



Effects of antisense p21 (WAF1/CIP1/MDA6) expression on the induction of differentiation and drug-mediated apoptosis in human myeloid leukemia cells (HL-60)

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The p21^{MDA6} gene product induces cell cycle arrest in p53-null human leukemic cells exposed to differentiation stimuli. We employed an HL-60 cell line stably transfected with a p21^{MDA6} antisense construct to compare the effects of p21^{MDA6} dysregulation on the response of myeloid leukemia cells to differentiating and cytotoxic agents. Antisense-expressing cells (HL-60/AS5) treated with 5 nM PMA for 24 h exhibited attenuated induction of p21^{MDA6} compared to empty vector controls (HL-60/V2). This phenomenon was accompanied by a reduction in the percentage of cells undergoing G₁ arrest (67.6 ± 4.7 vs 82.9 ± 1.3; *P* ≤ 0.01) and expressing the monocytic maturation marker cd11b (35.5 ± 2.8 vs 50.5 ± 2.4; *P* ≤ 0.005). Although HL-AS5 and HL-60/V2 cells did not exhibit obvious differences in the phosphorylation status of the retinoblastoma protein (pRB), in E2F complex formation, or in p27^{kip1} induction following PMA exposure, inhibition of activity of cyclin-dependent kinase-2 was attenuated in the antisense-expressing line. A 24-h exposure to 5 nM PMA also reduced the cloning efficiency of HL-60/V2 cells to a significantly greater extent than HL-60/AS5 cells (ie to 30.1 ± 7.0 vs 57.2 ± 5.6 of controls; *P* ≤ 0.01). In contrast to the disparate responses to PMA, HL-60/AS5 and HL-60/V2 cells treated with the antimetabolite 1-β-D-arabinofuranosylcytosine (Ara-C; 10 μM for 6 h) displayed equal susceptibility to G₁ arrest, apoptosis, and inhibition of clonogenicity, phenomena unaccompanied by p21^{MDA6} and p27^{kip1} induction, or pRB dephosphorylation. These observations indicate that dysregulation of p21^{MDA6} in p53-null human myeloid leukemia cells interferes with PMA-related G₁ arrest, CDK-2 inhibition, differentiation, and loss of clonogenic survival in the absence of obvious alterations in pRB phosphorylation status or E2F complex formation. They also provide functional evidence that p21^{MDA6} induction does not appear to be required for Ara-C-induced apoptosis, G₁ arrest, or the resulting reduction in the self-renewal capacity of HL-60 cells.

Keywords: p21; MDA-6; apoptosis; Ara-C; cell cycle arrest; HL-60 cells

Introduction

Human p21 melanoma differentiation-associated gene 6 (MDA6),¹⁻⁴ also referred to as wild-type p53 activated factor-1 (WAF1),⁵ cyclin-dependent kinase (CDK) interacting protein-1 (CIP1),^{6,7} and senescent cell-derived inhibitor-1 (SDI1)⁸ is an inhibitor of several CDKs. p21^{MDA6} appears to be a normal constituent of active cyclin/CDK complexes in cells expressing functional p53.^{9,10} The p53-dependent induction of p21^{MDA6} has been well characterized in response to DNA damage and differentiating agents.^{11,12} As the molar ratio of p21^{MDA6} to CDK increases above 1, the kinase activity of a number of cyclin/CDK combinations is inhibited,^{13,14} resulting in G₁ or G₂ cell cycle arrest.^{12,15} A certain percentage of these cells subsequently undergoes apoptosis. More recently, p53-inde-

pendent induction of p21^{MDA6} has been observed in response to both differentiating and DNA damaging agents.^{3,4,16-19} However, not all p53-negative cell lines express p21^{MDA6} in response to all stimuli. For example, following γ-irradiation, p45.41A mouse embryonic fibroblasts do not express p21^{MDA6},¹⁸ whereas KG-1 cells exhibit a robust induction.¹⁹ Additionally, different HL-60 sublines, when treated with retinoic acid or vitamin D₃ either fail to display p21^{MDA6} induction¹³ or exhibit strong up-regulation.¹⁷ Furthermore, cell lines lacking functional p53 appear to lack constitutive expression of p21^{MDA6} and are more sensitive to its inhibitory effects when induced via p53-independent mechanisms.^{6,19,20}

The role that p21^{MDA6} plays in apoptosis varies between cell lines and the inducing stimulus. For example, BAF3 murine hematopoietic cells undergoing apoptosis following interleukin-3 (IL-3) withdrawal or serum starvation do not express increased levels of p21^{MDA6}.^{21,22} In addition, RKO cells undergo apoptosis, but fail to undergo cell cycle arrest or induce p21^{MDA6} in response to phospholipase A₂ (PLA₂).²³ Furthermore, thymocytes and intestinal epithelial cells obtained from p21^{MDA6} knockout mice undergo apoptosis when irradiated.^{24,25} Conversely, numerous cell lines containing functional or mutant p53 respond to DNA damage by growth arrest in G₁ and apoptosis concomitant with p21^{MDA6} up-regulation.^{16,23} However, apoptosis can also be induced in these p53-positive cell lines in a p21^{MDA6}-independent manner.¹⁶ Thus, it is apparent that p21^{MDA6} expression is not necessary for apoptosis to proceed in all cell types, but its induction may be required in certain circumstances depending on the cell line and the provoking stimulus.

There are also unresolved questions regarding the role of p21^{MDA6} in apoptosis induced by antineoplastic agents in human myeloid leukemia cells. For example, studies demonstrating the failure of 1-β-D-arabinofuranosylcytosine (Ara-C) to induce p21^{MDA6} in HL-60 cells, imply, albeit indirectly, the absence of a role for p21^{MDA6} in drug-induced apoptosis.¹¹ On the other hand, Dou and Lui²⁶ described dephosphorylation of the retinoblastoma protein (pRb) in HL-60 cells exposed to similar concentrations of Ara-C. Since pRb dephosphorylation and G₁ arrest are known to be downstream consequences of p21^{MDA6} induction, the latter observation is potentially compatible with a role for p21^{MDA6} in drug-induced cell death.

Currently, little direct evidence exists linking p21^{MDA6} expression and cell death in human leukemia cells. The aim of this study was to gain further insight into the functional role of p21^{MDA6} with regard to both differentiation and drug-induced apoptosis in a human myeloid leukemia cell line (HL-60) known to be p53 null due to a major deletion in the p53 gene.²⁷ To this end, an HL-60 cell line stably transfected with and expressing antisense p21^{MDA6} was employed to characterize the effects of p21^{MDA6} dysregulation on responses to phorbol 12-myristate 13-acetate (PMA) and Ara-C. Our results indicate that p21^{MDA6} antisense-expressing cells display a reduced capacity to up-regulate p21^{MDA6} in response to PMA

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and that this phenomenon is accompanied by a partial reduction in cell cycle arrest, expression of differentiated features, and loss of clonogenic survival. In contrast, G₁ arrest, apoptosis, and inhibition of clonogenicity following Ara-C exposure are equivalent in both p21^{MDA6} antisense and control cells, providing direct evidence that cell death occurring in response to this agent proceeds via p21^{MDA6}-independent pathways.

Materials and methods

Drugs and reagents

Phorbol 12-myristate 13-acetate (Sigma Chemical, St Louis, MO, USA) was diluted in sterile DMSO. 1-β-D-arabinofuranosylcytosine (free base; Sigma) was diluted in sterile water. PMA was stored at -20°C under light-free conditions and diluted to appropriate final concentrations in media. Ara-C was stored at 4°C and diluted to appropriate final concentrations in media. Vehicle controls of water and DMSO (≤0.01%) were included in all experiments and consistently found to be equivalent to drug-free controls in gene expression, protein expression, and occurrence of apoptosis.

Cell culture and drug exposure

The human promyelocytic leukemia cell line, HL-60, was derived from a cell line originally described by Gallagher *et al.*²⁸ U937 monocytic leukemia cells were derived from a cell line as previously reported.²⁹ HL-60 cell lines expressing antisense p21^{MDA6} were established by introducing pREP4-p21^{MDA6}_{3,30} (pREP4 from Invitrogen, San Diego, CA, USA) into HL-60 cells via electroporation (275 V, 750 μF, R9) and selected by limiting dilution in 400 μg/ml hygromycin B (Boehringer Mannheim, Indianapolis, IN, USA). The pREP4-p21^{MDA6} plasmid is a eukaryotic expression vector in which the p21^{MDA6} antisense sequence is under the control of the Rous sarcoma virus long terminal repeat promoter. The approach employed utilized an antisense construct directed against the entire p21^{MDA6} coding region. This avoids targeting a specific region of the molecule that might result in ineffective suppression of p21^{MDA6}. The p21^{MDA6} antisense clones were screened by treating cells with 5 nM PMA for 24 h and evaluating p21^{MDA6} expression by Western blot. The clone of cells chosen for all subsequent experiments, HL-60/AS5 (antisense-5), exhibited the most attenuated induction of p21^{MDA6} in response to PMA. Empty vector-containing cells, HL-60/V2 (vector-2), were used as controls in each experiment. All cells were grown in RPMI 1640 (phenol red-free formulation; Sigma) supplemented with 0.2% sodium bicarbonate, 1.0% sodium pyruvate, non-essential amino acids, L-glutamine, PSN antibiotic mix (Sigma) and 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT, USA); empty vector and pREP4-p21^{MDA6} cells were also supplemented with 400 μg/ml hygromycin B. All cultures were maintained under a fully humidified atmosphere of 95% air and 5% CO₂ at 37°C. Cultures were routinely screened for mycoplasma contamination with a rapid hybridization assay for mycoplasma RNA (Gen-Probe, San Diego, CA, USA) and consistently found to be mycoplasma-free. Cell densities were determined by Coulter (Hialeah, FL, USA) counter and cell viability was assessed by hemacytometer and trypan blue exclusion. For experimental incubations, cells in log-phase growth were sus-

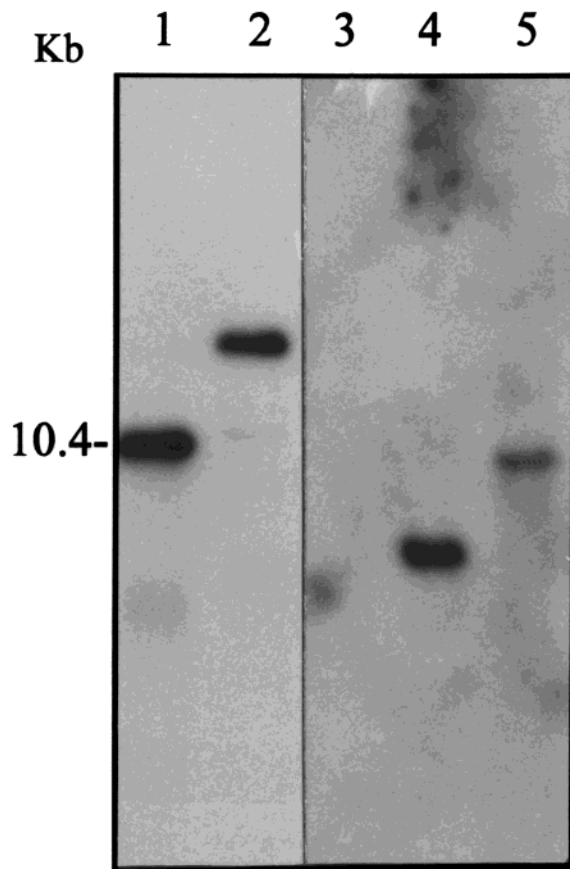


Figure 1 Southern blot of HL-60 DNA probed with the hygromycin B resistance gene. Genomic DNA was isolated, digested with *Bam*HI, and separated on a 0.7% agarose/TBE gel along with vector DNA. The DNA was transferred to nylon and probed as described in Materials and methods. Lane 1, pREP4 vector; lane 2, pREP4 vector with p21^{MDA6} antisense insert; lane 3, parental HL-60 DNA; lane 4, empty vector HL-60 transfectant; lane 5, p21^{MDA6} antisense HL-60 transfectant.

ended at a density of 4 × 10⁵ cells/ml containing the appropriate concentrations of inhibitor and maintained as described above. Incubations containing PMA were not exposed to direct light. Experimental incubations were terminated by pelleting cells at 400 g for 10 min and aspiration of the media. Cell pellets were subsequently prepared for procedures described below.

Southern analysis

Total cellular DNA from 1 × 10⁷ cells was isolated following a method described in *Current Protocols in Molecular Biology*.⁶⁶ DNA was quantitated and 10 μg was digested to completion with *Bam*HI at 37°C for 4 h. The DNA was separated on a 0.7% agarose/1 × TBE (45 mM Tris-borate, 10 mM EDTA, pH 8.0) gel at 100 V for 6 h. The DNA was blotted onto nylon (Schleicher and Schuell, Keene, MD, USA) by upward capillary transfer for 16 h with 0.5 M NaOH/1.5 M NaCl and then cross-linked to nylon by baking at 80°C for 2 h. Blots were then hybridized with a *Hinc*II fragment of the expression vector containing the hygromycin B resistance gene. The probe was nick-translated with α-dCTP³² (3000 Ci/mM) (New England Nuclear, Boston, MA, USA) using a kit and protocol from Life Technologies (Gaithersburg, MD, USA). Blots were

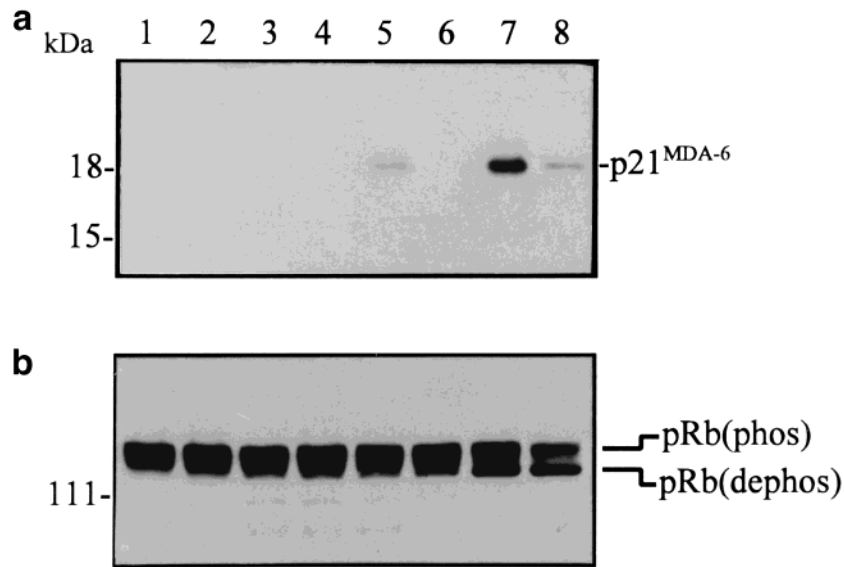


Figure 2 Western blot showing time course of (a) p21^{MDA6} and (b) pRb induction in response to 5 nM PMA in HL-60/V2 and HL-60/AS5 cells. Following 0, 6, 12 and 24 h exposure to PMA (HL-60/V2, lanes 1, 3, 5, 7; HL-60/AS5, lanes 2, 4, 6, 8, respectively), cells were washed, pelleted, and lysed as described in Materials and methods. Equivalent amounts of protein (25 μ g) were separated and transferred to nitrocellulose. The blots were probed with the appropriate primary antibody followed by a goat anti-mouse-peroxidase secondary antibody and developed by chemiluminescence. The position of prestained molecular weight markers is indicated on the left, while the positions of the identified proteins are on the right. Two additional experiments yielded similar results.

then washed extensively in $0.2 \times$ SSC/0.1% SDS ($1 \times$ SSC = 150 mM NaCl, 15 mM sodium citrate) at 65°C and placed on Fuji RX film (Fuji Photo Film, Tokyo, Japan) with intensifying screens and exposed at -90°C.

Western analysis

Whole cell pellets (1×10^7) were washed twice in PBS. Cell pellets were then resuspended in 50 μ l PBS and lysed by the addition of 50 μ l $2 \times$ loading buffer ($1 \times = 30$ mM Tris-base, pH 6.8, 2% SDS, 2.88 mM β -mercaptoethanol, 10% glycerol, 0.1% bromophenol blue). Lysates were boiled for 10 min, centrifuged at 12 800 g for 5 min, and quantified using Coomassie protein assay reagent (Pierce, Rockford, IL, USA). Equal amounts of protein (25 μ g) were separated by SDS-PAGE (5% stacker and 6% (pRb) or 12% (p21^{MDA6} and p27^{kip1}) and electroblotted to nitrocellulose. The blots were stained in 0.1% amido black and destained in 5% acetic acid to ensure transfer and equal loading. The blots were then blocked in TBS-Tween (0.05%) and 5% milk for 1 h at 22°C. Blots were incubated in fresh blocking solution and probed for 4 h with the appropriate dilution of primary antibody: pRb (1:500 (G3-245); Pharmingen, San Diego, CA, USA); p21^{MDA6} and p27^{kip1} (both 1:500; Transduction Laboratories, Lexington, KY, USA). Blots were washed 3×5 min in TBS-T and then incubated with a 1:1000 dilution of peroxidase-conjugated secondary antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) in TBS-T for 1 h at 22°C. Blots were again washed 3×5 min in TBS-T and then developed by enhanced chemiluminescence (Pierce). Autoradiographs were quantitated via laser densitometry (Molecular Dynamics, Sunnyvale, CA, USA).

DNA fragmentation

Quantitative spectrofluorophotometry of apoptotic DNA fragments was performed as previously described.³¹ Briefly, cell pellets (3×10^6) were lysed overnight at 4°C in 300 μ l of 0.1% Triton X-100, 5 mM Tris-HCl (pH 8.0), and 20 mM EDTA. The lysates were centrifuged at 48 000 g for 40 min at 4°C and the pellets discarded. The presence of low molecular weight DNA fragments in all lysates was determined by diluting 20 μ l samples in 980 μ l assay buffer (3 M NaCl, 10 mM Tris-HCl, 1 mM EDTA, 1 μ g/ml bisbenzimidazole trihydrochloride) and monitoring net fluorescence ($\lambda_{ex} = 365$, $\lambda_{em} = 460$). DNA values were calculated against a calf thymus DNA standard and expressed as ng DNA/ 10^6 cells.

Cell morphology

Following treatment, cytocentrifuge slides were prepared containing 8×10^4 cells per slide. Slides were stained with 20% Wright-Giemsa and photomicrographs taken at 500 \times showing typical cell morphology using a Polaroid Microcam and 331 film (Polaroid, Atlanta, GA, USA). The percentage of apoptotic cells was determined by evaluating approximately 500 cells per condition in triplicate.

Cell cycle analysis

Following drug treatment, cells were pelleted at 500 g and resuspended in 1 ml cell cycle buffer (0.38 mM sodium citrate, 0.1% Triton X-100, 7 Kunitz/ml RNase B, 50 μ g/ml propidium iodide) at a concentration of 1×10^6 cells/ml. Samples were placed on ice overnight and analyzed using a Becton-Dickin-

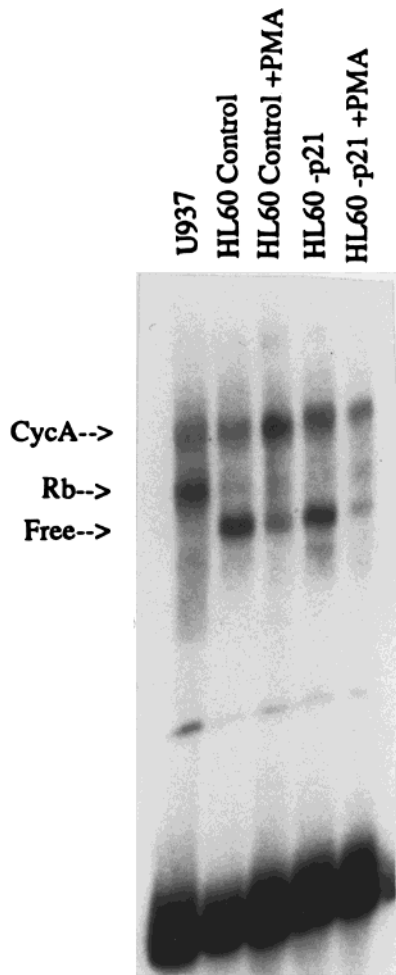


Figure 3 An electrophoretic mobility shift assay for E2F. Extracts from U937 or HL-60 cells were analyzed as described in Materials and methods. U937, untreated U937 cells; HL60 control, untreated HL-60/V2; HL60 control + PMA; HL-60/V2 treated with 5 nM PMA for 24 h; HL60-p21, untreated HL-60/A5; HL60-p21 + PMA, HL-60/A5 treated with 5 nM PMA for 24 h. The positions of free E2F as well E2F complexed with p107/cyclin A as well as pRb are shown on the left.

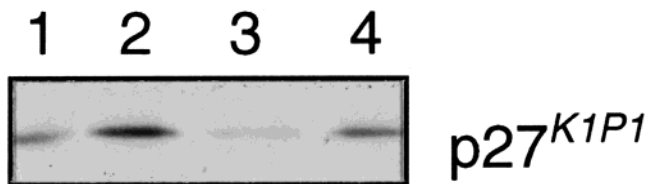


Figure 4 Cells were exposed to 5 nM PMA for 24 h after which expression of the CDK1 p27^{K1P1} was determined by Western analysis as described in the text. Lane 1, untreated V2 cells; lane 2, HL-60/V2 cells + PMA; lane 3, untreated HL-60/A5 cells; lane 4, HL-60/A5 cells + PMA.

son FACScan flow cytometer and Verity Winlist software (Verity Software, Topsham, ME, USA).

CD11b expression

After drug exposure, cells were pelleted at 500 g and resuspended in cold PBS at a concentration of 5×10^5 cells/ml. The

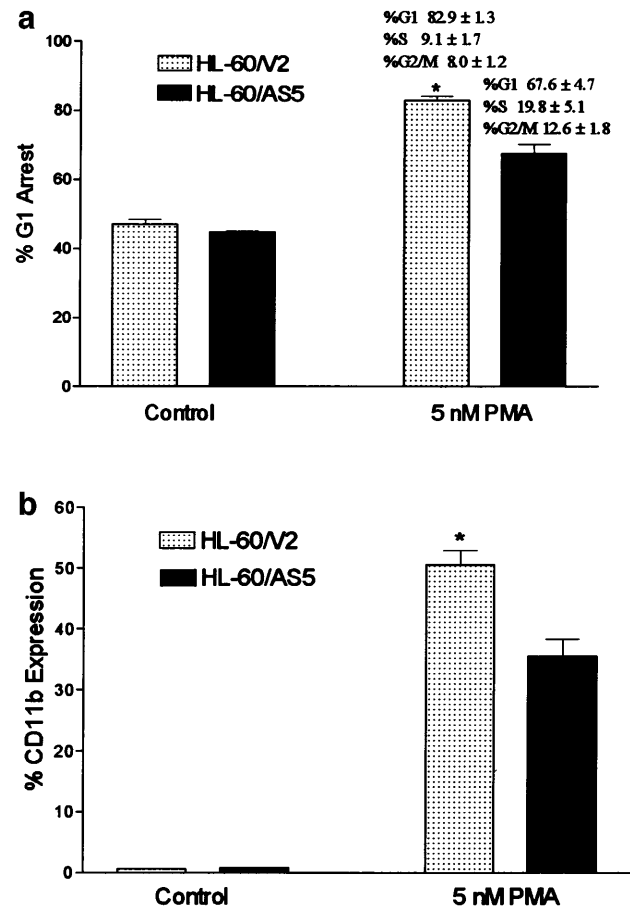


Figure 5 Changes in cell cycle distribution and CD11b expression following 24-h exposure to 5 nM PMA. Cells were pelleted and resuspended in either propidium iodide for cell cycle analysis or a fluorescein conjugated anti-CD11b antibody in PBS for CD11b analysis as described in Materials and methods. (a) *, a significantly larger percentage of HL-60/V2 cells arrested in G₀/G₁ (82.9 ± 1.3 vs 67.6 ± 4.7) compared to HL-60/AS5 cells ($P < 0.01$). (b) *, a significantly larger percentage of HL-60/V2 cells expressed the monocytic marker, CD11b (50.5 ± 2.4 vs 35.5 ± 2.8) when treated with PMA compared to HL-60/AS5 controls ($P < 0.01$). The results of cell cycle and CD11b expression studies represent the means for three replicate determinations ± s.e.

cell suspension (100 μ l) was mixed with 10 μ l of fluorescein isothiocyanate-labeled antibody (CD11b or IgG2 α control; Becton-Dickinson, Mountain View, CA, USA) and placed on ice for 20 min. Following this incubation, 900 μ l cold PBS was added to each sample and the cells were analyzed using a Becton-Dickinson FACScan flow cytometer and Verity Winlist software.

Clonogenic growth assay

Following drug treatment, cells were enumerated by hemacytometer, washed free of drug (3 \times) in 1 \times media with no FBS, and plated in triplicate in 12-well plates at 500 cells/ml in media containing 20% FBS and 0.3% agar. Plates were incubated in fully humidified atmosphere of 95% air and 5% CO₂ at 37°C and colonies consisting of more than 50 cells were counted 10–12 days after plating.

H1 kinase assay

Following treatment, cells were washed twice with PBS and resuspended in homogenization buffer (30 mM β -glycerophosphate, 5 mM NaF, 20 mM $MgCl_2$, 2.5 mM EGTA, 30 mM HEPES pH 7.4, and 10 μ g/ml each chymostatin, leupeptin, aprotinin, pepstatin and soybean trypsin inhibitor (CLAPS). The cell suspension was dounce homogenized, briefly sonicated, and centrifuged at 12800 *g* for 15 min at 4°C. The supernatant was transferred to a new tube and the protein concentration quantified using Coomassie protein assay reagent (Pierce). Equal amounts of protein (300 μ g) were incubated with 1 μ g anti-CDK2 antibody (Transduction Laboratories) overnight at 4°C and then 50 μ l of goat anti-mouse-IgG-Dynabeads (Dyna, Oslo, Norway) were added and incubated for an additional 4 h. The beads were washed 5 times (1 \times TBS, 25 mM β -glycerophosphate, 25 mM NaF, 0.25% Tween-20 and CLAPS) and resuspended in 20 μ l homogenization buffer. Kinase activity was assayed in a 25 μ l volume by mixing 5 μ l sample, 10 μ l 2.5 \times kinase buffer (1 \times = 20 mM Hepes, pH 7.4, 20 mM β -glycerophosphate, 15 mM $MgCl_2$, 1 mM EGTA, 0.4 mM dithiothreitol, 0.5 μ M protein kinase A inhibitor, 0.5 mg/ml histone H1), and 10 μ l ATP (0.3 μ Ci γ -ATP³²/ μ l in 50 μ M ATP) (6000 Ci/mmol; NEN DuPont, Wilmington, DE, USA) and incubating at 32°C for 15 min. Kinase reactions were performed in triplicate with and without substrate. The reactions were terminated by spotting 20 μ l on to phosphocellulose disks and washing twice for 5 min with 1% H_3PO_4 and twice with H_2O . Incorporated phosphate was determined by scintillation counting.

E2F electrophoretic mobility shift assay (EMSA)

An EcoRI–HindII fragment of the adenovirus E2 promoter containing two E2F binding sites (TTTCGCGC) was end-labeled using Klenow fragment and used as the probe for the gel-retardation assay.^{32,33} Eight micrograms of whole cell extracts from HL-60 cells were incubated in 13 μ l protein binding buffer for 15 min on ice and then mixed with 7 μ l of the probe mixture, which contains 0.2 ng ³²P-labeled E2F probe, 2 μ l 10 \times shift buffer (0.2 M HEPES pH 7.9, 0.4 M KC1, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM NaF, 1 mM Na_3VO_4), 1 μ g ssDNA (Sigma), 20 mg BSA (Boehringer Mannheim) and 0.1% NP-40. After an additional 15 min incubation at room temperature, the reaction products were separated on a 4% polyacrylamide gel in 0.25 \times TBE at 300 V for 2.5 h. The gel was dried and detected by autoradiography.

Results

HL-60 cells were transfected by electroporation with either the pREP4 vector or the pREP4 vector containing the p21^{MDA6} coding region in the antisense orientation. Transfected cells were selected for resistance to hygromycin by limiting dilution and analyzed by Southern blot for the presence of the hygromycin resistance gene (Figure 1). HL-60 cells do not express detectable levels of p21^{MDA6} constitutively, but express p21^{MDA6} in response to certain agents or conditions that induce differentiation (ie PMA). Stably transfected cells were analyzed further by Western blot for attenuated up-regulation of p21^{MDA6} in response to 5 nM PMA for 24 h (not shown). Following PMA exposure, a clone exhibiting the greatest attenuation of p21^{MDA6} was selected for all subsequent experiments (HL-60/AS5), as well as a clone containing the pREP4 empty vector (HL-60/V2).

The time-course of p21^{MDA6} expression as well as expression of the immediate downstream effector protein, pRb, was then examined in response to 5 nM PMA at 0, 6, 12 and 24 h (Figure 2). The induction of p21^{MDA6} in HL-60/V2 cells was observed first at 12 h and more prominently at 24 h. In contrast, p21^{MDA6} induction in HL-60/AS5 cells was not observed at 12 h and was expressed at \approx 15% of levels observed in HL-60/V2 cells at 24 h (Figure 2a). Because p21^{MDA6} binds to and inhibits the kinases that help maintain pRb in a hyperphosphorylated state,²⁹ we next examined the phosphorylation status of pRb. Interestingly, although the levels of p21^{MDA6} were greatly attenuated in the HL-60/AS5 cells in response to PMA, these cells exhibited dephosphorylation of pRb similar to treated control cells (Figure 2b).

One of the possible consequences of pRb dephosphorylation is binding to the E2F transcription factor, an event implicated in regulating the G₁–S transition.^{34–37} Moreover, it has recently been reported that interactions between E2F and the pRb-like protein p130 can be affected by p21^{MDA6}.³⁸ Hence, attempts were made to evaluate the status of E2F-containing complexes in cells expressing antisense p21^{MDA6} using an electrophoretic mobility shift assay^{33,39,40} (Figure 3). An E2F-pRb complex could be clearly seen in extracts of unstimulated U937 monocytic leukemic cells, but not in control HL-60 cells, a finding consistent with published reports.⁴¹ The majority of complexed E2F in both empty vector and p21^{MDA6} antisense cells migrated in the free form with the remainder in the position of a p107-cyclin A–E2F complex, as identified in previous gel-retardation assays.⁴¹ Upon stimulation with PMA, both cell lines displayed a reduction in free E2F. Moreover, the extent of this reduction was at least as great in the p21^{MDA6} antisense line as in empty vector controls. In both

Table 1 Clonogenic growth of p21^{MDA6} antisense and empty vector cells treated with 10 μ M Ara-C

Cell line	Empty vector HL-60/V2 (% control)	Antisense p21 HL-60/AS5 (% control)	P value
5 nM PMA	30.1 \pm 7.0	57.2 \pm 5.6	\leq 0.01
10 μ M Ara-C	42.6 \pm 10.3	49.4 \pm 11.9	>0.05

HL-60/V2 and HL-60/AS5 cells were either untreated or exposed to 5 nM PMA (24 h) or 10 μ M Ara-C (6 h). Cells were then washed free of drug and 500 residual cells/condition were plated in soft agar. The plates were incubated in a fully humidified atmosphere of 95% air and 5% CO₂ at 37°C and colonies consisting of groups of \geq 50 cells, scored 10–12 days after plating. Colony formation was expressed as the total number of colonies for each condition relative to untreated controls. Values represent the means for four (PMA) or seven (Ara-C) experiments performed in triplicate \pm 1 s.d.

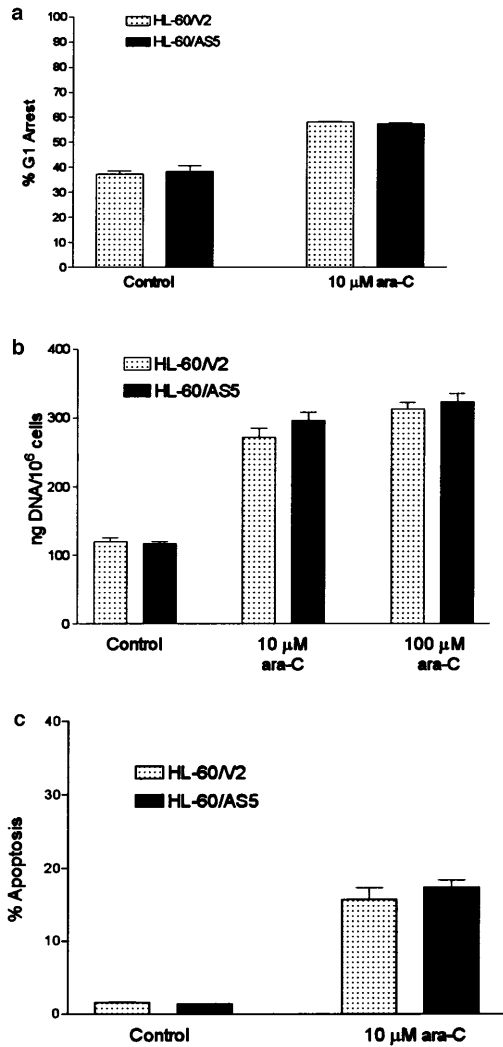


Figure 6 G₁ arrest and induction of apoptosis in HL-60/V2 and HL-60/AS5 cells following a 6-h exposure to Ara-C. (a) After treatment with 10 μM Ara-C, cells were pelleted and resuspended in propidium iodide solution for cell cycle analysis. The increase in the G₀/G₁ cell fraction following a 6-h exposure to 10 μM Ara-C was equivalent in antisense-expressing and controls cells (ie 17 and 18%, respectively; *P* > 0.05). Values represent the means for three separate experiments ± 1 s.d. (b) Spectrofluorometric analysis of low molecular weight DNA from cells treated with 10 or 100 μM Ara-C for 6 h. Values are expressed as ng DNA/10⁶ cells and represent means for triplicate determinations ± s.e. from a representative experiment; two additional studies yielded comparable results. (c) Following exposure of cells to 10 μM Ara-C for 6 h, Wright–Giemsa-stained preparations were monitored for apoptosis. Values are expressed as the percentage of apoptotic cells and represent the means for least 500 cells scored per condition from three replicate experiments ± 1 s.d.

cell lines, the bulk of E2F following PMA treatment migrated in the position of the cyclin A complex. Consequently, the impairment in p21^{MDA6} induction by PMA in antisense-expressing cells did not abrogate reductions in levels of free E2F. In separate studies, we also examined expression of the CDK inhibitor, p27^{kip1} which has been implicated in G₁ arrest of HL-60 cells following exposure to vitamin D₃ and PMA.^{37,42} Modest up-regulation of p27^{kip1} expression was observed in both antisense and empty vector cells following incubation with PMA (Figure 4).

Because of the apparent discordance between attenuated

up-regulation of p21^{MDA6} in HL-60/AS5 cells and dephosphorylation of pRb in both HL-60/AS5 and control cells, we monitored CDK-2 with represents one of the kinases responsible for maintaining pRb in the hyperphosphorylated state.^{43,44} Following treatment of HL-60/AS5 cells with PMA (5 nM; 24 h), CDK-2 activity did not change appreciably. However, activity in PMA-treated HL-60/V2 cells was 31.5 ± 9.3% lower than that observed in their antisense counterparts (*P* ≤ 0.05; data not shown). This finding is consistent with the attenuated induction of p21^{MDA6} in HL-60/AS5 cells.

The ultimate consequence of PMA treatment of HL-60 cells is the induction of cellular maturation. One of the critical events associated with differentiation is cell cycle arrest in G₀/G₁, which is thought to be mediated, at least in part, by p21^{MDA6}. Therefore, we next examined the cell cycle distribution of antisense expressing and control cells treated with PMA as well as the appearance of the monocytic differentiation marker, CD11b. While both HL-60/AS5 and control cells displayed evidence of G₁ arrest compared to untreated controls, the fraction of HL-60/AS5 cells arrested in G₀/G₁ was significantly less than that observed in HL-60/V2 cells (Figure 5a). A similar result was observed in the case of CD11b expression. Both antisense and control cell lines exhibited increases in the percentage of cells expressing CD11b, but this response was significantly attenuated in HL-60/AS5 cells (Figure 5b). In separate studies, the percentage of apoptotic cells following a 24-h exposure to 5 nM PMA was equivalent in the two cell lines (V2 = 8.7 ± 2.1%; V5 = 7.1 ± 1.6%; *P* > 0.05; data not shown). Thus, compared to the HL-60/V2 line, HL-60/AS5 cells were partially impaired in their ability to undergo G₁ arrest and express differentiation markers in response to PMA, but retained their capacity to undergo apoptosis.

Finally, the clonogenic growth of HL-60/V2 and HL-60/AS5 cells was compared following treatment with 5 nM PMA for 24 h (Table 1). While both cell lines showed a reduction in clonogenic potential, clonogenicity of empty vector cells was significantly lower than that of the antisense cells (30.1 ± 7.0% vs 57.2 ± 5.6% respectively; *P* ≤ 0.01). This finding is consistent with the notion that a block in G₁ arrest leads to attenuation of terminal differentiation, which is in turn accompanied by preservation of self-renewal capacity.

Having demonstrated diminished induction of p21^{MDA6} in response to a differentiation stimulus, we next examined the effect of dysregulated p21^{MDA6} expression on various aspects of drug-induced apoptosis. HL-60/AS5 and empty vector cells were treated with 10 μM Ara-C for 6 h and the cell cycle distribution was examined. Both antisense and empty vector cells underwent G₁ arrest to an equivalent extent, ie to ≈20% above levels in untreated cells (Figure 6a). Ara-C treatment also produced an equivalent sub-G₀/G₁ peak in both antisense and empty vector cells indicative of apoptosis (not shown). Subsequently, the extent of apoptosis resulting from Ara-C treatment was examined more rigorously by monitoring low molecular weight DNA fragmentation⁴⁵ and morphologic changes in cells exposed to 10 and 100 μM Ara-C for 6 h. Both cell lines exhibited equivalent degrees of DNA fragmentation following treatment with both concentrations of Ara-C (Figure 6b). Furthermore, when Wright–Giemsa-stained slides of HL-60/V2 and HL-60/AS5 cells treated with 10 μM Ara-C were examined for cyto-architectural features of apoptosis, the extent of cell death observed in the two cell lines was equivalent (Figures 6c and 7). These findings were confirmed by TUNEL analysis (not shown).

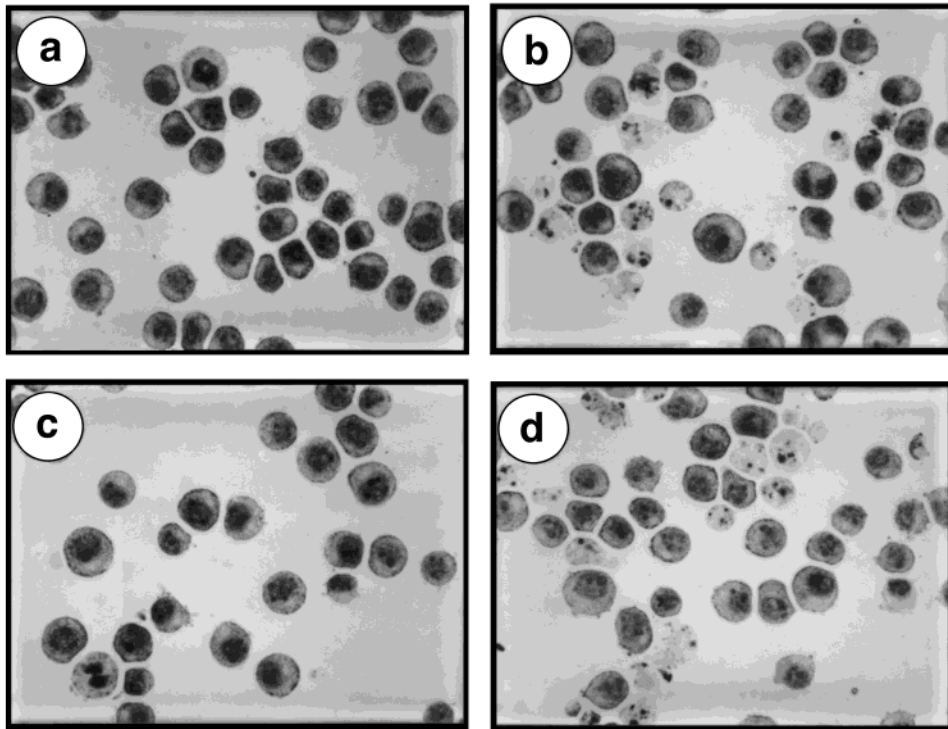


Figure 7 Wright-Giemsa-stained preparations of cells following a 6-h exposure to 10 μM Ara-C. (a) HL-60/V2 controls; (b) HL-60/V2 cells exposed to Ara-C; (c) HL-60/A5 controls; (d) HL-60/A5 cells exposed to Ara-C.

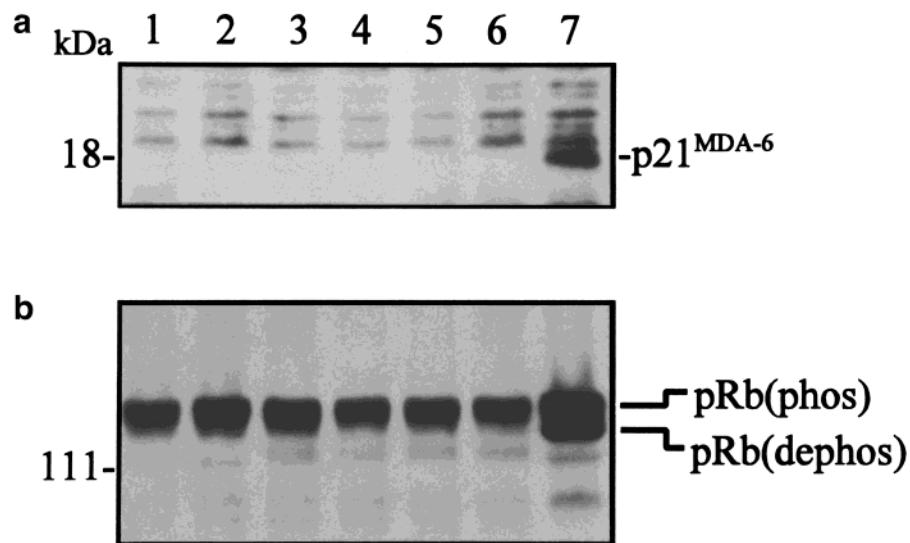


Figure 8 Western blot displaying changes in levels of (a) p21^{MDA6} and (b) pRb in response to 10 and 100 μM Ara-C for 6 h in HL-60/V2 and HL-60/A5 cells. Following treatment with 0, 10 and 100 μM Ara-C (HL-60/V2, lanes 1, 3, 5; HL-60/A5, lanes 2, 4, 6, respectively), cells were washed, pelleted and lysed as in Figure 1. Lane 7 contains lysate from HL-60/V2 cells treated with 5 nM PMA for 24 h and is included as a positive control for p21^{MDA6} induction and pRb dephosphorylation. Equivalent amounts of protein (25 μg) were separated and transferred to nitrocellulose. Blots were probed with the appropriate primary antibody followed by a goat anti-mouse-peroxidase secondary antibody and developed by chemiluminescence. The position of prestained molecular weight markers is indicated on the left, while the positions of the identified proteins are on the right. Two additional experiments yielded similar results.

Because both HL-60/V2 and HL-60/A5 cells exhibited increases in the percentage of cells in G₀/G₁ in response to Ara-C, we next examined the status of p21^{MDA6} as well as the downstream effector protein, pRb. Cells were treated with 10 μM Ara-C for 6 h and total cell lysates were obtained. Despite the apparent G₁ arrest, p21^{MDA6} was not induced by

Ara-C exposure (Figure 8a), a finding consistent with the results of an earlier report.⁸ Moreover, there was no evidence of pRb dephosphorylation (Figure 8b) an event associated with G₁ arrest and differentiation.³⁶ Ara-C treatment also did not lead to induction of p27^{KIP1} in either cell line (data not shown). Collectively, these findings indicate that dysregul-

ation of p21^{MDA6} in HL-60 cells does not alter the susceptibility of HL-60 cells to Ara-C-induced apoptosis nor does it attenuate Ara-C-mediated G₁ arrest.

Lastly, the clonogenic growth of HL-60/V2 and HL-60/AS5 cells treated with 10 μ M Ara-C for 6 h was examined. Following this exposure, both cell lines exhibited an equivalent reduction in cell number relative to controls (ie 12.4 \pm 2.2 vs 11.5 \pm 1.6% respectively; $P \geq 0.05$). Furthermore, when residual cells were plated in soft agar, empty vector and antisense-expressing cells exhibited 42.6 and 49.4% reductions in colony formation respectively ($P \geq 0.05$; Table 1). In separate studies, the S-phase fractions of unperturbed HL-60/V2 and HL-60/AS5 were found to be identical (35.9 \pm 2.6% vs 35.6 \pm 2.9%; $P \geq 0.05$). Thus, the failure of p21^{MDA6} dysregulation to modify Ara-C-induced apoptosis was accompanied by undiminished sensitivity of clonogenic cells to this agent.

Discussion

The induction of p21^{MDA6}, an inhibitor of G₁ cyclin-dependent kinases, is associated with cell cycle arrest in both p53-positive and -negative cells that have sustained DNA damage.^{16,23} In addition, p21^{MDA6} is thought to play a critical role in mediating G₁ arrest in cells undergoing differentiation (reviewed in Ref. 37). For example, increased expression of p21^{MDA6} has been reported in p53-null human leukemia cells induced to differentiate by PMA.^{11,17} Inhibition of CDKs leads to hypophosphorylation of pRb, which is then free to bind to, and thereby inactivate, the transcription factor, E2F.⁴⁶ Inactivation of E2F prevents transcription of diverse genes involved in cell cycle progression, including *c-myc*, dihydrofolate reductase, thymidine kinase, and ornithine decarboxylase, among others.^{34,36,47} The arrest of cells in G₁ is believed to be essential for certain pre-differentiation events to proceed, and for normal maturation to occur.⁴⁸ The relationship between p21^{MDA6} and apoptosis is less clear, and conflicting evidence on this point has been presented. For example, expression of p21^{MDA6} appears to be required for some cell types to undergo apoptosis,^{16,23} but not others.^{21,22,25,49} In human fibroblasts, expression of p21^{MDA6} antisense RNA induced cells in G₀ to progress through the cell cycle but did not trigger apoptosis.⁵⁰ On the other hand, attenuation of p21^{MDA6} in MCF-7 breast cancer cells by antisense constructs has been demonstrated to promote prostaglandin A₂-mediated apoptosis.^{23,51} More recently, antisense oligonucleotides directed against p21^{MDA6} have been shown to promote apoptosis in neuroblastoma cells,⁵² and enforced expression of p21^{MDA6} found to oppose cell death in differentiating myocytes.⁵³ Collectively, the latter studies suggest an anti-apoptotic role for this gene. Indirect evidence for this view has been provided by Zhang *et al*,⁵⁴ who reported that high levels of p21^{MDA6} expression in leukemic blasts obtained from patients with AML correlated with a poor response to chemotherapy. Since induction of leukemic cell maturation (eg by PMA) has been shown to reduce the susceptibility of leukemic cells to drug-induced cell death,⁵⁵ it is possible that upregulation of p21^{MDA6} represents a component of the differentiation response that antagonizes apoptosis.

Currently, little direct information is available concerning the role that p21^{MDA6} may play in the response of cells to differentiating agents or cytotoxic compounds, and essentially none in p52-negative human leukemic cells. For example, in the studies alluded to above, PMA induced p21^{MDA6} in HL-

60 cells, but the functional significance of this phenomenon was not specifically addressed.^{11,17} Similarly, PMA, okadaic acid, and IFN γ were found to induce p21^{MDA6} in three p53-null myeloid leukemia cell lines (HL-60, U937 and K562); moreover, transfection of K562 cells with p21^{MDA6} sense (but not antisense) constructs reduced cloning efficiency.⁵⁶ In studies involving p53-positive human colon carcinoma cells, Waldman and coworkers¹⁵ reported loss of p21^{MDA6} abrogated the G₁ arrest response to doxorubicin. However, to the best of our knowledge, analogous studies in human leukemia cells lacking p53 have not been conducted. To address this issue, an HL-60 cell line stably expressing a p21^{MDA6} antisense construct was generated, and these cells exhibit an unambiguously attenuated induction of p21^{MDA6} in response to the differentiation-inducing agent PMA. This model system provides a basis for characterizing the functional role that p21^{MDA6} plays in p53-independent pathways of leukemic cell maturation and drug-induced apoptosis.

The results of this study indicate that human myeloid leukemia cells expressing an antisense p21^{MDA6} construct are partially impaired in their ability to undergo cell cycle arrest and maturation in response to PMA. Previous studies have demonstrated that in p53-null HL-60 cells, PMA administration upregulates p21^{MDA6}, possibly through activation of the mitogen-activated protein kinase cascade.⁵⁷ This, in turn, results in pRb hypophosphorylation, cell cycle arrest in G₁, and the acquisition of differentiated features.³⁶ Consistent with this model, HL-60/AS5 cells exposed to PMA displayed decreases in the induction of p21^{MDA6}, expression of the maturation marker CD11b, the percentage of cells arrested in G₁, and in the degree of inhibition of CDK-2. However, despite the marked attenuation of p21^{MDA6} expression, HL-60/AS5 cells exposed to PMA did not appear to be impaired in their capacity to dephosphorylate pRb compared to empty vector controls, nor were there obvious perturbations in E2F complex formation. These phenomena may reflect (1) the presence of subtle changes in pRb and/or E2F status that could not be identified by the present methods and/or (2) the involvement of alternative cell cycle regulatory proteins, such as CDK4 and CDK6, which have also been implicated in pRb phosphorylation.⁵⁸ In this context, it has been proposed that perturbations in the cyclin A/CDK-2 complex may lead to qualitative alterations in pRb phosphorylation status that could exert effects on cell cycle arrest and related events.⁵⁹ Alternatively, p27^{Kip1} has been implicated in the maturation and G₁ arrest response of HL-60 cells to certain differentiation-inducing stimuli (eg dihydroxyvitamin D₃).⁴³ However, the failure to detect clear differences in p27^{Kip1} induction in empty vector and antisense-expressing cells argues against the contribution of this CDK inhibitor to the differential response to PMA.

In contrast to results obtained with PMA, dysregulation of p21^{MDA6} did not prevent the antimetabolite Ara-C from inducing cell cycle arrest; moreover, HL-60/AS5 cells were fully capable of mounting an apoptotic response to this agent. The latter finding, along with evidence that Ara-C fails to induce p21^{MDA6} in the HL-60 cell line,¹¹ indicates that Ara-C-mediated apoptosis proceeds along p53- and p21^{MDA6}-independent pathways in these cells. The inability of p21^{MDA6} dysregulation to influence drug-induced cell cycle block stands in contrast to recent reports demonstrating impairment in doxorubicin-induced G₁ arrest in a p21^{MDA6}-deficient colon tumor cell line HCT116.¹⁵ Similarly, McDonald *et al*⁶⁰ reported that HCT116 cells bearing a p21^{MDA6} carboxyl-terminal truncation mutant exhibited diminished DNA repair following exposure to UV light or cis-platinum. Discrepancies

between these and the present results could reflect multiple factors, including differences in the cell types examined (eg colon adenocarcinoma vs leukemia), p53 status (eg wild-type vs null), the method used to disturb p21^{MDA6} function (eg homologous recombination vs antisense expression), or the inciting stimulus (eg doxorubicin, UV light, cis-platinum vs Ara-C). For example, it is unlikely that DNA repair processes play a major role in determining the sensitivity of leukemic cells to agents such as Ara-C. In addition, although Ara-C induced G₁ arrest equally in HL-60/AS5 and control cells, this response was unaccompanied by alterations in pRb phosphorylation status in either cell line. It has recently been reported that induction of apoptosis in HL-60 cells leads to the accumulation of the hypophosphorylated pRb species,²⁶ and that pRb is proteolytically degraded by an ICE-like protease.⁶¹ Our inability to detect these events raises the possibility that such phenomena are subline-specific. In any event, the failure of Ara-C, in contrast to PMA, to induce pRb hypophosphorylation, and the differential effect of p21^{MDA6} status on Ara-C- vs PMA-mediated G₁ block suggest that drug- and phorboid-induced cell cycle arrest involve separate if inter-related pathways. Finally, the effects of p21^{MDA6} dysregulation on Ara-C-associated differentiation induction are currently under investigation.

In view of the heterogeneous behavior of HL-60 sublines, it is possible that the properties of HL-60/AS5 could be unique to this particular clone. However, we have recently stably transfected human monocytic leukemic (U937) cells with a p21^{MDA6} antisense-expressing vector, and these cells display impaired differentiation responses similar in most respects to those of HL-60/AS5 cells (unpublished observations). This would suggest that the effects of p21^{MDA6} dysregulation on differentiation do not represent an isolated phenomenon, at least as far as p53-null human myeloid leukemic cell lines are concerned.

In summary, the results of the present study indicate that dysregulation of p21^{MDA6} in human leukemia cells leads to impairment of the differentiation response, but does alter the susceptibility of cells to Ara-C-induced apoptosis. The observation that p21^{MDA6} is rarely mutated⁶² is consistent with the notion that this gene plays an important role in cellular maturation. However, the ability of cells from p21^{MDA6} knockout mice to differentiate normally²⁴ suggests the presence of redundant pathways capable of subserving some of the functions of p21^{MDA6}. It is possible that in leukemic cells, the ability of such alternative pathways to initiate a full differentiation program is lost. Finally, several reports indicate that exposure of leukemic cells to DNA damaging agents followed by a differentiation stimulus leads to a dramatic potentiation of apoptosis.^{63,64,65} The availability of a cell line exhibiting dysregulation of p21^{MDA6} and an impaired differentiation response will help to determine whether, and to what extent, p21^{MDA6} is involved in this phenomenon. Such studies are currently in progress.

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