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Roles of p38 and JNK mitogen-activated protein kinase pathways during cantharidin-induced apoptosis in U937 cells

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Abstract

Cantharidin is an active compound from blister beetles traditionally used for the treatment of cancer. It is known to exert its antitumor activity by inducing apoptosis in cancer cells. However, its signaling pathway still remains unclear. Therefore, we investigated the roles of the mitogen-activated protein kinases (MAPKs) and the tumor suppressor gene, p53, during cantharidin-induced apoptosis in U937 human leukemic cells. Cantharidin effectively activated ERK-1/2, p38 and JNK in U937 cells in a time- and dose-dependent manner. Cantharidin also exhibited a strong cytotoxicity and induced apoptosis in U937 cells. For the evaluation of the role of MAPKs, PD98059, SB202190 and SP600125 were used as MAPK inhibitors for ERK-1/2, p38 and JNK. PD98059 did not affect cantharidin-induced cytotoxicity and apoptosis, whereas SB202190 and SP600125 significantly interfered with cytotoxic and apoptotic activities induced by cantharidin. Cantharidin alone induced the apoptosis by phosphorylation of p53, up-regulation of downstream target genes, MDM2 and p21 and also cleaved caspase-3, whereas SB202190 and SP600125 caused the down-regulation of p53, MDM-2, p21 and cleaved caspase-3 after a co-treatment with cantharidin. Similarly, SB202190 and SP600125 significantly disturbed the caspase-3 activity after a co-treatment with cantharidin by colorimetric assay. Taken together, these results suggest that cantharidin can induce apoptosis by activation of p38 and JNK MAP kinase pathways associated with p53 and caspase-3.
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Keywords: Cantharidin; MAP kinase inhibitors; Apoptosis; p53; Caspase-3

1. Introduction

Cantharidin, an active ingredient of the blister beetle (*Mylablis phalerata* Pall. and *Mylablis cichorii* Linn.), was known as an inhibitor of PP2A [1] that plays a role in control of cell cycle [2,3], growth [4], and cell fate determination [5]. Cantharidin halted the mesenchymal cell cycle progression and reduced the branching morphogenesis in a fetal rat lung explant culture [3]. Moreover, cantharidin was reported to induce apoptosis via cytochrome c release, increase of caspase-3 activity and DNA fragmentation in the pancreatic beta cell line, HIT cells [6].

A group of cysteine proteases denoted ‘‘caspases’’ play key roles in apoptosis. Caspases are synthesized as proenzymes, which are activated by cleaving the prodomain at a specific aspartic acid cleaving site. One of the upstream signaling pathways leading to caspase activation involves the release of cytochrome *c* and the other apoptogenic factors from the injured mitochondria [7,8]. Cytochrome *c*

Abbreviations: PP2A, protein phosphatase type 2A; JNK, c-*jun* N-terminal kinase; ERK, extracellular signal regulated kinase; MAPK, mitogen-activated protein kinase; z-VAD-FMK, z-Asp-Glu-Val-Asp-fluromethylketone; XTT, [2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2*H*-tetrazolium hydroxide]; DMSO, dimethyl sulphoxide; PI, propidium iodide

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release and caspase activation are mediated by the translocation of cytosolic Bax to the mitochondria in response to the various apoptotic stimuli [9,10]. Several recent studies suggest that the mitochondrial integrity is regulated by a tumor suppressor protein, p53 [11,12]. The p53 protein plays an important role in the cellular response to various cellular stresses [13]. After DNA damage, p53 is phosphorylated, especially at serine 15, and subsequently the binding MDM2 to p53 results in increasing p53 accumulation [14]. The up-regulation of p53 also can induce cell growth arrest (p21/WAF1) and apoptosis [14,15]. p53 phosphorylation is mediated by protein kinases, including the mitogen-activated protein (MAP) kinase, i.e. the ERK-1/2, p38 kinase and c-Jun N-terminal kinase [16,17]. MAP kinases participate in the transmission of signals to the cell nucleus. MAPKs are activated by dual phosphorylation of thr and tyr within the motif Thr-Glu-Tyr (ERK) [18,19] or Thr-Gly-Tyr (p38) [20] or Thr-Pro-Tyr (JNK) [21] in subdomain VIII of the protein kinases. This phosphorylation is mediated by a protein kinase cascade that consists of a MAPK kinase kinase (MKKK) that phosphorylates and activates one or more MAPK kinases (MKK) [18–21]. In general, the ERK cascade is activated by growth factors and is critical for proliferation and survival [22]. On the other hand, the p38 and JNK pathways are stimulated by genotoxic agents and apoptosis [23–28]. However, controversial evidence has indicated that more complex roles of these pathways exist to transmit other ultimately distinct cellular effect in different cell lineages. For example, the persistent activation of ERK mediates growth arrest or differentiation signals in muscle cells and leukemic cells [29–31]. In contrast, transient p38 and JNK induction could provide a survival signal, whereas persistent activation induces apoptosis [32,33].

Although it appears that cantharidin can induce cytotoxicity and apoptotic cell death, the underlying mechanism of this process has not been well characterized. Therefore, we tried to investigate the role of MAP kinase pathways associated with p53 and caspase-3 in cantharidin-induced apoptosis in U937 human leukemic cells.

2. Materials and methods

2.1. Materials

Antibodies to human phospho p53 (Serine 15), cleaved caspase-3, anti-phospho ERK-1/2, p38, JNK, anti-ERK, p38, and JNK were purchased from Cell Signaling Technologies. The MDM2 and p21/WAF1 were purchased from Oncogene. The caspase-3 substrate, Ac-Asp-Glu-Val-Asp-chromophore *p*-nitroaniline (Ac-DEVD-pNA) was obtained from R&D Systems. Caspase-3 inhibitor, z-VAD-FMK was purchased from ICN Pharmaceuticals. The MEK inhibitor PD98059, JNK inhibitor SP600125 and p38 inhibitor SB202190 were bought from Calbiochem. Cantharidin was purchased from Sigma Chemical.

2.2. Cell culture

U937 human myelocytic leukemic cells (ATCC CRL) were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL of penicillin–streptomycin (Gibco BRL) in a humidified atmosphere of 5% CO_2, 95% air at 37°. Exponential growing U937 cells were diluted at 3×10^6 cells in T 75 cm^2 culture flasks (Nunc) and subsequently used for the experiments.

2.3. Treatment

Cantharidin was dissolved in DMSO. The stock solution of 20 mM cantharidin was diluted with media and sterilized by filtration. Cells were incubated with 20 μM cantharidin for 0.5–48 hr in serum-free medium. In the dose–response study, cells were treated with cantharidin (5, 10, 20 and 40 μM) in serum-free medium for 24 hr at 37°. Untreated control cells were incubated with DMSO (0.025, 0.05, 0.1, 0.2%) in serum-free medium. In experiments to determine the effects of PD98059, SB202190, SP600125 on MAPKs, cytotoxicity and apoptosis, cells were pretreated with these kinase inhibitors for 1 hr and then co-exposed to cantharidin for 24 hr.

2.4. Cytotoxicity assay

Cytotoxicity was assessed by XTT colorimetric assay. The assay is based on the cleavage of the yellow tetrazolium salt XTT to form an orange formazan dye by proliferative and viable cells. Briefly, 100 μL of 3×10^4 cells per well were seeded in 96-well microplates with 20 μM cantharidin in the presence or absence of PD98059 (10, 20, and 40 μM), SB202190 (10, 20 and 30 μM) and SP600125 (10, 20 and 30 μM) in a volume of 100 μL. After a 6, 12, 24 and 48 hr incubation at 37°, 20 μL of XTT (1 mg/mL) was added to each well, and incubated for 3 hr at 37° again. The plates were measured using an ELISA reader at 450–650 nm wavelength. Results were calculated as a percentage of cantharidin-treated cells relative to untreated control.

2.5. Measurement of apoptotic cells by flow cytometry

For flow cytometric analysis of sub G1 cell counting with fragmented DNA, 5×10^5 cells per well onto 6-well plates were collected at different concentrations and for various times. The cells were harvested and incubated with 1 mL of 75% cold ethanol for 2 hr at −20° and then washed with PBS. Cell pellets were incubated with 10 μg/mL RNase at 37° for 30 min before adding 50 μg/mL PI. Samples were analyzed on a FACSort flow cytometer using

the Cell quest analysis program (Becton Dickinson), which is also used to determine the percentage of sub G1 cells. PI was excited at 488 nm, and fluorescence was analyzed at 620 nm wavelength.

2.6. Western blotting

Cells were prepared by extracting proteins using a lyses 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 inhibitors (10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL pepstatin A, and 1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride) and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). After centrifugation, the pellet was resuspended in the loading buffer containing 50 mM Tris–HCl (pH 6.8), 4% glycerol, 2 mM EDTA, 3% SDS, and 0.01% bromphenol blue. The proteins were size-fractionated by SDS–polyacrylamide gel electrophoresis and transferred to Hybond-C nitrocellulose membranes for 2 hr at 300 mA using a transfer system. Membranes were blocked with 5% nonfat dry milk in TBS-T (0.05% Tween-20, 138 mM NaCl, and 25 mM Tris base). Protein expression was determined using antibodies purchased from the following sources: mouse anti-phospho p53 (Ser 15), MDM2, p21/WAF1, rabbit anti-cleaved caspase-3, anti-phospho ERK-1/2, p38, JNK, anti-ERK, p38, and JNK. The blots were incubated with a horseradish peroxidase-conjugated goat anti-mouse IgG and enhanced with chemiluminescence (ECL) detection system (Amersham Life Science).

2.7. Caspase-3 activity assay

Caspase-3 activity was determined by measuring the absorbance at 405 nm after cleavage of synthetic substrate Ac-Asp-Glu-Val-Asp-chromophore *p*-nitroaniline (Ac-DEVD-pNA). Briefly, cells were collected by pipetting in cold phosphate-buffered saline and brief centrifugation. The pellet was lysed on ice for 30 min in the cell lysis buffer with the R&D system colorimetric assay kit. The lysates (50 μg) were reacted with 50 μM Ac-DEVD-pNA in a reaction buffer (1% NP-40, 20 mM Tris–HCl, 137 mM NaCl, 10% glycerol, 10 mM dithiothreitol, and protease inhibitors, pH 7.4). The mixtures were maintained at 37° for 2 hr and subsequently analyzed in an enzyme-linked immunosorbent assay reader (Molecular device). The enzyme activity was calculated on the basis of a standard curve prepared using *p*-nitroanaline. The relative levels of pNA were normalized against the protein concentration of each extract.

2.8. Statistical analysis

The results were expressed as means ± SEM calculated from the specified numbers of determination. A Student's *t*-test was used to compare individual data with control value. A probability of $P < 0.05$ was taken as denoting a significant difference from control data.

3. Results

3.1. Effect of cantharidin on the activation of MAPKs

The phosphorylated status of the MAPKs were evaluated after incubation with cantharidin in U937 cells. In the time-course study, the level of the phosphorylated form of ERK MAPK increased 3–4-fold within 1 hr after being exposed cantharidin and its activity was sustained for 48 hr. The p38 MAPK level increased 23-fold 6 hr after being exposed cantharidin and retained its activity for 48 hr after the cantharidin treatment. The JNK level increased 9.5-fold within 3 hr after cantharidin exposure and sustained its activity up to 12 hr after the cantharidin treatment (Fig. 1A and B).

3.2. Effect of cantharidin on cytotoxicity in U937 cells

The cytotoxicity of cantharidin in U937 cells was examined. The relative survival rate of the cells treated with cantharidin was determined by XTT assay. The proliferation of U937 cells was significantly suppressed in a time- and dose-dependent manner following exposure to 20 μM cantharidin (Fig. 2A and B). Cantharidin inhibited the growth of U937 cells to 50% of control following 24 hr treatment, while 0.1% DMSO did not affect cell viability (Fig. 2A).

3.3. Effect of cantharidin on the apoptotic sub G1 cells

The next stage was to examine whether or not apoptosis was induced by cantharidin using flow cytometric analysis with propidium iodide staining. Cell death initiated from 6 hr exposure to cantharidin and significantly increased the proportion of apoptotic cells in a time-dependent manner (Fig. 3). Especifically, 38% of apoptotic cells were observed following exposure to 20 μM cantharidin for 48 hr (Fig. 3).

3.4. Effect of cantharidin and MAP kinase inhibitors on the cytotoxicity and apoptosis

The U937 cells were exposed to cantharidin in the presence of the MAP kinase inhibitors such as PD98059, SB202190 and SP600125 in order to identify the roles of ERK, p38 and JNK signaling pathways during cantharidin-induced apoptosis. The MAPK inhibitors alone did not exert any cytotoxicity on U937 cells, while cantharidin showed some cytotoxicity with an IC_{50} of 20 μM. SB202190 and SP600125 significantly enhanced the viability of U937 cells treated with 20 μM cantharidin in a dose-dependent manner, whereas PD98059 did not

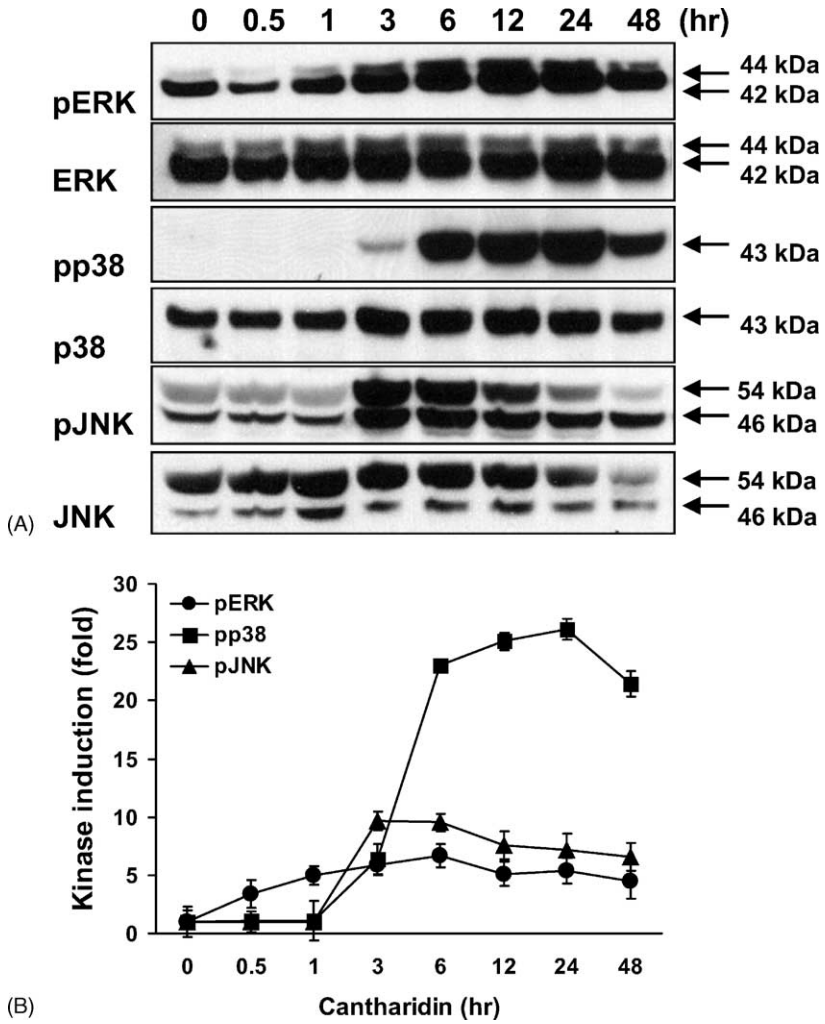

Fig. 1. Activation of ERK, p38 and JNK kinases in cantharidin-treated U937 cells. (A) Time course of MAP kinase activation. Cells were treated with 20 μM cantharidin for the indicated time periods. Expressions of ERK-1/2, p38 and JNK were determined by Western blot analysis. (B) Dose–response of MAP kinase activation. Kinase activity was measured by Western blot analysis of phospho ERK, p38 and JNK. The amount of phosphorylated kinase was quantified by densitometry of the phosphokinase band. The results represent the average of three distinct experiments. Bars represent SEMs.

affect the cytotoxicity of cantharidin (Fig. 4). In addition, flow cytometric analysis showed that, cantharidin significantly increased the apoptotic cells in a time-dependent manner, ~37% of cells undergoing apoptosis 48 hr exposure to 20 μM cantharidin. Co-administrating SB202190 with cantharidin and SP600125 with cantharidin markedly reduced the frequency of apoptotic cells. On the other hand, PD98059 did not affect the apoptotic cells (Fig. 5).

3.5. Effect of cantharidin and MAP kinase inhibitors on p53 phosphorylation and caspase-3 activation

In an attempt to understand the roles of MAP kinases with p53 and caspase-3 during cantharidin-induced apoptosis, cantharidin was added to U937 cells with the MAP kinase inhibitors including PD98059, SB202190 and SP600125. Cantharidin initiated the phosphorylation of p53, and increased the expression of MDM2, p21 and cleaved caspase-3 from 24 hr after treatment (Fig. 6A). PD98059 did not affect the apoptotic activity of cantharidin (Fig. 6B), whereas SB202190 and SP600125 disturbed the p53 phosphorylation, MDM2, p21 and caspase-3 cleavage during the apoptosis induced by cantharidin (Fig. 6C and D). A showed colorimetric assay that cantharidin significantly increased the caspase-3 activity (Fig. 7). In contrast, the inhibition of ERK-1/2 with PD98059 did not affect the caspase-3 activity induced by cantharidin, whereas the inhibition of p38/JNK with SB202190 and SP600125 significantly blocked the caspase-3 activation caused by cantharidin. However, z-Asp-Glu-Val-Asp-FMK, a caspase-3 inhibitor, completely blocked the caspase-3 activity induced by cantharidin (Fig. 7).

4. Discussion

Cantharidin, a 7-oxabicyclo[2.2.1]heptane-2,3-dicarboxylic acid, is a natural toxin extracted from blister

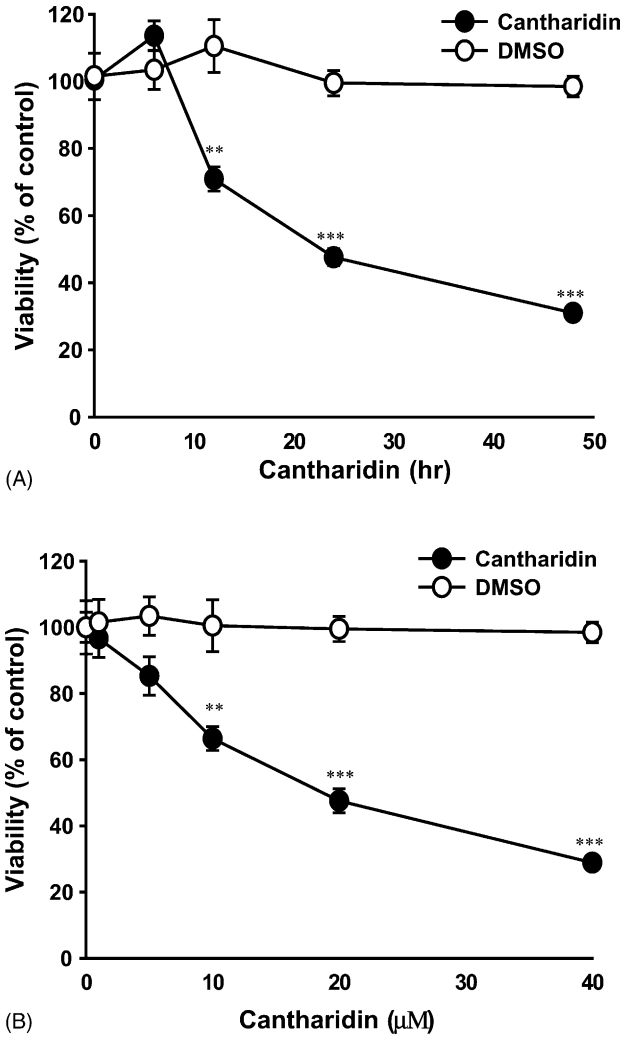

Fig. 2. Time- and dose-dependent effect of cantharidin on the viability of U937 cells. Cell viability was determined with the XTT assay. In brief, cells were treated with 20 μM cantharidin for various times (A) or with the indicated concentrations (B) of cantharidin for 24 hr. Data were obtained from three independent experiments and bars represent SEMs.

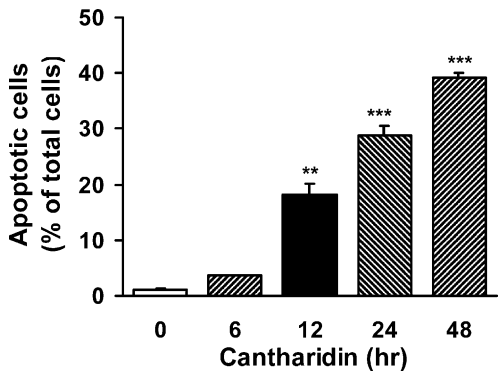

Fig. 3. Cantharidin caused the apoptosis of U937 cells. Apoptotic cells were quantified by FACSorter flow cytometer with propidium iodide (PI) staining. Cells were treated with 20 μM cantharidin for the specified time periods. Data were obtained from three independent experiments and bars represent SEMs. *P* value represents the significant difference between non-treated group and cantharidin-treated group ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

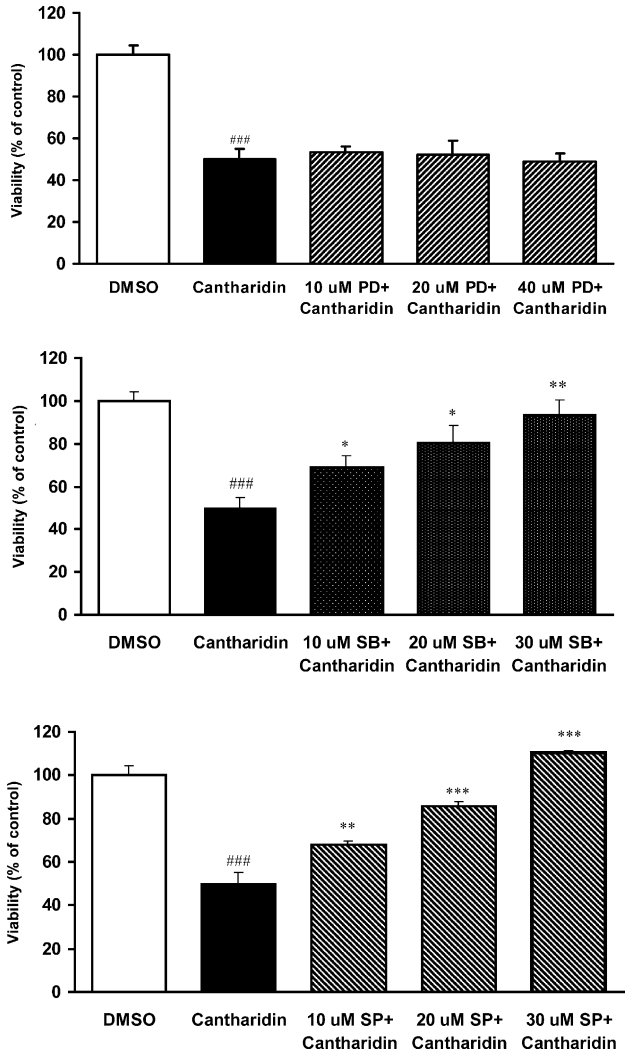

Fig. 4. Effects of MAP kinase inhibitors on the viability of cantharidin-treated U937 cells. 10, 20 and 40 μM PD98059 (PD), 10, 20 and 30 μM SB202190 (SB) or 10, 20 and 30 μM SP600125 (SP) were added to the cells 1 hr before cantharidin treatment and cultured for 24 hr. The viability was determined as described in Section 2. Results were obtained from three experiments and bars represent SEMs. $^{\#}P$ represents the significant difference between non-treated group and cantharidin-treated group. $^{*}P$ represents the significance between cantharidin-treated group and cantharidin and MAP kinase inhibitors (PD98059, SB202190 and SP600125) treated group by Student's *t*-test.

beetles or Spanish fly [1]. It was reported to inhibit the growth of some tumor cell types such as HeLa cells, murine ascites hepatoma, or reticular cell sarcoma [34]. Clinical trials suggested that cantharidin had effects on patients with a primary hepatoma. Its antitumor activity may be closely associated with the inhibition of the PP2A. However, its underlying mechanism through signal transduction pathway is unclear. Therefore, the present study focused on the elucidating the role of the MAPKs and the tumor suppressor gene, p53 during the apoptosis induced by cantharidin in U937 human leukemic cells.

We found that ERK-1/2, p38 and JNK MAPKs were activated prior to the development of apoptosis in U937 cells exposed to cantharidin. However, their activation

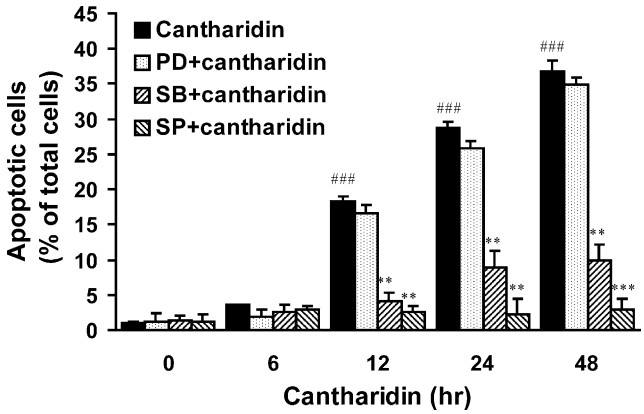

Fig. 5. Effects of MAP kinase inhibitors on cantharidin-induced apoptosis in U937 cells. Cells treated with 20 μM cantharidin were cultured for indicated times in the absence or presence of 40 μM PD98059 (PD), 20 μM SB202190 (SB), and 20 μM SP600125 (SP). The results represent the average of three distinct experiments. $^{\#}P$ represents the significant difference between non-treated group and cantharidin-treated group. $^{*}P$ represents the significance between cantharidin-treated group and cantharidin and MAP kinase inhibitors (PD98059, SB202190 and SP600125) treated group by Student's *t*-test.

pattern was different among the three MAPK families. In the time-course study, ERK was phosphorylated earlier than p38 and JNK after cantharidin exposure, and its activity was sustained in a time-dependent manner. p38 was activated later than the other MAPKs from 6 hr after cantharidin exposure (Fig. 1). The sustained activation of the ERK pathway has been reported to be associated with stimulation by growth factors such as the nerve growth factor [35], EGF and the hepatocyte growth factor [36]. Cantharidin may activate all the MAP kinase pathways in a similar way that paclitaxel induces apoptosis [37] by activating signal transduction pathways including ERK [38,39], p38 [39,40] and JNK [40] in all.

Cantharidin had a strong cytotoxicity on U937 cells in a dose- and time-dependent manner. However, the effective time for cytotoxicity was found over 12 hr after the cantharidin exposure on U937 cells (Fig. 2). PD98059, SB202190 and SP600125 were used as MAPK inhibitors for ERK-1/2, p38 and JNK in cytotoxicity and apoptosis assays to evaluate the roles of MAPKs. In the cytotoxicity assay PD98059 had no effect on the cantharidin-induced cytotoxicity on U937 cells, whereas SB202190 and SP600125 significantly interfered with the cytotoxicity induced by cantharidin suggesting that the cytotoxic activity of cantharidin may be closely related to the activation of p38 and JNK (Fig. 4). Similarly, flow cytometric analysis using PI staining revealed that cantharidin significantly increased the apoptotic population in a dose- and time-dependent manner (Fig. 3). However, PD98059 did not suppress the cantharidin-induced apoptosis, while SB202190 and SP600125 disturbed cantharidin-induced apoptosis (Fig. 5). These results suggest that p38 and JNK kinase pathways may mediate the cytotoxicity and apoptosis induced by cantharidin.

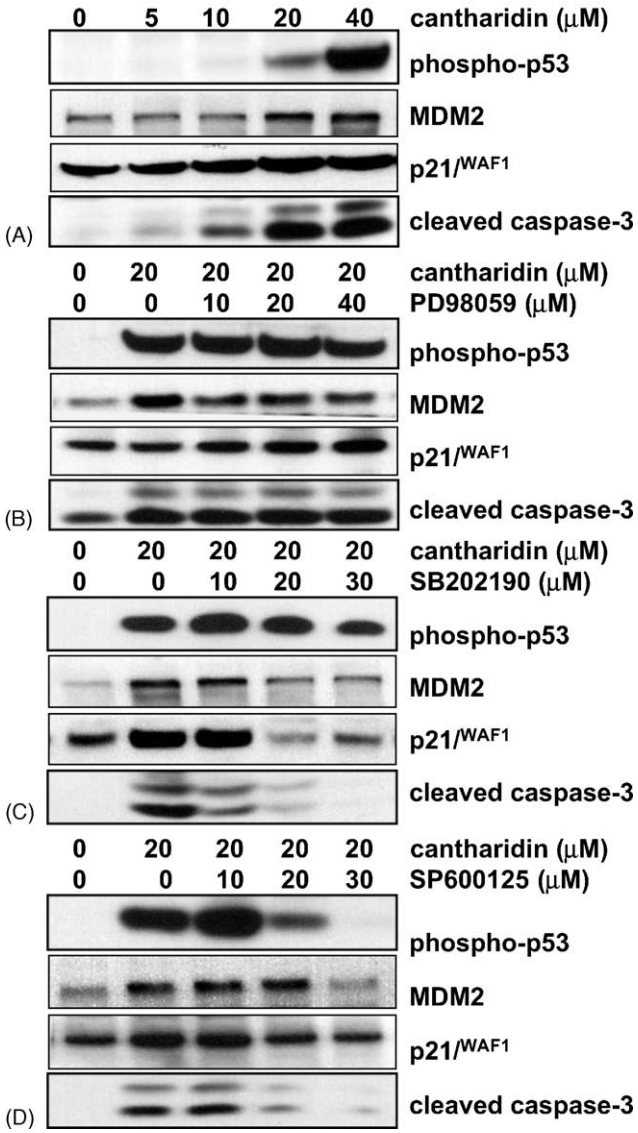

Fig. 6. Effects of MAP kinase inhibitors on the expressions of p53, MDM2, p21 and caspase-3 in cantharidin-induced apoptosis. (A) Cells were treated with 20 μM cantharidin for the different time periods (upper panel) or with the indicated concentrations of cantharidin for 24 hr (lower panel). (B), (C) and (D) Cells were treated with 20 μM cantharidin for 24 hr in the presence of various concentrations of PD98059 (B) or SB202190 (C) or SP600125 (D). The expressions of active caspase-3 and p53 were evaluated by Western blot analysis.

We confirmed that cantharidin caused apoptosis by p53 phosphorylation at serine 15, increasing the expression of downstream signal such as MDM2 and p21 in U937 leukemic cells in a dose- and time-dependent manner (Fig. 6A), which is similar to Krautheim's data that cantharidin induced caspase-3 activation, cytochrome *c* release and DNA fragmentation in HIT fibroblast cells [8]. However, in the comparative study with MAPK inhibitors, SB202190 and SP600125 caused the down-regulation of p53, MDM2, p21 and cleaved caspase-3 after a co-treatment with cantharidin, while PD98059 did not affect the expression levels of p53, MDM2, p21 and caspase-3 during the apoptosis induced by cantharidin,

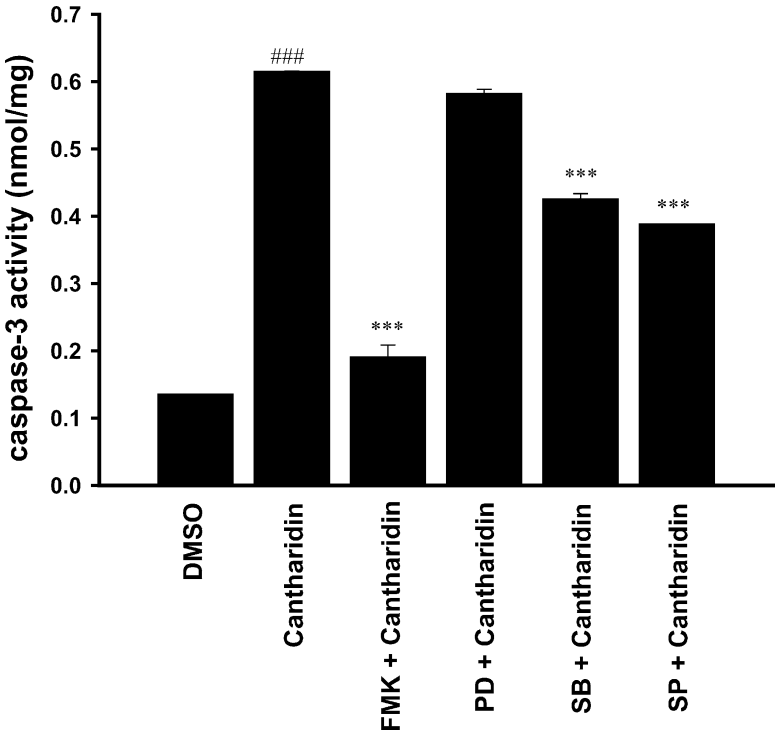

Fig. 7. Effect of MAP kinase inhibitors on caspase-3 activity induced by cantharidin. Cells were treated with 20 μM cantharidin for 24 hr in the absence or presence of indicated concentrations of 100 μM z-VAD-FMK (FMK), 40 μM PD98059 (PD), 20 μM SB202190 (SB) or 20 μM SP600125 (SP). Caspase-3 activity was determined by colorimetric assay. The results represent the average of three distinct experiments. $^{\#}P$ represents the significant difference between nontreated group and cantharidin-treated group. $^{*}P$ represents the significant difference between cantharidin-treated group and cantharidin and MAP kinase inhibitors (PD98059, SB202190 and SP600125) or z-VAD-FMK-treated group by Student's *t*-test.

which indicates p38 and JNK are closely associated with p53 phosphorylation, and caspase-3 activation. The phosphorylation of p53 usually can be induced at serine 15 or 18 or both 15 and 18, replaced by alanine. Especially, the phosphorylation of p53 at serine 15 was reported to be a key phosphorylation target during the p53 activation process to apoptosis [14]. Of the MAP kinase families, p53 can be phosphorylated either directly or indirectly by p38 kinase [16,41–43] and JNK [15,44]. Compared to numerous studies on the role of p38 kinase or JNK in p53 regulation, there is less evidence to indicate phosphorylation and/or accumulation of p53 by ERK-1/2. Similarly, in the present study, the close relationship between ERK-1/2 and p53 could not be found, while p38 and JNK could mediate p53 phosphorylation and caspase-3 activation to undergo apoptosis by cantharidin. SB202190 and SP600125 significantly disturbed the caspase-3 activation induced by cantharidin suggesting the roles of p38 and JNK in the control of caspase-3 activation (Fig. 7). This study also confirmed that cantharidin induced apoptosis through caspase-3-dependent pathway, since the caspase-3 inhibitor, z-VAD-FMK significantly reduced the caspase-3 activation by cantharidin (Fig. 7).

In conclusion, cantharidin can induce apoptosis through p53 phosphorylation and caspase-3 activation. However, of the MAPK families, p38 and JNK can play important roles in mediating the apoptotic signal including p53 phosphorylation and caspase-3 activation during cantharidin-induced apoptosis. Overall, these results suggest that cantharidin can induce apoptosis by activating the p38 and JNK MAP kinase pathways associated with p53 and caspase-3.

Acknowledgments

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