

Continuous Exposure to Low-Dose Cisplatin and Apoptosis

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A high concentration of cisplatin induces apoptosis in many tumor cell lines. Whether cisplatin induces apoptosis even in a controlled release formulation has not been determined. We therefore studied the relationship between the dosing regimen of cisplatin and the induction of apoptosis in rat hepatoma AH-109A cells. A colorimetric assay was used to quantify cell proliferation and viability, and caspase activity was determined using an exogenous fluorogenic peptide substrate. When delivered as a single dose, cisplatin caused a dose-dependent inhibition of AH-109A growth and enhancement of caspase-3 activity. Also, DNA laddering was detected in cells that had elevated caspase-3 activity. However, caspase-3 activity was low and DNA laddering and a sub-G1 population were not detected when cells were treated with a combination of cisplatin and the caspase inhibitor Z-VAD-FMK. These results suggest that cisplatin is cytotoxic in AH-109A cells because it induces apoptosis. We next examined intermittent exposure to cisplatin to estimate the effects of continuous exposure by a controlled release formulation. Cisplatin was divided into equal parts and was added intermittently into the medium resulting in the same final concentration as the single dose. The individual additions alone were not cytotoxic, but all of the doses together had a similar cytotoxic effect as a single exposure of cisplatin. The intermittent exposure resulted caspase-3 activity even higher than a single dose. These findings indicate that cisplatin induces apoptosis in AH-109A cells when delivered continuously even at the concentration that alone have no activity.

Key words apoptosis; low-dose; cisplatin (CDDP)

Cisplatin (CDDP) is widely used for the treatment of a variety of human malignancies.¹⁾ CDDP is a well-known DNA-damaging agent, and it is currently thought that DNA platination is an essential first step in its cytotoxic activity.^{2–5)} Recent evidence has shown that apoptosis is a marker of tumor cells that have been exposed to CDDP.^{6–8)} In addition, CDDP has been reported to induce apoptosis at higher concentrations than the clinical dose.

The CDDP is a type of drug whose effects depends on the area under the concentration–time curve (*AUC*),^{9,10)} which means that continuous exposure low concentrations of the drug have the same effect as a single short-term exposure at a high concentration. Continuous infusion or multiple administrations of low-dose CDDP is an excellent regimen for cancer patients.^{11,12)} For this reason, sustained release formulations of CDDP or CDDP derivatives have been developed.^{13–16)} However, it has not been clear whether continuous exposure with low-dose CDDP can induce apoptosis as effectively as a single high-dose exposure CDDP.

We therefore compared the ability of CDDP to induce apoptosis when delivered as a single high-dose exposure or as multiple low-dose exposures. In these studies, the induction of apoptosis was assessed by measuring caspase-3 activity, DNA fragmentation, and the percent of cells in the sub-G1 phase.

MATERIALS AND METHODS

Cell Culture Rat ascites hepatoma AH-109A cells were serially transplanted by intraperitoneal injection in Donryu rats. The cells taken from ascites were grown in RPMI-1640 medium containing 20% fetal bovine serum.

Drug Exposure Cells were seeded into 25 cm² flasks at 4 × 10⁵ cells/ml. Single exposures to CDDP (1.0 μg/ml, Kyowa Hakko Kogyo, Tokyo, Japan) were carried out at the starting point (0 h). When CDDP was delivered as 8 low-con-

centration exposures, the cells were dosed every 2 h between 0 to 9 h and between 24 to 33 h, and when 16 low-concentration doses, it was delivered every 1.5 h between 0 and 10.5 h and between 24 to 34.5 h.

Growth Inhibition Assay Cells were seeded into 96-well plates at 4 × 10⁵ cells/0.1 ml/well following single or multiple exposures to CDDP. To determine the growth inhibitory effect, 20 μl of a solution containing 2 mM WST-1 (Dojindo, Kumamoto, Japan) and 0.2 mM 1-methoxy methylphenazinium methylsulfate was added to each well followed by incubation for 6 h. The absorbance of each well was measured at 450 nm with a reference wavelength of 650 nm using a Model 550 microplate reader (BIO-RAD, Tokyo, Japan).

DNA Fragmentation Assay DNA laddering in apoptotic cells was detected using an ApopLadder Ex kit (Takara Bio, Shiga, Japan). Briefly, cells were suspended in lysis buffer and centrifuged. The supernatants were mixed with 10% Sodium dodecylsulfate (SDS) and Enzyme A and then incubated at 56 °C for 1 h. Enzyme B was then added, and the mixture was incubated for an additional 1 h at 37 °C. To precipitate DNA fragments, the preparation was mixed with precipitant and ethanol and then stored for 15 min at –20 °C. DNA pellets were washed with 80% ethanol and suspended in Tris-borate-EDTA buffer. DNA fragmentation was visualized by electrophoresis on a 2% agarose gel containing ethidium bromide.

Caspase Assay Caspase activity was measured using the Caspase Fluorometric Assay Kit (MBL, Tokyo, Japan). Briefly, cells were suspended in lysis buffer and centrifuged. The protein concentration of the supernatants was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL U.S.A.). Samples (12.5 μg protein) were incubated in reaction buffer containing a specific substrate for caspase-3 (Ac-DEVD-AFC) at 37 °C for 1 h. After an additional 1-h incubation at room temperature, AFC liberated from each substrate was measured using a Versa Fluor fluorometer (Bio-Rad,

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Cell Cycle Analysis Cells were washed and resuspended in phosphate buffered saline (PBS). DNA was stained with 4,6-diamidino-2-phenylindole, and cell cycle analysis was performed using a Cell Counter Analyser (Partec, Münster, Germany). Each analysis was done with using about 1×10^4 counts of cells.

RESULTS

Apoptosis Following a Single Exposure to CDDP The growth rate, caspase-3 activity, and DNA fragmentation of AH-109A cells are shown in Fig. 1. CDDP inhibited the growth of AH-109A cells in a concentration-dependent manner. CDDP had an IC_{50} of approximately $0.25 \mu\text{g/ml}$. At concentrations greater than $1 \mu\text{g/ml}$, it completely inhibited cell growth, significantly increased the caspase-3 activity, and caused DNA fragmentation as visualized as a ladder pattern on agarose gels. The intensity of the laddering pattern and the caspase-3 activity increased in parallel.

Effects of a Caspase Inhibitor We next examined the effect of the caspase inhibitor Z-VAD-FMK on the activation of caspase-3 and enhancement of DNA fragmentation caused by $4 \mu\text{g/ml}$ of CDDP (Fig. 2). The increase of caspase-3 activity was inhibited by Z-VAD-FMK at concentrations above $0.2 \mu\text{M}$. This compound also dose-dependently inhibited CDDP-induced DNA fragmentation. No DNA fragmentation was detected when AH-109A cells were exposed to CDDP in the presence of $20 \mu\text{M}$ of Z-VAD-FMK. Also, the sub-G1 population induced by CDDP disappeared when added along with $20 \mu\text{M}$ of Z-VAD-FMK (data not shown).

Comparison of Cytotoxic Activities The cytotoxic activities of CDDP using three different exposure regimens were measured by WST-1 assay (Fig. 3). Control cells were

exposed to each vehicle. A single exposure to CDDP ($1 \mu\text{g/ml}$) completely prevented the growth of AH-109A cells. When the same total dose of CDDP was delivered as 8 exposures ($0.125 \mu\text{g/ml}$ per dose), it was effective as a single

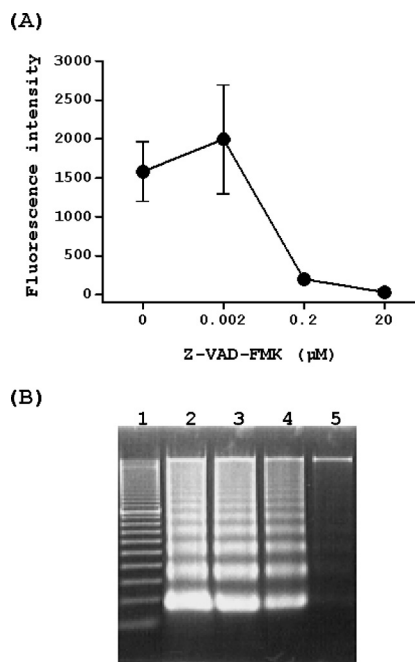


Fig. 2. Effects of Z-VAD-FMK on Caspase-3 Activity and DNA Fragmentation

Cells were preincubated Z-VAD-FMK for 1 h and then treated for 48 h with CDDP ($4 \mu\text{g/ml}$). (A) Caspase-3 activity. Data represent the means \pm S.D. from three independent experiments. (B) DNA fragmentation as determined by agarose gel electrophoresis. DNA fragmentation was visualized as the formation of DNA ladders. Lane 1, marker; lane 2, control (no Z-VAD-FMK); lane 3, $0.002 \mu\text{M}$ Z-VAD-FMK; lane 4, $0.2 \mu\text{M}$ Z-VAD-FMK; lane 5, $20 \mu\text{M}$ Z-VAD-FMK.

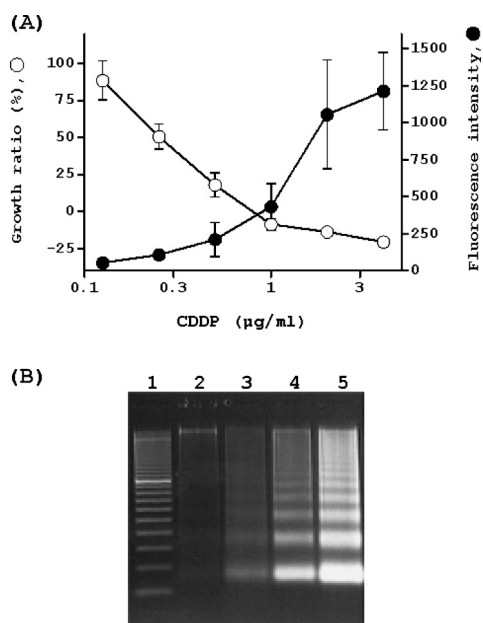


Fig. 1. Growth Inhibition and Apoptosis in AH-109A Cells

Cells were incubated with CDDP for 48 h. (A) Measurement of growth ratio (\circ) and activation of caspase-3 (\bullet). Data represent the means \pm S.D. for three individual experiments. (B) Fragmentation of DNA as determined by agarose gel electrophoresis. DNA fragmentation is visualized as the formation of DNA ladders. Lane 1, marker; lane 2, control; lane 3, CDDP $1 \mu\text{g/ml}$; lane 4, $2 \mu\text{g/ml}$; lane 5, $4 \mu\text{g/ml}$.

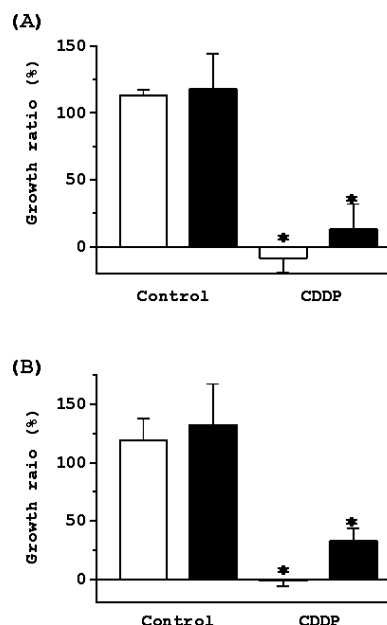


Fig. 3. Effects of Different Exposure Regimens on the Growth Inhibitory Effect of CDDP

Growth inhibitory effect was assessed by WST-1 assay 48 h after the beginning of the exposure to CDDP. Data represent the means \pm S.D. for three independent experiments. (A) Comparison of the effects of a single exposure at $1 \mu\text{g/ml}$ (\square) and 8 exposures at $0.125 \mu\text{g/ml}$ (\blacksquare). (B) Comparison of the effects of a single exposure at $1 \mu\text{g/ml}$ (\square) and 16 exposures at $0.0625 \mu\text{g/ml}$ (\blacksquare).

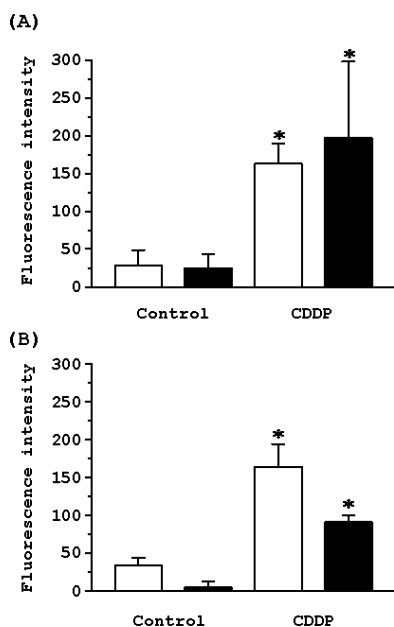


Fig. 4. Effect of the Exposure Regimen on Caspase-3 Activation by CDDP

Caspase-3 activity was measured using a fluorometric assay 48 h after the beginning of the exposure to CDDP. Data represent the means \pm S.D. for three independent experiments. (A) Comparison of the effects of a single exposure at 1 μ g/ml (\square) and 8 exposures at 0.125 μ g/ml (\blacksquare). (B) Comparison of the effects of a single exposure at 1 μ g/ml (\square) and 16 exposures at 0.0625 μ g/ml (\blacksquare).

dose at inhibiting cell growth, but when delivered as 16 exposures (0.0625 μ g/ml per dose), it was slightly less effective than the single exposure.

Activation of Caspase-3 We also examined the effect of the three exposure regimens on the activation of caspase-3 by CDDP. Caspase-3 activity was measured using a synthetic fluorescent substrate (Fig. 4). Control cells were exposed to each vehicle. Regardless of the exposure regimens, CDDP treatments (1 μ g/ml) significantly increased the caspase-3 activity. The effect of 8 exposures of 0.125 μ g/ml per dose was effective as a single exposure of 1 μ g/ml, but the effect of 16 exposures of 0.0625 μ g/ml per dose was slightly less effective as a single exposure. Interestingly, the effect of vehicle alone on caspase-3 activity was lower when delivered as 16 exposures than when delivered as a single exposure. Thus, the net effect of the different dosing regimens on caspase-3 activity did not differ.

Appearance of the Sub-G1 Population The cell cycle of AH-109A cells following exposure to CDDP (1 μ g/ml) is shown in Fig. 5. Compared with the control, CDDP caused an obvious disruption of the cell cycle and the sub-G1 population. The accumulation of sub-G1 cells tended to decrease as the time of exposure to CDDP increased.

DISCUSSION

There is a great deal of evidence that CDDP induces apoptosis in tumor cells.⁶⁻⁸ In most of these reports, the CDDP concentration was a few μ g/ml. In the clinic, CDDP has been administered by infusion to avoid severe toxicity,^{11,12} but whether CDDP induces apoptosis in cancer tissues in a clinical setting has not been determined. Therefore, we examined the ability of continuous low-dose CDDP to induce apoptosis

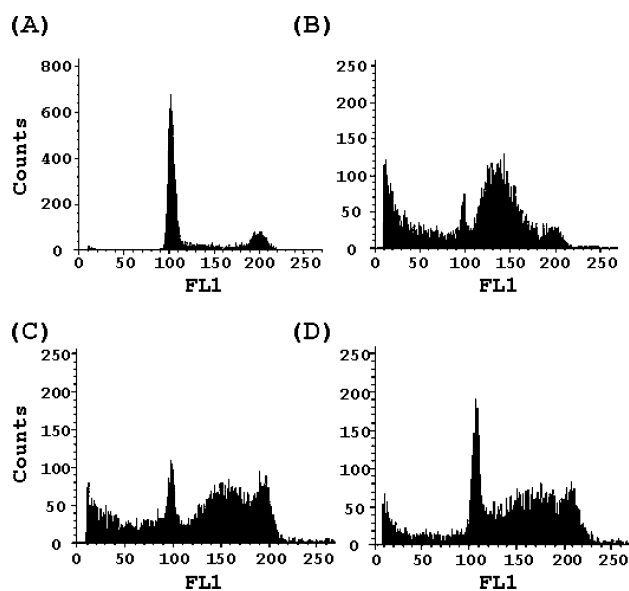


Fig. 5. Cell Cycle in AH-109A Cells Following CDDP Exposure

(A) Control. (B) A single exposure to 1 μ g/ml of CDDP. (C) Eight exposures of 0.125 μ g/ml. (D) 16 exposures at 0.0625 μ g/ml.

in cultured cells. We have previously used rat ascites hepatoma AH-109A cells to assess the effect of a sustained release preparation for the treatment of liver cancer.^{14,17,18} In those studies, we found that continuous exposure to a CDDP derivative causes a dose-dependent elevation of intracellular and DNA-bound platinum in AH-109A cells. In the current study we compared the ability various dosing regimens, including a single exposure and multiple exposures, to induce apoptosis.

We found that the activation of caspase-3 in AH-109A cells following a single exposure to CDDP is in direct proportion to the inhibition of cell growth. At a concentration resulting in obvious caspase-3 activation, we also detected DNA fragmentation as a ladder pattern on agarose gels and as sub-G1 population in flow cytometric analyses. Furthermore, a caspase inhibitor caused a parallel reduction in DNA fragmentation and caspase-3 activation. Even at concentrations less than 1 μ g/ml, of CDDP sufficiently inhibited the growth of AH-109A cells, though the increase of caspase-3 activity was not enough to detect DNA fragmentation. These results suggest that CDDP induces DNA fragmentation in AH-109A cells *via* the activation of caspase-3. In fact, caspase-3 has been specifically implicated as the effector caspase responsible for cleavage of the human DNA fragmentation factor, which subsequently activates the DNA endonuclease that is required for formation of apoptotic DNA ladders.¹⁹⁻²¹

The specific mechanism(s) that trigger apoptosis in response to CDDP injury have not yet been defined.⁶⁻⁸ Jamieson and Lippard reported that HMG proteins binding to CDDP-DNA adducts could modulate cell cycle events after DNA damage and trigger apoptosis.²² Cell cycle arrest and a specific cell cycle checkpoint at the G2/M boundary are thought to be involved in apoptosis in a variety of CDDP-treated cells. In the presence of Z-VAD-FMK, the sub-G1 population disappeared, but the cell cycle was still disturbed. In our study, Z-VAD-FMK could partially inhibit the appear-

ance of some of the morphological and biochemical features of apoptosis, but it may have no effect on the pathways upstream of the caspase cascade. Thus, the cytotoxic effect of CDDP on AH-109A cells may not always be due to the induction of apoptosis.

Ozawa *et al.* demonstrated that the ability of non-cell cycle-specific agents, such as CDDP or nitrogen mustard, to kill cells depends on the concentration \times time or AUC.^{9,10} For this reason, we compared the ability of three different regimens of CDDP dosing CDDP to inhibit cell growth, prevent caspase-3 activation, and reduce the sub-G1 population. To mimic continuous exposure, the CDDP was divided into multiple intermittently delivered doses: 8 exposures to 0.125 μ g/ml or 16 exposures to 0.0625 μ g/ml. It has been reported that the half-life of free CDDP was about 2 h for protein-binding reaction.²³ In this study cells were exposed to low dose of CDDP every 1.5 or 2 h. Thus, the concentration of free CDDP would remain non-toxic as a single exposure. As a single dose, each of these concentrations does not inhibit the growth of AH-109A cells, but the multiple exposures to these concentrations did inhibit cell growth. Also, renal toxicity of CDDP depends on its Cmax.²⁴ These multiple exposures would not increase in side effects. Interestingly, CDDP was less effective when delivered as 16 exposures than when it was delivered as 8 exposures. That might be due to too low each dose added into medium to form CDDP-DNA adducts effectively. Morazzoni *et al.* reported that continuous intravenous infusion of CDDP resulting in a concentration of 0.11 μ g/ml in ultrafiltered plasma enhances the anticancer effect of radiation.¹¹ Thus, the AUC-dependent effect of CDDP may require a certain threshold concentration that may be 0.1 μ g/ml.

Induction of apoptosis following multiple exposures to CDDP was determined by an assay of caspase-3 activity and by measurement of the sub-G1 population. All dosing regimens caused DNA fragmentation by a caspase-3-dependent pathway. Delivery of CDDP as 16 doses of 0.0625 μ g/ml was less cytotoxic and induced less apoptosis than a single dose or 8 doses at 0.125 μ g/ml. Thus, CDDP might be less effective on the formation of CDDP-DNA adducts when delivered as 16 exposures than when it was delivered as 8 exposures. Tacka *et al.* demonstrated that the amount of platination of nuclear DNA by CDDP is proportional to the AUC.²⁵ Recognition of CDDP-induced DNA damage produces a signal for the induction of apoptosis.^{8,22,26} Thus, apoptosis can be induced to the same extent by various dosing schedules as long as the final concentration of CDDP is the same. This suggests that continuous exposure to CDDP may induce apoptosis in tumor cells by causing the formation of a threshold level of DNA-platinum adducts.

In the present study, we assessed the relationship between the exposure regimen of CDDP and the induction of apoptosis in AH-109A cells. Delivery of CDDP as a single high

concentration dose activated caspase-3 and induced DNA fragmentation in these cells. Multiple exposures resulting in the same final concentration of CDDP caused the same level of caspase-3 activation and sub-G1 population even though each of the individual doses alone had no activity. Therefore, our results suggest that apoptosis following CDDP treatment is independent on the exposure regimen of CDDP and should be used as a guide to construct the appropriate regimen.

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