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Advances in Brief

Cyclin-dependent Kinase Inhibitor p27 as a Mediator of the G_1-S Phase Block Induced by 1,25-Dihydroxyvitamin D_3 in HL60 Cells[1]

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Abstract

Progression of mammalian cells through G_1 is controlled by the concerted action of protein kinases, the activities of which are modulated in both positive (cyclins) and negative [cyclin-dependent kinase inhibitors (CDIs)] manners by families of regulatory proteins. In differentiation of leukemia cells, a G_1 arrest is a common, if not invariable, occurrence and takes place after the appearance of markers of monocytic differentiation in human leukemia HL60 cells treated with 1,25 dihydroxyvitamin D_3 ($1,25D_3$) at low to moderately high concentrations (F. Zhang *et al.*, Cell Proliferation *27*: 643–654, 1994). In the present study, we investigated the protein levels of several G_1 regulatory proteins that are potential mediators of the $1,25D_3$-induced G_1 block. During the first 24 h of exposure to a high concentration (4×10^{-7} M) of $1,25D_3$, no increase was noted in the immunodetectable levels of cyclins D1 or E, or CDIs $p16^{Ink4}$, $p21^{Cip1/Waf1}$, or $p27^{Kip1}$, even though monocytic differentiation markers were evident, and a prolongation of G_1 was noted. After 48 h of exposure 4×10^{-7} M to $1,25D_3$, a G_1 to S-phase block progressively increased in parallel with the abundance of the $p27^{Kip1}$ CDI. A transient increase in $p21^{Cip1/Waf1}$ was noted only at 48 hr. The increase in $p27^{Kip1}$ protein level was dependent on the concentration of $1,25D_3$ and was accompanied by an increase in cyclin D and E proteins, which normally peak in mid-G_1 and at the G_1 to S-phase transition, respectively. These results indicate that $p27^{Kip1}$ protein is a strong candidate for the cell cycle regulator that blocks the entry into the S-phase in $1,25D_3$-treated HL60 cells.

Introduction

Currently, chemotherapy of cancer is based principally on agents that are toxic to the cells. There are indications, however, that induction of cell differentiation may supplement the use of cytotoxic drugs in several forms of neoplasia, such as the successful use of retinoic acid in the treatment of acute promyelocytic leukemia (1) or of oral leukoplakia (2). Interest is also developing in the use of deltanoids (*i.e.*, derivatives of vitamin D_3) for the chemoprevention and treatment of human malignancies (3–6). Although it is clear that deltanoids are powerful differentiation-inducing agents, the knowledge of the mechanisms by which cell growth is arrested after exposure to these compounds is quite fragmentary (7).

The physiological deltanoid $1,25D_3$[3] induces monocytic differentiation in human acute promyelocytic cell line HL60 (8, 9) without associated toxicity and in a manner that resembles hematopoietic stem cell differentiation in that several cell divisions can take place after markers of monocytic differentiation are expressed (10, 11). Furthermore, like some normal macrophages, the quiescent, noncycling, differentiated HL60 cells can reenter the proliferative cycle under appropriate conditions (12, 13). Thus, these properties and the wide use of HL60 cells for studies of neoplastic cell growth make this system an important paradigm for mechanistic studies of differentiation therapy.

The principal block to cell cycle progression in $1,25D_3$-treated human cells is known to occur in G_1 (14, 15). Recently, seemingly conflicting evidence was presented on the role of $p21^{Cip1/Waf1}$ CDI protein in this block (16, 17). We have therefore studied the $1,25D_3$-HL60 cell system to determine which of the several candidate cell cycle-controlling proteins is mainly responsible for the G_1 block produced by concentrations of $1,25D_3$ up to 4×10^{-7} M, the concentration used by Jiang *et al.* (16). The results suggest that the accumulation of the CDI protein $p27^{Kip1}$ causes the definitive G_1 block and also indicate that the block occurs in late G_1, most likely at the G_1 to S-phase transition.

Materials and Methods

Cell Culture. Human promyelocytic leukemia HL60-G cells, a clone of early-passage HL60 cells (4), were grown in RPMI 1640 (Mediatech, Washington, DC) containing 10% complement-inactivated FCS (Hyclone Laboratories, Logan, UT), 100 IU/ml penicillin and streptomycin (Mediatech), and 2 mM L-glutamine (Mediatech) at 37°C in 5% CO_2. The cell numbers were counted using a Neubauer hemocytometer (Thomas Scientific Co., Swedesboro, NJ), and cell viability was determined by 0.4% trypan blue exclusion. The experiments were initiated using cultures at a density of 3×10^5 cells/ml of fresh medium containing the desired concentrations of $1,25D_3$ (Hoffmann-LaRoche, Inc., Nutley, NJ) or an equivalent volume of sterile-filtered ethanol, which was the vehicle for $1,25D_3$.

Determination of Differentiation Markers. The extent of monocytic differentiation induced in HL60-G cells by $1,25D_3$ was determined by monitoring the NSE activity and the presence of the CD14 surface marker by flow cytometry. The procedure for NSE reaction was described previously (10). To detect the expression of the CD14 cell surface marker, aliquots of 3×10^6 HL60 cells were harvested at various time points, centrifuged, and washed twice with 1× PBS. The cell pellet was resuspended in 100 μl of PBS and 0.2 μl of monoclonal antibody specific for CD14, My4 (Coulter Electronics, Hialeah, FL) was added, and the mixture was incubated in the dark at room temperature for 45 min. The excess antibody was washed off with 1× PBS, and the pellet was resuspended in 500 μl of 1× PBS. The cells were analyzed by Epics Profile II flow cytometer (Coulter).

Cell Cycle Distribution. To evaluate the cell cycle profile, 3×10^6 cells were washed twice with cold 1× PBS. The cell pellet was resuspended in 1 ml of 1× PBS, and 1 μl of RNase (Boehringer Mannheim, Indianapolis, IN) was added. After a gentle vortexing, the mixture was placed on ice for 30 min. The cells were pelleted after this incubation, washed with 1× PBS, resuspended in propidium iodide (10 μg/ml, containing 0.1 M sodium citrate and 0.1% Triton X-100), and incubated at 4°C for at least 2 h. The cells were analyzed by Epics Profile II flow cytometer (Coulter), and cell cycle parameters were obtained using the Multicycle software package (Phoenix Flow Systems, San Diego, CA).

Preparation of Cell Extracts and Immunoblotting. Whole cell extracts were prepared by lysing 30×10^6 cells with extraction buffer (20 mM Tris-HCl-0.25 M sucrose-1 mM phenylmethylsulfonyl fluoride-2 mM EDTA-10 mM EGTA-10 μg/ml leupeptin-2 μg/ml aprotinin). After a brief

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[3] The abbreviations used are: $1,25D_3$, 1,25 dihydroxyvitamin D_3; CDI, cyclin-dependent kinase inhibitor; NSE, nonspecific esterase; TBS-T, Tris-buffered saline–0.01% Tween 20; pRb, retinoblastoma protein.

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Table 1 *Kinetics of the induction of differentiation markers and of the cell cycle prolongation and block by $1,25D_3$*

Time (h)	4	4	8	8	12	12	24	24	48	48	96	96
$1,25D_3$[a]	−	+	−	+	−	+	−	+	+	+	+	+
CD14	1.5 (1.0)	5.4 (3.2)	2.7 (1.3)	42.2 (3.6)	4.7 (2.6)	58.8 (5.1)	1.9 (0.9)	79.2 (3.2)	4.3 (2.1)	88.4 (7.1)	0.7 (0.5)	85.8 (5.1)
NSE	3.2 (0.4)	4.2 (0.5)	5.4 (1.1)	4.8 (1.2)	0.6 (0.1)	2.2 (0.7)	2.2 (0.1)	30.0 (0.3)	5.0 (2.1)	79.8 (9.7)	4.4 (1.3)	97.2 (5.2)
G_1	40.5 (3.1)	39.9 (0.7)	42.6 (2.1)	51.3 (6.1)	40.4 (3.1)	51.6 (2.7)	43.4 (3.9)	51.2 (1.6)	45.5 (1.6)	64.2 (1.8)	48.7 (1.4)	76.4 (3.8)
S	47.7 (2.1)	47.6 (3.8)	46.3 (3.5)	39.3 (4.6)	47.9 (2.1)	36.5 (1.9)	45.6 (3.8)	39.3 (2.7)	46.7 (1.9)	28.5 (0.9)	41.6 (2.3)	10.8 (5.1)
G_2-M	11.7 (1.3)	12.5 (2.1)	11.1 (0.4)	9.4 (1.6)	11.8 (2.8)	11.98 (0.9)	11.0 (2.6)	9.5 (2.6)	7.8 (1.0)	7.3 (1.7)	10.7 (0.9)	12.8 (0.5)

[a] HL60-G cells exposed to $1,25D_3$ (4×10^{-7} M) were sampled at intervals shown. CD14 markers of monocyte/macrophage differentiation and the cell cycle parameters were determined by flow cytometry, and NSE by cytochemistry, as described in "Materials and Methods." The mean and SD (in parentheses) of four experiments are shown.

sonication, the extracts were ultracentrifuged for 60 min at 42,000 rpm at 4°C. In some experiments, nuclear extracts were also prepared by the procedure of Andrews and Faller (18). The protein concentration was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA). Aliquots of the supernatant were mixed with equal amount of 3× SDS sample buffer (150 mM Tris-30% glycerol-3% SDS-1.5 mg/100 ml-bromophenol blue dye-100 mM DTT) and denatured at 90–100°C for 4 min. Thirty μg of total protein from treatment and control groups were resolved on 13% SDS-PAGE gels (19) along with rainbow-colored protein molecular weight markers (Amersham, Buckinghamshire, England). Gels were transferred to Hybond nitrocellular membranes as described (20). Upon completion of transfer, Ponceau S staining was performed to verify that equal amounts of total proteins were present in all the lanes (21). The stain was then washed off, and the blots were blocked with 5% milk (Carnation nonfat dry milk; Carnation Co., Los Angeles, CA) in TBS-T for 1 h. Membranes were washed with three changes of TBS-T for 30 min and incubated with primary antibodies at a dilution from 1:100 to 1:2000 for 1 h at room temperature. After blotting with a primary antibody, the membranes were washed three times and briefly blocked in 5% milk for 10 min. The blocking buffer was then washed off, and the membranes were blotted with an appropriate horseradish peroxidase-linked secondary antibody at 1:2000 dilution for 1 h at room temperature. After final washes in TBS-T, proteins were visualized with a chemiluminescence assay system (Amersham).

Antibodies. Mouse antihuman cyclin E, mouse antihuman $p21^{Cip1/Waf1}$, mouse antihuman Rb, and rat antihuman cyclin D1 antibodies were purchased from Oncogene Science (Uniondale, NY). Rabbit antihuman $p16^{Ink4}$ and rabbit antihuman $p27^{Kip1}$ antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Antimouse, antirabbit, and antirat horseradish peroxidase-linked secondary antibodies were purchased from Amersham.

Results

Induction of the Monocytic Phenotype and Cell Cycle Arrest by $1,25D_3$. It has been shown previously that when HL60-G cells are exposed to $1,25D_3$ at concentrations of 1×10^{-7} M or lower, the expression of markers of monocytic differentiation can be detected before the onset of the G_1 block (11). However, because the induction of CIP1/WAF1 expression in $1,25D_3$-treated HL60 cells was reported in cultures exposed to 4×10^{-7} M $1,25D_3$ (16), we asked whether the kinetics of the appearance of these parameters is accelerated at this concentration of the differentiation inducer. In this series of experiments, the expression of the CD14 surface marker of monocyte/macrophage differentiation was markedly elevated after 8 h of exposure to 4×10^{-7} M $1,25D_3$, and the cytoplasmic enzyme NSE became detectable at about 24 h (Table 1). A higher proportion of cells in G_1, apparently at the expense of cells in S phase, was also noted at 8 h of treatment (Table 1), but this proportion of G_1 cells did not increase during the first 24 h of $1,25D_3$ treatment. This indicates a prolongation of the traverse of G_1 rather than a block at a restriction point because a block would result in an increasing proportion of cells as they reach the block during the cell cycle traverse. The prolonged G_1 produces increased doubling time of $1,25D_3$-treated cells, but logarithmic growth continues for a variable period of time, as reported previously (4). In the present experiments, a progressively increasing percentage of cells in G_1 was noted at 48 h of exposure to 4×10^{-7} M $1,25D_3$, accompanied by an equally marked reduction of cells in S phase, but with little change in the proportion of cells in G_2-M (Table 1). This is consistent with the onset of a G_1 to S-phase block at approximately 48 h.

Levels of Immunodetectable CDI Proteins. Several inhibitors of cyclin-dependent kinases have been described that regulate the G_1 traverse and the transition into the S phase, as reviewed recently (22, 23). Of these, the mRNA steady-state levels of $p21^{Cip1/Waf1}$ have been reported to increase within a few hours after the addition of 4×10^{-7} M $1,25D_3$ to HL60 cells (16). We therefore determined the levels of the $p21^{Cip1/Waf1}$ protein in HL60 cells exposed to this concentration of $1,25D_3$ but did not observe immunodetectable protein levels during the first 24 h of $1,25D_3$ exposure (data not shown). Fig. 1 shows that at 48 h after addition of $1,25D_3$, $p21^{Cip1/Waf1}$ was detected, but the induction appeared to be transient, and of lesser intensity than that elicited by 12-*o*-tetradecanoylphorbol-13-acetate, as reported recently (17). The immunodetectable $p16^{Ink4}$ protein levels were also in low abundance in untreated cells and did not appear to be induced by $1,25D_3$ (data not shown). In contrast, the levels of $p27^{Kip1}$ protein were clearly increased at 48 h, and the increase continued until at least 96 h, at which time the increase was quite marked (Fig. 2). This coincided with the appearance of the G_1 block.

Dose Dependence of $1,25D_3$ Up-Regulation of G_1 Phase-Regulatory Proteins. We also examined the effect of $1,25D_3$ at two lower concentrations on the protein levels of $p27^{Kip1}$; the G_1 cyclins, the activity of which it controls (*i.e.*, cyclins D1 and E); and on the phosphorylation level of pRb because pRb is one of the target proteins of the G_1 cyclin-dependent kinase-cyclin complexes (24). The lowest concentration of $1,25D_3$ used, 10^{-9} M, had a small but significant ($P < 0.05$) effect on the differentiation (CD14 = 21.2% at 96 h) and on the cell cycle progression of HL60 cells (G_1 = 65.9%, S phase = 24.1% at 96 h). Similarly, this concentration of $1,25D_3$ elevated the levels of $p27^{Kip1}$ and of cyclins D1 or E (Fig. 3 *A–C*) only slightly, whereas the highly phosphorylated form of pRb, typical of the G_1 phase (25), appeared essentially unchanged (Fig. 3*D* and data not shown). However, when the concentration of $1,25D_3$ was raised to 10^{-7} M, the levels of $p27^{Kip1}$, cyclin D1, and cyclin E proteins were markedly increased (Fig. 3, *A–C*), and only the hypophosphorylated form of pRb was observed (Fig. 3*D*).

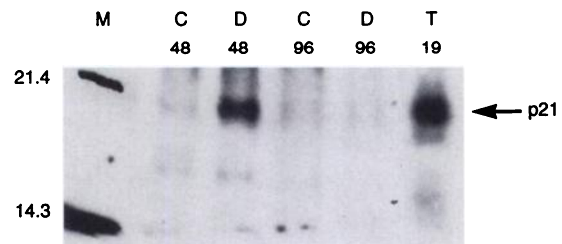

Fig. 1. Immunoblot analysis of $p21^{Cip1/Waf1}$ protein abundance in HL60-G cells treated with $1,25D_3$ or TPA for the indicated times in hours. *M*, size marker proteins, the size of which is shown in thousands; *C*, untreated cells exposed to ethanol vehicle; *D*, cells treated with 4×10^{-7} M $1,25D_3$ for the times shown; *T*, cells treated with 30 nM TPA for 19 h.

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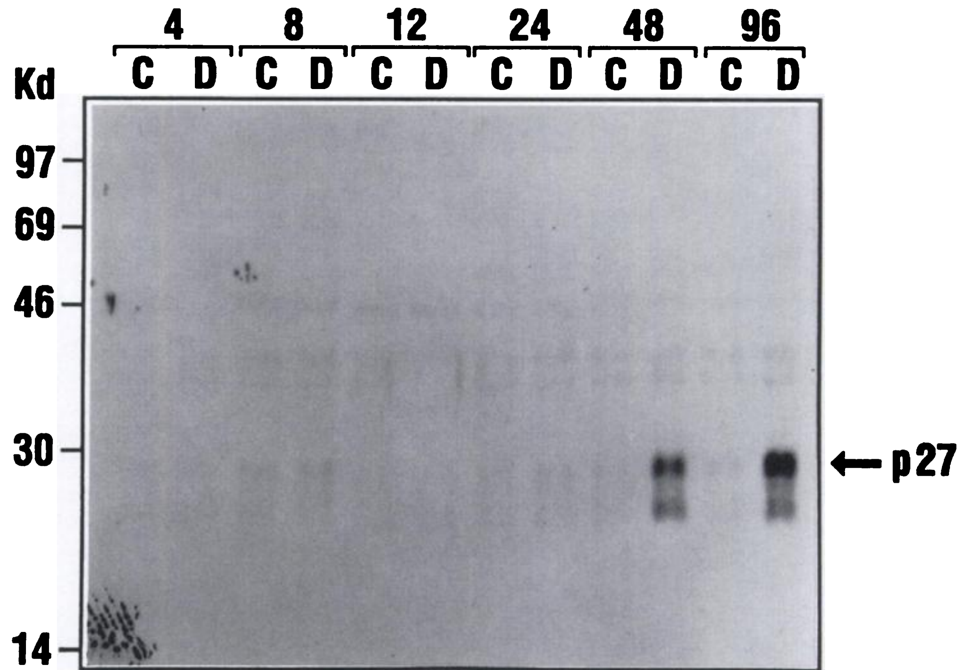

Fig. 2. Immunoblot analysis of $p27^{Kip1}$ protein. The annotations are explained in the legend to Fig. 1.

Discussion

The G_1 block in HL60 cells exposed to high concentrations of $1,25D_3$ becomes pronounced at 48 h and is essentially maximal 96 h after the addition of this steroid hormone (Table 1). At this time, a marked increase in the cellular content of $p27^{Kip1}$ is also observed (Fig. 2), supporting the hypothesis that the CDI $p27^{Kip1}$ protein is one of the principal mediators of the antiproliferative action of $1,25D_3$ on HL60 cells.

Recent publications have linked the up-regulation of the *Cip1/Waf1* gene to HL60 cell differentiation (16, 17, 26). A marked rise in $p21^{Cip1/Waf1}$ mRNA levels was reported to take place as an immediate early response to multiple differentiation-inducing agents, including a high concentration (4×10^{-7} M) of $1,25D_3$ (16, 26). However, another group using an even higher concentration of $1,25D_3$ (1.25×10^{-5} M), failed to detect induction of $p21^{Cip1/Waf1}$ at either the protein or the mRNA level (17). Although different methodologies cannot be excluded as the reason for these conflicting results, HL60 cells are subject to development of new characteristics in long-term culture (e.g. Refs. 4, 27), so different laboratories often have different sublines. In our experiments, the induction of $p27^{Kip1}$, but not of $p21^{Cip1/Waf1}$, correlates with the onset of the definitive G_1 to S-phase block.

The data also show that the cellular content of cyclins D1 and E is increased, although the cells cease to traverse G_1 (Fig. 3). This strengthens the notion that $1,25D_3$ exerts its antiproliferative effect not by forcing cell quiescence in G_0 but by the activation of CDIs, principally $p27^{Kip1}$ aided by $p21^{Cip1/Waf1}$ and perhaps other kinase inhibitors, which poise the cell in late G_1. This may account for the reversibility of $1,25D_3$-induced differentiation (12, 13). The upstream events that up-regulate the *KIP1* gene or unmask the preexisting

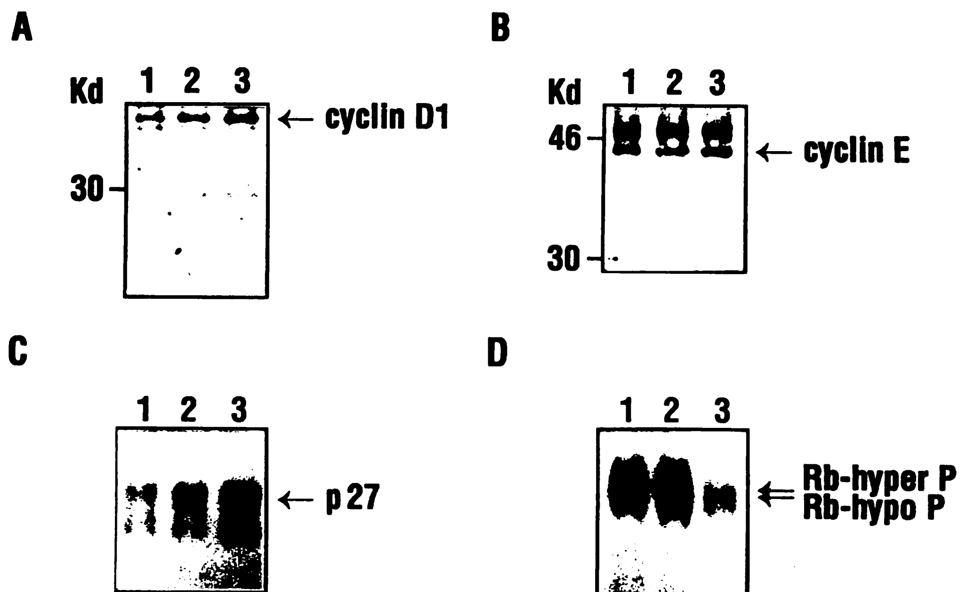

Fig. 3. Immunoblot analysis of cyclin D1, cyclin E, $p27^{Kip1}$ (p27), and Rb. The cells were exposed to ethanol control *(Lane 1)*, 10^{-9} M *(Lane 2)*, or 10^{-7} M *(Lane 3)* $1,25D_3$ for 96 h. Note that only the more rapidly migrating component of the two incompletely resolved Rb bands is present after exposure to 10^{-7} M $1,25D_3$. *Rb-hyper P*, hyperphosphorylated form of the Rb; *Rb-hypo P*, hypophosphorylated form of the Rb.

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intracellular $p27^{Kip1}$ protein present an interesting area for future investigation.

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