Biochemical Pharmacology 77 (2009) 1328–1336

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Trichostatin A sensitizes human ovarian cancer cells to TRAIL-induced apoptosis by down-regulation of c-FLIP$_L$ via inhibition of EGFR pathway

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ARTICLE INFO

Article history:
Received 14 August 2008
Accepted 24 December 2008

Keywords:
Trichostatin A
TRAIL
Apoptosis
EGFR
c-FLIP$_L$

ABSTRACT

TRAIL-resistant cancer cells can be sensitized to TRAIL by combination therapy. In this study, we investigated the effect of trichostatin A (TSA), a histone deacetylase inhibitor, to overcome the TRAIL resistance in human ovarian cancer cells. Co-treatment of human ovarian cancer cells with TSA and TRAIL synergistically inhibited cell proliferation and induced apoptosis. The combined treatment of ovarian cancer SKOV3 cells with TSA and TRAIL significantly activated caspase-8 and truncated Bid, resulting in the cytosolic accumulation of cytochrome *c* as well as the activation of caspase-9 and -3. Moreover, we found that down-regulation of c-FLIP$_L$ might contribute to TSA-mediated sensitization to TRAIL-induced apoptosis in SKOV3 cells, and this result was supported by showing that down- or up-regulation of c-FLIP$_L$ with transfection of siRNA or plasmid sensitized or made SKOV3 cells resistant to TRAIL-induced apoptosis, respectively. TSA or co-treatment with TSA alone and TRAIL also resulted in down-regulation of EGFR1/2 and dephosphorylation of its downstream targets, AKT and ERK. Treatment of SKOV3 cells with PKI-166 (EGFR1/2 inhibitor), LY294002 (AKT inhibitor), and PD98059 (ERK inhibitor) decreased c-FLIP$_L$ expression and co-treatment with TRAIL further reduced the level of c-FLIP$_L$, respectively, as did TSA. Collectively, our data suggest that TSA-mediated sensitization of ovarian cancer cells to TRAIL is closely correlated with down-regulation of c-FLIP$_L$ via inhibition of EGFR pathway, involving caspase-dependent mitochondrial apoptosis, and combination of TSA and TRAIL may be an effective strategy for treating TRAIL-resistant human ovarian cancer cells.

1. Introduction

TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is being developed as an anticancer agent, since it induces apoptosis in a wide range of cancer cells but not in most normal cells [1,2]. TRAIL can interact with two specific cell surface death receptors TRAIL-R1 (DR4/KILLER) and TRAIL-R2 (DR5/TRICK2) [3] and initiate the extrinsic apoptotic cascade via activation of pro-caspase-8 [1]. Activated caspase-8 can either directly cleave downstream effector caspases such as caspase-3, -6, and -7 without involving mitochondria activation (type I cells) or can require the engagement of the mitochondria-mediated pathway (intrinsic pathway) through cleavage of the pro-apoptotic Bcl-2 family protein BID (type II cells) [4,5]. However, recent reports have demonstrated that many tumor cells including ovarian cancer cells acquire resistance to the apoptotic effects of TRAIL and this could limit the efficiency of TRAIL in cancer therapy [4,6]. One of the most important regulators of resistance to TRAIL, c-FLIP (FLICE-like inhibitory protein), is an apoptosis inhibitory protein with homologous to caspase-8 that lacks catalytic activity [7] and is highly expressed in ovarian cancer cell lines [8]. To overcome the resistance of cancer cells to TRAIL, the enhanced effects using combination of TRAIL and chemotherapy have been reported [9,10]. However, the sensitivity of human ovarian cancer cells to such combinations is not well known.

Recently, accumulating evidences have suggested that HDAC inhibitor (HDACI) can be used as a new class of anticancer drug due to their selective toxicity and synergistic activity with

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Abbreviations: EGFR, epidermal growth factor receptor; TSA, trichostatin A; HDACI, histone deacetylase inhibitor; PARP, poly(ADP-ribose) polymerase; ERK, extracellular signal-regulated kinase; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; DR4, death receptor 4; DR5, death receptor 5; c-FLIP, cellular FLICE-inhibitory protein; XIAP, X-linked inhibitor of apoptosis protein.

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doi:10.1016/j.bcp.2008.12.027

chemotherapeutic drugs [11,12]. Several structural classes of HDACI have been identified as including derivatives of short-chain fatty acids, hydroxamic acids, cyclic tetrapeptides, cyclic peptides, and benzamides. Trichostatin A (TSA), a metabolite first isolated from cultures of *Streptomyces hygroscopicus*, is a kind of hydroxamic acids that is emerging as a new class of anticancer agents [13]. Recent studies suggested that TSA has a promising therapeutic effect on cancer cells when combined with radiotherapy or chemotherapy [14,15].

The epidermal growth factor receptor (EGFR) plays an important role in the regulation of cell proliferation, differentiation, development, and oncogenesis [16,17]. Amplification of the *EGFR* gene occurs frequently in breast, lung, ovary, and prostate cancer, which is associated with reduced patient survival [18,19]. As such, EGFR is an important therapeutic target in a number of human cancers and also confers reduced responses of cancer cells toward drug or radiation [20]. The EGFR regulates downstream signal such as PI3K/AKT [20], resulting in inhibiting the apoptosis induced by conventional or investigational cytotoxic agents [21]. Several targeted strategies have been developed to specifically inhibit aberrant EGFR signaling in tumor cells. The blockade of EGFR by inhibitors of EGFR tyrosine kinase leads to inhibition of cell cycle progression, induction of apoptosis, and enhancement of chemosensitivity, but EGFR-signaling antagonists alone do not appear to be curative in some instances [22–24]. Therefore, additional EGFR-targeted strategies or combination with other therapeutic approaches will be required. Recently, strong synergistic anticancer effects have been observed when EGFR-signaling antagonists were combined with activation of death receptors or their cognate ligands, including Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [25,26].

In this study, we demonstrated that TSA overcame resistance to TRAIL via EGFR pathway-dependent down-regulation of c-FLIP$_L$, consequently leading to induction of caspase-dependent mitochondrial apoptosis pathway in TRAIL-resistant SKOV3 cells. Therefore, we suggest that TSA in combination with TRAIL could be valuable in the treatment of TRAIL-resistant ovarian cancers.

2. Materials and methods

2.1. Cell culture, materials, and antibodies

The human ovarian cancer cell lines, SKOV3 cells, were obtained from American Type Culture Collection (ATCC, Manassas, VA) and Hey8 cells were obtained from Dr. I. J. Fidler (University of Texas M. D. Anderson Cancer Center, Houston, TX). Cells were maintained in RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and penicillin–streptomycin (50 U/ml) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. In this study the following inhibitors were used: caspase inhibitor, z-VAD-fmk (R&D Systems, Minneapolis, MN), EGFR1/2 inhibitor PKI-166 (Novartis, East. Hanover, NJ), PI3K/AKT inhibitor LY294002 (Sigma, St. Louis, MO), and ERK inhibitor PD98059 (Calbiochem, San Diego, CA). The inhibitors were dissolved in dimethylsulfoxide (DMSO; Sigma, St. Louis, MO) and the final concentration of DMSO was 0.1%. TRAIL was purchased from R&D Systems (Minneapolis, MN). TSA was purchased from Sigma–Aldrich (St. Louis, MO). Anti-EGFR1 clone 1005, anti-EGFR2 clone F-11, anti-p-ERK clone E-4, anti-Bcl-x$_L$ clone S-18, anti-Bcl-2 clone N-19, anti-Bax clone N-20, anti-PARP clone H-250, anti-caspase-3 clone H-277, and anti-cytochrome *c* clone H-104 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-8 clone 1C12, anti-p-AKT clone D9E, and anti-XIAP clone 2042 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Anti-c-FLIP clone NF-6 antibody was purchased from Alexis Biochemicals (San Diego, CA). Anti-caspase-9 (AAP-109C) antibody was purchased from Stressgen Biotechnologies Corp (Victoria, BC Canada). Anti-Bid (AF860), anti-DR4 (AF347), and anti-DR5 (AF631) antibodies were from R&D systems (Minneapolis, MN). Anti-β-actin clone AC-74 antibody was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Annexin V analysis for determining apoptosis

Cells were seeded in 6-well plates at a concentration of 1×10^6 cells/well and treated with increasing concentrations of TRAIL in the absence or presence of TSA for 16 h. The cells were resuspended in 100 μl of staining solution containing FITC-conjugated annexin V (BD Biosciences, San Jose, CA) and propidium iodide (Sigma, St. Louis, MO) in a HEPES buffer. After incubation at room temperature for 20 min, annexin V-positive cells were analyzed by FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). To determine whether caspase is involved in apoptosis induced by combined treatment with TSA and TRAIL, the cells were pre-treated with 20 μM of caspase inhibitor z-VAD-fmk for 3 h prior to addition of TSA and TRAIL followed by annexin V assay as described above. To evaluate that EGFR1/2 pathway and its downstream targets, PI3K/AKT and ERK are involved in TRAIL resistance in SKOV3 cells, 10 μM PKI-166, 10 μM LY294002, and 10 μM PD98059 were used. Cells were pre-treated with these inhibitors for 3 h prior to addition of TRAIL. The cells were incubated for a further 16 h at 37 °C and processed for annexin V analysis as described above.

2.3. Methylene blue analysis for determining cell survival

The methylene blue analysis was performed as previously described [27]. Cells were seeded in 96-well plates at a concentration of 1×10^3 cells/well and treated with increasing concentrations of TRAIL in the absence or presence of TSA for 16 h. After treatment, the cells were fixed with 70% ethanol, stained with methylene blue, and then the absorbance of dye eluted from the fixed cells in each well was measured on an automated scanning photometer at a wavelength of 630 nm. Results are presented as percentage of survival, taking control as 100%.

2.4. Flow cytometric analysis for determining DR4 and DR5 expression

The cells from the culture media were spun down at 500 × *g*, washed with phosphate-buffered saline (PBS), and resuspended in 500 μl PBS. Cells were then incubated with 5 μl of goat IgG2a, or anti-DR4 and anti-DR5 polyclonal goat antibody (1:100), respectively, for 1 h. After washing with PBS, FITC-conjugated rabbit anti-goat polyclonal antibody (1:200, Sigma, St. Louis, MO) was added to the cell suspension and incubated for 1 h on ice followed by washing with PBS. After rinsing, the samples were analyzed by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA). The data were analyzed using the CellQuest program.

2.5. cDNA and siRNA transfection

The pcDNA3.1-CMV-FLIP$_L$ (kind gifts from Dr. Park SI, National Institute of Health, Seoul, South Korea) was transfected into SKOV3 cells using the Lipofectamine following the manufacturer's protocol (Invitrogen Life Technologies, Inc., Carlsbad, CA). The transfected cells were grown for 24 h before subjected to annexin V analysis and methylene blue analysis. The c-FLIP$_L$ (5′-AAGGAACAGCUUGGCG-CUCAAdT dT-3′), XIAP (5′-TGTAGACTGCGTGGCACTATTdTdT-3′), and control scrambled (5′-CUUCCCGAAAACUUGAGACdTdT-3′) siRNAs were synthesized. SKOV3 cells in exponential phase of growth were plated in 6-well plates at 5×10^5 cells/well, cultured for 24 h,