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ORIGINAL ARTICLE

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Cisplatin (CDDP) specifically induces apoptosis via sequential activation of caspase-8, -3 and -6 in osteosarcoma

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Abstract *Purpose*: Osteosarcoma is a common malignant tumor. The first choice of treatment plan for osteosarcoma is chemotherapy. In particular, preoperative chemotherapy is most important in clinical treatment in orthopedics. In these chemotherapies, multiple anticancer drugs such as Adriamycin (ADM), CDDP, cyclophosphamide (CPM), methotrexate (MTX) and vincristine (VCR) are commonly used in combination. Recently, anticancer drugs have been shown to trigger apoptosis in various cancer cells. However, many studies on this topic have been examined using leukemia cell lines, and many kinds of cancer cells established from solid tumor are resistant to the induction of apoptosis by anticancer drugs. So in this study, we examined the effects of the anticancer drugs ADM, CDDP, CPM, MTX and VCR on osteosarcoma cells in vitro. We also examined the signaling pathways of each anticancer drug by studying the induction of apoptosis and activation of caspases in the osteosarcoma cells. *Methods*: We examined the effects of the anticancer drugs ADM, CDDP, CPM, MTX and VCR, which are used clinically for the treatment of osteosarcoma, on cells of the human osteosarcoma (HOS) cell line. The cytotoxic effects of the anticancer drugs were evaluated using the MTT assay. We used both flow cytometry and activation of caspases to confirm the induction of apoptosis in the HOS cells. To dissect the pathway of the caspase cascade in apoptosis in HOS cells, we used the tetrapeptides YVAD-CHO, DMQD-CHO, VEID-CHO and IETD-CHO, which selectively inhibit caspase-1, -3, -6 and -8, respectively. *Results*: ADM, CDDP, CPM and VCR, but not MTX, induced death of HOS cells in a dose-dependent manner. CDDP at 10 μ*M*, CPM at 7.5 μ*M*, ADM at 20 μ*M* and VCR at 150 μ*M* caused 80% cell death of HOS cells after 12 h. However, the percentages of apoptotic cells were 5.6% (medium alone), 75.9% (CDDP), 20.0% (CPM), 22.2% (ADM), 20.5% (VCR) and 13.1% (MTX). In addition, direct measurement of caspase-3 activity revealed that CDDP but not the other drugs activated caspase-3 in HOS cells. These analyses revealed that only CDDP induced apoptosis of HOS cells via activation of caspases. Furthermore, DMQD-CHO, VEID-CHO and IETD-CHO inhibited CDDP-induced apoptosis of HOS cells, suggesting that caspase-3, -6 and -8 are involved in the signaling pathway of CDDP-induced apoptosis. In contrast, none of the caspase inhibitors inhibited cell death induced by the other anticancer drugs. *Conclusions*: This study demonstrates that CDDP specifically induces apoptosis via activation of caspases and the other anticancer drugs induce death of HOS cells via different signaling pathways. It also demonstrates that caspase-8 is a key molecule in the earliest stage of the signaling pathway of CDDP-induced apoptosis of HOS cells, and caspase-3 works downstream of caspase-8.

Key words Apoptosis · Caspases · Cisplatin · Osteosarcoma

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Introduction

Apoptosis is accompanied by biochemical and morphological changes that involve chromatin condensation and margination at the nuclear periphery, extensive double-stranded DNA fragmentation, cellular shrinkage and blebbing [1]. Apoptosis is a feature of programmed cell death and occurs via activation of intracellular death proteins. A common signaling pathway in cells under-

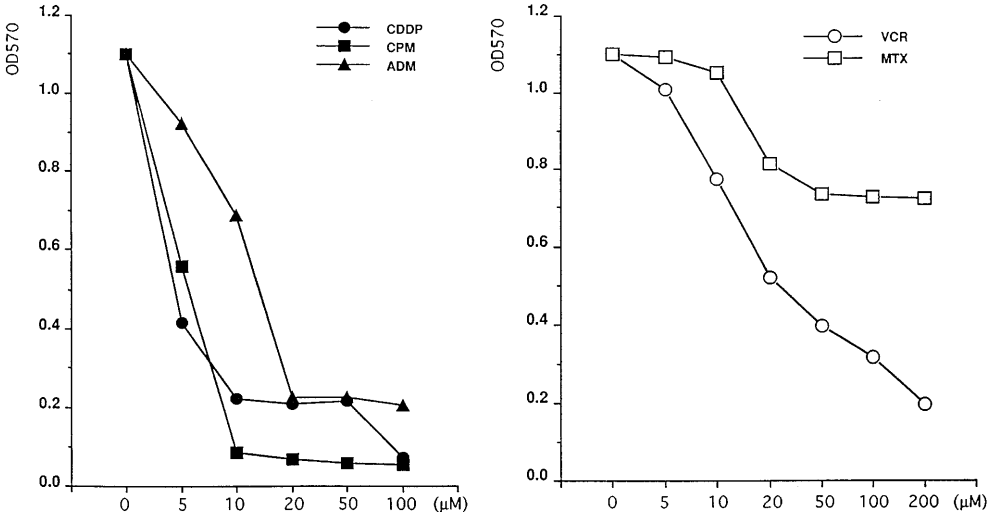


Fig. 1 The sensitivity of HOS cells to anticancer drugs. The ranges of concentration of each anticancer drug used in this experiment were 1–100 μ*M* (CDDP), 1–100 μ*M* (CPM), 1–100 μ*M* (ADM), 1–200 μ*M* (VCR) and 1–200 μ*M* (MTX). After 12 h of culture with each drug at the indicated concentrations, cell viability was determined using the MTT assay as described in Materials and methods. The results are expressed as OD_{570}. The means of duplicate determinations are shown

going apoptosis is the activation of caspases, a family of aspartic acid-directed proteases. Among ten or more caspases, caspase-3-like proteases activated by proteolytic cleavage are considered to play a critical role in the induction of apoptosis [2, 3]. In addition, apoptotic cells are phagocytosed by macrophages immediately, so the release of intracellular molecules which cause an inflammation or shock is limited to a low level compared with necrosis [4]. Thus, the induction of apoptosis in cancer cells by anticancer drugs is an appropriate aim in the therapy of malignant tumors [5].

Recently, anticancer drugs have been shown to trigger apoptosis in various cancer cells [6, 7]. However, in many studies on this topic leukemic cell lines have been used, and many kinds of cancer cells established from solid tumors are resistant to the induction of apoptosis by anticancer drugs [8–10]. In these studies, CDDP has been found to be effective against cancer cells derived from solid tumors such as hepatoma, thymoma and ovarian carcinoma [11–13]. Osteosarcoma is also a common malignant tumor. The first choice in the treatment plan for osteosarcoma is chemotherapy. In particular, preoperative chemotherapy is most important in clinical treatment in orthopedics. In these chemotherapies, multiple anticancer drugs such as Adriamycin (ADM), CDDP, cyclophosphamide (CPM), methotrexate (MTX) and vincristine (VCR) are commonly used in combination to increase the efficacy, decrease the toxicity toward the host and minimize the development of drug resistance. Recently, the target molecules of each anticancer drug have been revealed [14–17]. However, the mechanisms by which anticancer drugs induce cell death are not clearly defined.

In this study, we examined the effects of ADM, CDDP, CPM, MTX and VCR on osteosarcoma cells in vitro. We also examined the signaling pathways of each anticancer drug by studying the induction of apoptosis and activation of caspases in osteosarcoma cells.

Materials and methods

Cells and cell culture

The human osteosarcoma cell line HOS (ATCC, CRL1543) was maintained in monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) and 0.1 mg/ml kanamycin at 37 °C in a humidified atmosphere containing 5% CO_2.

Chemicals

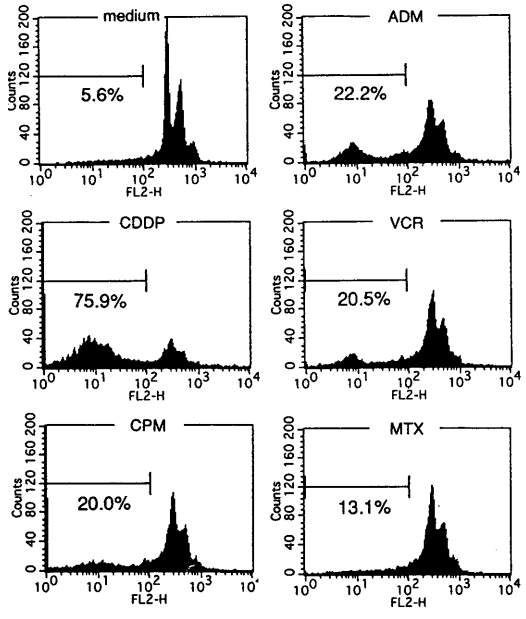
CDDP, MTX, ADM, VCR, CPM and propidium iodide (PI) were all purchased from Sigma (St. Louis, Mo.). The caspase-1 inhibitor YVAD-CHO was purchased from Bachem (King of Prussia, Pa.). The caspase-3 inhibitor DMQD-CHO and caspase-6 inhibitor VEID-CHO were purchased from Peptide Institute (Osaka, Japan). The caspase-8 inhibitor IETD-CHO was purchased from Biomol (Plymouth Meeting, Pa.). Anti-Fas monoclonal antibody was purchased from Serotec (Oxford, UK).

Measurement of cell viability

The sensitivity of HOS cells to anticancer drugs was determined using the MTT assay as described previously [18]. Briefly, 5×10^4 cells were cultured with various concentrations of each anticancer drug in a 96-well microtiter plate. After 12 h of culture, 10 μl of MTT solution (2.5 mg/ml) was added to each well and the cells were incubated for another 4 h. Then 100 μl of 0.04 *N* HCl-isopropanol was added and the absorbance at 570 nm was measured using a Titertek Multiscan Plus microplate reader (Flow General, McLean, Va.). The results are expressed as optical density (OD_{570}).

Detection of apoptosis by flow cytometry

We examined apoptosis by flow cytometry as described previously [19]. Briefly, cells were cultured in the presence of each anticancer drug with or without caspase inhibitors for 12 h. Then, the cells were collected by centrifugation and each pellet was dissolved in 1.5 ml hypotonic fluorochrome solution (PI 50 μg/ml in 0.1% sodium citrate containing 0.1% Triton X-100). The samples were placed in the dark overnight and the PI fluorescence of individual nuclei was measured using a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Calif.). The data were plotted on a logarithmic scale.



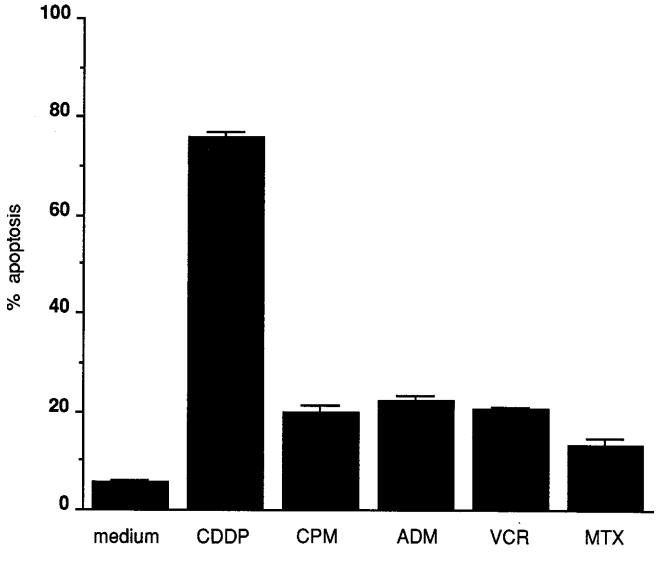


Fig. 2 Induction of apoptotic cell death in drug-treated HOS cells. DNA fragmentation was measured by flow cytometric analysis after PI staining. The concentrations of the anticancer drugs were 10 μ*M* (CDDP), 7.5 μ*M* (CPM), 20 μ*M* (ADM), 150 μ*M* (VCR) and 150 μ*M* (MTX). After 12 h of culture with each drug, 1×10^6 cells were stained with PI and used for flow cytometry. The percentages of apoptotic cells are indicated as the proportion of cells that contained subG1 DNA. The means ± SD of triplicate determinations are shown

Analysis of intracellular caspase-3 activity

Intracellular caspase-3 activity was measured using a caspase-3 cellular assay kit (Biomol, Plymouth Meeting, Pa.) according to the manufacturer's manual. Briefly, cells were cultured in the presence of 20 μ*M* CDDP with or without each caspase inhibitor for 12 h, and collected by centrifugation, washed twice with phosphate-buffered saline (PBS) and resuspended in cell lysis buffer (50 m*M* HEPES, pH 7.4, 0.1% CHAPS, 1 m*M* dithiothreitol (DTT) and 0.1 m*M* EDTA). The cells were then centrifuged again at 10,000 *g* at 4 °C for 10 min and the supernatant was used for the following assay. Supernatant (10 μl) was mixed with 80 μl assay buffer

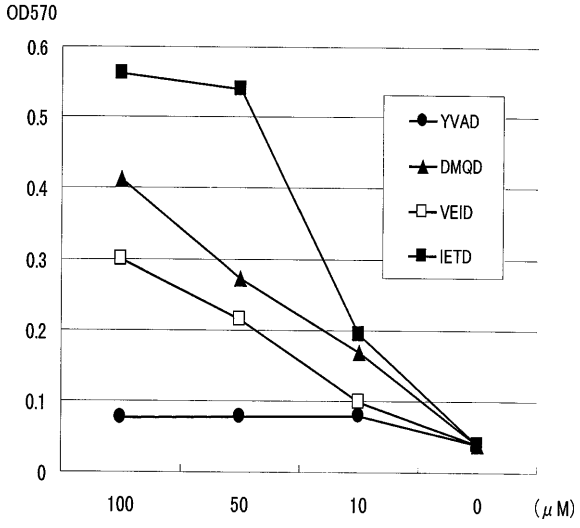


Fig. 3 The effects of caspase inhibitors on CDDP-induced death of HOS cells. Cells were cultured with 20 μ*M* CDDP and the caspase inhibitors YVAD-CHO, DMQD-CHO, VEID-CHO and IETD-CHO at the indicated concentrations. After 12 h of culture, cell viability was measured using the MTT assay and the results are expressed as OD_{570}. The means of duplicate determinations are shown

(50 m*M* HEPES, pH 7.4, 100 m*M* NaCl, 0.1% CHAPS, 10 m*M* DTT, 1 m*M* EDTA and 10% glycerol) in each well of a microtiter plate and incubated at 37 °C for 10 min. Finally, 10 μl Ac-DEVD-pNA substrate was added and after 12 h, the absorbance at 405 nm was measured using a Titertek Multiscan Plus microplate reader. The results are expressed as optical density (OD_{405}).

Results

HOS cells were exposed to various concentrations of anticancer drugs for 12 h. The cytotoxicity of the drugs was determined using the MTT assay as described in Materials and methods. The ranges of concentration of each anticancer drug used in this experiment were 1–100 μ*M* (CDDP), 1–100 μ*M* (CPM), 1–100 μ*M* (ADM), 1–200 μ*M* (VCR) and 1–200 μ*M* (MTX). As shown in Fig. 1, cell death was induced in a dose-dependent manner by CDDP, CPM, ADM and VCR. However, MTX induced growth inhibition of HOS cells but not cell death.

CDDP at 10 μ*M*, CPM at 7.5 μ*M*, ADM at 20 μ*M* and VCR at 150 μ*M* caused 80% cell death of HOS cells after 12 h of culture. To determine whether the cell death was apoptosis, we measured fragmented DNA by flow cytometry as described in Materials and methods. The percentages of apoptotic cells were determined from the number of cells in the subG1 area of the DNA histograms. As shown in Fig. 2, the percentages of cells containing subG1 DNA were 5.6% (medium alone), 75.9% (CDDP), 20.0% (CPM), 22.2% (ADM), 20.5% (VCR) and 13.1% (MTX). We also evaluated the apoptotic cells after 72 h of culture with each concentration of the anticancer drugs. The results were almost the same as those obtained following 12 h of culture (data

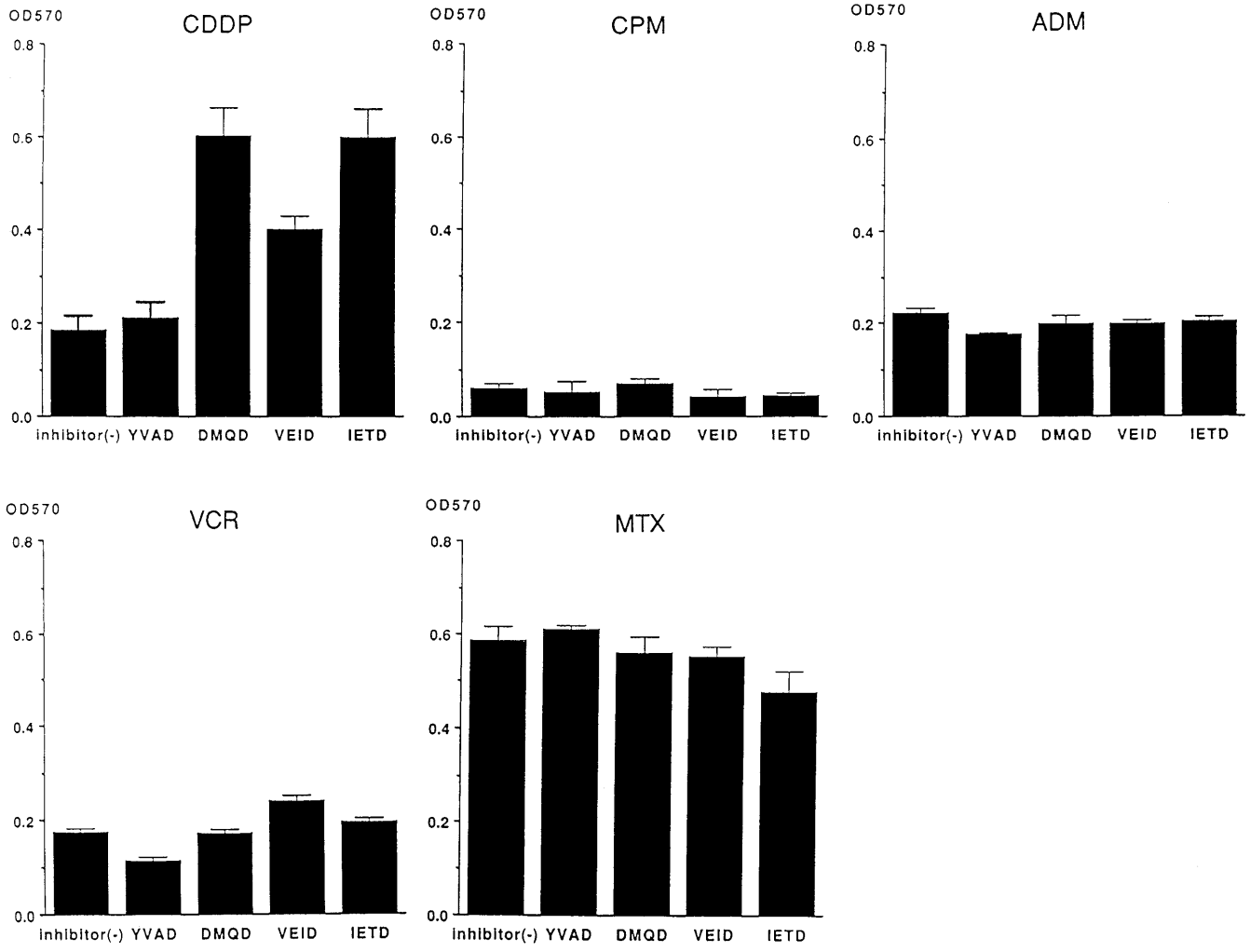


Fig. 4 The effects of caspase inhibitors on anticancer drug-induced death of HOS cells. Cells were cultured with each drug in the presence of each caspase inhibitor. After 12 h of culture, cell viability was measured using the MTT assay and the results are expressed as OD_{570}. The concentrations of each anticancer drug were 20 μM (CDDP), 20 μM (CPM), 20 μM (ADM), 150 μM (VCR) and 150 μM (MTX). The concentration of each caspase inhibitor used in this experiment was 100 μM. The means $\pm$ SD of triplicate determinations are shown

not shown). Thus, HOS cells underwent apoptosis mainly rather than necrosis upon stimulation with CDDP. This result also demonstrates that CPM, ADM, and VCR induced the death of HOS cells mainly by necrosis rather than by apoptosis as indicated by the results shown in Fig. 1.

YVAD-CHO, DMQD-CHO, VEID-CHO and IETD-CHO are specific inhibitors of caspase-1, caspase-3, caspase-6 and caspase-8, respectively. The effects of caspase inhibitors on CDDP-induced death of HOS cells were examined using the MTT assay. As shown in Fig. 3, DMQD-CHO, VEID-CHO and IETD-CHO inhibited CDDP-induced death of HOS cells in a dose-dependent manner, with the inhibition by IETD-CHO being the most efficient, suggesting that caspase-8 plays a critical role in the death-inducing pathway. We next investigated the effects of these caspase inhibitors on cell death caused by other anticancer drugs using the MTT assay. As shown in Fig. 4, YVAD-CHO did not inhibit cell death induced by CDDP or by any of the other anticancer drugs. DMQD-CHO, VEID-CHO and IETD-CHO inhibited only CDDP-induced cell death. These results confirm that the CDDP-induced death of HOS cells was due to apoptosis via activation of caspases.

We studied further the effects of various caspase inhibitors on CDDP-induced apoptosis of HOS cells by flow cytometry as described previously. As shown in Fig. 5, apoptosis of HOS cells was inhibited completely by DMQD-CHO and efficiently by VEID-CHO and IETD-CHO, but not at all by YVAD-CHO. These results indicate that caspase-3, -6 and -8 are involved in the signaling pathway in CDDP-induced apoptosis of HOS cells.

Caspase-3 has been reported to be commonly activated during the signaling events in apoptosis. Finally, we determined the signaling pathway in CDDP-induced apoptosis of HOS cells by measuring the activity of caspase-3. As shown in Fig. 6, caspase-3 was activated in CDDP-treated HOS cells. In addition, the caspase-8

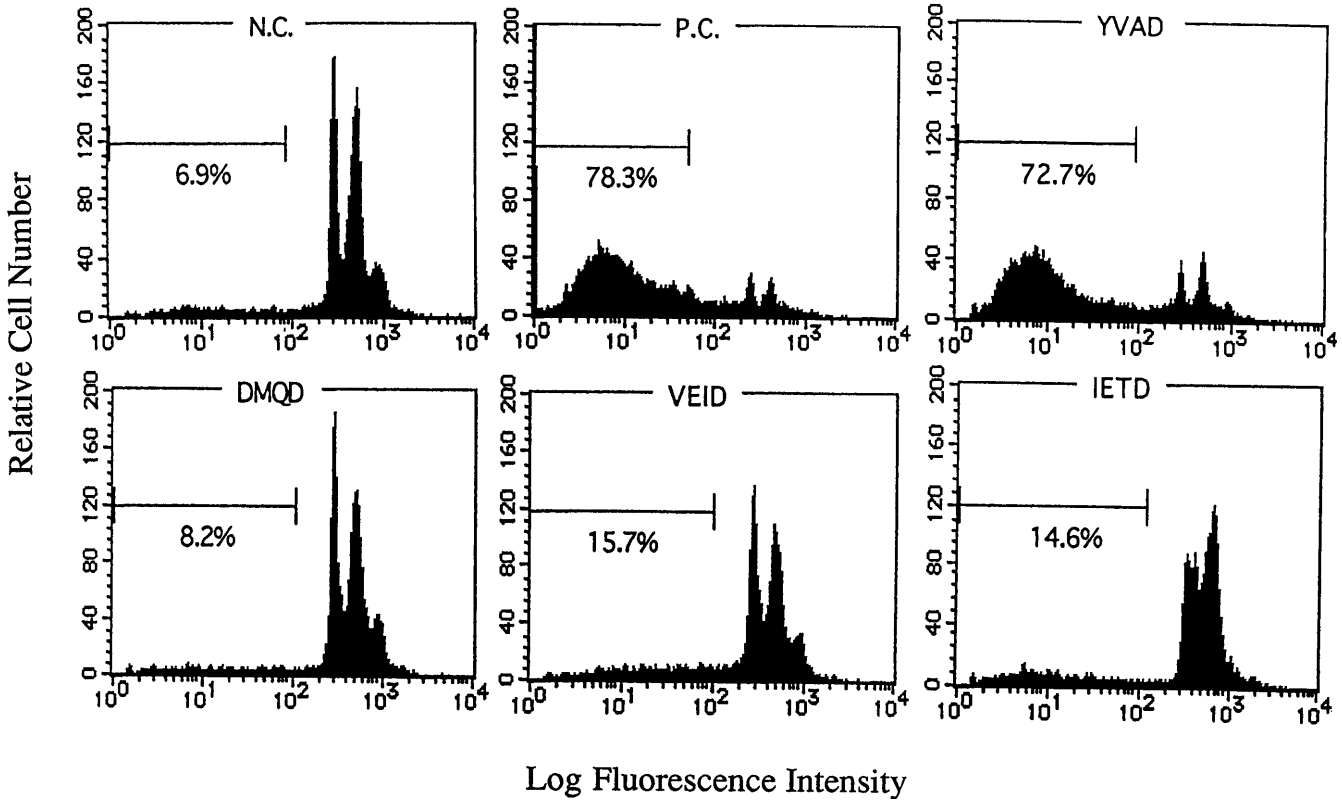


Fig. 5 The effects of caspase inhibitors on CDDP-induced apoptosis of HOS cells. The concentration of each caspase inhibitor used in this experiment was 100 μ*M*. After 12 h of culture with 20 μ*M* CDDP in the presence of each caspase inhibitor, 1×10^6 cells were stained with PI and used for flow cytometry. The percentages of apoptotic cells are indicated as the proportion of cells that contained subG1 DNA (*N.C.* and *P.C.* indicate the normal and positive controls cultured with medium alone or with CDDP without caspase inhibitors, respectively). A representative of three experiments is shown

inhibitor IETD-CHO dramatically inhibited the activation of caspase-3 induced by CDDP, but not the activity of purified caspase-3 directly. However, the caspase-6 inhibitor VEID-CHO did not inhibit either the activation of caspase-3 induced by CDDP or the activity of purified caspase-3. These results demonstrate that caspase-8 is activated upstream of caspase-3, followed by activation of caspase-3 and -6, in the signaling pathway in CDDP-induced apoptosis of HOS cells.

Discussion

Anticancer drugs are traditionally classified either by their origin or their target molecules. Of the anticancer drugs used in this study, the cytotoxic mechanism of VCR is known to be associated with M-phase arrest through the inhibition of spindle formation due to the destruction of microtubules in mitotic cells [14]. CDDP and CPM are alkylating agents which react with DNA and cellular proteins. Their primary mode of action is crosslinking of DNA, inhibiting replication of DNA and transcription of RNA [15]. ADM stabilizes topoisomerase II-DNA complexes and is also considered to inhibit the activity of topoisomerase II [16]. MTX inhibits DNA synthesis of cancer cells and induces arrest in early S-phase [17]. Thus, many studies of anticancer drugs have been concerned with the cell cycle. On the other hand, it has been recently reported that various anticancer drugs induce apoptosis in cancer cells [5, 7, 8]. In this study, we demonstrated that CDDP, but not CPM, ADM, VCR or MTX, specifically induced apoptosis via activation of caspases in osteosarcoma cells. CPM, ADM and VCR induced death of HOS cells mainly by necrosis rather than by apoptosis. In addition, MTX induced inhibition of growth but did not induce the death of HOS cells. These findings indicate that the mechanisms of cell death induced even by the same anticancer drug are different both in terms of the process of inducing cell death and between individual cell lines.

Although many investigators have studied the signaling pathway from the target molecules of anticancer drugs to cell death, it has not yet been clarified. In recent years, however, the details of the sequential activation of proteins belonging to the caspase family during apoptosis have been defined [20]. Their activation has been mainly studied upon triggering of death receptors, such as Fas, the TNF receptor, DR3 or the TRAIL receptor, which recruit caspase-8 as the most proximal effector to the receptor complex. It has been reported that the Fas/FasL system is involved in anticancer drug-induced apoptosis in solid tumor and leukemia cells [21, 22]. In our

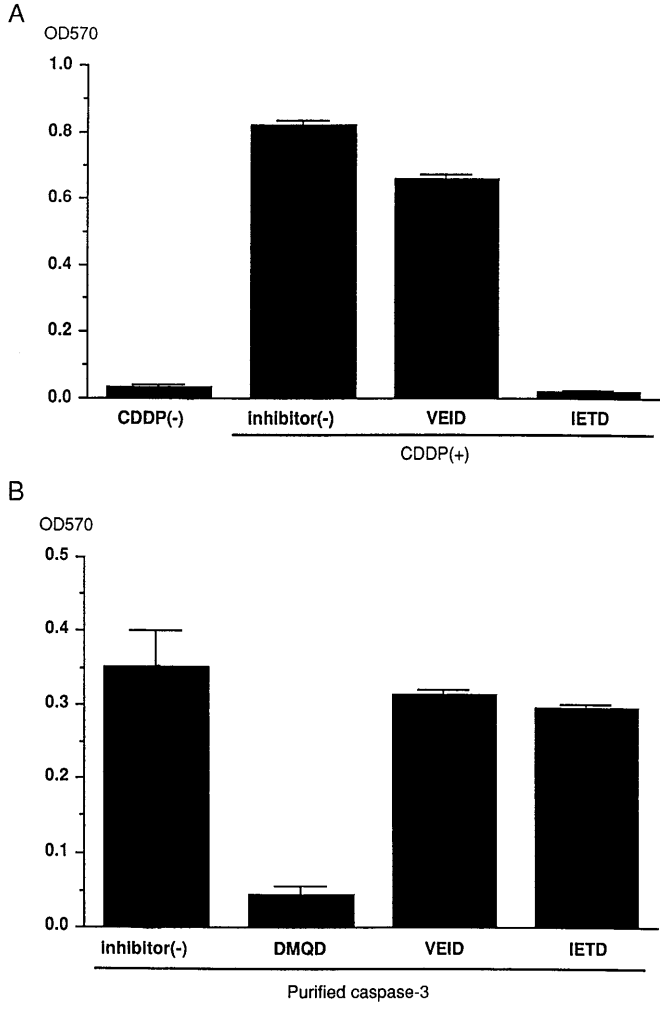


Fig. 6A,B Activation of caspase-3 in HOS cells induced by CDDP. **A** Cells were cultured with 20 μM CDDP and 100 μM of each caspase inhibitor. The cytosol was then extracted and caspase-3 activity was measured as described in Materials and methods. **B** Purified caspase-3 was used as a control for caspase-3 activity and the direct effects of each caspase inhibitor on caspase-3 were examined by the same method as described for **A**. The means ± SD of triplicate determinations are shown in both **A** and **B**

study, however, anticancer drug-treated HOS cells expressed neither Fas nor FasL, suggesting that the Fas/FasL system is not required for anticancer drug-induced death of HOS cells (data not shown). However, receptor-independent apoptosis induced by anticancer drugs via activation of caspase-8 has also been reported [23]. As well as the apoptosis studied in our system, doxorubicin-induced apoptosis in human T-cell leukemia is mediated by cell surface receptor-independent mechanisms with sequential activation of caspases [24]. These findings suggest that proteins belonging to the caspase family are widely involved in apoptosis induced by various stimuli such as anticancer drugs.

In the present study, we demonstrated that only CDDP among the anticancer drugs tested induced apoptosis in HOS cells. We also found that caspase-8, -3 and -6 but not caspase-1 are sequentially activated in CDDP-induced apoptosis. According to the studies of receptor-mediated apoptosis, activation of caspase-8 is the earliest event in the caspase cascade, followed by activation of the intracellular protein Bid, which induces release of cytochrome c from mitochondria and sequentially activates Apaf-1, caspase-9 and caspase-3 [25–28]. However, caspase-8 knockout mice have revealed that caspase-8 is not necessarily required for the induction of apoptosis [29]. In those studies, etoposide, which is one of the most well-known anticancer drugs, can also induce apoptosis without activation of caspase-8 [29]. Moreover, it has been reported that a new family of MAPK is associated with a signaling pathway independent of caspases in etoposide- and camptothecin-induced apoptosis [30]. However, we found in this study that CDDP induced the activation of caspase-8 in HOS cells, confirming the findings of other studies.

Generally, proteins belonging to the Bcl-2 family inhibit the caspase cascade mainly by suppressing the release of cytochrome c from mitochondria [31, 32]. SCC25 cells, which were selected for their resistance to CDDP, overexpress Bcl-xL proteins [33]. Ectopic overexpression of Bcl-xL in parental U87MG cells also results in the suppression of both apoptosis and caspase activation induced by CDDP [34]. These findings confirm the signaling pathway from caspase-8 to caspase-3 via the mitochondrial pathway induced by CDDP. On the other hand, caspase-8 can directly activate caspase-3 without mediation via the mitochondrial pathway [35]. Recently, FLIP and XIAP proteins, which directly inhibit the activation of caspase-8 and caspase-3, have been defined [36, 37]. These findings indicate the possibility that overexpression of proteins such as FLIP and XIAP, as well as of Bcl-2 proteins, in cancer cells causes the resistance to anticancer drugs.

Currently, combination therapy with multiple anticancer drugs is common in the treatment of cancer. The purpose of using anticancer drugs in combination is to increase the therapeutic efficacy, decrease toxicity toward the host and minimize or delay the development of drug resistance. For example, the combination of ADM, CDDP and MTX is clinically the most effective chemotherapy against osteosarcoma. On the other hand, the main cause of therapeutic failure might be the resistance of tumor cells to anticancer drugs including CDDP [38]. Many approaches to overcoming the drug resistance have been tried. Combining anti-Fas antibody with CDDP overcomes resistance in the treatment of bladder cancer [39]. Caffeine enhances the effect of CDDP not only by inhibiting DNA repair but also by inducing apoptotic cell death [40]. In this study we demonstrated that CDDP specifically induces apoptosis via activation of caspases, a mechanism different from that involved in the induction of the death of osteosarcoma cells by other anticancer drugs, suggesting that the sensitivity of HOS cells to CDDP is partially dependent on the signaling pathway inducing apoptosis as reported

previously for another system [41]. This study may provide a cue for the analyses of the resistance of cancer cells to anticancer drugs. To overcome these problems, the way in which caspases are activated and apoptosis induced should be investigated further.

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