FEBS Letters 399 (1996) 158–162 FEBS 17917

Failure to activate interleukin 1β-converting enzyme-like proteases and to cleave retinoblastoma protein in drug-resistant cells

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Received 26 September 1996

Abstract We previously found that retinoblastoma (RB) is cleaved at the initiation of apoptotic execution. Here we report that when an HL-60 cell line resistant to cytosine arabinoside (Ara-C) was exposed to this anticancer drug, neither RB cleavage nor apoptosis was detected. Consistent with that, processing of interleukin 1β-converting enzyme (ICE) and CPP32 (an ICE-like protease) was also prevented in these cells. In contrast, treatment of the HL-60-Ara-C-resistant cells with etoposide induced all of these apoptotic events. Furthermore, the etoposide-induced RB cleavage was inhibited by a specific tetrapeptide ICE-like inhibitor. Our results demonstrate that activation of the RB cleavage enzyme, an ICE-like protease, is required for overcoming drug resistance.

Key words: Apoptosis; Cancer; Cleavage; Drug resistance; Interleukin 1β-converting enzyme; Retinoblastoma

1. Introduction

Despite the achievements in the design and use of chemotherapeutic agents, the majority of human cancers at present are resistant to therapy [1–3]. It has been suggested that proteins mediating cancer cell sensitivity to chemotherapy may also play a role in regulating processes of proliferation and apoptosis (or programmed cell death) [1–3]. For example, resistance to anticancer drugs and radiation is associated with inactivation of the tumor suppressor protein p53 [1,2], or overexpression of Bcl-2 oncoprotein [1,3].

Apoptosis is an active process of cell death, which occurs in two physiological stages, commitment and execution [4]. Little is known about molecular controls of the apoptotic commitment in mammalian cells. It has been proposed that induction of p53 might be sufficient to commit cells to undergo apoptosis [5], whereas overexpression of Bcl-2 oncoprotein can block this process [6]. Apoptotic execution in mammalian cells is initiated by specific proteases of the interleukin 1β-converting enzyme (ICE) family [4]. The active ICE enzyme consists of two subunits, p20 and p10, processed from a 45 kDa precursor [7]. CPP32 is another member of the ICE family [8]. The full-length 32 kDa form of CPP32 (pro-CPP32) represents an inactive zymogen. Upon stimulation of apoptosis, pro-CPP32 is processed to its active form, p20 and p11 subunits [8,9]. It has also been found that at the initiation of apoptotic execution, the activated CPP32 cleaves poly(ADP-ribose) polymerase (PARP) [9]. The importance of PARP cleavage to the death process remains unknown.

We previously found that when human leukemia cells were treated with a variety of anticancer agents, retinoblastoma (RB) became dephosphorylated [10], and then immediately cleaved [11]. Two major cleavage fragments of RB, p48 and p68, were detected by monoclonal RB antibodies G3-245 (which recognizes an epitope between amino acids 300 and 380 [12]) and XZ55 (which recognizes epitopes between 444 and 621 and between 646 and 665 [13]), respectively. Here, we report that HL-60 cells resistant to cytosine arabinoside (HL-60-Ara-C-resistant cells [14]), when exposed to Ara-C, failed to cleave RB, did not activate ICE and CPP32, and did not induce apoptosis. However, treatment of the Ara-C-resistant cells with etoposide (VP-16), another anticancer agent that acts through a different mechanism, induced all of these apoptotic events. Furthermore, the VP-16-induced RB cleavage in these cells was blocked by a specific tetrapeptide inhibitor of ICE-like proteases.

2. Materials and methods

2.1. Materials

Mouse monoclonal culture supernatant to human RB, XZ55, was a kind gift from Drs. N. Dyson and E. Harlow (Massachusetts General Hospital Cancer Center, Charlestown, MA). Purified mouse monoclonal antibody to human RB, G3-245, was purchased from PharMingen; purified rabbit polyclonal antibody to p20 subunit of human ICE from Upstate Biotechnology Inc.; purified mouse monoclonal antibody to p20 subunit of human CPP32 from Transduction Laboratories. The specific tetrapeptide ICE-like inhibitor, acetyl-YVAD-chloromethyl ketone (YVAD-CMK), was from Bachem. VP-16, Ara-C and other chemicals were from Sigma.

2.2. Cell culture

HL-60-Ara-C-resistant cell line was a gift originally from Dr. K. Bhalla (Medical University of South Carolina). Both HL-60 and HL-60-Ara-C-resistant cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Sigma), 100 unit/ml of penicillin, 100 μg/ml of streptomycin and 2 mM L-glutamine.

2.3. Whole cell extracts and Western blot assay

Whole cell extraction [11] and the enhanced chemiluminescence Western blot assay [15] were performed as described previously.

3. Results

3.1. Failure to cleave RB in HL-60-Ara-C-resistant cells

We had found that when HL-60 cells were exposed to Ara-C or VP-16, RB was first dephosphorylated and then immediately cleaved. This RB cleavage was accompanied by the internucleosomal fragmentation of DNA (a 180 base-pair DNA ladder) [11]. We speculated that if RB cleavage is critical for the initiation of apoptotic execution, failure to cleave RB should be associated with drug resistance. In this study, we used a pair of HL-60 cell lines that were sensitive [10] or resistant [14] to the anticancer drug Ara-C. Both HL-60 and HL-60-Ara-C-resistant cells were treated with Ara-C, followed

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PII S0014-5793(96)01311-7

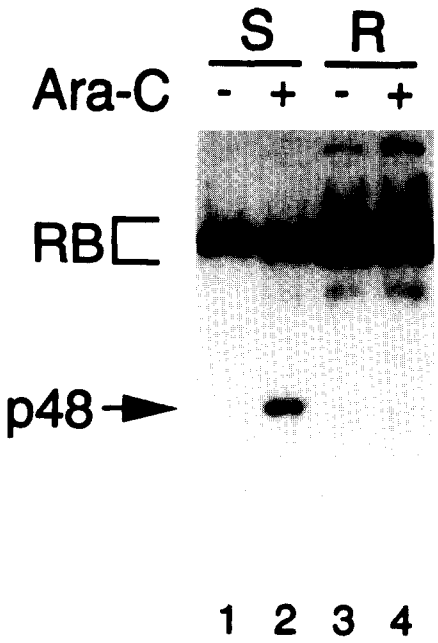

Fig. 1. Failure to cleave RB in HL-60-Ara-C-resistant cells. Both HL-60 (S) and HL-60-Ara-C-resistant (R) cells were treated with 10 μM Ara-C (+) or an equal percentage (0.02%) of PBS (−) for 4 h, followed by preparation of whole cell extracts and Western blot analysis using a purified monoclonal antibody to human RB, G3-245. Bands of RB and its cleavage fragment, p48, are indicated.

by the measurement of RB cleavage (by Western blot assay) and apoptosis (by DNA fragmentation assay).

When HL-60 cells were exposed to Ara-C for 4 h, an abundant band with molecular mass 48 kDa (p48) was detected by purified monoclonal RB antibody G3-245 (Fig. 1, lane 2), suggesting RB cleavage. At the same time, a 180 base-pair DNA ladder was detected [10]. The p48 fragment of RB was not found in HL-60 cells that had been treated with the drug-vehicle, PBS (Fig. 1, lane 1), demonstrating that RB cleavage is Ara-C-induced.

In contrast, When HL-60-Ara-C-resistant cells were treated with Ara-C for 4 h, neither the p48 fragment of RB (Fig. 1, lane 4) nor DNA fragmentation [16] was detected. Therefore, failure to cleave RB is associated with failure to induce apoptosis in these cells.

3.2. *VP-16 induces RB cleavage in HL-60-Ara-C-resistant cells, which is inhibitable by YVAD-CMK*

We had found that HL-60-Ara-C-resistant cells were sensitive to treatment of VP-16 (see Fig. 2), another anticancer agent that acts via a different mechanism (as an inhibitor of topoisomerase II [17]). We investigated whether exposure to VP-16 of HL-60-Ara-C-resistant cells induced RB cleavage. When the Ara-C-resistant cells were treated with VP-16 for 3–4 h, RB was converted from the hyperphosphorylated (p120/hyper) to a hypophosphorylated form (p115/hypo) (Fig. 2, lanes 9,10 vs. lanes 7,8). Furthermore, after 4 h VP-16 treatment, bands of 68 kDa (p68) were detected by the monoclonal RB antibody XZ55 (Fig. 2, lane 10). The level of the p68 fragment of RB was increased afterwards (Fig. 2, lane 11). The p48 fragment of RB was also generated according to similar kinetics, detected by the monoclonal RB antibody G3-245 (data not shown). These data demonstrate that VP-16 induces the process of RB cleavage in HL-60-Ara-C-resistant cells.

Associated with cleavage of RB, internucleosomal DNA fragmentation was detected in the VP-16-treated Ara-C-resistant cells [16]. Neither RB cleavage nor DNA fragmentation was found in HL-60-Ara-C-resistant cells treated with di-

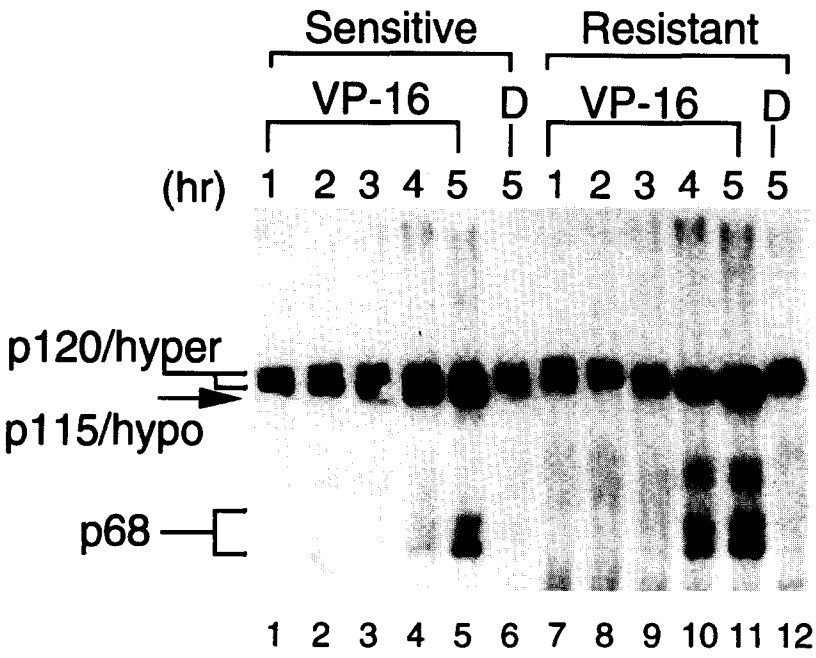

Fig. 2. Induction of RB cleavage by VP-16 in HL-60-Ara-C-resistant cells. Both HL-60 (Sensitive) and HL-60-Ara-C-resistant (Resistant) cells were treated with 20 μM VP-16 or an equal percentage (0.01%) of dimethyl sulfoxide (D) for the indicated hours. Western blot assay was performed using a mouse monoclonal culture supernatant to human RB, XZ55. The hyperphosphorylated form (p120/hyper), hypophosphorylated form (p115/hypo) and a cleavage fragment (p68) of RB are indicated.

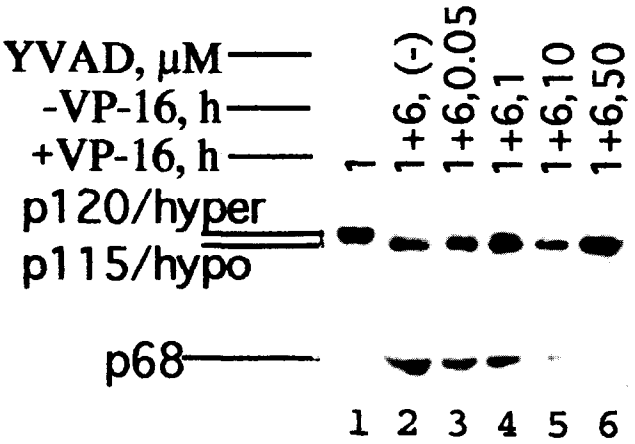

Fig. 3. Inhibition of the VP-16-induced RB cleavage by YVAD-CMK in HL-60-Ara-C-resistant cells. Ara-C-resistant cells were treated with 10 μM VP-16 (+VP-16) for 1 h and then incubated in drug-free medium (−VP-16) for an additional 6 h in the absence (−) or presence of various concentrations of YVAD-CMK as indicated. Western blot assay was performed using RB antibody XZ55. The p120/hyper, p115/hypo and the p68 fragment of RB are indicated.

methyl sulfoxide, the solvent of VP-16 (Fig. 2, lane 12). These data strongly suggest that induction of RB cleavage is correlated with overcoming drug resistance in these cells.

As a control, treatment of normal HL-60 cells with VP-16 for 4–5 h also induced RB dephosphorylation and cleavage (Fig. 2, lanes 4, 5). The VP-16-induced RB changes occurred earlier in HL-60-Ara-C-resistant cells than in HL-60 cells (Fig. 2), indicating that HL-60-Ara-C-resistant cells are more sensitive to treatment with VP-16. These results also indicate that pathways for induction of RB cleavage are intact in the Ara-C-resistant cells.

To investigate whether VP-16 activates an ICE-like protease that mediates RB cleavage in HL-60-Ara-C-resistant cells, we used YVAD-CMK, a well-characterized specific tetrapeptide inhibitor of ICE-like proteases [7]. The Ara-C-resistant cells were pretreated with VP-16 for 1 h, washed and then incubated in drug-free medium for an additional 6 h. Using this procedure we found that transient exposure to VP-16 for 1 h was sufficient to generate readily detectable p68 fragment of RB (Fig. 3, lane 2 vs. lane 1). Addition of YVAD-CMK at the start of the 6 h incubation effectively blocked production of the p68 fragment: 50% at 50 nM, 80% at 10 μM and 100% at 50 μM (Fig. 3, lanes 3–6 vs. lane 2). Inhibition of RB cleavage by YVAD-CMK was accompanied by an increase in the level of p115/hypo/RB (Fig. 3, lane 6 vs. 2), supporting the idea that p115/hypo/RB is the substrate of the RB cleavage enzyme. In contrast, addition of tosyl-L-lysine chloromethyl ketone, a non-specific ketone protease inhibitor, at 50 μM had no effect (data not shown). These data indicate that a VP-16-induced ICE-like protease mediates the process of RB cleav-

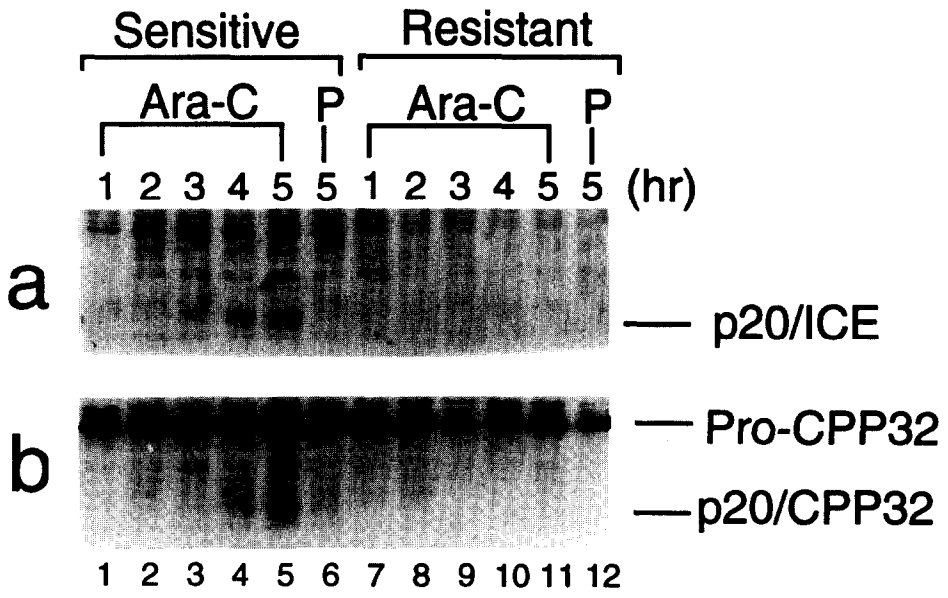

Fig. 4. Failure to activate ICE and CPP32 in HL-60-Ara-C-resistant cells. Both HL-60 (Sensitive) and HL-60-Ara-C-resistant (Resistant) cells were treated with 10 μM Ara-C or an equal percentage (0.02%) of PBS (P) for the indicated hours, followed by preparation of whole cell extracts. Western blot analysis was performed by using purified antibodies to p20 subunit of human ICE (a), or p20 subunit of human CPP32 (b). Bands of p20/ICE, p20/CPP32 and the full length of CPP32 (Pro-CPP32) are indicated.

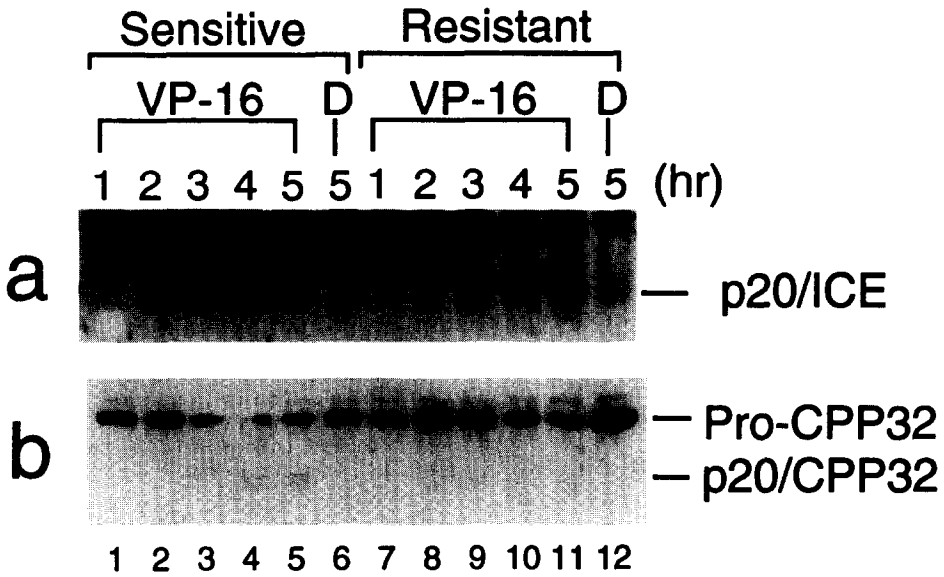

Fig. 5. Activation of ICE and CPP32 by VP-16 in HL-60-Ara-C-resistant cells. Both HL-60 (Sensitive) and HL-60-Ara-C-resistant (Resistant) cells were treated with 20 μM VP-16 or an equal percentage (0.01%) of dimethyl sulfoxide (D) for the indicated hours. Western blot assay was performed by using purified antibodies to p20/ICE (a) or p20/CPP32 (b). Bands of p20/ICE, p20/CPP32 and the full length of CPP32 (Pro-CPP32) are indicated.

age in HL-60-Ara-C-resistant cells. Activation of the ICE-like protease and consequent RB cleavage is, therefore, associated with overcoming drug resistance.

3.3. *VP-16 but not Ara-C activates ice and CPP32 in HL-60-Ara-C-resistant cells*

Because cleavage of RB is mediated by an ICE-like protease activity (Fig. 3), failure to cleave RB in HL-60-Ara-C-resistant cells (Fig. 1) might be due to inhibition of the ICE-like protease activation. To test this hypothesis, we treated both HL-60 and HL-60-Ara-C-resistant cells with Ara-C for up to 5 h, and measured levels of p20/ICE and p20/CPP32, the active forms of these enzymes [7,9], by using their specific monoclonal antibodies. When HL-60 cells were exposed to Ara-C for 3 h, a faint band of 20 kDa (p20/ICE) was detected by its specific antibody (Fig. 4a, lane 3). The level of p20/ICE was further increased afterwards (Fig. 4a, lanes 4,5). The level of p20/CPP32 was increased after 4–5 h treatment with Ara-C (Fig. 4b, lanes 4,5), detected by a purified antibody to the p20 subunit of CPP32. Neither p20/ICE nor p20/CPP32 was found in the control-treated HL-60 cells (Fig. 4a,b, lane 6). The observation that processing of ICE preceded that of CPP32 (Fig. 4a vs. b) is consistent with the idea that activated ICE cleaves and activates CPP32 during cellular apoptosis [18]. The kinetics of ICE/CPP32 processing is comparable to that of RB cleavage (Fig. 4 vs. Fig. 1 and [11]), supporting the hypothesis that an ICE-like protease cleaves RB.

In contrast, when HL-60-Ara-C-resistant cells were exposed to Ara-C for up to 5 h, neither p20/ICE nor p20/CPP32 was observed (Fig. 4a,b, lanes 7–12). Therefore, inhibition of ICE/CPP32 activation is associated with failure to cleave RB in these Ara-C-resistant cells (Figs. 1 and 4).

We predicted that treatment of HL-60-Ara-C-resistant cells with VP-16 would activate ICE and CPP32. Indeed, when these Ara-C-resistant cells were treated with VP-16 for 3–5 h, p20/ICE became detectable (Fig. 5a, lanes 9–11). At the same time, CPP32 was also activated because the level of pro-CPP32 was decreased and a faint band of p20/CPP32 was observed (Fig. 5b, lanes 9–11). The kinetics of ICE/CPP32 activation is correlated well with that of RB cleavage in HL-60-Ara-C-resistant cells treated with VP-16 (compare Fig. 5 to Fig. 2). These results further support the hypothesis that VP-16 activates an ICE-like protease that cleave RB in the Ara-C-resistant cells.

When normal HL-60 cells were treated with VP-16 for up to 5 h, p20/ICE also became detectable (Fig. 5a, lanes 1–6). CPP32 also became activated in these cells, as demonstrated by a decreased level of pro-CPP32 and the appearance of a faint p20/CPP32 band (Fig. 5b, lanes 1–6). Taken together, these results demonstrate that signal transduction pathways for ICE/CPP32 activation are intact in the Ara-C-resistant cells.

4. Discussion

We found that when normal HL-60 cells were induced to undergo apoptosis by Ara-C, RB cleavage occurred (Fig. 1), accompanied by processing and activation of ICE and CPP32 (Fig. 4). In contrast, HL-60-Ara-C-resistant cells, when exposed to Ara-C, failed to induce these events and apoptosis (Figs. 1 and 4). However, treatment of HL-60-Ara-C-resistant cells with VP-16 induced RB cleavage, ICE/CPP32 activation and apoptosis (Figs. 2 and 5). Furthermore, the VP-16-induced RB cleavage in the Ara-C-resistant cells was blocked by a specific ICE-like protease inhibitor, YVAD-CMK (Fig. 3).

Although both Ara-C and VP-16 are DNA-damaging agents, they act through different mechanisms. It has been suggested that cellular deoxycytidine kinase activates Ara-C, which inhibits DNA polymerase [19]. In addition, incorporation of Ara-C into cellular DNA results in premature chain termination which may also count for the Ara-C-mediated

cytotoxicity [20]. In contrast, VP-16 inhibits the catalytic activity of topoisomerase II [17]. More importantly, this inhibition leads to stabilization of the normally transient covalent intermediate formed between the DNA substrate and the enzyme [17]. It has been reported that the HL-60-Ara-C resistant cells used in our studies had a low level of deoxycytidine kinase and decreased ability to transport Ara-C [14]. Therefore, these resistant cells are unable to efficiently activate Ara-C and/or incorporate Ara-C into DNA, resulting in insufficient DNA damage. This is probably responsible for the failure to cleave RB, to activate ICE/CPP32 and to induce apoptosis in HL-60-Ara-C-resistant cells (Figs. 1 and 4). Because these cells also failed to dephosphorylate RB (Fig. 1 and [16]), failure to cleave RB in these cells, therefore, may be due to inhibition of both the substrate production and protease activation.

In contrast to Ara-C, VP-16 effectively induced RB cleavage, ICE/CPP32 activation and apoptosis in the Ara-C-resistant cells (Figs. 2 and 5). Because pretreatment of these cells with VP-16 for 1 h was sufficient to induce these events (Fig. 3), VP-16 must be able to generate sufficient DNA damage in these cells for induction of these apoptotic events.

The concept of apoptosis inhibition could be of great importance since the consequence of apoptosis inhibition is the resistance of cancer cells to chemotherapy. Abolishment of apoptosis inhibition, therefore, could be a novel strategy for treatment of drug-resistant cancers. With further understanding of the molecular mechanisms of apoptosis it may eventually be possible to develop novel cancer therapies that specifically seek to modulate the physiologic cell death pathway.

Acknowledgements: We thank Dr. K. Bhalla for providing HL-60-Ara-C-resistant cells, Drs. N. Dyson and E. Harlow for RB monoclonal culture supernatant XZ55, V.W.Y. Lui for technical assistance, and C.L. Fattman for critical reading of the manuscript. This research was supported in part by a James A. Shannon Director's Award from the National Institutes of Health and by National Institutes of Health Grant AG13300 (to Q.P.D.).

References

[1] Harrington, E.A., Fanidi, A. and Evan, G.I. (1994) Curr. Opin. Gene Dev. 4, 120–129.
[2] Kellen, J.A. (1994) Anticancer Res. 14, 433–435.
[3] Reed, J.C. (1995) Hematol. Oncol. Clin. North Am. 9, 451–473.
[4] Steller, H. (1995) Science 267, 1445–1449.
[5] Hartwell, L.H. and Kastan, M.B. (1994) Science 266, 1821–1828.
[6] Reed, J.C. (1994) J. Cell Biol. 124, 1–6.
[7] Thornberry, N.A. et al. (1992) Nature 356, 768–774.
[8] Fernandes-Alnemri, T., Litwack, G. and Alnemri, E.S. (1994) J. Biol. Chem. 269, 30761–30764.
[9] Nicholson, D.W. et al. (1995) Nature 376, 37–43.
[10] Dou, Q.P., An, B. and Will, P.L. (1995) Proc. Natl. Acad. Sci. USA 92, 9019–9023.
[11] An, B. and Dou, Q.P. (1996) Cancer Res. 56, 438–442.
[12] Ludlow, J.W., DeCaprio, J.A., Huang, C.M., Lee, W.H., Paucha, E. and Livingston, D.M. (1989) Cell 56, 57–65.
[13] Hu, Q.J., Bautista, C., Edwards, G.M., Defeo-Jones, D., Jones, R.E. and Harlow, E. (1991) Mol. Cell. Biol. 11, 5792–5799.
[14] Bhalla, K., Nayak, R. and Grant, S. (1984) Cancer Res. 44, 5029–5037.
[15] Dou, Q.P., Levin, A.H., Zhao, S. and Pardee, A.B. (1993) Cancer Res. 53, 1493–1497.
[16] Dou, Q.P. and Lui, V.W.Y. (1995) Cancer Res. 55, 5222–5225.
[17] Long, B.H. (1992) Semin. Oncol. 19, 3–19.
[18] Enari, M., Talanian, R.V., Wong, W.W. and Nagata, S. (1996) Nature 380, 723–726.
[19] Graham, F.L. and Whitmore, G.F. (1970) Cancer Res. 30, 2636–2644.
[20] Kufe, D.W., Major, P.P., Egan, E.M. and Beardsley, G.P. (1980) J. Biol. Chem. 255, 8997–9000.