Expression of Wild-Type p53 Increases Etoposide Cytotoxicity in M1 Myeloid Leukemia Cells by Facilitated G₂ to M Transition: Implications for Gene Therapy\(^1\)

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**Abstract**

We have evaluated the role of p53 in the induction of cell death by the DNA topoisomerase II inhibitor etoposide in M1 myeloid leukemia cells. Three different clones of M1 cells were used: S6, which lacks p53; Phe-132, which expresses mutant p53 constitutively; and LTR-13, which expresses mutant protein at 37°C and wild-type p53 at 32°C. As described previously, LTR-13 cells undergo rapid apoptosis upon induction of wild-type p53 at 32°C. Multiparameter flow cytometric analysis showed that etoposide treatment (0.5 μg/ml) of all three cell lines at 37°C is associated with a block in the G₂ phase of the cell cycle, whereas the cells preferentially die out of the next S phase. Induction of wild-type p53 in LTR-13 cells is associated with a loss of cells in late S and G₂-M phase, and the cells die out of the early S phase. Interestingly, the simultaneous induction of apoptosis by both pathways (wild-type p53 and etoposide) leads to suppression of the etoposide-induced G₂ block. To determine the effect of p53 on the G₂ transition, LTR-13 cells were incubated with etoposide for 24 h at 37°C and then either maintained for an additional 12 h at 37°C or shifted to 32°C to activate wild-type p53. The expression of wild-type p53 resulted in an increase in mitosis-specific phosphorylation, as determined by the MPM-2 antibody as well as the formation of mitotic spindles. This was associated with an important augmentation of the cytotoxic effect of etoposide. In contrast, a similar temperature shift of Phe-132 cells, which express mutant p53, had no effect on either immunostaining with MPM-2 or the cytotoxicity. Taken together, our results indicate that wild-type p53 can override the etoposide-induced G₂ block in at least some cell types. These data propose a new role for p53 in the cell death induced by chemotherapeutic agents and may have important implications for gene therapy.

**Introduction**

p53 is a tumor suppressor gene product involved in the cellular response to DNA damage (1–3). Although wt\(^3\) protein is present in most normal human tissues, the p53 gene is absent or mutated in a high proportion of human tumors (4). p53 gene mutations usually lead to expression of a full-length protein that is deficient in certain features, such as specific DNA binding (5). It has been suggested that restoration of wt p53 function may represent a new strategy for cancer treatment.

Genotoxic lesions leading to DNA strand breaks result in a rapid increase of p53 protein levels (2). The induction of p53 might lead to growth arrest or to apoptosis, depending on p53 levels, cell type, and the cellular environment (6–10). The p53 protein appears, at least in part, to exert its activity through activation or suppression of gene transcription (4). The role of p53 in G₂ arrest has been associated with increased expression of the DNA damage-inducible gene GADD45 (11) and p21/WAF1/CIP1, which encodes a potent inhibitor of the cyclin-dependent kinases (12, 13). Loss of a p53-associated G₂ checkpoint has been linked to increased gene amplification and aneuploidy (14) but does not seem to result in decreased cell survival following DNA damage (15). More recently, expression of p53 has been associated with growth arrest in the G₂-M phase of the cell cycle (3, 16, 17), whereas others report a positive role of p53 in G₂ exit following γ irradiation (18).

The influence of p53 on the cellular sensitivity toward irradiation and DNA-damaging agents is not clear. At least in some systems, inactivation of p53 results in increased resistance, suggesting that some of the pathways leading to cell death are p53 dependent (3, 19, 20). However, in other cases, the disruption of p53 function may lead to increased sensitivity (21–23).

The present work was undertaken to clarify the influence of p53 on cell death induced by the DNA topoisomerase II inhibitor etoposide. Exposure of tumor cells to etoposide leads to two distinct modes of cell death, apoptosis and mitotic cell death (24–26). Apoptosis is an internally programmed, physiological process in which cells undergo shrinkage, chromatin condensation, cytoplasmic vacuolization, plasma membrane blebbing, activation of proteases and endonucleases, and finally disintegration into plasma membrane-bound apoptotic bodies (27).

Mitotic cell death occurs after release from G₂ arrest and is associated with the formation of enlarged cells containing multiple nuclear fragments (micronuclei), which are readily distinguished from apoptotic nuclear fragments (24–26). After a prolonged incubation period, multinucleated cells become morphologically similar to apoptotic cells (25, 28).

In these studies, we have used different cell lines derived from M1 myeloid leukemia cells expressing either no, mutant, or wt p53 protein. In this cell type, expression of wt p53 leads to cell death by apoptosis (7). Exposure to therapeutic doses of etoposide also leads to apoptosis independent of p53 status (29). We have determined how induction of apoptosis by the two pathways (wt p53 and etoposide) affects cell cycle progression and DNA fragmentation. Our results indicate that even short-term expression of wt p53 can override the etoposide-induced G₂ block, resulting in an important increase in the cytotoxic effect of the drug. These data propose a new role for p53 in the cell death induced by chemotherapeutic agents and may have important implications for gene therapy.

**Materials and Methods**

**Chemicals and Reagents.** Etoposide was a kind gift from Bristol Myers-Squibb. RPMI 1640, FCS, and antibiotics were from Life Technologies, Inc.
Cell Culture and Drug Treatment. The M1 murine myeloid leukemia cell line (clone S6) and its transfectants LTR-5, LTR-13, and Phe-132 were characterized earlier (7). The parental M1 cells, which express neither p53 protein nor mRNA, were stably transfected with a temperature-sensitive p53 mutant (LTR-13: Ala \rightarrow Val at position 135), which shows wild-type conformation at 32°C and mutant conformation at 37°C. The same parental line was also transfected with a different plasmid encoding a constitutively expressed p53 mutant protein (Phe-132, Cys \rightarrow Phe at position 132). Cells were grown in RPMI 1640 supplemented with 10% FCS and antibiotics (0.1 \mu g/ml streptomycin and 100 IU/ml penicillin) at 37°C in a humidified 5% CO₂-air atmosphere.

Viability and Cell Cycle Distribution. All cell cycle distribution measurements were made on a Coulter EPICS Profile II flow cytometer equipped with an argon laser to give 488 nm light. Cell viability was determined after 5 days incubation by a PI exclusion assay as described (30). Cells were briefly (about 5 min) exposed to 20 \mu g/ml PI and immediately analyzed by flow cytometry. For cell cycle distribution studies, cells were fixed in 70% ethanol and stained with PBS containing PI (20 \mu g/ml) and ribonuclease A (100 \mu g/ml) for 30 min at room temperature. Data from 10^4 cells were collected and analyzed by Multicycle software (Phoenix Flow Systems, San Diego, CA).

TdT Assay. Cells were prepared according to methods published previously (31). Following drug treatment, cells were collected by centrifugation and fixed in 1% formaldehyde in PBS for 15 min on ice. After centrifugation, the pellet was washed in PBS and resuspended in 70% ethanol; samples were stored overnight at 4°C. After rehydration in PBS, cells were resuspended in 50 \mu l of reaction buffer containing 5 units of terminal transferase, 2.5 mM cobalt chloride, 0.2 mM sodium cacodylate, 25 mM Tris·HCl (pH 6.6), 0.25 mg/ml bovine serum albumin, and 1 \mu g of biotin-dUTP (Boehringer Mannheim, Mannheim, Germany) and incubated at 37°C for 30 min. The pellet was washed with rinsing buffer (PBS containing 0.1% Triton X-100 and 0.5% bovine serum albumin), resuspended in 100 \mu l of staining buffer that contained 2.5% FITC-avidin, 4X SSC buffer (1X SSC buffer = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7), 0.1% Triton X-100, and 5% w/v nonfat dry milk and incubated for 30 min at room temperature in the dark. The pellets were then washed twice with rinsing buffer, resuspended in PBS containing 5 \mu g/ml PI and 100 \mu g/ml RNase A, and incubated at room temperature for 30 min. The red (PI) and green (fluorescein) fluorescence were measured with an EPICS Profile II flow cytometer; the data from 10^4 cells were collected and analyzed by Multigraphics software.

Immunocytochemistry. For detection of MPM-2 epitopes by indirect immunostaining, cells were fixed in 70% ethanol at −20°C overnight, rehydrated in ice-cold PBS for 10 min, and permeabilized in 0.25% Triton X-100/PBS for 5 min on ice. Cells were washed with PBS and blocked in 1% BSA/PBS for 15 min at room temperature. After washing with PBS, cells were incubated for 1 h at room temperature with MPM-2 antibodies diluted in 0.5% BSA/PBS at 1:100. Samples were then washed with PBS and incubated with anti-mouse IgG-FITC (Sigma Chemical Co.) at 1:50 in 0.5% BSA/PBS for 30 min at room temperature in the dark. DNA was stained in PBS containing 10 \mu g/ml PI and 0.1 mg/ml RNase A for 30 min at room temperature and analyzed by flow cytometry as described above.

For simultaneous staining of DNA and \( \beta \)-tubulin, cells were fixed onto microscopy cover glass by a Cytospin 2 centrifuge (Shandon, Inc., Pittsburgh, PA) and permeabilized for 20 s with tubulin stabilizing buffer [80 mM PIPES (pH 6.9), 5 mM EGTA, 1 mM magnesium chloride, and 0.1% Triton X-100], after which glutaraldehyde (final concentration, 0.5%) was added under gentle mixing. After fixation (10 min at room temperature), cells were washed in PBS for 10 s, quenched for 6 min with 1 mg/ml sodium borohydride in PBS, washed once with PBS, blocked with 1% BSA in PBS for 30 min, and incubated with anti-\( \beta \)-tubulin antibodies (1:100, 1 h). After three washes with 0.2% Tween 200/0.5% BSA/PBS, cells were incubated with anti-mouse IgG-FITC (1:50, Sigma Chemical Co.) in 0.5% BSA/PBS for 30 min at room temperature in the dark. Cells were then washed with 0.2% Tween 200/0.5% BSA/PBS, stained with 4,6-diamindino-2-phenylindole (1 \mu g/ml) for 10 s, washed with distilled water, dried, and mounted in 90% glycerol in 10 mM Tris·HCl (pH 8) containing 22 mM 1,4-diazobicyclo(2,2,2)-octane. Photographs were taken using an Olympus fluorescence microscope and Polaroid type 667 film.

Results and Discussion

Etoposide and p53 Have Different Effects on Cell Cycle Progression and DNA Fragmentation. Previous results show that apoptosis can be induced by both p53-dependent (expression of wt p53) and -independent (exposure to the topoisomerase II inhibitor etoposide) pathways in M1 myeloid leukemia cells (7, 29). In the current study, different cell lines derived from M1 cells were used to determine how the two apoptotic pathways affect cell cycle progression and DNA fragmentation. This includes S6 cells, which are p53 null. LTR-13 cells, which express mutant p53 at 37°C and wt protein at 32°C, and Phe-132 cells, which express mutant p53 constitutively. Exposure of LTR-13 cells to 0.5 \mu g/ml etoposide for 12 h at 37°C resulted in a delayed S-phase progression and arrest in the G₂-M phase of the cell cycle (Fig. 1B). After 24-h etoposide treatment, most cells were blocked at the G₂-M phase (Fig. 1C). Similar results were found for S6 and Phe-132 cells (results not shown). Microscopic examination of nuclear morphology as well as staining with the MPM-2 antibody, which specifically recognizes phosphoepitopes present in mitotic cells (32), showed that etoposide-treated cells were in the G₂ phase (results not shown). After prolonged drug incubation (24 h and longer), G₂ arrest was accompanied by an increase in the sub-G₀/G₁ region of cells with a fractional DNA content, which is typical for later stages of apoptosis (Fig. 1C).

Fig. 1. Cell cycle distribution determined by flow cytometry in M1 cells treated with etoposide. A, untreated LTR-13 cells at 37°C; B, untreated LTR-13 cells incubated with 0.5 \mu g/ml etoposide for 12 h at 37°C; C, LTR-13 cells incubated with 0.5 \mu g/ml etoposide for 24 h at 37°C; D, untreated LTR-13 cells incubated for 12 h at 32°C; E and F, LTR-13 cells incubated with 0.5 \mu g/ml etoposide for 12 and 24 h at 32°C.
Fig. 2. DNA fragmentation determined by the TdT assay in M1 cells treated with etoposide. A, untreated LTR-13 cells at 37°C; B, LTR-13 cells incubated for 12 h at 32°C; C, LTR-13 cells treated with 0.5 μg/ml etoposide for 48 h at 37°C; D, LTR-13 cells treated with 0.5 μg/ml etoposide for 12 h at 32°C.

At 32°C, activation of wt p53 induced apoptosis of LTR-13 cells. This was accompanied by an apparent arrest in early S phase and loss of cells in late S phase as well as G2-M (Fig. 1D). LTR-13 cells incubated with etoposide at 32°C showed a cell cycle distribution similar to what was observed in the absence of drug, except for a higher G2 fraction (Fig. 1, E and F).

To determine if the DNA fragmentation observed in the treated cells occurred in a particular phase of the cell cycle, the TdT assay was carried out, in which fragmented DNA is labeled with biotinylated dUTP and terminal transferase, followed by staining with a streptavidin-FITC conjugate. Two-parameter flow cytometry analysis showed that LTR-13 cells treated with etoposide at 37°C gave a TdT-positive signal in the S-phase region (Fig. 2C). Similar results were observed for etoposide-treated S6 and Phe-132 cells (results not shown).

Fig. 3. The effect of a transient exposure of LTR-13 cells to wt p53 on cell cycle progression (A) and mitosis-specific phosphorylation as determined by immunostaining with the MPM-2 antibody (B). A, cells were incubated in the absence (i) or presence of 0.5 μg/ml etoposide for 12 h at 37°C (ii) and postincubated at either 37°C (iii) or 32°C (iv) for an additional 12 h. B, cells were incubated in the absence (iii) or presence of 0.5 μg/ml etoposide for 24 h at 37°C and postincubated at either 37°C or 32°C for the times indicated.
The results in Fig. 1 showed that etoposide-treated LTR-13 cells contain relatively few cells in G2-M when wt p53 is expressed compared to the almost complete G2 block observed when p53 has mutant conformation (Fig. 1, compare B and C with E and F). Part of this could be due to lack of recruitment of cells into the later stages of the cell cycle because wt p53 preferentially killed cells out of early S phase (Fig. 2B). However, it was also possible that wt p53 had an effect on the etoposide-induced G2 arrest. To investigate this possibility, LTR-13 cells were treated with etoposide at 37°C for 12 h (Fig. 3A, ii) and then either maintained at the same temperature (Fig. 3A, iii) or shifted to 32°C (Fig. 3A, iv) for an additional 12 h to activate wt p53. The results show that the transient expression of wt p53 resulted in the appearance of a G1 peak (Fig. 3A, iv) compared to the almost complete G2 block observed when cells were kept at 37°C where p53 has mutant conformation (Fig. 3A, iii). The effect on cell cycle progression was not due to the temperature as such because the majority of both S6 cells (no p53) and Phe-132 cells (mutant p53) remained arrested in G2, even after 24 h postincubation at 32°C (results not shown).

Topoisomerase II is required for chromosome condensation during mitosis. For this reason, it is not possible to get an accurate estimate of the M-phase fraction by conventional mitotic spreads, which relies on the visualization of highly condensed chromosomes, if the cells have been treated with topoisomerase II inhibitors such as etoposide (34). We, therefore, used immunostaining with the MPM-2 antibody, which has been shown to specifically recognize phosphoepitopes present in mitotic cells (32), to determine the entry of cells into mitosis. MPM-2 staining of LTR-13 cells pretreated with etoposide for 24 h at 37°C and then either kept at 37°C or shifted to 32°C to activate wt p53 show that the expression of wt p53 leads to an increase in mitosis-specific phosphorylation as early as 1 h after the temperature shift. The fraction of MPM-2-positive cells had increased to 12% of the total cell population by 3 h compared to about 2% for cells kept at 37°C (Fig. 3B). At the same time, mitotic spindles were apparent, as shown by staining with antibodies directed against β-tubulin (Fig. 4). Interestingly, the presence of cycloheximide (1 μg/ml), which results in 96% inhibition of protein synthesis, prevented the
p53-induced G2-to-M transition as well as the p53-induced apoptosis. At this dose, cycloheximide by itself had no effect on either cell viability or cell cycle distribution (results not shown). This suggests that in these cells, protein synthesis is required both to override the p53-induced G2 block and to induce p53-dependent apoptosis.

Recent studies where a battery of different p53 mutants were microinjected into primary mouse embryonic fibroblasts derived from p53 null animals showed conclusively that the transcriptional activity of p53 is essential for both G1 arrest and apoptosis. However, the transcriptional pathways for these two processes were different (35). Our results suggest that the p53-dependent G2-to-M transition also relies on transcriptional activation by p53, although it remains to be determined which genes are involved. One possibility is increased transcription of genes acting on p34cdc2 kinase because etoposide-induced G2 arrest is associated with hyperphosphorylation of p34cdc2 (36). Other studies have shown that activation of p53 in M1 cells results in a modest (about 2-fold) increase in the transcription of cyclin B1. In addition, the transcription of cyclin G, a cyclin with thus far unknown function, was increased 30-fold (37).

**Transient Expression of wt p53 Increases the Cytotoxic Effect of Etoposide.** It is currently accepted that arrest in the G2 phase of the cell cycle decreases the cytotoxic effect of a given agent by providing the cell time to repair its lesions prior to mitosis and the start of a new cell cycle. To determine the effect of the p53-induced G2-M transition on the cytotoxicity of etoposide, LTR-13 or Phe-132 cells were treated with etoposide for 24 h at 37°C, either maintained at 37°C or shifted to 32°C for 12 h, and then postincubated at 37°C for an additional 5 days. The results show that the viability of cells expressing mutant p53 was the same with and without temperature shift (Fig. 5). By itself, the expression of wt p53 for 12 h reduced the viability only by 18%. However, etoposide exposure associated with expression of wt p53 for 12 h increased the overall cytotoxicity by a factor of 10. Therefore, even a transient expression of wt p53 increases the cytotoxic effect of etoposide by inducing an accelerated G2-M transition. In contrast, 1 mM caffeine, which is often used to abrogate the G2 block, had no effect on the etoposide-induced G2 arrest in M1 cells when given either during drug treatment or after induction of the G2 block (results not shown).

A similar effect of p53 on the G2 exit has been reported recently for G2 arrest induced by γ-irradiation (18). Therefore, the p53-induced G2-to-M transition is not limited to etoposide but is likely to be relevant for a variety of DNA-damaging agents. Furthermore, these findings are not restricted to cell lines with ectopic expression of p53 because studies with myeloblast-enriched bone marrow cells obtained form wt or p53 null mice also show that wt p53 positively modulates the exit from a γ-irradiation induced G2 block (18). Our results are in apparent contradiction with other reports showing a preferential effect of abrogaters of the G2 checkpoint such as 7-hydroxystauroporine (UCN-01) and caffeine on cancer cells with disrupted p53 function (38, 39). However, it should be noted that the above studies were carried out with cell lines where activation of p53 results in G1 and G2 arrest. In contrast, the p53-induced G2-to-M transition has been observed in cells where activation of p53 also leads to apoptosis (18) and this study. This suggests that p53 might have a dual function including either its classical role as “guardian of the genome,” where the induction of G1 and G2 arrest tends to protect genomic integrity, or alternatively, a much more destructive role in which p53 drives the cells into S or M phases, where the probability of cell death is higher.

Our results have several important implications for gene therapy with p53 or other treatments aiming at restoring p53 function in tumor cells to induce apoptosis. Because both commitment and the subsequent cell death appear to be cell cycle dependent, the expression of p53 alone will probably only have an important cytotoxic activity toward tumors with a high proportion of proliferating cells. Therefore, p53-directed therapies are likely to be combined with other treatment modalities. In this respect, it is particularly interesting that even a transient expression of p53 is highly efficient in increasing the cytotoxic effect of DNA-damaging agents such as γ-irradiation and etoposide by modulation of cell cycle progression on the G2-M border.

Taken together, our data propose a new role for p53 in the cell death induced by chemotherapeutic agents, which may have important implications for gene therapy.

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**References**


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