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Organophosphorus pesticides induce apoptosis in human NK cells

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Abstract

We found previously that organophosphorus pesticides (OPs) significantly inhibited natural killer (NK) activity. To explore the mechanism of OP-induced inhibition of NK activity, we investigated whether OPs can induce apoptosis in NK cells in the present study. We used NK-92CI and NK-92MI cells, which are interleukin-2 independent human NK cell lines. NK-92CI and/or NK-92MI were treated with dichlorvos (DDVP) or chlorpyrifos (CP) at 0–100 ppm for 1–72 h at 37 °C *in vitro*. Apoptosis induced by DDVP and CP was determined by FITC-Annexin V staining and the intracellular level of active caspase-3 was analysed by flow cytometry. We found that DDVP and CP significantly induced apoptosis in NK-92CI and NK-92MI cells in a dose- and time-dependent manner. DDVP also induced an increase of intracellular active caspase-3 in NK-92CI in a dose- and time-dependent manner, and a caspase-3 inhibitor, Z-DEVD-FMK, significantly inhibited DDVP-induced apoptosis, suggesting that this apoptosis is partially mediated by activation of intracellular caspase-3. The pattern of apoptosis induced by CP differed from that induced by DDVP. CP showed a faster response than DDVP at higher doses; whereas, DDVP showed a slower, but stronger apoptosis-inducing ability than CP at lower doses. Moreover, the response to OPs differed between NK-92CI and NK-92MI cells, and NK-92CI was more sensitive to OPs than NK-92MI. This is similar to the inhibition of NK activity induced by DDVP, in which NK-92CI was more easily inhibited by DDVP than NK-92MI. Taken together, these findings suggest that OP-induced inhibition of NK activity may be at least partially mediated by OP-induced apoptosis in NK.

Keywords: Annexin-V; Apoptosis; Caspase-3; Chlorpyrifos; Dichlorvos; NK-92; NK activity

1. Introduction

We found previously that organophosphorus pesticides (OPs) significantly inhibited natural killer (NK) activity (Li et al., 2002, 2005, 2006; Li and Kawada, 2006; Li, 2007), which is mediated by the following mechanisms: (1) OPs impair the granule exocytosis pathway of NK, LAK, and CTL cells by inhibiting the activity of granzymes (Li et al., 2002) and by decreasing the intracellular level of perforin, granzyme A, and granulysin, which was mediated by inducing degranulation of NK cells, and by inhibiting the transcription of mRNAs of perforin, granzyme A, and granulysin (Li et al., 2005, 2006); (2) OPs impair the FasL/Fas pathway of NK, LAK, and CTL cells, as investigated by using perforin-knockout mice, in which the granule exocytosis pathway of NK cells does not function and only the FasL/Fas pathway remains functional (Li et al., 2004). However, the above two mechanisms cannot completely explain the entire mechanism of OP-induced inhibition of NK activity and there must be other mechanisms involved. This encouraged us to speculate that OPs may induce apoptosis in NK, LAK, and CTL and

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ultimately inhibited the cytolytic activity of effectors. Thus, we first investigated the apoptosis-inducing activity of organophosphorus pesticide, chlorpyrifos (CP) in a human monocyte cell line, U937, and found that CP significantly induced apoptosis in the U937 cell line by activating caspase 3 (Nakadai et al., 2006). In the present study, CP and dichlorvos (DDVP) as the two most widely used OP compounds were employed to examine their apoptotic-inducing potential in human NK cells using NK-92MI and NK-92CI cells, which are interleukin-2-independent human NK cell lines, that express CD56, perforin, granzyme A, and granulysin and are highly cytotoxic to K562 cells in the chromium release assay (Li et al., 2005, 2006). Although, it has been reported that CP induced apoptosis in human U937 monocytes (Nakadai et al., 2006) and that paraoxon and parathion induced apoptosis in murine EL4 T-lymphocytic leukemia cell lines (Saleh et al., 2003a,b), there have been no reports on human NK cells.

2. Materials and methods

2.1. Reagents

Alpha minimum essential medium (α-MEM) without ribonucleosides and deoxyribonucleosides, inositol, 2-mercaptoethanol (2-ME), folic acid, and glutamine were obtained from Sigma (St. Louis, MO). RPMI 1640 medium was purchased from Nissui Pharmaceutical (Tokyo, Japan). FBS was purchased from JRH Biosciences (Lenexa, KS), and heat-inactivated at 56 °C for 30 min prior to use. Dimethyl 2,2-dichlorovinyl phosphate (DDVP, dichlorvos) and 3,5,6-trichloro-2-pyridyl diethyl phosphorothionate (chlorpyrifos, CP) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Fluorescein isothiocynate (FITC)-anti human Annexin V and FITC-anti human active caspase-3, Z-DEVD-FMK (a caspase-3 inhibitor), Z-FA-FMK (a negative control for Z-DEVD-FMK), and Cytofix/cytoperm solution were purchased from BD PharMingen (San Diego, CA). CP, Z-DEVD-FMK, and Z-FA-FMK were prepared as stock solutions in DMSO. Sodium ^{51}Cr-chromate solution was obtained from Perkin-Elmer (Boston, MA).

2.2. Cells

NK-92CI and NK-92MI cell lines were obtained from ATCC (Manassas, VA) and were maintained in α-MEM without ribonucleosides and deoxyribonucleosides with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 0.2 mM inositol, 0.1 mM 2-ME, 0.02 mM folic acid, and 10% FBS at 37 °C in a 5% CO_2 incubator. K-562 target cells were also obtained from ATCC and were maintained in RPMI 1640 medium containing 10% FBS.

2.3. OP-induced apoptosis in NK-92 cells determined by FITC-Annexin V staining

It has been reported that Annexin-V staining is able to detect apoptosis at an early stage based on alterations to the cell membrane (Dong et al., 2005; Nakadai et al., 2006). Therefore, we used Annexin-V staining assessed by flow cytometry to investigate OP-induced apoptosis.

The NK-92 cells at 2×10^5 ml^{-1} were treated with DDVP at 0–100 ppm or CP at 0 (0.1% DMSO)–100 ppm for 1–72 h at 37 °C in a 5% CO_2 incubator, and then harvested and washed twice with PBS. The treated cells were suspended in binding buffer at 3×10^5 cells/100 μl, and supplemented with 5 μl FITC-Annexin-V and 5 μl PI, and incubated for 15 min at room temperature in the dark. Flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) as described previously (Nakadai et al., 2006).

2.4. Determination of intracellular active caspase-3 in NK-92CI by flow cytometry

NK-92CI cells at 2×10^5 ml^{-1} were incubated with DDVP at 0, 12.5, 25, 50, or 100 ppm for 16 or 24 h at 37 °C in a 5% CO_2 incubator, and then harvested and washed twice with PBS. The cells were fixed/permeablized with Cytofix/cytoperm solution for 20 min at 4 °C, and then intracellular active caspase-3 was stained with FITC-anti human active caspase-3 for 30 min at room temperature according to the manufacturer's instructions (BD PharMingen). Flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson) as described previously (Nakadai et al., 2006).

2.5. Inhibition of DDVP-induced apoptosis by caspase-3 inhibitor in NK-92CI

NK-92CI cells at 2×10^5 ml^{-1} were preincubated with Z-DEVD-FMK, an inhibitor of caspase-3, or Z-FA-FMK, a negative control for Z-DEVD-FMK, at 20 μM for 30 min and then treated with DDVP at 0, 25, 50, or 100 ppm for 24 h, and then harvested and washed twice with PBS. The treated cells were suspended in binding buffer at 3×10^5 cells/100 μl and supplemented with 5 μl FITC-Annexin-V and 5 μl PI, and incubated for 15 min at room temperature in the dark. Flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson) as described previously (Nakadai et al., 2006).

2.6. Inhibition of DDVP-induced active caspase-3 by caspase-3 inhibitor in NK-92CI

NK-92CI cells at 2×10^5 ml^{-1} were preincubated with Z-DEVD-FMK or Z-FA-FMK at 20 μM for 30 min, then treated with DDVP at 50 ppm for 24 h, and subsequently harvested and washed twice with PBS. The cells were fixed/permeablized with Cytofix/cytoperm solution for 20 min at 4 °C, and then intracellular active caspase-3 was stained with FITC-anti human active caspase-3 for 30 min at room temperature.

Flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson) as described previously (Nakadai et al., 2006).

2.7. DDVP inhibits cytolytic activity of NK-92CI and NK-92MI

The NK-92 cells at 2×10^5 ml^{-1} were treated with DDVP at 0–50 ppm for 16 h at 37 °C in a 5% CO_2 incubator, and then harvested and washed twice with PBS. A standard microtiter ^{51}Cr-release assay was used to measure NK activity as described previously (Li et al., 2002, 2006). Briefly, K-562 target cells were labeled with sodium ^{51}Cr-chromate solution for 60 min at 37 °C in 5% CO_2 and washed four times in RPMI 1640 containing 10% FBS. The target cells were plated into round-bottomed 96-well microplates, then NK-92CI at effector/target (E/T) ratios of 2.5/1, 5/1, and 10/1 or NK-92MI cells at E/T ratios of 0.625/1, 1.25/1, and 2.5/1 were added to the wells in triplicate. Following a 4 h incubation, the microplates were centrifuged and 0.1 ml of supernatant from each well was collected and counted in a gamma counter. The cytolytic activity induced by NK-92CI or NK-92MI cells was calculated by averaging cpm for triplicate wells as described previously (Li et al., 2002, 2006).

2.8. Statistical analyses.

For statistical analyses, *F*-tests were performed to determine whether the groups showed equal variances. Since the variances of all data in the present study were equal, statistical analyses were performed using the unpaired *t*-test (two-tailed). Linear correlation analysis was also used. The significance level for *p* values was set at <0.05.

3. Results

3.1. OP-induced apoptosis in NK-92 cells

As shown in Fig. 1, DDVP (A and B) and CP (C and D) significantly induced apoptosis in NK-92CI cells in a

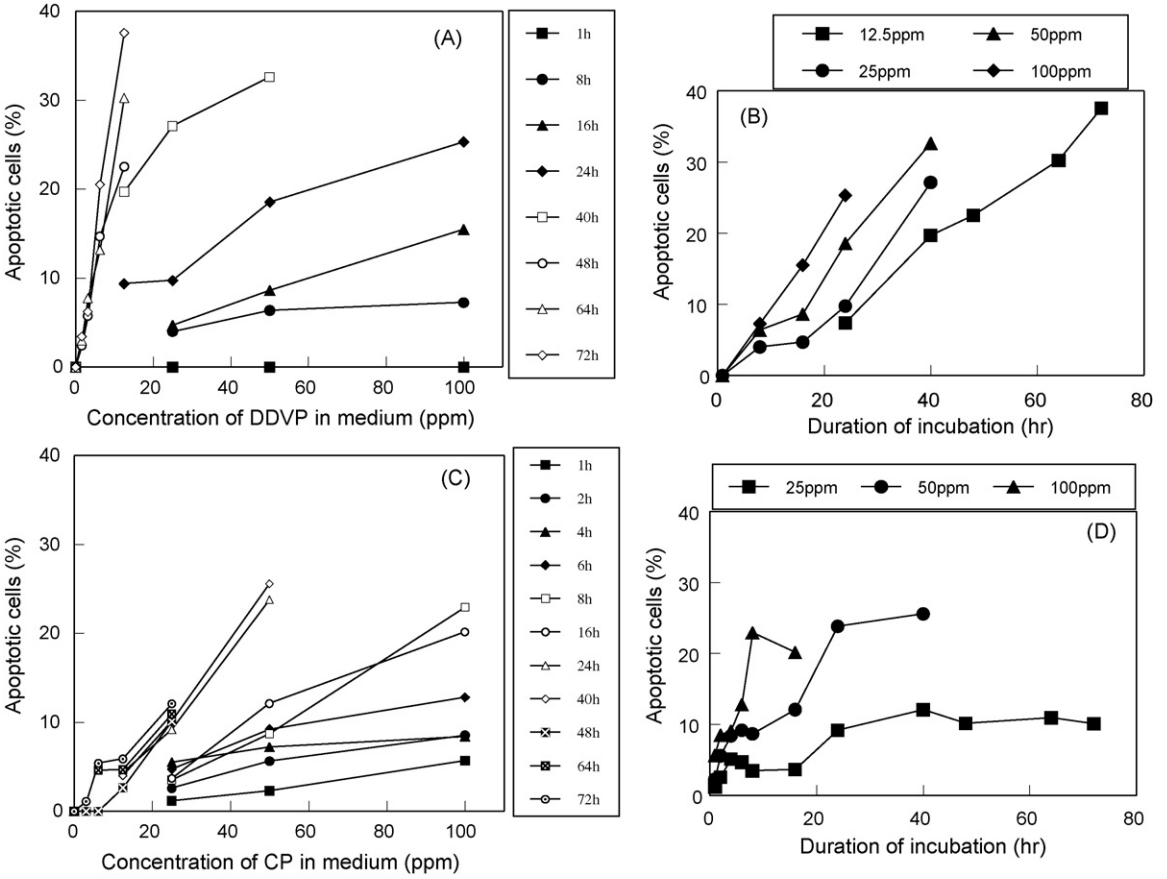

Fig. 1. DDVP (A and B) and CP (C and D)-induced apoptosis in NK-92CI cells in dose-dependent (A and C) and time-dependent (B and D) manner determined by FITC-Annexin-V staining detected by flow cytometry. The correlation coefficients between the rate of apoptotic cells and the dose of OPs were $r = 0.994$ ($n = 5$, $p < 0.01$) for DDVP at 72 h, and $r = 0.990$ ($n = 4$, $p = 0.01$) for CP at 8 h; the correlation coefficients between the rate of apoptotic cells and the period of treatment were $r = 0.992$ ($n = 5$, $p < 0.01$) for DDVP at 12.5 ppm, and $r = 0.857$ ($n = 11$, $p < 0.01$) for CP at 50 ppm. Experiments were repeated at least three times with very similar results.

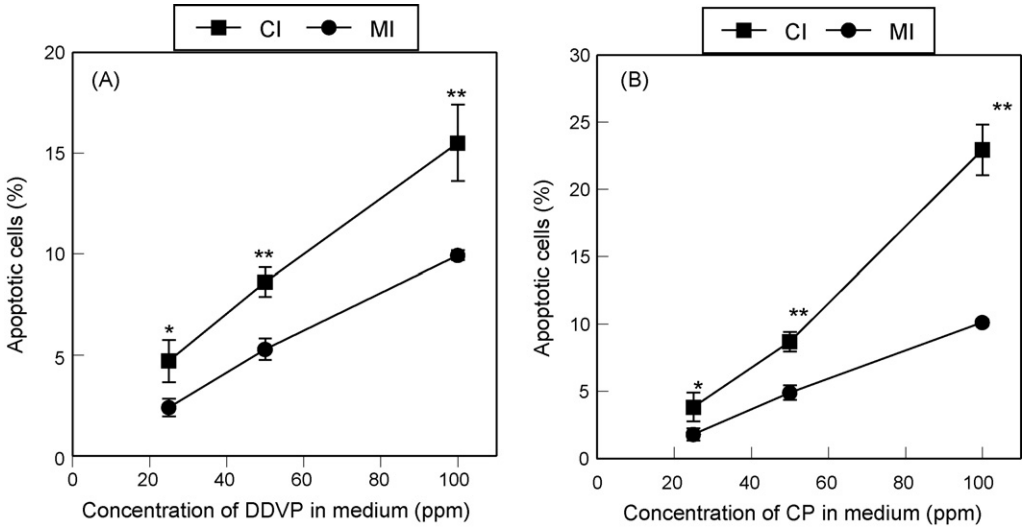

Fig. 2. Different response to DDVP (A) and CP (B) in apoptosis between NK-92CI and NK-92MI cells. NK-92CI and NK-92MI cells were treated with DDVP for 16 h or with CP for 8 h. Data are presented as the mean ±S.D. ($n = 3$). *$p < 0.05$, **$p < 0.01$, significantly different from NK-92MI cells by unpaired t-test.

dose (A and C)- and time (B and D)-dependent manner. The correlation coefficients between the rate of apoptotic cells and the dose of OPs were $r = 0.994$ ($n = 5$, $p < 0.01$) for DDVP at 72 h, and $r = 0.990$ ($n = 4$, $p = 0.01$) for CP at 8 h; the correlation coefficients between the rate of apoptotic cells and the period of treatment were $r = 0.992$ ($n = 5$, $p < 0.01$) for DDVP at 12.5 ppm, and $r = 0.857$ ($n = 11$, $p < 0.01$) for CP at 50 ppm. The pattern of apoptosis induced by CP differed from that induced by DDVP. At higher doses (25–100 ppm), CP showed a faster response than DDVP; whereas, at lower doses (1.56–12.5 ppm), DDVP showed a slower, but stronger apoptosis-inducing ability than CP. Similar results also were obtained in NK-92MI cells (data not shown).

3.2. Difference on OP-induced apoptosis between NK-92CI and NK-92MI cells

As shown in Fig. 2, the apoptosis induced by DDVP (A) or CP (B) differed between NK-92CI and NK-92MI cells, and NK-92CI cells were more sensitive to OPs than NK-92MI cells.

3.3. Mechanism of DDVP-induced apoptosis in NK cells

To explore the mechanism of OP-induced apoptosis in NK cells, we investigated whether DDVP affected the intracellular level of active caspase-3 in NK-92CI cells. As shown in Fig. 3, DDVP induced a significant increase in active caspase-3 in NK-92CI cells in a dose- and time-dependent manner. We also found that an inhibitor of active caspase-3 could partially, but significantly inhibit DDVP-induced apoptosis (Fig. 4). Moreover, the inhibitor of caspase-3 also partially, but significantly inhibits the DDVP-induced increase of active caspase-3 in NK-92CI cells (Fig. 5). These findings strongly suggest that DDVP induced apoptosis at least partially *via* the caspase-3 pathway.

3.4. Differential response to DDVP between NK-92MI and NK-92CI in cytolytic activity

As shown in Fig. 6, DDVP significantly inhibited cytolytic activity in both NK-92CI (A) and NK-92MI (B) cells in a dose-dependent manner; however, the strength of inhibition differed between two cells, and NK-92CI cells was more sensitive to DDVP than NK-92MI cells.

4. Discussion

Organophosphorus pesticides (OPs) are used widely throughout the world as insecticides in agriculture and/or eradicating agents for termites around homes (Richardson, 1993a,b; Ellenhorn and Barceloux, 1988). There is still a large quantity of OPs on the market in Japan (Japan Plant Protection Association, 2003). OPs are potent inhibitors of serine esterases such as acetylcholinesterase and serum cholinesterase (Richardson, 1993a,b; Ellenhorn and Barceloux, 1988; Pope, 1999). The main toxicity of OPs is neurotoxicity, which

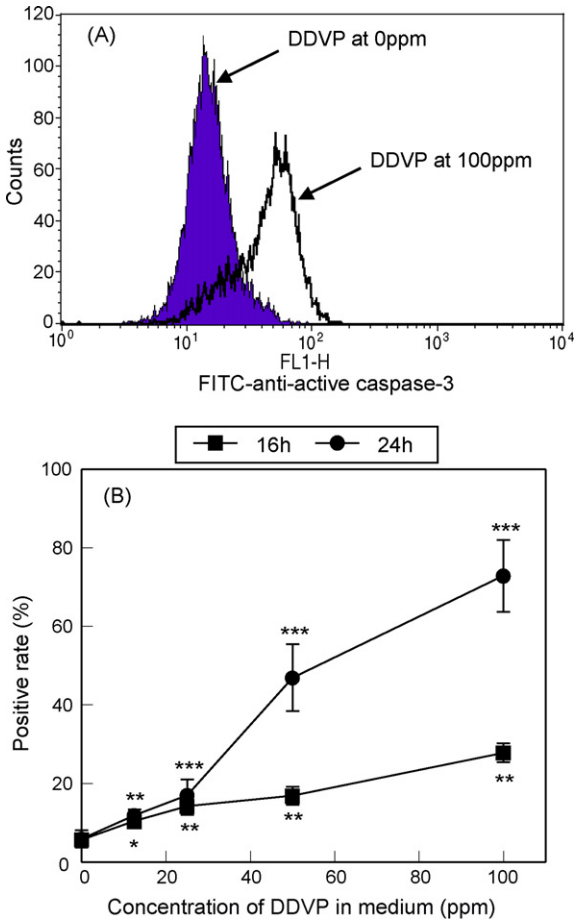

Fig. 3. DDVP-induced increase in active caspase-3-positive NK-92CI cells. (A) the shaded histogram shows the control cells (DDVP at 0 ppm) and the open histograms show the cells treated with DDVP at 100 ppm for 24 h stained with FITC-rabbit anti-human active caspase-3. (B) Data are presented as the mean ±S.D. ($n = 3$ for 16 h, $n = 5$ for 24 h). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, significantly different from 0 ppm by unpaired t-test.

is caused by the inhibition of acetylcholinesterase (Richardson, 1993a,b; Ellenhorn and Barceloux, 1988; Pope, 1999; Bajgar, 2004). We found previously that OPs significantly inhibit NK, LAK, and CTL activities by impairing the granule exocytosis pathway and the FasL/Fas pathway of NK, LAK, and CTL cells (Li et al., 2000, 2002, 2004, 2005, 2006; Li and Kawada, 2006; Li, 2007). However, the above two mechanisms cannot entirely explain all the mechanism of OP-induced inhibition of NK activity and there must be other mechanisms involved (Nakadai et al., 2006; Li and Kawada, 2006; Li, 2007). This encouraged us to speculate that OPs may induce apoptosis in NK cells and ultimately inhibit NK activity. Thus, in the present study, DDVP and CP as the two most widely used OP compounds were employed

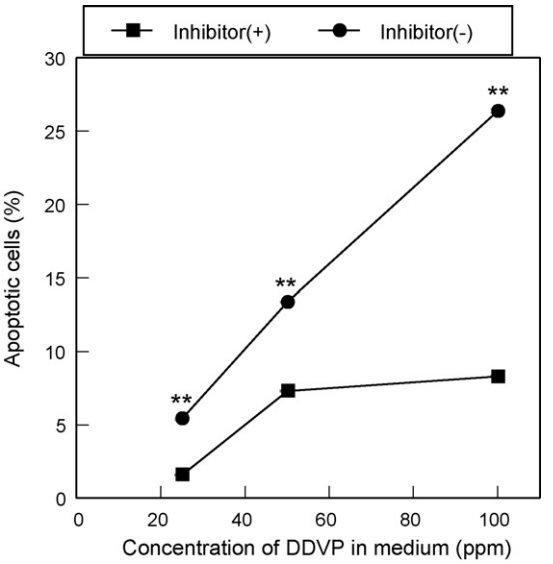

Fig. 4. Effect of Z-DEVD-FMK on DDVP-induced apoptosis in NK cells using FITC-Annexin-V staining by flow cytometry. Data are presented as the mean ±S.D. ($n = 3$). **$p < 0.001$, significantly different from the responses with inhibitor by unpaired t-test.

to examine their apoptotic-inducing potential in human NK cells.

The findings of Annexin-V staining indicated that DDVP and CP-induced cell death consisting of apoptosis. Both DDVP and CP significantly induced apoptosis in human NK cells in a dose- and time-dependent manner. This is the first report on OP-induced apoptosis in human NK cells, although it has been reported that OPs

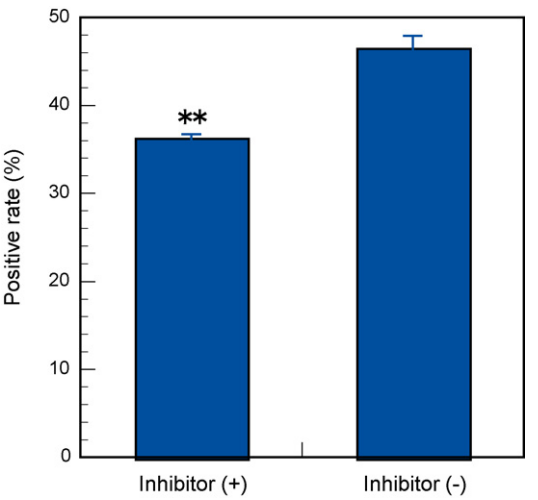

Fig. 5. Effect of Z-DEVD-FMK on DDVP-induced increase of intracellular active caspase-3 in NK cells by flow cytometry. Data are presented as the mean ±S.D. ($n = 3$). **$p < 0.001$, significantly different from the responses without inhibitor by unpaired t-test.

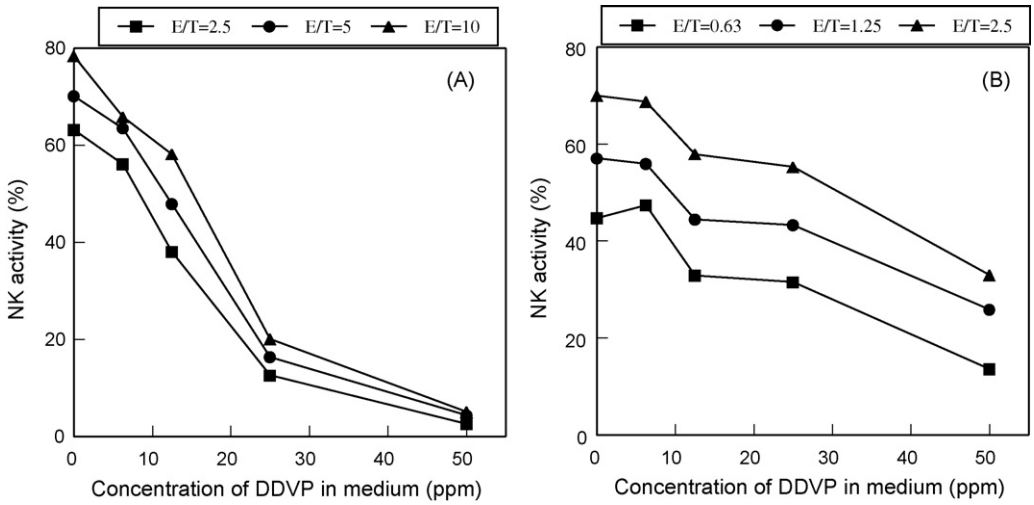

Fig. 6. Different response to DDVP in cytolytic activity between (A) NK-92CI and (B) NK-92MI cells. Experiments were repeated at least three times with very similar results.

induced apoptosis in a human monocyte cell line, U937 (Nakadai et al., 2006), and a murine EL4 T-lymphocytic leukemia cell line (Saleh et al., 2003a,b).

The pattern of apoptosis induced by CP differed from that induced by DDVP. At higher doses, CP showed a faster response than DDVP, whereas, at lower doses, DDVP showed a slower, but stronger apoptosis-inducing ability than CP. We cannot explain the difference between the two OPs, but maybe the different water solubility partially influences the apoptosis-inducing ability.

Moreover, the apoptosis induced by OPs differed between NK-92CI and NK-92MI cells, and NK-92CI cells were more sensitive to OPs than NK-92MI cells. This is similar to the inhibition of NK activity induced by DDVP, in which NK-92CI cells were more easily inhibited by DDVP than NK-92MI cells (Fig. 6), strongly suggesting a relationship between DDVP-induced apoptosis and the inhibition of cytolytic activity in NK cells.

The caspase family of cysteine proteases plays a key role in apoptosis and inflammation. Caspase-3 is a key protease that is activated during the early stages of apoptosis and, like other members of the caspase family, is synthesized as an inactive proenzyme that is processed in cells undergoing apoptosis by self-proteolysis and/or cleavage by another protease (Patel et al., 1996). To explore the mechanism of OP-induced apoptosis in NK cells, we examined the intracellular active caspase-3 in DDVP-treated NK-92CI cells and found that DDVP induced a significantly higher level of intracellular active caspase-3. We also found that Z-DEVD-FMK significantly inhibited DDVP-induced apoptosis. Moreover, Z-DEVD-FMK also significantly decreased the level of intracellular active caspase-3 induced by DDVP. These findings suggest that DDVP induces apoptosis *via* the caspase-3 pathway. However, the caspase-3 inhibitor only partially inhibited DDVP-induced apoptosis, suggesting that other pathways are also involved. We also found previously that CP-induced apoptosis in U937 cells were partially mediated by activation of intracellular caspase-3 (Nakadai et al., 2006). Further studies will be necessary to explore the effect of DDVP on the mitochondrial pathway by determining the mitochondrial membrane potential and cytochrome C release, etc., under similar exposure conditions. Saleh et al. (2003a) have reported that paraoxon induces apoptosis in EL4 cells *via* the activation of mitochondrial pathways. On the other hand, Caughlan et al. (2004) reported that CP induces apoptosis in rat neurons *via* a mechanism that is regulated by the balance between p38 and extracellular signal-regulated protein kinase (ERK)/c-Jun NH2-terminal protein kinase (JNK) MAP kinases.

Taken together, the present findings indicate that DDVP and CP can induce apoptosis in human NK cells, and the apoptosis is at least partially mediated by activation of intracellular caspase-3. These findings suggest that OP-induced inhibition of NK activity may be at least partially mediated by OP-induced apoptosis in NK cells.

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