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p53-Mediated Cell Death: Relationship to Cell Cycle Control

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M1 clone S6 myeloid leukemic cells do not express detectable p53 protein. When stably transfected with a temperature-sensitive mutant of p53, these cells undergo rapid cell death upon induction of wild-type (wt) p53 activity at the permissive temperature. This process has features of apoptosis. In a number of other cell systems, wt p53 activation has been shown to induce a growth arrest. Yet, wt 53 fails to induce a measurable growth arrest in M1 cells, and cell cycle progression proceeds while viability is being lost. There exists, however, a relationship between the cell cycle and p53-mediated death, and cells in G_1 appear to be preferentially susceptible to the death-inducing activity of wt p53. In addition, p53-mediated M1 cell death can be inhibited by interleukin-6. The effect of the cytokine is specific to p53-mediated death, since apoptosis elicited by serum deprivation is refractory to interleukin-6. Our data imply that p53-mediated cell death is not dependent on the induction of a growth arrest but rather may result from mutually incompatible growth-regulatory signals.

The p53 phosphoprotein is the product of a tumor suppressor gene, whose inactivation may play a role in the development and progression of many types of cancer (reviewed in references 5, 26, 35, and 43). In most cases, inactivation is achieved through missense mutations, leading to the expression of a full-length protein which is deficient in functions maintained by its wild-type (wt) counterpart. Such events are required not only for the induction, but also for the maintenance of the neoplastic state (69). Attempts to elucidate the biological activities of wt p53, whose loss presumably confers upon the cell an increased neoplastic potential, have been based on the reintroduction of functional wt p53 into transformed cells which had lost it. These studies have indicated that the most frequent in vitro outcome of reconstituted wt p53 expression is the induction of a growth arrest (11, 16, 38–40). Characteristically, such cells accumulate in an apparent G_1 state. Hence, wt p53 can function as an inhibitor of cell cycle progression, presumably acting at some stage before the G_1-to-S transition. It is noteworthy that the growth-inhibitory effects of wt p53 are preferentially exerted on transformed cells lacking wt p53 expression, whereas cells which still maintain an active wt p53 gene are more refractory to the addition of extra wt p53 (3, 6, 7).

In addition to interfering with cell proliferation, wt p53 has recently been shown to promote differentiation (56, 57). It is conceivable that these two activities are coupled, as p53-mediated differentiation is preceded by an extension of the cell cycle (56).

Experiments employing the M1 mouse myeloid leukemia cell line (clone S6) have revealed a third biological manifestation of wt p53 activity (67). These cells do not express any p53 at all. However, they can be induced to express wt p53 activity after stable transfection with a temperature-sensitive (ts) mouse p53 mutant. This mutant, p53val135, resembles other p53 mutants at 37.5°C, as determined both biologically and biochemically (18, 40, 41, 46). However, when shifted to 32.5°C, the protein assumes a wt-like conformation and activity. In fibroblasts, this is reflected by its ability to impose a reversible block of cell proliferation at this permissive temperature (16, 38, 40). In M1 cells, on the other hand, p53val135 induces more dramatic changes, culminating in cell death (67). This process possesses features considered to be indicative of apoptosis (67). Significantly, p53-mediated cell death can be effectively inhibited in this system by exposing the cells to interleukin-6 (IL-6). Recently, wt p53 has also been found to induce death in tumor-derived cells of human epithelial origin (58); in this case, too, an apoptotic process has been implicated. These findings raise the possibility that p53, and perhaps also the products of other tumor suppressor genes, are playing a role in regulating the dependence of certain cell types on survival factors. In the absence of such factors the cells will normally die. Abrogation of wt p53 activity may presumably allow the retention of viability under undesirable circumstances.

In an attempt to determine the basis for p53-mediated M1 cell death, we analyzed the behavior of such cells in the absence or in the presence of IL-6. The results indicate that cell death does not represent a secondary consequence of a p53-induced growth arrest and therefore support a direct linkage between wt p53 activity and the loss of viability. The data further suggest that this death process may be a result of mutually incompatible signals, which are reconciled in the presence of the appropriate survival factor. Given that apoptosis may be employed physiologically to eliminate cells in which a growth deregulation has occurred, these findings suggest that loss of wt p53 function may prevent this protective response, thereby contributing to the development of cancer.

MATERIALS AND METHODS

Cells and plasmids. The generation of cell lines LTR-6 (identical to the original LTR-1), LTR-13, and SV-1 has been described before (67). Cells were maintained in RPMI 1640 medium, containing 10% fetal calf serum. For serum deprivation studies, cells were washed and resuspended in serum-

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free medium for the indicated periods. Cell viability was determined by the ability to exclude trypan blue. For complement-mediated killing, log-phase cells were seeded at a final concentration of 10^7 cells per ml and incubated at 37.5°C with various dilutions of normal human serum. Viability was determined 20 h later, at which time DNA was extracted for analysis.

Plasmid pMcmyc-54 was the generous gift of K. Marcu (StonyBrook). A 1.5-kb *Sac*I-*Hin*dIII fragment derived from this plasmid served as a murine c-*myc*-specific probe. Mouse GAPDH cDNA was obtained from D. Givol (Rehovot, Israel).

Flow cytometric analysis and cell fractionation. Flow cytometric analysis was carried out in a fluorescence-activated cell sorter (FACStar plus; Becton Dickinson). To monitor DNA synthesis, determination of bromodeoxyuridine (BrdU) incorporation was performed essentially as described elsewhere (32). Briefly, cells were labeled for 15 min with 5 mM BrdU, fixed in 70% cold ethanol for 30 min, and rehydrated overnight in phosphate-buffered saline at 4°C. Staining with anti-BrdU antibodies was performed after acid denaturation and was followed by a fluorescein isothiocyanate-conjugated second antibody. DNA was counterstained with Hoechst (2 μg/ml) as previously described (32) at least 1 h before flow cytometric analysis. Dead cells were rejected for analysis. Cell fractionation by centrifugal elutriation was as described before (21).

Protein and nucleic acid analysis. Cells were labeled with [^{35}S]methionine and subjected to p53 analysis as described before (37). DNA fragmentation was measured as detailed earlier (67). Analysis of RNA by Northern (RNA) blotting was as described elsewhere (19).

RESULTS

Kinetics of p53-mediated cell death. In the present study, we employed primarily two M1-derived clones expressing ts p53: LTR-13, which produces high levels of p53val135, and LTR-6, which is essentially identical to the previously described LTR-1 (67) and produces more moderate levels of the protein. LTR-13 cells tend to lose viability faster, with the majority of cells being dead within 24 h of incubation at 32.5°C (67). On the other hand, over 50% of LTR-6 cells are still apparently viable after 24 h of exposure to the permissive temperature (Fig. 1a). In agreement with observations made with other lines (67), this process is greatly inhibited by the presence of IL-6 (Fig. 1a).

Like in many well-characterized apoptotic scenarios (1, 61), wt p53-mediated cell death is also accompanied by a typical pattern of DNA fragmentation (67). In order to determine the temporal relationship between this fragmentation and cell death, total DNA was prepared from LTR-6 cells at various times following a shift to 32.5°C and subjected to gel electrophoresis. As seen in Fig. 1b, a very small extent of DNA fragmentation was already discernible after as early as 6 h at 32.5°C. After 16 h of exposure to the wt-like activity of p53, a prominent "ladder" became visible, whereas the extent of cell death as defined by trypan blue uptake was still very modest. At 24 h, even though the majority of cells could still exclude trypan blue, almost all their DNA had already been converted into low-molecular-weight forms. Hence, DNA fragmentation precedes overt death by several hours. This is in agreement with earlier observations with a variety of cell types induced to undergo apoptosis (1, 61).

p53-mediated cell death is not coupled with a growth arrest.

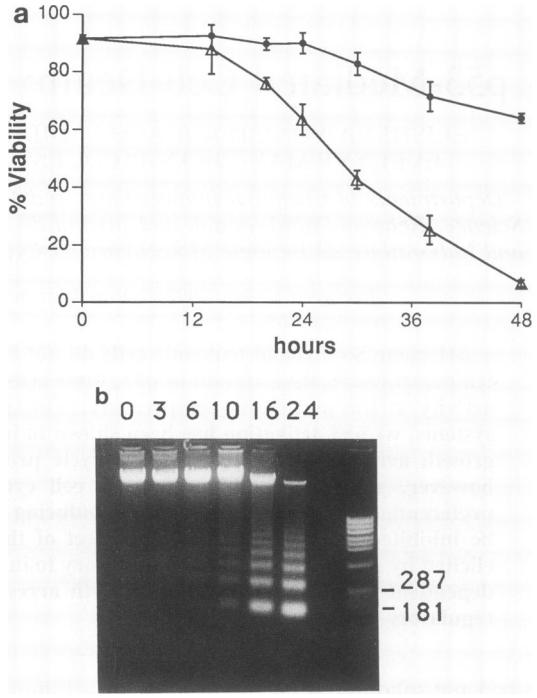

FIG. 1. Kinetics of cell death (a) and DNA fragmentation (b) in LTR-6 cells at 32.5°C. Parallel log-phase cultures of LTR-6 cells (8×10^5 cells per ml), grown at 37.5°C, were shifted down to 32.5°C and maintained in the absence (△) or presence (●) of 12.5 ng of recombinant IL-6 (Interpharm; 1.6×10^7 U/mg) per ml. At the indicated times (in hours) samples were withdrawn for analysis. Viability was determined by the ability to exclude trypan blue, and total DNA was prepared and analyzed on an agarose gel as described before (67).

Apoptosis is often preceded by a growth arrest, sometimes in the G_1 phase of the cell cycle (2, 62). A G_1 growth arrest is also a frequent result of the induction of wt p53 activity in transformed cells (11, 38–40). It therefore appeared plausible that in M1 cells, too, the primary effect of wt p53 activity was to establish a G_1 arrest, with cell death eventually ensuing as a secondary consequence. To test this notion, cells undergoing p53-mediated death at 32.5°C were subjected to cell cycle analysis at various times during this process. In each case, dead cells were eliminated by an appropriate window in the FACS, and only viable cells were subjected to analysis. Surprisingly, no evidence for any G_1-type growth arrest could be found in LTR-6 cells (Fig. 2), even after 20 h at 32.5°C, when cell death was already well in progress. In fact, these cells exhibited a distribution characteristic of actively proliferating populations. A substantial fraction of the cells were apparently in S phase, and there was actually even an increase in the proportion of S-phase cells at later times (35% at 10 h versus over 50% at 20 h). Similar results were obtained when dead cells were removed on a Ficoll gradient prior to FACS analysis (data not shown).

To further establish whether wt-like p53 activity indeed failed to elicit a G_1 arrest, the same cultures were assayed for the incorporation of BrdU, serving as a measure for DNA synthesis (Fig. 2A). Active S phase was evident even at late times, when overt cell death was already taking place. It is nevertheless noteworthy that, when compared with LTR-6 cells maintained at 37.5°C, the pattern of BrdU incorporation after 15 or 20 h at 32.5°C was rather irregular (Fig. 2). This

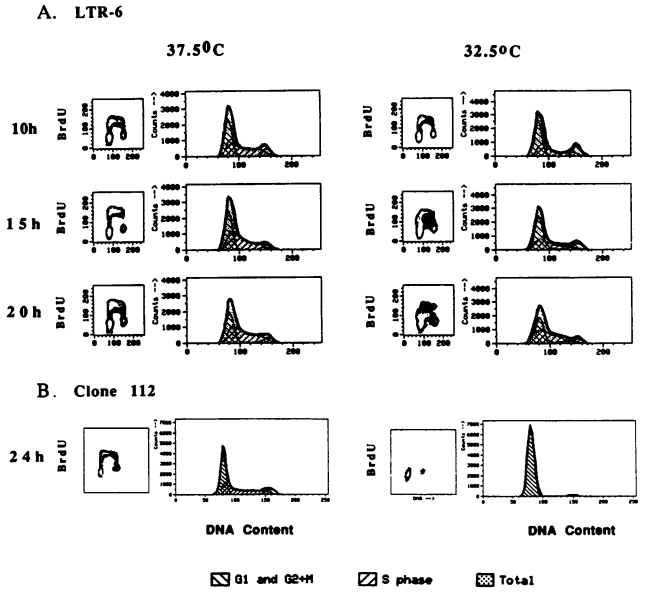

FIG. 2. Cell cycle distribution and BrdU incorporation in cells undergoing p53-mediated cell death (A) or p53-mediated growth arrest (B). (A) Asynchronous cultures of log-phase LTR-6 cells were either shifted to 32.5°C or maintained at 37.5°C. Samples were withdrawn 10, 15, and 20 h thereafter and subjected to cell cycle analysis in a FACS. In parallel, BrdU incorporation was determined on the corresponding cultures as detailed in Materials and Methods. The percentages of dead cells rejected for analysis were as follows. 37.5°C: 10 h, 6%; 15 h, 3%; 20 h, 4%. 32.5°C: 10 h, 19%; 15 h, 67%; 20 h, 75%. (B) A similar analysis was carried out on cells of the fibroblastic clone 112 (40), proliferating at 37.5°C or growth arrested by cultivation for 24 h at 32.5°C. BrdU incorporation is shown in units of fluorescence intensity.

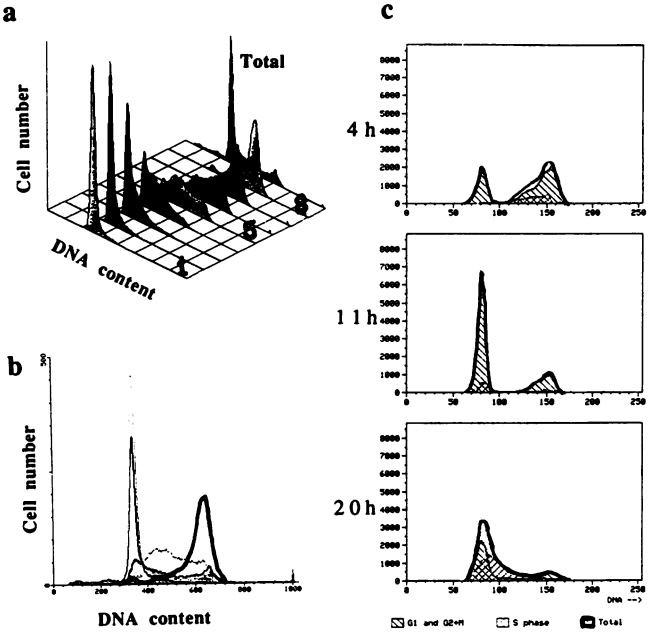

FIG. 3. Cell cycle analysis of centrifugally elutriated cells undergoing p53-mediated death or growth arrest. LTR-6 cells grown at 37.5°C were fractionated by centrifugal elutriation, as described in Materials and Methods. (a) DNA content distributions in the eight consecutive fractions collected by elutriation. (b) DNA content profiles of the total starting population (shaded area), as well as of fractions 1, 5, and 8 of panel a (representing predominantly G_1, S, and G_2+M cells, respectively). The elutriated G_2+M fraction (fraction 8) is accentuated. (c) Fraction 8 was placed at 32.5°C, and samples were taken for cell cycle analysis 4, 11, or 20 h later. Percentages of dead cells rejected for analysis were as follows: 4 h, 3%; 11 h, 4%; and 20 h, 70%.

probably reflects the fact that many of the S-phase cells were already undergoing extensive DNA fragmentation, which may have resulted in aberrant and heterogeneous rates of BrdU incorporation and DNA synthesis. Essentially similar conclusions were derived from measurements of radioactive thymidine incorporation (data not shown). By contrast, a typical G_1 arrest could easily be observed at 32.5°C when cells of the rat fibroblastic clone 112, carrying the same ts mutant (40), were analyzed under similar conditions (Fig. 2B).

The ability of cells exposed to wt-like p53 activity to move continuously through the cell cycle was also tested directly, as shown in Fig. 3. LTR-6 cells, growing at 37.5°C, were subjected to centrifugal elutriation (see Materials and Methods). Populations representing different phases of the cell cycle were purified (Fig. 3a), transferred to 32.5°C, and monitored for their cell cycle distribution at various times thereafter. Fraction 8 contained predominantly cells in G_2+M (Fig. 3a and b). As seen in Fig. 3c, by 11 h at 32.5°C the majority of these cells had completed cytokinesis and entered G_1. Yet, the cells did not arrest at this stage. Rather, they went on through the cycle, and by 20 h many of them had moved out of G_1 and into S. It is noteworthy that at this time cell death was already beginning to become apparent in this particular population, with viability having dropped from about 95 down to 85% (see Fig. 4). All these different approaches clearly indicate that, in transfected M1, p53-mediated cell death is not preceded by a G_1 arrest and most probably also not by any other sort of inhibition of cell proliferation. Thus, at least in this particular system, the activity of wt p53 appears to be directly coupled with the induction of cell death.

Commitment to death is cell cycle related. The failure of the transfected M1 to undergo a growth arrest does not necessarily imply that there is no relationship whatsoever between the position of a given cell within the cell cycle and the way it responds to wt p53. To address this issue more directly, LTR-6 cells were fractionated by centrifugal elutriation as shown in Fig. 3a, and the kinetics of death of each representative fraction was monitored. The results are displayed in Fig. 4. It is evident that the different populations exhibited distinct rates of cell death. Thus, cells which were predominantly in G_1 at the time of shift to 32.5°C (fraction 1 in Fig. 3a) lost viability first, whereas cells elutriated as being in S phase were the slowest to respond. This observation suggests that the commitment to undergo p53-mediated cell death takes place preferentially in G_1 or at the G_1/S boundary. Accordingly, the longer the time needed for a cell to reach the critical point in G_1, the later will its death program be executed.

The protective effect of IL-6 is specific for p53-mediated cell death. Exposure to IL-6 can effectively protect transfected M1 cells from wt p53-mediated cell death (Fig. 1a). In an attempt to elucidate the basis for this protective effect, we next asked whether IL-6 exhibited any specificity for p53 or rather acted as a general blocker of cell death in these cells.

Parental M1 cells, as well as p53val135 transfectants

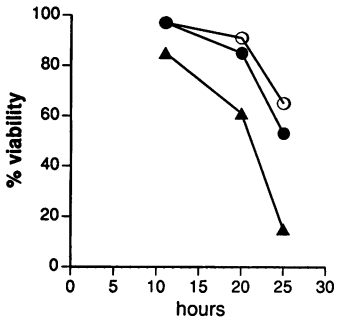

FIG. 4. Kinetics of cell death as a function of cell cycle position. LTR-6 cells grown at 37.5°C were fractionated by centrifugal elutriation, as shown in Fig. 3. Cells elutriated as predominantly in G_1 (▲; fraction 1 [Fig. 3a]), S (○; fraction 5), or G_2+M (●; fraction 8) were shifted to 32.5°C, and residual viability was monitored at various times thereafter.

maintained at 37.5°C, gradually lose viability in the absence of serum. This process also possesses features of apoptosis, as revealed by the induction of internucleosomal DNA fragmentation (Fig. 5a and b). It is noteworthy that the kinetics of cell death in the absence of serum is slower than in the case of wt p53 induction, and this difference is most pronounced when one compares the rates of DNA fragmentation in the two systems (Fig. 1b and 5). Furthermore, no significant differences in the patterns of DNA fragmentation upon serum starvation could be observed between the various populations obtained by centrifugal elutriation (Fig. 5b). More importantly, unlike p53-mediated cell death, the process induced by serum deprivation did entail a growth arrest. As depicted in Fig. 5c for the fraction elutriated as G_2+M, 48 h after serum depletion there was hardly any BrdU incorporation into S-phase cells, and most of the cells accumulated in G_1. Essentially similar pictures were obtained for cells elutriated as predominantly G_1 or S (data not shown).

It is also noteworthy that induced M1 cell death does not necessarily always occur through apoptosis. Thus, when subjected to complement-mediated cell killing, such cells exhibited no evidence of internucleosomal DNA fragmentation (Fig. 5a, lane 4).

To determine whether IL-6 could act as a nonspecific blocker of M1 cell death, we assessed the ability of IL-6 to protect cells from death caused by the absence of serum. As shown in Fig. 6 for LTR-6 cells maintained at 37.5°C, serum deprivation resulted in 80% of these cells being dead within 48 h. Addition of IL-6 had absolutely no protective effect. This finding militates against the possibility that IL-6 is a general inhibitor of M1 cell death. Rather, it suggests that this cytokine interferes selectively with the ability of wt p53 to trigger cell death.

IL-6 does not act by preventing p53 expression. One mechanism through which IL-6 could inhibit p53-mediated cell death is by a direct reduction of p53 levels. This could, for instance, result from an ability of IL-6 to down-regulate transcription from the promoters driving p53 expression in this system. Such a mechanism, not involving the authentic p53 gene promoter, would be of very little physiological relevance. The protective effect of IL-6 has previously been observed in cells in which p53 expression was under the control of either a Moloney leukemia virus long terminal repeat (LTR) or the simian virus 40 early promoter (67). The

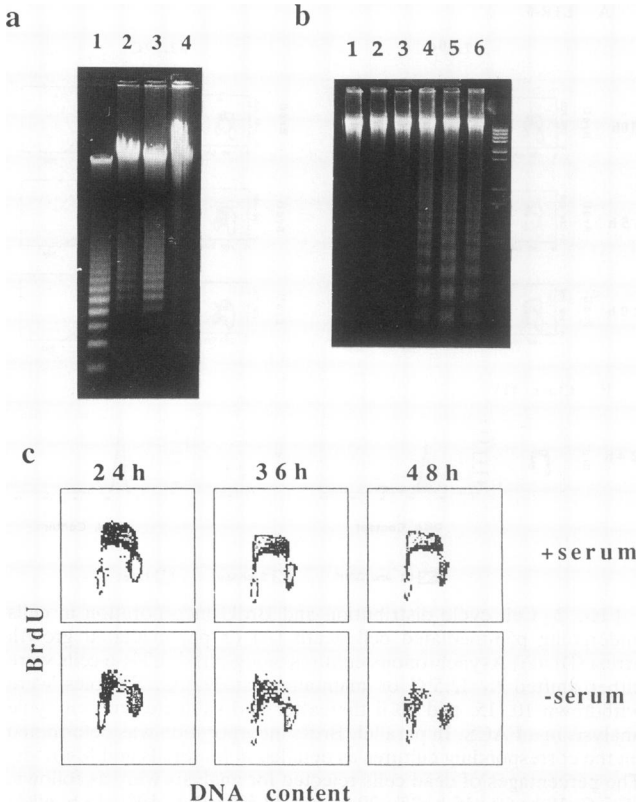

FIG. 5. Features of cell death induced by serum deprivation or exposure to complement. (a) Cells of clone LTR-13 were either transferred to 32.5°C for 24 h (lane 1) or left at 37.5°C and subjected to either serum deprivation (lanes 2 and 3) or exposure to the cytolytic action of complement (lane 4) (see Materials and Methods). DNA was extracted and analyzed as for Fig. 1b. Lanes 2 and 3 correspond to 24 and 48 h without serum, respectively. Percentages of viable cells for each of the treatments were 10, 80, 50, and 30%, respectively. (b) Cells of clone LTR-6 were fractionated by centrifugal elutriation and then maintained at 37.5°C either in the presence (lanes 1 to 3) or in the absence (lanes 4 to 6) of serum. DNA was extracted after 48 h and subjected to agarose gel electrophoresis. Lanes 1 and 4, G_1 cells; lanes 2 and 5, S cells; lanes 3 and 6, G_2+M cells. (c) Cells of clone LTR-6, enriched for G_2+M phase by centrifugal elutriation (b), were maintained at 37.5°C either in the presence or in the absence of serum. At the times indicated on top of each panel, samples were withdrawn and assessed for BrdU incorporation as for Fig. 2b.

response of both these promoters to IL-6 was therefore tested. As seen in Fig. 7, IL-6 reduced the expression of p53 when the latter was driven by the Moloney leukemia virus LTR at 37.5°C but had no effect at 32.5°C—the temperature at which it inhibits cell death. The reason for the difference between the two temperatures is not known. However, it is apparently not related to the presence of wt p53 activity, since it was also seen with the non-ts p53phe132 mutant (Fig. 7). In addition to its failure to reduce LTR-driven p53 expression at 32.5°C, IL-6 actually caused an increase in the rate of p53 synthesis when expression was under the simian virus 40 early promoter (data not shown). Yet, with both promoters, IL-6 could effectively inhibit p53-mediated death (67). Thus, the protective effect of IL-6 must stem from an interaction with p53-dependent processes rather than from a shut-off of p53 expression.

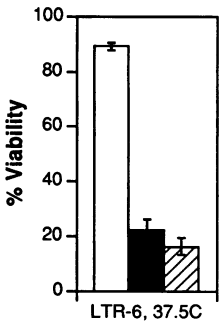

FIG. 6. Effects of IL-6 on cell death induced by serum deprivation. LTR-6 cells were brought to a density of 8×10^5 cells per ml at 37.5°C and maintained at the same temperature thereafter (□). For serum deprivation analysis, cells were washed and subsequently maintained in serum-free medium (■). IL-6 (12.5 ng/ml) was added as indicated (▨). Viability was scored by trypan blue exclusion 48 h later.

Cells exposed to wt p53 plus IL-6 become irreversibly factor dependent. The fact that IL-6 can induce both differentiation and protection from cell death (28, 36, 52) could imply that differentiated M1 cells are intrinsically resistant to the apoptosis-like effects of wt p53. If this were indeed the case, then cells which had escaped p53-mediated cell death thanks to the action of IL-6 and had gone into terminal differentiation would be expected to eventually become independent of IL-6. To test this notion, LTR-6 cells were maintained at 32.5°C for 3 days in the presence of IL-6. Viable cells were purified on a Ficoll gradient and then monitored for survival under various conditions (Fig. 8). When the cells were returned to 32.5°C in the absence of IL-6, a very rapid loss of viability was observed. This indicated that even a long exposure to the cytokine did not render the cells factor independent. Most surprisingly, however, these cells had

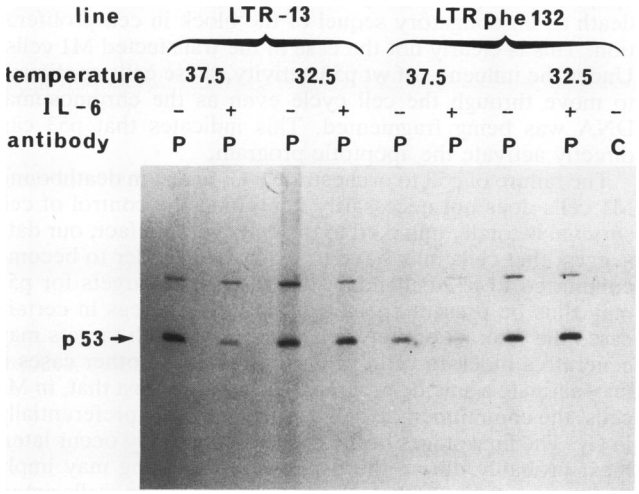

FIG. 7. Effects of IL-6 on the expression of p53 in transfected cell clones. Cells of clones LTR-13 and LTRphe132 were incubated with (+) or without (−) IL-6 (12.5 ng/ml) for 15 h at 37.5 or 32.5°C and then metabolically labeled with [^{35}S]methionine for 2 h. Cell extracts were prepared and subjected to immunoprecipitation with the p53-specific monoclonal antibody PAb122 (P) or the simian virus 40 large T-specific monoclonal antibody PAb419 (C) as described before (37).

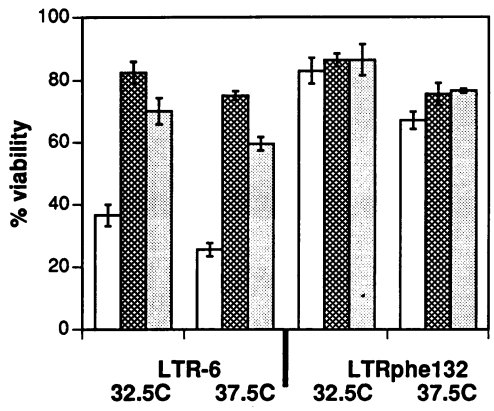

FIG. 8. Factor dependence of cells exposed to pretreatment with IL-6 in the presence of wt p53. LTR-6 and LTRphe132 cells were incubated at 32.5°C for 72 h in the presence of IL-6. Viable cells were then purified on a Ficoll gradient, washed, and returned to the indicated temperature for an additional 24 h, in the presence (▩) or absence (□) of IL-6 (12.5 ng/ml) or recombinant IL-3 (▨; British Biotechnology, 10 ng/ml). Viability was determined by trypan blue exclusion.

now become IL-6 dependent even in the absence of wt p53 activity, as reflected by their rapid loss of viability when placed in cytokine-free medium at 37.5°C. Thus, exposure to wt p53 and IL-6 had caused the cells to become irreversibly factor dependent. That this effect required previous exposure to wt p53 is evident from the fact that when cells carrying a non-ts p53 mutant (LTRphe132) were taken through the same treatment, they did not exhibit a comparable dependence on IL-6. Similarly, when cells carrying the ts p53 were pretreated with IL-6 at 37.5°C rather than at 32.5°C, they failed to exhibit enhanced cell death upon removal of the cytokine (data not shown). Most interestingly, cells exposed for 3 days to the combined activity of wt p53 and IL-6 could now be rescued not only by IL-6 but also by IL-3, whereas the latter cytokine had no protective effect when applied without prior IL-6 exposure (Fig. 8). Protection by IL-3 could also be achieved by incubating LTR-6 cells with IL-6 at 37.5°C for as little as 16 h before shifting them down to 32.5°C in the presence of IL-3 alone (data not shown). This observation probably reflects a need for IL-6 in order to induce the expression of IL-3 receptors, as demonstrated previously for a different subclone of M1 (36). Hence, both IL-6 and IL-3 have the potential of serving as survival factors in M1 cells subjected to wt p53 activity, provided the cognate receptors are present. The most likely explanation for the observations depicted in Fig. 8 is that exposure to wt p53 activity turns on a cell death program in M1, but the completion of this program is inhibited by the presence of appropriate survival factors. The data suggest that, once a commitment to cell death has been induced by wt p53, the latter is no longer necessary in order to maintain this commitment. Survival factors therefore presumably block the apoptotic process at a stage which is downstream to wt p53.

TGF-β enhances p53-mediated cell death. IL-6 imposes a G_1 growth arrest on M1 clone S6 cells (48, 49). A seemingly similar type of arrest is also elicited by transforming growth factor β (TGF-β) (49). Within the time frame needed for efficient induction of cell death by wt p53, TGF-β alone does not exert significant effects on viability (data not shown). To determine whether the mere induction of a G_1 arrest is

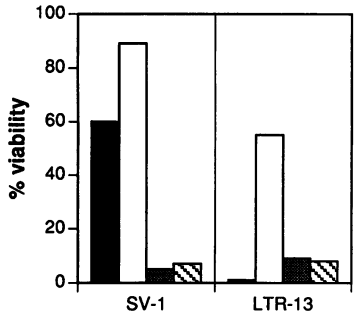

FIG. 9. TGF-β enhances p53-mediated cell death. Cells of clones LTR-13 and SV-1, containing p53val135 under the Harvey sarcoma virus LTR and the simian virus 40 early promoter, respectively, were maintained at 32.5°C for 24 h in the absence (■) or presence (□) of IL-6 (12.5 ng/ml) or TGF-β (▩; R&D systems, 3 ng/ml) or both (▧). Viability was determined by trypan blue exclusion.

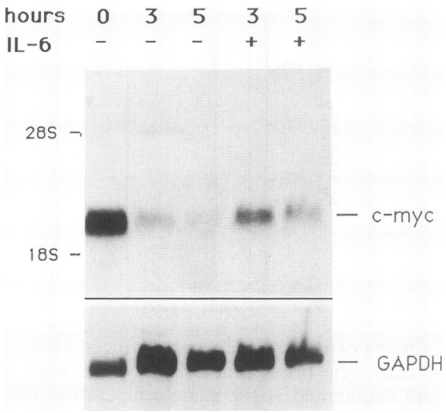

FIG. 10. Effects of wt p53 activity on c-*myc* mRNA levels. LTR-6 cells were transferred to 32.5°C, either in the absence or in the presence of IL-6. Portions of the culture were taken for the extraction of total cytoplasmic RNA at the indicated times following the down-shift. Each lane corresponds to 20 μg of RNA. The blot was hybridized sequentially with a mouse c-*myc* cDNA probe and with a mouse GAPDH cDNA probe.

sufficient to render M1 cells more refractory to the apoptotic activity of wt p53, we assessed the effect of TGF-β on this process. As seen in Fig. 9, the effect of TGF-β was actually opposite to that of IL-6. In the presence of TGF-β, p53-dependent death was faster; furthermore, the protective effects of IL-6 were at least partially abolished. One possible interpretation is that TGF-β provides distinct apoptosis-promoting signals, cooperating with the ones elicited by wt p53. Alternatively, TGF-β may modulate directly the biochemical properties of the p53 protein itself. Either way, the apoptotis-promoting effects of TGF-β in this system do not appear to be blocked by IL-6.

Rapid c-*myc* mRNA reduction precedes p53-mediated cell death. A growing number of reports now suggest that p53 possesses transcription regulatory activities (13, 14, 17, 31, 46, 47, 53, 64, 66, 68). It was therefore of interest to determine whether wt p53 activation had any measurable effects on the levels of specific transcripts. One obvious candidate was c-*myc*. A close correlation has been shown to exist in M1 cells between the biological activities of IL-6 and alterations in c-*myc* gene expression (25, 48, 50, 54). Steady-state levels of c-*myc* mRNA were therefore monitored in LTR-6 cells after exposure to wt p53 activity. It was found (Fig. 10) that c-*myc* mRNA levels dropped rapidly following down-shift to 32.5°C, with a marked decrease seen already after 3 h. Hence, repression of c-*myc* expression is an early event in this apoptotic paradigm. Interestingly, the presence of IL-6 appeared to delay partially the reduction in c-*myc* mRNA levels. It is noteworthy that IL-6 alone actually also causes a marked drop in c-*myc* mRNA, but with much slower kinetics than does wt p53 (25, 50).

The effect of wt p53 on the levels of c-*myc* mRNA was not due to a general transcriptional repression. Thus, no comparable reduction was found when steady-state levels of c-*jun* mRNA were determined (data not shown), even though the c-*jun* promoter has been shown to be down-regulated by overexpressed wt p53 in fibroblasts (17). Similarly, there was no reduction in histone H1° mRNA, at least within the first 5 h after the induction of wt p53 activity (data not shown). Thus, the activation of wt p53 in M1 cells appears to lead to a selective repression of c-*myc* expression. Additional genes, yet to be identified, may also be similarly down-regulated.

DISCUSSION

In the present study, we analyzed in detail the process of cell death induced by wt p53 in a murine myeloid leukemic cell line. Like in many other cases in which apoptosis has been shown to take place (1, 61), here too DNA fragmentation precedes formal cell death by several hours.

In a variety of apoptotic systems, cell death is preceded by a growth arrest, often (but not necessarily) in G_1 (1, 2, 61, 62). One could argue that in particular cell types, apoptosis occurs by default whenever a G_1 arrest is imposed. The expression of wt p53 can often elicit an arrest in G_1 (11, 38–40, 42). In most cases studied thus far, this arrest is reversible and is not followed by a significant loss of viability. However, in cells in which growth arrest is coupled with apoptosis, wt p53 activity could be expected to lead to cell death as an obligatory sequel of the block in cell proliferation. This is clearly not the case in the transfected M1 cells. Under the influence of wt p53 activity, these cells continued to move through the cell cycle even as the chromosomal DNA was being fragmented. This indicates that p53 can directly activate the apoptotic program.

The failure of p53 to orchestrate a G_1 arrest in deathbound M1 cells does not necessarily imply that the control of cell survival is totally unlinked to the cell cycle. In fact, our data suggest that cells may have to reach G_1 in order to become committed to p53-dependent death. Critical targets for p53 may thus be present specifically in G_1. Whereas in certain cases the interaction between wt p53 and such targets may generate a block to cell cycle progression, in other cases it may activate a suicide program. Our data suggest that, in M1 cells, the commitment to cell death takes place preferentially in G_1. The final stages of the process apparently occur later, most probably during the S phase. This timing may imply that once sufficient DNA cleavage has occurred, cells entering S cannot successfully exit it. It is of note that apoptotic cell death in S phase has been described before (10).

How does wt p53 contribute to the induction of cell death? Recent data suggest that p53 acts as a transcriptional modulator, upregulating the expression of some genes while repressing that of many others (13, 14, 17, 31, 46, 47, 53, 64, 66, 68). Positive regulation may rely on the ability of p53 to

engage in sequence-specific DNA binding (13, 15, 31, 68). Most importantly, transcriptional modulation is exerted only by wt p53, but not by mutants of the various types found in tumor cells. wt p53 may therefore promote cell death by activating genes required for the induction of apoptosis (4, 27, 44) or by repressing genes such as *bcl-2*, whose products suppress apoptotic processes (22–24, 55, 60, 65).

Certain cells appear to have a salient commitment to apoptosis, which needs to be continuously overriden by survival signals (1, 45). Such cells thus possess a functional molecular circuitry poised to execute the apoptotic process. It is conceivable that p53 may be a component of this circuitry. The propensity of a cell to undergo apoptosis is often dictated by its state of differentiation and maturation (1, 20). The suggestion that p53 is involved in regulating these processes (29, 56, 57) provides a potential link between p53 and the control of cell death. Whatever the mechanism, if wt p53 is indeed necessary for allowing particular cells to become committed to apoptosis, then its loss will allow the illegitimate survival of such cells, thereby promoting neoplasia.

It is possible that the link between wt p53 activity and cell death exists only in transformed cells. It has been suggested that apoptosis may serve as a means for "executing" cells in which growth control has been grossly disturbed (63). Such cells may be subjected to conflicting signals, especially when environmental cues call for a cessation of proliferation. This will create an imbalance between the growth-inhibitory signals and the constitutive activation of growth-promoting pathways. Recent evidence strongly indicates that such imbalance may indeed trigger apoptosis, as seen when cells carrying a deregulated c-*myc* gene are deprived of factors essential for maintaining their proliferation (2, 12). M1 cells induced to express wt p53 may be another such example. These cells may harbor one or more activated oncogenes which provide constitutive growth-promoting signals. Like in cells with deregulated c-*myc* (2, 12), the trigger to activate the suicide program in M1 cells may be provided by their inability to arrest properly in G_1. It appears, however, rather unlikely that constitutive c-*myc* overexpression plays a key role in mediating cell death in M1, given that c-*myc* mRNA levels are in fact rapidly reduced by wt p53.

A connection between p53 and apoptosis may also be predicted from the recent proposal that wt p53 is involved in maintaining the stability of the genome (30, 33, 34). DNA damaging agents can activate a suicidal response, which may serve to prevent the accumulation of irreparable genetic alterations (8, 61). It is tempting to speculate that wt p53 may activate the apoptotic program once the genetic damage is too extensive to be repaired.

Our findings do not prove that p53 normally plays a role in apoptosis in vivo. Nevertheless, several recent findings are consistent with this possibility. Hence, in both involuting mouse mammary epithelium (59) and prostate epithelium of castrated rats (9), increased p53 expression is seen early after exposure to apoptosis-inducing manipulations. In addition, in normal human myeloid blasts the resident wt p53 was reported to exhibit a mutant conformation (51). This suggests that the ongoing proliferation and survival of such cells in vivo require that p53 be kept in an inactive state. Hence, the fact that wt p53 renders myeloid M1 cells IL-6 dependent for survival may bear physiological relevance. Clearly, future work should address more directly the role of p53 in normal apoptotic processes.

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