Cell Cycle Blockade and Differentiation of Ovarian Cancer Cells by the Histone Deacetylase Inhibitor Trichostatin A Are Associated with Changes in p21, Rb, and Id Proteins

Kevin A. Strait, Bashar Dabbas, Elizabeth H. Hammond, C. Terry Warnick, Sarah J. Istrup, and Clyde D. Ford

Abstract

Inhibitors of histone deacetylase activity are emerging as a potentially important new class of anticancer agents. In the current studies, exposing A2780 ovarian cancer cells to the histone deacetylase inhibitor trichostatin A (TSA) produced a marked change in cellular morphology, proliferation, and differentiation. Within 24 h of TSA treatment, there was a morphological transformation of the cells, with increased cytoplasm, a more epithelial-like columnar appearance, and the emergence of distinct cellular boundaries. Commensurate with the morphological transformation, TSA also inhibited cell proliferation; cells treated with TSA for 72 h increased to 110% of the initial cell numbers versus control cell numbers of 62%, with a corresponding reduction in mitotic activity and a flow cytometry S-phase fraction of 3.9% in TSA-treated cells versus 28.8% for control. TSA also induced epithelial-like differentiation with increased cytokeratin expression from 2% of controls to 22–25% of TSA-treated cells and the reappearance of intercellular plasma membrane junctions and primitive microvilli. Immunocytochemical analyses indicate the molecular mechanism underlying the actions of TSA on A2780 cell cycle progression and differentiation involves reexpression of the CDK inhibitor p21. Elevated levels of p21, in TSA-treated cells, were associated with a reduction in the phosphorylation of the cell cycle regulator retinoblastoma protein (Rb). TSA also caused a decrease in the helix-loop-helix inhibitor of differentiation/DNA binding protein Id1, with no change in Id2 levels. In conclusion, the observed TSA-induced changes in p21, Rb, and Id1 are consistent with cell cycle senescence and differentiation of A2780 ovarian cancer cells.

Introduction

Ovarian cancer is the leading cause of gynecological cancer deaths in the United States (American Cancer Society). The vast majority of these cancers (85%) arise from specialized epithelial cells that cover the surface of the ovary (1). Over the past several years, a number of important oncogenes and oncosuppressor genes have been identified whose activities are linked to tumorigenesis. Many of these genes are aberrantly expressed or silenced in cancer cells due to widespread epigenetic modifications of the tumor cell genome (2). Because complex cellular processes involving the cell cycle, differentiation, and apoptosis often have overlapping pathways, identifying individual control genes or gene products for targeted therapies is difficult.

The recent identification of gene-regulatory chemotherapeutic agents that can reverse the epigenetic modifications present in the tumor cell genome is an area of intensive investigation (3). One mechanism through which the activity of multiple genes can be affected is modification of the structure of the DNA chromatin complex. In particular, post-translational modifications of the core histone proteins that comprise the nucleosomal complex are known to dramatically influence the transcription of genes in response to cellular signals (4). Recent interest has focused on the acetylation of the histone proteins, which neutralizes their net positive charge, leading to a loosening of the histone-DNA nucleosomal structure (5) and increased transcriptional activity (4).

The net acetylation state of histones is the function of diametrically opposed histone acetyltransferase and HDAC enzyme families. Histone acetyltransferases have been identified as transcriptional coactivators in a number of species (6, 7). Conversely, HDACs have been shown to interact with several known transcriptional repressors, including Sin3, SMRT, and NCoR, where they function as corepressors (8). Changes in histone acetylation via recruitment of HDAC enzymes appear to be targeted to specific genes because microarray analysis of mRNA expression profiles indicate that HDAC inhibitors affect a small percentage (1–2%) of the total genes expressed (9).

The association of HDAC activity with known cellular oncogenes has led to speculation that histone deacetylation is a potential mechanism to account for the uncontrolled cell proliferation associated with cancers. Support for this hypothesis was provided in studies of acute promyelocytic...
leukemia cells, where a fusion protein of PML and a RA receptor repressed gene transcription by recruiting HDAC enzymes (10). PML-RA receptor fusion protein recruitment of HDAC produced sustained cell proliferation that was resistant to the differentiation actions of RA (10). Pretreating these cells with HDAC inhibitors relieved the transcriptional blockade and allowed them to undergo normal differentiation (11).

Given the close association between HDAC activity and repression of transcription in tumors, inhibitors of HDAC are emerging as a potentially important new class of anticancer agents. TSA is a potent specific inhibitor of HDAC activity through a mechanism that involves Zn⁺ ion chelation (12). In the past several years, studies have shown that TSA induces growth arrest, differentiation, and/or apoptosis in a number of cancers, including breast (13), colon (14), lung (15), and leukemic cells (16).

How TSA’s inhibition of HDAC activity leads to cell cycle arrest and differentiation is an area of intense investigation. Several reports indicate that HDAC inhibitors block cell cycle progression by reexpressing inhibitors of the CDKs, such as p21 (17). p21 inhibition of CDK activity blocks cell cycle progression by inducing hypophosphorylation of the CDK substrate retinoblastoma protein (Rb; Ref. 18). Hypophosphorylation of Rb blocks cell cycle progression by binding and sequestering cell cycle cycle control factors, including the transcription factor E2F (19).

Another group of proteins associated with the morphogenesis of several cell types, including epithelial cells, are the inhibitors of differentiation/DNA binding proteins (Id proteins; Ref. 20). The Id proteins belong to the basic HLH family of transcription factors, characterized by their ability to form dimers through interaction of their basic HLH regions and bind response element sequences via a DNA-binding domain (21). The Id proteins (Id1–4) are structurally and biologically distinct from the other HLH proteins in that they lack a DNA-binding domain (22). As such, they function as negative regulators of the other HLH proteins through the formation of heterodimers that are unable to bind DNA, and they play an essential role in embryogenesis by stimulating cell proliferation and inhibiting premature differentiation (21). Given their effects on proliferation and differentiation, recent studies have focused attention on their potential role in cancer, where an association between the loss of appropriate regulation of Id expression and tumorigenesis has emerged (23).

In the present studies, we examined the effects of TSA on the morphology, proliferation, and differentiation of A2780 ovarian cancer cells. Our data indicate that TSA caused a significant alteration in cellular morphology, the result of inhibition of cell cycle progression and stimulation of epithelial-like differentiation. Immunocytochemical analyses indicated that the morphological, proliferative, and differentiation effects of TSA occur commensurate with changes in p21, Rb, and Id, consistent with an important role for these proteins in the observed cell cycle inhibition and epithelial-like differentiation.

Materials and Methods

Cell Culture and Reagents. The human ovarian cancer cell line A2780 was obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom). A2780 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Cultures were grown at 37°C in a humid 95% air:5% CO₂ chamber and periodically tested to ensure they remained free of Mycoplasma infection during the course of the experiments. TSA was obtained from Sigma (St. Louis, MO).

TSA Treatment. Twenty-four h before TSA addition, A2780 cells were subcultured onto either fresh 100-mm tissue culture plates or Lab-Tek chamber slides. The following day, the media were replaced with fresh media containing 100 ng/ml TSA, and the cells were allowed to incubate overnight. The following day, the TSA was removed by replacing the media, and the cells were again returned to the incubator for an additional 24–48 h. At various times during the experimental period (as noted), cells were removed from the plates or fixed to the Lab-Tek slides and examined for TSA-induced changes.

Preparation of Paraffin Blocks from Cultured Cells. Cultured A2780 cells were fixed and embedded in paraffin blocks as described previously (24). Briefly, cultures were trypsinized and removed from the culture dishes upon dilution with 2 volumes of media +10% FBS, centrifuged at 150 × g for 5 min, washed once in culture medium to remove the excess trypsin, and recentrifuged. The final pellet was resuspended in 0.25 ml of medium, and 0.5 ml of human plasma was added, followed by a similar volume of thromboplastin, as described previously (25). The mixture was agitated for 2 min until coagulation occurred, followed by addition of 10% buffered formalin for 2 min. The clotted sample of cells was removed, placed in a VIP Tissue Processor (SAKURA), and allowed to process overnight. The following day, the processed sample was embedded in a paraffin block, and 4-μm sections were cut and mounted on slides.

H&E Staining. Cells grown on microscope slides (Lab-Tek) were washed twice with PBS, fixed with 4% paraformaldehyde (5 min, room temperature), dehydrated through a series of ethanol washes, and stained with H&E (26).

Immunohistochemistry. Paraffin-embedded fixed cells were immunostained for Ki67, CAM 5.2, or AE1/AE3. Immunostaining of p53, p21, p27, Rb, and Id proteins was performed on cells grown on Lab-Tek chamber slides and fixed in 100% methanol for 10 min. Both paraffin-embedded and methanol-fixed cells were stained using an immunoperoxidase procedure, as described previously (27) with 3,3’-diaminobenzidine as the substrate. Staining was performed on a DakoAutoStainer (DAKO). Briefly, mouse monoclonal antibodies against human Ki67 (DAKO; 1:10 dilution); CAM 5.2 (Becton Dickinson; prediluted); AE1/AE3 (DAKO; prediluted); p53 (DAKO; 1:200); p21<sub>WAF1/Cip1</sub> (DAKO; 1:50); p27<sup>Kip1</sup>, Rb, and Phos-Rb (ser 795; Cell Signaling Technology; 1:100 dilution); and Id1 and Id2 (Santa Cruz Biotechnology; 1:200 dilution) were used as the primary antibody. The secondary antibody was a biotinylated goat antimouse antibody (DAKO; prediluted). Antibody binding was detected using horseradish peroxidase (LSAB2 System; DAKO). The following control tissues were stained simultaneously to act as positive controls for the antisera: Ki67 (tonsil); AE1/AE3 (squamous cell carcinoma of skin); p21 (colon); p27 (tonsil);
and p53 (p53 positive tumor). For the Rb and Id antisera, control A2780 cells were included on each slide to act as positive controls. The samples were counterstained with hematoxylin (DAKO), dehydrated, and mounted. The percentage of positively stained cells was determined from five separate areas of the slide using an automated cell imaging system (Chromvision). The automated cell imaging system combines automated microscopy to record and assemble hundreds of individual captured fields with computerized image analysis to simultaneously determine both staining intensity and percentage of stained cells for control and TSA-treated cells on the same slide.

**Flow Cytometry.** The percentage of cells in G₁, S, and G₂-M phase of the cell cycle was determined by a modified Vindelov propidium iodide DNA staining procedure (28). Briefly, cells were isolated from the culture dishes with trypsin, washed once in fresh media, and resuspended at a concentration of 1 x 10⁶ cells/ml. The cell suspension (200 µl) was then incubated with 500 µl of a propidium iodide solution [0.006 g of PI, 70 units of RNase, 0.058 g of NaCl, 0.121 g of Trisma base, and 0.1 ml of NP40; volume to 100 ml (pH 8.0)] overnight at 4°C. The following day, the stained cell nuclei were run on a Coulter Epix XL flow cytometer. The un gated histogram was evaluated using the Phoenix flow DNA modeling system to determine the percentage of cells in G₁, S, and G₂-M phase.

**EM.** Cells were fixed and sectioned for EM as described previously (29). Briefly, cultured cells were rinsed twice in 5 ml of PBS and then gently scraped from the cultures plates in 1 ml of PBS using a Teflon cell scraper. The cells were spun at 1000 rpm (5 min) to form a cell pellet in the bottom of a 15-ml conical tube. The supernatant was removed and carefully replaced with 1 ml of 4% glutaraldehyde so as not to disrupt the pellet and allowed to fix for 1.5 h. The fixed cell pellet was then washed twice in PBS for 30 min, followed by the addition of 2 ml of 2% Osmium and an additional 30-min incubation. The pellet was again rinsed twice (10 min) in normal saline, dehydrated stepwise with acetone (30%, 70%, 95%, and 100%; 5 min each), and embedded in an epoxy resin (Embed) at room temperature in a 1:1 dilution of acetone:Embed for 15 min. The excess solution was removed, and the pellet was embedded in a Beem capsule and allowed to polymerize overnight at 85°C. The polymerized cell pellets were subsequently sectioned on an Ultratome (LKB), affixed to 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined on the JEM1010 JEOL electron microscope.

**Results**

Previous studies using inhibitors of HDAC in other cancers indicate that these compounds reduce cell proliferation and induce differentiation or apoptosis. Therefore, we initially examined whether addition of the HDAC inhibitor TSA to A2780 cultures produced any effect on cellular morphology. Fig. 1 is a photomicrograph composite of A2780 cells incubated in the presence (100 ng/ml, 24 h) or absence (control) of TSA. Control A2780 cells (Fig. 1, left panels) appear as undifferentiated small round clumps of cells with indistinct cell borders. Examining these same cells on successive days indicated that they remained undifferentiated and were rapidly dividing, reaching confluence by the third day.

Photomicrographs in the right panels of Fig. 1 (+TSA) show the same A2780 cells treated with TSA (100 ng/ml, 24 h). As is apparent from the figure, within 24 h, TSA had a dramatic effect on the morphology of the cells. The original small round clumps of cells (day 0) spread out in monolayer on the surface of the plate. There was an increase in the amount of cytoplasm, with the appearance of numerous cytoplasmic projections and well-defined cellular borders. Examining the cells 24–48 h after removal of TSA (days 2 and 3) showed a persistent effect of TSA on morphology.

H&E staining (Fig. 2) of the cells confirmed the morphological changes observed using phase-contrast microscopy. Cells were stained on day 0 and on day 2, after ±TSA treatment. Control A2780 cells appear as small, round, undifferentiated cells that grow in clusters, with round to slightly oval nuclei and very little cytoplasm. A majority of the TSA-transformed cells had a significant increase in cytoplasmic content, resulting in a stellate-like appearance, often with multiple cytoplasmic projections radiating out from the cell body and well-defined nuclei with prominent nucleoli.
treated cultures also had a significantly reduced cell density as compared with control cultures. To test whether the reduced cell density after TSA treatment is the result of changes in proliferation or apoptosis, we determined both the cell numbers and viability of our TSA-treated cultures (Fig. 3). As indicated in the figure, TSA addition to the media affects the proliferation of the A2780 cells, producing a significant ($P < 0.05$) reduction in cell numbers within the first 24 h [+TSA: 105 ± 13% of the starting cell numbers as compared with control cells [226 ± 23%]] that persisted for at least another 48 h after TSA was removed from the culture medium [day 3: 110 ± 12% (TSA) and 622 ± 37% (control; $P < 0.01$)]. The difference in cell numbers in control versus TSA-treated cultures is directly attributable to a complete cessation of cell proliferation in the TSA-treated cultures over the entire 3-day experimental period (initial cell numbers = 100; +TSA: day 1, 105%; day 2, 84%; and day 3, 110%).

To determine whether the reduction in the number of cells in the TSA-treated cultures was the result of an increase in apoptotic cell death, we examined both the cell culture medium and the trypsinized cells for viability using trypan blue exclusion. At no time in the 3-day experimental period did we observe a loss of cells in the TSA-treated cultures, due either to dissociation from the culture dish or decreased cell viability, in excess of that observed in the A2780 control plates (<1% of cells; data not shown). In addition, we also stained control and TSA-treated cells with annexin V to identify phosphatidylserine translocation to the external plasma membrane, an early event in apoptosis. In two separate experiments, annexin V bound to <1% of the cells in both the control and TSA-treated cultures (data not shown).

Evidence to support a TSA-mediated arrest in A2780 cell proliferation was obtained by immunohistochemical analysis, using an antibody (Ki67) that only stains mitotically active cells (Fig. 4). In control cultures, Ki67 stained nearly all of the cells (89.7 ± 2.1%). By contrast, the presence of TSA in the media resulted in a significant ($P < 0.01$) reduction in the levels of Ki67 staining to 54 ± 7.5% of the cells.

Flow cytometry analysis of propidium iodide-stained cells indicated that TSA’s inhibition of A2780 proliferation is the result of cell cycle blockade (Fig. 5). The histogram of DNA content in the control cultures (Fig. 5A) showed a majority of cells in G1 (56.0%), with a significant S-phase fraction of 28.8%. The remaining control cells (16.2%) were found to be in G2-M phase. TSA treatment produced a shift in the DNA content histogram (Fig. 5B). Whereas the G1-phase fraction remained essentially unchanged after TSA treatment (59.2%), the S-phase fraction decreased significantly ($P < 0.01$) to 3.9%. The decrease in the S-phase fraction was offset by a rise in the G2-M-phase fraction (36.9%), the result of an arrest in the cell cycle at the G2-M checkpoint.

To examine whether the changes in morphology and proliferation documented above are a reflection of cell differentiation, we examined our A2780 cultures for expression of genes associated with an epithelial phenotype. Control and TSA-treated cells were immunostained with antibodies (AE1/AE3 and CAM 5.2) directed against several cytokeratin proteins (Fig. 6). As anticipated, control A2780 cells were virtually devoid of cytokeratin staining (2% of cells showed weak immunoreactivity against either antisera). TSA treatment triggered reexpression of the cytokeratins, with immunostaining increasing significantly ($P < 0.01$) to 22% (AE1/AE3) and 25% (CAM 5.2) of cells.

Control and TSA-treated cells were further examined ultrastructurally to look for subcellular changes commensurate with epithelial-like differentiation (Fig. 7). Photomicrographs of control A2780 cells (Fig. 7A) show that the plasma membranes of adjacent cells abut without forming intercellular junctions (Fig. 7A, open arrow). Occasional intercellular luminal spaces were present (labeled L), which contained rare,
abortive microvilli. After treatment with TSA (Fig. 7B), intercellular junctional fusions were clearly present between adjacent cells (filled arrows), resembling zona occludens. In addition, the intercellular luminal spaces (labeled L) were more frequent and contained primitive, epithelial-like surface microvilli.

Having established that TSA induced cell cycle arrest and differentiation, we refocused our studies to investigate potential mechanisms underlying the actions of TSA. Control and TSA-treated cells were examined for the presence of selective inhibitors of cell cycle progression (Fig. 8). TSA treatment caused a marked fall in p53 expression within 24 h, from 52% of control cells to 8% of TSA-treated (B) cultures. The percentage of cells staining for Ki67 was determined by counting five separate areas of each slide using a Chromavision automated cell imaging system. Data are presented as means ± SD. Significance (P < 0.05) was determined by unpaired Student’s t-test (original magnification, ×200).

Fig. 4. TSA decreases Ki67 staining in A2780 cells. In vitro cultures of A2780 cells were treated with the absence (control) or presence of TSA (100 ng/ml, 24 h). On day 2, the cells were removed from the culture dishes with trypsin, pelleted, formalin fixed, paraffin embedded, and stained for the presence of the Ki67 antigen (see “Materials and Methods”). The photomicrographs are representative of two independent experiments and show the presence of Ki67 staining as punctate brown dots located within the nuclei of A2780 cells from control (A) and TSA-treated (B) cultures. The percentage of cells staining for Ki67 was determined by counting five separate areas of each slide using a Chromavision automated cell imaging system. Data are presented as means ± SD. Significance (P < 0.05) was determined by unpaired Student’s t-test (original magnification, ×200).

Fig. 5. Induction of A2780 cell cycle arrest by TSA. In vitro cultures of A2780 cells were treated with the absence (A) or presence (B) of TSA (100 ng/ml, 24 h). On day 2, the cells were removed from the culture and analyzed by fluorescence-activated cell sorting for cell cycle distribution. A representative histogram from three independent experiments is shown. Insets show the percentage of cells in each phase of the cell cycle (G1, S, and G2-M). The coefficient of variance for G1, S, and G2-M ranged from 1.8 to 2.2.

ence in control (76%) and TSA-treated (69%) cells, an indication that the effects of TSA on p21 expression were not due to a generalized increase in all CDK inhibitors.

Because p21 is an inhibitor of CDK-mediated phosphorylation, we examined the effects of TSA on the phosphorylation status of a CDK substrate involved in cell cycle regulation, the Rb protein (Fig. 9). Immunostaining of A2780 cells with an antibody that recognizes the Rb protein only when phosphorylated at serine 795 [Fig. 9, Rb(795)] was significantly diminished in TSA-treated cells (28%) compared with controls (77%; P < 0.05), with a concomitant 60% reduction in the intensity of staining. To ensure that the loss of immunostaining was the result of a reduction in Rb phosphorylation, we stained similarly treated cells with an antibody capable of detecting total Rb protein regardless of phosphorylation status (Fig. 9, Rb). These experiments confirmed that total Rb protein was unaffected by TSA treatment as determined by similar immunoreactivity in 82% of control cells and 81% of TSA-treated cells.

We also examined TSA-treated cells for expression of the Id proteins (Fig. 9), which have previously been shown to play
a critical role in cell cycle regulation and the normal cellular differentiation of a number of cell types, including epithelial cells (30). In control A2780 cells, Id1 protein was abundantly expressed with intense nuclear staining in 82% of the cells. TSA significantly reduced Id1 expression to 28% of the cells (P < 0.05), with a 70% reduction in the intensity of staining. Similar staining with an Id2-specific antibody also showed high levels of expression in the control cells (92%); however, there was no effect of TSA on Id2 expression (85%, TSA-treated cells).

Discussion

The present studies provide evidence that alterations in histone acetylation have a dramatic effect on the proliferation and differentiation of ovarian cancer cells. In the current studies, within 24 h of TSA exposure, A2780 cells demonstrated measurable changes in both cellular morphology and proliferation (Figs. 2 and 3). Diminished proliferation of ovarian cancer cell lines has previously been reported using aromatic fatty acid analogues as inhibitors of HDAC activity (31). In the present studies, we have extended this observation to show that diminished cell cycle progression is the result of a reduction in the S-phase fraction [28.8% (control) versus 3.9% (TSA-treated cells); Fig. 5] due to cell cycle blocks at the G1 and G2-M checkpoints, consistent with an association between HDAC activity and cell cycle control genes (32).

Our studies also provide evidence that TSA may produce cell cycle blockade through a mechanism that involves the p21 Rb/E2F cell cycle-regulatory system. Treating A2780 cells with TSA causes an increase in the CDK inhibitor p21, by a p53-independent mechanism (Fig. 8). Commensurate with p21 inhibition of CDK activity, we observed a measurable reduction in phosphorylated Rb protein (serine 795) in TSA-treated cells, with no change in total Rb protein (Fig. 9). The significance of a change in the phosphorylation state of Rb relates to its ability to bind the cell cycle control factor E2F. CDK phosphorylation of Rb, at any of several sites within the large A/B pocket region (including serine 795), prevents Rb binding to E2F (33), allowing E2F to stimulate cell cycle progression. By analogy, in TSA-treated A2780 cells...
cells, diminished phosphorylation of Rb would increase Rb-E2F binding, resulting in the observed cell cycle arrest (Fig. 5).

The current studies also provide the first demonstration that TSA down-regulates the expression of Id1, one of the inhibitors of differentiation/DNA binding proteins, with no change in Id2 levels (Fig. 9). A number of tumors exhibit aberrant expression of the Id genes (23). Evidence indicates that reducing Id1 protein expression results in suppression of cell proliferation (34), whereas overexpression of Id1 prevents cell cycle arrest and differentiation (35). These observations are in accord with our present findings that Id1 expression falls in A2780 cells after TSA exposure, commensurate with cell cycle blockade and differentiation (Fig. 9).

The role the various Id proteins play in regulating the activity of HLH proteins during normal cell cycle progression and differentiation is presently unknown. However, among the Id proteins, Id1 is unique in that it alone can bind to mouse Id-associated protein 1 (36). Id1 binding facilitates the interaction of mouse Id-associated protein 1 with DNA elements within the promoter region of several known growth factor/cytokine genes, inducing their expression to stimulate cell proliferation (36). Other data indicate that Id1 may also directly stimulate cell proliferation by binding to the HLH transcription factor E2A, rendering E2A incapable of inducing the expression of the cell cycle inhibitor p21 (37). Thus, in the present studies, the fall in Id1 expression in TSA-treated A2780 cells is consistent with the observed increase in p21 expression (Fig. 8) and the reduction in cell proliferation (Fig. 5). Finally, the fall in Id1 expression occurs commensurate with epithelial-like differentiation (Figs. 6 and 7) and might indicate that Id1 functions as an inhibitor of ovarian epithelial cell differentiation, similar to reports in other cell lines (21), by sequestering HLH-containing transcription factors necessary for the expression of genes associated with a terminally differentiated phenotype.

In A2780 cells, Id2 proteins were unaffected by TSA (Fig. 9). The disparity in Id1 versus Id2 protein responses to TSA may relate to recent data that indicate these proteins appear to be functionally distinct (38). Id2 proteins, but not Id1, are
able to disrupt HLH-containing proteins from binding to the E-box sequences of promoters from genes involved in the G1 to S-phase transition (39). In this same series of experiments, the Id2 protein was also shown to be a substrate for phosphorylation by the G1-specific CDK2 enzyme, which was not able to phosphorylate Id1 (39). The use of mutant Id2 isoforms, which are incapable of being phosphorylated by CDK2, indicates that phosphorylation blocks Id2 binding to proteins involved in cell cycle regulation, analogous to phosphorylation of Rb by CDKs, and is required for cell cycle progression through the G1-S checkpoint (39). Therefore, in TSA-treated A2780 cells, it appears plausible that p21 inhibition of CDK activity also produces hypophosphorylated Id2 proteins, which facilitate cell cycle blockade by Rb by sequestering additional cell cycle control factors.

Id2 proteins may also play a role in A2780 cell differentiation; however, unlike the Id1 proteins that block differentiation, the available data indicate that Id2 proteins facilitate terminal differentiation (21). For example, in myeloid cells, the presence of Id2 protein was shown to be an absolute requirement for differentiation (40, 41). Particularly relevant to the present studies, in which Id2 proteins remain elevated during TSA-mediated cell cycle inhibition/differentiation, studies using antisense transcripts have shown that Id2 expression was essential for normal mammary epithelial cell differentiation (42). In these same cells, differentiation was accompanied by a fall in Id1 (42) that overexpression studies indicated was required for the cessation of cell proliferation and induction of differentiated phenotypic gene expression (42). Thus, the presence of Id2 proteins, concurrent with a loss of Id1 expression, during normal mammary epithelial cell differentiation mirrors the changes observed with TSA-induced differentiation of A2780 cells (Fig. 9).

In epithelial cells, cytokeratin proteins are a significant structural component of the cytoskeleton and are often used as a cytologic marker in the diagnosis and classification of epithelial malignancies (43). TSA induced the reexpression of cytokeratin proteins in A2780 cells (Fig. 6), consistent with epithelial-like differentiation. Reexpression of silenced genes has also been reported in other cancer cell lines (44), including epithelial cancers of the breast, where inhibition of HDACs resulted in increased expression of milk fat globule proteins (45). TSA also induced ultrastructural changes in A2780 cells (Fig. 7), including the formation of primitive intercellular junctions resembling zonulae occludens and maculae occludens junctions, and increased the numbers of intercellular luminal spaces containing primitive microvilli. The reexpression of cytokeratins and formation of intercellular junctions provide convincing evidence that TSA induces epithelial-like differentiation of A2780 cells, possibly as the result of a loss of Id1 inhibition of phenotypic gene expression required for epithelial differentiation.

Finally, TSA exposure in another ovarian cancer cell line, SK-OV-3, produced morphological, cell cycle, and differentiation actions similar to those observed in A2780 cells. However, when we examined ovarian cell lines with known resistance to cisplatinum (OVCAR-3 or A2780/CP), we observed none of the morphological or cell cycle-regulatory factor changes observed in the A2780 cells. Instead, TSA induced a strong apoptotic response, as determined by elevated annexin V binding and induction of caspase 3 activity (data not shown). Studies are currently under way to examine
the relationship between TSA-induced apoptosis and cisplatinum resistance in these cell lines.

In conclusion, the actions of TSA in ovarian tumor cells are a clear indication that epigenetic modifications that result from hypoacetylation of histone proteins may play a critical role in the etiology of ovarian cancers by altering the expression of genes involved in cell cycle control and differentiation.

Acknowledgments
We thank Janet Hansen for assistance with the immunohistochemical and EM analyses, Susan Wall for preparation of the paraffin-embedded A2780 cell blocks, Jeffrey G. Anderson for technical assistance in performing flow cytometry, and Dale Kern for computer support in preparation of the manuscript.

References
Molecular Cancer Therapeutics

Cell Cycle Blockade and Differentiation of Ovarian Cancer Cells by the Histone Deacetylase Inhibitor Trichostatin A Are Associated with Changes in p21, Rb, and Id Proteins

1

Kevin A. Strait, Bashar Dabbas, Elizabeth H. Hammond, et al.


Updated version
Access the most recent version of this article at:
http://mct.aacrjournals.org/content/1/13/1181

Cited articles
This article cites 43 articles, 16 of which you can access for free at:
http://mct.aacrjournals.org/content/1/13/1181.full#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/1/13/1181.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://mct.aacrjournals.org/content/1/13/1181.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.