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Norcantharidin induces apoptosis in human prostate cancer cells through both intrinsic and extrinsic pathways

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ABSTRACT

Background: Norcantharidin, a modified pure compound from blister beetles, was previously demonstrated to induce apoptosis of cancer cells. This study investigated its anti-cancer activity in prostate cancer cells and the mechanisms involved.

Methods: Two human prostate cancer cell lines, 22Rv1 and Du145, were treated with norcantharidin at concentrations ranging from 3 to 30 μg/ml. Cytotoxic effect of norcantharidin was determined by use of the 3-(4,5-dimethylthiazol-yl)-diphenyl tetrazoliumbromide (MTT) assay. The effects of apoptosis were evaluated by cell death assay, Caspase-3, -8, -9 activity and cytochrome *c* release. The apoptotic related protein expressions (Bcl-2 family and inhibitor of apoptosis proteins) were determined using western blotting.

Results: An MTT assay revealed that norcantharidin induced cytotoxicity against both prostate cancer cells in dose- and time-dependent manners. Treatment with norcantharidin at 3 μg/ml or higher significantly increased oligonucleosomal formation with concomitant appearance of PARP cleavage, implicating the induction of apoptosis. Norcantharidin intrinsically elevated cytosolic cytochrome *c* levels and activated caspase-3, -8, and -9. Extrinsically, it upregulated the expression of not only the death receptors Fas and DR5 in 22Rv1 cells, but also of RIP and TRADD adaptor proteins in Du145 cells. Mechanistically, norcantharidin increased ratios of pro-/anti-apoptotic proteins and decreased expression of IAP family member proteins, including cIAP1 and survivin, regardless of the distinct status of androgen receptor expression in both cells.

Conclusions: Norcantharidin exhibited cytotoxicity against 22Rv1 and Du145 prostate cancer cells by inducing both intrinsic and extrinsic apoptotic pathways and could thus potentially be a remedy for prostate cancer.

Introduction

Prostate cancer (PCa) is the most common malignant tumor in men in the United States. In 2014, approximately 233,000 new cases and 29,480 deaths were estimated in USA [1]. Currently, the major strategies for treating PCa encompass surgery, radiation and hormonal therapy. Hormonal therapy is frequently used in combination with radiotherapy and postoperative cancer recurrence, but it often leads to severe complications that affect life quality of patients, and eventually develops to castration-resistant prostate cancer (CRPC). Accordingly, chemotherapy becomes the sole treatment modality for CRPC. Since 2004, docetaxel has become the treatment of choice in metastatic CRPC, but it presents severe limitations including drug resistance and disease relapse [2].

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Norcantharidin, a synthetic demethylated analog of cantharidin isolated from blister bettles (*Mylabris phalerata* Pall.). It has the advantages of easy synthesis, lower intrinsic toxicity and retaining anticancer activity [3]. Norcantharidin has been shown to inhibit the growth of numerous cancer cell lines via apoptosis, autophagy and cell cycle arrest, including oral cancer [4], hepatoma [5–8], leukemia [9], colorectal adenocarcinoma [10–12], melanoma [13,14], glioblastoma [15], gallbladder carcinoma [16] and PCa [17–19]. *In vivo* studies using animals bearing tumor xenografts have demonstrated that norcantharidin is able to prolong host survival rate. It inhibits angiogenesis, reduces pulmonary metastatic capacity, inhibit tumor growth by down-regulating NF-κB p65 expression and decrease plasma VEGF level, but without renal and liver toxicity [5,16,20–22]. The molecular mechanisms underlying norcantharidin-elicited cytotoxicity involve inhibition of cell adhesion, caspases activation, inhibition of invasion and metastasis, activation of the MAPK and protein kinase C (PKC) pathways, regulation of Bcl-2 family proteins and overcoming multidrug resistance [6,10,12,13,23–26].

Our previous *in vitro* studies have demonstrated that norcantharidin can induce apoptosis in human colorectal and breast cancer cell lines [12,25]. To extend its applicability, the current study aimed to investigate whether norcantharidin can inhibit PCa cell growth. And to elucidate the mechanisms involved. The cytotoxic effect of norcantharidin was tested in two human PCa cell lines with opposite status of androgen dependency (i.e., androgen-dependent 22Rv1 and androgen-independent Du145 cells). The norcantharidin-interrupted expression of apoptotic mediators in both intrinsic and extrinsic pathways was also analyzed.

Materials and methods

Chemical reagents

Norcantharidin was purchased from Sigma (Sigma, St. Louis, MO, USA) and prepared by serial dilutions in culture medium. Primary antibodies against Bax, Bak, Bad, Bid, Bcl-2, Bcl-xL, Mcl-1, Fas, RIP, TRADD, DR5, XIAP, survivin, cIAP1, cIAP2, poly (ADP-ribose) polymerase (PARP) and goat-anti-rabbit secondary antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies against actin and was from Millipore Biotechnology (Billerica, MA, USA). The goat-anti-mouse secondary antibody was from PerkinElmer (Wellesley, MA, USA).

Cell culture

Human PCa cell lines, 22Rv1 and Du145, were purchased from the National Health Research Institute Cell Bank, Taiwan. 22Rv1 cells were grown in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. Du145 cells were grown in 90% Minimum essential medium Eagle with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. The media of both cell lines contained 50 μg/ml gentamicin and 10% fetal bovine serum. The cell cultures were maintained in a humid chamber at 37 °C under 95% humidified air/5% CO_2 atmosphere.

Evaluation of antitumor activity

Norcantharidin antitumor activity was evaluated using a microculture tetrazolium test (MTT) (Sigma). Briefly, PCa cells (about 5000 cells in 100 μl complete medium per well) were seeded into a 96-well plate (Nunc, Roskilde, Denmark). After incubation at 37 °C for 24 h, 100 μl of culture medium with or without norcantharidin was added to each well in triplicate for consecutive 24, 48, and 72 h of incubation at 37 °C. Then 50 μl MTT solution (Sigma) was added to each well. Following incubation for an additional 4 h at 37 °C, supernatants were removed and 100 μl DMSO was added to dissolve the MTT-formazan product. Absorbance was read using a microplate reader (Labsystems, Helsinki, Finland) at 550 nm. Cell growth inhibition at each norcantharidin concentration was measured and the IC_{50} values for all incubation periods were calculated and normalized by setting the untreated control group as 100%.

Cell death determination

Human PCa cells were seeded into 96-well plates and cultured for 24 h. Cells were treated with or without norcantharidin for another 24 h. After treatment, DNA fragmentation was determined by examining the cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) using a Cell Death Detection ELISA Plus kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, cell lysates from untreated and norcantharidin-treated cells were transferred to a streptavidin-coated microplate. A mixture of biotinylated anti-histone- and horseradish peroxidase (HRP)-conjugated anti-DNA antibodies were added to cell lysates and incubated for 2 h. The complex was then conjugated to form an immune complex on the plate, which then was read for optical density at 405 nm. The enrichment of mono-oligonucleosomes in cell lysates was calculated as absorbance of norcantharidin-treated cells/absorbance of untreated controls.

Caspase activity assay

PCa cells treated with or without norcantharidin were subjected to caspase activity assay according to the manufacturer's protocol (R&D systems, Minneapolis, MN, USA). Briefly, cell lysates (100 μg total protein) were added to the reaction mixtures (final volume of 50 μl) containing colorimetric substrate peptides specific for caspase-3 (DEVD-pNA), caspase-8 (IETD-pNA), or caspase-9 (LEHD-pNA). The reaction was performed at 37 °C for 2 h. Absorbance at 405 nm was determined with a microplate reader.

Cytochrome c assay

The cytochrome *c* ELISA kit (Assay Designs, Ann Arbor, MI, USA) was used to determine whether cytochrome *c* was released during norcantharidin-induced apoptosis. Briefly, after treatment with norcantharidin, cells were collected and washed with PBS, resuspended in Digitonin cell permeabilization buffer, and incubated for 5 min on ice. After centrifugation, supernatants were collected for the determination of cytochrome *c* in the cytosol. All samples were diluted and added into the cytochrome *c* 96-well plate. After incubation for 1 h at 500 rpm, the cytochrome *c* conjugate was added into each well and incubated for another 30 min at 500 rpm. After the substrate solution was added to each well, the plate was incubated for 45 min at room temperature. The cytochrome *c* level was determined after adding the stop solution by reading absorbance at 405 nm with a microplate reader.

Western blot analysis

PCa cells treated with or without norcantharidin for 24 h were lysed with RIPA protein lysis buffer and protein concentration was determined using the BCA protein assay kit (Novagen, Madison, WI, USA). Equal amounts of protein lysates were separated by 10% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a nitrocellulose membrane. After blocking