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ORIGINAL ARTICLE

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The role of caspase family protease, caspase-3 on cisplatin-induced apoptosis in cisplatin-resistant A431 cell line

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Abstract *Purpose*: Cisplatin (cis-diamminedichloroplatinum(II), CDDP) has been reported to induce apoptosis in cancer cells, the mechanism of the apoptosis in cancer cells induced by CDDP is still unclear. Recent studies have revealed that caspase family of cystine proteases play an important role in the regulation of several apoptotic processes. In this study, whether apoptosis induced by CDDP could be mediated by the activation of caspase-3, a caspase family protease, was investigated. *Methods*: The CDDP-resistant subline A431/CDDP2 from the previously established human epidermoid carcinoma cell line A431 was used. The parent A431 cells (A431/P) and the A431/CDDP2 were exposed to CDDP with or without a caspase family protease inhibitor (Z-Asp-CH_2-DCB), and cellular sensitivity to CDDP was determined. DNA fragmentation was then analyzed, and the caspase-3 protein levels determined by Western blotting following exposure of the cells to CDDP with or without Z-Asp-CH_2-DCB. *Results*: In the A431/P cells, the cytotoxicity of CDDP was clearly reduced by Z-Asp-CH_2-DCB compared with its cytotoxicity in A431/CDDP2 cells. Furthermore, quantitative analysis of DNA fragmentation revealed that Z-Asp-CH_2-DCB inhibited DNA fragmentation induced by CDDP in A431/P cells, but not in A431/CDDP2 cells. Western blotting analysis demonstrated a marked reduction in procaspase-3 protein levels in A431/P cells treated with Z-Asp-CH_2-DCB. In the A431/CDDP2 cells, procaspase-3 protein levels were no different with and without Z-Asp-CH_2-DCB. *Conclusions*: These findings suggest that caspase-3 may mediate apoptosis induced by CDDP, and its induction could represent a novel approach to the effective treatment of malignant tumors.

Key words Caspase family protease · Caspase-3 · Cisplatin resistance · Apoptosis · A431

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Introduction

Apoptosis plays a very important role in cancer and cancer therapy [8, 15, 16]. Cisplatin (cis-diamminedichloroplatinum(II), CDDP) induces apoptosis in some cancer cell lines [2, 18]. However, the molecular mechanism whereby CDDP induces apoptosis is still unclear. Furthermore, since the majority of cells in which the phenomenon of apoptosis induced by CDDP has been described were derived from the hemopoietic system, it is even more important to clarify the molecular mechanism of CDDP-induced apoptosis in solid tumors [1, 5, 19].

Notably, interleukin-1β-converting enzyme (ICE) is a mammalian homologue of CED-3, a positive regulator of apoptosis in the nematode *Caenorhabditis elegans* [23]. During the onset of drug-induced apoptosis, ICE/CED-3 (caspase) family proteases are activated in cancer cells [22]. Caspase-3 (also called CPP32, Yama or apopain), a caspase family protease, appears to play a key role in driving apoptosis [11]. Recently, Mashima et al. have reported an actin cleavage activity in human myeloid leukemia U937 cells during apoptosis induced by antitumor agents. The actin cleavage activity could be attributed to a caspase family protease, because the activity is inhibited by an inhibitor of caspase family proteases, Z-Asp-CH_2-DCB (benzyloxycarbonyl-Asp-$CH_2OC(O)$-2,6,-dichlorobenzene) [12]. Z-Asp-CH_2-DCB is an inhibitor of a broad spectrum of caspase-like proteases [6].

However, after the initial cellular damage, the subsequent cellular responses that result in caspase-3 activation remain largely unknown. Our previous study has shown that reduction of apoptosis may be one of the pathways

for CDDP resistance [14], although the mechanism of reduced apoptosis is not yet fully understood.

In the present study, the role of caspase-3 in CDDP-induced apoptosis in the human epidermoid carcinoma cell line A431 (A431/P) and the CDDP-resistant cell line, A431/CDDP2 was investigated. Whether the caspase-3 family protease inhibitor, Z-Asp-CH_2-DCB, could regulate CDDP-induced apoptosis was also investigated in both cell lines.

Materials and methods

CDDP-resistant A431 cell lines and cell culture

The CDDP-resistant subline A431/CDDP2 previously established from the human epidermoid carcinoma cell line A431 was used [13]. A431/CDDP2 cells, a cell line created by mutagenic induction with mutagen, have 2.7 times more resistance to CDDP than the parent A431 cells (A431/P) in terms of IC_{50}. The cells were cultured in DMEM/F-12 (Life Technologies, Rockville, Md.) containing 5% FBS (Life Technologies) and 1% penicillin-streptomycin at 37 °C in an atmosphere containing 5% CO_2.

Materials

Z-Asp-CH_2-DCB was obtained from TaKaRa Company, Tokyo, Japan. CDDP was kindly provided by Nippon Kayaku, Tokyo, Japan.

In vitro drug sensitivity assay

A431/P and A431/CDDP2 cells (1×10^4/well) were seeded in a 24-multiwell dish. After 24 h, various concentrations (0.01, 0.05, 0.1, 0.2, 0.5, 0.8 and 1.0 μg/ml) CDDP and 100 μg/ml Z-Asp-CH_2-DCB were added to the medium. After an additional 72 h of culture, the viable cells were counted. All counts were done in triplicate, and the viability was assessed by trypan blue dye exclusion assay.

Time-course of drug sensitivity assay

A431/P and A431/CDDP2 cells (1×10^4/well) were seeded in a 24-multiwell dish. CDDP (1.0 μg/ml) and Z-Asp-CH_2-DCB (100 μg/ml) were added to the medium. After various culture periods (1, 3, 5, 7, 12, 24, 48 and 72 h), the viable cells were counted. All counts were done in triplicate, and the viability was assessed by trypan blue dye exclusion assay.

DNA fragmentation assay with ELISA

The release of fragmented DNA into the cytoplasm during apoptotic cell death was measured with a cellular DNA fragmentation ELISA kit (Boehringer Mannheim, Meylan, France). In brief, exponentially growing A431/P and A431/CDDP2 cells were labeled with 10 μ*M* BrdU for 18 h at 37 °C in a humidified atmosphere. After labeling, the cells were resuspended in culture medium. The cells were adjusted to 1×10^5 cells/ml and 100 μl/well were transferred to a 96-well microtiter plate to replicate wells containing CDDP (1.0 μg/ml) and Z-Asp-CH_2-DCB (100 μg/ml) to yield a final volume of 200 μl/well. The cells were incubated for 24, 48 or 72 h at 37 °C in a humidified atmosphere containing 5% CO_2. After incubation, the cells in the microtiter plate were lysed for 30 min at room temperature. The microtiter plate was centrifuged at 250 *g* for 10 min and the supernatant was collected as samples for assay. The samples were transferred to the wells of a precoated microtiter plate. The samples were incubated for 90 min at room temperature. After washing, the samples were denatured and fixed by microwave irradiation (650 W) for 5 min. After cooling the microtiter plate for 10 min at −20 °C, anti-BrdU peroxidase conjugate solution was added and the plate was incubated for an additional 90 min at room temperature. After washing, immunocomplexed anti-BrdU peroxidase was detected and measured at 450 nm using a spectrophotometer (Microplate Reader; Bio-Rad Laboratories, Hercules, Calif.).

Detection of caspase-3 protein by Western blotting

A431/P and A431/CDDP2 cells were cultured at 1×10^6 cells/90-mm dish. These cells were treated with CDDP (1.0 μg/ml) and Z-Asp-CH_2-DCB (100 μg/ml) for 1 h, and were then incubated in CDDP-free medium for 24, 48, or 72 h. Cells were lysed in lysis buffer (1% SDS, 1.0 m*M* sodium vanadate, 10 m*M* Tris, pH 7.4) and centrifuged at 10,000 *g* for 10 min at 4 °C, and the amount of protein was determined using the Bio-Rad DC protein assay (Bio-Rad Laboratories). The lysates were boiled for 3 min, separated on 12.5% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore, Bedford, Mass.) in transblotting buffer (25 m*M* Tris, 192 m*M* glycine, 20% v/v methanol, pH 8.3). The membranes were incubated with blocking buffer comprising 5% non-fat milk and 0.1% Tween 20 in TBS (TBS-T) for 1 h, and then with monoclonal antibodies to mouse anti-caspase-3 (CPP32; Transduction Laboratories, Lexington, Ky.) diluted 1:1000 in the same buffer for 1 h. The membranes were washed three times with TBS-T and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Capel, Durham, N.C.) diluted 1:1000 in TBS-T for 1 h. After the membranes had been washed three times with TBS-T, the signal was visualized by enhanced chemiluminescence with an ECL system (Amersham Corporation, Buckinghamshire, UK).

Results

Drug sensitivity

The IC_{50} of A431/P cells treated with CDDP alone was 0.096 μg/ml, but the IC_{50} A431/P cells also treated with Z-Asp-CH_2-DCB was 0.29 μg/ml. This represents a threefold decrease in resistance to CDDP following treatment with Z-Asp-CH_2-DCB. The IC_{50} of A431/CDDP2 cells treated with CDDP alone was 0.26 μg/ml, but the IC_{50} of A431/CDDP2 cells also treated with Z-Asp-CH_2-DCB was 0.46 μg/ml. This represents a 1.77-fold decrease in resistance to CDDP following treatment with Z-Asp-CH_2-DCB (Fig. 1). In both cell lines, cell death was reduced by treatment with Z-Asp-CH_2-DCB. The time-course of the drug sensitivity assay showed that the sensitivity of A431/P cells treated with Z-Asp-CH_2-DCB was significantly different from that of A431/P cells treated with CDDP alone after 7 h, but the sensitivity of A431/CDDP2 cells treated with Z-Asp-CH_2-DCB was only slightly different from that of A431/CDDP2 cells treated with CDDP alone (Fig. 2). In brief, treatment with Z-Asp-CH_2-DCB increased resistance to CDDP in A431/P cells to a greater extent than in A431/CDDP2 cells.

DNA Fragmentation with ELISA

To quantify the apoptotic response to treatment with CDDP in A431/P and A431/CDDP2 cells, we used an

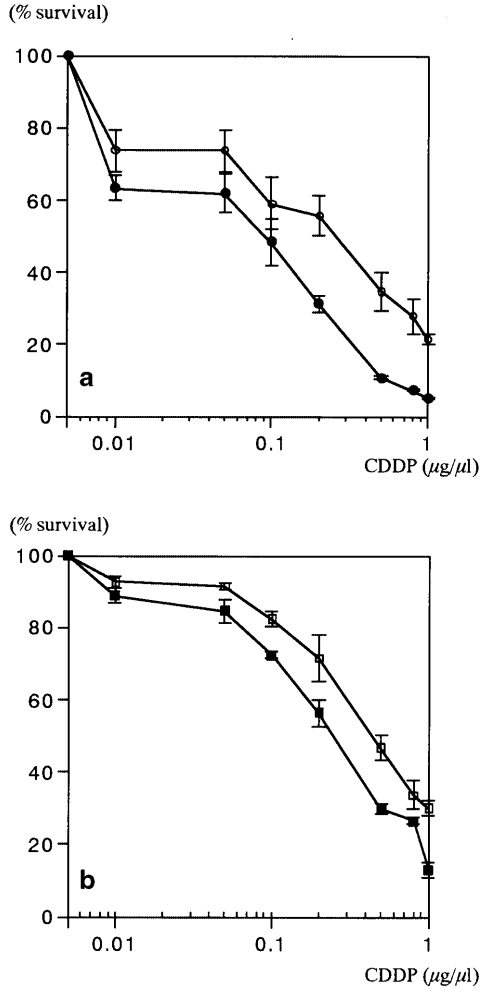

Fig. 1a, b Dose-response curves for CDDP with or without Z-Asp-CH_2-DCB in A431/P cells (**a**) and A431/CDDP2 cells (**b**). **a** ● A431/P cells without Z-Asp-CH_2-DCB, ○ A431/P cells with Z-Asp-CH_2-DCB; **b** ■ A431/CDDP2 cells without Z-Asp-CH_2-DCB, □ A431/CDDP2 cells with Z-Asp-CH_2-DCB. Points and bars represent the means ± SD from three determinations

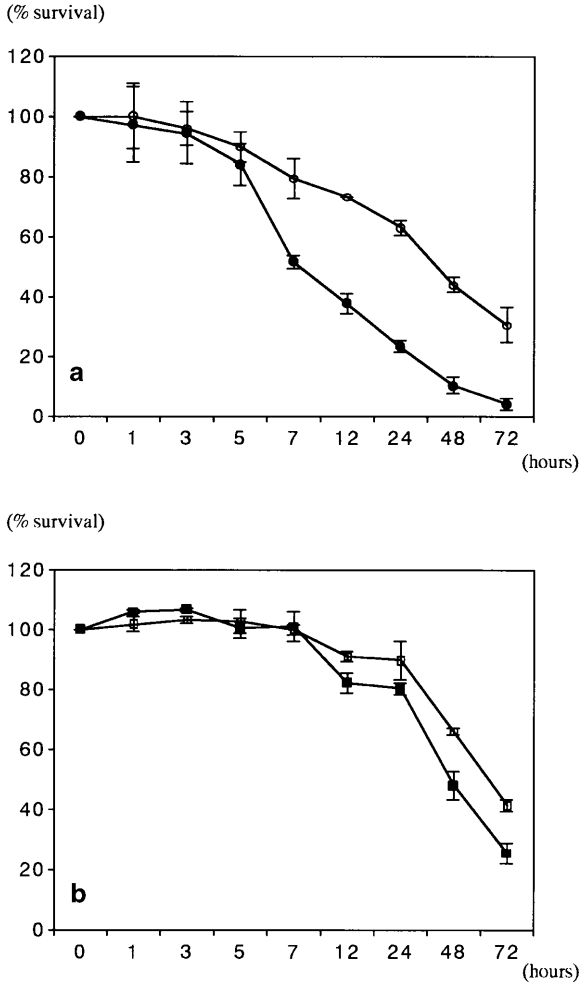

Fig. 2a, b Time-course of CDDP sensitivity assay with or without Z-Asp-CH_2-DCB in A431/P cells (**a**) and A431/CDDP2 cells (**b**). **a** ● A431/P cells without Z-Asp-CH_2-DCB, ○ A431/P cells with Z Asp-CH_2-DCB; **b** ■ A431/CDDP2 cells without Z-Asp-CH_2-DCB, □ A431/CDDP2 cells with Z-Asp-CH_2-DCB. Points and bars represent the means ± SD from three determinations

ELISA that allowed the specific detection and quantification of BrdU-labeled DNA fragments. Figure 3 shows the time-course of quantitative DNA fragmentation in CDDP and Z-Asp-CH_2-DCB-treated cells. The amount of DNA fragmentation in A431/P cells treated with CDDP increased in a time-dependent manner. This shows that treatment of A431/P cells with Z-Asp-CH_2-DCB clearly reduced but did not completely block CDDP-induced apoptosis. On the other hand, DNA fragmentation in A431/CDDP2 cells was not significantly different from that in A431/CDDP2 treated with Z-Asp-CH_2-DCB.

Detection of caspase-3 protein by Western blotting

Western blotting analysis of procaspase-3 protein levels is shown in Fig. 4. Expression of procaspase-3 protein was detected in A431/P cells at all the experimental time-points. The expression in A431/P cells treated with Z-Asp-CH_2-DCB was clearly weaker than in untreated A431/P cells. CDDP-induced apoptosis in A431/P cells was closely correlated with procaspase-3 protein levels. In contrast, procaspase-3 protein expression in A431/CDDP2 was weaker than in A431/P, or not detected. The procaspase-3 protein expression levels were similar in A431/CDDP2 cells treated with Z-Asp-CH_2-DCB.

Discussion

Apoptotic cell death has been shown to play a key role in many fundamental and physiological processes in the development of multicellular organisms and the immune system [3, 7]. Apoptosis is an active process of cell death which occurs in two physiological stages, commitment and execution [4]. Apoptotic execution in mammalian cells is initiated by specific cystine proteases of the ICE

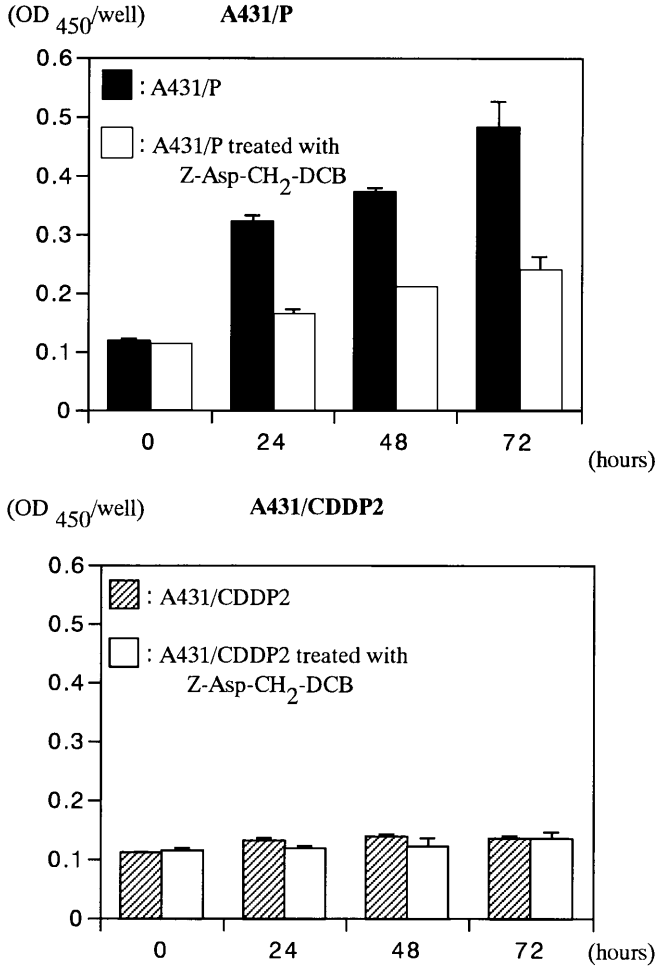

Fig. 3 DNA fragmentation ELISA assay with or without Z-Asp-CH_2-DCB in A431/P cells (**a**) and A431/CDDP2 cells (**b**). Each value is the mean ± SD of three determinations

family [23]. The caspases, members of the cystein protease family, which are homologous to the *Caenorhabditis elegans* death gene CED-3, are a common and critical component of the cell death pathway [22]. In clinical oncology, the success of chemotherapy is determined by the effectiveness of activation of apoptosis in

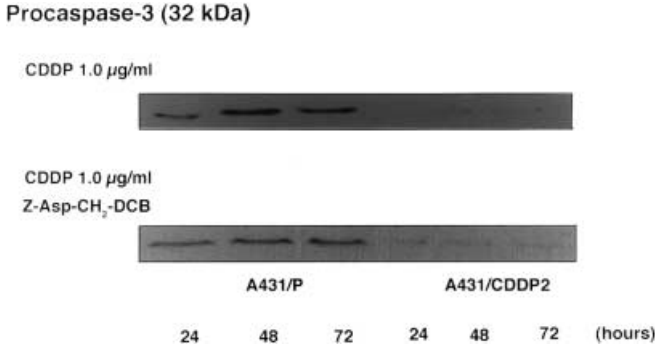

Fig. 4 Detection of procaspase-3 protein in A431/P cells and A431/CDDP2 cells with or without Z-Asp-CH_2-DCB by Western blotting

tumor cells in response to the DNA damage [8]. However, the precise molecular mechanisms regulating apoptosis induced by DNA damage are unknown. To improve the success of cancer treatment, it is important to determine how apoptosis provoked by DNA damage is regulated [9]. We have previously reported that a CDDP-resistant subline, A431/CDDP2, shows a reduction in apoptosis induced by CDDP [14].

Caspase-3, a caspase family protease, is a common regulator of chemotherapy-induced apoptosis in tumors [1]. In the study reported here, the effects of the caspase family protease inhibitor, Z-Asp-CH_2-DCB, on apoptotic induction in A431/P and A431/CDDP2 cells treated with CDDP were investigated. A431/P cells were induced to undergo apoptosis by treatment with CDDP, accompanied by expression of caspase-3, but Z-Asp-CH_2-DCB treatment prevented both apoptosis and the expression of caspase-3 in these cells. In contrast, A431/CDDP2 cells treated with CDDP did not show an increase in apoptotic DNA fragmentation at 24, 48 and 72 h, although CDDP achieved significant cytotoxicity between 24 and 72 h in this cell line. Z-Asp-CH_2-DCB did not affect the apoptosis and expression of procaspase-3 in A431/CDDP2 cells (Fig. 4). These results suggest that escape from apoptosis mediated by caspase-3 may be one of the molecular mechanisms involved in CDDP resistance in epidermoid cell carcinoma.

Recent studies suggest that all caspase family members are Asp-based proteases [20, 21], and that cells may contain a common substrate which, when cleaved by an appropriate Asp-based protease, can cause apoptosis [10]. Caspase family proteases may cause cell death directly by cleaving proteins, and the activation of caspase family proteases may represent a final common pathway of apoptosis [17]. However, it has been reported that caspase-3 may not be the only protease that triggers apoptosis in cells treated with CDDP [21]. In fact, Z-Asp-CH_2-DCB did not completely inhibit apoptosis in A431/P cells, and did not influence apoptosis in A431/CDDP2 cells. Therefore, there may be other proteases that function separately in the induction of apoptosis in cells treated with CDDP.

Inhibition of apoptosis could be of great importance in cancer chemotherapy because it may lead directly to the resistance of cancer cells to CDDP. In conclusion, we found in the present study that inhibition of apoptosis mediated by caspase-3 is one of the important mechanisms of CDDP resistance. Our results indicate that the abolition of apoptosis inhibition may be a novel strategy for treatment of CDDP-resistant cancers. Additional extensive studies are needed to establish an effective therapy to suppress the inhibition of apoptosis induced by CDDP.

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