G1	S	G2/M
Neo	5	44	29	18	7	41	29	21
Neo - E	11	26	30	30	36	26	20	16
Neo - I	6	44	29	17	7	43	28	20
Neo - IE	11	30	30	27	40	23	20	15
IWAF	10	33	33	21	11	42	26	18
IWAF - E	12	28	31	27	43	17	23	16
IWAF - I	9	57	22	12	9	68	12	9
IWAF - IE	15	40	22	23	30	33	17	19

Profiles were determined by propidium iodide staining 24 (day one) or 48 (day two) hr after etoposide addition. DNA contents in G1, S, G2/M, or subdiploid (Frag.) compartments were calculated using the ModFit program (Coulter, Inc, Miami, FL). The decrease in G1-phase of IWAF cells exposed to etoposide is most evident at day two (bolded). Neo or IWAF cells were exposed to IPTG(I) to etoposide (E) or to both (IE) as indicated.

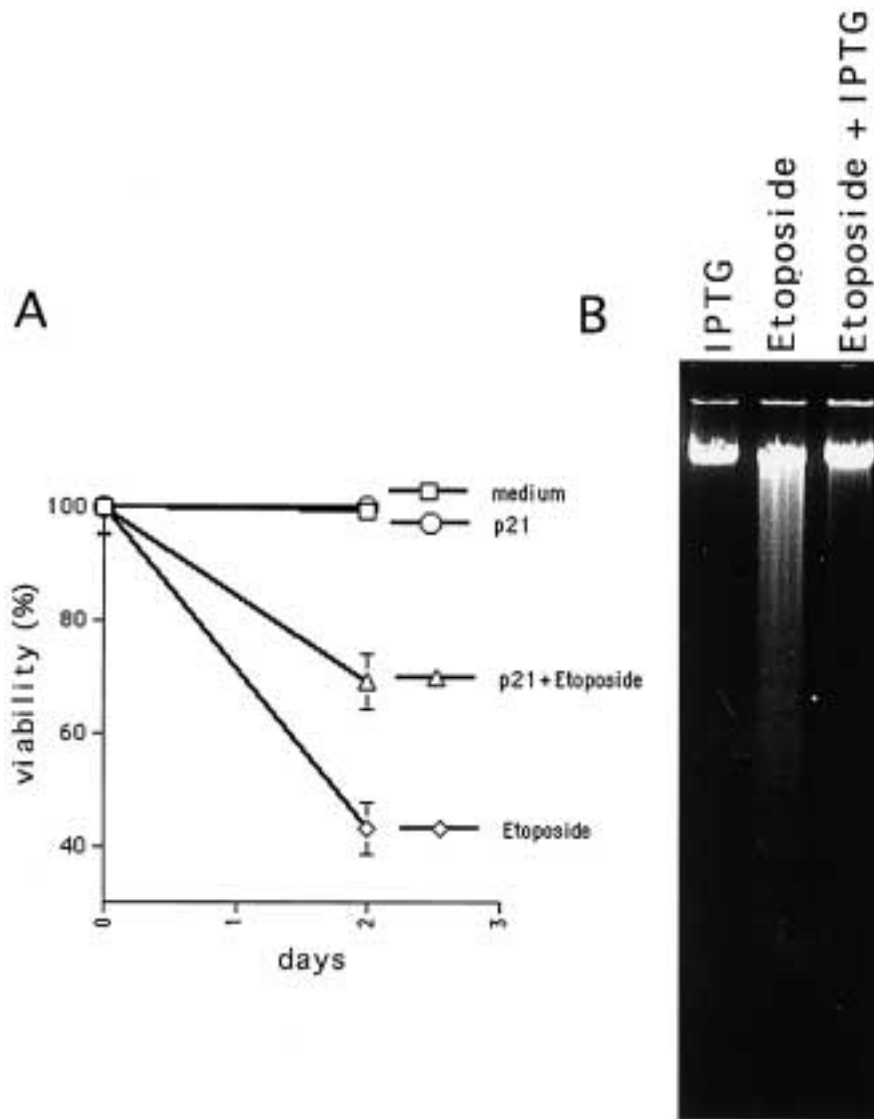


Fig. 3. Protection from apoptosis by p21WAF1. Inducible p21WAF1-vector (IWAF) cells were induced by p21WAF1 inducer (IPTG) to express p21WAF1 or uninduced (medium) for 24 hr. Cells were subsequently exposed to 100 μ M etoposide

for 48 hr, after which viability was assessed by trypan blue exclusion (Fig. 3A, $n = 3$). Apoptosis was demonstrated by fragmentation of genomic DNA after the addition of IPTG, etoposide, or both as indicated.

p21WAF1 Decreased Cleavage of Caspase Targets

Etoposide-generated DNA damage results in activation of the “executioner” caspases-3 and -7 (36). These downstream participants in the apoptotic cascade cleave multiple cellular proteins (37,38). In K562 cells, etoposide was shown to cause cleavage of the caspase-3 substrate poly (ADP-ribose) polymerase (PARP) (29). We examined whether PARP cleavage was altered by p21WAF1 induction. Interestingly, although procaspase-3 cleavage was not altered by p21WAF1 expression, the generation of PARP cleavage fragments was reduced markedly in p21WAF1-expressing cells (Fig. 5).

We then examined whether cleavage of lamin B was altered by p21WAF1 expression. The pathway for lamin cleavage differed from that for PARP (39), involving caspase-6 (40,41). Only a small proportion of lamin was cleaved after etoposide addition to K562, but this also was inhibited by p21WAF1.

Cleavage of Rb was described in association with rapid apoptosis of HL60 cells exposed to etoposide (42). The addition of etoposide also causes Rb to be cleaved in K562 cells, generating a 48 kD fragment that previously was described (42,43), as well as a prominent 28 kD band (Fig. 5). The generation of these

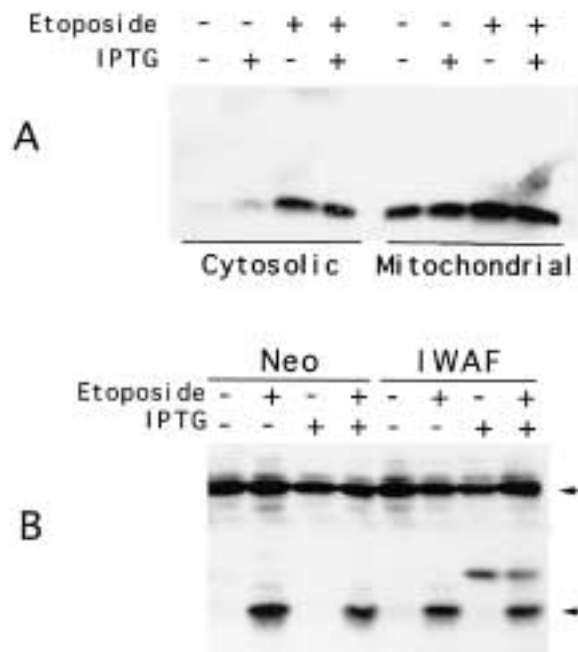


Fig. 4. Lack of effect of p21WAF1 on sensing of DNA damage. (A) Inability of induced p21WAF1 to alter cytochrome c release into the cytosol. Inducible p21WAF1-vector (IWAF) cells either expressing (IPTG+) or not expressing (IPTG-) p21WAF1 were exposed to etoposide or control for 48 hr. Harvested cells then were separated into cytosolic and mitochondrial fractions and cytochrome c levels determined by Western blots analysis. (B) Inability of induced p21WAF1 to alter procaspase-3 cleavage. Cells transfected with control vector (Neo) or with the inducible p21WAF1 vector (IWAF) were treated with etoposide for 48 hr after preincubation with p21WAF1 inducer (IPTG) as indicated. Harvested cells were blotted with polyclonal anti-caspase-3 antibody. Uncleaved and cleaved procaspase-3 products are indicated by the upper and lower arrows, respectively. A slight residual band from p21WAF1 blotting is evident in IWAF cells exposed to IPTG.

cleavage products, like PARP and lamin, was decreased by p21WAF1 expression.

Etoposide Post-transcriptionally Down-modulates c-IAP1 Levels, an Effect Opposed by p21WAF1

The above experiments indicated that p21WAF1 decreased the death of etoposide-exposed K562 cells by interfering with apoptotic machinery distal to cytochrome c release and procaspase-3 cleavage, but proximal to cleavage of distal caspase substrate proteins. We hypothesized that inhibitor of apoptosis proteins

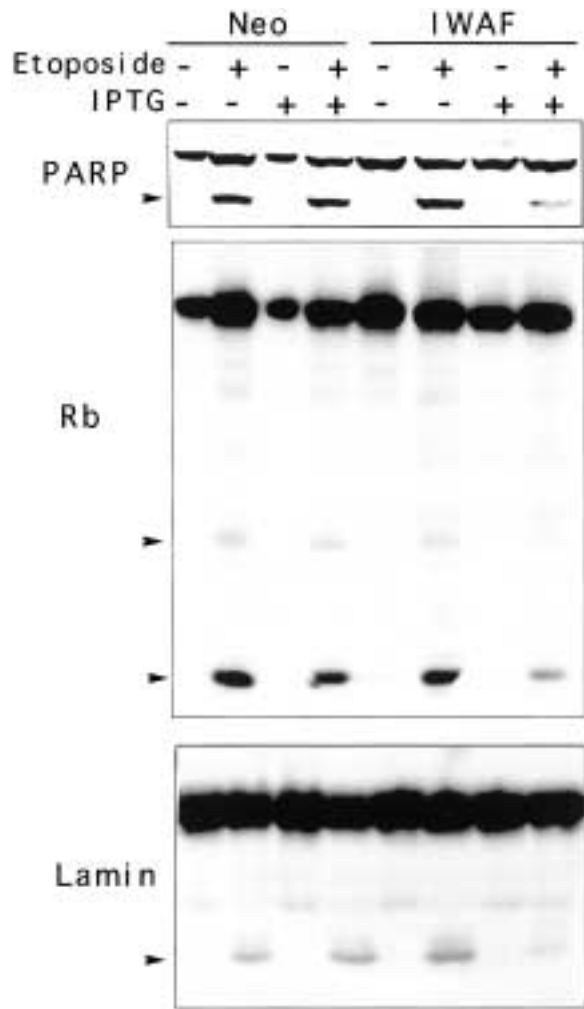


Fig. 5. Cleavage of cellular caspase targets induced by etoposide is inhibited by p21WAF1. Cells treated as in Figure 4 were harvested and blotted for protein targets as indicated. Generation of the 85 kD poly (ADP-ribose) polymerase (PARP) fragment by etoposide is evident, and is decreased by p21WAF1. Retinoblastoma (Rb) cleavage fragments of 48 and 28 kD and lamin cleavage are similarly affected. Cleavage products are indicated by arrows.

(IAPs) might mediate the anti-apoptotic effect of p21WAF1. IAP proteins directly bind to and inhibit distal caspases in the apoptotic pathway, so might be involved in p21WAF1 inhibition of cleavage of distal caspase substrates. Of three IAPs investigated—c-IAP1, c-IAP2 and h-ILP—c-IAP1 was most informative. As shown in Figure 6A, the addition of etoposide markedly decreased the levels of c-IAP1 protein in the K562 cells. Down-modulation of c-IAP1 preceded PARP cleavage, suggesting that the decrease in c-IAP1 may accelerate the effects of executioner caspases. Interestingly, this de-

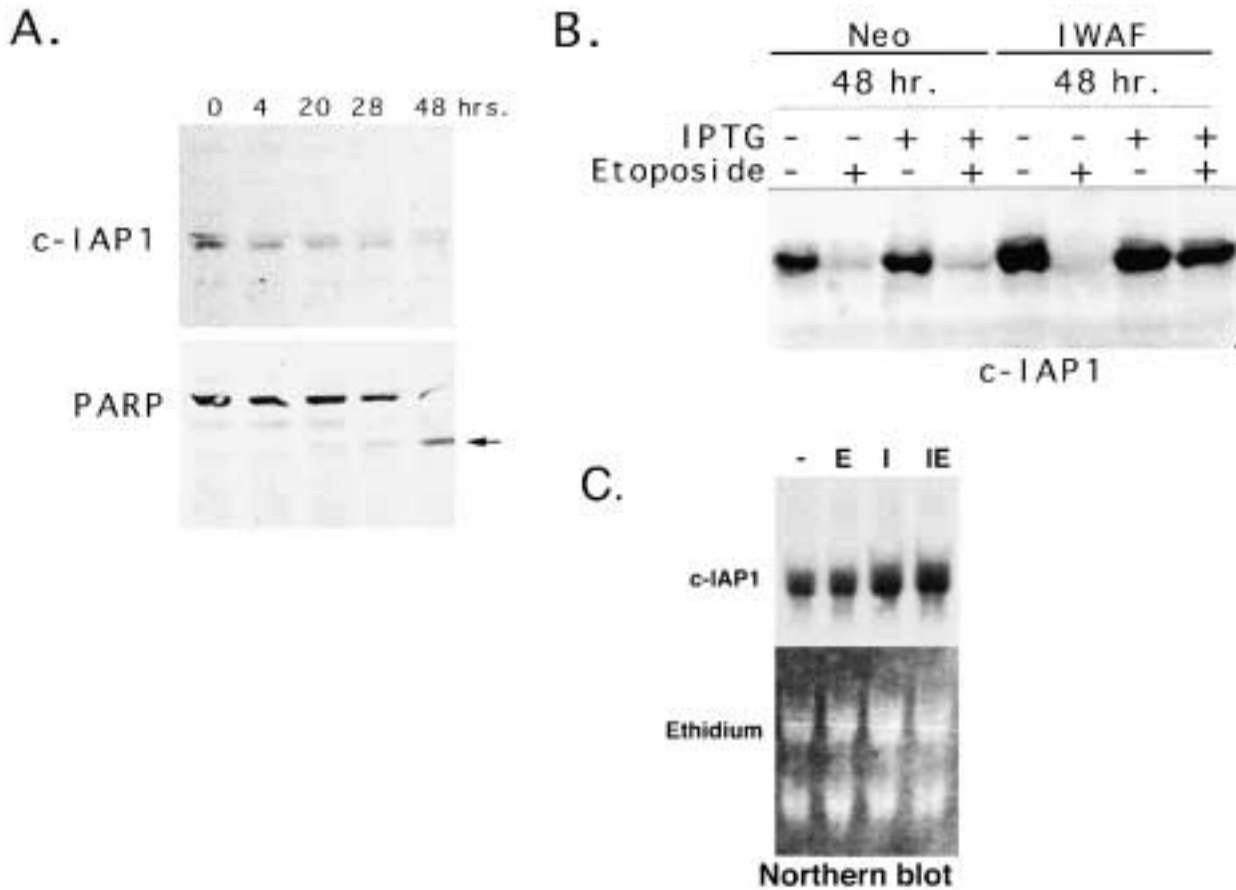


Fig. 6. c-IAP1 loss follows etoposide treatment and is inhibited by p21WAF1. (A) Kinetics of (c-IAP1) down-modulation by etoposide. K562 control transfectants were exposed to etoposide for 0 to 48 hr and expression of c-IAP1 and poly (ADP-ribose) polymerase (PARP) determined by Western blots analysis. (B) Prevention of c-IAP1 loss by p21WAF1. Representative results of three independent experiments are shown. Control transfectants (Neo) and p21WAF1-inducible transfectants (IWAF) were preincubated in p21WAF1 inducer (IPTG) as indicated, then etoposide (+, 100 μ M) or carrier (-, 0.1% DMSO-) were added as shown. Cells

were harvested after 48 hr and 50 μ g of protein for each condition was immunoblotted with polyclonal anti-c-IAP1 (shown). Equivalent loading in each lane was evident upon Ponceau S staining. The immunogen used for the c-IAP1 antibody shown (Santa Cruz-1867: KTASQRLFPGPSYQNIKSIC) is not shared with (CIAP-2). Monoclonal anti-c-IAP1 gave the same results. (C) c-IAP1 message is not altered by etoposide. Northern blots of cells exposed for 48 hr to medium (-), etoposide (E), IPTG (I) or both (IE). The slight increase in signal in the two right lanes reflects loading differences. DMSO, dimethylsulfoxide; IPTG, p21WAF1 inducer.

crease in c-IAP1 levels was prevented by p21WAF1 expression (Fig. 6B). Northern blot analysis showed that the down-modulation of c-IAP1 and its continued expression in the presence of p21WAF1 could not be accounted for on a transcriptional basis (Fig. 6C).

C-IAP1 LOSS DID NOT INVOLVE CASPASES AND MAY INVOLVE THE PROTEASOME. Conceivably, c-IAP1 could be degraded by caspases - etoposide caused the caspase apoptotic pathway to be activated. Several anti-apoptotic proteins are inactivated by caspase cleavage, notably p35 and CrmA (44). In Figure 7A, we tested

whether etoposide-induced c-IAP1 loss could be mediated by caspases. K562 cells were preincubated 2 hr with DMSO vehicle (-), a cdk2 inhibitor (roscovitine), or caspase inhibitors, and etoposide was then added for 24 hr. The caspase inhibitors used were z-DEVD-FMK, which inhibits caspase-3, and z-VAD-FMK, a pancaspase inhibitor. Both were used in excess at a concentration of 80 μ M. Interestingly, neither inhibitor prevented c-IAP1 loss in the presence of etoposide. Reprobing of this blot with anti-PARP antibody showed that z-DEVD-FMK and z-VAD-FMK totally prevented the appearance of the PARP cleavage.

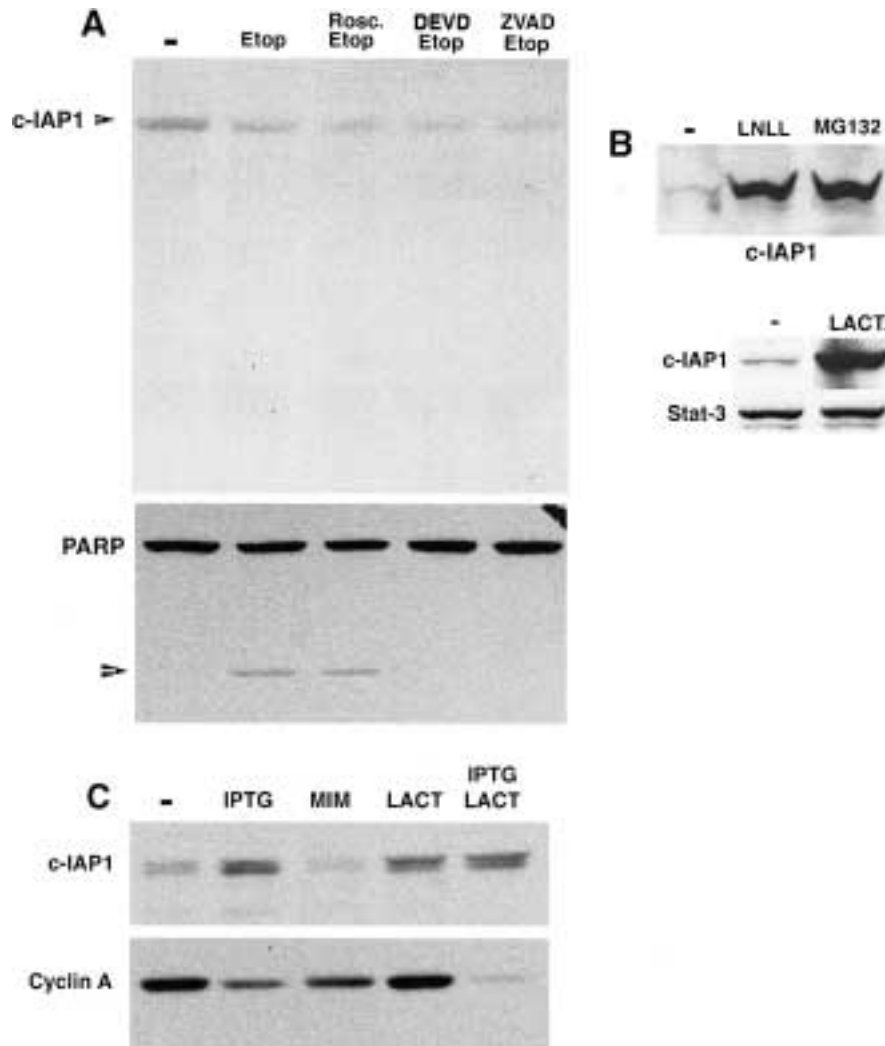


Fig. 7. Caspase and proteasome pathway involvement in c-IAP1 expression. (A) Etoposide-initiated (c-IAP1) loss is not prevented by caspase or (cdk2) inhibitors. K562 cells incubated for 16 hr in the presence of dimethylsulfoxide (DMSO) vehicle (-) or etoposide (100 μ M) as indicated. Caspase inhibitors (DEVD; 80 μ M) or (ZVAD; 80 μ M) and cdk2 inhibitor, roscovitine (8 μ g/ml), were added 2 hr prior to etoposide. (B) Increase in c-IAP1 levels by proteasome inhibitors. K562 cells were exposed for 24 hr to proteasome inhibitors (LNLL; 75 μ M) or (MG132; 40 μ M). The

specific proteasome inhibitor lactacystin (lact) also enhanced c-IAP1 expression. Equal loading is demonstrated by blotting for Stat 3. (C) Mimosine does not protect against etoposide-mediated c-IAP1 loss. K562 cells were exposed to etoposide for 48 hr in the presence or absence of pre-incubation with p21WAF1 inducer (IPTG), 250 μ M mimosine (MIM), 5 μ g/ml β clasto-lactocystin (LACT), or lactocystin and IPTG combined, as shown. Protein loading was equivalent by Ponceau S staining. The membrane was sequentially probed with c-IAP1 and with cyclin A antibodies.

Figure 7A also demonstrates that the cdk2 inhibitor roscovitine (at a dose that inhibits cell growth) did not prevent the c-IAP1 decrease, implying that p21WAF1 protection may not occur through cdk2 inhibition.

PROTEASOME. Another mechanism of c-IAP1 loss could be through proteasome-mediated degradation. Proteasome inhibitors were shown

to oppose etoposide-initiated apoptosis (45). As shown in Figure 7B, peptide aldehyde proteasome inhibitors (LNLL) and (MG132) both enhanced the expression of c-IAP1 in K562 cells. c-IAP1 expression also was enhanced markedly by incubation of cells for 24 hr in the presence of the specific proteasome inhibitor lactacystin. These findings indicated that c-IAP1 degradation may be mediated

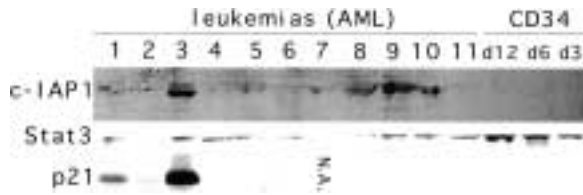


Fig. 8. Heightened expression of c-IAP1 in myeloid leukemias. 40 μ g protein extracts of untreated (#1,2,3,4,5,6,8,9,10) or relapsed (#7 and 11) myeloid leukemias or of CD34+ hematopoietic progenitor cells exposed for 3–12 days to granulocyte colony stimulating factor (G-CSF), interleukin-3 (IL-3) and stem cell factor (SCF) were blotted for c-IAP1 expression. A parallel gel was blotted for p21WAF1 expression. The leukemias consisted of >90% blasts. Equal loading was evident on total protein staining of membrane. Blotting for (Stat3) highlights the presence of protein in CD34 samples.

through the proteasome. To see whether the increased c-IAP1 levels observed following lactocystin and after p21WAF1 induction were additive, lactocystin and IPTG were added together (Figure 7C). It was apparent that lactocystin did not increase c-IAP1 in IPTG-exposed cells, suggesting that p21WAF1 and lactocystin worked along the same pathway.

Figure 7C also shows whether another cell cycle blocker can mimic the ability of p21WAF1 to sustain c-IAP1 expression in etoposide-exposed cells. We preincubated K562 cells for 16 hr in the G1-blocker mimosine at a concentration that we found to block cell growth. Subsequent addition of etoposide for 48 hr decreased c-IAP1 levels in mimosine-exposed cells, as in control cells. This indicated that the cell cycle phase alone was not responsible for protection of c-IAP1 expression. Figure 7C also indicates that c-IAP1 levels were not coupled to the cell cycle, as reflected in cyclin A expression.

Enhanced Expression of c-IAP1 in Myeloid Leukemia Blasts

To gauge whether altered expression of c-IAP1 in these experiments using the K562 leukemic cell line had physiological significance, we examined c-IAP1 expression in normal myeloblasts induced to differentiate along the myeloid lineage, and in 11 samples of leukemic blasts from patients. c-IAP1 message was shown to be expressed at a low level in peripheral blood leukocytes (46), but we did not detect c-IAP1 protein in extracts from differentiating cells

consisting of metamyelocytes and granulocytes (CD34, day 12; Fig. 8). c-IAP1 was also undetectable at earlier stages of myelopoiesis, including extracts of myeloblasts and promyelocytes (CD34, day 3). In contrast, c-IAP1 was present at least at a low level in 8/11 myeloid leukemia samples and was elevated markedly in four of those samples (Fig. 8). p21WAF1 expression, also shown, was markedly elevated in one sample expressing high levels of c-IAP1.

Discussion

p21WAF1 expression decreases apoptosis of K562 cells when exposed to etoposide. p21WAF1 appears to enhance cellular viability by opposing the action of distal caspases on cellular substrates. We note that p21WAF1 stabilizes the level of the inhibitor of apoptosis c-IAP1. Interestingly, in the absence of p21WAF1, c-IAP1 expression is decreased markedly by etoposide. This suggests that IAPs (inhibitor of apoptosis proteins) may constitute novel targets of chemotherapeutic agents. Because IAP proteins oppose the actions both of regulatory (procaspase-9) and executioner (caspase-3 and -7) caspases (44,47), IAP down-modulation may be necessary for chemotherapeutic agents to induce apoptosis successfully. Conversely, stabilization of IAPs by p21WAF1 would be expected to promote chemoresistance.

c-IAP1 was shown to inhibit apoptosis in transfection experiments by two laboratories (44,48). Our data supports a critical role of c-IAP1 in protecting against apoptosis by demonstrating that down-modulation of c-IAP1 precedes the cleavage of caspase targets, such as PARP (Fig. 6). Down-modulation of c-IAP1 by etoposide constitutes a novel pathway through which a pro-apoptotic agent overcomes inherent protective mechanisms within the cell. It will be of interest to characterize what other chemotherapeutic agents down-modulate c-IAP1 and to identify other antiapoptotic proteins that could similarly be targeted.

Our data indicates that the anti-apoptotic effects of p21WAF1 in K562 cells could result, in a large part, from stabilization of c-IAP1 expression. The degree of protection against apoptosis conveyed by exogenous p21WAF1 (roughly 60%, Fig. 3) is similar to that afforded by exogenous expression of c-IAP1 (44,48). Moreover, p21WAF1 action maps to a point in the apoptotic cascade that is consistent with it acting through c-IAP1. c-IAP1 inhibition of cas-

pase-3 action could account for the decreased proteolysis of PARP following p21WAF1 induction. p21WAF1 also reduces cleavage of lamin B, a caspase-6 substrate. Although activated caspase-6 is not inhibited in vitro by c-IAP1, this may reflect c-IAP inhibition of the proximal protease caspase-8 (47,48) on processing of procaspase-9 (47). However, we did not detect any effect of c-IAP1 stabilization on procaspase-3 cleavage, which might be expected if c-IAP1 was acting on upstream caspases. We interpret this as evidence that endogenous c-IAP1 primarily inhibits activated caspase cleavage products; whereas, at high levels attained in transfections or in vitro experiments, it can also inhibit upstream procaspases. Decreased lamin cleavage following p21WAF1 induction could be a marker of decreased caspase-3 and caspase-6 cross-activation (49).

It is likely that c-IAP1 stabilization does not comprise the sole mechanism of p21WAF1 protection against apoptosis and that different mechanisms predominate in different cells. Suzuki et al. (7) demonstrated that p21WAF1 could form a complex with procaspase-3 in Hep G2 cells and sterically inhibited cleavage of this procaspase to form activated caspase-3. We do not, however, observe an effect of p21WAF1 on procaspase-3 cleavage. It is possible that this reflects differences in apoptotic pathways in different cell types; we were not able to demonstrate a direct interaction of p21WAF1 with procaspase-3 or with c-IAP1 in K562 cell extracts (data not shown).

Like Roy et al. (44) we find that c-IAP1 is not a target of caspase cleavage. In addition to their demonstration that recombinant caspases-3,-6,-7, and -8 do not cleave c-IAP1, we find no evidence for a c-IAP1 cleavage product concomitant with its down-modulation by etoposide. Moreover, we could not prevent c-IAP1 down-modulation with z-VAD-FMK, a pancaspase inhibitor.

Our data supports a model in which c-IAP1 is cleaved by the proteasome following etoposide exposure. Our results suggest that endogenous levels of c-IAP1 may be regulated by the proteasome. Both peptide aldehyde inhibitors of the proteasome and the direct proteasome subunit inhibitor, lactacystin, increase the expression of c-IAP1. Proteasome-mediated degradation of c-IAP1 would constitute an additional and novel mechanism through which proteasome and caspase pathways intersect. c-IAP1 may not be the only node at which an-

tiapoptotic proteins are linked with the proteasome pathway. Recently, a 530 kD ubiquitin-conjugating enzyme was shown to contain a baculovirus IAP repeat (BIR)-repeat related to IAPs and to inhibit apoptosis (50,51). That giant protein may serve to couple IAP and proteasome pathways.

While this manuscript was under revision, Yang et al. (52) reported proteasome-mediated down-modulation of c-IAP1 in thymocytes exposed to etoposide. They observed that the (RING) domain of c-IAP1 was required for proteasomal degradation. p21WAF1 appears to act on the proteasome pathway to stabilize c-IAP1. We found that p21WAF1 and the proteasome-specific inhibitor, lactocystin, both sustained c-IAP1 expression at an equivalent level, and that they were not additive in up-regulating c-IAP1 in etoposide-exposed cells (Figure 7). The definitive mechanism through which p21WAF1 sustains c-IAP1 remains to be determined, however. It must account both for the lack of effect of p21WAF1 on basal c-IAP1 expression and for the equivalent effect of p21WAF1 and lactocystin on c-IAP1 in etoposide-exposed cells. Our data does indicate that p21WAF1 prevents c-IAP1 degradation through a mechanism distinct from cell cycle arrest. Mimosine, which blocks cells in the G1 phase, did not protect against c-IAP1 loss after etoposide addition (Figure 7). Moreover, the G1-phase checkpoint induced by p21WAF1 in the absence of etoposide was disrupted by etoposide. The ability of p21WAF1 to provide protection against apoptosis, despite the loss of the G1 checkpoint, supports the view that the anti-apoptotic effect of p21WAF1 is not an epiphenomenon of cell cycle arrest.

A complete understanding of the basis of p21WAF1-anti-apoptotic effects is biologically significant because diverse cancers express high levels of p21WAF1 and may, therefore, be chemoresistant (21). Indeed, p21WAF1 expression was shown to be a negative prognostic indicator in leukemias (1). A switch between anti-apoptotic and pro-apoptotic effects of p21WAF1 may be a modulated process in normal development. In the setting of leukemia, anti-apoptotic effects of p21WAF1 are likely to convey a selective advantage.

The finding of c-IAP1 elevation in myeloid leukemia samples and not in normal myeloid precursors is intriguing. c-IAP1 may convey a selective advantage to leukemic cells even prior to treatment. Modulation of IAP protein

levels is likely to be a significant means through which cancers avoid apoptosis. The recently cloned IAP, survivin (53), is expressed at elevated levels in a range of solid tumors and in 50% of high-grade non-Hodgkin's lymphoma (53–55). Survivin expression is associated with a negative prognosis in colon cancer (55). Similar analyses of other IAPs have not been reported, but our finding of heightened c-IAP1 expression in leukemia suggests that the expression levels of other IAPs, in addition to survivin, may have clinical importance. Recently, decreased caspase activation was associated with poor prognosis in AML (56). This suggests that c-IAP1 overexpression also will be associated with a poor prognosis in leukemia, because it opposes caspase activation. Because c-IAP1 may be absent in normal myeloid cells, therapies directed at lowering c-IAP1 levels in leukemia are unlikely to be myelotoxic.

Our findings in K562 cells suggest that, although p21WAF1 does not affect basal expression of c-IAP1, it does stabilize its expression in the presence of etoposide and, possibly, other chemotherapeutic agents. Such a mechanism may explain why p21WAF1 expression is associated with chemoresistance and a negative prognosis in myeloid leukemia (1).

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