Effect of berberine on p53 expression by TPA in breast cancer cells

SANGMIN KIM, JEONGHUN HAN, NAM-YOUNG KIM, SE KYUNG LEE, DONG HUI CHO, MIN-YOUNG CHOI, JEE SOO KIM, JUNG-HAN KIM, JUN-HO CHOE, SEOK JIN NAM and JEONG EON LEE

Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, Republic of Korea

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Abstract. Berberine (BBR), an isoquinoline derivative alkaloid compound, has been reported to have anti-oxidant and anti-carcinogenic effects. A loss of functional p53 is involved with an increased risk of cancer proliferation and metastasis. Here, we investigated the effect of BBR on the transcriptional activity and the protein expression of p53 in p53-positive (wild-type, MCF7 cells) and p53-negative (mutant, MDA-MB231 cells) human breast cancer cells. Our results showed that the basal level of p53 mRNA and protein expression was increased by BBR treatment. However, tumor promoter, TPA, decreased the level of p53 mRNA and protein expression in MCF7 cells with wild-type p53. In addition, TPA-induced down-regulation of p53 mRNA and protein expression was increased by UO126, but not by SP600125 and SB203580. To verify the regulatory mechanism of p53 protein expression, we investigated the effects of proteasomal inhibitors (ALLN and MG132) or a lysosomal inhibitor (chloroquine) on TPA-induced down-regulation of p53. We observed that TPA-induced down-regulation of p53 protein was prevented by ALLN and MG132, but not by chloroquine. Further, we investigated the effect of BBR on TPA-induced down-regulation of p53 mRNA and protein levels. Interestingly, the levels of TPA-induced down-regulation of p53 mRNA and protein were prevented by BBR, but MDA-MB231 cells with mutated p53 were not affected. In addition, TPA-induced down-regulation of p53 mRNA was also prevented by BBR. Taken together, we suggest that BBR may be used as an effective ingredient for anticancer products, which trigger the transcriptional activity and the inhibition of the degradation of p53, a tumor suppressor gene, in human breast cancer.

Introduction

Berberine, an isoquinoline derivative alkaloid, was originally isolated from *Berberis aquifolium* (Oregon grape), *Berberis aristata* (tree turmeric), *Berberis vulgaris* (barberry), and *Hydrastis canadensis* (goldenseal) (1). Berberine, the major ingredient in these herbs, interacts with nucleic acids, especially DNA *in vitro*, and inhibits DNA, RNA, and protein synthesis; as a result, berberine causes cell cycle arrest and has anti-cancer effects (2-4). In recent studies, we reported that berberine suppresses TNF-α- and TPA-induced MMP-9 expression in gastric cancer cells and human primary keratinocytes (5-7). In addition, UV-induced MMP-1 and TPA-induced MMP-9 expression, and cell invasion are inhibited by berberine in human dermal fibroblasts and primary keratinocytes (6,8). However, the effect of berberine as a regulator on the p53 expression in breast cancer cells is not fully understood.

The tumor suppressor protein p53 is a transcription factor that can trigger either growth arrest and apoptosis (9). The function of p53 is highly regulated through several mechanisms, including phosphorylation, acetylation, methylation, and ubiquitination (10). MDM2 can bind directly to p53 and promote its ubiquitination, and subsequently its degradation by the proteasome (11,12). The induction of p53 by a wide variety of cellular stress, such as UV, hypoxia, and oncogene expression, is involved in accelerated cellular senescence (13) as well as the G1/S and G2/M cell cycle checkpoint activation (14,15). Somatic missense mutations of p53 are found in approximately 50% of human cancers (16). Some p53 mutations are capable of conferring increased tumorigenicity, metastasis, and tumor invasion (17,18).

The aim of this study was to evaluate a novel function of berberine on the level of p53 mRNA and protein expression in human breast cancer cells. We have found that berberine suppresses TPA-induced the down-regulation of p53 mRNA and protein in MCF7 human breast cancer cells.

Materials and methods

Reagents and cell cultures. Dulbecco's modified Eagle's medium (DMEM) and antibiotics were purchased from Life Technologies (Rockville, MD, USA). Ten percent fetal bovine serum (FBS) was purchased Thermo Fisher Scientific (Waltham, MA, USA). Mouse monoclonal anti-p53 and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). TPA was purchased from R&D Systems (Minneapolis, MN, USA). Berberine was purchased from Sigma (St. Louis, MO, USA).

Correspondence to: Dr Jeong Eon Lee, Department of Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Irwon-dong, Gangnam-gu, Seoul 135-710, Republic of Korea
E-mail: paojlus@hanmail.net

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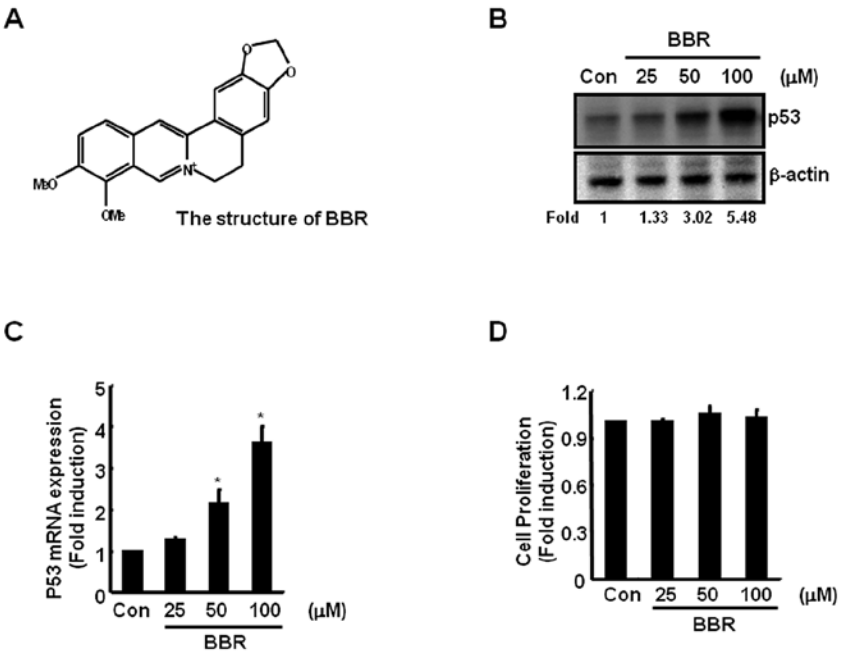

Figure 1. Berberine increases the level of p53 mRNA and protein expression in MCF7 human breast cancer cells in a dose-dependent manner. (A) The chemical structure of berberine. (B, C and D) After serum-starvation for 24 h, cells were treated with berberine at the concentrations indicated for 24 h. The levels of p53 and β-actin protein expression were analyzed by Western blotting (B). The level of p53 mRNA was analyzed by real-time PCR (C). (D) Cell proliferation was analyzed by Quick Cell Proliferation Assay Kit II, as described in Materials and methods. The results were representative of three independent experiments. The values shown are the means ± SEM. *P<0.05 vs. control. Con; control.

The secondary peroxidase-conjugated antibodies and ECL reagents were from Amersham (Buckinghamshire, UK).

Cell culture. The human breast cancer cell lines, MCF7 and MDA-MB231, were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37˚C in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Each cell line was maintained in culture medium supplemented without FBS for 24 h.

Berberine and chemical treatment. Cells were maintained in culture medium without FBS for 24 h, then the culture media were replaced with fresh media without FBS and the cells were further incubated with the indicated concentrations of berberine for 24 h. In experiments involving berberine, ALLN, MG132, and chloroquine, cells were pretreated for 60 min prior to treatment with TPA (20 nM).

Cell proliferation assay. Total cell numbers were evaluated by Quick Cell Proliferation Assay Kit II (BioVision, Mountain View, CA) according to the manufacturer's protocol. Briefly, MCF7 breast cancer cells ($5x10^4$/well) were cultured in a 96-well plate in 100 μl/well of culture media in the absence or presence of the indicated concentration of berberine. After incubating the cells for 24 h, 10 μl WST reagent was added to each well. Viable cells were quantified photometrically at 480 nm.

Western blotting. Cell lysates were used in immunoblot analysis for p53, p-ERK, p-JNK, p-p38, and β-actin. Proteins were boiled for 5 min in Laemmli sample buffer and electrophoresed in 10% SDS-PAGE gels. Proteins were transferred to PVDF membranes and the membranes were then blocked with 10% skim milk in TBS with 0.01% Tween-20 for 15 min. The blots were incubated with anti-p53 and β-actin antibodies (1/1,000 dilution) in 1% TBS/T buffer (0.01% Tween-20 in TBS) at 4˚C overnight. Blots were washed 3 times in TBS with 0.01% Tween-20 and subsequently incubated in anti-rabbit peroxidase-conjugated antibody (1/2,000 dilution) in TBS/T buffer. After 1-h incubation at room temperature (RT), the blots were washed three times and ECL reagents (Amersham Bioscience) were used for development. Signal densities were quantified using a densitometric program (Bio 1D; Vilber Lourmat, Marne La Vallec, France).

Real-time PCR. Total RNA was extracted from cells by using the TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Isolated RNA samples were then used for RT-PCR. Samples (total RNA, 1 μg) were reverse-transcribed into cDNA in 20-μl reaction volumes using a first-strand cDNA synthesis kit for RT-PCR, according to the manufacturer's instructions (MBI Fermentas, Hanover, MD, USA).

Gene expression was quantified by real-time PCR using SensiMix SYBR Kit (Bioline Ltd., London, UK) and 100 ng of cDNA per reaction. The sequences of the primer sets used for this analysis are as follows: human p53 (forward, 5'-GGC CCA CTT CAC CGT ACT AA-3'; reverse, 5'-AAG CGA GAC CCA GTC TCA AA-3') and β-actin as an internal control (forward, 5'-CTG GCA CCC AGC ACA ATG-3'; reverse, 5'-GCC GAT CCA CAC GGA-3'). An annealing temperature of 60˚C was used for all of the primers. PCRs were performed in a standard 384-well plate format with an ABI 7900HT real-time PCR detection system. For data analysis, the raw threshold cycle

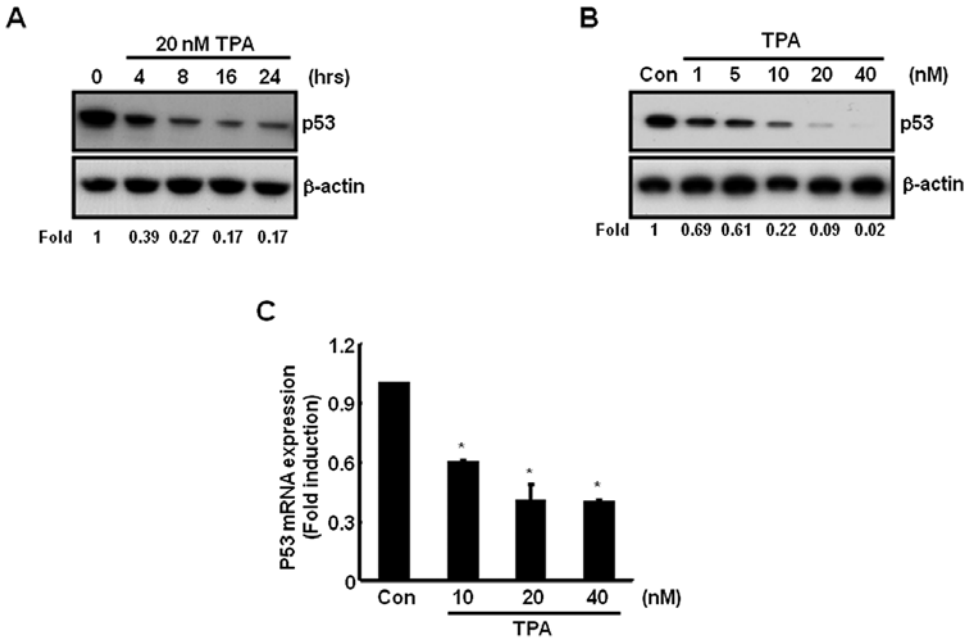

Figure 2. The expression of p53 mRNA and protein is decreased by TPA in a time- and dose-dependent manner in MCF7 human breast cancer cells. (A) After serum-starvation for 24 h, cells were treated with 20 nM TPA for the times indicated. (B and C) After serum-starvation for 24 h, cells were treated with TPA at the concentrations indicated for 24 h. The levels of p53 and β-actin protein expression were analyzed by Western blotting. The level of p53 mRNA was analyzed by real-time PCR. The results are representative of three independent experiments. The values shown are the means ± SEM. *P<0.05 vs. control. Con; control.

(C_T) value was first normalized to the housekeeping gene for each sample to get ΔC_T. The normalized ΔC_T was then calibrated to control cell samples to get $\Delta\Delta C_T$.

Statistical analysis. Statistical significance was determined using the Student's t-test. Results are presented as means ± SEM. All quoted P-values are two-tailed and differences were considered significant at P<0.05.

Results

Berberine increases the level of p53 mRNA and protein expression in MCF7 human breast cancer cells in a dose-dependent manner. In previous studies, berberine-induced cancer cell growth inhibition and apoptosis was mediated through p53-dependent mechanism in human osteosarcoma (19) and prostate cancer cells (20). Here, to determine the effect of berberine on the expression of p53, we treated MCF7 cells with the indicated doses of berberine for 24 h. The structure of berberine is provided in Fig. 1A. As shown in Fig. 1B, berberine increased the expression of p53 in a dose-dependent manner. The level of p53 protein expression was dose-dependently increased to 1.33-, 3.02-, and 5.48-fold of the control level by 25, 50, and 100 μM berberine treatment, respectively (Fig. 1B). In addition, the level of p53 mRNA expression was significantly increased to 1.28-, 2.18-, and 4.85-fold of the control level by 25, 50, and 100 μM berberine treatment, respectively (Fig. 1C). Under the same conditions, cells were not affected by berberine treatment at the indicated concentrations (Fig. 1D).

The expression of p53 mRNA and protein is decreased by TPA in a time- and dose-dependent manner in MCF7 human breast cancer cells. To verify the level of p53 mRNA and protein expression by TPA, we treated cells for the indicated times and at the indicated doses. The level of p53 protein expression was decreased by TPA treatment in a time- (Fig. 2A) and dose-dependent manner (Fig. 2B). Twenty-four hours following 20 nM TPA treatment, the level of p53 protein expression was significantly decreased (0.17-fold of the control level; Fig. 2A). In p53-negative (mutant, MDA-MB231 cells) human breast cancer cells, the level of p53 expression was not changed by TPA (data not shown). In addition, the expression level of p53 was decreased by 0.09- and 0.02-fold of control level at 20 and 40 nM TPA treatment, respectively (Fig. 2B). In addition, the level of p53 mRNA was also decreased by TPA in a dose-dependent manner (Fig. 2C). Based on these results, we suggested that TPA down-regulates the level of p53 mRNA and protein expression in MCF7 breast cancer cells with wild-type p53.

The down-regulation of p53 mRNA and protein by TPA is mediated by MEK/ERK-dependent mechanism in MCF7 breast cancer cells. To investigate the signaling mechanism involved in the TPA-induced the down-regulation of p53, MCF7 with wild-type p53 breast cancer cells were pretreated with a MEK1/2 inhibtor, UO126, a JNK inhibitor, SP600125, and a p38 inhibitor, SB203580 for 30 min prior to TPA treatment and then treated with 20 nM TPA for 24 h. As shown in Fig. 3A, the TPA-induced down-regulation of p53 mRNA expression was reversed by UO126, but not by SP600125 and SB203580. The level of p53 mRNA expression was significantly decreased to 0.3-fold of the control level by 20 nM TPA treatment (Fig. 3A). In contrast, the TPA-induced down-regulation of p53 mRNA was increased to 0.8-fold of the control level by UO126 (Fig. 3A). Under the same condi-

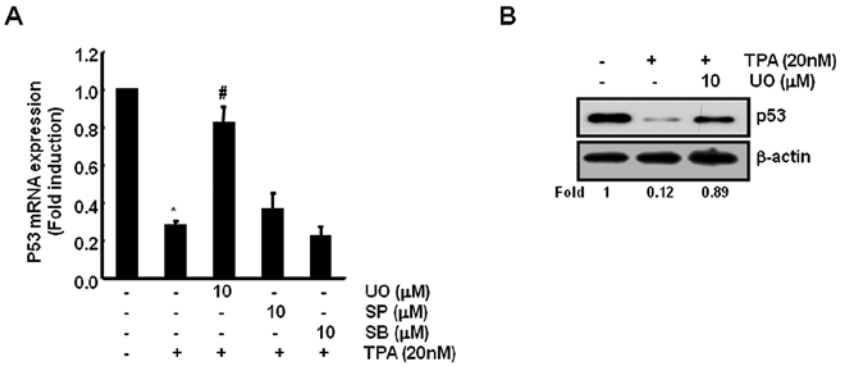

Figure 3. The down-regulation of p53 mRNA and protein by TPA is suppressed by MEK inhibitor, UO126 in MCF7 human breast cancer cells. (A and B) After serum-starvation for 24 h, cells were pretreated with 10 μM UO, SP, and SB for 30 min, respectively, and then treated with 20 nM TPA for 24 h. The level of p53 mRNA was analyzed by real-time PCR (A). The levels of p53 and β-actin protein expression were analyzed by Western blotting (B). The results are representative of three independent experiments. The values shown are the means ± SEM. *P<0.05 vs. control. #P<0.05 vs. TPA-treated cells. Con; control, UO; UO126, SP; SP600125, SB; SB203580.

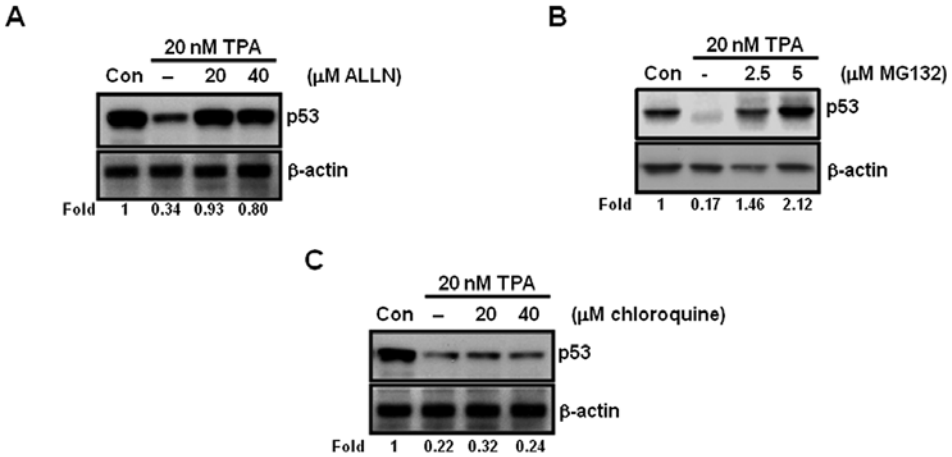

Figure 4. The down-regulation of p53 by TPA is mediated by a proteasomal-dependent pathway, but not by a lysosomal-dependent pathway in MCF7 human breast cancer cells. After serum-starvation for 24 h, cells were pretreated with ALLN (A), MG132 (B), and chloroquine (C) at the concentrations indicated for 60 min and then treated with 20 nM TPA for 24 h. The levels of p53 and β-actin protein expression were analyzed by Western blotting. The results are representative of three independent experiments. Con; control.

tion, the TPA-induced down-regulation of p53 protein was prevented by UO126 (Fig. 3B). Therefore, we demonstrated that the TPA-induced down-regulation of p53 mRNA and protein were mediated by MEK/ERK-dependent mechanism in MCF7 breast cancer cells.

The down-regulation of p53 by TPA is mediated by a proteasomal-dependent pathway, but not by a lysosomal-dependent pathway in MCF7 human breast cancer cells. Next, we investigated the down-regulation pathway of p53 protein by TPA in MCF7 breast cancer cells. Our results showed that the TPA-induced down-regulation of p53 protein was increased by the proteasomal inhibitors, ALLN and MG132 in a dose-dependent manner (Fig. 4A and B). The p53 expression in response to TPA was decreased by 0.34-fold of the control level (Fig. 4A). In contrast, TPA-induced p53 down-regulation was increased by 0.93- and 0.80-fold of the control level by 20 and 40 μM ALLN, respectively (Fig. 4A). TPA-induced p53 down-regulation was also increased by 1.46- and 1.48-fold of the control level by 2.5 and 5 μM MG132, respectively (Fig. 4B). However, TPA-induced p53 down-regulation was not affected by the lysosomal inhibitor, chloroquine (Fig. 4C). Therefore, we demonstrated that the TPA-induced down-regulation of p53 protein was mediated by a proteasomal-dependent pathway, but not by a lysosomal-dependent pathway in MCF7 breast cancer cells with wild-type p53.

Berberine prevents the TPA-induced down-regulation of p53 mRNA and protein in MCF7 human breast cancer cells in a dose-dependent manner. We next investigated the effects of berberine treatment on TPA-induced the down-regulation of p53 in MCF7 and MDA-MB231 cells. TPA treatment significantly decreased p53 protein expression by 0.33-fold of the control level (Fig. 5A) in MCF7 cells. In contrast, TPA-induced p53 degradation was prevented to 0.82- and 1.95-fold of the control level by 50- and 100-μM berberine treatment, respectively (Fig. 5A). The TPA-induced down-regulation of p53 mRNA was also prevented to 0.60- and 1.77-fold of the control level by 50 and 100 μM berberine treatment,

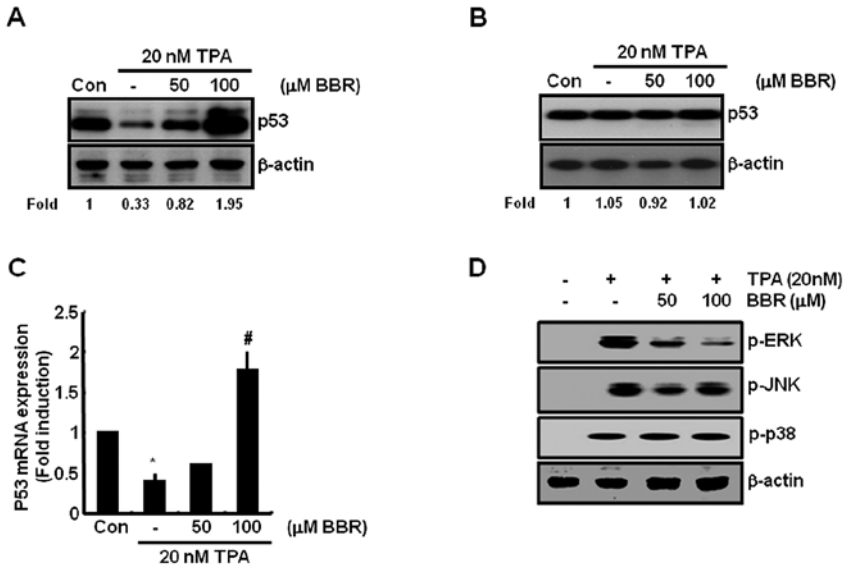

Figure 5. Berberine prevents TPA-induced down-regulation of p53 mRNA and protein in MCF7 human breast cancer cells. After serum-starvation for 24 h, MCF7 cells with wild-type p53 (A and C) and MDA-MB231 cells with mutated p53 (B) were pretreated with berberine at the concentrations indicated for 60 min and then treated with 20 nM TPA for 24 h. (D) After serum-starvation for 24 h, MCF7 cells with wild-type p53 were pretreated with berberine at the concentrations indicated for 60 min and then treated with 20 nM TPA for 30 min. The levels of p53, p-ERK, p-JNK, p-p38 and β-actin protein expression were analyzed by Western blotting. The level of p53 mRNA was analyzed by real-time PCR. The results are representative of three independent experiments. The values shown are the means ± SEM. *P<0.05 vs. control. Con; control.

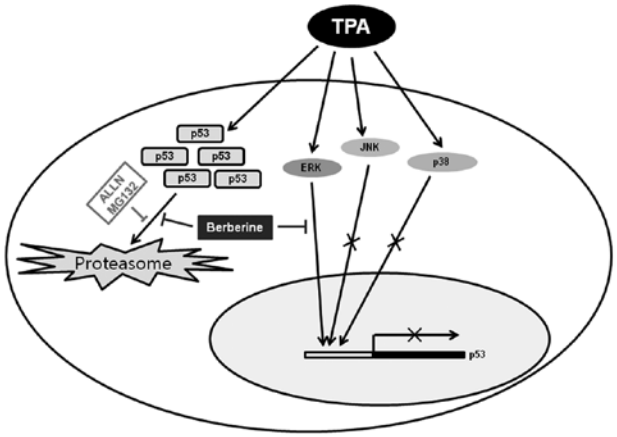

Figure 6. Schematic model.

respectively (Fig. 5B). However, we found that TPA did not affect the down-regulation of p53 protein in MDA-MB231 cells with mutant p53 (Fig. 5C).

We also showed that the phosphorylation of ERK1/2, JNK, and p38 was significantly increased by TPA. In contrast, TPA-induced phosphorylation of ERK was decreased by berberine in a dose-dependent manner in MCF7 breast cancer cells (Fig. 5D). Therefore, we demonstrated that berberine prevents the TPA-induced down-regulation of p53 mRNA and protein through the inhibition of MEK/ERK pathway.

Discussion

Berberine, one of the major alkaloids, has a variety of pharmacological effects including inhibition of protein synthesis, cell cycle progression and apoptosis in a variety of cancer cell lines such as hepatoma, leukemia and breast cancer cells (3,4,7). Zhang *et al* also reported that berberine triggers apoptosis in acute lymphoblastic leukemia cells through down-regulating the MDM2 (21). Because the role of p53 has been emerging in cell cycle and apoptosis of cancer cells, we also investigated the effect of berberine on p53 mRNA and protein expression in human breast cancer cells.

The level of p53 expression is regulated by p53-interacting protein, MDM2 (an E3 ubiquitin ligase) that promotes ubiquitination and subsequent proteasomal degradation of p53 (22). The function of p53 is known in a variety of cell types including the arrest of cell cycle, cellular senescence, and apoptosis (19,21). p21 and Polo-like kinase 1 are important targets of p53 for G1/S and G2-M cell cycle checkpoint activation, respectively (15). It has been reported that berberine inhibits growth of the breast cancer cells (23) and acute lymphoblastic leukemia cells through the induction of p21 (21). Generally, many cancer cells have the function of self-prevention by conventional chemotherapy. In the present study, our results showed that the level of p53 protein expression is increased by berberine in a dose-dependent manner. Based on these results, we believe that berberine is a potent drug for treatment of breast cancer patients through the regulation of p53 mRNA and protein expression.

TPA is a well known tumor promoter and may induce the generation of ROS in a PKC-dependent fashion (24). TPA may act as a potential inducer of tumor invasion and migration in various tumor cells including breast cancer and prostate cancer cells (25,26). In addition, TPA-induced PKC mediates EMT and cell migration coupled with gene expression of MMP-9 (27,28). Our results showed that the phosphorylation of ERK is increased by TPA. The levels of p53 mRNA and protein expression were significantly suppressed by TPA treatment. These effects were prevented

by the MEK inhibitor UO126. Therefore, we demonstrate that TPA-induced down-regulation of p53 mRNA and protein is mediated through MEK/ERK dependent pathway in MCF7 breast cancer cells with wild-type p53.

The viral E6 protein derived from tumor associated papillomavirus types principal target is the cellular suppressor protein p53 (29) and the complex formation between E6 and p53 triggers the ubiquitin-mediated degradation (30). In addition, Mantovani and Banks reported that the inhibition of E6 mediated degradation of p53 results in an accumulation of p53 expression, while mitomycin-induced the p53 expression is further increased by proteasome inhibition (31). Consistent with these reports, our results also showed that the level of p53 protein expression was significantly decreased by TPA. TPA-induced down-regulation of p53 expression was suppressed by proteasome inhibitors, ALLN and MG132. Although we did not investigate the role of berberine as the proteasome inhibitor, we suggest that berberine may act as a proteasomal inhibitor like the ALLN and the MG132 in breast cancer cells.

In this study, we found that the level of wild-type p53 mRNA and protein expression is dose-dependently increased by berberine treatment. In contrast, TPA significantly decreased the level of wild-type p53 expression in MCF7 cells but not the level of mutant p53 in MDA-MB231 cells. As shown in Fig. 6, TPA suppresses the transcriptional activity of p53 through MEK/ERK dependent pathway and mature p53 protein degradation by TPA mediates through proteasome pathway in MCF7 breast cancer cells. Interestingly, we observed that the TPA-induced inhibition of transcriptional activity and the degradation of p53 are prevented by berberine. Therefore, we suggest that berberine may be used as an effective ingredient for anti-cancer products, which trigger the transcriptional activity and the inhibition of p53 degradation, a tumor suppressor gene, in human breast cancer.

Acknowledgements

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