Defects in p21<sub>WAF1/CIP1</sub>, Rb, and c-myc Signaling in Phorbol Ester-resistant Cancer Cells

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ABSTRACT

Growth arrest and differentiation of leukemic cells by phorbol 12-myristate 13-acetate (PMA) is accompanied by p53-independent activation of p21<sub>WAF1/CIP1</sub> and c-myc down-regulation. We show that despite p21 induction in 7 of 12 human cancer cell lines treated with PMA, growth inhibition was observed only in two cell lines (SKBr3 breast and LNCaP prostate cancer cells). Treatment of SKBr3 and LNCaP cells with PMA was followed by Raf-1 hyperphosphorylation, p21 induction, Rb hypophosphorylation, c-myc down-regulation and growth inhibition. The 10 remaining PMA-resistant cell lines were comprised of 5 that failed to induce p21 and 5 that induced p21 but had defects in steps putatively downstream of this (Rb hypophosphorylation and c-myc down-regulation). Exogenous expression and subsequent failure to down-regulate c-myc protein expression in SKBr3 and LNCaP cells was correlated with acquisition of resistance to the growth inhibitory effect of PMA. Exogenous p21 expression down-regulated c-myc protein in PMA-sensitive cancer cells. Our findings suggest that induction of p21 and down-regulation of c-myc may be necessary steps in a PMA-induced growth-inhibitory pathway in cancer cells.

INTRODUCTION

The phorbol ester PMA<sup>2</sup> activates PKC, which in turn directly activates Raf-1 kinase (1) and the MAPK pathway. Activation of this pathway by growth factors leads to growth stimulation, and overexpression of various mediators in the pathway can lead to cellular transformation (reviewed in Refs. 2 and 3). Growth stimulation induced by PMA has been observed in fibroblasts, epidermis, and lymphocytes, whereas PMA has been found to induce growth arrest and differentiation in certain leukemia cells (reviewed in Refs. 4–6). Recent work has implicated p21<sub>WAF1/CIP1</sub>, a cell cycle inhibitor, as possibly signaling the growth arrest associated with monocytic-macrophage differentiation following exposure of leukemic cells to PMA (7–9). It has been shown that induction of p21 by PMA depends on Raf-1 and MAPK signaling (10), and involves transcriptional activation of the p21 promoter (11). p21 is believed to inhibit cell cycle progression through its interaction with cyclin-CDK complexes and the proliferating cell nuclear antigen (12, 13). Cyclin-CDK kinase activity appears to be required for various cell cycle transitions. This is in part accomplished through phosphorylation of the Rb family of proteins in G<sub>i</sub>. Release of the E2F family of transcription factors has been shown to induce expression of a number of genes required for S phase, including c-myc (reviewed in Refs. 14 and 15).

PMA-induced leukemia cell differentiation has been found to involve both Rb hypophosphorylation (9) and c-myc down-regulation (6). p21 levels have been found to rise, independent of p53, and remain elevated as the cells growth arrest and differentiate following exposure to PMA (7–11). Moreover, p21 induction, c-myc down-regulation, and growth arrest are tightly correlated during differentiation in leukemia cells (16). We hypothesized that PMA may signal growth arrest through Raf-1 > MAPK >> p21 > Rb and that c-myc down-regulation may be a late step in the growth arrest program coupled to differentiation. We have found previously that epithelial cancer cells are resistant to PMA (11), and these cancer cells did not induce p21. Here we attempted to evaluate the PMA sensitivity in cancer cells that may respond to PMA by p21 induction and identify the level of the signaling defects in PMA-resistant cancer cells. We tested 12 cancer cell lines and found that only 2 of them (SKBr3 breast and LNCaP prostate cancer cells) were sensitive to growth inhibition by PMA. Following exposure of these cells to PMA, p21 was induced, Rb was hypophosphorylated, and c-myc was down-regulated. The ten remaining PMA-resistant cell lines were comprised of five that induced p21 and five that failed to induce p21 following PMA treatment. Of the five PMA-resistant lines that induced p21, one had no Rb and two appeared to have little change in Rb phosphorylation. All 10 PMA-resistant cell lines failed to down-regulate c-myc, suggesting that this may be a later event in the signaling pathway. To further investigate this possibility, we generated c-myc-expressing SKBr3 and LNCaP cells and correlated their failure to down-regulate c-myc with increased resistance to PMA. Our findings suggest that induction of p21, hypophosphorylation of Rb, and finally c-myc down-regulation may be necessary events in the PMA-induced growth inhibition cascade.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The human colon cancer cell line HCT116 was obtained from Dr. Bert Vogelstein (Johns Hopkins University) and maintained in supplemented McCoy’s 5A as described. The human glioblastoma cell line GM was a gift from Dr. W. Edward Mercer (Thomas Jefferson University) and was passaged as described previously (17). HL60 cells were obtained from Dr. Michael B. Kastan (Johns Hopkins University). The UP293 cell line was obtained from the Cell Center at the University of Pennsylvania. All other cell lines were obtained from American Type Culture Collection and maintained in the recommended media. PMA (Sigma Chemical Co.) was dissolved in DMSO as a 2 mM stock solution. Adenovirus expressing wild-type p21<sub>WAF1/CIP1</sub> (Ad-p21) was prepared as described (18).

Western Blot Analysis. Immunoblotting for p53, p21<sub>WAF1/CIP1</sub>, Raf, C-myc was carried out using anti-human p53 pAb1801 (Oncogene Science), anti-human WAF1 pAb1 (EA10; Oncogene Science), anti-human RB (LM95.1; Oncogene Science), anti-human c-myc mouse monoclonal (9E10) and rabbit anti-Raf-1 polyclonal (C19; Santa Cruz Biotechnology, Inc.) as described previously (19).

Determination of Cell Cycle Arrest. The growth-inhibitory effects were assessed by measuring loss of thymidine incorporation into newly synthesized DNA as described previously (11).

Transfection and Isolation of PMA-resistant SKBr3 and LNCaP Cells. To generate SKBr3 and LNCaP clones, previously PMA-sensitive cells, which did not down-regulate c-myc expression, were transfected with a human c-myc (driven by its own promoter) mammalian expression plasmid. The c-myc expression plasmid was a generous gift from Dr. William M. F. Lee (University of Pennsylvania). Cotransfections were carried out by electroporation of 10<sup>6</sup> cells in DMEM at 1160 Farads at 340 V with equal amounts (5 μg) of the
c-myc plasmid and pCMV-neo-Bam vector (to confer G418 resistance to transfectected cells) as described previously (11). pCMV-neo-Bam was a generous gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Following transfections, cells were selected in the presence of 500 μg/ml G418 (Life Technologies, Inc.) for 21 days, and individual G418-resistant clones were isolated and tested for their c-myc protein levels before and after exposure to PMA, as shown in Fig. 6.

RESULTS

Prostate Cancer Cells May Proliferate despite p21<sup>WAF1/CIP1</sup> Induction by PMA. We began our evaluation of the relationship between PMA sensitivity and putative mediators of growth-inhibitory signals in a series of human prostate cancer cell lines with diverse biological characteristics. We compared PMA sensitivity versus MitC sensitivity of hormone-dependent wild-type p53-expressing LNCaP cells, hormone-independent mutant p53-expressing DU145 cells (20), and PC3M cells, which express no p53 protein (Ref. 20; Fig. 1).

In the absence of any treatment, p53 protein was only detected in DU145 cells (Fig. 1A, Lane 1); consistent with prolongation of its half-life due to mutation, p21 was only detected in LNCaP cells (Fig. 1A, Lane 4), and c-myc was detected in all three cell lines (Fig. 1A, Lanes 1, 4, and 7). MitC only induced p53 and p21 in wild-type p53-expressing LNCaP cells (Fig. 1A, Lane 6), and also inhibited c-myc expression. c-myc has been described previously as a target for transcriptional repression by p53 (21). p21 was induced in all three prostate cancer cell lines following treatment with PMA (Fig. 1A, Lanes 2, 5, and 8). There was no evidence for p53 induction by PMA in LNCaP cells (Fig. 1A, Lane 5). PMA-induced p21 expression was clearly p53-independent because it also occurred in DU145 and PC3M cells (Fig. 1A, Lanes 2 and 8).

Despite p21 induction in all three cell lines following PMA treatment, only LNCaP cells were sensitive to PMA (Fig. 1C). All three cell lines were equally likely to undergo growth arrest following MitC (Fig. 1B), apparently independent of p53 status.

Sensitivity of Epithelial Cancer Cells to PMA Was Not Confirmed by Wild-Type p53. We and others have found previously that leukemic cells are growth inhibited following exposure to PMA, possibly through effects of the p21<sup>WAF1/CIP1</sup> cell cycle inhibitor, regardless of their p53 status. Because LNCaP cells express wild-type p53, the results shown in Fig. 1 could not rule-out the possibility that PMA sensitivity was in some way related to p53 status in epithelial cancers. To further investigate this possibility, we measured the growth-inhibitory effects of PMA in breast cancer cell lines (Fig. 2). Two wild-type p53-expressing cell lines (MCF7 and ZR75-1) and mutant p53-expressing SKBr3 breast cancer cells (22) were tested for their sensitivity to the growth-inhibitory effects of PMA. The wild-type p53-expressing MCF7 and ZR75-1 cells appeared to be more resistant to growth inhibition by PMA as compared with mutant p53-expressing SKBr3 cells (Fig. 2). Thus, in epithelial cancer cells, PMA sensitivity does not appear to be conferred by wild-type p53.

PMA-sensitive Prostate and Breast Cancer Cells Induced p21 and Down-Regulated c-myc Expression. In our initial survey of prostate cancer cell sensitivity, we noted that LNCaP cells down-regulated c-myc protein expression following exposure to PMA (Fig. 1A, Lanes 5 versus Lane 4). Because LNCaP cells contain wild-type p53 and c-myc was down-regulated following p53 induction by MitC,
Table 1  Effects of PMA in human cancer cells

<table>
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*The p53 status was taken from Refs. 6, 20, 22, and 28. wt, wild type; mt, mutant.

+ , p21 induction, Rb hypophosphorylation, or c-myc down-regulation following PMA treatment; - , absence of p21 induction, absence of Rb hypophosphorylation, or absence of c-myc down-regulation following PMA treatment; +/- , little change in the case of Rb phosphorylation.

+ S, >95% inhibition of [3H]thymidine incorporation following a 24-h incubation with 50 nM PMA; R, <50% inhibition of [3H]thymidine incorporation following a similar exposure to PMA.

Expresses no endogenous Rb (Fig. 4).

Transient induction at 4 h (data not shown).

HL60 is shown as an example of leukemia cells (6—9, 16). AML, acute myelogenous leukemia.
Fig. 5. Infection with p21-expressing adenovirus (Ad-p21) resulted in Rb dephosphorylation and c-myc down-regulation. Uninfected SKBr3 cells (Lane 1), cells infected by control adenovirus (Ad-β-Gal), see Ref. 18; Lane 2), and SKBr3 cells infected with Ad-p21 (Lane 3) were harvested after 24 h. c-myc and Rb protein were determined by Western blot as described in "Materials and Methods."

Table 1 shows a composite result of PMA sensitivity and resistance in our series of cancer cell lines, along with proposed defects in the putative p21 > Rb > c-myc signaling pathway. c-myc is the latest event because all PMA-resistant cancer cells failed to down-regulate its expression despite p21 induction or Rb hypophosphorylation. The failure of DU145 cells to down-regulate c-myc, despite elevated p21 levels after PMA, suggested that Rb function may be critical, and that other Rb family members such as p107 could not substitute for Rb. As a test of the hypothesis that following p21 induction in PMA-sensitive cells Rb hypophosphorylation is accompanied by c-myc down-regulation, we infected SKBr3 cells with p21-expressing adenovirus and examined the consequences with respect to Rb and c-myc. Following infection by Ad-p21, Rb was found in a hypophosphorylated state, and c-myc levels were decreased as compared with uninfected or Ad-LacZ infected cells (Fig. 5).

Abrogation of PMA-induced Growth Arrest in c-myc-transfected Cells. To further test the hypothesis that c-myc down-regulation was required for PMA-induced growth arrest, we introduced c-myc by transfection into PMA-sensitive SKBr3 breast cancer cells. Following G418 selection, we isolated 10 SKBr3 clones that still down-regulated c-myc and remained sensitive to PMA treatment (Fig. 6 and data not shown) and one clone (C5B) that did not down-regulate c-myc and became resistant to growth inhibition following exposure to PMA. All SKBr3 clones, including C5B, up-regulated p21 expression following PMA treatment (data not shown). The correlation between acquired resistance of C5B cells to PMA and their failure to down-regulate c-myc suggested that this may have been the underlying mechanism for their resistance to p21. Similar results were obtained with the LNCaP cell line in which failure to down-regulate exogenous c-myc correlated with relative resistance to growth arrest in c-myc-transfected clones (Fig. 6D). One of the clones (no. 3) exhibited 90–100% resistance to the DNA synthesis inhibitory effect of PMA (Fig. 6D). A 2–3-fold greater amount of thymidine incorporation was observed in clones nos. 2 and 4 as compared with neo-transfected clone 1 (Fig. 6D) or the parental cells (Fig. 1). It appears that at least for LNCaP clone no. 2, there was some down-regulation of c-myc and a lower c-myc expression level following PMA as compared with clones nos. 3 and 4. It is unclear at present why clone...
thereby blocking cell proliferation. Defects in this proposed signaling cascade have been identified in 10 PMA-resistant human cancer cell lines. We showed that PMA induced growth arrest in LNCaP prostate and terminal differentiation, associated with c-myc down-regulation (4). The acquisition of PMA resistance in SKBr3 and LNCaP cells that failed to down-regulate c-myc suggested that c-myc down-regulation was more than just a marker of PMA sensitivity and that c-myc function may have been required to achieve the resistant state. It may, therefore, be useful to include screening for c-myc down-regulation in studies of cancer cell drug resistance, because continued c-myc expression provides a bypass of growth inhibition by multiple tumor suppressors (25–27).

It is not clear where the defect exists in PMA-resistant cancer cells that did not up-regulate p21 expression, because Raf-1 activation was short-lived (Fig. 4). Different isoforms of PKC and also activation of parallel pathways may have contributed to the observed differences in p21 induction. Identification of these upstream defects should be aided by the identification of the cellular factors that mediate p53-independent p21 expression following treatment with PMA. Future experiments will determine the effects of the cyclin-CDKs on other Rb family members following PMA treatment.

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REFERENCES

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