Activation of Actin-cleavable Interleukin 1β-converting Enzyme (ICE) Family Protease CPP-32 during Chemotherapeutic Agent-induced Apoptosis in Ovarian Carcinoma Cells

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ABSTRACT

We have previously reported that actin cleavage activity (ACA) by interleukin 1β-converting enzyme (ICE) family protease was elevated during anticancer drug-induced apoptosis in human leukemia U937 cells. In this study, the involvement of ACA in the drug-induced apoptosis in solid tumor cells was investigated. Human ovarian carcinoma OVCAR-3 cells undergo apoptotic cell death when cells are treated with chemotherapeutic agents such as cisplatin and etoposide. The induction of the actin cleavage activity accompanied the development of apoptosis. ICE/ced-3 family protease inhibitors such as Z-VAD-CH₂DCB and Z-EVD-CH₂DCB at 100 μg/ml prevented both the emergence of ACA and the morphological change, characteristics of apoptosis, in cisplatin-treated OVCAR-3 cells. The ACA in apoptotic OVCAR-3 cell lysate was greatly adsorbed by antibody against CPP-32, an ICE family protease. Furthermore, the immunoprecipitated CPP-32 from OVCAR-3 lysate could cleave actin to generate a 15-kDa fragment, as did the apoptotic OVCAR-3 cell lysate, indicating that CPP-32 is a major protease responsible for the ACA. The activation of CPP-32 in the drug-treated cell lysate was verified with Western blot analysis. Our present results indicate that CPP-32, an actin cleavage ICE/ced-3 family protease, could be a common mediator involved in the process of chemotherapy-induced apoptosis of cancer cells.

INTRODUCTION

Apoptosis, as a genetically controlled program, has important roles in a variety of biological processes (1–5). Morphologically, it is different from necrosis in many of its characteristic changes, including nuclear chromatin condensation, cytoplasmic blebbing, and packaging of nuclear fragments into small apoptotic bodies (1). The realization that chemotherapy can also induce apoptosis in some cancer cells both in vitro (6, 7) and in vivo (8, 9) indicates that apoptosis may play a very important role in cancer and cancer therapy. However, the molecular mechanism whereby anticancer drugs induce apoptosis is still unclear. Furthermore, since the majority of cells in which the phenomenon of drug-induced apoptosis has been described are derived from the hemopoietic system, it is even more important to clarify the molecular mechanism of anticancer drug-induced apoptosis in solid tumors.

Although the mechanism of apoptosis is not yet fully understood, the morphological alterations are accompanied by a variety of biochemical changes. Several investigators have reported that activation of intracellular proteases is a crucial event in apoptosis (10–13). Notably, a series of ICE/ced-3 family protease genes have been isolated, and apoptosis was induced by the overexpression of these proteases in a number of cells (14–19). Some cellular proteins, such as PARP (20), Ul-70kD (21), Lamin B (22), DNA protein kinase (23), and RB (24), have been cleaved by such ICE/ced-3 family proteases during apoptosis. These results suggest that ICE/ced-3 family proteases may be positive regulators of apoptosis. However, as shown in the recent report using ICE knockout mice (25), ICE itself is not always indispensable in the induction of apoptosis. It is still uncertain how these proteases participate in a common signal pathway of apoptosis triggered by various stimuli.

Recently, we reported that there is an actin cleavage activity which occurs in human myeloid leukemia U937 cells during apoptosis induced by antitumor agents, and the activity could be attributed to an ICE/ced-3 family protease because ACA is inhibited by Z-Asp-CH₂-DCB (benzyloxycarbonyl-Asp-CH₂OC(O)-2,6-dichlorobenzene), an inhibitor of ICE family protease (26). Almost at the same time, another group reported that actin could be cleaved by ICE (27). However, the ACA is distinct from ICE itself because ICE is not activated in apoptotic U937 cells. In the present study, we showed that chemotherapeutic agents could induce apoptosis in human ovarian carcinoma (OVCAR-3) cells. The occurrence of ACA accompanied the development of apoptosis and was inhibited by ICE/ced-3 family protease inhibitors. The examination with fluorogenic substrates DEVD-MCA and YVAD-MCA and the immunoprecipitation study showed that CPP-32 was a major component responsible for the ACA. These results indicate that CPP-32, an actin cleavage ICE/ced-3 family protease, could play an important role in chemotheraphy-induced apoptosis in solid tumor cells.

MATERIALS AND METHODS

Materials. cDDP and VP-16 were generous gifts from Bristol-Myers Squibb Co., Ltd. Tokyo, Japan). Z-VAD-CH₂DCB and Z-EVD-CH₂DCB were synthesized as described previously (28). A fluorogenic substrate for CPP-32 protease, DEVD-MCA, and a fluorogenic substrate for the ICE protease YVAD-MCA were purchased from the Peptide Institute (Osaka, Japan).

Cell Lines and Cell Culture. Human ovarian carcinoma cell lines OVCAR-3 and OVCAR-8 were supplied by the National Cancer Institute (NIH, Bethesda, MD). All cells were maintained in RPMI 1640 (Nissui Co., Ltd. Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum and 100 μg/ml of kanamycin in a humidified atmosphere of 5% CO₂ and 95% air.

Drug Treatment. Logarithmically growing OVCAR-3 or OVCAR-8 cells were harvested by trypsinization and seeded at an initial density of 2 × 10⁴ cells in 10 ml of fresh medium in a 10-cm dish. After overnight incubation, cDDP or VP-16 was added from 1000-fold concentrated stocks in DMSO with a final concentration of 0.1% DMSO in medium, which had no influence on cells. After various periods of incubation, floating cells and trypsinized adherent cells were combined and sedimented at 800 × g for 10 min, and then ACA, flow cytometry, and nuclear staining assays were performed.

Flow Cytometry and Nuclear Staining Assays. After treatment of OVCAR-3 or OVCAR-8 cells with cDDP or VP-16, cells were harvested by trypsinization and fixed in 70% ethanol. Following treatment with RNAse (1 mg/ml in 0.1 M phosphate buffer, pH 7.0), the cells were stained in propidium iodide solution (50 mg/ml in 0.1% sodium citrate, 0.1% NP40) and then analyzed by flow cytometry using a FACScan (Becton Dickinson, Sunnyvale, CA). Staining of the nuclear DNA was carried out according to the method of Vistica et al. (29). Briefly, cells were stained with propidium iodide (1 mg/ml) for 30 min at room temperature. For metaphase analysis, cells were stained with 2 μg/ml of DAPI (4',6-diamidino-2-phenylindole).
analyzed using a Becton Dickinson FACScan flow cytometer (Braintree, MA). To assay the nuclear morphology, harvested cells were washed with PBS, fixed with a methanol-acetic acid solution (3:1) for 30 min, placed on slides and allowed to stand for 30 min, and then stained with 1 mg/ml of DAPI for 30 min. The nuclear morphology of cells was visualized using a fluorescence microscope (UXF-II A; Nikon, Tokyo, Japan).

**ACA Assay.** An ACA assay was carried out as described previously (26). In brief, rabbit muscle actin was labeled with biotin by incubation with 100 μg/ml of sulfo-scinicinimidyl-6-(biotinamido) hexanoate-biokin (Pierce, Rockford, IL) at 25°C for 40 min in 1 mM HEPES (pH 8.0). The drug-treated cells were harvested, washed with PBS, and then resuspended in the suspension buffer [10 mM Tris-HCl (pH 8.1), 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml of leupeptin, and 1 μg/ml of pepstatin], freeze-thawed four times, and finally centrifuged for 20 min at 70,000 × g. The biotinylated actin (0.2 μg/assay) was incubated with cell cytosolic fractions at 37°C for 3 h in the ICE assay buffer [20 mM HEPES (pH 7.5), 2 mM DTT, and 10% (v/v) glycerol], and then the reaction mixtures were separated by 15–25% gradient polyacrylamide gels (Daiichi Chemical, Tokyo, Japan). The electrophoresed proteins in the polyacrylamide gel were transblotted onto a nitrocellulose membrane. After blocking, the blotted membrane was incubated with a peroxidase-conjugated avidin (ABC kit; Vector Laboratories, Burlingame, CA) at 25°C for 1 h, washed several times with PBS-0.5% Tween 20, soaked in an enhanced chemiluminescence mixture (ECL; Amersham, Buckinghamshire, United Kingdom), and exposed to Kodak X-Omat AR film.

**Assay of CPP-32 and ICE Proteolytic Activity.** The cDDP-treated cell lysate (10 μg of protein) was incubated with 1 mM DEVD-MCA or YVAD-MCA at 37°C for 30 min, and the release of amino-4-methylcoumarin was monitored by a spectrofluorometer (Hitachi F-2000), using an excitation wavelength of 380 nm and an emission wavelength of 460 nm. One unit was defined as the amount of enzyme required to release 1 pmol amino-4-methylcoumarin/min at 37°C.

**Western Blot Analysis.** CPP-32 Western blot analysis was performed by using anti-CPP-32 monoclonal antibody (Transduction Laboratories, Lexington, KY) according to the manufacturer. In brief, the cDDP-treated cell lysate was electrophoresed by SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking, the membrane was incubated with anti-CPP-32 antibody for 2 h at 25°C. Immunodetection was performed using the enhanced chemiluminescence kit for Western blotting detection (Amer sham).

**Immunoprecipitation.** For the immunoprecipitation study, anti-CPP-32 antibody was bound with protein A-Sepharose at 4°C for 1 h. The antibody/protein A-Sepharose complex was then incubated with cDDP-treated OVCAR-3 cell lysate at 4°C for 1 h. After centrifugation at 15,000 × g for 10 min, the supernatant was collected as the immunoadsorbed supernatant, and the precipitated pellet was washed several times with ICE assay buffer. The ACA assay was performed as described above by incubating the supernatant or the pellet with actin for 3 h at 37°C.

**RESULTS**

**Chemotherapeutic Agent-induced Apoptosis in OVCAR-3 Cells.** When human ovarian carcinoma OVCAR-3 cells were treated with cDDP or VP-16, cell growth was significantly inhibited, and cell death was dose dependent. To elucidate whether this drug-induced cell death was apoptosis, we examined the nuclear changes using DAPI staining. Fig. 1 represents fluorescence microscopy of DAPI staining and similar results were obtained (data not shown). The FACScan analysis showed that the apoptotic cell population in cDDP- and VP-16-treated cells were about 28% and 15%, respectively (Fig. 1B).

This apoptotic process is time and dose dependent, as shown in Fig. 2. The time course of the cDDP treatment at 10 μg/ml indicated that no significant change was apparent in OVCAR-3 cell morphology at 4 h after cDDP treatment. Morphological change was observed after 12 h, and about 15% of the cells appeared to detach from the cell layer with apoptotic characteristics as determined by flow cytometric analysis. By 24 h, these changes were more pronounced, and after 48 h of drug treatment, the apoptotic cells increased to about 55%, although some cells were still attached to the tissue culture plate. To confirm the progression of apoptosis, we measured the apoptotic cell population by counting those with condensed and fragmented nuclei after DAPI staining and similar results were obtained (data not shown).
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sequence of the 15-kDa fragment, which is a potential ICE/ced-3 family protease cleavage site (26). After cDDP treatment for 24 to 48 h, the ACA in the OVCAR-3 cell lysate increased according to the increased population of apoptotic cells. A similar result was obtained when OVCAR-3 cells were treated with VP-16 (Fig. 3B).

Fig. 4 represents the difference between the apoptosis-sensitive OVCAR-3 cells and apoptosis-resistant OVCAR-8 cells. When OVCAR-3 cells were treated with 3 μg/ml of cDDP for 2 days, about 28% underwent morphological apoptosis (see Figs. 1 and 2), and a relevant ACA was detected in the OVCAR-3 cell lysate (Fig. 4). On the contrary, when OVCAR-8 cells were treated in the same way and at the same time, the cells showed no morphological changes characteristic of apoptosis (see Fig. 1), and no ACA activity was detected in the cell lysates (Fig. 4). The results indicate that OVCAR-8 cells are resistant to chemotherapy-induced apoptosis compared with OVCAR-3 cells, and the activation of ACA correlates well with the progression of apoptosis induced by these agents.

Inhibition of ACA and apoptosis by ICE/ced3 family protease inhibitors. To characterize the apoptosis-associated ACA activity, we tested the effect of ICE/ced-3 family protease inhibitors, such as Z-EVD-CH2DCB and Z-VAD-CH2DCB, on the development of apoptosis and ACA. The inhibitors at 100 μg/ml completely prevented cDDP-induced morphological changes of apoptosis (Fig. 5A) and ACA in the cytosolic fraction (Fig. 5B), which suggested that the ICE/ced-3 family protease plays an important role in these activities. We further examined the effect of Z-EVD-CH2DCB and Z-VAD-CH2DCB on the ACA in the cDDP-treated OVCAR-3 cell lysate. Z-EVD-CH2DCB at 0.01 μM and Z-VAD-CH2DCB at 0.1 μM completely inhibited the ACA in the cell extracts (Fig. 5C), indicating that an ICE/ced-3 family protease is responsible for ACA in OVCAR-3 cells.

CPP-32 protease is a major component of ACA. To identify the protease species responsible for ACA, the cleavage of the fluoro-
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Fig. 5. Effect of ICE/ced-3 family protease inhibitors on morphological change of apoptosis (A) and actin cleavage activity (B and C) in cDDP-treated OVCAR-3 cells. A and B. OVCAR-3 cells were incubated without (panel 1) or with 10 µg/ml of cDDP for 36 h in the absence (panel 2) or presence of 100 µg/ml of Z-EVD-CH₂DCB (Z-EVD; panel 3) or Z-VAD-CH₂DCB (Z-VAD; panel 4) which was added just before cDDP treatment. Cell morphology was determined by fluorescence microscopy after staining with DAPI (A) and ACA was examined (B). C. ACA of cDDP-treated OVCAR-3 cell lysate was examined in the absence or presence of Z-EVD-CH₂DCB (Z-EVD) or Z-VAD-CH₂DCB (Z-VAD) which was added just before incubation with actin.

genic substrates, DEVD-MCA for CPP-32 protease and YVAD-MCA for ICE protease, were examined. The cleavage activity for DEVD-MCA was marginal in the control OVCAR-3 cell lysate, but the activity increased up to about 300-fold in cDDP-treated OVCAR-3 cell lysate. On the other hand, YVAD-MCA cleavage activity was negligible in both the control and the cDDP-treated cell lysates (Fig. 6A). These results suggest that ACA is a CPP-32-like protease. Western blot analysis verified that the CPP-32 protease was processed to a 17-kDa fragment, indicating that the CPP-32 was activated in the cDDP-treated OVCAR-3 cell lysate (Fig. 6B).

To confirm that CPP-32 was a component of the ACA, an immunoprecipitation study was carried out. After immunoadsorbance with the antibody against CPP-32, ACA in the cDDP-treated OVCAR-3 cell lysate was attenuated (Fig. 7). Furthermore, the immunoprecipitated CPP-32 could cleave actin to generate a 15-kDa fragment, as did the cDDP-treated cell lysate (Fig. 7). These results indicate that CPP-32 is a major protease responsible for the ACA in the cDDP-treated OVCAR-3 cell lysate.

DISCUSSION

In this report, we investigated the mechanism of apoptosis induced by chemotherapeutic agents in solid OVCAR-3 cells and found that ACA was associated with the development of apoptosis. ACA was initiated in OVCAR-3 cells by chemotherapeutic drugs such as cDDP and VP-16, and the cells showed the morphological changes characteristic of apoptosis. Inhibitors of ICE/ced-3 family proteases, such as Z-VAD-CH₂DCB and Z-EVD-CH₂DCB, prevented development of ACA and morphological apoptosis caused by the anticancer drugs. They also inhibited ACA in vitro. These results indicate that the ACA initiation is a critical event in the process of chemotherapy-induced apoptosis in OVCAR-3 cells.

Recently, actin was reported to be cleaved by ICE (27), but this is not the case with our system. We previously reported that the ACA was activated in human myeloid leukemia U937 cells during chemotherapy-induced apoptosis and that the activity was not due to ICE (26). Recently, we found that a member of ICE/ced-3 family proteases, CPP32, is responsible for the ACA in the apoptotic U937 cells. In this study, we identified that CPP-32 is also a major protease responsible for ACA in OVCAR-3 cells undergoing chemotherapy-induced apoptosis. These results suggest that CPP-32 could be a common regulator of chemotherapy-induced apoptosis in tumors.

During apoptosis, several proteins, such as PARP, U1-70kD, and

\[ \text{T. Mashima et al., unpublished results.} \]
with DEVD-MCA or YVAD-MCA as described in “Materials and Methods.” Bar, SD. B, cell lysate. The cDDP-treated OVCAR-3 cell lysate was immunoprecipitated with mouse CPP32 in the apoptotic process. However, it is known that actin is a functional relevance of these protein cleavages in apoptosis remains unknown. RB, are cleaved by ICE/ced-3 family proteases. However, the functional relevance of these protein cleavages in apoptosis remains unknown. At present, we do not know the role of actin cleavage by CPP32 in the apoptotic process. However, it is known that actin is a major structural component of all cell types. In addition, actin is involved in many cellular functions, like cytokinesis, membrane ruffling, cell locomotion, attachment of cells to a substratum, and maintenance of cell shape. Thus, actin and actin-containing structures appear to have a variety of functions within a cell. Therefore, actin cleavage could result in morphological changes typically observed in apoptotic cells. In addition to the structural functions, actin is also a natural inhibitor of DNase I (29), and the cleaved fragments do not exhibit the inhibitory activity (27). Furthermore, actin was selectively repressed during the programmed death of insect skeletal muscle (30). According to these observations, we speculate that actin and its cleavage could have a regulatory function in apoptosis.

Solid tumors are often refractory and difficult to cure with chemotherapy. One of the important reasons may be that solid tumors are more resistant to apoptosis than leukemic cells. It should be noted that the development of apoptosis in OVCAR-3 cells takes longer (1–2 days) as compared with leukemic cells which undergo apoptosis within several hours after drug treatment. Solid tumor cells could have the survival advantage because of the slow progression of apoptosis, during which time cells could develop protective responses and repair damages caused by the anticancer drugs. We believe that elucidation of the molecular mechanism of chemotherapy-induced apoptosis in solid tumors can enhance the development of cancer chemotherapy.

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Fig. 6. Activation of CPP-32 protease in cDDP-treated OVCAR-3 cells. A, proteolytic activities in the cell lysate treated with 10 µg/ml of cDDP for 0 and 48 h were measured with DEVD-MCA or YVAD-MCA as described in “Materials and Methods.” Bar, SD. B, CPP-32 Western blot analysis was carried out with cell lysate treated without (Lane 1) or with 10 µg/ml of cDDP for 48 h (Lane 2).

Fig. 7. Actin cleavage by immunoprecipitated CPP-32 from cDDP-treated OVCAR-3 cell lysate. The cDDP-treated OVCAR-3 cell lysate was immunoprecipitated with mouse IgG (Lane 1), anti-CPP-32 antibody (Lane 2), and PBS (Lane 3), and then the ACA in the supernatant and the pellet was analyzed.

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