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Enforced expression of the tumor suppressor p53 renders human leukemia cells (U937) more sensitive to 1-[β-D-arabinofuranosyl]cytosine (ara-C)-induced apoptosis☆

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Abstract

The effects of enforced expression of p53 on the sensitivity of $p53^{-/-}$ human monocytic leukemia cells (U937) to apoptosis following exposure to the S-phase-specific antimetabolite 1-[β-D-arabinofuranosyl]cytosine (ara-C) were examined. Cells were stably transfected with a plasmid containing a chimeric DNA construct encoding a temperature-sensitive p53 variant ($135_{ala \Rightarrow val}$), which transactivates at 32° but is non-functional at 37°. A significant reduction in the S-phase population was observed in ptsp53 mutants incubated at 32°. Nevertheless, while vector controls did not exhibit differential sensitivity to ara-C at 32° versus 37°, temperature-sensitive p53 mutants displayed a significant increase in apoptosis at the permissive temperature. This was not accompanied by increased ara-CTP formation, DNA incorporation of [^{3}H]ara-C, or altered expression of Bcl-2 or Bax. Enhanced sensitivity was associated with increased mitochondrial injury (e.g. cytochrome *c* release), caspase activation, and loss of clonogenic survival. Significantly, ptsp53 cells synchronized in S phase were markedly more sensitive to ara-C-mediated mitochondrial injury and apoptosis at 32°, indicating that wild-type p53 specifically enhances the susceptibility of this subpopulation to ara-C lethality. Consistent with these results, transient transfection of human wild-type p53 cDNA rendered parental U937 cells more sensitive to ara-C-mediated cell death. Collectively, these findings indicate that p53 expression renders S-phase U937 cells more susceptible to ara-C-mediated mitochondrial dysfunction, cytochrome *c* release, apoptosis, and loss of clonogenic survival without enhancing ara-C metabolism. Such findings raise the possibility that loss of functional p53 activity allows leukemia cells to circumvent ara-C lethality.

Keywords: Leukemia; Apoptosis; Ara-C; p53

1. Introduction

Although p53 activity is lost in the majority of all human cancers, mutations in the *p53* gene are relatively rare in hematopoietic malignancies [1]. However, more than 90% of AML cell lines assayed are $p53^+$ [2], indicating that loss of this gene is not of primary importance in the establishment of immortalized cell lines. Nevertheless, p53 mutations in patient samples predict for poor clinical outcome and response to therapy involving nucleoside analogues [3,4]. Such findings raise the possibility that loss of functional p53 may provide a mechanism by which leukemic cells avoid the lethal actions of certain antileukemic agents.

The specific role of p53 activation in the modulation of cellular drug response, particularly in the case of antimetabolites, has not been elucidated fully. The presence of p53 has been variously reported to enhance or diminish the lethal effects of antineoplastic agents [2,5,6]. For example, under some circumstances, the cell cycle arrest functions of the DNA damage response may predominate, thereby attenuating activation of the apoptotic cascade. One important mechanism by which p53 regulates cell cycle progression is by direct induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ [7], which inhibits the cyclin D/cdk4

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Abbreviations: 7-AAD, 7-amino-actinomycin D; FBS, fetal bovine serum; PI, propidium iodide; pRb, retinoblastoma protein.

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complex [8] as well as CDK1 and CDK2 [9]. The subsequent dephosphorylation of pRb and the inactivation of E2F prevent progression of cells into the S phase. The resulting growth arrest protects some cell lines from apoptosis in response to DNA damage, but this phenomenon can be overridden in other cell lines [10]. The observation that U937 leukemia cells stably expressing a p21 antisense construct are more susceptible to apoptosis induced by the antimetabolite 1-[β-D-arabinofuranosyl]cytosine (ara-C) [11] argues for a protective role of G_1 arrest in the response of human leukemia cells to certain chemotherapeutic drugs.

Under other circumstances, i.e. those characterized by more extensive DNA damage, p53-related pro-apoptotic actions may supersede cell cycle regulatory functions. In this case, inappropriate passage through the cell cycle may promote apoptosis, rather than repair or senescence, in response to cellular stress [12]. Such a model may account for the paradoxical ability of p53 to abrogate the G_1 checkpoint by triggering the caspase-mediated cleavage of Rb [13–15]. This can lead to cell death by two mechanisms: (a) passage of cells through the G_1 checkpoint which, in the absence of repair, results in replication of damaged DNA and (b) unscheduled (inappropriate) activation of the E2F transcription factor, which can induce apoptosis directly [16]. Alternatively, expression of p53 may directly induce apoptosis in some cell types [17]. In this context, p53 is known to increase expression of the pro-apoptotic protein Bax [18] and decrease that of the cytoprotective protein Bcl-2 [19], leading to cytochrome *c* release and an apoptotic cell death. Defects in the *bax* gene correlate with drug resistance in non-neoplastic fibroblasts [20], and interference with Bax expression reduces the p53-dependent apoptotic response to several agents in a variety of neoplastic cell lines including B-CLL cells [21,22]. The results of other studies, however, found no correlation between Bax status and drug response in $p53^+$ cancer cells [23,24].

The deoxycytidine analog ara-C is one of the most active agents in the treatment of acute leukemia [25]. It is phosphorylated intracellularly to its triphosphate derivative, ara-CTP, which represents the lethal form of the drug [26]. The major mechanism of ara-C lethality appears to involve incorporation of the drug into DNA during replication [27], leading to a slowing of S-phase traverse and interference with chain elongation and chain termination, followed by apoptosis, a process that occurs exclusively in the S phase [28]. Currently, information regarding the functional role that p53 plays in ara-C lethality in human leukemic cells is largely lacking. Furthermore, a systematic analysis of the effects of p53 expression on various determinants of ara-C cytotoxicity, particularly in cells displaying an isogenic background, has not been performed. To address these issues, we have employed a murine temperature-sensitive mutant p53 construct to express wild-type p53 activity conditionally in human monocytic leukemia cells (U937) that are otherwise p53 null due to a large deletion in the *p53* gene [1]. The present results indicate that enforced expression of wild-type p53 significantly increases ara-C-mediated mitochondrial dysfunction (i.e. cytochrome *c* release) and apoptosis despite promoting induction of $p21^{WAF1/CIP1}$ and subsequent G_1 arrest. These events are not accompanied by changes in the relative levels of Bcl-2 and Bax. Notably, cells synchronized in the S phase were significantly more sensitive to ara-C-induced lethality when p53 was in the wild-type conformation. Together, these findings suggest that the presence of wild-type p53 renders leukemic cells intrinsically more sensitive to ara-C-mediated lethality by lowering the threshold for mitochondrial damage in the S-phase population. They also support the notion that loss of functional p53 may provide a mechanism by which leukemic cells escape the lethal consequences of ara-C exposure.

2. Materials and methods

2.1. Cell lines

U937 [29] cells were obtained from the ATCC and cultured in RPMI 1640 medium supplemented with sodium pyruvate, minimal essential medium, essential vitamins, L-glutamate, penicillin, streptomycin, and 10% heat-inactivated FBS (Gibco). p53 temperature-sensitive cells, designated ptsp53, were transfected with an expression vector containing a murine mutant p53 construct ($135_{val \Rightarrow ala}$) under the control of the HMSV long terminal repeat and stable cell lines established as described [30].

2.2. Reagents

Ara-C (Sigma) was freshly prepared in sterile water. 7-AAD (Sigma) was prepared as a DMSO stock and diluted in PBS before use.

2.3. Assessment of apoptosis

Cell cultures were prepared by cytocentrifugation (400 *g* for 30 min at room temperature), stained with a Diff-Quick stain set (Dade Diagnostic), and viewed by light microscopy. Experiments were performed independently in triplicate, and for each, five randomly selected fields were evaluated for characteristic apoptotic morphology (chromatin condensation and fragmentation, membrane blebbing).

Alternatively, cell death was assessed by monitoring the uptake of 7-AAD by flow cytometry as previously described [31]. In addition, in some cases apoptosis was confirmed by flow cytometric analysis of annexin V- and PI-stained specimens (Sigma) according to the instructions of the manufacturer. For assessment of cell death, the percentage of cells displaying uptake of annexin V alone (early apoptosis), as well as both annexin V and PI (late apoptosis), were determined and combined.

2.4. Western blot analysis

Treated cells were washed in cold PBS and lysed by the addition of SDS sample buffer (60 mM Tris, pH 6.8, 4% sodium dodecyl sulfate, 5.76 mM β-mercaptoethanol, 10% glycerol) and briefly sonicated. In the case of cytochrome *c*, cytosolic S-100 fractions were isolated using previously described methods [11]. Protein levels were quantified using Coomassie protein assay reagent (Pierce). Extracts were boiled for 10 min, and 25 mg of total protein was fractioned by SDS–PAGE and transferred to Optitran nitrocellulose (S and S), and immunoblotted for Bcl-2 (Dako), Bax (Pharmingen), cytochrome *c* (Pharmingen), and poly(ADP ribose) polymerase (PARP) (Biomol). Blots were then incubated with horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories), washed, and developed by enhanced chemiluminescence (Pierce). In the case of p53, blots were probed with sheep polyclonal p53, secondary biotinylated rabbit anti-sheep, and streptavidin-horseradish peroxidase (Oncogene Research Products). After development, blots were re-probed for actin (Sigma) to ensure equal protein loading.

2.5. Immunoprecipitation

Following treatment, cells were washed twice in PBS and lysed by passage through a 21 gauge needle in Nonidet P-40 (NP-40)/SDS buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS, pH 7.4) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mg/mL of aprotinin, 5 mg/mL of trypsin inhibitor, 1 mg/mL of leupeptin, 1 mg/mL of chymostatin, 1 mg/mL of pepstatin) (Sigma) on ice. Lysates were centrifuged at 15,000 *g* at 4° for 30 s, and the supernatants were quantified for protein content as described above. Equal quantities of protein were incubated with antibody specific to either mutant [clone PAb240, Calbiochem (Ab3)] or wild-type [clone Pab246, Calbiochem (Ab4)] conformation and then with secondary goat anti-mouse antibody bound to paramagnetic beads (Dynal). Immunoprecipitants were concentrated with a magnetic particle collector and analyzed by Western blotting as described above.

2.6. Assessment of mitochondrial membrane permeability transition

Cells were harvested and incubated with 40 nM 3,3-dihexyloxacarbocyanine ($DiOC_6$, Molecular Probes) for 15 min at room temperature and analyzed by flow cytometry on a Becton Dickinson FACScan. The percentage exhibiting low fluorescence, reflecting loss of inner mitochondrial membrane potential, was determined by comparison with the untreated control using CELLQuest software (Becton Dickinson).

2.7. Cell cycle analysis and synchronization

Cells treated for the times indicated were fixed in cold 70% ethanol, pelleted, resuspended in staining buffer (3.8 mM sodium citrate, 0.5 mg/mL of RNase A, 0.01 mg/mL of PI), and incubated on ice for 4 hr. After resuspension in PBS, fluorescence data were collected on a Becton Dickinson FACScan, and cell cycle distribution was determined with ModFit LT (v2.0, Verity Software). For S-phase synchronization, cells were exposed to 0.15 mg/mL of aphidicolin (Sigma) for 24 hr, washed three times, and resuspended in fresh medium before use. In all experiments, >80% of cells were in S phase immediately after the removal of aphidicolin with no measurable toxicity by dye exclusion.

2.8. HPLC

After treatment, cells were counted and equal numbers (20×10^6) were washed in cold PBS, lysed in 0.6 N trichloroacetic acid, and extracted in trioctylamine:1,1,2-trichlorotrifluoroethane (1:3.5) (Sigma); the aqueous phase was stored at −80°. Prior to column addition, samples were thawed and sequentially extracted with 0.5 M sodium periodate, 4 M methylamine, and 1 M rhamnose to convert the NTPs to their respective bases. Extracts were run on a Waters radial-pak 10 mm SAX cartridge, monitored at 280 nm on a Beckman 160 detector, and analyzed with a Bio-Rad model 700 Chromatography Workstation (v3.63). Samples were run at 3 mL/min for 22 min in 25% ammonium phosphate (0.75 M, pH 3.7):75% ammonium phosphate (5 mM, pH 2.8) increasing to 100% (0.75 M) over 40 min. Peaks were identified by relative retention time compared to ara-C triphosphate and deoxycytidine triphosphate controls (Sigma).

2.9. Incorporation of ara-CTP into DNA

Cells were treated with ara-C and [^{3}H]ara-C to a final concentration of 1 mM, and the DNA was extracted with the DNeasy Tissue Kit per the instructions of the manufacturer (Quiagen). DNA concentration was determined by A_{260}, purity was assessed by A_{260}/A_{280}, and the radioactivity was monitored by liquid scintillography.

2.10. Clonogenic potential

Following drug exposure, cells were washed three times in drug-free medium, counted, and plated in triplicate at 500/well in 1 mL of medium + 20% FBS, 0.3% Bacto agar (Difco). Plates were incubated at 37°, and colonies of >50 cells were scored 14 days after plating.

2.11. Transient transfections

U937 cells were transfected (Multiporator, Eppendorf) with pEGFP-N1 (Clontech), and an 8-fold excess of either

human wild-type or Trp^{248} mutant p53 cDNA (provided by Dr. J.R. Bertino, Memorial Sloan Kettering Cancer Center) was cloned into pcDNA3.1 (Invitrogen). Viability was assessed by flow cytometry following treatment at 24 hr post-transfection by equilibrating with 5 μg/mL of 7-AAD (FACScan, Becton Dickinson).

3. Results

To examine the effects of wild-type p53 expression in $p53^{-/-}$ U937 cells, a temperature-sensitive p53 mutant was used to generate a U937 cell line with selectively active p53. The murine $p53^{val135}$ mutant has been shown to be non-functional [32] and cytoplasmic at physiologic temperatures but is nuclear and transactivates in a yeast-based assay at 32° [33]. This murine construct was stably transfected into U937 cells with a selectable vector control and clonally selected by limiting dilution [30]. Prior characterization of this cell line has been described [34].

To confirm wild-type p53 activity at the permissive temperature, conformation-specific antibodies for p53 [35] were utilized to characterize clonal populations of transfectants and vector controls. Cells were incubated at the physiologic or permissive temperature, lysed, and immunoprecipitated with p53 antibodies specific for either wild-type or mutant protein. Precipitates were subjected to western analysis with a non-specific p53 antibody (Fig. 1). In the ptsp53 cell line, the p53 protein precipitated predominantly with the wild-type conformation-specific antibody at 32° and with the mutant-specific antibody at 37° (Fig. 1A). The vector control lines exhibited no precipitable p53 protein, as anticipated. Western analysis of $p21^{WAF1/CIP1}$ expression in whole cell lysates after incubation at the permissive temperature exhibited a substantial induction of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ in the temperature-sensitive cell line but not in the vector control (Fig. 1B), indicating that the transactivating functions of wild-type p53 are intact at 32°, but not at 37°. Western blotting demonstrated no observable change in the basal levels of Bax or Bcl-2 protein in untreated ptsp53 cells (data not shown), a finding compatible with evidence that the effects of p53 on Bax and Bcl-2 are cell type- and stimulus-specific [20,36,37].

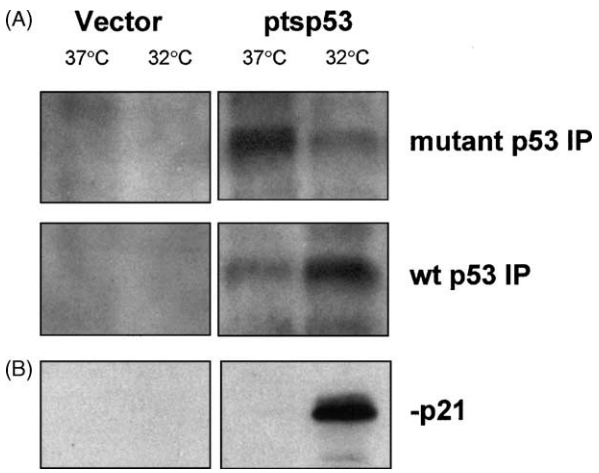

Fig. 1. U937 cells stably transfected with a temperature-sensitive mutant p53. (A) ptsp53 cells and vector controls were incubated at 37° or 32°, and cell lysates were immunoprecipitated with conformation-specific antibodies directed against either mutant or wild-type p53. Immunoprecipitates were subjected to Western blotting using a non-specific p53 antibody. Each lane was loaded with 20 μg of precipitated protein. (B) Whole cell lysates from the same experiment were analyzed by Western blotting as described in Section 2 for induction of $p21^{WAF1/CIP1}$. Each lane was loaded with 20 μg of protein. Two additional studies yielded equivalent results.

To investigate the effects of enforced expression of wild-type p53, and subsequent transactivation of $p21^{WAF1/CIP1}$, on cell cycle progression, ptsp53 and vector control cell lines were incubated for 24 hr at 37° or 32° and then analyzed for DNA content. A representative experiment is shown (Fig. 2A), as well as the cell cycle distribution obtained from five independent experiments (Fig. 2B). Whereas the temperature shift had no effect on the cell cycle distribution in the vector control cell line, ptsp53 cells incubated at 32° exhibited a significant increase in the G_0/G_1 fraction with a corresponding decrease in the S-phase population ($P < 0.05$ relative to values obtained in ptsp53 cells incubated at 37° in each case). There was no discernible change in the G_2/M phase population (data not shown). These observations are consistent with a p53-mediated arrest in G_1, a known downstream consequence of p53-mediated induction of $p21^{WAF1/CIP1}$ [7].

To determine whether the expression of wild-type p53 enhanced the apoptotic response to ara-C, ptsp53 cells and vector controls were exposed to ara-C (1 μM, 18 hr) and incubated at both permissive and non-permissive temperatures (Fig. 3A). Ara-C induced apoptosis in both vector control and ptsp53 cells, as well as in parental U937 cells. While there was no difference in ara-C-mediated cell death in the vector control cell line at either temperature, ptsp53 cells displayed a modest but statistically significant ($P < 0.05$) increase in apoptosis at the permissive temperature when p53 was in the wild-type conformation. Furthermore, this increase in apoptosis was observed over a range of ara-C concentrations (1–100 mM) and treatment intervals (6–24 hr) (data not shown). Significantly, the p53-mediated potentiation of ara-C lethality occurred despite the demonstrated decline in the S-phase fraction (Fig. 2A), the phase of the cell cycle in which ara-C is known to act [28].

Cells were subsequently examined with respect to loss of the electrochemical potential gradient across the inner mitochondrial membrane (mitochondrial permeability transition, $\Delta\psi_m$, Fig. 3B). These studies revealed that the enhancement of the ara-C-induced apoptotic response in the presence of wild-type p53 was accompanied by a significant ($P < 0.05$) increase in mitochondrial dysfunction.

To determine whether the capacity of p53 to potentiate ara-C-induced apoptosis would lead to a reduction in

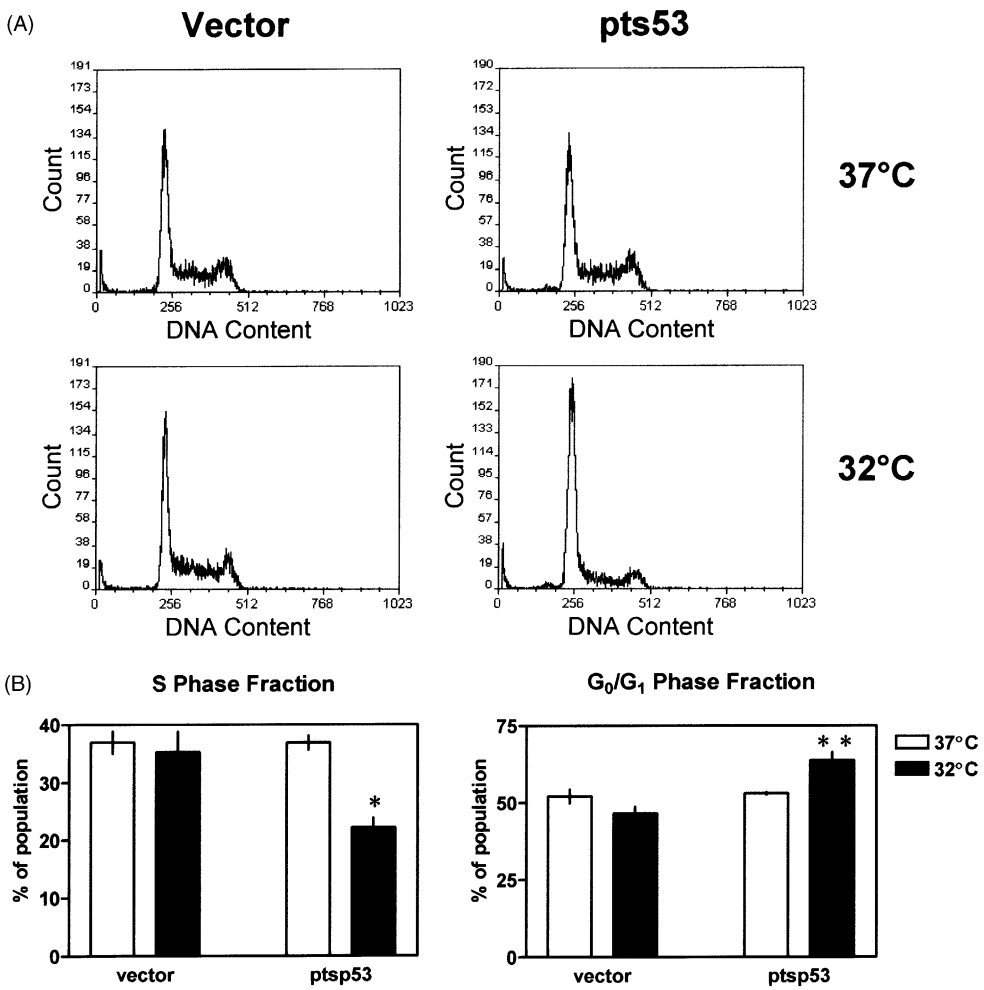

Fig. 2. Wild-type p53-mediated induction of G_0/G_1 arrest. (A) ptsp53 cells and empty-vector controls were incubated at 32° or 37° for 24 hr, stained with PI, and assayed for DNA content by flow cytometry. (B) Cell cycle populations were determined by flow cytometric analysis as described in Section 2; values correspond to the results for five independent experiments. The changes in population in the G_0/G_1 and S-phase compartments are shown as mean values (±SEM). Key: (*) significantly less than values for 37°, $P < 0.05$; and (**) significantly greater than values for 37°, $P < 0.05$.

leukemic cell self-renewal capacity, clonogenic assays were performed. Cell lines were exposed to ara-C at either temperature, washed, and equivalent numbers of cells were plated in soft agar at 37°, after which colonies (50 cells) were scored at day 14 (Fig. 4). While incubating ara-C-treated vector control cells at 32° did not modify clonogenic potential relative to cells treated at 37°, ara-C-treated ptsp53 cells incubated at the permissive temperature exhibited a significant ($P < 0.05$) decrease in colony formation compared to vector controls. Thus, the increased apoptotic response to ara-C in the presence of enforced expression of wild-type p53 was accompanied by a significant decline in the long-term clonogenic growth of U937 cells.

To determine whether p53 modulated ara-C-induced apoptosis by altering ara-C metabolism, ptsp53 and vector control lines were exposed to ara-C and assayed for formation of the lethal metabolite ara-CTP, which correlates with toxicity [27]. Specifically, cells were exposed to ara-C (1 μM, 18 hr) at either temperature, after which ara-CTP levels were monitored by HPLC. Results were then plotted as the ratio of ara-CTP formed at 32° to that formed at 37° (Fig. 5A). When ptsp53 cells were exposed to ara-C under conditions permitting expression of wild-type p53 and enhanced susceptibility to apoptosis, no increase in ara-CTP formation was noted. Identical results were obtained for two additional clonal populations of ptsp53 cells (data not shown).

To examine whether expression of p53 increased the incorporation of ara-CTP into DNA, which has also been shown to correlate with ara-C lethality [38], cells were exposed to [^{3}H]ara-C (1 μM total ara-C, 18 hr), and the DNA was extracted. The concentration of ara-C incorporated into DNA was then calculated at both permissive and non-permissive temperatures (Fig. 5B). Neither vector control nor ptsp53 cells exhibited a significant increase in [^{3}H]ara-C incorporation when incubated at 32°. Taken together with the previous results, these data indicate that enforced expression of the wild-type conformation of p53, which resulted in a significant increase in ara-C-mediated apoptosis in U937 cells, did not act by potentiating ara-C

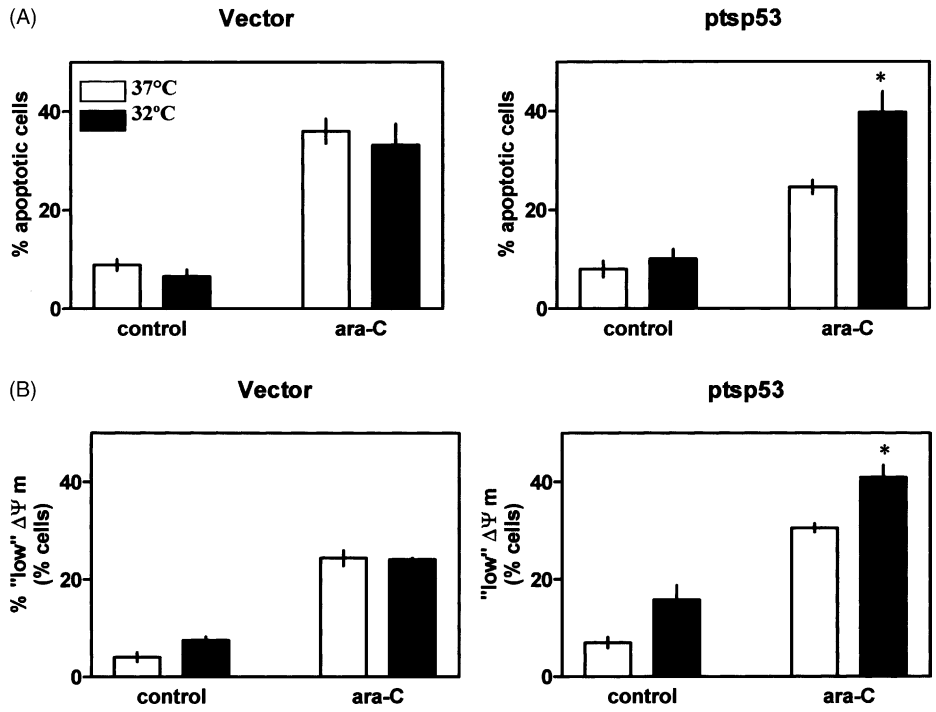

Fig. 3. Increase in apoptosis and mitochondrial dysfunction in response to ara-C in wild-type p53-expressing leukemia cells. (A) ptsp53 cells and vector controls were exposed to ara-C (1 μM, 18 hr) at physiologic or permissive temperature, and the extent of apoptosis was determined as described in Section 2. (B) Cells from the same experiments were equilibrated with $DiOC_6$ and analyzed for reductions in $\Delta\psi_m$ by flow cytometry. In each case, values, corresponding to the percentage of cells exhibiting low $DiOC_6$ uptake, represent the means ± SEM for three separate experiments. Key: (*) significantly greater than values for 37°, $P < 0.05$.

metabolism. Instead, they suggest that the increase in cell death in cells expressing wild-type p53 represents a diminished cellular threshold for ara-C-mediated apoptosis.

Because enforced expression of p53 induced an increase in the apoptotic response to ara-C, while at the same time diminishing the susceptible S-phase population [28], an attempt was made to examine the lethal effects of ara-C on a cell population specifically enriched for S-phase cells. To this end, ptsp53 and vector control cells were exposed to aphidicolin (0.15 mg/mL, 24 hr) to arrest cells at the G_1/S boundary, and the aphidicolin was subsequently washed out to release the cells into the S phase. The cells were then treated with ara-C (1 μM, 18 hr) and incubated at permissive and non-permissive temperatures. For each experiment, synchronization of >80% of cells in G_1 and subsequent release into the S phase were confirmed by flow cytometric cell cycle analysis. Fig. 6 demonstrates that ptsp53 cells enriched for S phase were substantially more sensitive to ara-C-mediated apoptosis when incubated at the permissive temperature. Moreover, the extent of enhancement was significantly greater than that observed in asynchronous populations, consistent with the hypothesis that p53-mediated G_1 arrest in the latter cells opposes the apoptotic response to ara-C. Equivalent results were obtained when apoptosis in S-phase synchronized cells was monitored by annexin V/PI analysis (i.e. ara-C-induced apoptosis was significantly greater in ptsp53 cells at the permissive than the non-permissive temperature, $P < 0.01$; data not shown).

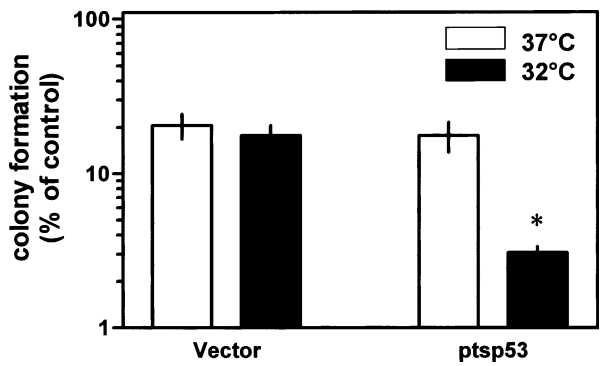

Fig. 4. Loss of long-term clonogenic potential in U937 cells exposed to ara-C in cells expressing wild-type p53. ptsp53 cells were exposed to ara-C (1 μM, 18 hr) at 32° or 37°, washed free of drug, and 500 cells/well were plated in soft agar at the latter temperature for 14 days. The number of colonies (50 cells) were scored and expressed relative to colony formation in untreated controls. Values represent the means (±SEM) for three independent experiments; triplicate samples were plated and counted for each experiment. Key: (*) significantly less than values obtained at 37°, $P < 0.05$.

To investigate whether p53 enhanced the ara-C-mediated mitochondrial injury and caspase activation of S-phase cells, cytosolic S-100 fractions obtained from treated cells were subjected to western analysis to monitor expression of cytochrome *c* and the active 17 kDa fragment of caspase-3 (Fig. 7A). Ara-C-induced apoptosis was

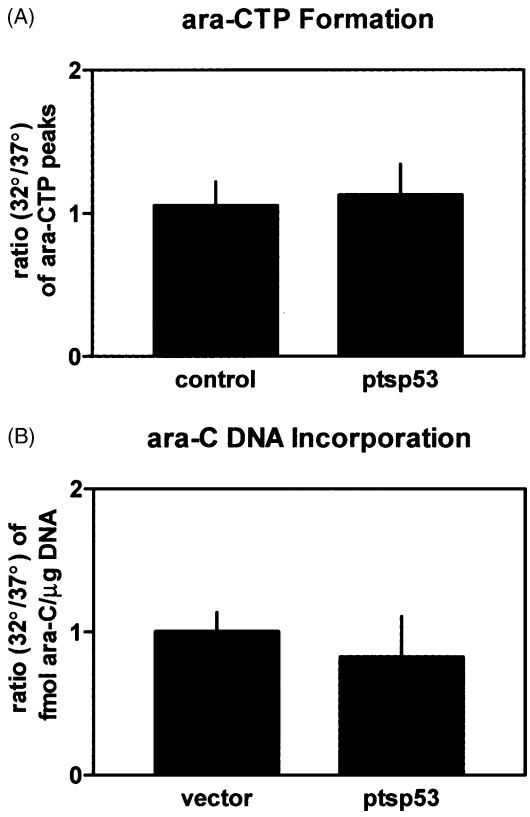

Fig. 5. Ara-C pharmacodynamics in cells expressing wild-type or mutant p53. (A) ptsp53 and vector control cells were treated with ara-C (1 μM, 18 hr) at 32° or 37°, and the triphosphates were extracted from equivalent numbers of cells in three separate experiments. Ara-CTP formation was quantified by HPLC by comparing peak areas to those of known standards; values (means ± SD) represent the ratios of ara-CTP formed at 32° to that formed at 37° for each condition. (B) ptsp53 cells were treated with [^{3}H]ara-C, and genomic DNA was extracted. Total DNA was quantified by monitoring absorbance at A_{260}, and radioactivity was quantified by liquid scintillography. Values represent the ratios of ara-C incorporated relative to DNA extracted at the permissive and non-permissive temperatures, and correspond to the mean values for three experiments ± SEM.

accompanied by the appearance of cytochrome *c* and active caspase-3 in the cytoplasmic fraction; moreover, in the presence of wild-type p53, there was an increase in both cytochrome *c* release and caspase activation relative to that observed in vector controls. Thus, wild-type p53 specifically enhanced ara-C-mediated cytochrome *c* release and procaspase-3 cleavage in S-phase cells.

Because p53 has been reported to induce apoptosis by inducing expression of Bax [18], or by decreasing cellular Bcl-2 protein [19], the possibility that p53 enhanced the cellular apoptotic response to ara-C by modulating levels of these apoptotic regulators was examined. ptsp53 and vector control cell lines were synchronized in S phase as described above and treated with ara-C (1 μM, 18 hr). Whole cell lysates were subjected to western analysis for Bcl-2 and Bax (Fig. 7B) and revealed no discernible alteration in either protein in the presence of wild-type p53.

To rule out the possibility that the temperature-sensitive murine p53 might not fully recapitulate the activities of human wild-type p53, the previous findings were verified using a human wild-type p53 model. Parental U937 cells were transiently transfected with either a wild-type or a non-functional mutant (Trp^{248}) [39] human p53 cDNA. Transfectants were exposed to ara-C (1 μM, 18 hr) and stained with 7-AAD, which is actively excluded from living cells with intact plasma membranes [31]. Transfected cells were assayed for viability by flow cytometry; the results of a representative experiment, in which 45% of GFP$^+$ cells expressing wild-type p53 were apoptotic versus 25% of cells expressing mutant p53, are shown in Fig. 8A. In addition, the mean values for three separate experiments are shown in Fig. 8B. Cells transfected with wild-type p53 cDNA were significantly ($P < 0.05$) more sensitive to the lethal effects of ara-C than those transfected with a non-functional mutant. Untreated wild-type p53 transfectants exhibited viability equivalent to those transfected with the

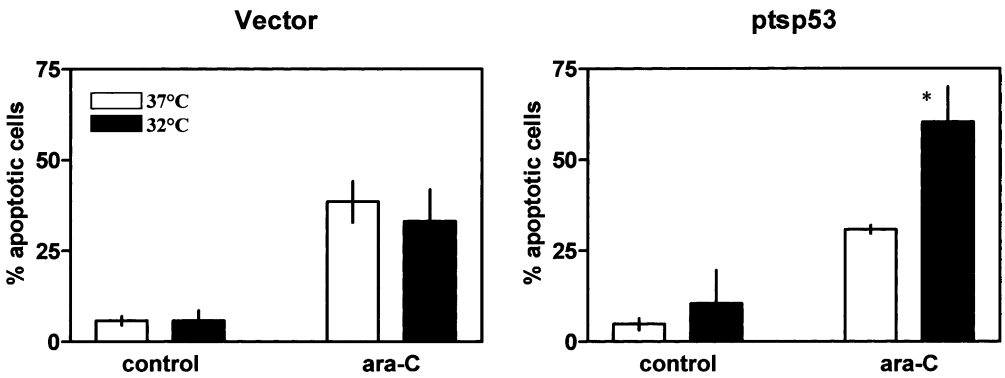

Fig. 6. Specific enhancement of the susceptibility of S-phase cells to ara-C-induced apoptosis by wild-type p53. ptsp53 and vector control cell lines were exposed to aphidicolin (0.15 mg/mL, 24 hr, 37°) to synchronize the populations in the G_1 phase. Cells were washed three times to release the cell cycle block, and aliquots of the cell suspension were treated with ara-C (1 μM) after which they were incubated for 18 hr at 32° or 37°. Flow cytometric analysis confirmed that for each sample >80% of cells were in the S phase at the time ara-C was added. At the end of the incubation period, the extent of apoptosis was determined as described in Section 2. Values represent the means ± SEM for three separate experiments. Key: (*) significantly greater than values obtained at 37°, $P < 0.05$.

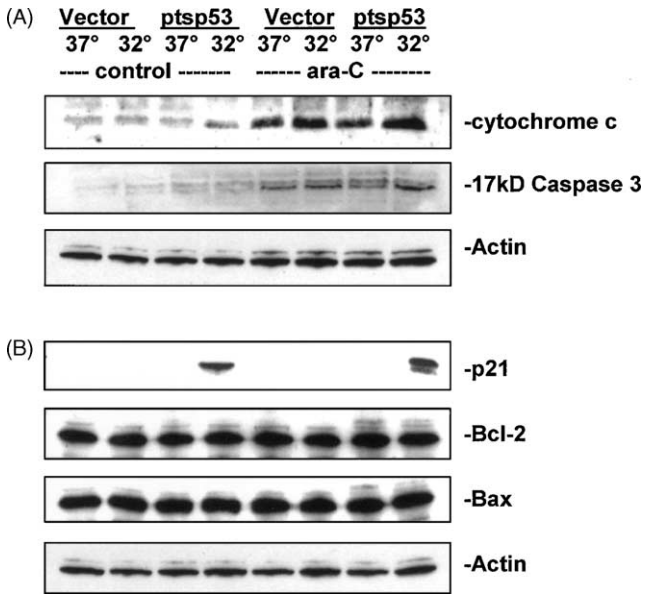

Fig. 7. Effects of wild-type p53 expression on ara-C-induced cytochrome *c* release and Bax/Bcl-2 expression. (A) ptsp53 and vector control cells were synchronized with aphidicolin, released into the S phase, and treated with ara-C (1 μM, 18 hr), after which cytosolic fractions were analyzed for cytochrome *c* release and expression of the 17 kDa active fragment of caspase-3 by Western blotting. Each lane was loaded with 20 μg of protein. (B) Whole cell lysates from the same experiment were analyzed for expression of $p21^{WAF1/CIP1}$, Bcl-2, and Bax. Actin was used as a control to ensure equivalent loading and transfer. Two additional experiments yielded similar results.

mutant. Taken together with the previous results, these data provide further evidence that enforced expression of wild-type p53 protein significantly increases the apoptotic response of myelomonocytic leukemia cells to ara-C-exposure.

4. Discussion

There are several mechanisms by which the presence of wild-type p53 could modulate the cellular apoptotic response to DNA damage. For example, induction of G_1 arrest mediated by the downstream p53 target $p21^{CIP1/WAF1}$ has been implicated in preventing apoptosis in response to DNA damage [10]. However, p53 is also capable of facilitating passage through this checkpoint, preventing the protective arrest and repair response [14]. Additionally, p53 may directly induce apoptosis by altering the ratio of pro- and anti-apoptotic Bcl-2 family proteins [18,19]. Moreover, p53 may influence the cellular apoptotic threshold, possibly by altering the expression of one or more p53-inducible genes (*PIGs*) [40]. Finally, p53 could, by virtue of its 3′,5′-exonuclease activity, remove mismatched nucleotides from DNA [41]. In this context, transfection of p53-null ML-1 leukemic cells with wild-type p53 has been shown to increase their sensitivity to the nucleoside analog gemcitabine, although excision of analog residues was limited [42]. While the precise mechanism by which wild-type p53 expression might sensitize leukemic cells to ara-C lethality remains to be determined, the present results support the notion that p53 acts, at least in part, by lowering the apoptotic threshold, rather than, or in addition to, disrupting DNA damage checkpoints or by altering levels of pro- and anti-apoptotic proteins.

The observation that wild-type p53 expression potentiated ara-C lethality in myeloid leukemia cells is consistent with results of some previous studies in the literature, but at variance with others. Because of the potentially disparate outcomes of the p53-mediated DNA damage response on drug-induced lethality, the impact of p53 expression on the responses of neoplastic cells to a particular chemotherapeutic agent can be difficult to predict. For example, apoptosis following radiation-induced DNA damage has been shown to be increased in the presence of p53 [5]. In contrast, doxorubicin and camptothecin-induced cell death was shown to be diminished in wild-type p53 erythroblastic leukemia cells [2], but that induced by vincristine was increased in the same cells. Similarly, doxorubicin-mediated cell death was enhanced in colon carcinoma cells lacking p53, while 5-fluorouracil (5-FU) sensitivity was diminished markedly [6]. Whether the finding that p53 promotes ara-C-induced apoptosis in U937 leukemia cells reflects a stimulus- or a cell type-specific response is unclear, although the possibility exists that both factors influence the ultimate impact of p53 expression on drug-related lethality.

In view of the observation that enforced expression of p53 potentiated ara-C-mediated mitochondrial damage, it is tempting to speculate that p53 actions stem from alterations in the relative levels of pro- and anti-apoptotic proteins, particularly in view of evidence that such proteins play a critical role in regulating mitochondrial integrity [43]. In this regard, activation of p53 has been shown to induce an increase in Bax and a decrease in Bcl-2 protein levels by binding to and modulating their promoters [18,44]. While $p53^+$ neoplastic cells often exhibit higher levels of Bax and lower levels of Bcl-2 than their p53-null counterparts, correlations between reductions in Bcl-2 protein levels and p53-dependent drug sensitivity have not been documented [45]. In contrast, defects in the *bax* gene correlate well with drug resistance in non-neoplastic fibroblasts [20], and interference with Bax expression has been shown to diminish the p53-dependent apoptotic response to several agents in a variety of neoplastic cell lines [21]. Interestingly, Bax levels also correlate with cell death in response to DNA damaging agents in B-CLL cell lines in which p53 status does not predict for drug sensitivity [22], raising the possibility that the two are independent factors. However, in other studies, no correlation between Bax status and drug response in $p53^+$ cancer cells could be found [23,24]. Moreover, analogous to our results, restoration of p53 function has been shown to sensitize MCF-7 breast cancer cells to apoptosis in the

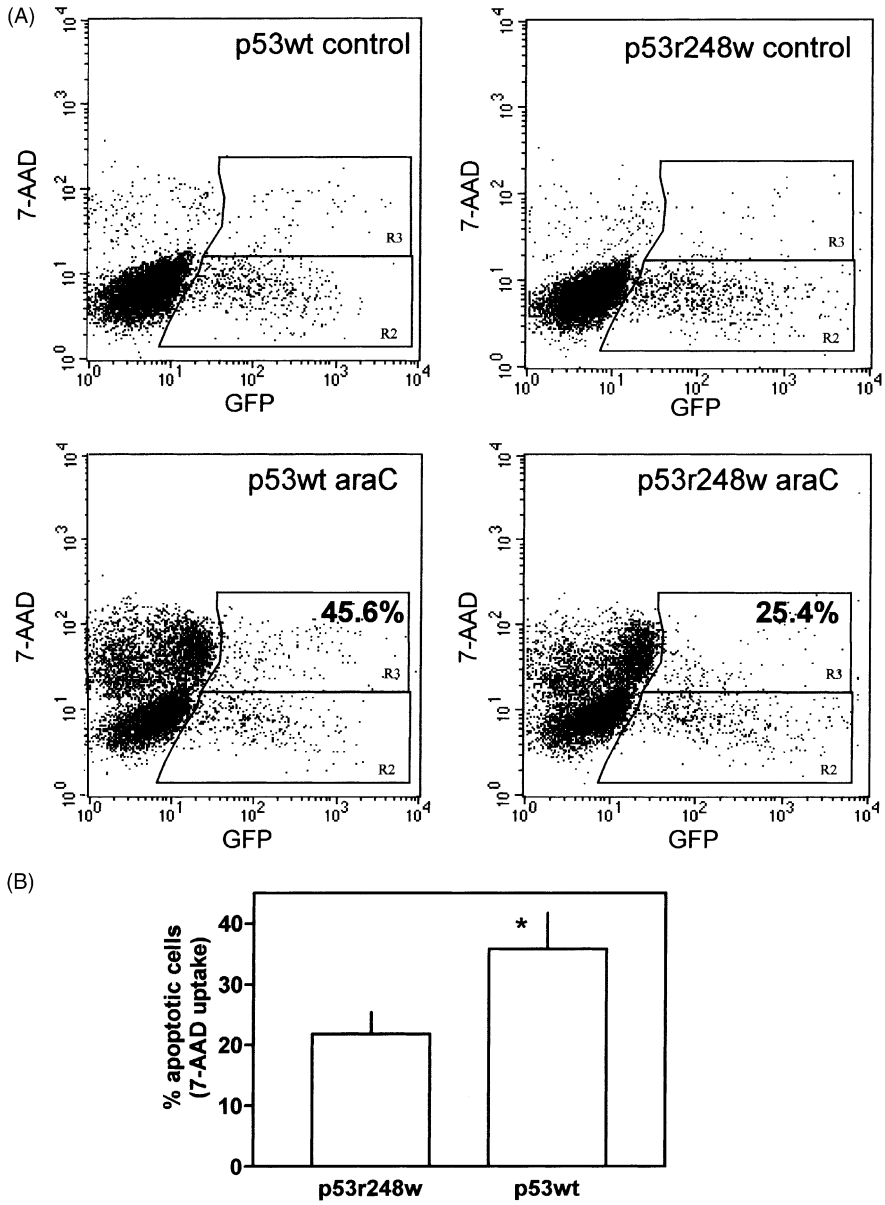

Fig. 8. Effect of transient transfection of wild-type p53 on ara-C-mediated cytotoxicity in U937 cells. Parental U937 cells were transiently transfected with a plasmid encoding a green fluorescent protein and an excess of a plasmid encoding either wild-type human (p53wt) or mutant (p53r248w) p53 cDNA. Following treatment with ara-C (1 μM, 18 hr), cells were stained with 7-AAD and analyzed by flow cytometry. A representative histogram is shown in (A); GFP^+ cells appear in windows R2 and R3. (B) The percentage of GFP^+ cells displaying uptake of 7-AAD, reflecting apoptosis, following ara-C treatment. Values represent the means (±SEM) for three independent experiments. Key: (*) significantly greater than values for mutant p53, $P < 0.05$.

absence of changes in Bcl-2 or Bax protein levels [46]. In any case, the failure to detect changes in Bax and Bcl-2 levels in $p53^+$ U937 cells argues strongly against the possibility that such alterations contributed to the increase in ara-C-induced lethality observed in the present studies.

Effects of p53 on cell cycle progression following genotoxic stress are complex, and may contribute to conflicting findings regarding the influence of p53 on drug sensitivity. For example, the classic, tumor suppressive, p53-dependent response to DNA damage is mediated through induction of the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$. By inhibiting the cyclin D/cdk4 complex, p53 indirectly mediates the dephosphorylation of the retinoblastoma protein, resulting in binding and inactivation of the E2F transcription factor [47]. It is thought that G_1 arrest in response to genomic injury allows the cell to exit the replicating population and to initiate the repair process [48]. While p53 is also capable of triggering cell cycle arrest in the G_2 compartment by induction of $p21^{CIP1/WAF1}$ as well as *GADD45* [49], it also acts as a mediator of G_1 arrest [50]. However, in contrast to its G_1 checkpoint role, p53 may also facilitate the progression of cells into the S phase by enhancing the caspase-dependent cleavage of the retinoblastoma protein (Rb) [13], leading to activation of the E2F transcription factor and cell cycle progression. Accelerated entry into the S phase due to disruption of the

Rb/E2F interaction is a potent stimulus for apoptosis [51]. Thus, pRb cleavage, which has also been observed in cells treated with the topoisomerase II inhibitor etoposide [13], may serve as an amplification mechanism, particularly in cells subjected to irreparable DNA damage. The possibility also arises that the balance between these events (i.e. induction of $p21^{CIP1/WAF1}$ and G_1 arrest versus degradation of pRb and S-phase entry) may determine the net effect of p53 expression on the response of cells to genotoxic agents such as ara-C.

The present findings demonstrate that enforced expression of wild-type p53 increases the sensitivity of U937 cells to ara-C in the absence of perturbations in ara-C metabolism. Furthermore, in ptsp53 cells treated at the permissive temperature, the enhanced apoptotic response occurred despite several events that might be predicted to diminish lethality, including: (a) $p21^{CIP1/WAF1}$ induction by p53; (b) arrest of cells in G_1; and (c) a reciprocal reduction in the S-phase fraction. The present results bear comparison to those of Bunz *et al*. [6], who reported that dysregulation of the p53/$p21^{CIP1/WAF1}$ axis increased the sensitivity of HCT116 cells to various agents, including those that damage DNA, by uncoupling S phase and mitosis. However, in that study, enhanced apoptosis triggered by genotoxic agents occurred at relatively late intervals (e.g. >40 hr), and only after the cells were able to progress through the cell cycle. The present results also differ in some respects from those described in our earlier report, in which stable transfection of p53 null U937 cells with a $p21^{CIP1/WAF1}$ antisense construct rendered them more sensitive to early ara-C lethality [11]. The results of this study and of the study by Bunz *et al*. are consistent with the notion of a cytoprotective role for $p21^{CIP1/WAF1}$, at least as far as drug-induced lethality is concerned. However, in the present studies, enforced expression of p53 resulted in a significant increase in ara-C-associated apoptosis despite the accompanying increase in $p21^{CIP1/WAF1}$ expression. This suggests that the putative cytoprotective effects of $p21^{CIP1/WAF1}$, which may stem from cytosolic inactivation of caspases [52], are outweighed or rendered inoperative by the pro-apoptotic actions of p53. Alternatively, the possibility exists that under some circumstances $p21^{CIP1/WAF1}$ can exert pro-apoptotic actions, a phenomenon described by our group and others [53,54].

The notion that p53 expression enhances the intrinsic susceptibility of U937 cells to ara-C is supported further by the observation that synchronization of ptsp53 cells in the S phase enhanced the apoptotic response to ara-C, an effect that was significantly greater than that observed in asynchronous populations. Specifically, expression of wild-type p53 potentiated the ability of ara-C to trigger cytosolic release of cytochrome *c* and to induce loss of $\Delta\psi_m$ in S-phase cells despite failing to alter levels of Bcl-2 or Bax proteins. It is important to note that induction of $p21^{CIP1/WAF1}$ was also quite pronounced in S-phase cells, as it was in the non-synchronized population, indicating that this cyclin-dependent kinase inhibitor was unable to protect cells that had already progressed through G_1.

In summary, the present findings indicate that restoration of wild-type p53 in p53-null human leukemia cells significantly increases their susceptibility to mitochondrial damage and apoptosis induced by the antimetabolite ara-C. Furthermore, this phenomenon occurs in the absence of perturbations in Bcl-2/Bax protein levels or ara-C pharmacodynamics, and despite a reduction in the S-phase cell fraction. Instead, the presence of p53 appears to render leukemic cells undergoing DNA replication (i.e. those in S phase) intrinsically more sensitive to ara-C-mediated mitochondrial injury. A corollary of these findings is that loss of p53 might afford leukemia cells protection from the lethal effects of ara-C, and may also contribute to the association between mutant p53 status and poor clinical outcome in leukemia [3]. A key question to be addressed is which of the many p53-regulated target genes is responsible for these events. Accordingly, studies addressing this issue are currently in progress.

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