Cell Cycle Analysis of p53-Induced Cell Death in Murine Erythroleukemia Cells

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A temperature-sensitive mutant of murine p53 (p53^{Val-135}) was transfected by electroporation into murine erythroleukemia cells (DP16-1) lacking endogenous expression of p53. While the transfected cells grew normally in the presence of mutant p53 (37.5°C), wild-type p53 (32.5°C) was associated with a rapid loss of cell viability. Genomic DNA extracted at 32.5°C was seen to be fragmented into a characteristic ladder consistent with cell death due to apoptosis. Following synchronization by density arrest, transfected cells released into G₁ at 32.5°C were found to lose viability more rapidly than did randomly growing cultures. Following release into G₁, cells became irreversibly committed to cell death after 4 h at 32.5°C. Commitment to cell death correlated with the first appearance of fragmented DNA. Synchronized cells allowed to pass out of G₁ prior to being placed at 32.5°C continued to cycle until subsequently arrested in G₁; loss of viability occurred following G₁ arrest. In contrast to cells in G₁, cells cultured at 32.5°C for prolonged periods during S phase and G₂/M, and then returned to 37.5°C, did not become committed to cell death. G₁ arrest at 37.5°C, utilizing either mimosine or isoleucine deprivation, does not lead to rapid cell death. Upon transfer to 32.5°C, these G₁ synchronized cell populations quickly lost viability. Cells that were kept density arrested at 32.5°C (G₀) lost viability at a much slower rate than did cells released into G₁. Taken together, these results indicate that wild-type p53 induces cell death in murine erythroleukemia cells and that this effect occurs predominantly in the G₁ phase of actively cycling cells.

Not surprisingly, perhaps, it is becoming increasingly apparent that carcinogenesis is the result of the deregulation of the intricate control mechanisms which have evolved in multicellular organisms to control normal cell growth and differentiation. The protein products of oncogenes, first identified as the acute transforming genes transduced by retroviruses, are a group of dominantly acting genes (including growth factors and their receptors, as well as second messengers and mitogenic nuclear proteins activated by growth factors) involved in cell division. The molecular cloning and characterization of the tumor suppressor genes encoding p53 and the retinoblastoma protein show that both of the proteins suppress cell growth (17, 33). It has recently become apparent that normal hematopoiesis is dependent not only on pathways which positively or negatively regulate cell growth but also on factors which regulate apoptosis, or programmed cell death. Growth factors are necessary for both lymphoid and myeloid progenitor cell survival, and certain cellular and viral transforming genes function to prevent apoptosis (52, 54).

Friend virus-induced murine erythroleukemia cell (MEL cell) lines are a useful model system for the study of leukemia and hematopoietic differentiation (32, 50). Provirus integration adjacent to either the cellular *Spi-1* gene or the *Fli-1* locus has been associated with erythroleukemia induction (2, 39). The envelope protein of the Friend spleen focus-forming virus, gp55, can bind to the erythropoietin receptor of infected cells and stimulate growth (28, 46). Furthermore, MEL cell lines can be induced to terminally differentiate by a number of chemical agents, including

dimethyl sulfoxide (DMSO) (19). We and others have previously shown that constitutive expression of either *c-myc* or *c-myb* prevents MEL cell differentiation (6, 7, 12, 24, 42), and expression of *c-myb* late in the process of differentiation is sufficient to block terminal differentiation (8, 34). In many MEL cell lines, there is a lack of expression of the p53 (reviewed in reference 27) tumor suppressor gene or expression of a mutant p53 protein (29, 41). Furthermore, wild-type p53 could not be stably expressed by a p53-negative MEL cell line (22).

Myeloid leukemic cell lines frequently fail to express p53, in contrast to lymphoid leukemic cell lines, which characteristically overexpress p53 (9, 26, 43). However, p53 is often detectable in primary human myeloid leukemic blasts, though the level of expression is low (23, 47). p53 expression is also seen in normal human blast cells and increases with maturation (45). The myeloid leukemia cell line ML 1, which has wild-type p53 genes, lacks expression during logarithmic growth but shows increased expression with induced differentiation (23). MEL cell lines expressing mutant p53 typically show a decrease in expression with DMSO-induced differentiation (44). Taken together, these findings suggest a role for p53 in the control of proliferation of early myeloid precursors and possibly in myeloid differentiation. Whether p53 induces cell death *in vivo* is not yet known, although expression of wild-type p53 in the myeloid leukemia cell line M1 has recently been associated with apoptosis (54).

To begin to understand the role of p53 in mouse erythroleukemia, we have introduced a temperature-sensitive mutant of p53 (37) into DP16-1, a MEL cell line which has no endogenous expression of p53 protein (40). This mutant p53 contains a single-base-pair mutation (alanine→valine at position 135) and can cooperate with Ras in transforming

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primary rat embryo fibroblasts at 37.5°C. At 32.5°C, this mutant behaves as does wild-type p53 and inhibits oncogene-mediated transformation. At 37.5°C, p53^{Val-135} is predominantly cytoplasmic and is associated with the cellular heat shock protein hsc70; at 32.5°C, p53^{Val-135} becomes located in the nucleus (18). In this report, we demonstrate that DP16-1 clones expressing p53^{Val-135} are able to grow at 37.5°C but lose viability when placed at 32.5°C. Analysis of genomic DNA isolated from dying cells at 32.5°C indicates that the cells are undergoing apoptosis. Cell cycle analysis indicates that expression of p53^{Val-135} at 32.5°C induces cell death of DP16-1 cells specifically in the G₁ phase of the cell cycle.

MATERIALS AND METHODS

Plasmid construction. p53^{ts}neo was constructed from plasmid pLTRp53cG (15). A *Bam*HI site found in p53 intron sequences of pLTRp53cG was destroyed by partial digestion with *Bam*HI and blunt ending with the large fragment of *Escherichia coli* DNA polymerase I in a buffer containing all four deoxynucleotides. The plasmid was then recircularized with T4 DNA ligase. A 2.7-kb *Nde*I-*Bam*HI fragment from pSV-2neo (48), containing the neomycin resistance gene, was inserted into the remaining *Bam*HI site of pLTRp53cG, using *Bam*HI linkers. The 5' *Bam*HI site was subsequently destroyed; p53^{ts}neo contains the coding sequence of the neomycin resistance gene inserted in the same orientation as the p53 coding sequences.

Cell culture and DNA transfection. DP16-1 MEL cells were grown in Dulbecco modified minimal essential medium (DMEM) supplemented with 10% calf serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 µg of amphotericin B per ml. DNA transfections were performed by electroporation as previously described (42). Transfection mixes consisted of 10⁷ cells in 400 µl of transfection buffer (272 mM sucrose, 7 mM phosphate [pH 7.4], 1 mM MgCl₂) with either 10 µg of p53^{ts}neo or 10 µg of pSV-2neo, linearized with *Bam*HI. Transfected cells were selected in medium containing 1 mg of geneticin (G418; GIBCO) per ml. Single-cell clones were isolated by limiting cell dilution in 96-well microtiter plates. To induce differentiation, logarithmically growing cells were grown in medium containing 1.6% DMSO (Sigma) for a total of 5 days. Media were changed every other day. Viable cells were isolated by centrifugation through Ficoll-Paque (Pharmacia). Benzidine staining was performed as previously described (6).

Nucleic acid and protein analysis. Total RNA was isolated from clones by the guanidinium thiocyanate-phenol extraction method (5). Northern RNA blotting and hybridization were performed as previously described (6). To detect exogenous p53 expression, the 3.5-kb *Sma*I fragment of p53^{ts}neo, labeled with [α -³²P]dCTP (Amersham) by random priming using T7 DNA polymerase (Stratagene), was used as a probe.

Genomic DNA was prepared as previously described (6). Briefly, cells were suspended in a buffer consisting of 20 mM Tris (pH 7.5), 10 mM EDTA, and 300 mM NaCl, lysed by adding sodium dodecyl sulfate (SDS) to 0.5%, digested with proteinase K, and then subjected to organic extraction and ethanol precipitation. DNA samples (3 µg) were analyzed by electrophoresis through a 1.5% agarose gel containing 0.5 µg of ethidium bromide per ml or through a nondenaturing 5% polyacrylamide gel.

Cellular proteins were labeled by incubating cells (10⁷) for 3 h with 0.2 mCi of [³⁵S]methionine in 1 ml of methionine-free DMEM. Cells were lysed in lysis buffer (150 mM NaCl,

1% Triton X-100, 50 mM Tris [pH 8.0], 0.5 mM phenylmethylsulfonyl fluoride) and precleared by overnight incubation at 4°C with 50 µl of normal goat serum; then protein G-Sepharose beads (Pharmacia) were added, and the cells were centrifuged. The supernatant was immunoprecipitated with a mixture of PAb421 (20), a pan-specific monoclonal antibody against p53, and PAb248, a murine-specific monoclonal antibody against p53. Immune complexes were collected with protein A-agarose beads (Bethesda Research Laboratories), washed three times in lysis buffer, and suspended in 25 µl of Laemmli sample buffer. Samples were electrophoresed through 10% polyacrylamide in the presence of SDS, and dried gels were used for autoradiography.

To prepare cells for immunohistochemical staining, approximately 2.5 × 10⁵ cells were suspended in buffer (Ca²⁺- and Mg²⁺-free phosphate-buffered saline [PBS], 1% bovine serum albumin fraction V, 0.5% Tween 20, 5 µM EDTA) and pelleted on a microscope slide, using a cytospin centrifuge. Cells were fixed with methanol for 60 min at 4°C and then washed twice with PBS. Forty microliters of a mixture of PAb421 and PAb248 was added to each slide, and the slides were allowed to stand for 60 min at room temperature. Following a PBS wash, 25 µl of goat anti-mouse fluorescein isothiocyanate conjugate (Tago) was added for 60 min at room temperature. After a final PBS wash, the cells were counterstained with Evans blue (Sigma) and viewed through fluorescence microscopy.

Cell cycle analysis. Cells were synchronized in G₀/G₁ by density arrest (typically at a concentration of 1 × 10⁶ to 2 × 10⁶ cells per ml) for 48 to 72 h. Cells were stimulated to begin cycling by replating into fresh medium at a 1:10 split ratio. Growth arrest with mimosine (Sigma) was accomplished by the addition of 300 µM mimosine to logarithmically growing cells for a period of 24 h. Isoleucine-deficient medium was prepared by using a DMEM Select-Amine kit (GIBCO). Logarithmically growing cells were arrested by transfer to isoleucine-deficient medium for a period of 18 to 24 h. Cell cycle analysis was performed by fixing cells (1 × 10⁶ to 2 × 10⁶ cells in PBS) with methanol, collecting the cells by centrifugation, and resuspending the cells in propidium iodide (Sigma) stain (propidium iodide [50 µg/ml], 0.05% Triton X-100, EDTA [18 µg/ml] and RNase A [100 U/ml] in PBS). Avian erythrocytes (10⁶) were used as an internal standard. After a 30-min incubation at room temperature, DNA content was determined by quantitative flow cytometry (38).

RESULTS

Integration and expression of p53 in DP16-1 cells. Given the lack of expression of wild-type p53 in many MEL cell lines, the effect of p53 expression was studied by using a temperature-sensitive p53 mutant. DP16-1 MEL cells were transfected by electroporation with linearized p53^{ts}neo or pSV-2neo and subsequently selected by growth in medium containing G418 (1 mg/ml). Individual clones were obtained by limiting dilution. Total cellular RNA was purified from clones growing at 37.5°C and hybridized to the 3.5-kb *Sma*I fragment of p53^{ts}neo by Northern blotting. Figure 1A shows expression of p53-related message in the unselected p53^{ts}neo-transfected bulk culture as well as in three individual clones, C2, C4, and C9, isolated from the bulk culture. A transcript of approximately 2.1 kb is seen in the transfected clones. This transcript is not seen in RNA from parental cell line DP16-1.

To confirm the expression of p53 protein in transfected

FIG. 1. RNA and protein analysis of p53^{ts}neo transfectants. (A) Northern blot of 20 μ g of total RNA (7.5 μ g for clone 2) hybridized with the *Sma*I fragment of p53^{ts}neo. The parental line DP16-1 (lane a), clone 2 (lane b), clone 4 (lane c), clone 9 (lane d), and the unselected p53^{ts}neo bulk culture (lane e) are shown. Positions of the 28S and 18S rRNA bands are shown, and the expected 2.1-kb transcript is seen in the transfected clones. (B) Immunoprecipitation of p53 by using monoclonal antibodies PAb421 and PAb248. The indicated cells were incubated for 3 h in medium containing [³⁵S]methionine. Cells were then lysed, and lysates were precleared by incubation with protein G-Sepharose. The cleared lysates were then immunoprecipitated with protein A-agarose beads and anti-p53 antibodies or control antibodies. Shown are clone C9 immunoprecipitated with p53 antibody (lane a), parental line DP16-1 immunoprecipitated with p53 antibody (lane b) and control monoclonal antibody (lane c), and clone C9 immunoprecipitated with control monoclonal antibody (lane d). Molecular sizes are shown. The 53-kDa p53 protein is seen only in the transfected cells. (C) Immunohistochemistry showing the cellular location of p53 at 37.5 and 32.5°C. DP16-1 cells (left) and DP16-1 cells transfected with p53^{ts}neo grown at 37.5°C (middle) and 32.5°C (right) were collected onto a microscope slide by cytocentrifugation, fixed, stained with anti-p53 antibody and fluorescein-labeled anti-mouse immunoglobulin G, and photographed by using a fluorescence microscope. Note that control DP16-1 cells are not stained (left), whereas p53 was detected in the cytoplasm (white arrowheads) in p53^{ts}neo-transfected cells grown at 37.5°C (middle) and had translocated to the nucleus (white arrowheads) in cells grown at 32.5°C (right). For panels A and B, the Adobe Photoshop and Adobe Illustrator programs were used.

FIG. 2. Loss of viability induced by wild-type p53. At 0 h, logarithmically growing cells were transferred from 37.5 to 32.5°C. Symbols: □, Parental line DP16-1; ◆, clone C4; □, clone C9. Viability was determined by trypan blue staining. Note the rapid loss of viability of the p53-transfected cells grown at 32.5°C.

clones, cellular protein was labeled with [³⁵S]methionine and immunoprecipitated with PAb421 and PAb248, monoclonal antibodies which both detect murine p53. The p53 protein is clearly seen in the transfected clone C9 but not in parental line DP16-1 or in immunoprecipitations with a control monoclonal antibody (Fig. 1B). Finally, immunohistochemistry shows that the p53 protein is located in the cytoplasm of the transfected cells, but not the parental cells, grown at 37.5°C and is translocated to the nucleus when cells are incubated at 32.5°C (Fig. 1C).

Wild-type p53 induces cell death. Expression of wild-type p53 has been associated with reversible inhibition of cell growth in a number of solid tumor cell lines (1, 11, 36). Use of a temperature-sensitive p53 mutant allowed study of the effect of wild-type p53 expression in the leukemic cell line DP16-1. While clones expressing p53^{ts}neo grew rapidly at 37.5°C, transfer to 32.5°C led to growth arrest and subsequent loss of viability. This occurred over a 48-h period (Fig. 2). All six p53^{ts}neo clones examined, including clones C2, C4, and C9 as well as the uncloned G418-resistant bulk culture, showed similar kinetics following the transfer to 32.5°C (Fig. 2 and data not shown). Six pSV-2neo-transfected clones, as well as the parental cell line DP16-1, showed no loss of viability when grown at 32.5°C (data not shown). Immunohistochemical staining with PAb421 documented that transfer to 32.5°C was accompanied by movement of p53 protein from a cytoplasmic location into the nucleus (Fig. 1C). To determine whether the loss of viability seen at 32.5°C was through the process of apoptosis, genomic DNA was isolated from clones incubated for 20 h at 32.5°C. DNA from apoptotic cells gives a characteristic fragmentation pattern secondary to internucleosomal cleavage (53). This is clearly seen in DNA from clone C9 (Fig. 3), while only high-molecular-weight DNA is extracted from similarly treated DP16-1 cells. This finding is consistent with an effect of wild-type p53 to induce apoptosis in DP16-1 cells.

Cell cycle analysis of p53-induced cell death. The level of p53 protein varies in a cell cycle-dependent manner in MEL cells (25). Low levels are seen early in G₁, with a subsequent increase at the G₁/S transition and a moderate increase occurring during the remainder of S phase. A constant level is seen during G₂/M, with a return to low levels following

FIG. 3. DNA fragmentation in p53-induced apoptosis. Genomic DNAs (3 µg) isolated from clone 9 cells (lane a) and DP16-1 cells (lane b) were extracted from cells after incubation at 32.5°C for 20 h and then analyzed by electrophoresis through a 1.5% agarose gel. *Hind*III-digested lambda DNA and *Hae*III-digested φX174 DNA molecular weight markers are shown (lane c). Note the ladder of degraded C9 cell DNA characteristic of cells undergoing apoptosis.

division. We therefore studied the relationship of p53-induced cell death to the cell cycle in one of our transfected clones. Clone C9 was synchronized in G₀ by allowing cells to density arrest for 48 to 72 h at 37.5°C. Following release from density arrest by splitting cells 1:10 in fresh medium, the cells were placed at 37.5 and 32.5°C. At 37.5°C, the cells progressed into S and G₂/M after a delay of approximately 20 h (Fig. 4A). Cells released from density arrest at 32.5°C failed to begin cycling secondary to a very rapid decrease in viability (Fig. 4B). By 12 h after release, the majority of cells remained in G₀/G₁, although the sharpness of the G₀/G₁ peak was lost as cell viability decreased. Comparison of the kinetics of cell death with that of a randomly growing population of clone C9 cells placed at 32.5°C indicates that cells released into G₁ following density arrest undergo cell death at an accelerated rate (Fig. 5). Almost complete cell death occurs by 15 h at 32.5°C, as opposed to 36 to 48 h for a randomly growing population. This result suggested that cells released into G₁ may be preferentially susceptible to wild-type p53-induced cell death. Similar results were obtained with three other clones tested.

To explore this possibility, medium was added to density-arrested cells, and the cells were incubated for various times at 37.5°C to allow progression out of G₀. They were then placed at 32.5°C, and viability was monitored. Cells released from density arrest at 37.5°C for at least 12 to 15 h prior to being placed at 32.5°C began to show a survival advantage over cells released directly at 32.5°C. This advantage was more pronounced for cells allowed to progress for 24 to 48 h at 37.5°C (Fig. 5). Analysis of DNA content by quantitative flow cytometry of C9 cells released from density arrest at 37.5°C for 36 h prior to placement at 32.5°C is shown in Fig. 6. At the time the cells were downshifted to 32.5°C (0 h), the majority of cells had progressed into S phase. However, after 6 h at 32.5°C, the percentage of S phase cells had fallen significantly. This is attributable to a progression of some cells into G₂/M, while at the same time the percentage of cells found in G₁ also rises, indicating a failure of cells to progress from G₁ to S. By 15 h, a majority of cells have

FIG. 4. Cell cycle analysis by quantitative flow cytometry of clone C9. (A) Following density arrest for 48 to 72 h, cells were split 1:10 and kept at 37.5°C. Cell cycle analysis was performed at various time points after release from density arrest and showed the following distributions: 0 h (top left), 85% G₁, 11% S, and 4% G₂/M; 6 h (top right), 75% G₁, 23% S, and 2% G₂/M; 20 h (bottom left), 55% G₁, 44% S, and 1% G₂/M; and 26 h (bottom right), 50% G₁, 44% S, and 6% G₂/M. (B) Following density arrest for 48 to 72 h, cells were split 1:10 and transferred to 32.5°C, and cell cycle analysis was done 6 (top left), 9 (top right), and 12 (bottom) h after release from density arrest. The DNA contents of avian erythrocytes (vertical line) and MEL cells in the G₁ (1), S (2), and G₂/M (3) phases of the cell cycle are shown. Fluorescence-activated cell sorting analysis of DNA content shows that there is a progressive degradation of the cells' DNA at 32.5°C.

accumulated in G₁. It is at this point that cell viability begins to decrease (Fig. 5). Cycling cells therefore progress through S phase and G₂/M and accumulate in G₁ prior to cell death occurring. To rule out the possibility that G₁ arrest alone induces cell death, clones C9, C2, and C1 were growth arrested with either mimosine (300 μ M) (51) or isoleucine deprivation (21) at 37.5°C, both of which result in late G₁ arrest. These G₁-arrested cells do not undergo rapid cell death, although viability decreases to approximately 70% after 3 to 4 days of arrest (data not shown). Viability falls to less than 5% within 36 h in cells arrested in G₁ with either

FIG. 5. Loss of viability induced by wild-type p53. Clone C9 cells were density arrested, split 1:10, and placed at 37.5°C for various time periods before transfer to 32.5°C. Time at 37.5°C was 0 ($\square$), 6 ($\blacklozenge$), 9 ($\diamond$), 15 ($\square$), or 28 h ($\blacksquare$). Viability was determined by trypan blue staining. By 28 h, most cells have progressed into S phase, resulting in increased viability upon exposure to wild-type p53.

mimosine treatment or isoleucine deprivation and subsequent culture at 32.5°C, indicating that cell death is specifically induced by wild-type p53 expression.

G₀ and differentiated cells are not subject to p53-induced cell death. To determine whether release into G₁ was a necessary event for p53 to induce apoptosis, noncycling G₀ cells were placed at 32.5°C while still density arrested. Simultaneously, density-arrested cells were kept at 37.5°C, and cells released into G₁ (split 1:10) were placed at both 37.5 and 32.5°C. Viability was then monitored. While cells released into G₁ at 37.5°C grew well and maintained a high viability, cells released at 32.5°C had the expected rapid loss

FIG. 6. Cell cycle analysis by quantitative flow cytometry. Clone C9 cells were released from density arrest and placed at 37.5°C for 36 h before transfer to 32.5°C. Cell cycle analysis of cells grown at 32.5°C for various times showed the following distributions: 0 h (top left), 36% G₁, 61.5% S, and 2.5% G₂/M; 6 h (top right), 47% G₁, 33% S, and 20% G₂/M; 15 h (bottom left), 66.5% G₁, 9% S, and 24.5% G₂/M; and 20 h (bottom right), 81% G₁, 8% S, and 11% G₂/M. The DNA contents of avian erythrocytes (vertical line) and MEL cells in the G₁ (1), S (2), and G₂/M (3) phases of the cell cycle are shown.

TABLE 1. Time course of commitment to p53-induced cell death

Expt	Time at 32.5°C (h)	Viability at 18 h (%)
A ^a	1	83
	2	79
	3	67
	4	30
	6	12
B ^b	4	>90
	6	>90
	8	>90
	10	>90
	18	68

^a Density-arrested clone C9 cells were split 1:10 and placed at 32.5°C for various periods of time before transfer to 37.5°C. Viability was determined 18 h after release from density arrest.

^b Density-arrested clone C9 cells were split 1:10, allowed to cycle for 36 h at 37.5°C, and then placed at 32.5°C for various periods of time prior to transfer to 37.5°C. Viability was determined 18 h after placement at 32.5°C.

of viability. At 37.5°C, density-arrested p53^{ts}neo cells will lose viability slowly over time, and cell death is not accelerated at 32.5°C. The same cells split 1:10 undergo a much more rapid decline (data not shown).

MEL cells can also exit the cell cycle by being stimulated to terminally differentiate. DMSO was used to induce differentiation of p53^{ts}neo clones at 37.5°C. After 5 days exposure to 1.6% DMSO, viable cells were isolated by centrifugation through Ficoll-Paque. The cells obtained were terminally differentiated, as shown by (i) high benzidine positivity (70 to 80%) and (ii) failure to proliferate when placed at 37.5°C in DMSO-free media. These cells were placed at 32.5°C, and viability was monitored. Such cells maintained viability over several days, while control randomly growing cultures underwent the expected cell death (data not shown).

Commitment to cell death is cell cycle dependent. To delineate the time course of commitment to cell death, density-arrested C9 cells were split 1:10 in fresh medium and placed for various periods of time at 32.5°C. They were then returned to incubation at 37.5°C. Viability was checked 18 h after the cells had been released from density arrest. Cells which spent less than 3 h at 32.5°C maintained a high viability (Table 1). Between 3 and 4 h, however, the majority of cells became committed to cell death, as shown by a low viability at 18 h. By 6 h at 32.5°C, approximately 90% of the cells were committed to undergoing cell death. Cells which remained viable at 18 h were able to proliferate if kept at 37.5°C, indicating that they had escaped commitment to cell death.

In hematopoietic cells, p53 mRNA expression peaks in S phase (25). Clone C9 cells which, following release from density arrest, were allowed to cycle at 37.5°C for 36 h into S phase (Fig. 6) do not show an early commitment to cell death when placed at 32.5°C (Table 1). Although some cells have lost viability after 18 h at 32.5°C, if returned to 37.5°C, many of the surviving cells will continue to proliferate. In contrast to cells in G₁, which are committed to cell death after a brief exposure to p53, even a prolonged exposure to wild-type p53 during S phase and G₂/M does not appear to lead to an irreversible commitment to cell death.

DNA fragmentation occurs with commitment to cell death. To begin to understand the molecular events which lead to p53-induced MEL cell death, we analyzed the cells for the appearance of DNA fragmentation. Cells were released from density arrest at 32.5°C, and genomic DNA was isolated at

FIG. 7. Correlation of DNA fragmentation with commitment to cell death. Clone C9 cells were density arrested for 48 h, split 1:10 in fresh medium, and placed at 32.5°C for various periods of time prior to isolation of genomic DNA. DNA samples (3 µg) were then electrophoresed through a 1.5% nondenaturing agarose gel and stained with ethidium bromide. Lanes are labeled with the number of hours for which cells were cultured at 32.5°C prior to DNA isolation. Also shown is *Hind*III-digested lambda phage DNA and *Hae*III-digested ϕ X174 phage DNA used as a molecular weight marker (M). In this experiment, low-molecular-weight DNA fragments are seen after culturing for 6 h at 32.5°C, with a progressive increase with longer incubation times. For this figure, the Adobe Photoshop and Adobe Illustrator programs were used.

various time points. Commitment to cell death correlates with the initial appearance of low-molecular-weight DNA fragments (Fig. 7). This occurs after 4.5 to 6 h at 32.5°C (although cell viability remains greater than 90% at this time) in density-arrested cells released into G₁. Although appearing to be normal when viewed by light microscopy, the majority of these cells will lose viability despite being returned to 37.5°C.

DISCUSSION

p53-induced apoptosis is cell cycle dependent. These results demonstrate that expression of wild-type p53 protein in MEL cells is associated with growth arrest. In addition, cell death is seen to occur rapidly following p53-induced G₁ growth arrest. The cell death induced by wild-type p53 is accompanied by endonucleolytic cleavage of genomic DNA characteristic of apoptosis (Fig. 3) and occurs within 48 h (Fig. 2). As has been found for other tumors, our results show that wild-type p53 can affect hematopoietic tumor cells in blocking passage through the cell cycle specifically in G₁. Additionally, in actively cycling populations of MEL cells,

p53 induces cell death, predominantly in the G_0 - G_1 transition and G_1 phase of the cell cycle. There are several lines of evidence which support this hypothesis. Figure 4B demonstrates that cells released into G_1 from density arrest lose viability rapidly prior to progressing out of G_1 . In contrast, cell populations largely in S phase (Fig. 6) lose viability only after progressing through S phase and accumulating in G_1 . Furthermore, S-phase populations of cells exposed to wild-type p53 for up to 10 h show little or no commitment to cell death (Table 1); conversely, the majority of cells released into G_1 from density arrest show commitment to cell death after only 4 h of exposure to wild-type p53. This finding suggests that these cells may be preferentially sensitive to the effects of wild-type p53, possibly reflecting an alteration in p53 itself (such as phosphorylation level). When noncycling density-arrested or differentiated cells are exposed to wild-type p53, they do not become committed to cell death. Control cells released from density arrest into G_1 at 37.5°C (in the presence of mutant p53) maintain a high viability (>90%), demonstrating that the cell death seen in these experiments is not a result of density arrest.

p53-induced cell death and p53-induced cell cycle arrest are separate functions. The finding of cell cycle specificity in wild-type p53-induced cell death of cycling MEL cells suggests that p53 has two related but separable roles as a cell cycle regulatory protein. The expression of p53 during normal hematopoiesis implies that the ability of wild-type p53 to induce cell death either is an *in vitro* phenomenon or, more likely, is effectively regulated *in vivo*. Importantly, p53-induced cell death is not solely a function of its ability to block cells in G_1 . G_1 arrest by either isoleucine deprivation or mimosine exposure does not lead to rapid cell death in any of three p53^{ts}neo-transfected DP16-1 clones tested. This finding indicates that the cell death occurring in wild-type p53-arrested cells is uniquely induced by p53 and not the result of G_1 arrest alone. Cells arrested with mimosine or isoleucine deprivation undergo accelerated cell death when transferred to 32.5°C, suggesting that wild-type p53 expression affects cell viability independent of growth arrest directly attributable to p53. It is not known whether cells arrested in other phases of the cell cycle would be susceptible to p53-induced cell death; experiments utilizing other inhibitors of the cell cycle were not feasible secondary to the excessive toxicity of the drugs themselves to DP16-1 cells (data not shown).

The response of other cell lines to expression of wild-type p53 adds credence to the hypothesis that the growth arrest and cell death induction properties of the tumor suppressor gene are separable. Indeed, in a glioblastoma cell line and in an osteosarcoma line, expression of wild-type p53 caused a reversible growth arrest secondary to an inability of cells to progress into S phase (11, 36).

Mechanism of p53-induced apoptosis. The time that MEL cells commit to cell death is accompanied by the appearance of the typical apoptotic ladder (Fig. 7). In agreement with others, we found that the fragmented DNA is present in the cytoplasm, not the nucleus of the dying cells. This finding suggests that p53 may play a role in activating a nuclease and that the resultant degradation of cellular DNA is the irreversible event in p53-induced cell death. There are several mechanisms by which the nuclease might be activated. Since apoptosis requires synthesis of new proteins, and p53 has been shown to activate transcription, it is tempting to speculate that one of the genes transactivated by p53 is this nuclease. Of course, activation of the nuclease might be due to other processes, such as a change in the compartmental-

ization of the nuclease allowing it to degrade the nuclear DNA, activation of the nuclease by a cofactor, or a modification such as phosphorylation.

Significance of the cell cycle specificity of p53-induced cell death. p53 mRNA and protein expression is characteristically regulated during the cell cycle, as is phosphorylation of p53 by the cell cycle control protein p34^{cdc2} (49). It is likely that p53 is phosphorylated throughout the cell cycle with the exception of G_1 (3). p53 is also phosphorylated by casein kinase II, whose activity is increased in proliferating cells (35). In analogy to the retinoblastoma protein (4, 10), underphosphorylated p53 may play a role in cell cycle regulation as a G_1 block. In tumor cell lines lacking p53 expression, reintroduction of wild-type p53 activity has been associated with growth arrest (1, 11, 36).

Expression of wild-type p53 in the myeloid leukemia cell line M1 has recently been associated with apoptosis, which is prevented when cells are incubated with growth factors (54). Apoptosis (reviewed in reference 14) can also be seen in both normal and malignant hematopoietic cells deprived of growth factors (30, 52). In normal hematopoiesis, the vast majority of hematopoietic stem cells are quiescent in G_0 . Thus, these quiescent stem cells would not be susceptible to p53-induced apoptosis. On the other hand, progenitor cells are rapidly cycling and appear to be quite sensitive to growth factor deprivation-induced cell death. Thus, it is conceivable that p53 is involved in fine regulation of hematopoiesis as a homeostatic regulator of absolute hematopoietic cell numbers.

Role of p53 in carcinogenesis. The MEL cell lines are derived from mouse hematopoietic cells infected with the Friend murine erythroleukemia virus complex. The initial event in MEL cell transformation is the activation of the erythropoietin receptor via the association of the viral envelope with the receptor (25). The erythropoietin receptor usually transduces signals for cell growth and terminal differentiation (26); why the differentiation signal is absent in MEL cells is unknown. The virus-transformed MEL cells invariably have a deletion or inactivation of the p53 tumor suppressor gene (27). However, p53 had no effect on interleukin-6-induced differentiation of the M1 leukemia cell line (54). Thus, p53 mutations might help to explain the immortalization of MEL cells but probably do not explain the dissociation of proliferation from differentiation. Here we show that commitment to cell death occurs at the time when endonuclease cleavage of DNA is first detectable (Fig. 7), suggesting that this is likely the irreversible step ultimately resulting in loss of viability.

It is notable that the only functions identified for p53 are its role in growth regulation. Surprisingly, when both alleles of the gene were deleted through targeted disruption of p53 in mouse embryonic stem cells, the homozygous p53-expressing mice developed normally but tended to die at a young age secondary to the development of cancers (13). Since p53 expression is often tightly linked to the G_1 /S-phase cell cycle, we speculate that the gene acts as a mechanism to ensure that genes which induce cell growth are correctly regulated. For example, p53 transcription might be transactivated by some factor(s) which also transactivates one or more of the oncogenes and/or cell cycle control genes which are also expressed at the G_1 /S cell cycle transition. Often, oncogene protein expression is regulated posttranscriptionally. Thus, if a mutation arises which deregulates the hypothetical common p53/oncogene transcription transactivator, only the p53 protein might be expressed in G_1 since, unlike the postulated oncogene partner, it need not be regulated

posttranscriptionally by the same factors affecting the oncogene. The cell would then undergo a "fail-safe check" in G₁, and if only p53, but not the oncogene, is expressed, the apoptosis pathway is activated. Another possible way in which p53 might act as a fail-safe check is through transactivation by one of the cellular oncogenes which are normally expressed at the G₁/S transition, for example, *c-myc*. If *c-myc* were aberrantly expressed in G₁, it could induce p53 protein expression, which is not phosphorylated in G₁, and commit the cells to die.

Since both p53 and *c-myc* appear to be involved in apoptosis (16, 54), a hypothesis that the two proteins interact with each other is intriguing. One possibility is that they interact to induce apoptosis. However, unlike p53-induced cell death, *c-myc*-dependent apoptosis is a stochastic process which occurs when cells are blocked at any point in the cell cycle. Alternatively, p53 might be inactivated either directly or indirectly by cell cycle-regulatory oncogenes such as *c-myc*. This latter hypothesis is bolstered by the fact that when mouse cells are cotransfected with *ras* and *myc*, the resultant tumors have wild-type p53 genes (31).

Conclusions. Our data show that p53-induced apoptosis is cell cycle dependent and occurs in G₁ or the G₁/S boundary of the cell cycle. This view is consistent with a model in which p53 serves as a fail-safe mechanism which kills cells in which the normal cell cycle-regulatory pathways have been disturbed. The data are also consistent with a model in which p53 regulates the survival of normal hematopoietic progenitor cells, thus modulating the production of mature blood cells. Experiments are currently under way to test these various models.

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