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Elafin selectively regulates the sensitivity of ovarian cancer cells to genotoxic drug-induced apoptosis

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Abstract

Objective—Elafin has been reported to be abundantly expressed in human epithelial ovarian carcinoma (EOC), however, its functions are poorly understood. Here, we evaluated the role of elafin in modulating the sensitivity of human EOC cells to chemotherapeutic drugs.

Methods—Elafin expression was determined by ELISA in 9 established human EOC cell lines. A lentivirus encoding elafin-specific shRNA was used to down-regulate elafin expression in OVCAR3 and OV433 cells, and a plasmid encoding elafin was used to ectopically express elafin in elafin-negative SKOV3 cells. Sensitivity to cisplatin and other genotoxic agents and to paclitaxel, an inhibitor of microtubule depolymerization, was examined in OVCAR3, OV433 and SKOV3 sublines. Cell viability was determined by the MTT assay, apoptosis by annexin V/7-AAD staining and caspase activation by fluorimetric assay.

Results—Knockdown of the elafin gene decreases cisplatin IC₅₀ by at least 2-fold in OVCAR3 and OVCAR433 cells ($p < 0.01$) but does not affect paclitaxel IC₅₀. The sensitivity to other genotoxic agents such as carboplatin, cyclophosphamide and 5-fluorouracil was also increased by silencing the expression of elafin. Apoptosis and caspase-3 activation was significantly augmented in cisplatin-treated OVCAR3 cells with silenced elafin. Overexpression of elafin in SKOV3 cells made them more resistant to cisplatin and decreased cisplatin-induced apoptosis and caspases activation ($p < 0.01$).

Conclusions—Expression of elafin decreases the sensitivity of human EOC cells to several genotoxic agents, which may have an important implication in predicting the response of patients with EOC to chemotherapy in the clinic.

Keywords

Ovarian cancer; Elafin; Drug resistance; Genotoxic drugs

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Conflict of interest statement

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Introduction

Epithelial ovarian carcinoma (EOC) is the leading cause of death from gynecologic malignancies in the United States and is the fourth most common cause of cancer death in women [1]. Over 70% of women with EOC present with advanced stage disease and tumor dissemination throughout the peritoneal cavity [2]. Despite the standard therapy with surgical cytoreduction and the combination of cisplatin and paclitaxel, the treatment efficacy is significantly limited by the frequent development of drug resistance [3]. Novel therapeutic targets are urgently needed to improve ovarian cancer treatment efficacy.

Elafin, also known as skin-derived antileukoprotease (SKALP) or peptidase inhibitor 3 (PI3), is encoded by a gene belonging to the whey acidic protein (WAP) family [4-6] and is related to human epididymis protein 4 (HE4), one of the best diagnostic markers for ovarian carcinoma [7,8]. Elafin is an inhibitor of serine proteases such as elastases and neutrophil proteinase 3, exhibits anti-microbial and anti-inflammatory activities, and its expression is induced under conditions of inflammation and wound healing [4-6]. It is expressed in a significant number of squamous cell carcinomas [9-15], and a study on glioblastoma multiforme showed that elafin expression is correlated with poor outcome [16]. Recently, Clauss et al reported the elafin gene is overexpressed in serous EOC together with other members of the WAP family [17], including HE4 and secretory leukocyte protease inhibitor (SLPI), which all locate on chromosome 20q13.12, a region frequently amplified in serous EOC [18]. They further showed that elafin expression can be transcriptionally upregulated by inflammatory cytokines through activation of the nuclear factor κ B pathway and that patients with EOC expressing high levels of elafin did clinically worse and that EOC from patients with platinum-refractory disease expressed high levels of elafin [17].

However, more needs to be learned about the biological functions of elafin in ovarian cancer. In this study, we have investigated the role of elafin in modulating the sensitivity of EOC cells to several chemotherapeutic drugs including cisplatin and paclitaxel.

Materials and Methods

Cell lines and chemotherapeutic drugs

Nine established human EOC cell lines and one mouse EOC cell line were used to evaluate the expression of elafin. They included OVCA433, OVCAR-3, SKOV3, OVCAR-5 and OVCAR-10, which were obtained from American Type Culture Collection (ATCC, Manassas, VA), HE207, HE249, H4020 and H3639, which had been established in our laboratory from patients with stage III/IV OvC using published techniques [19] and ID8, which is a mouse EOC cell line obtained from Dr G. Coukos (University of Pennsylvania, Philadelphia, Pennsylvania). All cell lines were propagated in Iscove's Modified Dulbeccos Medium (IMDM; Thermo Scientific, Logan, UT) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a 5% CO₂-containing atmosphere. Cisplatin was obtained from APP Pharmaceuticals LLC (Schaumburg, IL), Carboplatin and paclitaxel/taxol from Hospira Inc (Lake Forest, IL), 5-fluorouracil and cyclophosphamide from Sigma (Louis, MO).

Measurement of elafin expression

The level of elafin was measured in culture supernatants using a commercially available ELISA kit (Raybiotech Inc, Norcross, GA). Wild type (WT) EOC cells, as well as EOC cells transfected with a lentivirus or plasmid specific for elafin or control were seeded into 6 well plates with 10⁶ cells per well at 37°C in 2 mL IMEM containing 10% FBS and 1% penicillin/streptomycin. Supernatants were harvested after 48 hours and assayed for elafin

secretion following the manufacturer's instructions. Results were expressed as pg/mL per million cells.

Immunoblot analysis

EOC cells were cultured as described above, 48 hours later, cells were lysed by 1x SDS sample buffer (100 μ L per well of 6-well plate; Santa Cruz) and sonicated for 15 seconds followed by heating for 10 minutes at 100°C. For detection of elafin in supernatants, EOC cells were grown in IMEM supplemented with 10% FBS and 1% penicillin/streptomycin to 80% confluence. The medium was then changed to serum-free media and the cells were cultured for an additional 48 hours. The cultured medium was then cleared by centrifugation and concentrated using a Millipore Amicon Ultra-5 centrifugal filter with a 10,000 molecular weight cutoff. The concentrates were then denatured in 5x SDS sample buffer for 10 minutes at 100°C. Equal volume of whole cell extracts or supernatants were separated by 4-20% Tris-HEPES-SDS precise protein gels (Thermo Scientific). After transfer, the blot was probed with primary rabbit polyclonal antibodies against β -actin or elafin (Santa Cruz) and followed by HRP conjugated anti-rabbit secondary antibody (Cell Signaling, Danvers, MA). Proteins were visualized by enhanced chemiluminescence (Cell Signaling).

Retrovirus-based RNA interference

Elafin shRNA lentiviral particles and control lentiviral particles were procured from Santa Cruz Biotechnology (Santa Cruz, CA). These lentiviral particles contain three to five expression constructs each encoding elafin-specific shRNA, whereas control lentiviral particles encode scrambled elafin sequences. The knockdown of elafin in OVCAR3 and OVCAR433 cells was performed using the manufacturer's protocol. Briefly, cells were transfected when they were 50% confluent. Polybrene was added at a final concentration of 5 μ g/mL, and elafin shRNA/control lentiviral particles (10-20 MOI) were added to cells. Medium was changed after 12 h, and cells were selected in IMEM containing 2 μ g/mL puromycin (Santa Cruz). Elafin knockdown was analyzed by ELISA 6-8 d after transduction.

Elafin overexpression

A pcDNA3.1 plasmid encoding elafin that had been constructed by Dr Drapkin's laboratory (Boston, MA) was obtained from Addgene (Cambridge, MA). SKOV3-elafin and control cell lines expressing elafin or pcDNA3.1 empty vector, respectively, were generated by transfection of the SKOV3 cells with 6 μ g of pcDNA3.1/enlafin or 6 μ g of pcDNA3.1 empty vector using Lipofactamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells expressing these vectors were selected in IMEM containing 0.4 mg/mL G418 (InvivoGen, San Diego, CA) for 2 weeks. Cells were expanded in culture medium supplemented with 0.2 mg/mL G418 and screened by ELISA for the expression of elafin.

Proliferation and cytotoxicity assays

Cell viability was evaluated with MTT assay following the protocol of the manufacturer (Promega, Madison, WI). Briefly, cells were plated at 20,000 cells/well in 96-well plates. For proliferation assay, cells were incubated for 72-96 h. For cytotoxicity assay, cells (confluence 60-70%) were treated on next day with serially diluted concentrations of cisplatin or paclitaxel incubated for 72 h. For other drugs, cells were treated with a fixed dose (as indicated) and incubated for 72 h. At the termination of the experiment, 20 μ L of MTT assay solution was added into 100 μ L of medium containing cells and incubated for 2 h. The absorbance of each well was determined using a microplate reader (Molecular devices, Sunnyvale, CA) at 492 nm with reference wavelength at 630 nm. The percentage of

cell survival was defined as the relative absorbance of untreated versus treated cells. All assays were performed in triplicate and repeated three times.

Annexin V and 7-aminoactinomycin D staining

Apoptotic cells were counted using FITC-conjugated Annexin V and 7-aminoactinomycin D (7-AAD) staining kit (Beckman Coulter, Inc, Fullerton, CA). Drug-treated cells were washed twice in cold PBS and resuspended in Annexin V-binding buffer at a concentration of 3×10^6 per mL. This suspension (100 μ L) was stained with 5 μ L of Annexin V-FITC and 5 μ L 7-AAD. 7-AAD is a nucleic acid dye that was used for the exclusion of nonviable cells. The cells were gently vortexed and incubated for 15 minutes at room temperature in the dark. After addition of 400 μ L of binding buffer to each tube, cells were analyzed by flow cytometry immediately.

Caspase-3 activity

The caspase-3 activity assay (Roche, Indianapolis, IN) was used to determine caspase-3 activity. Briefly, cells were washed in ice-cold PBS and then resuspended in lysis buffer (1 $\times$ dithiothreitol [DTT]) and incubated for 1 minute on ice. Supernatants were obtained after centrifugation at 14 000 rpm for 1 minute at room temperature. Supernatant was added to anti-caspase-3-coated wells and incubated at 37°C for 1 h. After 3 washing steps, substrate solution (Ac-DEVD-AFC) was added, and the wells were incubated for 2 h at 37°C. Fluorescence was measured with a 400-nm excitation filter and a 505-nm emission filter on a microplate reader (Molecular devices).

Statistical analysis

The data are presented as the means $\pm$ SEM from three independent experiments. Statistical comparisons between groups were performed by one-way ANOVA or paired Student's t-test using Prism 5.0 software. Statistical significance was indicated by $p < 0.05$.

Results

Cultured EOC cells release elafin into supernatants which can be prevented by silencing the elafin gene

We first screened the expression of elafin in 9 human EOC cell lines and 1 mouse EOC cell line. As shown in Fig. 1A, 6 human EOC cell lines secreted elafin into culture supernatants, although there was a substantial variation between individual lines. As expected, we did not detect human elafin in the mouse EOC ID8 cell line. The experiment was repeated twice with similar results. We also obtained the similar results by western blotting (Fig. 1A, right).

Supernatants from OVCAR3 and OVCAR433 cells contained high levels of elafin, and we selected these two EOC lines for experiments in which we silenced the elafin gene by using lentivirus-mediated shRNA interference. Fig. 1B shows that more than 70% of elafin expression was silenced in the supernatants of cultured OVCAR3 and OVCAR433 cells that had been transfected with lentivirus encoding shRNA specific for elafin (ELV), while supernatants of cells from the respective EOC cells that had been transfected with the control lentivirus (CLV) produced as much elafin as the wild type (WT) cells. We also confirmed that the elafin gene was silenced by using real-time PCR (supplementary Figure 1). Knockdown of the elafin gene did not affect cell proliferation in the presence or absence of serum (Fig. 1C).

Elafin knockdown sensitizes EOC cells to cisplatin and other genotoxic drugs but not to paclitaxel

Cisplatin and paclitaxel are both used in first-line therapy for EOC. Cisplatin is a genotoxic drug that produces DNA damage while paclitaxel inhibits microtubule depolarization. Although the mechanism of action of the two drugs differs, both of them ultimately induce an apoptotic cascade leading to cell death [20]. We assessed the effect of elafin down-regulation on the in vitro sensitivity of OVCAR3 and OVCAR433 cells to these two drugs by exposing cells with intact or silenced elafin to increasing concentrations of cisplatin and paclitaxel for 72 h. The fold difference in drug sensitivity was calculated by IC₅₀ where 50% cytotoxicity was observed for the cells with silenced elafin or their respective controls. The cytotoxicity of cisplatin was 2-fold greater when elafin was down-regulated in the OVCAR3 (9.971 μ M, 9.086 μ M and 3.274 μ M for WT, CLV and ELV respectively) and OVCAR433 (15.14 μ M, 15.08 μ M and 7.561 μ M for WT, CLV and ELV respectively) cells whereas their sensitivity to paclitaxel remained unchanged (Fig. 2A).

Next, using a similar approach, we examined the sensitivity of OVCAR3 and OVCAR433 cells with intact or silenced elafin to other genotoxic drugs (5-fluorouracil, carboplatin, and cyclophosphamide) versus paclitaxol which affects microtubule assembly. Sensitivity to the genotoxic drugs was significantly ($p < 0.01$) enhanced in elafin-silenced cells for all genotoxic agents while sensitivity to paclitaxol was not changed (Fig. 2B).

Elafin knockdown increases cisplatin-mediated apoptosis and caspases activation

Treatment of tumor cells with DNA-damaging agents such as cisplatin is associated with activation of the intrinsic apoptotic pathway [20]. We examined the effect of silencing elafin on cisplatin-induced apoptosis. Cells were treated with cisplatin (5 μ M), harvested 60 h later, and analyzed for apoptosis by Annexin V/7-AAD staining. After cisplatin treatment, the percentage of apoptotic cells in OVCAR3-ELV cells significantly increased to ~30% whereas control populations (OVCAR3-WT and OVCAR3-CLV) only increased to ~15% (Fig. 3B; $p < 0.01$). There was no difference in spontaneous apoptosis in these cells. Representative dotplots are shown in Fig. 3A. We next assessed caspase activity in response to cisplatin treatment in these cells using a fluorometric assay. Caspase-3 activity was 2-fold higher in OVCAR3-ELV cells compared to the control cells (Fig. 3C; $p < 0.01$). In contrast, exposure to paclitaxel did not increase caspase-3 activity in OVCAR3-E-LV cells when compared to control cells (Fig. 3C). Elafin knockdown did not alter the expression the levels of pro-caspase-9, pro-caspase-3 and PARP and apoptotic regulatory proteins Bcl-2, Bcl-XL and Bax in OVCAR3-ELV cells (supplementary Figure 2). Silencing of the elafin gene by itself does thus not increase apoptosis and caspases activation, while it enhances cisplatin-mediated apoptosis and caspases activation.

Elafin overexpression decreases the sensitivity of EOC cells to cisplatin but not to paclitaxel

To further confirm the selectivity of elafin on the sensitivity to cisplatin, SKOV3 cells, which do not express elafin, were transfected to stably express elafin (SKOV3-E) or an empty vector (SKOV3-C). Fig. 4A shows data from ELISA assays demonstrating that SKOV-E cells express a high level of elafin while SKOV3-C and OVCAR3-WT cells are negative. Elafin expression in SKOV3 did not have any significant effect on cell proliferation in vitro (Fig. 4B). SKOV3-E and SKOV3-C cells were then exposed to either cisplatin or paclitaxel. As shown in Fig. 4C, SKOV3-E cells were significantly ($p < 0.01$) more resistant than SKOV3-C or SKOV3-WT cells to cisplatin, while there was no difference in sensitivity of the three lines when they were exposed to paclitaxel (Fig. 4D). These findings further indicate that EOC cells expressing elafin are less sensitive to cisplatin.

Overexpression of elafin makes SKOV3 cells less sensitive to cisplatin induced apoptosis

We next examined whether SKOV3-E cells are more resistant to cisplatin-induced apoptosis and caspases activation than SKOV3-C and SKOV3-WT cells which do not express elafin. As shown in Fig. 5B, following cisplatin treatment, the percentage of apoptotic cells in SKOV3-WT and SKOV3-C) cells was ~20% as compared to less than 10% in SKOV3-E cells ($p<0.01$). Representative dotplots are shown in Fig. 5A. Consistent with this result, caspase-3 activity following cisplatin treatment was less in SKOV3-E cells than in the corresponding two control SKOV3 lines (Fig. 5C). Western blotting showed that the expression levels of pro-caspase-9, pro-caspase-3 and PARP and apoptotic regulatory proteins Bcl-2, Bcl-XL and Bax were similar in SKOV3-WT, SKOV3-Con and SKOV3-E cells (supplementary Figure 3).

Discussion

The efficacy of treatment for EOC is frequently limited by the rapid development of resistance to chemotherapeutic drugs. Therefore, the identification of molecules that can impact drug sensitivity is of clinical significance. We now report that elafin, which is expressed by most ovarian carcinomas, is correlated with a decreased sensitivity to cisplatin and several other genotoxic drugs while expression of elafin does not impact the sensitivity to paclitaxel, probably because the drugs operate via different mechanisms. In view of a great clinical interest in HE4 as a diagnostic marker [8], we tested whether expression of HE4 has an impact on drug sensitivity, but did not observe any significant effects (supplementary Figure 4).

We utilized two approaches to probe the relation between elafin expression and drug sensitivity. First, we transfected EOC cells that endogenously expressed high levels of elafin with shRNA to silence the elafin gene, and, second, we transfected elafin-negative EOC cells to stably express elafin. Silencing of the elafin gene promoted cisplatin-induced apoptosis and caspase activation in OVCAR3 and OVCAR433 cells. It also sensitized the EOC cells to all of three other genotoxic agents tested, cyclophosphamide, carboplatin and 5-fluorouracil. Conversely, elafin overexpression decreased cisplatin-mediated apoptosis and caspase activation in SKOV3 cells. In contrast, neither increase nor decrease of elafin expression affected the sensitivity to paclitaxel.

Our findings that elafin promotes the resistance of EOC cells to cisplatin and several other commonly used genotoxic drugs may explain, or at least contribute to, the correlation between high elafin expression in many tumors [7-13] and poor survival [16,17]. In difference to findings with some other tumors [21,22], expression or silencing of elafin had no direct effects on the proliferation (Fig. 1C and Fig. 4B) or intrinsic apoptosis of EOC cells (Fig.1C, Fig.3B and Fig.5B); elafin impacts the apoptosis of EOC cells only as a result of their exposure to cisplatin or a similar drug (Fig.3B and Fig.5B).

The mechanisms responsible for the elafin-related increased chemoresistance are unclear and deserve to be investigated, since they may reveal an important biological role of elafin in EOC cells, e.g. in relation to the production of cytokines and lymphokines as suggested by a recent studies [23]. Based on our findings we suggest that that EOC samples should be examined by immunohistology before and (when available) after chemotherapy for expression of elafin. If they confirm our findings, which were obtained by studying cultured EOC cells, one would be able to predict which patients are less likely to respond to cisplatin and similar drugs and act accordingly. Monitoring sera for elafin (in addition to CA125 and HE4) may also provide clinically applicable information, although elafin itself is not a highly specific and sensitive diagnostic biomarker for EOC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Cultured epithelial ovarian cancer (EOC) cells make large amounts of elafin.
- Elafin knockdown in EOC cells make them significantly more sensitive to genotoxic drug.
- Overexpression of elafin in EOC cells confers them more resistance to genotoxic drug.
- Our results support an important role of elafin on the sensitivity of EOC cells to the commonly used chemotherapy.

Fig. 1.

Elafin production by cultured EOC cell lines. (A) WT EOC cells were seeded into 6 well plates with 10^6 cells per well at 37°C in 2 mL IMEM containing 10% FBS. The supernatants were harvested after 48 h and assayed for elafin by ELISA (left). Results are expressed as pg/mL per million cells. The cell lysates and supernatant concentrates from selective EOC cell lines were also assayed for elafin expression by western blotting (left). (B) OVCAR3 and OVCAR433 cells transfected with lentivirus encoding shRNA specific for elafin (ELV) or control (CLV) were assayed for elafin release by ELISA. Results are expressed as pg/mL per million cells. (C) The indicated cells were plated in 96 well plates with 20000 cells per well and proliferation were determined by an MTT assay 72 h later. Values represent the mean $\pm$ SEM. * $p < 0.01$, compared with control.

Fig. 2. Effects of elafin knockdown on drug sensitivity of OVCAR3 and OVCAR433 cell lines. (A) Cells were incubated with increasing concentrations of cisplatin or paclitaxel and cell viability was determined by the MTT assay after 72 h. (B) Cells were treated with fixed concentrations of the indicated drug and their viability was determined. All tests were performed in triplicate and the data shown are representative of three independent experiments. Values represent the mean $\pm$ SEM. * $p < 0.01$, compared with control.

Fig. 3.

Effect of silencing the elafin gene on cisplatin-induced apoptosis and caspase activation. (A) EOC cells were treated with cisplatin (5 μ M) and the percentage of apoptotic cell was determined by measuring the percentage of 7-AAD/Annexin V-positive cells 60 h after the drugs had been added to the culture medium. Representative dotplots are shown for each group treated with cisplatin and for a group with untreated WT cells. (B) Summary of results from two experiments performed in duplicates. Values represent mean $\pm$ SEM. (C) Lysates from cells treated with cisplatin (5 μ M) or paclitaxel (10 nM) were assayed for caspase-3 activity by monitoring the fluorescence produced by hydrolysis of caspase-3 substrate DEVD-AFC. Results are expressed as fold increase relative to untreated cells. The relative fluorescence unit (RFU) of caspase-3 activity was normalized for the protein content of each extract. * $p < 0.01$, compared with controls (WT and CLV).

Fig. 4.

Effect of overexpression of elafin on the sensitivity of SKOV3 cells to cisplatin and paclitaxel. (A) Elafin expression was determined by ELISA. (B) The indicated cells were plated in 96 well plates with 20000 cells per well and proliferation were determined by the MTT assay 72 h later. (C) and (D) Cells were treated with cisplatin (10 μ M) or paclitaxel (10 nM or 2 μ M) for 72 h and their viability was determined by the MTT assay. All tests were performed in triplicate; the data shown are representative of three independent experiments. Values represent the mean $\pm$ SEM. * $p < 0.01$, compared with controls (WT and C).

Fig. 5. Effect of elafin overexpression on cisplatin-induced apoptosis and caspase activation in SKOV3 cells. (A) Cells were treated with cisplatin (10 μ M) and the percentage of apoptosis was determined by measuring the percent of 7-AAD/Annexin V-positive cells 72 h after adding drugs to the medium. The graphs show representative dotplots of each group treated with cisplatin including a dotplot for untreated WT cells. (B) Summary of results from two experiments each performed in duplicates. Values represent mean $\pm$ SEM. (C) Lysates from cells treated with cisplatin (10 μ M) were assayed for caspase-3 activity by monitoring the fluorescence produced by hydrolysis of caspase-3 substrate DEVD-AFC. Results are expressed as fold increase relative to untreated cells. The relative fluorescence unit (RFU) of caspase-3 activity was normalized for the protein content of each extract. * p <0.01, compared with controls (WT and C).