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Original Article

Disulfiram induces anoikis and suppresses lung colonization in triple-negative breast cancer via calpain activation

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ABSTRACT

Triple-negative breast cancers (TNBC) often exhibit an aggressive phenotype. Disulfiram (DSF) is an approved drug for the treatment of alcohol dependence, but has also been shown to kill TNBC cells in a copper (Cu)-dependent manner. Exactly how this occurs has not been clearly elucidated. We sought to investigate the mechanisms responsible for DSF/Cu-dependent induction of apoptosis and suppression of lung colonization by TNBC cells. DSF/Cu induced anoikis and significantly suppressed cell migration and invasion with negative effects on focal adhesions, coinciding with vimentin breakdown and calpain activation in TNBC cells. In a xenograft tumor model, DSF suppressed tumor growth and lung nodule growth, which was also associated with calpain activation. These findings warrant further investigation of disulfiram as a potential treatment for metastatic TNBC.

Introduction

Triple-negative breast cancer (TNBC) accounts for an estimated 10–15% of all breast cancers and is characterized by a lack of expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). TNBC is associated with aggressive metastasis and poor clinical outcomes due to a lack of effective treatment options [1,2]. New drugs that effectively target both cancer cell proliferation and metastasis are therefore needed to improve clinical outcomes.

Epithelial cells can undergo a form of apoptosis called anoikis, which is triggered by cell detachment from the extracellular matrix (ECM) and a concomitant reorganization of the cytoskeleton [3]. Anoikis can influence cell fate decisions and suppress metastatic spreading from the primary site to other organs. The term 'anoikis resistance' refers to the ability of cancer cells to circumvent barriers to migration and invasion, thereby promoting metastatic progression [4,5]. For this reason, the induction of anoikis in metastatic cancers may confer clinical benefit.

The calcium-dependent protease calpain has been implicated in numerous roles including cell morphology, migration, apoptosis, necrosis and autophagy [6]. Calpain also regulates cytoskeletal reorganization during cell motility and spreading. However, excessive activity can cause degradation of cytoskeletal (eg. microtubules and vimentin), and focal adhesion components [eg. focal adhesion kinase (FAK) and talin]. These events can promote collapse of the cytoskeleton and plasma membrane integrity, leading to apoptotic death [7–9].

Disulfiram (DSF) has an exceptional clinical safety profile and has been approved for the treatment of chronic alcohol dependence [10]. Several recent clinical studies have focused on evaluating the anti-cancer efficacy of DSF in solid tumors [11,12]. Various cellular mechanisms have been proposed for DSF-dependent induction of apoptosis including proteasome inhibition, suppression of NF-kB activity, and inhibition of HER2/Akt signaling [13–16]. Although the antitumor activity of DSF is known to be associated with apoptosis, the precise cellular mechanisms responsible remain to be elucidated. In the present study, we sought to characterize the mechanism of action of DSF during apoptotic induction in TNBC, as well its effects on cell migration, invasion and metastasis *in vitro* and *in vivo*.

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Materials and methods

Reagents and antibodies

Disulfiram, copper chloride, dimethyl sulfoxide (DMSO) and propidium iodide (PI) were obtained from Sigma–Aldrich (St Louis, MO). Phosphatase inhibitor and protease inhibitor cocktail tablets were purchased from Roche Applied Sciences (Penzberg, GER). ALLN and calpeptin, Z-VAD-FMK, RNase A, TEMED and fetal bovine serum (FBS) were purchased from Life Technologies (Carlsbad, CA). Antibodies against FAK, phospho-FAK (Tyr397), X-linked inhibitor of apoptosis (XIAP); vimentin, poly ADP-ribose polymerase (PARP), cleaved-PARP, cleaved-caspase-3, and cleaved-caspase-8 were from Cell Signaling (Beverly, CA); Bcl-2 and calpain from Santa Cruz Biotechnology (Santa Cruz, CA); β-actin and talin from Sigma–Aldrich; horseradish peroxidase (HRP)-conjugated anti-rabbit and mouse IgG were from Bio-Rad Laboratories (Hercules, CA); and Alexa Fluor-488 and -594 goat anti-mouse IgG were from Invitrogen (Carlsbad, CA).

Breast cancer cell culture

The TNBC cell lines MDA-MB-231, Hs578T (American Type Culture Collection), 4T1 (Japanese Collection of Research Bioresources Cell Bank) and MDA-MB-231-Luc-D3H1 cells (PerkinElmer, Inc. USA) were cultured in DMEM or RPMI containing 10% FBS, streptomycin-penicillin (100 U/ml) and Fungizone (0.625 μg/ml). Cells were incubated at 37 °C in an atmosphere of 5% CO_2.

Cell viability assay

Cell viability was measured using the CellTiter 96* Aqueous One Solution Cell Proliferation Assay [MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, WI, USA), as previously described [17].

Annexin V/PI and Sub-G1 assay

Cells were stained using a FITC-conjugated Annexin V apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's protocol. For Sub-G1 assay, cells were harvested and fixed with 95% ethanol containing 0.5% Tween-20 for 24 h, before washing with PBS and incubation with propidium iodide (PI, 50 μg/ml) and RNase (50 μg/ml) for 30 min. Stained cells were analyzed with a Beckman Coulter Expo flow cytometer (Brea, CA).

Immunofluorescence confocal microscopy

Cells on 8-well chamber slides (BD Biosciences, Franklin Lakes, NJ) were fixed with 4% paraformaldehyde, washed with PBS, and incubated with 0.2% Triton X-100 for 10 min. Primary antibodies in antibody-diluent (Dako, Glostrup, Denmark) were incubated overnight at 4 °C before incubation with fluorescence-conjugated secondary antibodies (Alexa-Fluor-594 or 488). Cells were counterstained with ProLong Gold Antifade Reagent with DAPI (Life Technologies, Carlsbad, CA). Images were acquired using a Carl Zeiss confocal microscope (Weimar, Germany). Intensity of fluorescence was analyzed using fluorescence profiling, as previously described [18].

Western blot analysis

The procedures were performed as previously described [17]. Primary antibody dilutions: [FAK (1:2000), phospho-FAK (Tyr397, 1:2000), calpain (1:2000), XIAP (1:2000), PARP (1:2000), cleaved-PARP (1:2000), pro-caspase-3 (1:1000), cleaved-caspase-3 (1:1000), cleaved-caspase-8 (1:1000), bcl-2 (1:1000), vimentin (1:2000), talin (1:2000) and actin (1:5000)], were followed by HRP-conjugated rabbit or mouse secondary antibodies (1:3000–1:10,000). Signal intensity was detected using an Enhanced Chemiluminescence Kit (Thermo Scientific Inc., Rockford, IL) and quantitated using AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

Wound healing assay

For kinetic migration analysis, cells were seeded to ~90% confluency in 96-well plates (Essen ImageLock, Essen Biosciences, USA). Wound areas were made with a 96-pin Wound Maker device and washed with PBS to prevent reattachment of dislodged cells. Cells were treated with DSF/Cu at various concentrations immediately after wound scratching, and images were automatically acquired every hour for 24 h with an IncuCyte™ ZOOM® Kinetic Imaging System. Relative wound density was analyzed using an IncuCyte™ Scratch Wound Cell Migration Software Module.

Invasion assay

Invasion chambers were coated with Matrigel matrix (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's recommendations. After treatment, cells (1×10^5) were trypsinized, washed, and suspended in the upper chamber with serum-free media. Migration-inducing media with 10% FBS was added to the lower chambers before incubation for 48 h at 37 °C in an atmosphere of 5% CO_2. Invaded cells were fixed and stained with Diff-Quik (Sysmex, Kobe, Japan) and counted under a BX51 microscope (Olympus, Tokyo, Japan).

Xenograft experiments

All animal procedures were carried out in accordance with guidelines approved by the Korea University Institutional Animal Care and Use Committee (IACUC). Five-week-old female BALB/c nude mice were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan) and housed in a pathogen-free environment. The animals were acclimated for 1 week prior to the study with free access to food and water. 5×10^5 MDA-MB-231-luc-D3H1 cells were injected through the tail vein of 6-week-old BALB/c nude female mice to create pulmonary metastasis, and mice were randomly divided into 2 groups (n = 8/each group). For the subcutaneous xenograft tumor model, 3×10^6 MDA-MB-231-luc-D3H1 cells in 100 μl of culture medium were mixed with 100 μl of Matrigel and implanted in the right flank of BALB/c nude mice (n = 6/each group). After 1 week, either control solvent (cremophor/DMSO/ethanol/PBS, 1.5:0.5:0.5:7.5) [14] or DSF (50 mg/kg/day, 5 times/week) was intraperitoneally administered for 4 weeks, respectively. Animals were anesthetized and subjected to NightOWL LB983 bioluminescence imaging (BLI) system (Berthold Technologies, TN, USA). D-luciferin sodium salt (Bio-Vision Inc. Milpitas, CA) at 150 mg/kg was administered intraperitoneally as a substrate before BLI imaging. The captured images were quantified using the IndiGo™ software package. For a syngeneic model of experimental metastasis, 1×10^5 4T1 cells in PBS were injected through the tail vein of BALB/c mice (n = 8/each group) and then either control solvent or DSF (50 mg/kg/day, 5 times/week) administrated for 14 days, respectively.

Immunohistochemistry and in-situ localization of apoptosis (TUNEL)

The procedures were performed as previously described [19]. Tissue sections with primary antibodies (Ki-67; 1:200, cleaved-caspase-3; 1:100, calpain; 1:100 or vimentin; 1:100) in antibody-diluent (Dako, Glostrup, Denmark) were incubated overnight at 4 °C. For secondary antibody reactions, the sections were incubated with the Alexa Fluor® 594 conjugated secondary antibody (Invitrogen, Carlsbad, CA) at RT for 2 h, and then incubated with ProLong gold antifade reagent with DAPI. In situ TUNEL staining was carried out on tissue sections using a TUNEL kit (Roche Applied Sciences, Penzberg, GER) in accordance with the manufacturer's instructions.

Statistical analysis

All data were analyzed using GraphPad Prism 5.0 statistical software (San Diego, CA). The results are presented as mean ± SEM of at least three independent replicates. Data were analyzed by student's t test, and one- or two-way ANOVA as appropriate. Significance between multiple experimental groups was determined using the Bonferroni post hoc test and defined at $p < 0.05$.

Results

DSF induces apoptosis in TNBC cell lines in a copper (Cu)-dependent manner in vitro and retards tumor growth in vivo

DSF/Cu-induced apoptosis was characterized by cell viability assays, Sub-G1 analysis and analysis of cell survival/apoptosis-related factors expression in MDA-MB-231 and Hs578T cells. Exposure to DSF/Cu (1 μM, at 1:1 M ratio) for 24 h significantly suppressed cell viability ($p < 0.001$), which did not occur with DSF or Cu treatment alone. DSF/Cu co-treatment (0.5–10 μM, 24 h) significantly suppressed cell viability in a dose-dependent manner ($p < 0.001$, Fig. 1A). A significant Sub-G1 accumulation was observed in response to DSF/Cu ($p < 0.05$, Fig. 1B), with apoptosis observed to be dependent on caspase-3 activation, as well as cleavage of PARP and XIAP ($p < 0.001$, Fig. 1C).

We next examined the influence of DSF on tumor growth and apoptosis *in vivo*. 3×10^6 MDA-MB-231 cells were inoculated in the right flank of BALB/c nude female mice. Over the course of 46 days, we observed that DSF administration resulted in a significant inhibition of tumor growth compared to control groups ($p < 0.05$, Fig. 1D). Bioluminescence imaging (BLI) analysis revealed a distinct reduction of the luminescence signal intensity in DSF-treated mice (Fig. 1E), accompanied by decreased ki-67 expression ($p < 0.001$, Fig. 1G) and increased apoptosis as determined by H&E staining (Fig. 1F), fluorescence IHC of cleaved-caspase-3 ($p < 0.01$, Fig. 1H) and TUNEL assay ($p < 0.05$, Fig. 1I), respectively.

DSF/Cu impairs cell migration and interferes with focal adhesions

Cell migration is an essential step occurring prior to tumor invasion and metastasis [20,21]. To explore whether DSF/Cu regulates