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Inhibition of EGFR/PI3K/AKT cell survival pathway promotes TSA's effect on cell death and migration in human ovarian cancer cells

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Abstract. Trichostatin A (TSA), a hydroxamate-type inhibitor of mammalian histone deacetylases, is emerging as one of a potentially new class of anticancer agents. TSA is known to act by promoting the acetylation of histones, leading to uncoiling of chromatin and activation of a variety of genes implicated in the regulation of cell survival, proliferation, differentiation, and apoptosis. In addition, there is an increasing appreciation of the fact that TSA may act through mechanisms other than induction of histone acetylation. Accumulated experimental data indicate that TSA activates phosphatidyl inositol-3-kinase (PI3K)/AKT signaling. Using human ovarian cancer cell line Caov3 cells, we observed that TSA induced cell death in a time- and dose-dependent manner and also inhibited cell migration. TSA transiently activated EGFR tyrosine phosphorylation and AKT activation in a time- and dose-dependent manner, which had been inhibited by EGFR inhibitor PD153035 and PI3 kinase inhibitor LY294002. We also observed that TSA transiently induced survivin expression that had been inhibited by PD153035 and LY294002, suggesting that TSA-induced survivin expression is mediated by EGFR/PI3 kinase pathway. Combination of EGFR inhibitor 153035 or PI3 kinase inhibitor LY294002 with TSA enhanced TSA-induced cell death and TSA reduction of cell migration. Collectively, our data demonstrate that TSA transiently activated EGFR/PI3K/AKT cell survival pathway, leading to expression of survivin. Inhibition of this pathway enhanced TSA-induced cell death and inhibited cell migration. Our data suggest that combination of EGFR/PI3K/AKT cell survival pathway inhibitors with TSA be a better approach to ovarian cancer treatment.

Introduction

Ovarian cancer is the fifth leading cause of cancer deaths among women in the United States with an incidence of about 23,000 new cases and 14,000 deaths annually (1,2). The vast majority of these cancers (85%) arise from specialized epithelial cells that cover the surface of the ovary. Most patients with ovarian cancer respond to first-line chemotherapy, but many relapse within 18-22 months. The development of efficacious salvage therapies that increase overall survival while maintaining quality of life remains a great challenge for the treatment of this disease (1,3,4).

Histone modification through acetylation and deacetylation is a key process in transcription, DNA replication, and chromosome segregation (5). The opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs) allow gene expression to be exquisitely regulated through chromatin remodeling. Aberrant transcription due to altered expression or mutation of genes that encode HATs, HDACs or their binding partners, is a key event in the onset and progression of cancer. Histone deacetylase inhibitors (HDAs) induce accumulation of highly acetylated histones by inhibiting the activity of histone deacetylase, and inhibit cell proliferation, induce differentiation, and promote apoptosis (6,7). Trichostatin A (TSA), a hydroxamate-type inhibitor of mammalian histone deacetylases, is emerging as one of a potentially important new class of anticancer agents (8,9). TSA acts by promoting acetylation of histones, leading in turn to uncoiling...
of chromatin and activation of a variety of genes implicated in the regulation of cell survival, proliferation, differentiation, and apoptosis. It has been demonstrated that TSA induces growth arrest and apoptosis and suppresses the invasion of cancer cells (10,11). In addition, there is an increasing appreciation of the fact that TSA may act through mechanisms other than induction of histone acetylation (12). Accumulated experimental data indicate that TSA activates phosphatidyl inositol-3-kinase (PI3K)/AKT signaling (13). Inhibition of protein kinase C abrogates TSA mediated up-regulation of nuclear factor κ transcriptional activity and p21 expression that is associated with profound induction of apoptosis in lung or esophageal cancer cells (14).

Receptor tyrosine kinase of the EGFR family regulates essential cellular functions, including proliferation, survival, migration, and differentiation, and appears to play a central role in the etiology and progression of solid tumors (15,16). Published data demonstrates that the effects of EGFR signaling on cell proliferation and survival are mediated by PI3K-AKT pathways (17,18). Since EGFR is frequently overexpressed in breast, lung, colon, ovarian, and brain tumors, EGFR signaling has become an important target in anticancer drug development due to its ability to suppress apoptosis and to control tumor cell proliferation and migration (19,20). Recently, we found that the inhibition of EGFR cell survival pathway enhanced betulinic acid-induced cell death in human melanoma cells (21).

We undertook the present study to investigate whether TSA transactivates EGFR/AKT pathway and up-regulates survivin expression in human ovarian cancer cells, and whether the inhibition of EGFR/AKT pathway could potentiate TSA's effect on ovarian cancer cell death. The data presented here demonstrate that TSA transiently activated EGFR/AKT cell survival pathway, leading to expression of survivin, and the inhibition of EGFR/AKT pathway sensitizes human ovarian cancer cells to TSA treatment. This suggests that a combination of EGFR inhibitor and TSA could provide better clinical treatment of human ovarian cancer.

Materials and methods

Cell culture. The human ovarian epithelial cancer cell line (Caov3 cells) was from University of Michigan Cancer Center. Human Caov3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (Hyclone), penicillin/streptomycin (1:100, Sigma) and 4 mM L-glutamine, in a humid atmosphere incubator with 5% CO2 at 37°C. Cells were reseeded twice a week at a density of 0.2 x 106 cells/ml with fresh complete culture medium. Unless otherwise indicated, cultures were grown to 70-80% confluence and then serum-starved overnight in DMEM medium prior to treatment. When inhibitors were added, the medium for the remainder of the experiment.

Reagents. TSA was from EMD Biosciences, Fibronectin and anti-ß-actin were from Sigma. Anti-phospho-EGFR (Tyr1068), phospho-AKT (Ser473), and AKT antibody were from Cell Signaling Technology (Beverly, MA). Anti-EGFR (1005), goat anti-rabbit IgG-HRP, and goat anti-mouse IgG-HRP antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). PD 153035, and LY294002 were from Calbiochem. Polybeads were from Polyscience.

Treatment. TSA solution was diluted in DMEM medium without serum to a final concentration of 100 ng/ml. In the time-dependent experiments, Caov3 cells were treated with 100 ng/ml of TSA, and harvested at 5, 15, and 30 min, 1, 2, 4, 8, and 24 h after TSA treatment. In the dose-dependent experiments, Caov3 cells were treated with 10, 100, and 1000 ng/ml of TSA for 0.5, 1, and 4 h, respectively. In experiments for exploration of cell proliferation and survival, and cell migration, Caov3 cells were treated with 10, 100, and 1000 ng/ml of TSA for 24 h, or with 100 ng/ml of TSA in the presence and absence of EGFR (PD153035, 20 μM) and PI3K/AKT (LY294002, 50 μM) inhibitors for 24 and 48 h. Cell densities and morphologies were photographed post treatment.

Western blot analysis. Cells with and without treatment were washed with cold phosphate-buffered saline (PBS: 50 mM phosphate, pH 7.4, 100 mM NaCl, and 10 mM KCl) and harvested by scraping into 0.2 ml of RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM NaF, 10 μM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (10 μg/ml leupeptin, 10 μg/ml aproatin, and 1 μg pepstatin). Cell lysates were incubated on ice for 30 min. After centrifugation at 14,000 rpm for 10 min at 4°C, the protein concentration was determined using a Bio-Rad protein assay (Bio-Rad, Hercules, CA). Proteins (50 μg) were denatured in 2X SDS-PAGE sample buffer for 5 min at 95°C. The proteins were separated by 12%, 10% or 7.5% SDS-PAGE and electro-transferred to Immobilon-P membrane (Millipore, Bedford, MA) for 2 h at 4°C. Non-specific binding was blocked with 10% dry milk in TBST (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 0.01% Tween-20) for 1 h at room temperature. With constant shaking, the membranes were incubated in primary antibodies in dilution buffer (2% BSA in TBST) overnight at 4°C. After washing with TBST three times, the membranes were incubated in secondary antibodies at room temperature for 1 h with constant shaking. The expression of targeted proteins was detected using an ECL kit (Amersham Biosciences) following the manufacturer’s instructions and visualized by autoradiography with Hyperfilm.

Phagokinetic track motility assay. The 12-well plates were placed by coating medium (20 μg/ml of fibronectin in PBS), and stored for at least 2 h at 37°C. After removing the coating medium by gentle suction with a Pasteur pipette, the wells were washed once with PBS and 2.4 ml of microsphere suspension (86 μl of stock microbeads in 30 ml PBS) was added per well. Then the plates were centrifuged at 1200 rpm at 4°C for 20 min and carefully transferred to an incubator for at least 1 h at 37°C. The medium (1.8 ml) was removed from each well and 1500 freshly trypsinized cells in 2 ml assay-medium (DMEM supplemented with a 0.05% fetal bovine serum) were seeded per well. Cells with or without treatments were cultured, and cell migration was photographed at 24 h and 48 h post treatment.
Results

Inhibition of cell proliferation and survival in human Caov3 cells by TSA. It has been shown that HDACIs inhibit proliferation, stimulate apoptosis, and induce cell cycle arrest in malignant cells (6,7,22). To verify whether TSA inhibits cell proliferation and survival in human ovarian cancer, we treated Caov3 cells with TSA. Cells were cultured in 12-well plates, and treated with TSA for various times and with various concentrations. Microscopic data indicated that TSA inhibited Caov3 cell proliferation and survival in a time-dependent manner. At the concentration of 100 ng/ml, only 60% of cells had survived 24 h after treatment and 45% of cells had survived 48 h after treatment (Fig. 1A). The effect of TSA on cell death was dose dependent (Fig. 1B).

Inhibition of cell migration in human Caov3 cells by TSA. HDACIs are known to exert anti-metastatic activity in vitro and in vivo (23-25). To investigate whether TSA inhibits cell migration in human ovarian cells in culture, we used phagokinetic motility assay as previously reported (21,26). Cells were placed on fibronectin and microbeads-coated plates and treated with TSA for various times and with various concentrations. Cell migration was monitored by microscope. The results showed that TSA inhibited Caov3 cell migration in a time and dose-dependent manner (Fig. 2).

Transactivation of EGFR in human Caov3 cells by TSA. Chemotherapeutic regimens are ultimately unsuccessful due to intrinsic or acquired drug resistance in ovarian cancer (27). Since overexpression and/or activation of EGFR has been correlated with tumor resistance to cytotoxic agents (28), EGFR might be a novel target for enhancing the efficacy of HDACIs in cancer therapy. To investigate whether TSA transactivates EGFR in human ovarian cancer cells, Caov3 cells were deprived of serum overnight and exposed to 100 ng/ml
of TSA and harvested at 5, 15, 30, 60, and 120 min post treatment. Western blot analysis using antibody recognizing phospho-EGFR indicated that TSA induced EGFR phosphorylation in a time-dependent manner. The phosphorylation of EGFR induced by TSA treatment started at 5 min post treatment, and the activity peaked at 30 min and then returned to basal level within 2 h (Fig. 3A). As shown (Fig. 3B), the phosphorylation of EGFR was maximum at a concentration of 100 ng/ml at 30 min after TSA treatment. Furthermore, pretreatment of Caov3 cells with EGFR inhibitor PD 153035 (20 μM) significantly inhibited TSA-induced EGFR expression (Fig. 3C).

**Inhibition of AKT activation induced by TSA in human Caov3 cells.** Previous data indicated that TSA activates phosphatidylinositol-3-kinase (PI3K)/AKT signaling in tumor cells (13). To investigate whether TSA induces AKT activation in human Caov3 cells, we treated cells with TSA at various concentrations and at various time-points. Western blot analysis indicated that 100 ng/ml of TSA transiently induced AKT activation, starting at 5 min post treatment and peaking at approximately 1 h (Fig. 4A). As shown in Fig. 4B, the activation of AKT was maximized at a concentration of 1000 ng/ml at 1 h after TSA treatment. EGFR signaling operates via activation of the PI3K-AKT pathways (29). In order to explore whether TSA-induced AKT signaling is mediated by EGFR activation, we used EGFR inhibitor PD153035 and PI3K/AKT pathway inhibitor LY294002. Human Caov3 cells were pretreated with PD153035 (20 μM) or LY294002 (50 μM) for 2 h, and then treated with 100 ng/ml of TSA for 1 h. Western blot analysis indicated that both PD153035 and LY294002 significantly inhibited TSA-induced AKT activation (Fig. 4C).
Inhibition of TSA-induced survivin expression in human Caov3 cells by EGFR and AKT pathway inhibitors. Survivin is unique for its expression in human malignancies but not in normal adult cells. It has been implicated in sensitization to chemotherapy and as a prognostic marker in several common cancers (30). Next, we examined whether TSA induces survivin expression in human Caov3 cells. Caov3 cells were treated with 100 ng/ml of TSA and harvested at 4, 8, and 24 h post treatment. Western blot analysis indicated that TSA induced-survivin expression peaked at 4 h (Fig. 5A). As shown in Fig. 5B, the expression of survivin was maximized at a concentration of 1000 ng/ml at 4 h after TSA treatment. To further examine whether TSA-induced survivin expression is mediated by EGFR/AKT signaling, we pretreated human Caov3 cells with 100 ng/ml of TSA and harvested at 4, 8, and 24 h post treatment. Western blot analysis indicated that TSA induced-survivin expression peaked at 4 h (Fig. 5A). As shown in Fig. 5B, the expression of survivin was maximized at a concentration of 1000 ng/ml at 4 h after TSA treatment. To further examine whether TSA-induced survivin expression is mediated by EGFR/AKT signaling, we pretreated human Caov3 cells.
with PD153035 (20 μM) or LY294002 (50 μM) for 2 h, and then treated cells with 100 ng/ml of TSA for 4 h. Western blot analysis indicated that both PD153035 and LY294002 significantly blocked TSA-induced survivin expression (Fig. 5C).

Enhancement of TSA-induced histone H4 acetylation in human Caov3 cells by inhibition of EGFR signaling. As widely believed, treating cells with TSA results in histone H4 hyperacetylation (31). To verify this result in ovarian cells in culture, we treated Caov3 cells with TSA at various time-points and concentrations. The results showed that TSA induced histone H4 acetylation in a time and dose-dependent manner (Fig. 6A and B). To further investigate whether inhibition of EGFR signaling modulates TSA-induced histone H4 acetylation in human Caov3 cells, cells were pretreated with PD153035 (20 μM) or LY294002 (50 μM) for 2 h, and then treated with 100 ng/ml of TSA for 4 h. Western blot analysis indicated that both PD153035 and LY294002 significantly enhanced TSA-induced histone H4 acetylation (Fig. 6C).

Figure 5. Inhibition of EGFR signaling blocked TSA-induced survivin expression in human Caov3 cells. Cells were deprived of serum overnight, exposed to 100 ng/ml of TSA, and harvested at 4, 8, and 24 h post treatment (A). Cells were treated with 10, 100, and 1000 ng/ml of TSA, and harvested at 4 h post treatment (B). Cells were treated with TSA (100 ng/ml) alone or in combination with PD153035 (20 μM) or LY294002 (50 μM), and harvested at 4 h post treatment (C). Survivin expression was analyzed by Western blotting.

Figure 6. Inhibition of EGFR signaling enhanced TSA-induced histone H4 acetylation in human Caov3 cells. Cells were deprived of serum overnight, exposed to 100 ng/ml of TSA, and harvested at 4, 8, and 24 h post treatment (A). Cells were treated with 10, 100, and 1000 ng/ml of TSA, and harvested at 4 h post treatment (B). Cells were treated with TSA (100 ng/ml) alone or in combination with PD153035 (20 μM) or LY294002 (50 μM), and harvested at 4 h post treatment (C). Survivin expression was analyzed by Western blotting.
Promotion of TSA-induced cell death in human Caov3 cells by inhibition of EGFR signaling. The above data indicate that inhibition of EGFR signaling not only blocked TSA-induced survivin expression but also enhanced TSA-induced histone H4 acetylation. To directly examine whether inhibition of EGFR signaling enhances TSA-induced cell death, cells were cultured in 12-well cell culture plates, TSA (100 ng/ml) alone or in combination with PD153035 (20 μM) or LY294002 (50 μM) was added to human Caov3 cells for 24 h. Microscopic data indicated that the reduction of cell viability induced by TSA treatment in combination with PD153035 or LY294002 was significantly increased in human Caov3 cells as compared with that caused by TSA alone (Fig. 7).

Enhancement of TSA-inhibited cell migration in human Caov3 cells by inhibition of EGFR signaling. To directly examine whether inhibition of EGFR signaling enhances TSA-induced down-regulation of cell migration in human Caov3 cells, cells
were cultured in 12-well cell culture plates and TSA (100 ng/ml) alone or in combination with PD153035 (20 μM) or LY294002 (50 μM) was added for 24 h. Phagokinetic motility assay results indicated that the cell migration caused by the combination of inhibitors with TSA was significantly reduced as compared with that caused by TSA alone (Fig. 8).

Discussion

As shown in studies of the biological activities of TSA, TSA induces growth arrest and apoptosis and suppresses migration in a number of cancer cells, including breast, colon, lung, and leukemic cancer cells. Treatment with TSA, which is followed by increased histone acetylation in the promoters, induces the expression of many genes that are suppressors of invasion and metastasis, including tissue inhibitors of metalloproteinase and nm23H1/H2, in addition to negative cell cycle regulators and apoptosis-related molecules (32-36). The present study provides evidence that alterations in histone acetylation have a dramatic effect on the proliferation and migration of ovarian cancer cells. Within 24 h of TSA exposure, Caov3 cell survival and proliferation (Fig. 1) and cell migration (Fig. 2) were dramatically inhibited.

Overexpression and/or activation of EGFR results in increased proliferation and migration of solid tumors including ovarian cancer (15,16,19,37). Moreover, it correlates with tumor resistance to cytotoxic agents (28). Activation of EGFR results in the activation of the lipid kinase, PI3K, generating the second messenger phosphatidylinositol 3,4,5-trisphosphate, which in turn activates AKT (38-40). It was reported that TSA activates phosphatidylinositol-3-kinase (PI3K)/AKT signaling (13). Our results also showed that TSA rapidly activated AKT signaling in human Caov3 cells (Fig. 4A and B).

Since ovarian cancer remains resistant to traditional and novel chemotherapeutic agents, relating, in part, to the activation of EGFR signaling, we further aimed to detect whether TSA could activate EGFR signaling. To our surprise, we found for the first time that TSA transiently activated EGFR phosphorylation in a time and dose-dependent manner (Fig. 3A and B). In addition, EGFR inhibitor PD153035 remarkably inhibited TSA-induced AKT activation, which suggested that TSA-induced AKT activation is mediated by EGFR signaling (Fig. 4C).

We present evidence that TSA induced survivin expression in Caov3 cells in a time and dose-dependent manner (Fig. 5A and B). Survivin is a novel anti-apoptotic protein that is highly expressed in cancer but is undetectable in most normal tissue. Survivin overexpression plays a pivotal role in the progression of ovarian tumors and provides an important prognostic implication for epithelial ovarian carcinomas (41). A novel antisense oligonucleotide targeting survivin expression induces apoptosis and sensitizes lung cancer cells to chemotherapy (42). It was reported that paclitaxel-mediated mitotic arrest of cancer cells is associated with survivin induction, which preserves a survival pathway and results in resistance to paclitaxel. Induction of survivin by paclitaxel is an early event (43). Our present study suggested that TSA-induced survivin expression might contribute to reduced sensitivity of human ovarian cancer cells to TSA. Furthermore, we found that EGFR inhibitor PD153035 and PI3K/AKT inhibitor LY294002 inhibited TSA-induced survivin expression (Fig. 5C). These results demonstrated that TSA-induced survivin expression is mediated by EGFR/AKT signaling, and inhibition of EGFR/AKT signaling might increase the sensitivity of human Caov3 cells to TSA.

Acetylation of nuclear histones, which is regulated by acetyltransferase and deacetylase (44-47), has been supposed to play a crucial role in gene expression because 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 (48-50). Furthermore, recent molecular and genetic approaches identified HATs and HDACs as transcriptional coactivators and transcriptional corepressors, respectively. These observations provide a molecular basis for regulation of transcription through acetylation of histones (51,52). Previous studies found that the acetylation levels of histone H4 inversely correlated to the depth of cancer invasion and pathological stage, and patients with higher levels of histone H4 acetylation had a better prognosis (53). As widely believed, treating cells with TSA results in histone H4 hyperacetylation and cell cycle arrest (31). In the present study, we demonstrated that TSA induced histone H4 acetylation in human Caov3 cells (Fig. 6A and B). Moreover, we found EGFR inhibitor PD153035 and PI3K/AKT inhibitor LY294002 significantly enhanced TSA-induced histone H4 acetylation (Fig. 6C). Therefore, we provided the first evidence that inhibition of EGFR/AKT signaling up-regulates the expression of histone H4 acetylation induced by TSA in human Caov3 cells.

It is conceivable that the ultimate role of HDACIs in cancer therapy will be as modulators of apoptosis induced by other cytotoxic agents. One particularly promising strategy involves attempts to combine HDACIs with other novel agents to promote tumor cell differentiation or apoptosis. In the current study, we showed that both the reduction of cell viability and the down-regulation of cell migration caused by TSA treatment in combination with EGFR inhibitor PD153035 or PI3K/Akt inhibitor LY294002 were significantly enhanced in human Caov3 cells as compared with that caused by TSA alone (Fig. 7). It appears that EGFR/AKT signaling might be a novel target for enhancing the efficacy of TSA in ovarian cancer therapy.

In conclusion, we present here that TSA transiently induced activation of EGFR/AKT signaling, leading to the expression of survivin and contributing to the reduced sensitivity of human ovarian cancer cells to TSA. Inhibition of EGFR/AKT signaling transiently activated by TSA not only blocked TSA-induced survivin expression but also enhanced TSA-induced histone H4 acetylation, which promoted cell death and inhibited cell migration in human Caov3 cells. Our data suggest that combination of EGFR inhibitor or PI3K/Akt inhibitor with TSA may provide a new approach to the design of chemotherapy strategies and the development of anticancer drugs.

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