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E1AF promotes mithramycin A-induced Huh-7 cell apoptosis depending on its DNA-binding domain

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ABSTRACT

Transcription factor E1AF is widely known to play critical roles in tumor metastasis via directly binding to the promoters of genes involved in tumor migration and invasion. Here, we reported for the first time the pro-apoptotic role of E1AF in tumor cells. The expression of E1AF at protein level was obviously increased during Huh-7 and Hep3B cells apoptosis induced by the anticancer agent mithramycin A. E1AF overexpression markedly enhanced mithramycin A-induced Huh-7 cell apoptosis and the expression of pro-apoptotic protein Bax depending on its DNA-binding domain. And, reduction of E1AF inhibited mithramycin A-induced Huh-7 cell apoptosis. Furthermore, reducing the expression of Bax significantly inhibited E1AF-increased Huh-7 cell apoptosis induced by mithramycin A. Taken together, E1AF increases mithramycin A-induced Huh-7 cells apoptosis and Bax expression depending on its DNA-binding domain, indicating that E1AF might contribute to the therapeutic efficiency of mithramycin A for hepatoma.

Apoptosis, which is a common cellular response to stress caused by environmental challenges [1], plays critical roles in cancer chemotherapy [2]. The anticancer antibiotic mithramycin A, which was originally isolated from *Streptomyces griseus*, has been used in cancer therapy in combination with hydroxyurea or a-IFN [3–8]. The mechanism of apoptosis induced by mithramycin A has been proposed to interact with GC-rich elements in genes promoters [9], leading to gene transcription modulation, such as multi drug resistance gene 1 [10], c-myc, or h-ras [11,12]. However, the underlying mechanisms of mithramycin A-induced cell apoptosis are largely unknown.

To a great extent, many transcription factors, including E2F1 and c-Myc, play an important role in drug-induced apoptosis [13,14]. E1AF, an *ets*-oncogene family transcription factor, is capable of regulating transcription by binding to the Ets-binding site in the promoters of its target genes [15], and is involved in a number of processes including neuronal pathfinding [16], mammary gland development and male sexual function [17,18]. And, E1AF plays an important role in HER2/Neu-mediated mammary oncogenesis and hepatocyte growth factor-induced cancer invasiveness and metastasis via directly binding to the promoters of genes involved in tumor migration and invasion [15,17,19–24], suggesting the contribution of E1AF in various malignant phenotypes of cancers cells. Consistent with this, we reported previously that E1AF overexpression promoted breast cancer cell cycle progression through up-regulation of Cyclin D3 transcription and induced glioma cell invasion in cooperation with Sp1 partly through up-regulation of β1, 4-galactosyltransferase V [25,26]. Interestingly, E1AF up-regulated p21 to induce cell cycle arrest in response to stress [15,27]. In spite of this knowledge, the contributions of E1AF in tumor development remain largely unknown.

The purpose of the present study was to investigate the contribution of E1AF in mithramycin A-induced cell apoptosis. Our results showed that the ectopic expression of E1AF in Huh-7 cells markedly increased mithramycin A-induced apoptosis and the expression of Bax depending on its DNA-binding domain. Furthermore, reducing the expression of Bax significantly inhibited the contribution of E1AF in mithramycin A-induced apoptosis. Taken together, our results reveal that E1AF increased Huh-7 cell apoptosis induced by mithramycin A and the expression of pro-apoptotic protein Bax depending on its DNA-binding domain, indicating that E1AF might contribute to the therapeutic efficiency of mithramycin A for hepatoma.

Materials and methods

Materials

Restriction enzymes, Trizol reagent and Lipofectamine reagent were purchased from Invitrogen. TaKaRa RNA PCR Kit (AMV Ver.2.1) was purchased from TaKaRa. Mithramycin A and Hoechst 33258 were purchased from Sigma Chemical Company.

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The anti-Bad, anti-Bak, anti-Bim, anti-Bax, anti-Bcl-2, anti-cleaved-caspase-3, anti-caspase-9, and anti-Bcl-xL antibodies were purchased from Cell Signaling Technology, Inc. The anti-GAPDH and anti-human-E1AF antibodies were purchased from Santa Cruz Biotechnology, Inc. The anti-GFP antibody was purchased from Roche. Anti-mouse-HRP secondary antibody and anti-rabbit-HRP secondary antibody were purchased from New England Biology. Other reagents were commercially available in China.

Cell culture and transfection

Huh-7, Hep3B, HeLa and H1299 cells were maintained in DMEM with 10% fetal bovine serum (FBS)[1] at 37 °C in a humidified atmosphere containing 5% CO_2. Cell transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Analysis of apoptosis by flow cytometry and Western blot analysis

Adherent and non-adherent cells were collected, washed twice in phosphate-buffered saline (PBS), and fixed with ice-cold 70% ethanol for at least 1 h. The fixed cells were washed and stained with propidium iodide. After incubation for 45 min at 37 °C, the DNA content was determined by quantitative flow cytometry with standard optics of FACScan flow cytometer (Becton-Dickinson FACStar). The percentage of apoptotic cell was quantified from sub-G1 events. Western blot was performed as previously described [28], using an antibody to GAPDH to ensure equivalent loading. Bands were quantified using Gel-Doc software (Biorad).

Analysis of nuclear morphology by fluorescence staining

Cells grown on the glass coverslips were fixed with 4% paraformaldehyde/PBS for 30 min, washed for 15 min in 0.1% Triton X-100/PBS, and incubated in dark with Hoechst 33258 (10 μg/ml) for 15 min. After the coverslips were washed in PBS, positive nuclei were counted. Normal nuclei and apoptotic nuclei (condensed new moon-type or fragmented chromatin) were easily distinguished. A minimum of 300 cells from four different microscopic fields were counted to obtain reliable estimates of cell apoptosis in three independent experiments.

Plasmids

Expression constructs for pSilencer-2.0, E1AF RNAi (shE1AF), HA-pcDNA3.0, pcDNA3.1-myc, pcDNA3.0-E1AF and EGFPN3 have been described previously [25,26,28–30]. GFP-tagged E1AF plasmid was constructed by inserting E1AF coding sequence into the HINDIII/XhoI site of EGFPN3 vector. The deletion mutations of E1AF were designated as ETS-GFP (amino acids from 341 to 421) and ▲ ETS-GFP (deletion of amino acids from 341 to 421). pLL3.7 plasmid was a kind gift from Prof. van Parijs L. (University of California) and pLL3.7-shBax plasmid was constructed as previously described [31]. The following sequences were targeted to silence Bax by shRNA expression: GCUCUGAGCAGAUCAUGAA.

Reverse transcription (RT)-PCR

Reverse transcription PCR were performed as previously described [30]. About 0.5 μg of RNA from each sample was reverse-transcribed into cDNA according to the manufacturer's manuals. The cDNA was amplified by PCR with the following primers. Their sequences were as follows: for E1AF, 5′-ATGGAGCGGAGGATG-3′ and 5′-CTGGGGGCTAGTAAGAG-3′; for Bax, 5′-CTGCAGAGGATGATTGCCG-3′ and 5′-TGCCACTCGGAAAAAGACCT-3′. GAPDH mRNA expression served as a loading control.

Results

The effect of mithramycin A, 5-FU and cisplatin on E1AF expression

To investigate the contribution of E1AF to cell apoptosis, we first examined the expression of E1AF in Hep3B cells treated with mithramycin A, 5-FU or cisplatin, which are widely used in the treatment of solid tumors and kill cells through the induction of apoptosis [32–34]. Western blot was performed to analyze the levels of E1AF protein expression in Hep3B cells untreated or treated with mithramycin A, 5-FU or cisplatin at the indicated concentrations. As depicted in Fig. 1A, the protein level of E1AF was significantly induced in response to mithramycin A in a dose-dependent manner; however, compared to controls, the levels of E1AF were no significantly altered in response to 5-FU or cisplatin. Consistent with this, mithramycin A-induced E1AF expression in Huh-7, HeLa and H1299 cells in a dose-dependent manner (Fig. 1B–D). To further clarify the mechanism of mithramycin A-induced E1AF expression, E1AF mRNA expression level in Hep3B and Huh-7 treated with or without mithramycin A was measured using RT-PCR assay. Interestingly, the expression of E1AF mRNA in Hep3B and Huh-7 was not significantly changed during mithramycin A-induced apoptosis (Fig. 1E).

E1AF overexpression promotes mithramycin A-induced apoptosis in Huh-7 cells

The effect of mithramycin A on E1AF expression motivated us to investigate the contribution of E1AF to mithramycin A-induced cell apoptosis. Huh-7 cells transiently transfected with pcDNA3.0 or E1AF plasmids were treated with or without mithramycin A (2 μg/ml) treatment for 48 h. Compared to control, the percentage of rounding and shrinkage cells was markedly increased in E1AF-overexpressed Huh-7 cells in the absence or presence of mithramycin A (Fig. 2A). In addition, the percentage of apoptotic cells in E1AF-overexpressed Huh-7 cells was markedly increased, compared to that of the controls by FACS assay (Fig. 2B). To further investigate the pro-apoptotic effect of E1AF on Huh-7 cells, Huh-7 cells transiently transfected with control or E1AF expression vector were treated with or without mithramycin A (2 μg/ml) for 48 h and cell nuclei was stained with Hoechst 33258. E1AF overexpression sensitized Huh-7 cells to mithramycin A-induced apoptosis as indicated by fragmented and condensed nuclei (Fig. 2C). Furthermore, down-regulation of E1AF mediated by E1AF RNAi decreased mithramycin A-induced E1AF expression (Fig. 2D) and reduced mithramycin A-induced Huh-7 cell apoptosis by FACS assay and Hoechst 33258 staining assay (Fig. 2E and F), indicating the involvement of E1AF in mithramycin A-induced cell apoptosis.

E1AF overexpression induced mithramycin A-induced cell apoptosis via up-regulation of Bax

To investigate the mechanism of E1AF-increased Huh-7 cell apoptosis, we explored whether E1AF influenced on the expression of the members of Bcl-2 and Caspase family which play key roles in the apoptosis progress [35]. As shown in Fig. 3A, E1AF overexpression increased the expression of Bax and cleaved Caspase-3 in the presence or absence of mithramycin A, without significantly changing the expression of the other examined proteins. To further investigate the mechanism of E1AF-induced Bax expression, the level of Bax mRNA expression in Huh-7 cells transiently transfected with control or E1AF in the presence or absence of mithramycin A was measured using RT-PCR assay. The ectopic expression of E1AF dramatically increased Bax mRNA expression in a dose-dependent manner (Fig. 3B).

To investigate the contribution of Bax in E1AF-increased cell apoptosis, shRNA targeted to Bax was constructed as previously described [31], and transiently transfected into Huh-7 cells (Fig. 3C). Compared to control, down-regulation of Bax mediated by shBax in Huh-7 cells decreased mithramycin A-induced cell apoptosis and blocked the positive effect of E1AF on mithramycin A-induced cell apoptosis (Fig. 3D and E).

[1] *Abbreviations used:* FBS, fetal bovine serum; PBS, phosphate-buffered saline; AD, acidic domain; Gln-rich domain, glutamine-rich domain.