Arch Pharm Res Vol 27, No 5, 554-561, 2004

Archives of Pharmacal Research
http://apr.psk.or.kr

Estrogen Receptor Enhances the Antiproliferative Effects of Trichostatin A and HC-toxin in Human Breast Cancer Cells

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(Received March 18, 2004)

Trichostatin A, an antifungal antibiotics, and HC-toxin are potent and specific inhibitors of histone deacetylase activity. Histone deacetylase inhibitors are new class of chemotherapeutic drugs able to induce tumor cell apoptosis and/ or cell cycle arrest. In this study, the antiproliferative activities of trichostatin A and HC-toxin were compared between estrogen receptor positive human breast cancer cell MCF-7 and estrogen receptor negative human breast cancer cell MDA-MB-468. Trichostatin A and HC-toxin showed potent antiproliferative activity in both MCF-7 and MDA-MB-468 cells. In MCF-7 cells that contain high level estrogen receptor, trichostatin A and HC-toxin brought about three-times more potent cell growth inhibitory effect than estrogen receptor negative MDA-MB-468 cells. Both trichostatin A and HC-toxin showed cell cycle arrest at G_2/M phases of MCF-7 and MDA-MB-468 cells in a dose- and time- dependent manner. Trichostatin A and HC-toxin also induced apoptosis from MCF-7 and MDA-MB-468 cells in a dose- and time-dependent manner. Results of this study suggested that antiproliferative effects of trichostatin A and HC-toxin might be involved in estrogen receptor signaling pathway, but cell cycle arrest and apoptosis of trichostatin A and HC-toxin might not be involved in estrogen receptor system of human breast cancer cells.

Key words: MCF-7, MDA-MB-468, Human breast cancer cell, Estrogen receptor, Trichostatin A, HC-toxin, HDAC

INTRODUCTION

Acetylation of nuclear histones, which is regulated by acetyltransferase and deacetylase, has been known to play a crucial role in gene expression (Boyes *et al.*, 1998; Pazin and Kodonaga, 1997; Grunstein, 1997; Kuo and Allis, 1998). Transcriptionally activated genes have been found to be associated with highly acetylated loci whereas transcriptionally inactive genes have been found to be associated with hypoacetylation (Bannistar and Miska, 2000; Ogryzko *et al.*, 1996; Laherty *et al.*, 1997). Recent molecular and genetic studies have been identified histone acetyltransferase and histone deacetylase (HDAC) as transcriptional coactivators and transcriptional corepressors, respectively (Spencer *et al.*, 1997; Ogryzko *et al.*, 1996). These observations provide a molecular basis for regulation of transcription through acetylation of histones (Bannistar and Miska, 2000; Laherty *et al.*, 1997). Although the precise molecular mechanism underlying cell cycle arrest or differentiation through histone acetylation has not been understood, sodium n-butyrate (NaBu), a HDAC inhibitor, has been known to arrest the cell cycle (Van *et al.*, 1996) and provide various differentiation phenotypes or revertant phenotypes of cancer cells including leukemias (Brehm *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Lin *et al.*, 1998; Grignani *et al.*, 1998), colorectal cancers (Archer *et al.*, 1998), bladder cancer (Tanaka *et al.*, 1995), breast cancers (Yang *et al.*, 2000), and fibroblasts transformed by an oncogene (Wahrman *et al*,, 1985; Wang and Goldberg, 1976). Thus, compounds possessing HDAC inhibiting activity have been thought to represent a novel class of agent with less toxicity, along with all-trans retinoic acid (Lin *et al.*, 1998), for treatment of human cancers. Induction of apoptosis by HDAC inhibitors has been reported in several human cancer cell lines but the mechanism underlying this effect is not well understood (Huang *et al.*, 1999; Medina *et al.*, 1997; Lee *et al.*, 1996; Bernhard *et al.*, 1999; Click *et al.*, 1999). One possibility is that histone acetylation relaxes chromatin and enhances

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accessibility of DNA to apoptotic endonuclease (Sealy and Chalkly, 1978; Lee *et al.*, 1996). Other studies suggest that HDAC inhibitor-induced apoptosis is related to effects on gene expression including p21, c-myc and gelsolin (Hoshikawa *et al.*, 1994; Sowa and Orita, 1999; Gray *et al.*, 1999; Koyama *et al.*, 2000; Futamura *et al.*, 1995). Interestingly, apoptosis of human lung cancer cells induced by TSA is greatly augmented in the presence of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (DAC) (Zhu *et al.*, 2001). DNA methylation status also has an influence on the level of local histone acetylation (Eden *et al.*, 1998). DNA methylation induces hypoacetylation of chromatin and the methyltransferase Dnmt1 interacts directly with HDAC to form a transcriptionally inactive chromatin structure (Robertson *et al.*, 2000; Fuks *et al.*, 2000; Rountree *et al.*, 2000). Recent studies indicate that concurrent treatment of cells with TSA and DAC can restore mRNA expression of methylated tumor suppressor genes (Cameron *et al.*, 1999). Recent studies indicated that ERá is directly hyperacetylated in response TSA treatment in MCF-7 breast adenocarcinoma cell line (Vigushin and Pael, 2001), while the another report showed that acetylation of ERá lysine residues in the hinge/ligand binding domain suppresses ligand sensitivity and regulates transcriptional activation by HDAC inhibitors (Wang *et al.*, 2001). Furthermore, conservation of the acetylated ERá motif in other nuclear receptors suggests that direct acetylation may play an important role in the regulation of diverse nuclear receptor signaling functions (Wang *et al.*, 2001). In ERá positive breast cancer cell lines, TSA treatment alone is sufficient to reactivate transcription of the methylated ER gene. In this study, we have investigated the effects of trichostatin A and HC-toxin on the proliferation and apoptosis of estrogen receptor positive MCF-7 and estrogen receptor negative MDA-MB-468 human breast cancer cells in order to examine if the ER is important for the action of HDAC inhibitor. We found that both trichostatin A and HC-toxin inhibited the proliferations of estrogen receptor positive MCF-7 than estrogen receptor negative MDA-MB-468 human breast cell lines more effectively.

METERIALS AND METHODS

Cell culture

The human breast cancer cell lines MCF-7 and MDA-MB-468 cells were obtained from American Type Culture Collection (Rockville, MD). MCF-7 Cells were maintained in MEM supplemented with 5% fetal bovine serum, insulin and penicillin-streptomycin at 37°C and in 5% CO_2. MDA-MB-468 cells were maintained in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin at 37°C and in 5% CO_2.

Cell proliferation

MCF-7 and MDA-MB-468 cells were plated in 96 well plates at a density of 10,000 cells per well. The following day, the cells were treated with various concentrations of the HDAC inhibitors for three days. The numbers of cells were measured based on the modified method of SRB assay (Soto *et al.*, 1995).

DAPI staining

MCF-7 cells were plated in 6 well plates and treated with various concentrations of the HDAC inhibitors for three days. Cells were washed with 1x PBS. After hypotonic swelling with 75 mM KCl for 1 min at room temperature, cells were fixed in ice-colded methanol:acetic acid solution (3: 1). Cells were air-dried and incubated with the DNA specific fluorochrome DAPI for 20 min. The excess of DAPI was removed and stained nuclei were visualized under a fluorescence microscopy.

Flow cytometry anlaysis

For flow cytometry analysis, cells were plated in 35 mm dishes and treated with DMSO or HDAC inhibitors. After 12, 24, or 72 h, the medium were removed and cells were detached using trypsin-EDTA, washed in PBS and fixed using 70% ethanol. After centrifugation, cell pellets were resuspended and treated with RNase A for 20 min at 37°C. The DNA content was evaluated in a FACScalibur (Becton-Dickinson, Mountain View, Cal., USA) after staining the cells with propidium iodide for 30 min at 37°C in the dark. Apoptosis was assayed by the appearance of a sub-G_1 (< 2N ploidy) population by ModiFit software.

Statistical analysis

Statistical analysis of data was carried out by Student's *t*-test.

RESULTS

Inhibition of proliferations by trichostatin A and HC-toxin in estrogen receptor positive and negative mammary tumor cells

The estrogen receptor positive human breast cancer cell line MCF-7 was cultured with increasing concentrations of TSA (10 nM - 10 μM). As shown in Fig. 1, concentrations of TSA over 0.1 μM caused a profound cell growth inhibition. The IC_{50} of 0.04 μM was calculated as 50% reduction in cell number after 72 h of continuous exposure to TSA (Table I). Various concentrations of TSA (10 nM-10 μM) were treated to estrogen receptor negative human breast cancer cell line MDA-MB-468 for 72 h. Fig. 1 showed inhibition of estrogen receptor negative human breast cancer cell MDA-MB-468 proliferation by TSA treatment. The IC_{50} of 0.107 μM was calculated as 50%

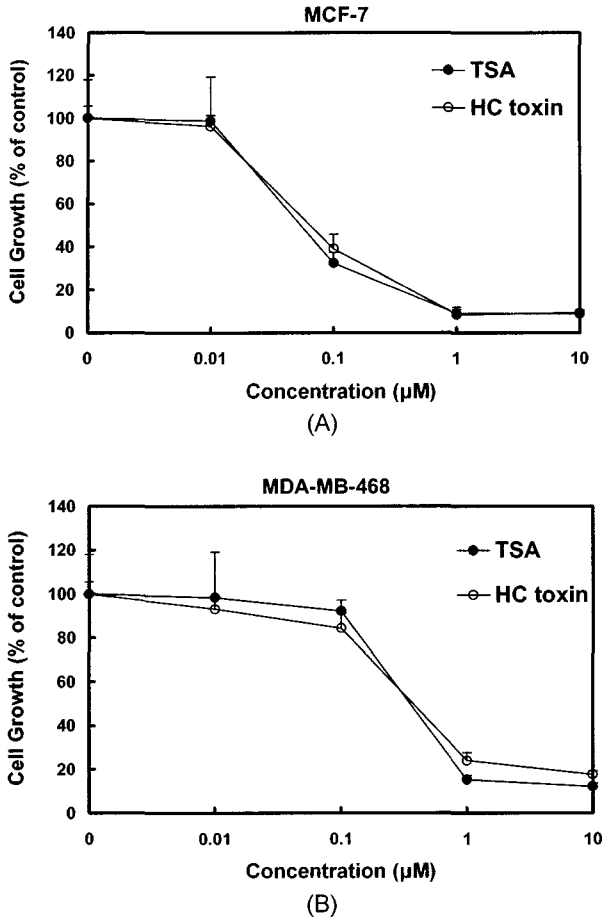

Fig. 1. Effect of HDAC inhibitors on the MCF-7 and MDA-MB-468 human breast cancer cell proliferation. MCF-7 (A) and MDA-MB-468 (B) cells were plated in 96 well plates at a density of 10,000 cells per well. The following day, cells were treated with increasing concentrations of the HDAC inhibitors, Trichostatin A (TSA), or HC-toxin for 72 hrs. Cell growth was determined by absorbance at 570 nm as described in Methods. Data represent the mean ± SD of three independent experiments. (●, Trichostatin A (TSA); ○, HC-Toxin)

Table I. Inhibition of cell proliferation (IC_{50}) of MCF-7 and MDA-MB-468 human breast cancer cells by HDAC inhibitors.

	IC_{50} (μM)	
	MCF-7	MDA-MB-468
Trichostatin A	0.04	0.107
HC-toxin	0.053	0.137

The IC_{50} indicate the concentrations of HDAC inhibitors (Trichostatin A, or HC-toxin) that resulted in 50% reduction of cell number. The IC_{50} was calculated by plotting the inhibition of cell growth. Data were expressed as the average of triplicate values from different experiment.

reduction in cell number after 72 h of continuous exposure to trichostatin A (Table I). Trichostatin A has three-times more potent antiproliferative effect on estrogen receptor positive MCF-7 cells than estrogen receptor negative MDA-MB-468 cells. ER-positive human breast cancer cell line MCF-7 was treated with various concentrations of HC-toxin (10 nM-10 μM) for 72 h. As shown in Fig. 1, HC-toxin was observed to have a potent anti-proliferative effect on MCF-7 cells. IC_{50} of 0.053 μM was calculated as 50% reduction in cell number after 72 hr of continuous exposure to HC-toxin (Table I). Various concentrations of HC-toxin (10 nM-10 μM) were treated to estrogen receptor negative human breast cancer MDA-MB-468 cells for 72 hrs. As shown in Fig. 1, HC-toxin inhibited MDA-MB-468 cell proliferation. IC_{50} of 0.137 μM was calculated as 50% reduction in cell number after 72 h of continuous exposure to HC-toxin (Table I). HC-toxin showed three-times more potent antiproliferative effects on estrogen receptor positive MCF-7 human breast cancer cells than estrogen receptor negative MDA-MB-468 breast cancer cells.

Trichostatin A and HC-toxin arrest cell cycle at G_2/M

We found that treatment of human breast cancer cell line MCF-7 with TSA for 12, 24, or 48 h induced cell cycle arrest (Fig. 2). Treatment of trichostatin A to MCF-7 human breast cancer cells resulted in concentration-dependent cell cycle arrest at G_2/M (Table II). Treatment of MCF-7 cells for 12, 24, or 48 h with HC-toxin also induced cell cycle arrest at G_2/M in a concentration - dependent manner (Table II). The estrogen receptor negative human breast cancer cell MDA-MB-468 cells were cultured with various concentrations (0.1 μM-10 μM) of either TSA or HC-toxin for 24 h. Result of FACS analysis showed that both trichostatin A and HC-toxin arrested cell cycle at G_2/M phase in a concentration-dependent manner (Fig. 2, Table II). Either 1 μM TSA or 1 μM HC-toxin was treated to estrogen receptor negative MDA-MB-468 cells for 12, 24, or 48 h. FACS analysis showed that the time- dependent arrest of cell cycle at G_2/M was observed (Table II). Existence of estrogen receptors in cells showed no effect on TSA- or HC-toxin-induced cell cycle arrest.

Trichostatin A- and HC-toxin-induced apoptosis of human breast cancer cells

Treatment with TSA or HC-toxin to either MCF-7 or MDA-MB 468 human breast cancer cells for 24 hrs induced apoptosis of cells in a concentration-dependent manner (Fig. 3). Cell death induced by TSA (0.1, 1.0, or 10 μM) was compared with that by HC-toxin (0.1, 1.0, or 10 μM) in MCF-7 human breast cancer cells. Significant cell death was observed after treatment with TSA or HC-toxin for 12 h and steadily increased over 48 h time period (Table II). Treatment with different concentrations of TSA (0.1, 1.0, or 10 μM) or HC-toxin (0.1, 1.0, or 10 μM) for 24 h resulted in a dose-dependent increase of apoptosis in MDA-MB-468 cells. Treatment with 1 μM TSA or 1 μM

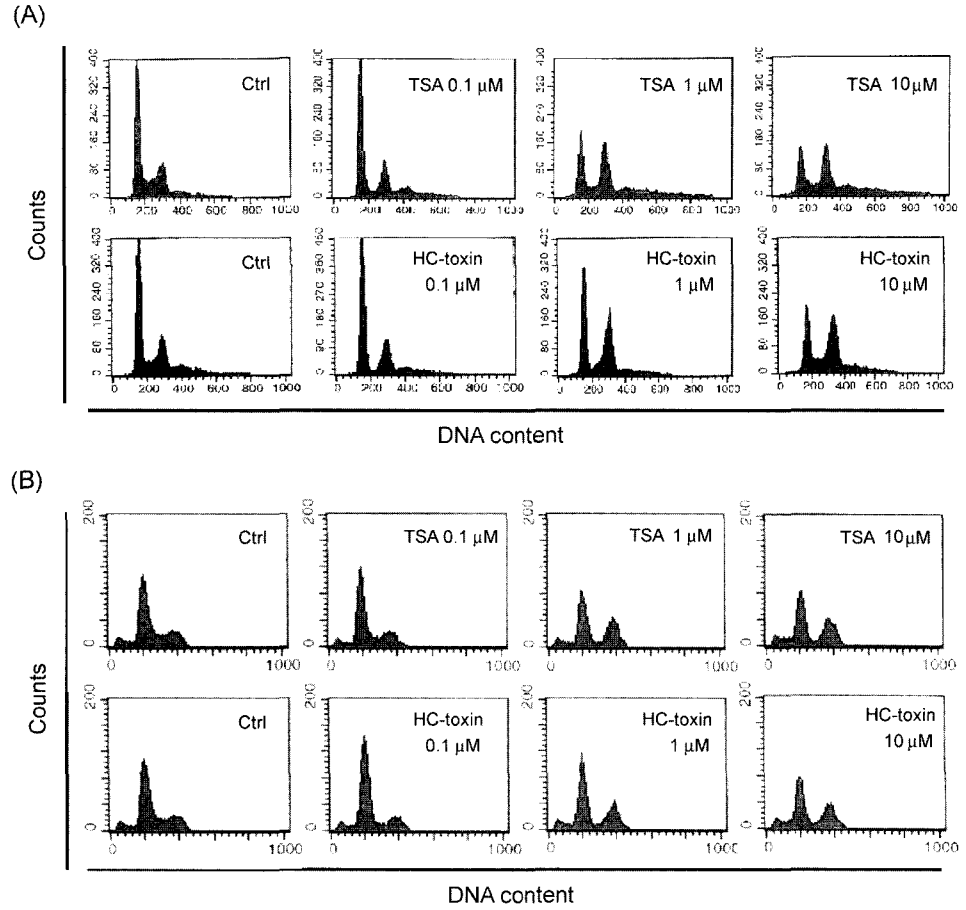

Fig. 2. Effect of HDAC inhibitors on the cell cycle distribution. Representative profiles of cell cycle status. MCF-7 (A) and MDA-MB-468 (B) cells were treated with vehicle solvent (0.1% DMSO) or HDAC inhibitors (trichostatin A or HC-toxin) at the indicated concentration for 12, 24, or 48 hrs, respectively. Cells were harvested, fixed, and stained with propidium iodide. Stained cells were subjected to flow cytometry analysis to determine the distribution of cells. The percentage of cells in G_0/G_1, S, and G_2/M phases was determined by ModiFit software. Cells in G_0/G_1 phase represent the first peak, and cells in the G_2/M phase represent the second peak. Cells in S phase are in the area between the G_0/G_1 and G_2/M phase peaks. Quantitation of cell cycle distribution is presented in Table II.

HC-toxin for 12, 24, or 48 h also showed time-dependent apoptosis of MDA-MB-468 cells (Table II). Similar results were obtained using PI staining and DAPI staining as a marker of cell death (Fig. 4).

DISCUSSION

HDAC inhibitors belong to a heterogeneous class of compounds that include derivatives of short chain fatty acids, hydroxamic acids, cyclic tetrapeptides, and benzamides. Among the hydroxamic acids, TSA and suberoylanilide hydroxamic acid (SAHA) are commonly used inhibitors of HDACs (Lin *et al.*, 1998; Finnin *et al.*, 1999). Numerous antiproliferative effects have been reported for TSA and SAHA, including induction of G_0/G_2 cell cycle arrest, differentiations, and selective apoptosis of transformed cells (Van *et al.*, 1996; Richon *et al.*, 1996; Nuse *et al.*, 1990; Greenspan *et al.*, 1985). SAHA, in particular, shows strong antiproliferative effects but low toxicity *in vivo* and is currently under clinical trials for the treatment of solid and hematological tumor (Lin *et al.*, 1998; Finnin *et al.*, 1999). Recently there has been strong interest in HDAC inhibitors as anticancer agents due to their selective toxicity against tumor cells and synergistic activity with existing therapeutic agents, including retinoic acid (Kitamura *et al.*, 2000), vitamin D analogues (Rashid *et al.*, 2001), and peroxisome proliferators-activated receptor ligands (Chang and Szabo, 2002). In this study, we have shown dose-dependent antiproliferative effects of trichostatin A and HC-toxin on estrogen receptor positive MCF-7 and estrogen receptor negative MDA-MB-468 human breast cancer cells. Our data showed a stronger antiproliferative effects of TSA and HC-toxin in estrogen receptor positive MCF-7 human breast cancer cells than in estrogen receptor negative MDA-MB-468 human breast cancer cells (Table I). TSA has been shown to arrest cells in G_1

Table II. Kinetic Analysis of Cell Cycle Distribution by HDAC inhibitors in MCF-7 and MDA-MB-468 cells

(A)

Treatment (μM)		Distribution (%)			
		Sub-G1	G0/G1	S	G2/M
12 h					
Control		0.07	52.16	32.38	15.48
TSA	0.1	0.72	28.01	21.43	50.57
	1	0.9	25.63	35.00	39.38
	10	1.07	31.09	24.52	44.40
HC-toxin	0.1	0.2	53.35	16.92	29.73
	1	0.4	34.63	28.04	37.33
	10	0.92	30.23	28.42	41.35
24 h					
Control		0.16	54.17	20.19	25.65
TSA	0.1	0.4	63.26	13.90	22.83
	1	2.88	34.43	21.90	43.64
	10	15.5	25.71	20.21	54.08
HC-toxin	0.1	0.25	69.01	7.66	23.33
	1	1.5	40.47	16.71	42.83
	10	1.87	27.06	27.30	45.64
48 h					
Control		0.56	52.43	32.37	15.21
TSA	0.1	1.68	43.9	39.69	16.42
	1	29.05	32.16	40.59	27.24
	10	36.33	32.43	39.75	27.82
HC-toxin	0.1	11.32	52.99	33.68	13.34
	1	44.13	13.83	53.60	32.57
	10	31.80	9.28	62.96	28.77

(B)

Treatment (μM)		Distribution (%)			
		Sub-G1	G0/G1	S	G2/M
12 h					
Control		1.48	47.08	35.86	17.06
TSA	1	1.38	53.93	22.69	23.38
HC-toxin	1	1.13	55.46	21.87	22.67
24 h					
Control		3.66	56.13	32.5	11.37
TSA	0.1	2.66	63.01	22.95	14.04
	1	5	57.28	5.4	37.32
	10	6.43	54.42	8.05	37.53
HC-toxin	0.1	3.66	77.35	11.04	11.61
	1	5.7	63.5	3.05	33.45
	10	6.49	57.78	8.4	33.83
48 h					
Control		5.15	56.04	27.64	16.32
TSA	1	12.42	52.29	28.15	19.55
HC-toxin	1	8.42	37.88	38.43	23.69

MCF-7 (A) and MDA-MB-468 (B) cells were treated with vehicle solvent (0.1% DMSO) or HDAC inhibitors (Trichostatin A or HC-toxin) for 12, 24, or 48 hrs at the indicated concentrations. Cell cycle distribution was measured by flow cytometry.

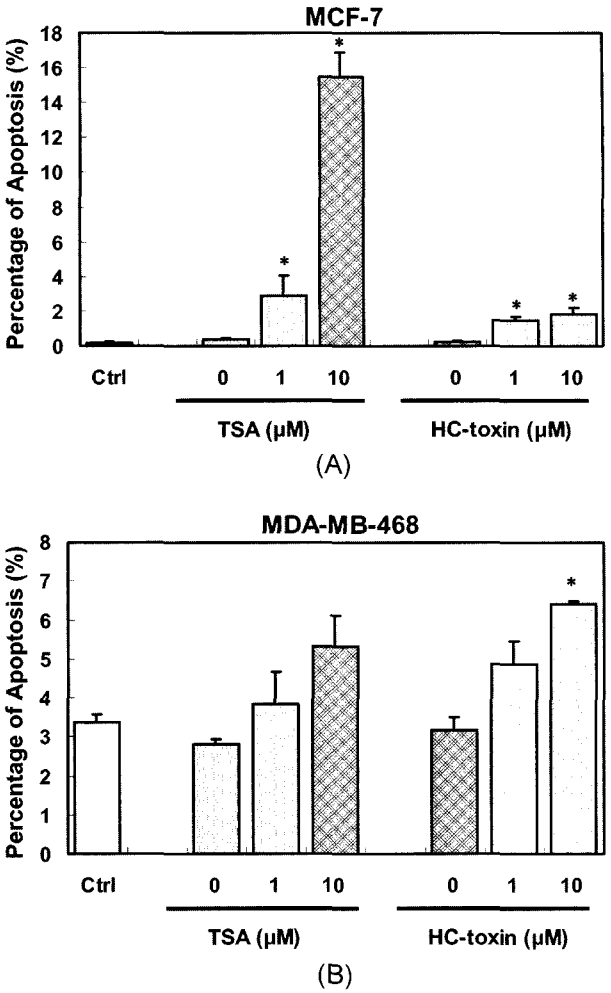

Fig. 3. Quantitative analysis of apoptosis. Quantitative analysis of apoptosis was done using FACS profiles as those shown in figure 2. Apoptosis was assessed by the appearance of a sub-G_1 (<2N ploidy) population by ModiFit software. MCF-7 (A) and MDA-MB-468 (B) cells were treated with vehicle solvent (0.1% DMSO) or HDAC inhibitors (trichostatin A or HC-toxin) at the indicated concentration for 24 hr. *: Significant different from control at $p < 0.05$. □, Control (0.1% DMSO); ▒, TSA; □, HC-toxin.

and G_2 phases of the cell cycle, induce differentiation, and reverses the transformed morphology of cells in culture (Yoshida *et al.*, 1995). The precise mechanisms underlying these cellular responses have got to be characterized. TSA and HC-toxin showed cell cycle arrest at G_2/M from both estrogen receptor positive human breast cancer cell line MCF-7 and estrogen receptor negative MDA-MB-468 cells (Fig. 2, Table II). These data suggested that presence of estrogen receptor in human breast cancer would not affect the sensitivity of histone deacetylase. Induction of apoptosis by histone deacetylase inhibitors has been reported in several human cancer cell lines but the mechanism underlying this effect is not well understood (Huang *et al.*, 1999; Media *et al.*, 1997; Click *et al.*, 1999).

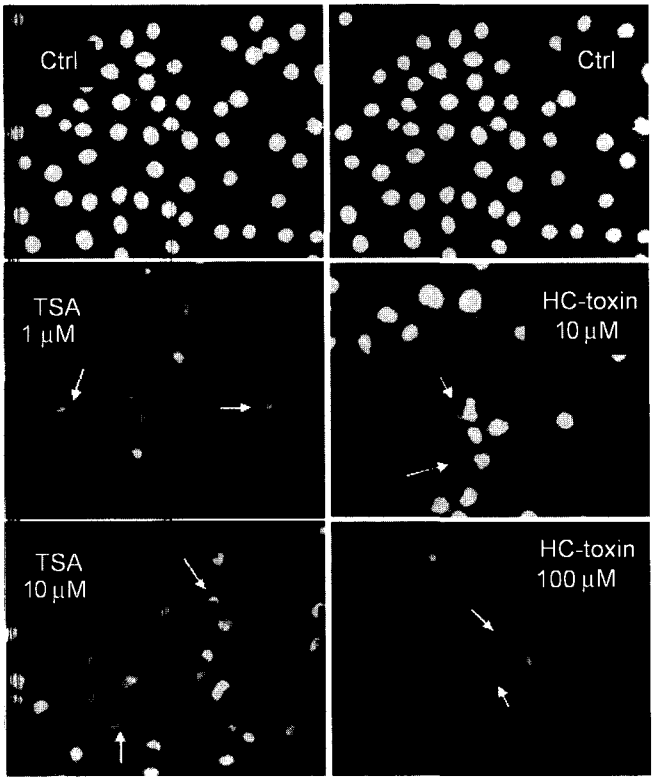

Fig. 4. Induction of apoptosis by HDAC inhibitors, determined by DAPI staining. Apoptosis induction by HDAC inhibitors, trichostatin A (TSA) or HC-toxin in MCF-7 human breast cancer cell line was analyzed by fluorescence microscopy. MCF-7 cells were treated with vehicle solvent (0.1% DMSO) or HDAC inhibitors (trichostatin A or HC-toxin) for 24 h and then stained with DAPI. Stained nuclei were visualized under a fluorescence microscopy (x400). The cell indicated by the arrow is example of a morphological characteristic of apoptosis (; marked chromatic condensation, apoptotic bodies, cytoplasmic condensations, and cellular shrinkages).

Although our results suggested that TSA and HC-toxin induced apoptosis in mammary tumor cells (Fig. 3), the action of cell death mechanism of these two compounds was not delineated. Taken together, results of this study indicated that TSA and HC-toxin inhibited cell proliferation and induced apoptosis of human breast cancer cells regardless of estrogen receptor status and that histone deacetylase inhibitors might be new therapeutic agents for the treatment of breast cancer.

ACKNOWLEDGEMENT

This research was supported by the grant NTP 238 from the KFDA of Korea.

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