p53-independent Induction of WAF1/CIP1 in Human Leukemia Cells Is Correlated with Growth Arrest Accompanying Monocyte/Macrophage Differentiation

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ABSTRACT

The p53 tumor suppressor gene plays a role in controlling a G1 phase checkpoint. The WAF1/CIP1 gene with encodes p21WAF1/CIP1 protein, an inhibitor of cyclin-dependent kinases, is a downstream mediator of p53 function. We examined expression of the WAF1/CIP1 gene and its relationship to growth arrest and differentiation in p53-null human leukemia cell lines. We show that p53-independent induction of WAF1/CIP1 occurs in human leukemia cells treated with 12-O-tetradecanoylphorbol-13-acetate, okadaic acid, or IFN-γ but not with retinoic acid, vitamin D3, or DMSO. Furthermore, WAF1/CIP1 induction correlates with growth arrest associated with monocyte-macrophage differentiation. The present studies support the idea that WAF1/CIP1 gene expression can be regulated through multiple mechanisms, suggesting that strategies may be designed to restore the G1 checkpoint controls in p53-null cells by targeting these p53-independent mechanisms of WAF1/CIP1 induction.

INTRODUCTION

p53 is a tumor suppressor gene thought to play a role in the multistep process of carcinogenesis (1, 2). p53 is mutated or abnormally expressed in some experimental animal tumors and in a variety of human cancers including leukemias and lymphomas (3–5). Additional evidence implicating p53 in carcinogenesis comes from studies of homozygous p53-null mice which exhibit a high incidence of malignancies, with a marked tendency toward lymphomas (6).

Ectopic expression of wild-type p53 in human tumor cell lines results in decreased tumor cell growth in vitro and in vivo (7–10). The growth inhibitory effect of p53 is thought to be mediated by its ability to arrest cells in G1 of the cell cycle (7–12). In addition to its role in cell cycle regulation, p53 has been implicated in DNA synthesis and repair, maintenance of genomic stability, cell differentiation, and programmed cell death (apoptosis; Refs. 8–12, for review see Ref. 13). Cellular context, interactions with cellular and viral oncoproteins, and changes in the physical conformation of p53 protein appear to influence these activities such that not all functions are active simultaneously.

The pleiotropic effects of wild-type p53 may be due in part to its ability to modulate gene expression. p53 protein is a transcription factor which can exert both positive and negative effects on the expression of genes (reviewed in Ref. 14). Activation of gene expression mediated by wild-type p53 protein involves specific interaction with DNA elements. A number of diverse genes has now been identified that contain wild-type p53-binding sequences including the MDM-2 oncogene (15), the muscle creatine kinase gene (16), the GADD45 gene (17), and the WAF1/CIP1 gene (18).

The WAF1/CIP1 gene encodes a Mr, 21,000 protein (p21WAF1/CIP1) which associates with cyclin-Cdk3 complexes and is capable of inhibiting kinase activity associated with these complexes (18–20). A major target of p21 inhibition is the cyclin-E-Cdk2 kinase complex whose activity is required for G1 progression into S phase (18–21). In addition, recent studies indicate that p21WAF1/CIP1 protein is able to inhibit DNA replication in vitro in what appears to be independent of cyclin-Cdk activity (22). The WAF1/CIP1 gene is transcriptionally activated in response to DNA damaging agents that trigger G1 arrest or apoptosis in cells with wide-type p53 but not in tumor cells harboring deletions or mutations of the p53 gene (21). Recent studies have revealed a p53-independent pathway of WAF1/CIP1 gene activation in primary embryonic fibroblasts derived from homozygous p53-null mice in response to individual growth factors such as platelet-derived growth factor or fibroblast growth factor, or following treatment with TPA, or okadaic acid (23).

A role for p53 in hematopoietic cell differentiation was suggested by the increased levels of p53 protein observed during maturation of normal bone marrow cells (24–26). For example, Kastan et al. (24) observed that p53 protein is not expressed at detectable levels in proliferating hematopoietic precursor cells but is detectable in the nonproliferative, mature cells of all lineages (B cell, T cell, granulocyte, and monococyte). Expression of wild-type p53 has been linked with apoptosis in murine M1 myeloid leukemic cells (27), DP16-1 erythroleukemia cells (28), and the murine hematopoietic cell line BAFl3 following exposure to ionizing radiation and interleukin 3 deprivation (21). Furthermore, restoration of wild-type p53 expression in an early pre-B lymphoid cell line (29) has been shown to advance the cells to a more mature differentiated phenotype with only a modest effect on cell growth. Recent studies have shown that introduction of wild-type p53 into the p53-null leukemia cell line HL60 is sufficient to induce granulocytic differentiation in the absence of detectable G1 arrest (30). Taken together, these observations indicate that the biological effects of p53 in hematopoietic cells are diverse and may be partitioned between growth arrest, apoptosis, and/or differentiation.

Since the WAF1/CIP1 gene is a key downstream mediator of wild-type p53 function in cell cycle control, we examined WAF1/CIP1 expression and its relationship to growth and differentiation in p53-null human leukemia cell lines. Introduction of wild-type p53 markedly elevated WAF1/CIP1 expression and suppressed proliferation. Furthermore, ectopic expression of the WAF1/CIP1 gene alone also suppressed proliferation. These data indicate that WAF1/CIP1 is an important mediator of the growth control function of p53 in human leukemia cells. In addition, our studies reveal that WAF1/CIP1 expression can be induced by TPA, okadaic acid, or IFNs in leukemia cells in the absence of p53. Taken together with previous observations

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2 The abbreviations used are: Cdk, cyclin-dependent kinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; BrdUrd, bromodeoxyuridine; Vit D3, vitamin D3; PKC, protein kinase C; IFN, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4—150 mM NaCl—4% calf serum—0.1% NaN3.

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(23), these results demonstrate that WAF1/CIP1 induction may occur under different physiological conditions. This suggests that alternative strategies may be designed to elevate the level of WAF1/CIP1 expression in cells harboring functionally inactive p53, perhaps restoring $G_1$ checkpoint growth controls.

MATERIALS AND METHODS

Cells and Plasmids. K562 chronic myelogenous leukemia cells, U973 monocytic leukemia cells, and HL60 acute myelogenous leukemia cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% FCS in a 37°C incubator containing 5% CO$_2$. All three cell lines do not express p53 mRNA or protein and are designated p53-null (5). pC-WAF-S (sense), pC-WAF1-AS (antisense), and pC-WAF1-ES (deletion mutant) expression plasmids have been described previously (18).

Reagents and Treatment. For okadaic acid experiments, okadaic acid (Sigma) was added to the aliquots of K562 or U973 cells at a final concentration ranging from 20 to 150 nM. The cells were incubated for 8 h. For the experiments in which K562, HL60, or U973 cells were treated with IFNs, TPA, retinoic acid, DMSO, or Vit D$_3$, the combination and duration of treatment are indicated in the legends of Figs. 1–8. The concentrations used: IFNs, 500 units/ml; TPA, 0.1 ng/ml; retinoic acid, 10 nM; DMSO, 1.2%; Vit D$_3$, 5 µg/ml; TPA, 20 ng/ml or 100 ng/ml as indicated.

Electroporation. Six million exponentially growing K562 cells were mixed with 10 µg of expression plasmid for wild-type or mutant p53, or WAF1/CIP1 in 0.45 ml of RPMI 1640 (without serum) at room temperature for 10 min. The cells were then pulsed (600 µF, 420 V) using a BTX600 transfection (BTX, Inc., San Diego, CA). After a 10-min incubation, the cells were incubated at 37°C with 5% CO$_2$. The transfected cells were collected at different times and lysed as described previously (31). Protein concentration was quantitated using the Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions.

Western Blotting. Forty µg of protein extract from cells were boiled in sample buffer (125 mM Tris-HCl, pH 6.8, 1% SDS, 2% β-mercaptoethanol, and 0.01% bromophenol blue) for 5 min and loaded onto a 10% SDS-polyacrylamide gel. After electrophoresis overnight at 45 V, the protein was transferred to an Immobilon polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), blocked with a solution composed of 50 µl Tris-HCl (pH 7.4), 0.9% NaCl, 3% nonfat dry milk, and 0.05% Tween 20 for 1 h, and then incubated overnight with anti-WAF1/CIP1 antibody (PharMingen, San Diego, CA), anti-p53 antibody DO-1 (Oncogene Science, Uniondale, NY), or anti-Rb antibody (Triton Bioscience, Inc., Alameda, CA). The levels of protein were analyzed using the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL) according to the manufacturer’s instructions.

Methylcellulose Assay. After electroporation, the cells were allowed to recover for 16 to 24 h. Viable cells (2 × 10$^6$) were mixed with 3 ml of liquid methylcellulose (Terry Fox Laboratory, Vancouver, British Columbia, Canada) containing 0.25 mg/ml hygromycin (GIBCO-BRL, Grand Island, NY). The cells were cultured in a humidified 5% CO$_2$ incubator at 37°C. After 2 weeks, colonies that consisted of more than 50 cells were counted.

Cell Cycle Assays. DNA synthesis was assessed by incorporation of BrdUrd and flow cytometric analysis essentially as described by Hoy et al. (32). Briefly, after incubation with 10 µM BrdUrd for 4 h, cells were fixed in 70% methanol, resuspended in 0.1 N HCl/0.7% Triton X-100 for 10 min at 4°C, washed excessively with PBS, and heated to 97°C for 10 min in deionized acidified water. The cells were then chilled on ice for 10 min and washed twice with IFA buffer containing 0.5% Tween 20. After incubation with 0.1 µl anti-BrdUrd-FITC isothiocyanate (Becton Dickinson) diluted 1:5 in IFA buffer for 30 min at 4°C, the cells were washed twice and then analyzed by flow cytometry.

Northern Blot Analysis. Total cellular RNA isolation and Northern blot analysis were carried out as described previously using approximately 15 µg RNA/jane (11). 32P-labeled human WAF1 and histone H3 hybridization probes were prepared as described previously (11, 18). Prehybridization, hybridization, and posthybridization washing conditions were by standard procedures.

Flow Cytometric Analysis. As marker of both granulocytic and monocytic differentiation the CD11b antigen was evaluated by fluorescence-activated cell sorting, according to Perussia et al. (33). Briefly, 3 × 10$^5$ cells were resuspended in PBS-1% human AB serum and the primary mAb anti-CD11b/B43.4, kindly provided by Dr. Perussia (Thomas Jefferson University, Philadelphia, PA), was added at a predetermined saturating concentration. After 30 min at 4°C, cells were washed three times and incubated with goat anti-mouse F(ab')$_2$ secondary antibody, FITC-conjugated (CAPPEL Laboratories, Cochranville, PA) for 30 min at 4°C. The samples were analyzed on an EPICS PROFILE II (Coulter Electronics, Hialeah, FL) and immunofluorescence was recorded on a logarithmic scale.

RESULTS

Wild-Type but not Mutant p53 Increases p21$^{WAF1/CIP1}$ Levels in p53-Null K562 Leukemia Cells. Previous studies have shown that wild-type p53 inhibits K562 cell proliferation whereas mutant p53 fails to do so (31). We sought to determine whether this was correlated with increased levels of p21$^{WAF1/CIP1}$. K562 cells were transfected with expression plasmids encoding wild-type p53 and various mutant p53 cDNAs. The level of p21$^{WAF1/CIP1}$ protein in cells was determined 48 h after transfection by Western blot assay. As shown in Fig. 1, wild-type p53 markedly increased the level of p21$^{WAF1/CIP1}$ protein in transfected cells. On the contrary, transfection of five frequently detected mutant p53 cDNAs and a C-terminal deletion mutant, p53(1–326), did not increase p21$^{WAF1/CIP1}$ protein level. The status of the RB1 protein was also examined in these experiments since recent in vitro studies suggest that RB1 protein is a substrate whose phospho-

![Fig. 1. Induction of p21 protein by wild-type p53. K562 cells were transfected with parental vector CMV.neo or expression plasmids containing either wild-type p53 or mutant p53 cDNAs. The p53 mutants are named based on the mutated codon followed by the amino acid. p53(1–326) is a mutant which has 67 amino acids deleted from the C terminus. Forty-eight h after transfection, cellular protein was extracted and p21$^{WAF1/CIP1}$ protein, p53 protein, and RB1 protein were analyzed by Western blot with specific antibodies.](attachment:image)
Two weeks after culture colonies composed of examination and counted.

Transfected cells were mixed with methylcellulose and selected in the presence of hygromycin. Two weeks after culture colonies composed of more than 50 cells were visualized by microscopic examination and counted.

**Ectopic WAF1/CIP1 Expression Is Sufficient to Inhibit Growth of K562 Cells.** We next sought to determine whether WAF1/CIP1 expression alone was sufficient to suppress the growth of K562 cells. We transfected expression plasmids containing human WAF1 sense, antisense, or a deletion mutant into cells. The transfected cells were mixed with methylcellulose and incubated in the presence of the drug hygromycin. As demonstrated in Fig. 2 and Table 1, transfection of WAF1 sense plasmid resulted in significantly fewer colonies, compared to cells transfected with WAF1 antisense or WAF1 mutant plasmids. Furthermore, p21WAF1/CIP1 protein was readily detectable in the nucleus of cells transfected with WAF1 sense plasmid by immunocytochemical staining using anti-WAF1 mAb (21). We could find no morphological signs of apoptosis in cytopsin preparations of cells expressing p21WAF1/CIP1 protein (data not shown).

**p53-independent Pathways of WAF1/CIP1 Induction in Leukemia Cell Lines.** To further characterize the behavior of the WAF1/CIP1 gene in leukemia cell lines; we sought to determine whether WAF1/CIP1 gene expression could be induced by mechanisms independent of p53 in leukemia cell lines. In these experiments, three p53-null human leukemia cell lines (K562, HL60, and U937) were examined. We tested a variety of agents which induce growth arrest or differentiation. Cells were exposed to okadaic acid, IFNs, TPA, DMSO, retinoic acid, Vit D3 alone, or in combination. The levels of the p21WAF1/CIP1 protein were evaluated by Western blot analysis.

In K562 cells, p21WAF1/CIP1 protein is induced by okadaic acid in a dose-dependent manner (Fig. 3). Okadaic acid treatment initially resulted in accumulation of the hyperphosphorylated p116Rbl form of the Rbl1 protein in cells that expressed only minimal levels of p21WAF1/CIP1 protein; however, accumulation p21WAF1/CIP1 protein was accompanied by a switch to underphosphorylated p110Rbl form.

The p21WAF1/CIP1 protein also accumulated with time in K562 cells treated with IFN-γ (Fig. 4, Lanes 1–4). In HL60 and U937 cells, the level of p21WAF1/CIP1 protein increased following exposure to TPA alone, or TPA plus IFN-α, or TPA plus interleukin 6 (Fig. 4, Lanes 10, 15, 16, and 17). On the contrary, exposure of cells to DMSO, Vit D3, or retinoic acid (Fig. 4) did not result in an increase in p21WAF1/CIP1 protein levels, at least within the time period evaluated. A slight but reproducible increase in p21WAF1/CIP1 protein levels was observed in HL60 cells treated with DMSO plus IFN-α (Fig. 4, Lane 7). These results indicated that the level of p21WAF1/CIP1 protein can be increased in human leukemia cell lines through p53-independent pathways.

**Induction of WAF1/CIP1 and Growth Arrest in HL60 and U937 Cells Treated with TPA.** The observation that p21WAF1/CIP1 protein levels increase in leukemic cells treated with TPA but not with Vit D3, DMSO, or retinoic acid, all of which are inducers of differentiation, prompted us to ask whether WAF1/CIP1 induction was correlated with growth suppression and/or with lineage-specific differentiation. For these experiments HL60 and U937 cells were treated with either TPA, which induces differentiation along the monocytic-macrophage pathway, or DMSO, which induces differentiation along the granulocytic pathway (34). WAF1/CIP1 mRNA expression was evaluated at different times after treatment by Northern blot analysis.

**Table 1** Colony formation of transfected K562 cells

<table>
<thead>
<tr>
<th>Transfectant</th>
<th>Colonies/dish</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAF1 (sense)</td>
<td>32, 36, 43, 46, 64, 74</td>
</tr>
<tr>
<td>WAF1 (antisense)</td>
<td>369, 738, 890</td>
</tr>
<tr>
<td>WAF1 (mutant)</td>
<td>529, 571, 726</td>
</tr>
</tbody>
</table>

Fig. 2. Overexpression of WAF1/CIP1 inhibits colony formation of K562 cells. K562 cells were transfected with expression plasmids containing WAF1 (sense), WAF1 (antisense), or WAF1 (mutant). Transfected cells were mixed with methylcellulose and selected in the presence of hygromycin. Two weeks after culture colonies composed of more than 50 cells were visualized by microscopic examination and counted.

Fig. 3. Induction of p21 protein by okadaic acid in K562 cells. p53-null K562 cells were exposed to increasing concentrations of okadaic acid: 20, 40, 50, 100, and 150 nM (Lanes 2–5). Untreated cells were used as a negative control, (Lane 1). Cells transfected with wild-type p53 were used as a positive control (Lane 7). The presence of p21WAF1/CIP1 protein and the state of Rbl phosphorylation were analyzed by Western blotting at 8–10 h after okadaic acid treatment or transfection.
Fig. 4. Induction of p21 protein by growth-inhibiting and differentiation-inducing agents. Three p53-null cell lines (K562, HL60, and U937) were treated and the presence of p21WAF1/CIP1 protein was analyzed by Western blotting. Anti-actin antibody was used to probe actin protein as a control for equal loading. Lane 1, untreated K562 cells (in this batch of cells, a low but detectable level of p21WAF1/CIP1 protein was detected); Lane 2, IFN-γ for 3 h; Lane 3, IFN-γ for 6 h; Lane 4, IFN-γ for 16 h; Lane 5, untreated HL60; Lane 6, 1.2% DMSO for 24 h; Lane 7, 1.2% DMSO for 24 h followed by IFN-α for 1.5 h; Lane 8, Vit D3 for 19 h; Lane 9, retinoic acid for 19 h; Lane 10, TPA for 19 h; Lane 11, IFN-α for 1 h; Lane 12, untreated U937 cells; Lane 13, Vit D3 for 16 h; Lane 14, interleukin 6 (300 units/ml) for 1 h; Lane 15, TPA for 19 h; Lane 16, TPA for 19 h followed by IFN-α for 1 h; Lane 17, TPA for 19 h followed by interleukin 6 for 1 h.

Fig. 5. Time course of WAF1/CIP1 mRNA expression in HL60 cells induced to differentiate with TPA. A, Northern blot analysis of WAF1/CIP1 mRNA expression at different times after treatment of exponentially growing cells with 20 nM TPA. Lane 1, untreated cells; Lanes 2–4, cells treated with TPA for 12, 24, and 48 h, respectively. B, total amounts of RNA loaded in each lane as demonstrated by ethidium bromide staining. C, evaluation of CD11b antigen expression in HL60 cells; untreated (□) or treated with TPA for 48 h (•). Cell numbers are shown on the ordinate and relative fluorescence intensity is shown on the abscissa.

Fig. 6. Time course of WAF1/CIP1 mRNA expression in U937 cells induced to differentiate with TPA. A, Northern blot analysis of WAF1/CIP1 mRNA and histone H3 expression at different times after treatment of exponentially growing cells with 20 nM TPA. Lane 1, untreated cells 24 h after plating; Lanes 2–4, cells treated with TPA for 12, 24, and 48 h, respectively. B, total amounts of RNA loaded in each lane as demonstrated by ethidium bromide staining. C, Evaluation of CD11b antigen expression in U937 cells; untreated (□) or treated with TPA for 48 h (•). Cell numbers are shown on the ordinate and relative fluorescence intensity is shown on the abscissa.
TPA. WAF1/CIP1 mRNA levels are in arbitrary WAF1/CIP1 mRNA levels and the percentage of HL60 and U937 cells with TPA. Shown are relative expression and DNA synthesis after treatment of cytometry. HL60, •; U937, •

conjugated anti-BrdUrd antibody staining and flow rated BrdUrd during a 4-h pulse at various times autoradiographs from Northern blot. HL60, O; U937, □

peak induction of WAF1/CIP1 mRNA expression occurs at 12 and 24 expression and cell cycle progression in HL60 and U937 cells. The effect of these agents on cell cycle progression was monitored simul different times after treatment with the differentiating agent. The CIP1 mRNA expression was examined by Northern blot analysis at different age of cells in S-phase declines as measured by incorporation of BrdUrd (Fig. 7). These results show that cessation of cell cycle progression coincides with WAF1/CIP1 induction in both HL60 and U937 cells induced to differentiate along the monocyte-macrophage pathway by treatment with TPA.

WAF1/CIP1 is Not Induced by Agents that Induce Differentiation Uncoupled from Early Growth Arrest. In these experiments HL60 cells were treated with retinoic acid, Vit D₃, or DMSO. WAF1/CIP1 mRNA expression was examined by Northern blot analysis at different times after treatment with the differentiating agent. The effect of these agents on cell cycle progression was monitored simultaneously by examining histone H3 RNA expression. Differentiation was evaluated by flow cytometric analysis of the CD11b marker. Fig. 8 shows the results of a typical experiment. WAF1/CIP1 mRNA expression is not induced in HL60 cells treated with retinoic acid, Vit D₃, or DMSO; although, as expected, it is induced by treatment with TPA (Fig. 8A). Histone H3 mRNA levels do not decrease in HL60 cells treated with retinoic acid, Vit D₃, or DMSO, indicating that, within the time frame evaluated, these agents do not have a significant effect on cell cycle progression.

DISCUSSION

This study demonstrates that WAF1/CIP1 gene expression can be activated in p53-null human leukemic cells by ectopic p53-dependent or p53-independent pathways. The novel findings of this study are: (a) Accumulation of p21WAF1/CIP1 protein is accompanied by increased levels of the underphosphorylated form of the retinoblastoma protein, RB1. (b) p53-independent induction of WAF1/CIP1 in leukemia cells occurs in response to differentiation-inducing agents that also produce early growth arrest but not by agents that produce differentiation uncoupled from early growth arrest. These findings will be discussed in the context of our current understanding of WAF1/CIP1-mediated growth control pathways.

Evidence for a link between the growth-suppressing activity of wild-type p53 and inactivation of cyclin-Cdks was provided by molecular cloning of the WAF1/CIP1 gene whose transcription is directly activated by wild-type p53 protein (18). The product of the WAF1/CIP1 gene, p21WAF1/CIP1 protein, binds to cyclin-Cdk complexes and inhibits the activity of multiple Cdks (including Cdk4, Cdk2, and cdc2), albeit with different efficiencies (19, 20). The cyclin-Cdks play an important role in controlling key cell cycle transition checkpoints which occur in G₁ and G₂-M (reviewed in Ref. 37). Once activated, G₁ cyclin-Cdks phosphorylate targets that include the retinoblastoma RB1 protein (38) and related proteins releasing bound inactive transcription factors such as E2F1 (39) and other related nuclear regulatory proteins (40) needed for the G₁ to S-phase transition and DNA replication. Phosphorylation of RB1 protein possibly mediated by cyclin D-Cdks (41, 42) creates a permissive state for the passage of cells from G₁ into S-phase. Studies in normal diploid fibroblasts following exposure to ionizing radiation have shown that p21WAF1/CIP1-mediated G₁ arrest is correlated with increased stability of wild-type p53 protein and inhibition of cyclin-Cdk activity associated with accumulation of the underphosphorylated form of RB1 protein (20). Thus, G₁ checkpoint controls appear to monitor the readiness of cell to progress into S-phase and start DNA replication.

Transfection of wild-type p53 into cells has previously been shown to reduce colony-forming efficiency (31). Here we show that p21WAF1/CIP1 protein levels are increased in K562 cell transfected with wild-type p53 but not mutant p53 expression plasmids. Furthermore, introduction of WAF1/CIP1 expression plasmids alone was sufficient to reduce colony formation in these cells. We could find no evidence of apoptosis in transfected cells which expressed increased levels of nuclear p21WAF1/CIP1 protein suggesting that programmed cell death was not responsible for reduced colony formation. Similar results have been reported for human brain, colon (18), and lung tumor cell lines, and normal human diploid fibroblasts (19). This suggests that

4 L. Grasso and W. E. Mercer, unpublished results.
WAF1/CIP1-mediated pathways represent a common mechanism of cell cycle control.

Increased levels of p21\textsuperscript{WAF1/CIP1} protein present in K562 cells transfected with wild-type p53 or treated with okadaic acid were accompanied by the accumulation of the underphosphorylated form of RB1 protein. It is worthwhile to mention that Hatzfeld et al. (43) have reported that inhibition of RB1 gene expression can release hematopoietic cells from a state of G0 arrest, implicating RB1 in hematopoietic cell cycle control. Furthermore, mice deficient for RB1 are nonviable and show defects in hematopoiesis and neurogenesis (44).

Taken together, these observations suggest that p21\textsuperscript{WAF1/CIP1} protein may be acting through RB1-mediated cell cycle control pathways to produce G1 growth arrest in human leukemia cells. Further studies will be required to test this hypothesis.

\textbf{WAF1/CIP1} gene expression has been shown to be induced by DNA damage induced by ionizing radiation that triggers G1 arrest or apoptosis in cells with a wild-type p53 gene, but not in tumor cells harboring deletions or missense mutations in the p53 gene (21). Recent studies have shown that WAF1/CIP1 expression can also be induced in embryonic fibroblasts derived from homozygous p53-null mice by the growth factors platelet-derived growth factor or fibroblast growth factor, or by treatment with TPA or okadaic acid revealing a p53-independent pathway (23). The present studies extend these observations to p53-null human leukemia cell lines. In HL60 and U937 cells treatment with TPA results in induction of WAF1/CIP1 gene expression and accumulation of p21\textsuperscript{WAF1/CIP1} protein accompanied by growth arrest and differentiation along a monocytic-macrophage lineage pathway. On the contrary, WAF1/CIP1 expression was not induced in HL60 cells treated with retinoic acid, Vit D3, or DMSO, which within the time frame evaluated did not produce growth arrest. The observation that the WAF1/CIP1 gene is not induced in HL60 cells induced to differentiate along a granulocytic pathway suggests that different signaling pathways may be involved. This idea is consistent with the observation that expression of c-jun is elevated in HL60 cells during monocytic but not granulocytic differentiation (45). Interestingly, WAF1/CIP1 expression was not induced in HL60 cells treated with Vit D3, although a differentiation characteristic of a monocytic phenotype uncoupled from growth arrest was observed. This suggests that WAF1/CIP1 expression in leukemic cells is correlated more with growth arrest than with a lineage-specific differentiation phenotype per se.

Okadaic acid treatment of U937 cells has been shown to be associated with induction of a differentiated monococyte-macrophage phenotype characterized by growth arrest, increased Mac-1 cell surface antigen expression, and down-regulation of c-myc gene expression (46). Similar effects have been observed in K562 cells (47). Treatment of K562 and U973 cells with okadaic acid also induces WAF1/CIP1 gene expression (present study). The mechanism by which okadaic acid or TPA induces WAF1/CIP1 gene expression in these human leukemic cell lines and in p53-null mouse embryo fibroblasts (23) is unknown. One may speculate that the effects of these agents most likely involves PKC-mediated phosphorylation pathways. PKC-mediated phosphorylation of target proteins occurs on serine and threonine residues (48). Okadaic acid is a potent and selective inhibitor of phosphatases 1 and 2A (49), the latter of which contribute to the dephosphorylation of phosphorylated PKC substrates (reviewed in Ref. 50). The notion that PKC pathways are involved in WAF1/CIP1 induction is supported further by the observation that the chemotherapeutic drug Adriamycin, which has been shown to activate PKC pathways in a variety of cell systems (51), is a potent inducer of WAF1/CIP1 in cells containing wild-type p53 (21) and is also capable of inducing WAF1/CIP1 albeit at higher doses in p53-null cells (23).

Finally, the identification of p53-dependent and -independent pathways of WAF1/CIP1 induction has several important implications. (a) WAF1/CIP1 inducibility by DNA damaging agents that rely on p53-dependent pathways may provide a molecular marker to assess the
functional status of the p53 gene in human tumors. (b) WAF1/CIP1 induction in p53-null tumor cells (p53-independent activation) may be useful as a molecular marker to screen for agents that can restore G1 checkpoint controls. (c) Alternative strategies may be used to induce WAF1/CIP1 gene expression in tumors with a functionally inactive p53 gene. Such strategies are likely to have clinical utility because as shown in this study they include some already in use as IFN therapy.

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p53-independent Induction of WAF1/CIP1 in Human Leukemia Cells Is Correlated with Growth Arrest Accompanying Monocyte/Macrophage Differentiation

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