SPANDIDOS PUBLICATIONS

Effects of trichostatin A, a histone deacetylase inhibitor, on the regulation of apoptosis in H-*ras*-transformed breast epithelial cells

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Abstract. This study examined the mechanism for the anti-cancer effects of histone deacetylase (HDAC) inhibitor trichostatin A (TsA) in H-*ras*-transformed human breast epithelial (MCF10A-*ras*) cells. The effects of TsA on anti-cancer effects of MCF10A-*ras* cells were determined by measuring the level of cell cycle regulator expression and apoptotic cell death using Western blotting and flow cytometry analysis, respectively. TsA induced morphological changes, apoptotic cell death and modulation of the cell cycle regulatory proteins in the MCF10A-*ras* cells. TsA increased the levels of acetylated histone H3 and H4 in MCF10A-*ras* cells. In addition, TsA markedly down-regulated the expression of cyclin D1 and CDK4, up-regulated the expression of $p21^{WAF1}$ and p53 and induced cell cycle arrest at the G_1 phase in MCF10A-*ras* cells. The levels of hyperphosphorylation of the Rb protein were lower in MCF10A-*ras* cells after the TsA treatment. Furthermore, the up-regulation of p53 promoted Bax expression, which led to the activation of pro-caspase-3 and eventually to apoptosis in MCF10A-*ras* cells. TsA significantly increased the levels of ERK1/2 phosphorylation in MCF10A-*ras* cells. Overall, the TsA-activated ERK pathway plays an important role in cell cycle arrest and apoptosis through the ERK-dependent induction of p21 in Ras-related human cancer cells.

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Introduction

Histone deacetylase (HDAC) inhibitors are considered as promising target molecules for cancer chemotherapy. Recently, an increasing number of structurally diverse HDAC inhibitors, including trichostatin A (TsA), suberoylanilide hydroxamic acid (SAHA), trapoxin, sodium butyrate, FK228 and MS-275 have been identified (1,2). These inhibitors have been reported to inhibit cancer cells proliferation by arresting the cell cycle (3,4), induce morphological changes in oncogene-transformed cells (5,6) and exhibit anti-tumor activity in a variety of cancers (7,8). TsA effectively inhibits the activity of HDAC enzymes at nanomolar concentrations, targeting the cell cycle progression of several cell lines and inducing cell growth inhibition at both the G_1 and G_2/M phases (4,9). Taken together, the potential use of HDAC inhibitor for cancer chemotherapy is currently being investigated in clinical trials and a well-tolerated safety profile has been demonstrated in the clinical studies (10,11).

There is considerable evidence suggesting that HDAC inhibitors alter the cellular morphology and cause cell cycle arrest in oncogene-transformed cells or cancer cells (12,13). However, the molecular mechanisms for the effects of HDAC inhibitors on the regulation of apoptosis through the Ras-activated ERK pathways are not completely understood. Several studies have shown that *ras*-transformed cells have a higher susceptibility to anticancer agents, such as etoposide (14) and depsipeptide FR901228 (15). In addition, it was reported that a culture of Ras-transformed cells in the presence of the HDAC inhibitor, apicidin, exhibited morphological reversion and cell cycle arrest in the G_1 phase (16). Therefore, HDAC inhibitors might alter the cell morphology of oncogene-transformed cells and increase the cell cycle arrest of transformed cells (2,17). However, the cellular effects of HDAC inhibitors in oncogene-transformed mammalian cells are unclear but they are believed to be a novel class of anticancer agents (3,18). The above studies highlight the need for a better understanding of the Ras-mediated signaling

cascade that leads to the increased cell susceptibility to HDAC inhibitors in *ras*-transformed cells.

Although TsA exhibits potent broad spectrum anti-protozoal activity against apicomplexan parasites and has anti-proliferative activity against many cancer cell lines (4,5), the precise mechanism of how TsA regulates apoptosis in ras-activated human cancer cells is not completely understood. For this study, stably H-*ras*-transformed human breast epithelial cells (MCF10A-*ras* cells) were used as target cells to determine how TsA regulates the Ras-mediated apoptotic pathways.

Materials and methods

Materials. Trichostatin A (TsA) was obtained from Sigma-Aldrich Biotechnology (Sigma-Aldrich, St. Louis, MO, USA). TsA was dissolved in dimethyl sulfoxide (DMSO) and diluted immediately prior to experiment with media containing 5% fetal bovine serum (FBS). The final DMSO concentration in each treatment was <0.01%.

Cell cultures. MCF10A cells, which is a spontaneously immortalized cell line derived from diploid primary human breast epithelial cells without viral or chemical intervention, and the MCF10A-*ras* cells were kindly provided by Dr A. Moon (Duksung Women's University, Seoul, Korea). The MCF10A and MCF10A-*ras* cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10 μg/ml insulin, 20 ng/ml epidermal growth factor (EGF), 0.1 μg/ml cholera enterotoxin, 100 U/ml penicillin-streptomycin, 2.5 mM L-glutamine and 0.5 μg/ml fungizone. Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were plated on plastic dishes and the culture medium was replaced every 2 days. For cell growth analysis the cells were cultured at a density of $1x10^4$ viable cells in 6-well plates. The cells were then trypsinized and the number of viable cells was counted daily over a 6 day period.

Ras activation assay. The levels of activated Ras in the cell lysates isolated from MCF10A-*ras* cells were determined using a Ras activation assay kit (Upstate, Billerica, MA, USA) with the horseradish peroxidase-conjugated anti-mouse antibody (Santa Cruz, CA, USA) according to the manufacturer's instructions.

Cytotoxicity assay. Cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The MCF10A and MCF10A-*ras* cells were cultured in 96-well plates at a density of $2x10^3$ cells per well for 48 h. After incubation, the cells were treated with a 5 mg/ml MTT solution at 37˚C for 4 h and then dissolved in DMSO. The ratio of viable cells was determined by measuring the absorbance per well at 540 nm using a VERSA@max Microplate Reader (Molecular Devices Corp, Sunnyvale, CA, USA).

DAPI staining of nuclei. The morphological changes in the nuclear chromatin of the cells undergoing apoptosis were observed by staining with the DNA binding fluorochrome, 4',6-diamidino-2-phenylindole (DAPI). The cells were grown in 6-well plates at a density of $2x10^4$ cells per well. After treatment, cells were fixed in absolute methanol and stained with 300 μl of a DAPI solution (1 μg/ml) at 37˚C for 15 min. The staining solution was removed and the cells were examined by fluorescence microscopy (Axiovert 200, Carl Zeiss, Jena, Thuringen, Germany).

Flow cytometry analysis. The MCF10A and MCF10A-*ras* cells were treated with TsA for 24 h. The cells were harvested and washed with 1% bovine serum albumin in PBS before being fixed with 70% ethanol containing 0.5% Tween-20 at 4˚C for at least 1 h. The fixed cells were then washed with 1% bovine serum albumin in PBS and suspended in a cold propidium iodine (PI) staining solution (100 μg/ml RNase A and 10 μg/ml PI in PBS) at 4˚C for 40 min. The stained cells were analyzed for their relative DNA content using a flow cytometry system (Beckman Coulter, Fullerton, CA, USA), which is based on measuring the increase in red fluorescence.

Western blot analysis. The total protein from control, TsA-treated cells were prepared using the following procedure. Briefly, the cells were harvested by trypsinization, washed with ice-cold PBS, centrifuged at 1,500 x g for 5 min and incubated for 20 min in 50 μl of lysis buffer I (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.5 mM PMSF). The mixture was then incubated for 10 min on ice and then centrifuged at 12,000 x g for 10 min. After collecting the supernatant containing the cytosolic fraction, the pellet was resuspended by syringe/needle in 30 μl of ice-cold buffer II (2.75% lysis buffer I and 0.05% NP-40) and incubated for 20 min on ice. After centrifugation at 12,000 x g for 10 min, the pellet was resuspended in 40 μl of lysis buffer III (5 mM HEPES, pH 7.9, 300 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF and 26% glycerol) for 40 min. After final centrifugation at 12,000 x g for 40 min, the supernatant (nuclear extract) was collected. The protein content from extracts was determined using the protein assay reagent (Bio-Rad).

Statistical analysis. Unless otherwise indicated, all data are expressed as the mean ± SD from triplicate experiments performed in a parallel manner. The statistical significance was analyzed using a Student's t-test. The comparisons are made relative to the untreated controls and the statistical significance is indicated as $^*p<0.05$.

Results

Cell viability, ras activation and histone protein acetylation. The MCF10A and MCF10A-*ras* cell lines were characterized by measuring the levels of activated Ras in their cell lysates using a Ras activation assay kit. The level of the activated Ras protein was higher in the MCF10A-*ras* cells than in the MCF10A cells (Fig. 1A). The effect of activated Ras on cell proliferation was determined by counting the number of MCF10A-*ras* cells daily over a 48 h period. As shown in Fig. 1B, the inhibition of MCF10A-*ras* cell proliferation was observed in a concentration- or time-dependent manner. The MCF10A and MCF10A-*ras* cells expressed low levels of

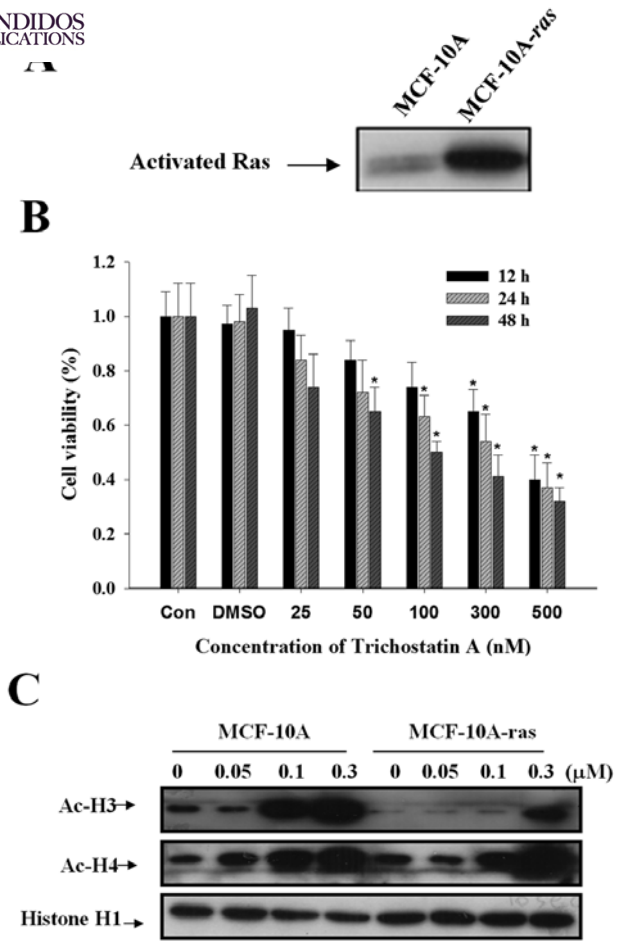

Figure 1. Effects of trichostatin A on ras oncogene expression, cell proliferation and histone protein acetylation in the stable transfection of MCF10A cells with the activated. (A) MCF10A and MCF10A-*ras* cells were treated with the vehicle alone (Con) or different concentrations of trichostatin A for 48 h. The levels of activated Ras in the cell lysates of the MCF10A-*ras* transfectant (MCF10A-*ras*) and untransfected control (MCF10A) cells were characterized using a Ras activation assay kit. (B) Effect of trichostatin A on MCF10A-*ras* cells cytotoxicity was measured using an MTT assay. Data are expressed as the mean ± SD. $^{*}P<0.05$, compared with the control cells. (C) Effect of trichostatin A treatment on acetylation of histone proteins in MCF10A and MCF10A-*ras* cells. The nuclear extracts were isolated from MCF10A and MCF10A-*ras* cells treated with trichostatin A for 24 h. Western blot analysis was carried out using anti-acetyl histone H3 and anti-acetyl histone H4 antibodies. Ac-H3, acetylated histone H3 and Ac-H4, acetylated histone H4.

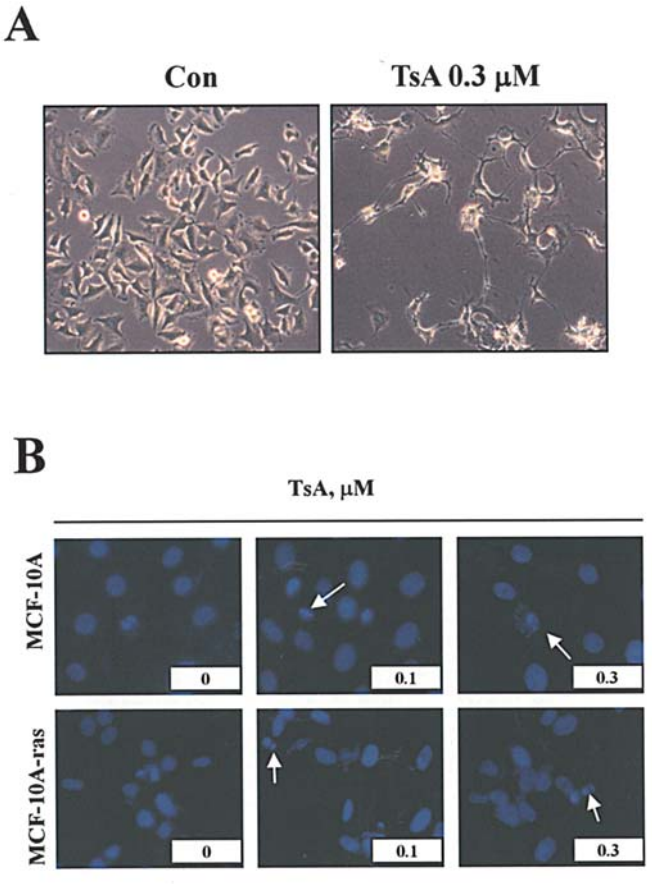

Figure 2. Morphological changes in the MCF10A-*ras* cells after the trichostatin A treatment. (A) MCF10A-*ras* cells were treated with trichostatin A (0.3 μM) for 24 h and the cell morphology was examined microscopically (x200). (B) MCF10A and MCF10A-*ras* cells were treated with the vehicle alone (Con) or with different concentrations of trichostatin A (0.1 and 0.3 μM) for 24 h and then stained with DAPI. The nuclear morphology was visualized using fluorescence microscope. The arrows indicate the apoptotic cells with condensed and fragmented nuclei.

acetylated H3 and H4 in the control cultures, whereas TsA treatment caused a significant concentration-dependent increase in the levels of acetylated H3 and H4 (Fig. 1C).

Morphological changes and apoptosis induction. An oncogenic transformation often causes dramatic morphological changes in the target cells (19). As shown in Fig. 2A, the morphology of MCF10A-*ras* cells in the culture changed from a rounded morphology to a spindle *ras*-transformed morphology. After exposure to TsA (up to 0.3 μM), the cell line showed dramatic changes to an elongated shape with filamentous protrusions. The MCF10A and MCF10A-*ras* cells were stained with DAPI, which enters the nuclear membrane and binds to DNA, in order to determine if the decrease in cell viability caused by TsA is associated with cellular apoptosis. As shown in Fig. 2B, the TsA treatment caused the production of cellular apoptotic bodies in both cell lines after TsA treatment for 24 h but not in the control cells. This suggests that TsA inhibits cell proliferation and induces apoptotic cell death.

Cell cycle distribution. Considering that TsA decreases the level of MCF10A-*ras* cell proliferation, its effect on the cell cycle distribution was also examined by flow cytometry. As shown in Fig. 3, TsA caused growth arrest in G_1 phase of the cell cycle in MCF10A-*ras* cells compared with their untreated counterpart cultures. Treating the cells with 0.3 μM TsA caused the accumulation of cells predominantly at the G_1 phase (83.2%) with a concomitant decrease in the number of cells in S phase.

Expression of cell cycle regulators. The mechanisms involved in the cell cycle arrest induced by TsA were examined by measuring the expression levels of the major cell cycle regulators (cyclin D1, CDK4, cyclin E, $p21^{WAF1/Cip1}$, p53, retinoblastoma (Rb) and Rb phosphorylation) by Western blot analysis. As shown in Fig. 4, TsA caused a decrease in the level of cyclin D1 and CDK4 expression in both cell lines

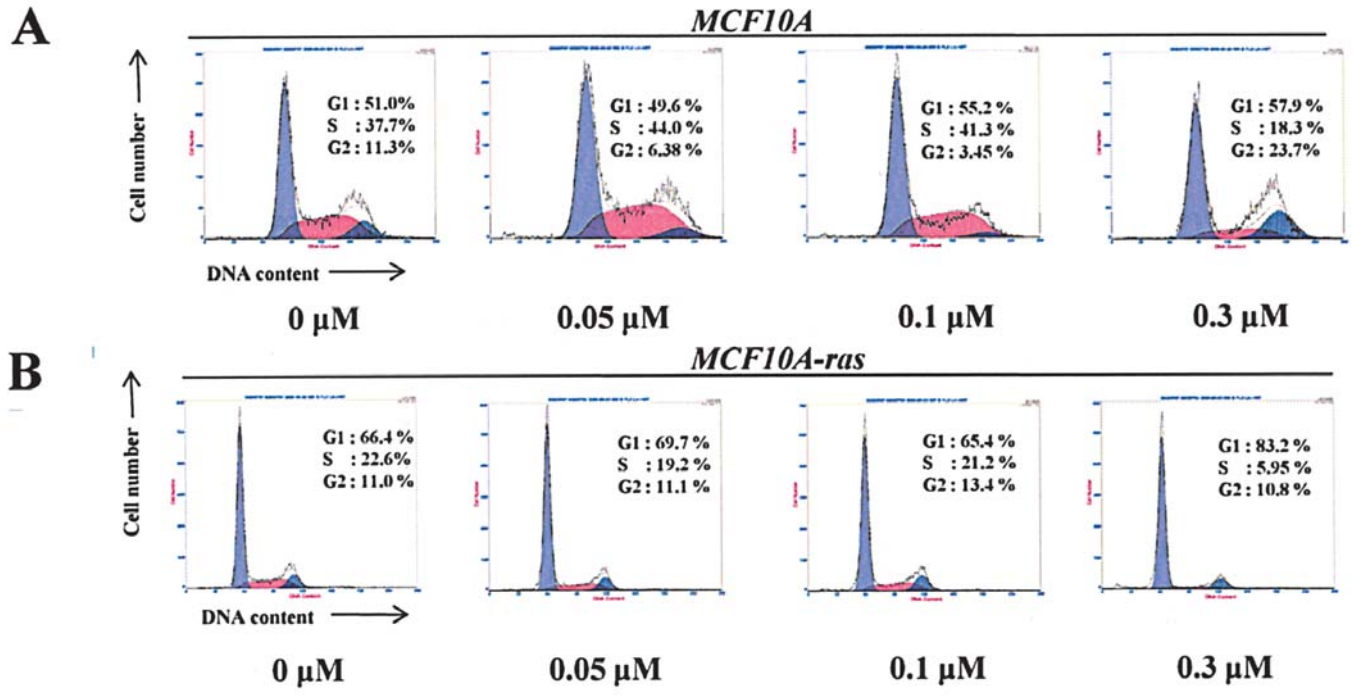

Figure 3. Effect of trichostatin A on the cell cycle phase distribution. The MCF10A (A) and MCF10A-*ras* (B) cells were treated with different concentrations (0.05, 0.1 and 0.3 μM) of trichostatin A for 24 h. The cell cycle distribution was analyzed by flow cytometry after staining the DNA with propidium iodide (PI).

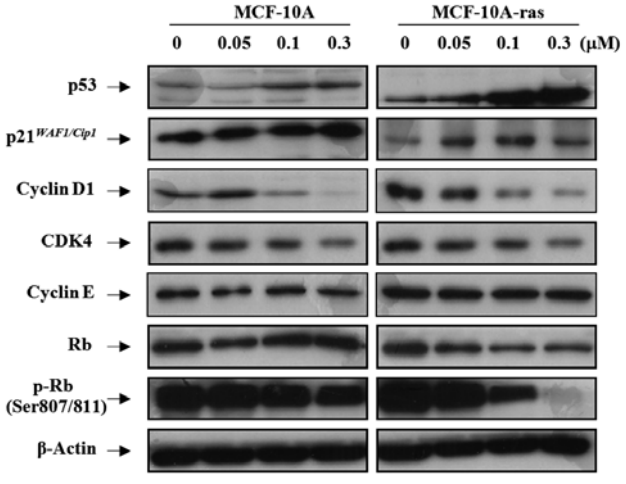

Figure 4. Effect of trichostatin A on the expression of the cell cycle regulatory proteins. The proteins were isolated from MCF10A and MCF10A-*ras* cells treated with trichostatin A for 24 h. The expression of cell cycle regulatory proteins was determined by Western blot analysis using the indicated antibodies. CDK, cyclin dependent kinase; p-Rb, phosphorylated Rb and Rb, retinoblastoma protein.

without altering the levels of cyclin E. In addition, TsA increased the levels of p53 and p21$^{WAF1/Cip1}$ expression in MCF10A-*ras* cells compared with control or MCF10A cells. After exposure to TsA, there was a significant decrease in the level of phosphorylated Rb (p-Rb) protein in MCF10A-*ras* cells, whereas TsA had almost no effect on the total Rb protein levels in either cell line.

Expression levels of apoptosis regulating genes. The effects of TsA on the expression of the Bcl-2 family genes were examined in order to determine the molecular basis for the TsA-induced apoptosis. As shown in Fig. 5A, TsA caused the clear down-regulation of the anti-apoptotic gene, Bcl-2, whereas the expression of the pro-apoptotic gene, Bax, was increased. The levels of procaspase-3 expression were also measured in order to clarify the TsA-induced apoptosis pathway. As expected, TsA markedly decreased the levels of procaspase-3 in MCF10A-*ras* cells. Furthermore, the level of ERK1/2 expression in the Ras-signaling pathway was measured after a 24 h TsA treatment. Similar to the levels of ectopically expressed H-Ras, the level of ERK1/2 phosphorylation was measured to determine if TsA activates the ERK pathway, the signaling pathway downstream of Ras. The TsA treatment dramatically increased the levels of phosphorylated ERK1/2 in MCF10A-*ras* cells (Fig. 5C).

Discussion

There has been considerable attention on the use of HDAC inhibitors as anticancer agents because they can induce either apoptosis or cell cycle arrest (20). Many studies have examined the ability of HDAC inhibitors to induce the expression of genes such as p21$^{Waf1/Cip1}$, p27^{Kip1} and p53, as well as induce cell cycle arrest at the G_1 or G_2/M phase (21,22). In this study, it was found that TsA induced apoptosis in MCF10A-*ras* cells through cell cycle arrest at the G_1 phase. Similar to the other HDAC inhibitors, TsA effectively blocked the deacetylation of the histone H3 and H4 proteins, which influenced the activation of *ras*-mediated transcriptional factors and inhibited the expression of cell cycle regulators. Furthermore, the TsA treatment produced cellular apoptosis in MCF10A-*ras* cells as a result of the induction of cell cycle arrest at G_1 phase. This suggests that TsA inhibits the MCF10A-*ras* cell growth by increasing the expression of

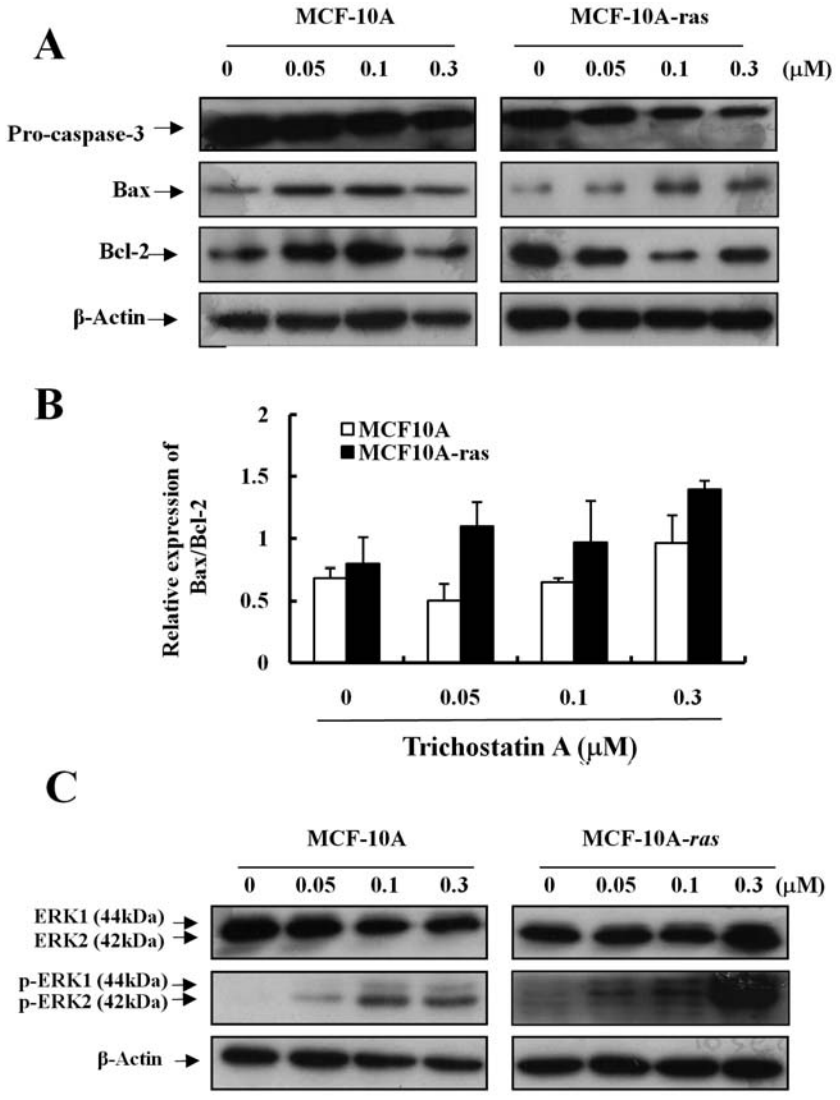

Figure 5. Effect of trichostatin A on the expression of the downstream proteins in apoptotic pathway. The cells were treated with vehicle alone or with 0.05, 0.1 and 0.3 μM of trichostatin A for 24 h. Western blot analysis was performed using the indicated antibodies. (A) The blots for procaspase-3, Bax and Bcl-2 are shown. The quantification of Bcl-2/Bax ratio is expressed. (B) Autoradiograms were scanned by densitometry. Data are expressed as the mean ± SD (n=3). (C) Effect of trichostatin A on ERK1/2 activation. The level of protein expression was determined by Western blot analysis with the antibodies directed against ERK1/2 and p-ERK1/2. ERK, extracellular signal-regulated kinase and p-ERK, phosphorylated ERK.

CDK inhibitors (such as $p21^{WAF1/Cip1}$ and $p27^{Kip1}$) and decreasing the expression of G_1 phase cell cycle regulators, such as cyclin D1 and CDK4 protein. Cyclin D1 plays an important role in the progression of mammalian cells through the G_1 phase in the cell cycle. Previous studies reported that TsA down-regulates cyclin D1 and CDK4, increases the p53 protein levels and effectively blocks G_1 phase of cell cycle, leading to apoptosis (19,23,24).

TsA increased significantly the level of p53 and $p21^{WAF1/Cip1}$ expression in MCF10A-*ras* cells compared with that observed in MCF10A cells. Therefore, TsA is likely to block cell cycle progression from G_1 to S phases. It was recently reported that cyclin D1 is a key mediator of the Ras-induced $p21^{WAF1/Cip1}$ stability in *ras*-transformed cells (25). In this study, TsA reduced the level of cyclin D1 expression and induced p53 expression in MCF10A-*ras* cells in a concentration-dependent manner, which was accompanied by up-regulation of $p21^{WAF1/Cip1}$. Rb is one of the targets of the cyclin/CDK complexes, which upon hyperphosphorylation dissociate from bound transcription factors, such as E2F, enabling them to activate the genes essential for DNA replication (26). The data in the current study showed that TsA causes a significant decrease in the level of p-Rb expression in MCF10A-*ras* cells, whereas the levels of cyclin E and CDK4 proteins were attenuated in the TsA-treated MCF10A cells without altering p-Rb expression.

Moreover, downstream of the p53 pathway, Bax, a direct target of p53, plays an important role in TsA-induced apoptosis. Bax promotes apoptosis through an interaction with the anti-apoptotic members in the mitochondria, such as Bcl-2 (27). These interactions have been reported to activate caspases. Bax and Bcl-2 are homologous proteins that have opposite effects on the cell fate; Bax promotes apoptosis and Bcl-2 prolongs cell survival (9). In this study, up-regulation of Bax induced the activation of pro-caspase-3 in TsA-treated MCF10A-*ras* cells. This shows that TsA has a significant anti-proliferation effect by activating the apoptotic processes

characterized by the activation of pro-caspase-3 through a p53-dependent mechanism. However, the TsA-induced apoptosis observed in MCF10A cells was independent of caspases that are generally accepted as being effectors of the apoptotic process.

A previous study reported the regulation of p53 expression through the Ras-activated RAF/MEK/ERK pathway in the human fibrosarcoma cell line, HT1080 (25). Once the ERKs are activated by TsA, the up-regulation and activation of p53 are the key downstream events, as reported previously (28,29). The level of ERK1/2 expression in the Ras-signaling pathway was measured to determine the molecular basis for the TsA-induced cell cycle arrest. TsA treatment of MCF10A-*ras* cells increased the levels of phosphorylated ERK1/2. Therefore, the ERK pathway downstream of Ras is involved in mediating the signals that modulate cell cycle arrest and apoptosis in MCF10A-*ras* cells. Similar to these results, previous studies reported that cisplatin- and quercetin-induces apoptosis in human cancer cells through the activation of ERK (30,31). Pettersson *et al* (32) also suggested that the activation of ERK was essential for the retinoid-induced apoptosis of breast cancer cells. Furthermore, an HDAC inhibitor, valproic acid, increased the level of ERK 1/2 phosphorylation in human umbilical vein endothelial cells (3).

In summary, TsA causes morphological changes and induces cell cycle arrest in MCF10A-*ras* cells by up-regulating p53 and $p21^{WAF1/Cip1}$. The effect of TsA appears to be associated with the down-regulation of phosphorylated Rb, whose expression appears to be under the control of cyclin D1/CDK4 complex. In addition, TsA inhibits the growth of MCF10A-*ras* cells through cell cycle arrest at G_1 phase by activation of ERK1/2, which helps inhibit cell growth and the induction of apoptosis through the activation of pro-caspase-3 in MCF10A-*ras* cells.

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