p21^{WAF1} is associated with CDK2 and CDK4 protein during HL-60 cell differentiation by TPA treatment

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Abstract. TPA-treated HL-60 cells are mainly arrested in G1 by p21^{WAF1} accumulation. We investigate the downstream changes following such accumulation. Increased p21^{WAF1} is associated with CDK2 and CDK4. pRb is dephosphorylated in the presence of p21–CDK2/4 complexes, and the Rb–E2F1 complex increases after TPA treatment, whereas the Rb–HDAC1 complex decreases slightly. Our results suggest that increased p21^{WAF1} is associated with CDK2/4, and that these complexes induce pRb dephosphorylation. In turn, hypophosphorylated pRb are mainly complexed with E2F1, but HDAC1 appears not to be a key component in this process.

INTRODUCTION

HL-60 cells – human promyelocytic leukaemia cells – are widely used for studying differentiation (Koeffler 1983). TPA (12-o-tetradecanoylphorbol-13-acetate) is a potent activator of PKC (protein kinase C) and affects a wide array of biological processes involved in HL-60 cell differentiation (Breitman 1990). The induction of differentiation by TPA results in growth arrest of HL-60 cells in the G1 phase (Burger *et al.* 1994; Horiguchi-Yamada *et al.* 1994). p21^{WAF1} is regarded as a key CKI (cyclin-dependent kinase inhibitor) for G1 arrest in cells (Jiang *et al.* 1994; Steinman *et al.* 1994). We previously reported that p21^{WAF1} appeared to be an immediate early response gene, and that the p21^{WAF1} level was maximally accumulated 24 h after TPA treatment. In addition, pRb (retinoblastoma tumour suppressor protein) was dephosphorylated following p21^{WAF1} accumulation (Suh *et al.* 1997; Cho *et al.* 1999). In the case of 1,25-dihydroxyvitamin D3-treated HL-60 cells, p27^{KIP1} plays a key role in G1 arrest mainly through binding CDK6 (cyclin dependent kinase 6) (Wang *et al.* 1997). However, in TPA-treated HL-60 cells, it has not been reported which CDKs are complexed with p21^{WAF1} to arrest at the G1 phase. Furthermore, it has recently been reported that pRb are complexed with E2F1 transcription factor (E2F1) and HDAC1 (histone deacetylase 1) for the regulation of E2F1-responsive genes (Brehm *et al.* 1998; Magnaghi-Jaulin *et al.* 1998; Iavarone & Massague 1999). In the present study, we investigated

the downstream changes following p21^{WAF1} accumulation in TPA-treated HL-60 cells, including the possible formation of the Rb–HDAC1–E2F1 triple complex.

MATERIALS AND METHODS

Cell culture

HL-60 cells were cultured in RPMI-1640 medium (Gibco BRL, Germany) supplemented with 10% FBS (foetal bovine serum, Hyclone, Life Science Co., USA). Cells were maintained at a cell density between 1 and 2×10^5 per ml. Differentiation to macrophage-like cells was induced by exposure of the cells to 32 nM TPA (Sigma Co., USA) for the indicated times.

Western blot analysis

Cells were lysed in lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, PMSF (phenylmethylsulphonyl fluoride, 10 µg/ml), aprotinin (10 µg/ml), leupeptin (10 µg/ml), 5 mM phenanthroline and 28 mM benzamidinium-HCl] for 30 min in ice. Lysates were clarified by centrifugation. Lysates were quantified using the Bradford assay (Bio-Rad Co., USA) with bovine serum albumin as a reference standard. Lysates (35 µg) were resolved by SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gels) and transferred to PVDF (polyvinylidene difluoride membrane, Millipore Co., USA). After incubation with primary antibodies, proteins were visualized by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Co., USA), followed by ECL (enhanced chemiluminescence) kit according to the manufacturer's instructions (Amersham Co., USA). Primary antibodies against Rb (G3-245: PharMingen Co., USA), CDK2 (M2: Santa Cruz Co., USA), CDK4 (C-22: Santa Cruz Co., USA), p21^{WAF1} (F-5: Santa Cruz Co., USA), E2F1 (KH95: Santa Cruz Co., USA), HDAC1 (C-19: Santa Cruz Co., USA) and β -actin (I-19: Santa Cruz Co., USA) were applied at optimized concentrations.

Immunoprecipitation

For immunoprecipitation of p21, cells were lysed in ice-cold lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 0.5% NP-40, 100 mM NaF, 200 µM sodium orthovanadate, PMSF (10 µg/ml), aprotinin (10 µg/ml), leupeptin (10 µg/ml), 5 mM phenanthroline and 28 mM benzamidinium-HCl] for 20 min in ice. Lysates (200 µg) were clarified by centrifugation at 12 000 rpm for 20 min at 4 °C. After pre-clearing with protein A-sepharose beads (Sigma), the supernatants were then immunoprecipitated with the anti-p21^{WAF1} antibody for 2 h and then protein A-sepharose beads for 1 h at 4 °C. Immunoprecipitated complexes were washed five times with lysis buffer and boiled in sample buffer for 5 min. Samples were resolved by 12% SDS–PAGE.

For immunoprecipitation of Rb, cells were washed with cold PBS and lysed in EBC lysis buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 0.5% NP-40, 100 mM NaF, 200 µM Sodium orthovanadate, PMSF (10 µg/ml), aprotinin (10 µg/ml), leupeptin (10 µg/ml), 5 mM phenanthroline and 28 mM benzamidinium-HCl]. Lysates were pre-cleared, and equal concentrations (1 µg) of lysates were immunoprecipitated with anti-Rb antibody and resolved by 6.5% SDS–PAGE.

Flow cytometric analysis

For flow cytometric analysis of DNA content, approximately 10^6 cells were fixed in 80% ethanol at –20 °C for 24 h. Ethanol-fixed cells were stained with propidium iodide (PI) staining solution [PI (50 µg/ml), RNase A (0.1 mg/ml), 0.1% NP-40, 0.1% trisodium citrate] for 30 min and then analysed by a FACS analyser (Becton-Dickinson Co., USA).

DNA fragmentation assay

Cells were harvested at the indicated times by centrifugation and lysed in ice for 20 min by the addition of 20 μ l lysis buffer consisting of 20 mM EDTA, 100 mM Tris (pH 8.0), and 0.8% (w/v) sodium lauryl sarcosine. RNase A (0.5 mg/ml) and proteinase K (1 mg/ml) were added and incubated at 37 °C for 1 and 2 h, respectively. Total lysates were loaded onto 1.5% agarose gel and separated at 50 mV for 2 h. DNA fragments were visualized after staining with ethidium bromide by transillumination with UV light.

Enzymatic cleavage activity of caspase-3

Cells were lysed in lysis buffer (50 mM Tris (pH 7.5), 0.03% NP-40, 1.0 mM DTT) for 30 min on ice. After centrifugation at 12 000 rpm for 30 min at 4 °C, the supernatant was quantified using the Bradford assay kit with bovine serum albumin as a reference standard. Lysates (20 μ g) were incubated with 0.2 mM ac-DEVD-pNA (Enzyme System product Co., USA) in a total volume of 0.1 ml. Assays were performed twice and the results are presented as the average increase in absorbance at 405 nm.

RESULTS

Effects of TPA on cell morphology and cell cycles

HL-60 cells were cultured for 24 h in RPMI-1640 media containing 32 nM TPA. Figure 1a shows that the cells were differentiated into macrophage-like cells in the presence of TPA. Flow cytometric analysis showed that the population of cells in the G1 phase increased, whereas the number in the S phase markedly decreased 24 h after TPA treatment (Fig. 1b).

Figure 1. The effect of TPA on cell morphology and cell cycle in HL-60 cells. HL-60 cells were treated with 32 nM TPA for 24 h. Note the dramatic change of HL-60 cell morphology (a) and cell cycle arrest in the G1 phase after TPA treatment (b). Photographs were taken after crystal violet staining ($\times 100$).

Figure 2. Association of p21^{WAF1} with CDK2 and CDK4 in HL-60 cells after 32 nM TPA treatment. Lysates from cells were immunoprecipitated (I.P.) with anti-p21^{WAF1} antibody followed by Western blotting (W.B.) with anti-p21^{WAF1}, anti-CDK2, and anti-CDK4 antibodies.

p21^{WAF1} is associated with both CDK2 and CDK4 after TPA treatment

Immunoprecipitation–Western blot analysis using anti p21^{WAF1} antibody was used to investigate the downstream changes following p21^{WAF1} accumulation in TPA-treated HL-60 cells. Figure 2 shows that the level of p21^{WAF1} protein increased remarkably 24 h after TPA treatment. Furthermore, this increased level of p21^{WAF1} was associated with CDK2 and CDK4.

Effect of TPA on the Rb–E2F1–HDAC1 triple complex

We then investigated the phosphorylation status of pRb in the presence of p21^{WAF1}–CDK2/4 complexes, and whether pRb is complexed by E2F1 and HDAC1 after TPA treatment. Figure 3a shows that pRb was remarkably dephosphorylated 12 h after TPA treatment, and that the hypophosphorylated pRb–E2F1 complex increased 12 h after TPA treatment, whereas the concentration of the hypophosphorylated pRb–HDAC1 complex was slightly reduced (Fig. 3b). These results indicate that pRb–E2F1 complexes may be significantly involved in the process leading to the G1 arrest in TPA-treated HL-60 cells, whereas it is likely that pRb–HDAC1 complexes are not significantly involved.

Comparison of G1 cell cycle regulatory proteins between adherent and floating cells in the presence of TPA

After TPA treatment, the majority of HL-60 cells were differentiated into macrophage-like cells (adherent cells). However, a fraction of the cells were not differentiated and remained as floating cells, even in the presence of TPA. We hypothesized that failure of p21^{WAF1} accumulation in these cells after TPA treatment may have been the cause of this differentiation failure. To elucidate the cause of this failure, we compared the cell cycle regulatory proteins of adherent and floating cells (Fig. 4). Floating cells proved to have remarkably lower p21^{WAF1} levels than the adherent cells, and consistent with this result, pRb was not fully dephosphorylated in floating cells. Furthermore, the level of E2F1 was significantly higher in floating cells than in adherent cells. However, levels of CDK2, CDK4 and HDAC1 were similar in adherent and floating cells.

Figure 3. Immunoprecipitation–Western blot analysis of pRb, E2F1, and HDAC1 after 32 nM TPA treatment. (a) Total cell lysates (25 µg) were resolved on a 6.5% SDS–PAGE gel and immunoblotted with anti-Rb, anti-E2F1 and anti-HDAC1 antibodies. (b) Total cell lysates (1 mg) were immunoprecipitated (I.P.) with anti-Rb antibody followed by Western blotting (W.B.) with anti-E2F1 and anti-HDAC1 antibodies. pRb: hypophosphorylated Rb; ppRb: hyperphosphorylated Rb.

Figure 4. Comparison of G1 cell cycle regulatory proteins of adherent and floating cells. Total cell lysates (25 µg) were immunoblotted with anti- $p21^{WAF1}$, anti-Rb, anti-E2F1, anti-HDAC1, anti-CDK2 and anti-CDK4 antibodies. The same results were obtained from three independent experiments.

Figure 5. Apoptotic cell death of floating HL-60 cells. (a) DNA fragmentation analysis, (b) flow cytometric analysis, and (c) analysis of caspase-3 enzymatic activity were performed as described in Materials and Methods to confirm the apoptotic cell death of floating cells.

Apoptotic cell death of floating cells

To determine the destination of floating cells, we investigated floating cell viability using the DNA fragmentation assay, flow cytometric analysis and caspase-3 activity analysis. Figure 5a shows that distinct apoptotic DNA ladders were observed in floating cells, but not in adherent

cells. Consistent with these data, floating cells also showed a markedly increased sub-G1 population and caspase-3 activity in a time-dependent manner compared to adherent cells (Figs 5b and c). These results indicate that the floating HL-60 cells are not induced to differentiate but are committed to the apoptotic pathway.

DISCUSSION

In this study, we present a G1 arrest mechanism underlying HL-60 cell differentiation by TPA. Our results suggest that p21^{WAF1} protein plays a crucial role in the dephosphorylation of Rb protein by association with CDK2 and CDK4. Recently, several groups have reported that pRb interacts simultaneously with E2F1 and HDAC1 (Histone deacetylase 1) (Brehm *et al.* 1998; Magnaghi-Jaulin *et al.* 1998; Iavarone & Massague 1999), and that this triple complex should be regarded as an important factor in cell cycle control, particularly in the regulation of S phase specific genes. In this study, we investigated whether this triple complex contributes to the G1 phase arrest in HL-60 cell differentiation induced by TPA. Even though HDAC1 was complexed with pRb in proliferating HL-60 cells, its association with pRb was slightly reduced after TPA treatment. Additionally, the level of HDAC1 was not increased in TPA-treated HL-60 cells. We previously reported that cyclin E mRNA was not significantly decreased during the 12 h following TPA treatment. Burger *et al.* (1994) also reported that the down-regulation of cyclin A, B and E genes is not a prerequisite for the induction of terminal differentiation and the concomitant cell cycle block at G1. Thus it seems likely that HDAC1 is not a major component of the pRb complex for the regulation of S phase specific genes in TPA-induced HL-60 cell differentiation.

Several groups have reported that HL-60 cells are not differentiated but undergo apoptosis even in the presence of TPA. In this study, cells that failed to differentiate (floating cells) showed a low p21^{WAF1} level even in the presence of TPA. Mizuno *et al.* (1997) reported that fragments of nPKC (protein kinase C) isotypes induced apoptotic cell death in floating cells after TPA treatment. In addition, Aihara *et al.* (1991) reported that the sustained activation of PKC is essential for HL-60 cell differentiation into macrophage. Considering that p21^{WAF1} is a major target of the PKC-mediated inhibition of cell cycle progression (Barboule *et al.* 1999; Black 2000), perturbation of the PKC signalling pathway may cause differentiation failure in TPA-treated HL-60 cells, due to insufficient p21^{WAF1} accumulation. Furthermore, Das *et al.* (2000) recently reported that inhibition of the ERK cascade inhibits HL-60 cell attachment following TPA treatment through the inhibition of p21^{WAF1} accumulation. This result is concordant with our data.

p21^{WAF1} can also block cdc2/cyclin B activity and prevent progression into the M phase (Tchou *et al.* 1996; Niculescu *et al.* 1998). In this study, a small number of G2/M phase cells were observed among the adherent cells. To investigate whether p21^{WAF1} may associate with cdc2 in adherent cells, immunoprecipitation-immunoblot analysis was performed using anti-p21^{WAF1} and anti-cdc2 antibodies. However, increased p21/cdc2 complexes in adherent cells were not detected in our system (data not shown).

Thus, our results suggest that the increased expression of p21^{WAF1} plays an important role in G1 arrest by associating with CDK2/4, and that a poor accumulation of p21^{WAF1} may result in differentiation failure. Increased concentrations of p21-CDK2/4 complexes induce dephosphorylation of pRb in TPA-treated HL-60 cells, and the hypophosphorylated pRb is mainly complexed with E2F1 transcription factor for the regulation of E2F1-responsive genes. HDAC1 is probably not a core component of this process.

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