Abstract. The effects of histone deacetylase (HDAC) inhibitors on epithelial-mesenchymal transition (EMT) differ in various types of cancers. We investigated the EMT phenotype in four colon cancer cell lines when challenged with HDAC inhibitors trichostatin A (TSA) and valproic acid (VPA) with or without transforming growth factor-β1 (TGF-β1) treatment. Four colon cancer cell lines with different phenotypes in regards to tumorigenicity, microsatellite stability and DNA mutation were used. EMT phenotypes were assessed by the expression of E-cadherin and vimentin using western blot analysis, immunofluorescence, quantitative real-time RT-PCR following treatment with TSA (100 or 200 nM) or VPA (0.5 mM) with or without TGF-β1 (5 ng/ml) for 24 h. Biological EMT phenotypes were also evaluated by cell morphology, migration and invasion assays. TSA or VPA induced mesenchymal features in the colon carcinoma cells by a decrease in E-cadherin and an increase in vimentin expression at the mRNA and protein levels. Confocal microscopy revealed membranous attenuation or nuclear translocation of E-cadherin and enhanced expression of vimentin. These responses occurred after 6 h and increased until 24 h. Colon cancer cells changed from a round or rectangular shape to a spindle shape with increased migration and invasion ability following TSA or VPA treatment. The susceptibility to EMT changes induced by TSA or VPA was comparable in microsatellite stable (SW480 and HT29) and microsatellite unstable cells (DLD1 and HCT116). TSA or VPA induced a mesenchymal phenotype in the colon carcinoma cells and these effects were augmented in the presence of TGF-β1. HDAC inhibitors require careful caution before their application as new anticancer drugs for colon cancers.

Introduction
Colorectal cancer (CRC) is the fourth most fatal cancer in Korea (1). Although the 5-year survival rate of CRC overall has been reported to be as high as 71.3% (1), the rate for patients with recurrence is <40% (2). Epithelial-mesenchymal transition (EMT) is critical for carcinogenesis, tumor invasion, metastasis, chemo-resistance and acquisition of stem cell properties. The EMT phenotype is indicative of a poor prognosis in CRC (3,4). Combined treatment to attenuate or reverse EMT enhances the sensitivity to conventional anti-neoplastic agents and improves oncologic outcomes (5).

Both genetic and epigenetic changes are required for the transformation, promotion and progression of CRC (6). Downregulation of tumor-suppressor genes due to unbalanced histone deacetylation (HDAC) hyperactivity has been reported in CRC. The histone acetylation status may play a role in colon tumorigenesis and re-expression of these genes can be induced by treatment with an HDAC inhibitor in CRC. HDAC inhibitors have been reported to be effective as anticancer agents and, in combination with conventional chemotherapeutics, have been reported to synergistically enhance growth arrest, apoptosis and differentiation in cancer cells (6). These effects in colon cancer cells have currently been demonstrated for many HDAC inhibitors including valproic acid (VPA) (7) and trichostatin A (TSA) (8-11). TSA, a hydroxamate, may have the highest potency against HDAC1, 3 and 8. VPA, a short chain fatty acid, appears to have the highest activity against HDAC1 and 2, but appears also to affect HDAC3, 4, 5 and 7 at higher doses, showing non-specific sensitivity to HDACs (12). TSA and VPA reduce the growth and survival, induce differentiation, enhance radiosensitivity and reduce chemoresistance (13).

Histone modification has been shown to play a key role in controlling EMT. Many studies have reported that various HDAC inhibitors reverse or attenuate EMT through the upregulation of E-cadherin in different solid tumors such as HCC (14), breast (15,16), esophageal cancer (17) and ovarian tumors (18). In addition, they attenuate TGF-β1-induced EMT in different cells: hepatocytes (19), retinal pigment epithelial cells (20), lens epithelial cells (21) and renal epithelial
cells (22). These results suggest that HDAC inhibitors may have therapeutic roles to reduce EMT. However, recent studies have also reported that HDAC inhibitors induce EMT, reverse stem cell properties and enhance metastasis and invasion in prostate cancer (23), head and neck squamous cell carcinoma (24) and nasopharyngeal cancer (25). Whether HDAC inhibitors inhibit or induce EMT in CRC has not yet been reported, and needs to be clarified.

We investigated the effect on the EMT of colon cancer cells induced by the HDAC inhibitors TSA or VPA, alone, and in combination with TGF-β1. TGF-β1 induced the EMT of colon cancer cells in DNA microsatellite stable (MSS) cells, whereas cells with DNA microsatellite instability (MSI) showed variable responses to TGF-β1 (26). Tumor cells with MSI having mutant TGF-β receptor type II (TGFBRII) exhibited less EMT and a more favorable prognosis compared to cells with MSS. We selected four CRC cell lines including MSI cells (DLD1 and HCT116) and MSS cells (HT29 and SW480). We evaluated the expression of E-cadherin as an epithelial marker, and vimentin as a mesenchymal marker. The EMT phenotype was also evaluated by cell morphology, migration and invasion assays.

Materials and methods

Cell lines and culture conditions. We used four colon carcinoma cell lines. DLD1, HT29, HCT116 and SW480 were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). These cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), containing 10% heat inactivated fetal bovine serum (FBS), L-glutamine 2 mM and sodium bicarbonate 20 mM. DLD1 and HCT116 were maintained in DMEM supplemented with 10% FBS, potassium penicillin 100 u/ml, streptomycin 100 g/ml, and 2% FBS and 2% antibiotics. The cells were seeded at a density of 10,000 cells/cm² in 24-well plates and incubated in 5% CO₂ and 95% humidity in a 37°C chamber. The growth medium was changed every 3 days.

Western blot analysis. Total protein was extracted, and the protein concentration was measured by the Bradford DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Then, 20-40 µg protein from each sample was separated on 12% Bis-Tris polyacrylamide gel through electrophoresis and blotted on immobilon transfer membranes (Millipore, Temecula, CA, USA). Blots were immunostained with vimentin (1:1,000; Sigma-Aldrich, St. Louis, MO, USA) and E-cadherin (1:1,000; Invitrogen, Eugene, OR, USA). The primary antibody was added at 4°C overnight, and then the secondary antibody at room temperature for 1 h. Proteins were visualized by using ECL Plus Western Blotting detection reagents (Intron Biotechnology, Seoul, Korea) and measured by image analysis of the Fuji Image Quant software (FujiFilm Las-3000 mini, Tokyo, Japan), according to the manufacturer’s instructions.

Quantitative real-time RT-PCR. The total RNA was isolated using the RNase Mini kit (Qiagen, Valencia, CA, USA). The residual DNA was removed using an RNase Free DNase kit (Qiagen). High capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) was used to reverse transcribe 1 µg of RNA into cDNA according to the manufacturer’s instructions. Real-time PCR was performed using specific primers to quantify gene expression using SYBR-Green RT PCR reagents (Applied Biosystems). The relative amount of mRNA was normalized to the expression of GAPDH. The primer sequences used in the present study were as follows; GAPDH primer sequences: forward, 5’-CATCAATGGAAA TCCCATCA-3’ and reverse, 5’-TTCTCCATGGTGTTGAA GAC-3’; E-cadherin primer pair: forward, 5’-TTCTGTGCTG TCTTGCCTGT'-3’ and reverse, 5’-TGCTCAAGTCAA GTCTGT-3; Vimentin primer pair: forward, 5’-GCCCTTA AAGGAAACAAATGA-3’ and reverse, 5’-AGCTTCAACGG CAAAGTTCT-3’. The PCR reactions were repeated three times in three independent experiments.

Immunofluorescence microscopy. Individual sterile coverslips were placed in the wells of a 4-well plate and colon carcinoma cells were added and incubated for 24 h. Subsequently, the cells were treated with 10 ng/ml TGF-β1, 100 or 400 nM TSA, 0.5 mM VPA diluted in DMEM media with 1% FBS for 48 h. After washing with PBS, the cells were fixed in 3.7% paraformaldehyde for 20 min at room temperature. The paraformaldehyde was removed and cells were washed with PBS. Blocking solution was then added for 30 min to prevent non-specific binding, and then incubation was carried out using anti-E-cadherin (1:100; Cell Signaling Technology, Danvers, MA, USA) and anti-vimentin (1:100; Leica Microsystems, Wetzelar, Germany) as the primary antibodies, overnight, at 4°C, on a rocking platform. The washed slides were incubated for 1 h at room temperature with 1:100 dilutions of Alexa-488 anti-rabbit IgG (H+L) (Molecular Probes Inc., Eugene, OR) secondary antibody, and Alexa-568 goat anti-mouse IgG(H+L) (Molecular Probes) secondary antibody. They were washed again, mounted with Vectashield mounting medium (Dako, Carpenteria, CA, USA), and examined using Leica Zeiss optics in the Core Facility of Chungbuk National University. We also captured immunofluorescence images at time-points of 6, 12, 24 and 48 h to evaluate the protein translocalization.

Cell invasion and migration assays. We evaluated cell invasion and migration ability in the MSI or MSS cell lines. DLD1 and SW480 were selected. DLD1 and SW480 cells were seeded in 96-well plates over a homogeneous thin layer of fibronectin (BD Biosciences, Bedford, MA, USA) in a Millicell cell culture insert (Millipore) that contained polycarbonate filter membranes with 8-mm diameter pores. Tumor cells from the control group were maintained in DMEM supplemented with 1% FBS and 1% antibiotics, and the HDAC inhibitor group received 100 nM of TSA or 0.5 mM VPA diluted in media. The lower chamber contained DMEM supplemented with 1% FBS and 1% antibiotics. The cells were incubated for 24 h at 37°C in a 5% CO₂-humidified incubator. Invasive cells in the lower chamber were stained with hematoxylin and eosin (H&E). Images were captured using a QImaging Exi Aqua monochrome digital camera attached to a Nikon Eclipse 80i microscope (Nikon Inc., Melville, NY, USA) and visualized using QCapture Pro software.

The cells were allowed to grow in 10% FBS containing DMEM to confluence in a 6-well plate. A central linear wound was made with a 200-ml sterile pipette tip and then the cells were washed with PBS twice. Afterwards, the HDAC inhibitor group received 100 nM of TSA or 0.5 mM VPA and 10 ng/ml TGF-β1 diluted in media with 1% FBS. Plates were photographed after 0, 24 and 48 h.
Cytotoxicity assay. Cell viability was assessed using Promega cell proliferation MTS assay (Promega, Madison, WI, USA). Briefly, CRC cells were seeded in 96-well plates at a density of 1x10^4/well and incubated with increasing concentrations of TSA, VPA and TGF-β1 for 48 h to determine a dose-response curve. Subsequently, dead cells were washed away, the attached cells were incubated with MTS, and cell viability was detected using microplate reader Model-680 (Bio-Rad Laboratories). All the experiments were conducted in triplicate.

Statistical analysis. The SPSS 16.0 software (IMB, Armonk, NY, USA) was used to analyze the data. The differences between groups were evaluated using the Mann-Whitney U test and Wilcoxon signed-rank test, with P<0.05 being considered to indicate a statistically significant result.

Results

Cell morphology. The four CRC cell lines were altered from round or rectangular-shaped cells to spindle-shaped cells with loose cell-cell contact following TSA or VPA treatment or TGF-β1 treatment. The response to TGF-β1 treatment in the DLD1 and HCT116 cells was not prominent compared to the SW480 and HT29 cells. However, the morphological changes induced by TSA and VPA treatment were similar in both the MSS and MSI cell lines. Morphological changes were most prominent in the SW480 cells, which were distributed as single round cells. However, the HT29 cells showed minor changes compared to the SW480 cells. DLD1 and HCT116 cells showed a wider gap between cells and were spindle-shaped. The morphological changes were similar following TSA or VPA single treatment or in combination with TGF-β1 treatment (Fig. 1).

Nuclear localization of E-cadherin and increased expression of vimentin in the colon cancer cells. The four CRC cell lines showed attenuation of membrane expression and nuclear translocation of E-cadherin and/or increased vimentin expression following TSA and VPA treatment in both the MSI and MSS cell lines (Fig. 2A-D). The effects of TSA and VPA treatment on the DLD1 cells differed; TSA induced changes in E-cadherin expression, whereas VPA had effects on vimentin expression. TGF-β1 induced a mesenchymal phenotype in the MSS cells (HT29 and SW480), whereas TGF-β1 treatment in MSI cells (DLD1 and HCT116) had variable results; a mesenchymal phenotype in DLD1 cells was noted and increased membrane expression of E-cadherin in the HCT116 cells was observed. Nuclear localization of E-cadherin and increased expression of vimentin occurred after 6 h and increased until 24 h in the HT29 cells (Fig. 2E).

mRNA expression of E-cadherin and vimentin in the colon cancer cell lines. The four colon cancer cell lines showed decreased E-cadherin or increased vimentin expression following TSA or TGF-β1 treatment. These effects were greater in the presence of TGF-β1. However, the mRNA expression among the four cell lines showed slight differences (Fig. 3).
Protein expression of E-cadherin and vimentin in the colon carcinoma cells. After treatment with HDAC inhibitors, a decrease in E-cadherin and an increase in S100A4 and vimentin expression were noted in the SW480 cells. These changes were enhanced by co-treatment with TGF-β1. A decrease in E-cadherin expression was demonstrated following TSA or VPA treatment in the HCT116 and DLD1 cells. Vimentin was increased by co-treatment with the HDAC inhibitors and with TGF-β1 in the four cell lines (Fig. 4).

Migration and invasion assays. Single treatment with TSA or VPA did not show a difference in effects compared to the controls. However, the migratory ability increased following VPA and TGF-β1 treatment in the DLD1 cells and it increased
following the TSA or VPA treatment combined with TGF-β1 in the SW480 cells (Fig. 5A). These results demonstrated that EMT was enhanced following TSA or VPA treatment in combination with TGF-β1.

Invasion was increased by TSA, VPA or TGF-β1 treatment compared to no treatment in the DLD1 cells (P=0.002, P=0.002 and P=0.001, respectively). However, invasive ability did not increase following co-treatment with HDAC inhibitors and TGF-β1 in the DLD1 cells. Invasion was increased following VPA treatment only when compared to no treatment in the SW480 cells (P=0.039) (Fig. 5B).

Cell proliferation. Cell proliferation of colon carcinoma cells was assessed following treatment with TSA, VPA or TGF-β1 (Fig. 6). TSA (100 nM) or VPA (0.5 mM) did not influence cell proliferation except in the SW480 cells, which exhibited slightly suppressed cell proliferation. TGF-β1 (5 ng/ml) did not have an influence on cell proliferation. However, TGF-β1 (10 ng/ml) enhanced cell proliferation compared to the control except for DLD1 cells, which showed suppressed cell proliferation.

Discussion

The present study demonstrated that HDAC inhibitors TSA and VPA induced EMT, which was enhanced following TGF-β1 co-treatment in the colon cancer cells. The mesenchymal phenotype was determined by reduced expression and nuclear translocation of E-cadherin, and decreased expression of vimentin in the MSS (SW480 and HT29) and MSI (DLD1 and HCT116) cells with variable susceptibility. TSA or VPA also increased cell migration and invasion ability.
EMT is a biological process that is encountered in primary mesenchyme, wound healing and carcinoma (27). A hallmark of EMT is the disintegration and disassembly of cell-cell junctions by loss of proteins associated with the polarized...
epithelial phenotype, which normally interacts with the basement membrane via its basal surface, initiating invasive and metastatic behavior. EMT is characterized by loss of proteins, including E-cadherin, ZO-1 and tight junctional molecules, whereas it is characterized by upregulation of vimentin, which is a biomarker of the mesenchymal phenotype.

Many studies have reported that HDAC inhibitors reverse or attenuate EMT through upregulation of E-cadherin in different solid tumors and attenuate TGF-β-induced EMT in normal epithelial cells. They suggest that HDAC inhibitors may have therapeutic roles in solid cancers and fibrotic disorders by inhibition of EMT.

However, several recent studies reported opposite results that HDAC inhibitors can induce EMT. HDAC inhibitors TSA and vorinostat (suberoylanilidehydroxamic acid, SAHA) were found to induce the EMT phenotype, increasing mesenchymal markers such as vimentin and N-cadherin, concomitant with an increase in the expression of Sox2 and Nanog, which is associated with cancer stem-cell characteristics in prostate cancer cells (23). They suggest that the mechanisms of EMT induction by HDAC inhibitors are through the activation of promoters of Snail, Slug and Twist1. Inhibition of HDAC by TSA also induced EMT in head and neck squamous cell carcinomas, accumulation of Bmi-1, an oncogene associated with tumor aggressiveness, and expression of vimentin (24). Another study showed that HDAC inhibitors induced fibroblast-like morphology, upregulated Snail and vimentin and downregulated E-cadherin in nasopharyngeal carcinoma cells following sodium butyrate (NaB) or SAHA treatment (25). HDAC inhibitor TSA enhanced vimentin gene expression requiring the proximal promoter region including GC-box1, a known Sp1/Sp3 binding site (28). A recent study revealed that SAHA induced EMT during human embryonic implantation (29).

The different results in regards to the EMT response to HDAC inhibitor treatment may be related to the balance of gene expression, which includes EMT inducers and EMT suppressors. Studies with cDNA arrays have shown that treatment with HDAC inhibitors leads to a 2-fold or greater change in the expression of ~7-10% of the genes examined (30). HDAC inhibitor was found to induce about as many genes as were repressed. Whether or not these changes in gene expression result in cell death probably depends upon the cellular context. In addition, the gene expression pattern caused by HDACs on tumors is relatively non-specific (12), which suggests that individual HDAC inhibitors show differing degrees of effectiveness in their action on EMT, even in the same cell type.

The present study found that TSA and VPA induced EMT-like phenotypes in all the CRC cell lines irrespective of MSI, mutation of TGFBR2, K-RAS or APC genes, and tumor aggressiveness. HCT116 cells have wild-type APC and higher malignant potential. DLD1, HT29 and SW480 cells have mutated APC and mutated P53. All cell lines, except HT29, also carry activating mutations in the KRAS gene. Malignant potential was also different in the four CRC cell lines; DLD1-1, HT29, HCT116 and SW480, in ascending order (31). The changes in EMT phenotype induced by TGF-β and HDAC inhibitors were variable according to the different cell type. Mutant or wild-type TGFBR2, APC, and K-RAS proteins were different in the four CRC cell lines. EMT induction by TGF-β1 was greater in the MSS (SW480 and HT29) cells than that in the MSI (DLD1 and HCT116) cells.

The expression of E-cadherin, which suppresses EMT, may prevent invasiveness and metastasis. However, recent studies showed that the shift of E-cadherin localization from the membrane to the cytoplasm or nucleus in CRC was associated with metastasis and poor outcomes in human tissues (32) and animal models (33). A colonic cancer metastasis model showed that E-cadherin proteolysis and nuclear localization were associated with aggressive growth foci in the peritoneal microenvironment. The localization of E-cadherin was also demonstrated in various tumors including Merkel cell carcinoma (34), stomach (35), renal (36), esophageal (37) and solid pseudo-papillary tumors of the pancreas (38). However, most reports of nuclear localization of E-cadherin in cancer concerned studies that utilized immunohistochemistry only, which must be interpreted with caution (39). The mechanism by which the E-cadherin molecule is translocated to the nucleus is currently not clear. The present study showed that TSA and VPA induced nuclear translocation of E-cadherin in the four CRC cell lines with preserved or reduced membrane expression. We suggest that the most important E-cadherin change during EMT process was not the mRNA or protein amounts but the location. The amount of E-cadherin reduction following treatment of HDAC inhibitors was not enough to reach statistical analysis in several cell lines in the present study. A similar result was noted in the previous study, which showed that SAHA treatment in breast cancer cells induced redistribution of E-cadherin from the cell surface to the cytoplasm while preserving total E-cadherin levels (40).

TSA was previously found to prevent TGF-β (5 ng/ml)-induced EMT in a concentration-dependent manner in human renal epithelial cells (22) and in mouse hepatocytes (41). TSA treatment induced a fundamental increase in the mRNA levels of ZO-1 and E-cadherin in a concentration-dependent manner, which started to be expressed at doses of 20 ng/ml of TSA and was most effective at the dose of 400 nM in mouse hepatocytes (41). Cell cytotoxicity and apoptosis were induced at higher doses (800 nM), and had no effect at lower doses (200 nM) (19). These results suggest that the effect of TSA on EMT begins at a low dosage, prior to apoptosis and inhibition of proliferation, although the dosage differs according to cell type and cancer progression. We used TSA (100 or 200 nM) at a level that induces mild suppression of cell proliferation while preserving cell viability. The concentration of VPA was fixed at 0.5 mM, which is in the range of low toxicity in the human (42).

Pharmacological inhibitors of class I and II HDAC activity (HDAC inhibitors) are potent inducers of growth arrest, differentiation and apoptosis of colon cancer cells in vitro and in vivo. Studies of HDAC inhibitors on colon cancer cells have attempted to identify the improvements in response, and potential efficacy with conventional chemotherapeutics and their tolerable cytotoxicity. Many studies have reported that HDAC inhibitors have an effective action against colon cancer cells with a synergistic effect together with radiation or targeted therapies (6). VPA and TSA inhibit proliferation in colon cancer cells and have been shown to have far greater selective toxicity to tumor cells (43). However, the present study demonstrated that HDAC inhibitor TSA or VPA can induce the EMT of CRC cells, alone or in combination with TGF-β1.
These results should be considered when using combination treatment of HDAC inhibitors and other chemotherapies.

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References


