Inhibitory effects of norcantharidin against human lung cancer cell growth and migration

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Received: 20 May 2009 / Accepted: 28 December 2009 / Published online: 20 January 2010
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Abstract The effects of norcantharidin (NCTD), an anticancer drug in China, on the growth and migration in human lung cancer cells were investigated by in vitro and ex vivo assays. NCTD significantly inhibited the in vitro and ex vivo growth of human non-small cell lung cancer A549 cells in dose- and time-dependent manners. Western blot analysis indicated that NCTD dose-dependently down-regulated the expression of anti-apoptotic protein Bcl-2 and up-regulated the level of pro-apoptotic protein Bax, eventually leading the reduction of ratio of Bcl-2/Bax proteins in A549 cells. Moreover, NCTD significantly suppressed the A549 cell migration in the case of without reducing the cell viability. More importantly, NCTD significantly enhanced the anticancer activity of anticancer agents such as trichostatin A (the histone deacetylase inhibitor), celecoxib (the inhibitor of cyclooxygenase-2) and lovastatin (the

inhibitor of HMG-CoA reductase) by strongly reducing the viability and/or the ratio of Bcl-2/Bax protein in A549 cells. Our findings suggest that NCTD may have the wide therapeutic and/or adjuvant therapeutic application in the treatment of human lung cancer.

Keywords Norcantharidin · Trichostatin A · Celecoxib · Lovastatin · Human lung cancer · Growth · Bcl-2/Bax · Migration

Abbreviations

NCTD	Norcantharidin
T	Trichostatin A
S	Celecoxib
luo	Lovastatin
NSCLC	Non-small cell lung cancer

Introduction

Lung adenocarcinoma is the leading cause of cancer mortality among both men and women in the world (Parkin et al. 2005; Jemal et al. 2008). Despite recent advances in diagnosis and treatment, the mortality rates with an overall 5-year survival of only 15%. This high mortality is probably attributable to early metastasis, which is related to abnormal growth, migration, invasion and angiogenesis in the cancer cells. The non-small cell lung cancer (NSCLC), which constitutes ~80% of lung cancer cases, remains an

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aggressive lung cancer associated with a poor patient survival. The patients with advanced disease have a median survival of approximately 10 months when treated with standard platinum-based therapy. Hence, there is an imminent need for better therapies and/or anticancer agents for NSCLC. To date, chemotherapy has been the most frequently used treatment for lung cancer and other cancers. However, some normal cells are destroyed as well by this method of treatment. Due to their wide range of biological activities and low toxicity in animal models, some natural products have been used as alternative treatments for cancers including lung cancer. Norcantharidin (NCTD) is the demethylated analog of cantharidin isolated from natural blister beetles. In China, NCTD as an anticancer drug is currently used to treat breast cancer, hepatoma, leukemia, colon cancer, etc. NCTD induced apoptosis in hepatoma cells, which is involved in a caspase activation pathway (Chen et al. 2002). NCTD suppressed the invasion and metastasis in CT26 colorectal adenocarcinoma cells (Chen et al. 2005). It also induced apoptosis in human leukemic Jurkat cells without affecting the viability of normal mononuclear cells (MNC) (Liao et al. 2007). However, whether or not NCTD has inhibitory activities against NSCLC is unclear. In this study, we investigated the *in vitro* and *ex vivo* effects of NCTD on the survival of human NSCLC A549 cell line and the migration of the lung cancer cell as well as its mechanisms of action. We show that NCTD significantly inhibited the *in vitro* and *ex vivo* growth of A549 cells and regulated the expressions of Bcl-2 and Bax proteins in the cancer cells in a dose-dependent manner. NCTD also significantly suppressed A549 cell migration and enhanced the anticancer activity of anticancer agents by strongly reducing the viability and ratios of Bcl-2/Bax proteins in A549 cells.

Materials and methods

Materials

Fibronectin and Boyden chambers were purchased from BD Inc. (Franklin Lakes, NJ) and Costar Corning (Corning Inc., Corning, NY), respectively. Norcantharidin (NCTD/N), trichostatin A (TSA/T) celecoxib (S), Bay 11-7082 (Bay), lovastatin (luo), RPMI 1640, penicillin, streptomycin, propidium

iodide (PI), trypsin/EDTA, fetal bovine serum (FBS), 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), and all other chemicals employed in this study were purchased from Sigma Chemical Co. (St. Louis, MO).

Animal experimentation and preparation of sera from NCTD-fed rabbits

These were done according to our published methods with slight modifications (Zhang et al. 2001). In brief, New Zealand White rabbits (3.5–4 kg; from Luye Pharmaceutical Company, Yantai, China) were treated in accordance with guidelines established by the Animal Care and Use Committee at Yantai University. NCTD was orally intubated to rabbits once daily at a dose of 10 mg/mL/kg body weight for 3 days. On the third day, the blood was then collected at 0, 15, 30, and 60 min from the rabbits (fasted for 16 h) after oral intubation of NCTD. The collected blood was left to clot for 2 h at room temperature and centrifuged twice at 3000×g at 4 °C for 20 min. The sera were sterilized by filtration and then heated at 56 °C for 30 min. The prepared sera were aliquoted, and stored at –80 °C until *ex vivo* cell growth assay.

Cell culture and *in vitro* and *ex vivo* cell growth assays

The A549 lung adenocarcinoma cell line was obtained from the American Type Culture Collection. The human cancer cell line was cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 µg/mL) at 37 °C in a humidified incubator with 95% air/5% CO₂ atmosphere. The *in vitro* and *ex vivo* assays were done according to our published methods (Zhang et al. 1999, 2001). The cells in control group were treated with DMSO (0.1%, final concentration). The cells were incubated in RPMI 1640 medium supplemented with 10% FBS (in the case of *in vitro* assay) containing different concentrations of NCTD or in the absence or presence of an existing anticancer agent (TSA, celecoxib, Bay, lovastatin), or 10% rabbits sera (in the case of *ex vivo* assay) obtained at different time points after NCTD was orally intubated to the rabbits. Cell viability was measured 24, 48, and 72 h after the treatments using MTT assay kit. Each experiment was repeated three times.

Morphological evaluation of apoptotic cells

This was done according to our published methods (Zhang et al. 2000). In brief, A549 cells at 70% confluence were respectively treated for 48 h with NCTD at concentrations of 0 (0.1% DMSO, vehicle as control), 1, and 2.5 $\mu\text{g/mL}$. The treated cells were fixed with 1% glutaraldehyde in PBS for 30 min at room temperature, washed in PBS, and stained with 1 mM Hoechst 33258 for 30 min at room temperature. The morphological changes in the nuclear chromatin were observed under a fluorescent microscope (Nikon, TE2000-U, Japan), using 40 $\times$ lens.

Western blot analysis

This was performed according to the method previously published by Chen et al. (2001), Zhang et al. (2009), and by Yu et al. (2009). In brief, A549 cells were treated with NCTD at different concentrations (0–10 $\mu\text{g/mL}$) in the absence or presence of trichostatin A (TSA, 20 $\mu\text{g/L}$) and celecoxib (S, 10 μM). The cells in control group were treated with DMSO (0.1%, final concentration). The treated cells were collected at 48 h. Equal amounts of cell extracts were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and probed with primary antibodies to human Bcl-2, Bax, and β -Actin and then with horseradish-conjugated secondary antibodies, respectively. Anti- β -Actin antibody was used as a loading control. Detection was done using an enhanced chemiluminescence system (GE Healthcare Life Sciences).

In vitro migration assay

This was performed according to the method previously published by Zhang et al. (2009) and by Liu et al. (2009). In brief, Cancer cell migration was measured by examining cell migration through fibronectin-coated polycarbonate filters, respectively using modified transwell chambers. In brief, A549 cells (5×10^4) were seeded onto the upper chamber in 200 μL of serum-free medium containing NCTD at the concentrations of 0–0.8 $\mu\text{g/mL}$, respectively. The cells in control group were treated with DMSO (0.1%, final concentration); the lower compartment was filled with 0.66 mL of RPMI 1640 medium supplemented with 10% of FBS (as a chemoattractant). After incubation for 6 h at 37 $^{\circ}\text{C}$, the cells that migrated

to the lower surface of the filter were fixed and stained using propidium iodide. The cells on the upper side of the filter were removed using a cotton swab. The migrated cells on the underside of the filter were counted and recorded for images under a fluorescent microscope (Nikon, TE2000-U, Tokyo, Japan). Each experiment was repeated three times.

Statistical analysis

The data were expressed as mean $\pm$ standard deviation (SD) and analyzed by the SPSS 13.0 software to evaluate the statistical difference. One-way or two-way ANOVA followed by the appropriate post hoc test (Bonferroni) was used to establish whether significant differences existed between groups. For confirming the synergistic effect between NCTD and TSA, S or luog, comparison was made by two-way ANOVA followed by Bonferroni post hoc test. Differences were considered significant at $P < 0.05$. Mean concentrations and cell viability or migration (%) are shown for each group; Asterisk $P < 0.05$, double asterisk $P < 0.01$. For all tests, P values less than 0.05 were considered statistically significant. All statistical tests were two-sided.

Results and discussion

In vitro and ex vivo effects of NCTD on the growth of A549 cells and the anticancer activity of anticancer agents against A549 growth

The in vitro cell growth assay showed that NCTD reduced the viability of human lung cancer cell line A549 in dose- and time-dependent manners after the cells were treated with NCTD at 1–10 $\mu\text{g/mL}$ for 24, 48 and 72 h, respectively (Fig. 1a). The ex vivo assay indicated that the rabbit sera obtained 15, 30 and 60 min after oral administration of NCTD significantly reduced the viability of A549 cells after the cells were treated with these sera for 24, 48 and 72 h, respectively (Fig. 1b). This result confirms that NCTD has a certain bioavailability by oral administration and the peak inhibition of the cancer cell growth is at 30–60 min after oral administration of NCTD in rabbits. There have been reports showing that the blood concentration of NCTD can be detected after intragastric administration of NCTD in mice (Wei et al.

Fig. 1 In vitro (a, c) and ex vivo (b) effects of norcantharidin (NCTD) and sera from NCTD-fed rabbits on the growth of human lung cancer A549 cells and the anticancer activity of anticancer agents against A549 cell growth. The cells were treated for 24, 48, and 72 h with NCTD at the concentrations indicated (a) or the sera taken from rabbits ($n = 6$ for each group at different time points) at 0 h (as the control group), 15, 30 and 60 min after oral administration of NCTD in rabbits (b), respectively. c In vitro effects of NCTD on the anticancer activity of anticancer agents against A549 cell growth. The cells were treated for 48 h with NCTD at 1–10 $\mu\text{g}/\text{mL}$ in the absence or presence of the anticancer agents trichostatin A at 10 and 20 $\mu\text{g}/\text{L}$ (TSA10 and TSA20, the histone deacetylase inhibitor), celecoxib at 10 and 20 μM (S10 and S20, the

inhibitor of cyclooxygenase-2), lovastatin at 1 and 2 μM (Iuo1 and Iuo2, the inhibitor of HMG-CoA reductase), and Bay at 2.5 μM (BAY2.5, the inhibitor of NF- κB). The in vitro and ex vivo effects on the A549 cell growth were examined by the MTT assay as described in the section of “Materials and methods”. The data are presented as the mean $\pm$ SD (Bar) for each group ($n = 6$). The figures (a–c) are the representative of 3 similar experiments performed. Statistical analysis was carried out using the ANOVA and Bonferroni test. * $P < 0.05$, ** $P < 0.01$. Values with different letters (a–f) differ significantly ($P < 0.05$). f/s, d/s, e/s and c/s represent the significant synergistic effects ($P < 0.001$, two-way ANOVA) compared with the treatment with its individual compound alone

2007); NCTD also appeared in human serum after oral administration of NCTD in human volunteers (Wei et al. 2008a). In the present study, we have also demonstrated that NCTD at the concentrations of 1 and 2.5 $\mu\text{g}/\text{mL}$ enhanced the anticancer activity of the anticancer agents trichostatin A at 20 $\mu\text{g}/\text{L}$ (a histone deacetylase inhibitor), celecoxib at 10 μM (an inhibitor of cyclooxygenase-2), and lovastatin at 1 μM (an inhibitor of HMG-CoA reductase) by strongly reducing the viability of A549 cells after the cells were treated for 48 h with the agents (Fig. 1c). These results partially explain why NCTD has its therapeutic and/or adjuvant therapeutic effects on treatment for some cancer patients.

NCTD induced apoptosis by reducing the Bcl-2/Bax protein ratio in A549 cells

To understand the mechanisms of action of NCTD on the growth in human lung cancer cells, we

investigated the effects of NCTD on apoptosis and the expressions of Bcl-2/Bax proteins in A549 cells. Hoechst 33258 staining showed that the typical morphological changes, such as formation of apoptotic bodies appeared in A549 cells after the cells were treated for 48 h with NCTD at 1 and 2.5 $\mu\text{g}/\text{mL}$, whereas the control cells without NCTD treatment did not show the evident apoptotic morphological changes (Fig. 2a). Furthermore, Western blot analysis confirmed that NCTD dose-dependently down-regulated the expression of anti-apoptotic protein Bcl-2 and up-regulated the level of pro-apoptotic protein Bax, eventually leading to a reduction of Bcl-2/Bax protein ratio in A549 cells (Fig. 2b). In addition, NCTD at 1 $\mu\text{g}/\text{mL}$ enhanced the anticancer activity of anticancer agents celecoxib at 10 μM and TSA at 20 $\mu\text{g}/\text{L}$ by reducing Bcl-2 level and increasing Bax expression, causing a reduction of the Bcl-2/Bax protein ratio in A549 cells (Fig. 2b). There have been studies showing that Bcl-2 and its dominant inhibitor

Fig. 2 In vitro effects of NCTD on induction of apoptosis (a) and reduction of ratios of Bcl-2/Bax proteins (b) in A549 cells by NCTD with its synergistic anticancer agents. **a** Induction of apoptosis in A549 cells by treatment for 48 h with NCTD at concentrations of 0 (0.1% DMSO vehicle as the control), 1 and 2.5 µg/mL. The treated cells were stained with Hoechst 33258 and the apoptotic morphological changes in the nuclear chromatin were observed under a fluorescent microscope as described in the “Materials and methods” section. **b** Reduction of Bcl-2/Bax protein ratios in A549 cells by treatment for 48 h with NCTD at the concentrations of 1 to 10 µg/mL (N1, N2.5, N5, and N10) in the absence or presence of celecoxib at 10 µM (S10, an inhibitor of cyclooxygenase-2), and trichostatin A at

20 µg/L (TSA20). The protein expressions of Bcl-2 and Bax were analyzed by Western blotting as described in the “Materials and methods” section. Anti-β-Actin antibody was used as a sample loading control. The ratio of Bcl-2 and Bax, (the ratio of relative density of each band normalized to β-actin), shown as mean ± SD (Bar) is relative to that of 0 (0.1% DMSO vehicle) as the control (100%). For one experiment, 3 assays were carried out and only one set of gels is shown. Values with *different letters* (a–f) differ significantly ($P < 0.05$); d/s and f/s represent the significant synergistic effect ($P < 0.001$, two-way ANOVA) compared with the treatment with its individual compound alone

Bax are key regulators of cell proliferation and apoptosis. Overexpression of Bcl-2 enhances cell survival by suppressing apoptosis, but overexpression of Bax accelerates cell death (Oltvai et al. 1993). Induction of apoptosis and decrease in the Bcl-2/Bax protein ratio by NCTD may be one of the important mechanisms of action of NCTD against the lung cancer cell growth.

In vitro effects of NCTD on the A549 cell migration

Abnormal growth and metastasis of cancer cells are regarded as the important biological characteristics of cancers. The presence of metastasis is the main cause of morbidity and mortality in millions of patients with cancer. During the complicated process of

Fig. 3 In vitro effects of NCTD on the migration of human lung cancer A549 cells. The A549 cell migration was examined by the migration assay as described in the section of “[Materials and methods](#)”. **a** The photos show the propidium iodide-stained A549 cells that migrated through the fibronectin-coated transwell chamber. The cells were treated for 6 h with either the vehicle (0.1% DMSO as the control) or NCTD at 0.2 µg/mL. **b** Suppression of A549 cell migration by NCTD. The cells

were treated for 6 h with NCTD at the concentrations indicated in the figure. Relative migration (%) $\pm$ SD ($n = 6$) is shown for the indicated NCTD concentrations and the cells in control group were treated with DMSO (0.1%, final concentration). The figure is the representative of 3 similar experiments performed. Statistical analysis was carried out using the ANOVA and Bonferroni test. * $P < 0.05$

metastasis, the invasion of cancer cells is the most important and characteristic step. The migration of cancer cells is one of the important steps during the invasion. Therefore, we examined the effects of NCTD on the migration in A549 cells. The migration assay indicated that NCTD at the concentrations of 0.2–0.8 µg/mL significantly reduced the A549 cell migration rate more than 65–52% after the cells were treated for 6 h with NCTD (Fig. 3). NCTD at the concentrations of 0.2–0.8 µg/mL did not reduce the viability of A549 cells after the cells were treated with NCTD for 24 h (data not shown). This suggests that the inhibition of A549 cell migration by NCTD is not the result from the reduction of A549 viability. There have been studies showing that activation of PI3 K/Akt and NF- κ B increases the migration of cancer cell lines such as A549 (Huang et al. 2009a) and MDA-MB-231 (Wei et al. 2008b) cells, which can be suppressed by Ly294002, the inhibitor of

PI3 K/Akt. Our previous results confirmed that NCTD at a low concentration without inhibiting the cell growth showed significant suppression of the phosphorylation of Akt and NF- κ B expression in MDA-MB-231 cells (Huang et al. 2009b). Our recent results have also confirmed that berbamine, an anticancer agent at a low concentration without inhibiting the cell growth showed significant suppression of the phosphorylation of Akt and NF- κ B expression as well as the cell migration in MDA-MB-231 cells (Wang et al. 2009). In the present study, suppression of A549 cell migration in the low concentrations without restraining the cell growth may be involved in inhibiting the phosphorylation of Akt and NF- κ B expression in the cancer cells. However, the mechanisms of action of NCTD against the A549 cell migration require further investigation.

NCTD has been used as an anticancer drug for many years in China. NCTD was reported to induce

apoptosis in human colorectal adenocarcinoma, hepatoma, and leukemia cells (Chen et al. 2005, 2002; Liao et al. 2007). NCTD also inhibited invasion and metastasis of human colon cancer cells (Chen et al. 2005). Our present results have confirmed that NCTD significantly reduced the growth of human lung cancer A549 cells in vitro and ex vivo. Furthermore, we have also demonstrated that the induction of apoptosis by reducing the Bcl-2/Bax protein ratio in A549 cells is one of the important mechanisms of action of NCTD against cancer cell growth. In addition, our result indicates that NCTD reduced the A549 cell migration rate by more than 65% at the low concentration without affecting the cell viability. More importantly, we show that NCTD enhanced the anticancer activity of the anticancer agents TSA, celecoxib, and lovastatin by reducing the viability and/or the Bcl-2/Bax protein ratio in A549 cells. All these findings suggest that NCTD may have a wide therapeutic and/or adjuvant therapeutic application in the treatment of human lung cancer.

Acknowledgments This work is supported in part by grants from the Ministry of Education of the People's Republic of China to G.Z, from the Ministry of Human Resources and Social Security of the People's Republic of China to G.Z, Projects of Yantai University to GZ, Project from the National Natural Science Foundation of China to GZ (No. 30973553), and grants from the Department of Science and Technology of Shandong Province to GZ (Y2008C71; 2009GG10002087).

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