Differential p53 phosphorylation and activation of apoptosis-promoting genes *Bax* and *Fas/APO-1* by irradiation and ara-C treatment

Tohru Kobayashi^{1,5}, Sanbao Ruan¹, James R. Jabbur¹,
Ugo Consoli², Katharina Clodi², Hiroshi Shiku⁵,
Laurie B. Owen-Schaub³, Michael Andreeff², John C. Reed⁶
and Wei Zhang^{1,4,7}

¹ Department of Neuro-Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030, USA

² Department of Hematology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030, USA

³ Department of Immunology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030, USA

⁴ Department of Tumor Biology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030, USA

⁵ The Second Department of Internal Medicine, Mie University School of Medicine, Tsu, Mie Prefecture, Japan

⁶ The Burnham Institute, La Jolla, California 92037, USA

⁷ corresponding author: Wei Zhang, Department of Neuro-Oncology, Box 316, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030. tel: (713) 792-3778; fax: (713) 745-1183; e-mail: 12507vl@audumla.mdacc.tmc.edu.

Received 10.7.97; revised 1.12.97; accepted 4.3.98

Edited by D. Green

Abstract

In this study, we examined the effects of radiation and ara-C on induction of apoptosis and on the apoptosis-promoting genes *p53*, *Bax* and *Fas/APO-1*, in BV173 human leukemia cells, which harbor the wild-type *p53* gene. It has been reported that *p53* upregulates *Fas/APO-1* and *Bax* expression. Both irradiation and ara-C treatment resulted in apoptosis and induction of *p53* proteins within hours. The *Bax* gene was activated in irradiated and ara-C-treated BV173 cells, but *Fas/APO-1* was induced only in irradiated BV173 cells. Radiation and ara-C treatment did not induce *Bax* or *Fas/APO-1* protein expression in *p53*-null HL60 cells. Radiation weakly induced *Fas/APO-1* expression in KBM-7 cells, which harbor a partially defective *p53* gene. Both HL60 and KBM-7 cells are more resistant to radiation- and ara-C-induced apoptosis than BV173 cells. These results suggest that functional *p53* is necessary for the activation of *Bax* and *Fas/APO-1* expression. However, elevated *p53* protein is not sufficient to activate *Fas/APO-1* gene expression in ara-C-treated cells. Using two-dimensional gel electrophoresis, we found that the *p53* proteins in irradiated and ara-C-treated BV173 cells have different isoelectric points; they converged to a single isoelectric point after *in vitro* treatment with phosphatase. These results suggest that different genotoxic treatments cause different phosphorylations of *p53*, which may account for the different levels of activation of *Fas/APO-1* expression.

Keywords: *p53* phosphorylation; apoptosis; irradiation; ara-C

Abbreviations: SDS–PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; ISEL, *in situ* end labeling

Introduction

Programmed cell death, or apoptosis, is an important cellular process that regulates normal homeostasis. Abnormally blocked cell death is one of the hallmarks of cancer. The effectiveness of radiotherapy and chemotherapy may rely on their abilities to induce apoptosis in tumor cells. The status of the tumor suppressor gene *p53* has emerged as a key determinant for the responsiveness of tumor cells to these treatments. Stabilization and elevation of the steady-state levels of the wild-type *p53* protein occur in response to radiation or DNA-damaging agents and result in apoptosis in some tumor cells (Clarke *et al*, 1993; Lowe *et al*, 1993a,b; Radinsky *et al*, 1994; Ramqvist *et al*, 1993; Shaw *et al*, 1992; Yonish-Rouach *et al*, 1991). Wild-type *p53* governs cellular activities by regulating downstream genes that control various cellular pathways (Baak *et al*, 1993; El-Deiry *et al*, 1993; Kastan *et al*, 1992; Kuerbitz *et al*, 1992; Owen-Schaub *et al*, 1995). Two genes involved in the apoptosis signal transduction pathway have been shown to be regulated by *p53*. It has been demonstrated that *p53* activates the expression of the *Bax* gene, a member of the *Bcl-2* family (Miyashita *et al*, 1994b; Selvakumaran *et al*, 1994; Zhan *et al*, 1994), through a specific DNA-responsive element (Miyashita *et al*, 1994a; Miyashita and Reed, 1995). The product of the *Bcl-2* gene promotes cell survival and inhibits apoptosis (Korsmeyer, 1992; Sentman *et al*, 1991; Strasser *et al*, 1991), and it has been proposed that the *Bax* gene product accelerates apoptosis (Boise *et al*, 1993; Oltvai *et al*, 1993). The *Bax* protein can form heterodimeric complexes with *Bcl-2*, and the relative levels of *Bax* and *Bcl-2* are believed to be important determinants of cell survival and cell death (Oltvai *et al*, 1993; Yang and Korsmeyer, 1996). Our recent studies showed that *p53* transcriptionally activates the expression of *Fas/APO-1* (Owen-Schaub *et al*, 1995). The cell-surface protein *Fas/APO-1* is a member of the tumor necrosis factor receptor superfamily. Overexpression of *Fas/APO-1* or engagement of *Fas/APO-1* by its ligand (or agonistic antibody) triggers a signal transduction pathway that leads to apoptosis (Itoh *et al*, 1991; Oehm *et al*, 1992; Owen-Schaub *et al*, 1992; Suda *et al*, 1993; Trauth *et al*, 1989).

To further understand the mechanism and role of the *p53*-*Bax/Bcl-2* and *p53*-*Fas/APO-1* pathways in the response of cancer cells to radio- and chemotherapy, we focused on the effects of radiation and ara-C treatment on

the expression of p53, Bax, and Fas/APO-1 and on cell death in wild-type *p53*-containing BV173 cells and two other cell lines, KBM-7 and HL60, which harbor defective *p53* and no *p53*, respectively. In BV173 leukemia cells, both radiation and ara-C treatment induced apoptosis, raised the levels of p53 protein, and stimulated Bax expression, whereas radiation but not ara-C induced Fas/APO-1 expression on the cell surface. These results suggest that elevation of p53 protein, although necessary, is not sufficient to activate *Fas/APO-1* expression. Our study of p53 post-translational modification using two-dimensional electrophoresis revealed that the p53 protein in irradiated BV173 cells had a different phosphorylation status than that in ara-C-treated cells, which may explain the different transcriptional activation functions observed for p53. Intriguingly, radiation did not induce expression of the Fas/APO-1 ligand in BV173 cells, suggesting that either Fas/APO-1 induction is futile or elevated Fas/APO-1 can function independent of its ligand.

Results

Induction of apoptosis in BV173 cells by radiation and Ara-C

BV173 cells (*p53* wt/wt), KBM-7 cells (*p53* mt/mt), and HL60 cells (*p53* -/-) were exposed to either 5 or 10 Gy irradiation or to 50 or 500 nM ara-C. Apoptosis of these cells was analyzed by ISEL using flow cytometry at different times after treatment. As summarized in Table 1 and shown in Figure 1, both radiation and ara-C treatment caused BV173 cells to exhibit abundant apoptotic and secondary necrotic cells. In comparison, HL60 and KBM-7 cells exhibited fewer apoptotic cells. A detailed time-course study showed that abundant apoptosis (41%) was detected as early as 4 h after irradiation in BV173 cells. The difference in sensitivity to ara-C among the three cell lines was lost when high-dose ara-C was used.

Treatment with high-dose ara-C (500 nM) resulted in abundant apoptosis in HL60 cells (90.8% at 48 h after exposure) and KBM-7 cells (67.3% at 48 h after exposure). Apoptotic cells were visualized and confirmed by the presence of morphological features such as nuclear condensation and breakage and cell shrinkage (data not shown).

Effect of radiation and ara-C treatment on the protein levels of p53 and expression of Fas/APO-1, Bax and Bcl-2

Our previous studies established the identities of p53 on Western blotting and Fas/APO-1 in flow cytometry assay (Kobayashi *et al*, 1995). The Bax or Bcl-2 expression vector was transfected into K562 or Jurkat cells. Two days after transfection, Bax and Bcl-2 proteins were analyzed by Western blotting. Parental Jurkat cells contained only low levels of the Bax protein, whereas increased amounts of Bax were detected in Bax-transfected Jurkat cells (Figure 2A). In Bax-transfected K562 cells, the original form of Bax, which we named Bax(L), increased, and a smaller form of Bax, which we named Bax(S), appeared (Figure 2A). Bcl-2 protein expression was very low in K562 cells but became easily detected after transfection with Bcl-2-pCI-neo (Figure 2A).

Consistent with previous findings (Kuerbitz *et al*, 1992; Lowe *et al*, 1993b), p53 protein levels were elevated rapidly in BV173 cells after irradiation (Figure 2B) or treatment with ara-C (Figure 2C). In BV173 cells, 8 h after irradiation or 24 h after ara-C exposure, the Bax(S) band was induced dose dependently and at later time points was enhanced, with little change in the level of Bax(L) (Figure 2B). A detailed time-course study showed that the Bax(S) band became detectable after 6 h of irradiation (data not shown). In *p53*-null HL60 cells or KBM-7 cells, which have a mutant p53, only small amounts of Bax(S) were detected after 24–48 h of treatment (Figure 2B and C). The Bcl-2 protein did not change after irradiation in all three cell lines (Figure 2B) and was only weakly induced 48 h after ara-C treatment in

Figure 1 Apoptosis in BV173, HL60, and KBM-7 cells induced by radiation or ara-C treatment. The percentage of apoptotic cells, as analyzed by ISEL flow cytometry assay, are shown in the gated area. The Y-axis shows the fluorescence intensity, which indicates apoptosis; the X-axis shows the side-scattered light intensity

Table 1 Apoptotic cell population after radiation or ara-C treatment

Dose of radiation or ara-C	Hours after treatment	Apoptotic cells (%)		
		BV 173	HL 60	KMB-7
Control	0	2.56	1.53	5.72
	24	1.54	1.47	4.06
	48	2.53	2.28	5.39
5-Gy	8	45.95	11.24	26.34
	24	33.71 ^a	28.05	27.14
10 Gy	8	73.45	28.74	32.05
	24	57.18 ^a	47.50	43.65
50 nM	8	13.02	1.75	6.16
	24	29.97	2.09	11.93
	48	64.53 ^a	5.39	16.18
	48	^b	27.43	34.34
500 nM	24	^b	90.79	67.30
	48	^b		

^aMany cell showed secondary necrosis and lysis

^bExtensive cell lysis was observed

Figure 2 Expression of p53, Bax, and Bcl-2 protein in cells irradiated or treated by ara-C. (A) K562 and Jurkat cells were transfected with pCI-neo, Bax-pCI-neo, or Bcl-2-pCI-neo vector by electroporation. Forty-eight hours later, proteins were extracted and analyzed for expression of p53, actin, Bax, and Bcl-2 on Western blots using specific antibodies. (B) BV173, HL60, and KBM-7 cells were exposed to radiation at three doses. Proteins were extracted 8 or 24 h after radiation and analyzed for expression of p53, Bcl-2, Bax and actin. (C) BV173, HL60, and KBM-7 cells were exposed to 50 or 500 nM ara-C for 24 or 48 h, and proteins were extracted and analyzed by Western blotting for levels of p53, Bcl-2, Bax, and actin protein. (D) BV173 cells were irradiated, metabolically labeled with ^{35}S -methionine and pulse chased with unlabeled methionine for the periods indicated. Proteins were isolated and immunoprecipitated with anti-Bax antibody. The precipitates were analyzed by SDS-PAGE and autoradiography.

BV173 cells (Figure 2C). To further establish the identity of Bax(S), 5 Gy-irradiated BV173 cells were metabolically labeled with ^{35}S -methionine for 2 h and pulse-chased with unlabeled methionine for different periods of time. Bax antibody was used to immunoprecipitate Bax(L) and Bax(S). We found that a decrease in Bax(L) reciprocally accompanied an increase in Bax(S), suggesting that Bax(S) is a cleaved product of the Bax(L) form (Figure 2D). Production of Bax(S) is dependent on the dose of radiation and ara-C (Figure 2B and C) and on the extent of apoptosis induced by these treatments.

Next we analyzed the expression of surface Fas/APO-1 by flow cytometry. As shown in Figure 3A, surface Fas/APO-1 was strongly induced in BV173 cells by 10 Gy radiation. Surprisingly, Fas/APO-1 was not significantly induced in ara-C-treated BV173 cells (Figure 3A), although p53 protein was induced (Figure 2B). Induction of surface Fas/APO-1 was not detected in HL60 or KBM-7 cells after either treatment (data now shown). We also analyzed the protein expression of p21/WAF1/Cip1, a downstream target gene of p53 (El-Deiry et al, 1993). In BV173 cells, p21/WAF1/Cip1 was induced by radiation, but not by ara-C (Figure 3B). In irradiated HL60 cells and

KBM-7 cells, p21/WAF1/Cip1 was not induced (data not shown). These findings showed that elevated p53 in ara-C-treated BV173 cells was not functional in activating the expression of downstream genes *p21/WAF1/Cip1* and *Fas/APO-1*.

To determine whether the protein induction reflected induction at the mRNA level, total cellular RNA was isolated from the treated cells and analyzed for the levels of *Bax* and *Fas/APO-1* mRNA. The levels of *GAPDH* were used as an internal control for quantitation. mRNA levels of both *Bax* and *Fas/APO-1* were increased in irradiated BV173 cells (Figure 4A). In BV173 cells treated with ara-C, *Bax* mRNA was also induced (Figure 4B). In contrast, the *Bax* mRNA level was decreased in KBM-7 and HL60 cells for reasons not yet known. Consistent with the data shown in Figure 3B, in BV173 cells, a very high level of *Fas/APO-1* mRNA was induced after radiation (Figure 4A), whereas very weak *Fas/APO-1* mRNA was induced after ara-C treatment (Figure 4B). In irradiated or ara-C-treated HL60 cells, *Fas/APO-1* mRNA was barely induced (Figure 4), and in irradiated or ara-C-treated KBM-7 cells, *Fas/APO-1* mRNA was relatively weakly induced (Figure 4) compared with the induction by irradiation in BV173 cells. Interest-

Figure 3 Detection of surface Fas/APO-1 protein expression by flow cytometry and of p21/WAF1/Cip1 expression by Western blotting after irradiation or ara-C treatment. (A) The Y-axis shows the relative cell number and the X-axis the fluorescence intensity of Fas/APO-1 staining. Mouse IgG was used as a control for Fas/APO-1 antibody. (B) BV173 and HL60 cells were exposed to radiation or ara-C. Proteins were analyzed for expression of p21/WAF1/Cip1

Figure 4 Expression of *Bax* and *Fas/APO-1* mRNAs in cells irradiated or treated with ara-C. (A) Total RNA was isolated from cells 4, 8, or 24 h after radiation, and the levels of *Bax* and *Fas/APO-1* were analyzed by Northern blotting using cDNA for *Bax* or *Fas/APO-1* as a probe. (B) Total RNA was isolated from ara-C-treated BV173, HL60, and KBM-7 cells; *Fas/APO-1* and *Bax* mRNA levels were analyzed by Northern blotting. A total of 15 mg RNA was loaded in each lane. The level of *GAPDH* mRNA was also analyzed as a control of RNA loading. The intensities of the *Fas/APO-1*, *Bax*, and *GAPDH* bands on the autoradiograph were scanned using a densitometer and the levels of *Fas/APO-1* and *Bax* were normalized against that of *GAPDH*. The control value was designated as 1.0, and the relative levels of *Fas/APO-1* and *Bax* were calculated and are shown under each band

ingly, a fourfold induction of *Fas/APO-1* mRNA was not sufficient to generate enough surface Fas/APO-1 protein to be detected by flow cytometry, probably reflecting the relatively low sensitivity of flow cytometry. *Bax* mRNA induction was not observed in irradiated or ara-C-treated HL60 and KBM-7 cells (Figure 4).

To investigate whether the Fas/APO-1 pathway contributes to irradiation-induced apoptosis of BV173 cells, we analyzed the levels of Fas/APO-1 ligand expression on Northern blotting because Fas/APO-1 may need its ligand to be fully active. No induction of ligand expression was detected (data not shown). We then tested whether the agonistic Fas/APO-1 antibody (APO-1, Kamiya Biomedical Co.) could engage the Fas/APO-1 receptor induced by radiation and further sensitize the BV173 cells to apoptosis. The result was again negative (data not shown), suggesting

that the Fas/APO-1 pathway was not active in radiation-induced apoptosis in BV173 cells. As a positive control, the agonistic Fas/APO-1 antibody induced drastic apoptosis in Jurkat cells.

Distinct phosphorylation of p53 protein in irradiated and ara-c-treated BV173 cells

The above results posed two problems to our previous findings that wild-type but not mutant p53 activates *Fas/APO-1* expression (Owen-Schaub *et al*, 1995). First, Fas/APO-1 was also induced in mutant p53-containing KBM-7 cells. Our previous studies showed that the p53 mutant in KBM7 cells retains the ability to bind DNA (Zhang *et al*, 1993, 1995). This mutant, therefore, is only partially defective, which may explain its weak ability to activate *Fas/APO-1*. Second, elevated p53 in ara-C-treated BV173 cells did not markedly

Figure 5 Phosphorylation of p53 protein in irradiated and ara-C-treated BV173 cells as analyzed by two-dimensional gel electrophoresis. Six hours after irradiation or ara-C treatment, BV173 cells were labeled with ^{35}S -methionine for 2 h. Total proteins were isolated, and p53 proteins were immunoprecipitated with a mixture of the p53 antibodies PAb421 and PAb1801. Half the immunoprecipitates were treated with phosphatase. Then the p53 precipitates with or without phosphatase treatment were analyzed in a two-dimensional gel electrophoresis. The two mobilities of p53 on SDS-PAGE represent the two polymorphic forms of p53 and serves as a reference point

activate *Fas/APO-1* expression. To understand the molecular basis for the second problem, we hypothesized that the p53 proteins in the irradiated and ara-C-treated cells had different posttranslational modifications. To test this, we labeled the irradiated and ara-C treated BV173 cells (after 6 h of treatment) with ^{35}S -methionine, and then the immunoprecipitated p53 proteins were subjected to two-dimensional gel electrophoresis, with one dimension separated by isoelectric points (isoelectrofocusing gel) and another dimension by size (SDS-PAGE). On the SDS-PAGE dimension, p53 proteins in BV173 cells exhibited two different mobilities. We showed previously that these two forms represent the two polymorphic forms of p53 at codon 73 (Zhang *et al*, 1992). Along the isoelectrofocusing dimension, p53 in irradiated BV173 cells existed as multiple forms, with a pI spanning from 5.75–5.95 (Figure 5C). In comparison, in ara-C-treated BV173 cells, the isoelectric points of p53 proteins shifted to lower values (Figure 5E). Gels were aligned in reference to the molecular weight marker and the nonspecific protein product at pI 5.2 observed on the gel (Figure 5A–F). Because phosphorylation is the key factor affecting the pI of proteins, we assume that p53 proteins with different pIs represent p53 proteins with different phosphorylation status. To test this hypothesis, we treated the immunoprecipitated p53 protein with phosphatase and then analyzed the treated p53 proteins on two-dimensional gel electrophoresis. This treatment converted most of the p53 proteins to a common form (Figure 5B, D and F), confirming that phosphorylation was the modification that resulted in the different pI values for p53 proteins from irradiated and ara-C-treated cells. p53 proteins in both control and ara-C-treated cells appeared to be more phosphorylated than that in irradiated BV173 cells. In contrast, p53 mutant proteins with virtually identical pI values were identified in control, radiation-treated and ara-C-treated KBM-7 cells (Figure 6). We attempted to identify the specific phosphorylation sites using conventional phosphopeptide

Figure 6 Patterns of isoelectric points of the p53 mutant protein in KBM-7 cells after irradiation or ara-C treatment. Six hours after irradiation or ara-C treatment, KBM-7 cells were labeled with ^{35}S -methionine for 2 h. Total proteins were isolated, and p53 proteins were immunoprecipitated with a mixture of the p53 antibodies PAb421 and PAb1801. The precipitates were analyzed in a two-dimensional gel electrophoresis

mapping and sequencing. However, our preliminary studies showed that metabolic labeling with ^{32}P subjected cells to radiation strong enough to abolish the difference in the pIs of p53 in the irradiated and ara-C-treated cells. Other non-isotopic approaches are needed to identify the specific sites of p53 phosphorylation.

Discussion

Radiation and chemotherapy are two major modalities for eradicating cancer cells. Many factors affect the response of cells to these treatments. The status of the p53 gene has been shown to be a crucial element in the sensitivity of hematopoietic malignant cells to treatment-induced apoptosis. Previous studies have shown that p53 protein levels are elevated by DNA-damaging treatment, and p53 can transcriptionally activate two genes that may promote apoptosis, *Bax* (Miyashita *et al*, 1994a; Selvakumaran *et al*, 1994; Zhan *et al*, 1994) and *Fas/APO-1* (Owen-Schaub *et al*, 1995). In this study, we investigated the effect of radiation and ara-C on the transcriptional activation of *Bax* and *Fas/APO-1* in a wild-type p53-containing cell line, BV173. Both treatments elevated p53 protein levels to a similar extent and induced apoptosis; however, the expression patterns of *Fas/APO-1* were different. *Fas/APO-1* was strongly induced by radiation but only weakly induced in ara-C-treated cells. Although the role of *Fas/APO-1* in radiation-induced BV173 cells is not apparent because no *Fas/APO-1* ligand was expressed, these results do demonstrate that radiation and ara-C elicit different molecular events in the activation of apoptosis and that elevated p53 protein is not sufficient to activate the *Fas/APO-1* gene. Similarly, ara-C-induced p53 protein also failed to activate expression of the cell cycle modulator p21/WAF1/Cip1. Nevertheless wild-type p53 is necessary for maximal *Bax* and *Fas/APO-1* induction because they were not induced in p53-null HL60 cells and *Fas/APO-1* was only weakly induced in irradiated KBM-7 cells, which contain a partially active p53 mutant.

Our results showed that the function of p53 protein is also determined at the epigenetic level in drug-treated cells. Using two-dimensional gel electrophoresis, we analyzed the p53 proteins isolated from irradiated or ara-C-treated BV173 cells. Different patterns in isoelectric points of p53 proteins were observed. In contrast, in KBM-7 cells virtually identical patterns in the isoelectric points of mutant p53 were observed after either radiation or ara-C treatment. Because p53 is a phosphorylated protein and phosphorylation has been shown to modulate the functions of p53 (Friscella *et al*, 1993; Lees-Miller *et al*, 1992; Milne *et al*, 1995; Zhang *et al*, 1994b), we hypothesized that the p53 proteins in irradiated and ara-C-treated BV173 cells were differentially phosphorylated. The experiments with phosphatase confirmed this hypothesis. The lower pI values of p53 proteins in ara-C-treated BV173 cells indicated that this treatment induced more extensive phosphorylation. Unique phosphorylation of p53 induced by ara-C may be the basis for its inability to transactivate *Fas/APO-1*. These results provide further evidence that phosphorylation is an important mechanism in the functional regulation of p53, especially under genotoxic stress conditions. Our initial attempt to identify the specific phosphorylation sites using

the conventional ^{32}P isotopic labeling approach failed because the labeling itself stabilized p53 protein and caused extensive cell death, which may have resulted in further change in the phosphorylation status of p53. Thus, a nonisotopic approach is needed to study p53 phosphorylation. By the same token, although ^{35}S -methionine labeling was not strong enough to diminish the difference between the effects of ara-C and radiation on p53, the results need to be further validated in the future.

This study also revealed some interesting aspects of *Fas/APO-1*'s functional role and of *Bax* metabolism during irradiation- and ara-C-induced apoptosis. Both *Fas/APO-1* and *Bax* proteins were induced in apoptotic BV173 cells by irradiation, whereas *Fas/APO-1* protein was not induced in apoptotic BV173 cells by ara-C. This result showed that *Fas/APO-1* was not required in ara-C-induced, p53-dependent apoptosis. In irradiated BV173 cells, although *Fas/APO-1* was strongly induced, the *Fas/APO-1* ligand was not produced. This result suggests either that elevated *Fas/APO-1* can participate in apoptosis induction independent of its ligand or that *Fas/APO-1* induction is futile in this system. An inactive *Fas/APO-1* pathway had also been observed previously in K562 cells, in which *Fas/APO-1* was induced by a temperature-sensitive mutant of human p53 (Kobayashi *et al*, 1995). It is possible that *Bax* or other unidentified factors mediate p53-induced apoptosis in irradiated BV173 cells.

Our experiments detected a shortened form of *Bax*, *Bax(S)*, which was associated with the presence of apoptotic cells in the culture. Because *Bax(S)* was first detected in irradiated BV173 cells 6 h after irradiation, which was 2 h after significant levels of apoptotic cells (41%) were detected, it is not very likely that *Bax(S)* is an active form of *Bax* in apoptosis induction. A more likely possibility is that *Bax(S)* is a specifically cleaved product of *Bax* created during apoptosis. Cleavage of other proteins during apoptosis was also reported (Liu *et al*, 1996; Wang *et al*, 1995; 1996). It would be interesting to investigate whether ICE or ICE-related protease is responsible for *Bax* cleavage, whether *Bax(S)* plays any active role in the apoptotic process, and whether *Bax(S)* may serve as a useful marker for a later stage of apoptosis.

Materials and Methods

Cell culture

K562, Jurkat and HL60 cell lines were obtained from American Type Culture Collection (Rockville, MD); the KBM-7 cell line was provided by Dr B Andersson (The University of Texas MD Anderson Cancer Center); the BV173 cell line was derived from a patient with chronic myelogenous leukemia (Pegoraro *et al*, 1983; Th'ng *et al*, 1987). K562, Jurkat, HL60 and KBM-7 cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum in a 37°C incubator containing 5% CO₂ and 95% air; 5% fetal calf serum was routinely used to maintain BV173 cells. BV173 cells contain the wild-type p53 gene, KBM-7 cells contain a homozygous p53 mutant at codon 215 (Zhang *et al*, 1993), and the p53 gene is deleted in HL60 cells (Wolf and Rotter, 1985).

Radiation and ara-C treatment

The radiation source was provided by a model E-0103 irradiator (U.S. Nuclear Co.). ara-C treatment was performed by continuous exposure of cells to 50 nM cytosine b-D-arabinofuranoside (ara-C) (Sigma).

DNA transfection

Bax and *Bcl-2* expression vectors were prepared by inserting *Bax* or *Bcl-2* cDNA into the *EcoRI* cloning site of the pCI-neo vector (Promega). Electroporation was used to transfect expression plasmids into K562 cells and Jurkat cells, under the conditions described previously (Maxwell and Maxwell, 1988; Ulrich and Ley, 1990). To ensure stable transfection, cells were selected in medium containing 0.8 mg/ml G418 (Life Technologies Inc.) for 4 weeks.

Western blotting

Total cellular protein was extracted and analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Stadler et al, 1994; Zhang et al, 1993). After being transferred to an Immobilon membrane (Millipore), the proteins were incubated overnight with antibodies against Bcl-2 (Dako Co.), Bax (Krajewski et al, 1994), p53 (Ab-6, Oncogene Science), and actin (Ab-1, Oncogene Science). The levels of protein were analyzed using the enhanced chemiluminescence system (Amersham Corp.) according to the manufacturer's instructions.

Northern blotting

Total cellular RNA was isolated using TRI reagent (Molecular Research Center, Inc.) and Northern blot analysis was performed as described previously (Owen-Schaub et al, 1995). The cDNA probes used were (i) a 1.3 kb *PstI* gene fragment of *glyceraldehyde-3-phosphate-dehydrogenase* (*GAPDH*); (ii) a 595 kb *EcoRI* fragment of human *Bax*, and (iii) a 1.4 kb *EcoRI* fragment of human *Fas/APO-1*. The intensities of *Bax*, *Fas/APO-1*, and *GAPDH* (linear range) on blots were scanned using a densitometer. The levels of *Bax* and *Fas/APO-1* were normalized against that of *GAPDH*.

Flow cytometry

To analyze the Fas/APO-1 expression on cells, the phycoerythrin-conjugated anti-Fas monoclonal antibody (CD95, PharMingen) was used. Phycoerythrin-conjugated mouse IgG₁ with irrelevant specificity was used as negative control. A minimum of 1×10^6 cells was incubated with each monoclonal antibody for 30 min at 4°C. After two washings, cells were resuspended in 500 ml of cold phosphate-buffered saline. Flow cytometric acquisition was immediately performed on a FACScan instrument (Becton Dickinson) and analyzed with LysisII software (Becton Dickinson).

In situ end labeling (ISEL) was used to measure apoptosis. To quantitate the number of apoptotic cells, we used a method of ISEL of DNA strand breaks (Gorczyca et al, 1993), which was slightly modified from a previously described method (Kobayashi et al, 1995).

Immunoprecipitation, phosphate treatment and two-dimensional gel analysis

After irradiation or ara-C treatment, cells were labeled with ³⁵S-methionine-containing medium for 2 h as described (Zhang et al, 1994a). Protein was extracted with Nonidet P40 (NP40) lysis buffer containing 0.5 mg/ml aprotinin, 0.1 mg/ml pepstatin A, 1 mM DL-

dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, and 12.5 mM sodium pyrophosphate. Protein extract was precleaned with mouse IgG and protein G agarose and immunoprecipitated with p53 antibodies (PAb421 and PAb1801, Oncogene Science). For phosphatase treatment, each protein sample on protein G agarose was incubated with 66 DEA units of bovine intestinal alkaline phosphatase (Sigma) and 0.8 units of sweet potato acid phosphatase (Sigma) in 50 mM Tris buffer (pH 7.4) at 37°C for 1 h. Immunoprecipitated protein samples that had or had not undergone phosphatase treatment were resuspended with standard urea solubilization buffer consisting of 54% urea, 4% NP-40, 2% ampholyte (pH 3–10, Sigma), and 2% 2-mercaptoethanol. Isoelectric focusing gel solution consisted of 8.25 g urea, 6.0 ml H₂O, 800 ml ampholyte (pH 3–10), and 2.0 ml 30% acrylamide/1.8% bisacrylamide; it was polymerized with 10 ml TEMED and 70 ml 20% ammonium persulfate. After pre-running for 1 h at 200 V, protein samples were loaded into tubes and run overnight at 800 V at room temperature. The gels were then soaked with equilibrating buffer containing SDS for 15 min at room temperature; SDS-PAGE (10% acrylamide) was performed as second-dimension electrophoresis. After SDS-PAGE, gels were fixed, soaked with Amplify (Amersham International), dried and exposed on X-ray film.

Acknowledgements

This work was partially supported by a BRSG grant from MD Anderson Cancer Center and NIH grants CA67987 and CA55164. Special thanks are extended to Ms. Kathryn Carnes and Leslie Wildrick for their editorial assistance. T. Kobayashi and S. Ruan contributed equally to this work.

References

- Barak Y, Juven T, Haffner R and Oren M (1993). mdm2 expression is induced by wild type p53 activity. *EMBO J.* 12: 461–468
- Boise LH, Gonzalez-Garcia M, Postema CE, Ding L, Lindsten T, Turka L, Mao X, Nunez G and Thompson CB (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. *Cell* 74: 597–608
- Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML and Wyllie AH (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* 362: 849–852
- El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW and Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* 75: 817–825
- Fiscella M, Ullrich SJ, Zambano N, Shields MT, Lin D, Lees-Miller SP, Anderson CW, Mercer WE and Appella E (1993) Mutation of the serine 15 phosphorylation site of human p53 reduces the ability of p53 to inhibit cell cycle progression. *Oncogene* 8: 1519–1528
- Gorczyca W, Gong J-P and Darzynkiewicz Z (1993) Detection of DNA strand breaks in individual apoptotic cells by the in situ terminal deoxynucleotidyl transferase and nick translation assays. *Cancer Res.* 53: 1945–1951
- Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y and Nagata S (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* 66: 233–243
- Kastan MB, Zhan Q, El-Deiry WS, Carrier F, Jacks T, Walsh WV, Plunkett BS, Vogelstein B and Fornace AJ (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell* 71: 587–597
- Kobayashi T, Consoli U, Andreeff M, Shiku H, Deisseroth AB and Zhang W (1995) Activation of p21^{WAF1/Cip1} expression by a temperature-sensitive mutant of human p53 does not lead to apoptosis. *Oncogene* 11: 2311–2316
- Korsmeyer SJ (1992) Bcl-2 initiates a new category of oncogenes: regulators of cell death. *Blood* 80: 879–886
- Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG and Reed JC (1994) Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. *Am. J. Pathol.* 145: 1323–1336

- Kuerbitz SJ, Plunkett BS, Walsh WV and Kastan MB (1992) Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proc. Natl. Acad. Sci. USA* 89: 7491–7495
- Lees-Miller SP, Sakaguchi K, Ullrich SJ, Appella E and Anderson CW (1992) Human DNA-activated protein kinase phosphorylation serines 15 and 37 in the amino-terminal transactivation domain of human p53. *Mol. Cell. Biol.* 12: 5041–5049
- Liu X, Kim CN, Yang J, Jemmerson R and Wang X (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome C. *Cell* 86: 147–157
- Lowe SW, Ruley HE, Jacks T and Housman DE (1993a) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 74: 957–967
- Lowe SW, Schmitt EM, Smith SW, Osborne BA and Jacks T (1993b) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* 362: 847–849
- Maxwell IH and Maxwell F (1988) Electroporation of mammalian cells with a firefly luciferase expression plasmid: kinetics of transient expression differ markedly among cell types. *DNA* 7: 557–562
- Milne DM, Cambell LE, Cambell DG and Meek DW (1995) p53 is phosphorylated in vitro and in vivo by an ultraviolet radiation-induced protein kinase characteristic of the c-Jun kinase, JNK1. *J. Biol. Chem.* 270: 5511–5518
- Miyashita T, Harigai M, Hanada M and Reed JC (1994a) Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res.* 54: 3131–3135
- Miyashita T, Krajewski S, Krajewski M, Wang HG, Lin HK, Liberman DA, Hoffman B and Reed JC (1994b) Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* 9: 1799–1805
- Miyashita T and Reed JC (1995) Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* 80: 293–299
- Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, Li-weber M, Richards J, Dhein J, Trauth BC, Ponsling H and Krammer PH (1992) Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. *J. Biol. Chem.* 267: 10709–10715
- Oltvai ZN, Millman CL and Korsmeyer SJ (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell* 74: 609–619
- Owen-Schaub LB, Zhang W, Cusack JC, Angelo LS, Santee SM, Fujiwara T, Roth JA, Deisseroth AB, Zhang W-W, Kruzel E and Radinsky R (1995) Wild-type human p53 and a temperature-sensitive mutant induce Fas/APO-1 expression. *Mol. Cell. Biol.* 15: 3032–3040
- Owen-Schaub LB, Yonehara S, Crump WL III and Grimm EA (1992) DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. *Cell. Immunol.* 140: 197–205
- Pegoraro L, Matera L, Ritz J, Lewis A, Palumbo A and Biagini G (1983) Establishment of a Ph1-positive human cell line (BV173). *J. Natl. Cancer Inst.* 70: 447–453
- Radinsky R, Fidler IJ, Price JE, Esumi N, Tsan R, Petty CM, Bucana CD and Bar-Eli M (1994) Terminal differentiation and apoptosis in experimental lung metastasis of human osteogenic sarcoma cells by wild type p53. *Oncogene* 9: 1877–1883
- Ramqvist T, Magnusson KP, Wang Y, Szelely L, Klein G and Wiman KG (1993) Wild-type p53 induces apoptosis in a Burkitt lymphoma (BL) line that carries mutant p53. *Oncogene* 8: 1495–1500
- Selvakumaran M, Lin H-K, Miyashita T, Wang HG, Krajewski S, Reed JC, Hoffman B and Libermann D (1994) Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. *Oncogene* 9: 1791–1798
- Sentman C, Shutter J, Hockenbery D, Kanagawa O and Korsmeyer SJ (1991) bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell* 67: 879–888
- Shaw P, Bovey R, Tardy S, Sahli R, Sordat B and Costa J (1992) Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proc. Natl. Acad. Sci. USA* 89: 4495–4499
- Stalder T, Hahn S and Erb P (1994) Fas antigen is the major target molecule for CD4+T cell-mediated cytotoxicity. *J. Immunol.* 152: 1127–1133
- Strasser A, Harris AW and Cory S (1991) bcl-2 transgene inhibits T cell death and perturbs thymic self-censorship. *Cell* 67: 889–899
- Suda T, Takahashi T, Goldstein P and Nagata S (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. *Cell* 75: 1169–1178
- Th'ng KH, Garewal G, Kearney L, Rassool F, Melo JV, White H, Catovsky D, Foroni L, Luzzatto L and Goldman JM (1987) Establishment and characterization of three new malignant lymphoid cell lines. *Int. J. Cancer* 3989–3993
- Trauth BC, Klas C, Peters AM, Matzku S, Moller P, Falk W, Debatin K-M and Krammer PH (1989) Monoclonal antibody-mediated tumor regression by induction of apoptosis. *Science* 245: 301–305
- Ulrich MJ and Ley TJ (1990) Function of normal and mutated gamma-globin gene promoters in electroporated K562 erythroleukemia cells. *Blood* 75: 990–999
- Wang X, Pai J-T, Wiedenfeld EA, Medina JC, Slaughter CA, Goldstein JL and Brown MS (1995) Purification of an interleukin-1 beta converting enzyme-related cysteine protease that cleaves sterol regulatory element-binding proteins between the leucine zipper and transmembrane domains. *J. Biol. Chem.* 270: 18044–18050
- Wang X, Zelenski NG, Yang J, Sakai J, Brown MS and Goldstein JL (1996) Cleavage of sterol regulatory element binding proteins (SREBPs) by CPP32 during apoptosis. *EMBO J.* 15: 1012–1020
- Wolf D and Rotter V (1985) Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells. *Proc. Natl. Acad. Sci. USA* 82790–82794
- Yang E and Korsmeyer SJ (1996) Molecular thanatopsis: a discourse on the BCL2 family and cell death. *Blood* 88: 386–401
- Yonish-Rouach E, Resnitzky D, Lotem S, Sachs L, Kimchi A and Oren M (1991) Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. *Nature* 352: 345–347
- Zhan Q-M, Fan S-J, Bae I, Guillouf C, Lieberman DA, O'Connor PM and Fornace AJ Jr (1994) Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. *Oncogene* 9: 3743–3751
- Zhang W, Funk WD, Wright WE, Shay JW and Deisseroth AB (1993) Novel DNA binding of p53 mutants and their role in transcriptional activation. *Oncogene* 8: 2555–2559
- Zhang W, Guo X-Y, Hu G-Y, Liu W-B, Shay JW and Deisseroth AB (1994a) A temperature-sensitive mutant of human p53. *EMBO J.* 13: 2535–2544
- Zhang W, Hu G and Deisseroth A (1992) Polymorphism at codon 72 of the p53 gene in human acute myelogenous leukemia. *Gene* 117: 271–275
- Zhang W, McClain CD, Gau J-P, Guo X-Y and Deisseroth AB (1994b). Hyperphosphorylation of p53 induced by okadaic acid attenuates its transcriptional activation function. *Cancer Res.* 54: 4448–4453
- Zhang W, Randhawa GS, Gau J-P, Shay JW and Deisseroth AB (1995) The first intron of human H-ras is regulated by p53: Mediation of specific activation by a p53-binding element. *Int. J. Oncol.* 7: 1021–1028