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The radiotherapy-sensitization effect of cantharidin: Mechanisms involving cell cycle regulation, enhanced DNA damage, and inhibited DNA damage repair

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ABSTRACT

Background: Cantharidin is an inhibitor of protein phosphatase 2 A (PP2A), and has been frequently used in clinical practice. In our previous study, we proved that cantharidin could arrest cell cycle in G2/M phase. Since cells at G2/M phase are sensitive to radiotherapy, in the present study, we investigated the radiotherapy-sesitization effect of cantharidin and the potential mechanisms involved.

Methods: Cell growth was determined by MTT assay. Cell cycle was evaluated by flow cytometry. DNA damage was visualized by phospho-Histone H2A.X staining. Expression of mRNA was tested by micro-array assay and real-time PCR. Clinical information and RNA-Seq expression data were derived from The Cancer Genome Atlas (TCGA) pancreatic cancer cohort. Survival analysis was obtained by Kaplan-Meier estimates.

Results: Cantharidin strengthened the growth inhibition effect of irradiation. Cantharidin drove pancreatic cancer cells out of quiescent G0/G1 phase and arrested cell cycle in G2/M phase. As a result, cantharidin strengthened DNA damage which was induced by irradiation. Moreover, cantharidin repressed expressions of several genes participating in DNA damage repair, including UBE2T, RPA1, GTF2HH5, LIG1, POLD3, RMI2, XRCC1, PRKDC, FANC1, FAAP100, RAD50, RAD51D, RAD51B and DMC1, through JNK, ERK, PKC, p38 and/or NF-κB pathway dependent manners. Among these genes, worse overall survival for pancreatic cancer patients were associated with high mRNA expressions of POLD3, RMI2, PRKDC, FANC1, RAD50 and RAD51B, all of which could be down-regulated by cantharidin.

Conclusion: Cantharidin can sensitize pancreatic cancer cells to radiotherapy. Multiple mechanisms, including cell cycle regulation, enhanced DNA damage, and inhibited DNA damage repair, may be involved.

Abbreviations: PP2A, protein phosphatase 2A; MTT, methyl thiazolyl tetrazolium; DMSO, dimethyl sulfoxide; NER, nucleotide excision repair; BER, base excision repair; MMR, mismatch repair; DSB, double-strand break; NHEJ, nonhomologous end-joining; HR, homologous recombination; HRR, homologous recombination repair; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia/Rad3-related kinase; FA, fanconi anemia; XRCC1, X-ray cross complementing group 1 protein; PARP, poly-ADP-ribose polymerase; FANCD2, fanconi anemia complementation group D2; FANCI, fanconi anemia complementation group I.

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1. Introduction

Pancreatic cancer is one of the most lethal human cancers. The one-year survival rate, for all stages of the cancer, is only 26% from the time of diagnosis, and the five-year survival rate falls to less than 5% [1]. These poor prognoses are mainly attributable to late diagnosis, and its resistance to current therapy [2–4]. Radiotherapy has been applied to the treatment for pancreatic cancers. However, both intrinsic and acquired radioresistance are major obstacles [5], and the survival and mortality rate in pancreatic cancer has remained relatively constant [6]. Therefore, it is imperative to identify novel agents, as effective sensitizers, to overcome this radioresistance, and establish better treatment strategies for further improving therapeutic outcomes.

Cytotoxic agents and radiotherapy killed only proliferating cancer cells and, in contrast, had little effect on quiescent G0 cancer cells [7]. After cessation of chemotherapy and radiotherapy, these therapeutic-resistant quiescent cancer cells restarted cycling, leading to a relapse situation. The cell cycle phase determines a cell's relative radiosensitivity, with cells being most radiosensitive in the G2/M phase, less sensitive in the G0/G1 phase, and least sensitive during the latter part of the S phase [8].

In our previous studies, we found that treatment with cantharidin, the active constituent of Chinese blister beetle [9], inhibited growth of pancreatic and breast cancer cells [10,11], offering a potential role for cantharidin in cancer treatment. Above all, cantharidin arrested G2/M cell cycle transition and decreased the proportion in G0/G1 and S phase [12]. The capability of accumulating pancreatic cancer cells in radiosensitive G2/M phase makes cantharidin a promising candidate for radiotherapy-sensitization. Therefore, in the present study, we tried to verify whether cantharidin could sensitize pancreatic cancer cells to radiotherapy. The potential mechanisms involved were also investigated.

2. Materials and methods

2.1. Cell line and cultures

The human pancreatic cancer cell line PANC-1 and CFPAC-1 were purchased from the American Type Culture Collection (ATCC, VA, USA) and maintained in Dulbecco's minimum essential medium (DMEM; Gibco, NY, USA), supplemented with 10% fetal calf serum (FCS; Hyclone, UT, USA), 100 units/ml penicillin, and 100 mg/ml streptomycin. The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells were passaged every 2–3 days to obtain exponential growth.

2.2. Viable cell growth assay

Cells were seeded into 24-well tissue culture plates at 5×10^4 cells per well. After treatment, cells were then gently washed twice with pre-warmed PBS, fixed with 1% glutaraldehyde/PBS (15 min, 20 B) and stained with 0.1% crystal violet (w/v in ddH2O) (30 min, 20a). After removal of the crystal violet solution, the plates were washed three times by immersion in a beaker filled with tap water. Plates were left to dry at 37° and 0.6 ml of crystal violet destaining solution (50% Ethanol, 0.1 M Sodium Citrate, pH 4.2) were then added to each well. The absorbance was measured at 550 nm using a microplate ELISA reader (Bio Rad Laboratories, CA, USA).

2.3. Cell cycle analysis

Cell cycle analysis using propidium iodide (PI) was performed as previously described [10]. Prior to treatment, cells were synchronized in the cell cycle by serum starvation for 24 h. To decrease the effect of serum on cell cycle distribution, treatments were performed in medium supplemented with 2.5% FBS. After treatment with cantharidin (Enzo Life Science International, PA, USA) for 24 h, the cells were fixed in 80% ice cold ethanol, and incubated with 0.5% Triton X-100 solution containing 1 mg/ml RNase A at 37 °C for 30 min. PI (Sigma) was added to a final concentration of 50 μg/ml followed by 30 min incubation in the dark. Cellular DNA content was analyzed by a Beckman Coulter FC500 dual-laser five-color flow cytometer (Beckman Coulter) and quantified with CXP software (Beckman Coulter).

2.4. BrdU and 7AADstaining

BrdU staining was conducted using the BrdU Flow Kit (BD Bioscience, San Jose, CA, USA) following the manufacturer's instruction. In brief, 10^6 cells were incubated with BrdU in the culture medium at the final concentration of 10 μM for 1 h and cells were then fixed in 100 μlBD Cytofix/Cytoperm buffer (BD Bioscience) at RT for 20 min. After washing three times with BD Perm/Wash buffer (BD Bioscience), DNase was added into the resuspended cells at the final concentration of 300 μg/ml at 37 °C for 1 h. After washing, cells were incubated with PE conjugated anti-BrdU antibody (eBioscience, San Diego, CA, USA) at RT for 20 min 7AAD (BD Bioscience) was added before flow-cytometry analysis. Apoptosis and necrosis analysis: To determine the profile of cell death after γ-irradiation, Annexin V staining was conducted at 0, 24, 48, 72 and 96 h after 6.5 Gy γ-irradiation. Annexin V-PE antibody (BD Bioscience) was used to stain the fixed cells following the manufacturer's protocol. 7AAD was added before flow-cytometry analysis. All flow-cytometry analyses were performed on a FACSAria II (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Statistical analysis was performed using Kaluza Analysis Software.

2.5. Western blot analysis

Total protein was extracted using a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, supplemented with protease inhibitor cocktail kit (Roche, Indianapolis, IN, USA) and phosphatase inhibitor cocktail kit (Roche). The protein extract was loaded, size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad Laboratories). After blocking, the membranes were incubated with primary antibodies at 4 °C overnight. Mouse anti-Ki-67 and mouse anti-β-actin antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). The protein expression was determined using horseradish peroxidase-conjugated antibodies followed by enhanced chemiluminescence (ECL; Millipore) detection. The intensity of the bands was captured by JS-1035 image analysis scanning system (Peiqing Science & Technology, Shanghai, China). β-actin was used as the internal control.

2.6. γ-H2A.X analysis by using flow cytometry

0.5 h post 20 Gy Cs^{137}-irradiation, the cells were harvested, fixed with 4% paraformaldehyde and permeabilized using 0.1% Triton X-100. After washed with PBS for three times, cells were incubated with PE conjugated phospho-histone H2A.X (Ser139) (20E3) rabbit mAb (Cell Signaling Technology, Beverly, MA, USA) for 30 min at 4 °C. Subsequently, the cells were analyzed using a Beckman Coulter FC500 dual-laser five-color flow cytometer (Beckman Coulter) and quantified with CXP software.

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