TPA-induced p21 expression augments G2/M arrest through a p53-independent mechanism in human breast cancer cells

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Abstract. The tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), has a differential role on the regulation of the cell cycle in a variety of tumor cells. The mechanism between TPA and the cell cycle in breast cancer is not fully understood. Therefore, we investigated the regulatory mechanism of TPA on control of the cell cycle of breast cancer cells. Our results showed that TPA increased the level of p21 expression in MCF-7 cells with wild-type p53 and MDA-MB-231 cells with mutant p53 in a dose-dependent manner. In contrast, TPA decreased the expression of p53 in MCF-7 cells, but did not affect MDA-MB-231 cells. We next examined the regulatory mechanism of TPA on p21 and p53 expression. Our results showed that the TPA-induced up-regulation of p21 and down-regulation of p53 was reversed by UO126 (a MEK1/2 inhibitor), but not by SP600125 (a JNK inhibitor) or SB203580 (a p38 inhibitor), although TPA increased the phosphorylation of ERK and JNK in MCF-7 cells. In addition, the TPA-induced arrest of the G2/M phase was also recovered by UO126 treatment. To confirm the expression of p21 through the MEK/ERK pathway, cells were transfected with constitutively active (CA)-MEK adenovirus. Our results showed that the expression of p21 was significantly increased by CA-MEK overexpression. Taken together, we suggest that TPA reciprocally regulates the level of p21 and p53 expression via a MEK/ERK-dependent pathway. The up-regulation of p21 in response to TPA is mediated through a p53-independent mechanism in breast cancer cells.

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Introduction

Phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), are natural molecules that are well-known tumor promoters and reversible activators of protein kinase C (PKC) (1). TPA may act as a potential inducer of tumor invasion and migration in various tumor cells, as well as a trigger of diverse cellular functions, including cell cycle arrest in a variety of tumor cells, such as breast and colon cancer cells (1-4). TPA induces the expression of cyclins A and B, which are involved in the S phase and G2/M transition during cell proliferation in pancreatic cancer cells (5). However, TPA increases the cell proliferation of fibroblasts and several cancer cell lines, such as KG-1 human leukemia cells and ZR75-1 human breast cancer cells (6,7). To date, the role of TPA is controversial and the exact mechanism by which TPA regulates the cell cycle in breast cancer cells has not been fully elucidated.

The cyclin kinase inhibitor, p21, a universal inhibitor of cyclin-dependent kinases, plays an important role in keeping cells alive after DNA damage and regulates the cell cycle subsequent to p53 induction (6,7). Induction of p21 results in a survival advantage in a wide variety of cells, such as glioblastoma cells (8), muscle cells (9), and macrophages (10). In contrast, the down-regulation of p21 by transfection of antisense oligonucleotides promotes apoptosis in both T47D and MCF-7 human breast cancer cells (11). The amino-terminal binding site of p21 binds to various cyclin/CDK complexes and is involved with cell cycle progression (12). In addition, the induction of p53 and one of its downstream targets, $p21^{waf1/cip1}$, is involved in accelerated cellular senescence (13), as well as activation of the G1/S and G2/M cell cycle checkpoints (14).

The tumor suppressor protein, p53, is a transcription factor that can trigger cell cycle arrest, DNA repair, replicative senescence, and apoptosis (15). The level of p53 expression is regulated in a wide variety of cellular stresses which are genotoxic (DNA alterations induced by irradiation, UV, carcinogens, and cytotoxic drugs) and not genotoxic (hypoxia, cell detachment, growth factor deprivation, and oncogene expression) (18). Somatic missense mutations of p53 are found in approximately 50% of human cancers (16) and are capable of conferring increased tumorigenicity, metastasis, and tumor invasion (17,18).

In the current study, we investigated the regulatory mechanism of p53 and p21 expression by TPA and the effect of TPA on the control of the breast cancer cell cycle. We showed that the level of expression of p53 and p21 in response to TPA is reciprocally regulated via a MEK/ERK-dependent pathway in MCF-7 breast cancer cells. In addition, TPA-induced p21 mRNA and protein expression was shown to be mediated through a p53-independent mechanism in breast cancer cells.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM) and antibiotics were purchased from Life Technologies (Rockville, MD, USA). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Rabbit polyclonal anti-p21 and β-actin antibodies were purchased from Abfrontier (Seoul, Korea). Mouse monoclonal anti-p53 and the secondary peroxidase-conjugated antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). UO126 and TPA were purchased from Tocris (Ellisville, MO, USA). ECLplus reagents were purchased from Amersham (Buckinghamshire, UK).

Cell cultures. MDA-MB-231 and MCF-7 cells were cultured in DMEM media and MDA-MB-453 cells were cultured in RPMI-1640 media supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Each cell culture was maintained in medium supplemented without FBS for 24 h.

Chemical and/or inhibitor treatment. For experiments, cells were maintained in culture medium supplemented without FBS for 24 h. Culture media were replaced with fresh serum-free media and further incubated with TPA and/or specific inhibitors at 37˚C for 24 h. UO126 (10 μM) (a MEK1/2 inhibitor), SP600125 (a JNK inhibitor), or SB203580 (a p38 inhibitor) was added 30 min prior to TPA treatment.

Western blotting. Cell lysates were used in immunoblot analysis for p53, p21, and β-actin proteins. Proteins were boiled for 5 min in Laemmli sample buffer and electrophoresed in 10% SDS-PAGE gels. Proteins were transferred to PVDF membranes and blocked in 10% skim milk in TBS with 0.01% Tween-20 (TBS/T) for 15 min. The blots were incubated with anti-p53, p21 (1/1,000), or β-actin antibodies (1/2,000) in TBS/T buffer at 4˚C overnight. Blots were washed 4 times for 10 min in TBS/T buffer, and subsequently incubated in anti-rabbit peroxidase-conjugated antibody (1/2,000 dilution) in TBS/T buffer for 1 h at room temperature (RT), then blots were washed 4 times in TBS/T buffer. ECLplus reagents were used for development.

RT-PCR. Total RNA was extracted from MCF-7 and MDA-MB-231 human breast cancer cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Extracted RNA was electrophoresed in 1% agarose gels to confirm quality and quantity. Equal amounts of RNA (1 μg) were reverse-transcribed using a first-strand cDNA synthesis kit (MBI Fermentas, Vilnius, Lithuania). Semi-quantitative PCR was performed using specific primers, as follows: human p53 (forward, 5'-GGC CCA CTT CAC CGT ACT AA-3'; reverse, 5'-AAG CGA GAC CCA GTC TCA AA-3'); human p21 (forward, 5'-GCG ATG GAA CTT CGA CTT TGT-3'; reverse, 5'-GGG CTT CCT CTT GGA GAA GAT-3'); and β-actin as an internal control (forward, 5'-AAA CTG GAA CGG TGA AGG TG-3'; reverse, 5'-CTC AAG TTG GGG GAC AAA AA-3'). The PCR conditions used were as follows: 1 cycle of initial denaturation (5 min at 94˚C); 20 cycles (β-actin) of amplification (1 min at 94˚C, 1 min at 60˚C, and 1 min at 72˚C), 30 cycles (human p53) of amplification (30 sec at 94˚C, 30 sec at 54˚C, and 1 min at 72˚C), or 25 cycles (human p21) of amplification (30 sec at 94˚C, 30 sec at 58˚C, and 1 min at 72˚C); and 1 cycle of final extension (10 min at 72˚C). Reaction products were electrophoresed in 2% agarose gels and visualized with ethidium bromide (EtBr).

Flow cytometry analysis (FACS). Cells were trypsinized and harvested by centrifugation at 1,500 rpm for 5 min. The cell pellets were then resuspended in 1 ml of PBS and fixed in 70% ethanol for 20 min at RT. The fixed cells were centrifuged and washed twice in PBS to wash out any apoptotic cells. The cells were resuspended in 1 ml of PBS with 100 μg/ml of DNase-free RNase A (Biopure, Canada), then incubated for 30 min in a 37˚C water bath. The cells were collected by centrifugation at 1,500 rpm, the cell pellets were washed twice with PBS, resuspended in PBS containing 50 μg/ml of propidium iodide (Sigma, St. Louis, MO, USA), then analyzed using FACS-vantage (Becton-Dickinson, San Diego, CA, USA).

Adenovirus transfer. The empty (*Lac Z*) and adenoviral human CA-MEK was the gift of Dr Ha Hyunil (Seoul National University, Korea). Recombinant adenovirus expressing human CA-MEK was reproduced into 293A cells. The expression of this construct was confirmed by Western blotting.

Results

TPA differentially regulates the expression of p53 and p21 in breast cancer cells. To investigate TPA-induced modulation of the cell cycle, we chose MCF-7 and MDA-MB-231 breast cancer cells. The properties of MCF-7 and MDA-MB-231 breast cancer cells are wild-type p53 and mutant p53, respectively.

To determine the expression of p53 and p21 by TPA, MCF-7 and MDA-MB-231 breast cancer cells were treated for the indicated times and concentrations. Our results showed that TPA decreased the level of expression of p53 protein in a time-dependent manner (Fig. 1A). In contrast, the level of expression of p21 protein was significantly increased by TPA in MCF-7 breast cancer cells with wild-type p53 (Fig. 1A). Furthermore, we examined the dose-response effect of TPA in MCF-7 breast cancer cells. As shown in Fig. 1B, the level of expression of p53 and p21 protein was reciprocally regulated by TPA. In addition, we investigated these effects in MDA-MB-231 breast cancer cells with mutant p53. We showed that TPA did not affect the level of expression of p53 protein, whereas the level of expression of p21 was significantly increased by TPA (Fig. 1C). Therefore, we demonstrated that the TPA-induced expression of p21 was regulated through a p53-independent mechanism in breast cancer cells.

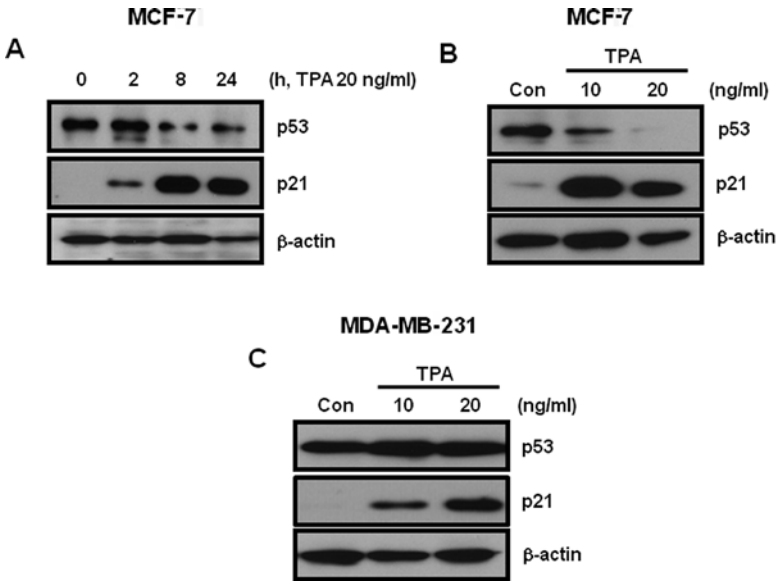

Figure 1. The expression of p53 and p21 in response to TPA in breast cancer cells. (A) After serum starvation for 24 h, MCF-7 breast cancer cells were treated with 20 ng/ml of TPA for the indicated times. After serum starvation for 24 h, MCF-7 (B) and MDA-MB-231 (C) breast cancer cells were treated with TPA at the indicated concentrations for 24 h. The expression levels of p53, p21, and β-actin protein were analyzed by Western blotting. The results are representative of three independent experiments. Con, control.

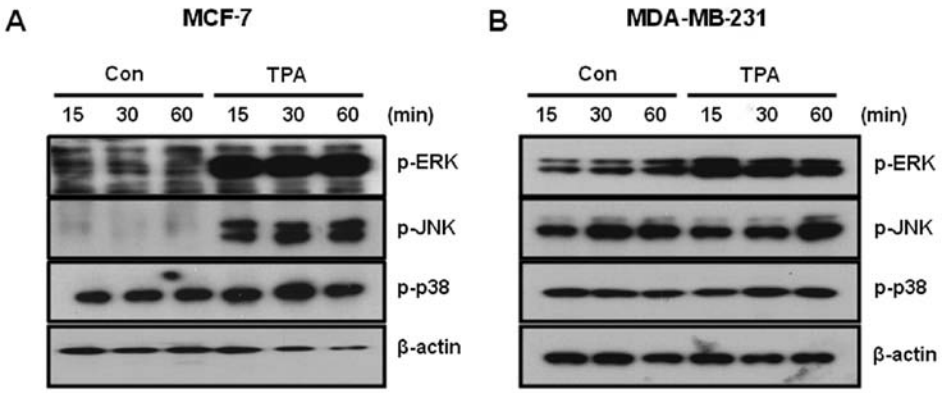

Figure 2. The phosphorylation of ERK1/2, JNK, and p38 in response to TPA in breast cancer cells. After serum starvation for 24 h, MCF-7 (A) and MDA-MB-231 (B) breast cancer cells were treated with 20 ng/ml of TPA for the indicated times. The levels of ERK1/2, JNK, and p38 phosphorylation were analyzed by Western blotting. The results are representative of three independent experiments. Con, control.

Phosphorylation of ERK1/2, JNK, and p38 in response to TPA in breast cancer cells. To verify the regulatory mechanism of p53 and p21, we treated cells with 20 ng/ml of TPA for the indicated times. We showed that the phosphorylation of ERK1/2 and JNK was maximally increased by TPA at 15 min. The phosphorylation of p38 was not affected by TPA in MCF-7 breast cancer cells (Fig. 2A). However, in MDA-MB-231 breast cancer cells, the phosphorylation of ERK1/2 was significantly increased by TPA, but not JNK and p38 (Fig. 2B). Therefore, we suggest that the MEK/ERK signaling pathway may play an important role on the expression of p53 and p21 in response to TPA in breast cancer cells.

Expression of p53 and p21 mRNA by TPA is mediated by a MEK/ERK-dependent mechanism in breast cancer cells. To determine the signaling mechanism involved in the TPA-induced up-regulation of p21 and the down-regulation of p53, MCF-7 with wild-type p53 and MDA-MB-231 with mutant p53 breast cancer cells were pre-treated with a MEK1/2 inhibitor (UO126), a JNK inhibitor (SP600125), or a p38 inhibitor (SB203580) for 30 min prior to TPA treatment, then treated with 20 ng/ml of TPA for 24 h. As shown in Fig. 3A, the TPA-induced up-regulation of p21 and down-regulation of p53 mRNA expression were reversed by UO126, but not by SP600125 and SB203580 in MCF breast cancer cells. In addition, TPA-induced p21 mRNA expression was decreased by UO126 in MDA-MB-231 breast cancer cells (Fig. 3B). However, the level of p53 mRNA expression was not changed by TPA and/or UO126 treatment (Fig. 3B). Therefore, we demonstrated that the TPA-induced up-regulation of p21 and down-regulation of p53 were mediated by a MEK/ERK-dependent mechanism, although the expression of p53

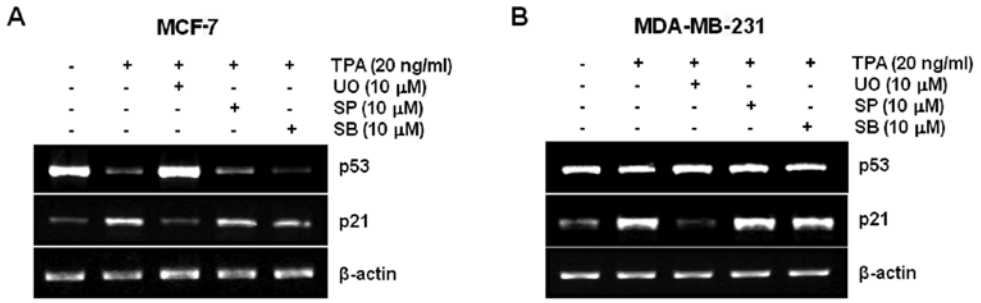

Figure 3. The expression of p53 and p21 mRNA by TPA is mediated by a MEK/ERK-dependent mechanism in breast cancer cells. After serum starvation for 24 h, MCF-7 (A) and MDA-MB-231 (B) breast cancer cells were pre-treated with 10 μM UO, SP, or SB for 30 min, then they were treated with 20 ng/ml of TPA for 24 h. The levels of p53 and p21 mRNA were analyzed by RT-PCR. The results are representative of three independent experiments. Con, control; UO, UO126; SP, SP600125; SB, SB203580.

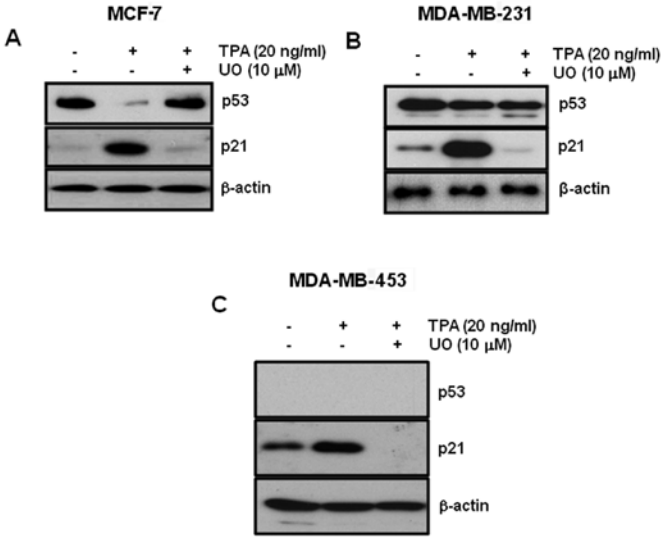

Figure 4. The TPA-induced up-regulation of p21 and down-regulation of p53 are reversed by UO126 in breast cancer cells. After serum starvation for 24 h, MCF-7 (A), MDA-MB-231 (B), and MDA-MB-453 (C) breast cancer cells were pre-treated with 10 μM UO for 30 min, then treated with 20 ng/ml of TPA for 24 h. The levels of expression of p53 and p21 protein were analyzed by Western blotting. The results are representative of three independent experiments. Con, control; UO, UO126.

mRNA was not affected by TPA in MDA-MB-231 breast cancer cells.

The TPA-induced up-regulation of p21 and down-regulation of p53 are reversed by UO126 in breast cancer cells. To confirm the level of p53 and p21 protein expression in response to TPA, MCF-7 with wild-type p53, MDA-MB-231 with mutant p53, and MDA-MB-453 with p53 null breast cancer cells were pre-treated with UO126 for 30 min prior to TPA treatment, then treated with 20 ng/ml of TPA for 24 h. Like the level of mRNA, the TPA-induced up-regulation of p21 was suppressed by UO126 in all of the cells (Fig. 4). In contrast, the TPA-induced down-regulation of p53 protein was increased by UO126 in MCF-7 breast cancer cells (Fig. 4A). However, the level of p53 protein expression was not changed by TPA and/or UO126 in MDA-MB-231 (mutant p53) and MDA-MB-453 (null p53) cells (Fig. 4B and C). In a previous study, Sheikh *et al* (19) reported that the $p21^{WAF1/CIP1}$ protein is a critical downstream effector of p53 and overexpression of the $p21^{WAF1/CIP1}$ suppressed the cell division of MCF-7 breast cancer cells. Therefore, we also suggest that the TPA-induced expression of p21 may trigger the arrest of the cell cycle in breast cancer cells.

The TPA-induced G2/M arrest of the cell cycle is recovered by UO126 in breast cancer cells. To determine the involvement of the MEK/ERK signaling pathway on TPA-induced growth arrest of breast cancer cells, we pre-treated cells with 10 μM UO126 prior to treatment of MCF-7 and MDA-MB-231 cells with 20 ng/ml of TPA. After 24 h, the cells were harvested and subjected to FACS analysis. Our results showed that TPA-treated cells significantly increased G2/M phase arrest compared with vehicle-treated control cells in MCF-7 and MDA-MB-231 cells (Fig. 5). The G2/M phase portion (30.5%) was significantly increased by 12.5% of the control level with TPA treatment in MCF-7 breast cancer cells (Fig. 5A). However, the TPA-induced G2/M phase arrest of the cell cycle was decreased by 10.2% of the control level with UO126 treatment (Fig. 5A). We also confirmed these effects in MDA-MB-231 breast cancer cells. Like the MCF-7 cells, the TPA-induced G2/M phase portion (39.4%) was significantly decreased by 7.8% of the control level with UO126 treatment (Fig. 5B). Therefore, we demonstrated that the TPA-induced G2/M phase growth arrest of the cell cycle was mediated through a MEK/ERK-dependent mechanism in breast cancer cells.

The levels of expression of p21 and p53 protein are regulated by CA-MEK in breast cancer cells. Finally, we investigated the involvement of the MEK/ERK signaling pathway on the expression of p21 and p53 in breast cancer cells. Thus, we transiently transfected MCF-7 and MDA-MB-231 cells with Ad-Lac Z and Ad-CA-MEK for 24 h, respectively, followed by further incubation for 24 h in serum-free media. Our results showed that the level of p53 protein expression was significantly decreased, whereas the level of p21 expression was increased by Ad-CA-MEK overexpression in MCF-7 breast cancer cells (Fig. 6A). In MDA-MB-231 cells, the level of expression of p53 protein was not changed by Ad-CA-MEK overexpression (Fig. 6B). However, under the same conditions, the expression of p21 protein was significantly increased

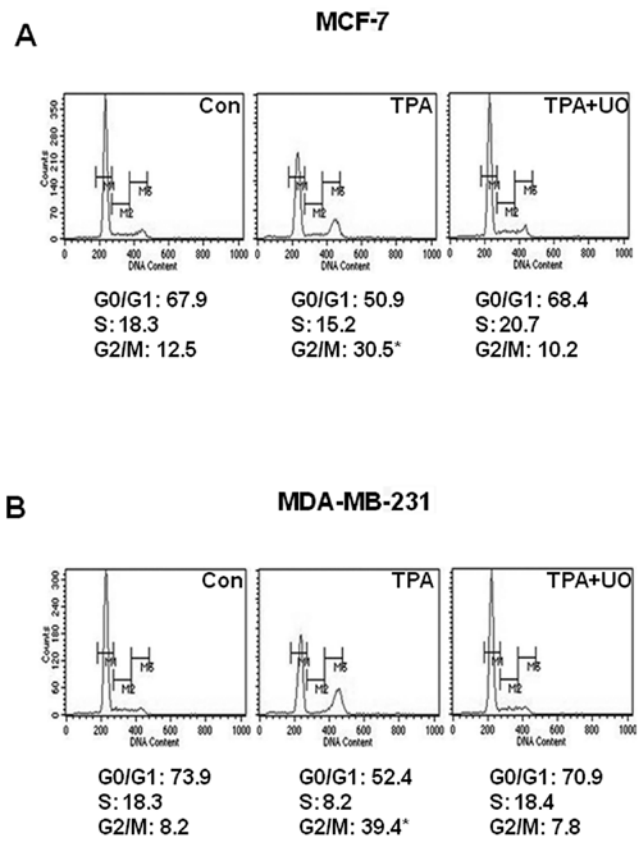

Figure 5. The TPA-induced G2/M arrest of the cell cycle is recovered by UO126 in breast cancer cells. After serum starvation for 24 h, MCF-7 (A) and MDA-MB-231 (B) breast cancer cells were pre-treated with 10 μM UO for 30 min, then treated with 20 ng/ml of TPA for 24 h. The cell cycle was analyzed by FACS analysis, as described in Materials and methods. The results are representative of three independent experiments. Con, control; UO, UO126.

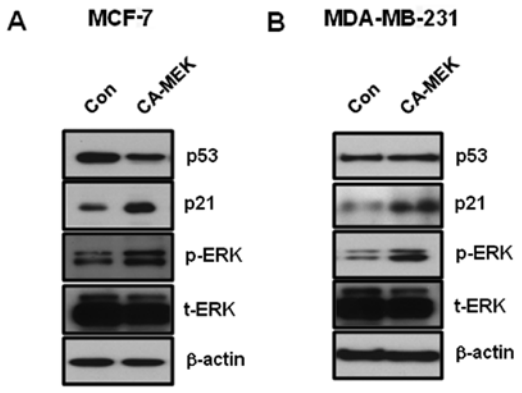

Figure 6. The levels of p21 and p53 protein expression are regulated by CA-MEK in breast cancer cells. After adenoviral (Ad)-Lac Z and Ad-CA-MEK infection for 24 h and serum starvation for 24 h, MCF-7 (A) and MDA-MB-231 (B) breast cancer cells were further incubated at 37˚C for 24 h in serum-free media. Using whole cell lysates, the expression of p53, p21, p-ERK1/2, and β-actin was measured by Western blotting. These results are representative of three independent experiments. Con, control (Ad-Lac Z); CA-MEK, constitutively active-MEK.

(Fig. 6B). Therefore, we demonstrated that the MEK/ERK signaling pathway directly regulates the level of expression in p21 and p53 in breast cancer cells.

Discussion

Phorbol esters, such as TPA, are natural molecules that are well-known tumor promoters and reversible activators of PKC (20). TPA has a differential effect on a variety of cells (21). TPA significantly increases cell proliferation in fibroblasts, epidermal cells, and several cancer cell lines, such as KG-1 human myeloblastic leukemia cells and ZR75-1 human breast cancer cells (22,23). However, TPA-induced activation of PKC suppresses the cell growth of non-small cell lung cancer cells and DanG pancreatic cancer cells (21,24). Although the role of TPA on the regulation of the cell cycle is controversial, we also found that TPA triggers the arrest of S to G2/M transition in MCF-7 and MDA-MB-231 breast cancer cells.

The induction of $p21^{waf1/cip1}$ expression during differentiation has been observed in monocytes and macrophages, such as U937, ML-1, and HL60 cells by TPA treatment (25,26). $p21^{waf1/cip1}$ is known to bind cyclin-cdk complexes to inhibit kinase activities and suppresses Rb protein phosphorylation to inhibit cell division (27,28). Overexpression of $p21^{waf1/cip1}$ has been reported to suppress the proliferation of cancer cells through the inactivation of cyclin D1-cdk in melanoma and prostate cancer cells (29,30). Consistent with these reports, our results showed that TPA significantly increases the level of p21 mRNA and protein expression in MCF-7, MDA-MB-231, and MDA-MB-453 breast cancer cells. Therefore, we demonstrated that enhanced p21 by TPA may augment the G2/M phase arrest of the cell cycle in MCF-7 and MDA-MB-231 breast cancer cells.

Induction of p21 by DNA damage is mediated through the binding of p53 in the p21 promoter (6). However, the level of p21 expression is greatly enhanced by PMA treatment in HL-60 leukemia cells lacking p53 (27). Our results showed that TPA-induced the up-regulation of p21 in both MDA-MB-231 with mutant p53 and MDA-MB-453 with p53 null, which is evidence that TPA-induced p21 expression may be regulated through a p53-independent mechanism.

The PMA-induced phosphorylation of ERK significantly increases AP-1 DNA binding activity, then promotes cell proliferation through the induction of cyclin D1 in CCL39 cells (31). However, the diterpene ester-induced activation of ERK is mediated by PKC activation and leads to cellular senescence through an ERK-dependent p21 induction in melanoma cells (32). Our results showed that the TPA-induced G2/M phase arrest and induction of p21 is reversed by a MEK inhibitor (UO126) in MCF-7 and MDA-MB-231 breast cancer cells. Therefore, we suggest that the cell cycle arrest and p21 induction by TPA is regulated through a MEK/ERK-dependent pathway in breast cancer cells.

In previous studies, Chen *et al* (33) reported that selenocystine (SeC)-induced S-phase arrest is associated with suppression of cyclin D1, and CDK4 and 6, with concomitant induction of p21, p27, and p53 through a PI3K/Akt- and MAPK-dependent pathway in MCF-7 breast cancer cells. The leptin-induced down-regulation of p53 is mediated through the JAK2/PI-3K and Akt-MEK/ERK pathways in ZR-75-1 breast cancer cells (33). In agreement with these reports, our results also showed that the TPA-induced down-regulation of p53 is suppressed by UO126 treatment, but not by SP600125 or SB203580. In contrast, the basal level of p53 expression was

significantly increased by CA-MEK overexpression in MCF-7 breast cancer cells. Therefore, we demonstrated that the MEK/ERK pathway directly regulates the transcriptional activity of p53 in MCF-7 breast cancer cells.

In the current study we determined the role of TPA in the regulation of the cell cycle and the regulatory mechanism of a differential expression of p53 and p21 by TPA in breast cancer cells. We found that the levels of expression of p53 and p21 are reciprocally regulated by TPA treatment in MCF-7 breast cancer cells with wild-type p53. In addition, the TPA-induced G2/M arrest, up-regulation of p21, and down-regulation of p53 was reversed by a MEK1/2 inhibitor (UO126), but not by a JNK inhibitor (SP600125), or a p38 inhibitor (SB203580). Taken together, we demonstrated that the opposite regulation of p53 and p21 by TPA is mediated through a MEK/ERK-dependent pathway in MCF-7 cells. Thus, TPA-induced p21 expression triggers G2/M phase arrest of the cell cycle in breast cancer cells.

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