

Apoptosis-inducing Activity of Cisplatin (CDDP) against Human Hepatoma and Oral Squamous Cell Carcinoma Cell Lines

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Abstract. *The sensitivity of human hepatoma (HepG2) and oral squamous cell carcinoma (HSC-2) cell lines against various apoptosis-inducing agents was compared. HepG2 cells were generally more resistant to an oxidant (H₂O₂), antioxidants (sodium ascorbate, gallic acid, epigallocatechin gallate) and anticancer drugs (doxorubicin, methotrexate, cisplatin (CDDP), etoposide, 5-fluoro-2,4(1H,3H)-pyrimidinedione (5-FU), peplomycin sulfate) as compared to HSC-2 cells. Lower concentrations of CDDP, but not other anticancer drugs, induced comparable cytostatic effects on both HSC-2 and HepG2 cells. CDDP induced internucleosomal DNA fragmentation and activation of caspases 3, 8 and 9 in HepG2 cells. On the other hand, CDDP did not induce DNA fragmentation and activated caspase 3 only marginally in HSC-2 cells. Combination treatment with CDDP (10 µM) and 5-FU (100 µM) additively activated all three caspases in HepG2 cells, but not in HSC-2 cells. The present study demonstrated the chemotherapeutic potential of combined treatment of CDDP and 5-FU against hepatoma cells and the considerable variation of drug sensitivity between cancer cell lines.*

Cisplatin (CDDP) is a popular anticancer agent clinically used for the treatment of various malignant tumors, such as head and neck cancer, gastric cancer, bladder cancer, prostate cancer, esophageal cancer and osteosarcoma (1). Many anticancer drugs have induced several apoptosis-associated characteristics, morphologically and biochemically similar to those observed during the developmental programmed cell death of harmful or unnecessary organs and tissues. It is therefore very important to clarify the mechanism by which anticancer agents induce apoptotic cell

death for the further improvement of treatment consequences. We investigated here the cytotoxic activity of various anticancer agents against human hepatoma (HepG2) and oral squamous cell carcinoma (HSC-2) cell lines. Since only CDDP, among these agents, showed comparable cytostatic effects on both HepG2 and HSC-2 cells, we next investigated whether CDDP alone, or in combination with 5-fluoro-2,4(1H, 3H)-pyrimidinedione (5-FU), can induce several apoptosis markers, such as internucleosomal DNA fragmentation and caspases 3, 8 and 9 activation (2).

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies : DMEM (Gibco BRL, NY, USA); fetal bovine serum (FBS)(JRH, Bioscience, Lenexa, KS, USA); sodium ascorbate, gallic acid (Tokyo Kasei Kogyo Ltd., Tokyo, Japan); hydrogen peroxide (H₂O₂), (-)-epigallocatechin gallate (EGCG) (Wako Pure Chemical Industries, Ltd. Tokyo, Japan); methotrexate (amethopterin, Nacalai Tesque, Inc., Kyoto, Japan); etoposide, doxorubicin, 3-[4.5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma Chem., St. Louis, USA); 5-FU (Kyowa, Tokyo, Japan); cisplatin (CDDP) (Briplatin injection, Bristol Pharmaceutical Co., Tokyo, Japan); peplomycin sulfate (Nihonkayaku, Tokyo, Japan); RNase A, proteinase K (Boehringer Mannheim) ; caspases 3, 8, 9 colorimetric protease assay kit (MBL, Nagoya, Japan).

Cell culture. HSC-2 and HepG2 cells were cultured at 37°C in DMEM medium supplemented with 10% heat-inactivated FBS under a humidified 5% CO₂ atmosphere.

Assay for cytotoxic activity. Near confluent cells were treated for 24 hours with various concentrations of test samples. The relative viable cell number of adherent cells was then determined by the MTT method. In brief, cells were incubated for 4 hours with 0.2 mg/mL MTT in the culture medium. After washing once with phosphate-buffered saline without Mg and Ca (PBS), the cells were lysed with DMSO and the absorbance at 540 nm of the cell lysate was measured by a microplate reader (Labsystems Multiskan, Biochromatic Labssystem, Osaka, Japan) with Star/DOT Matrix printer JL-10. The 50% cytotoxic concentration (CC₅₀) was determined from the dose-response curve.

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Assay for DNA fragmentation. The pelleted HL-60 cells or the attached HepG2 or HSC-2 cells were lysed and digested with RNase and proteinase K. The DNA was isolated and assayed for DNA fragmentation by 2% agarose gel electrophoresis (3). The DNA from apoptotic cells induced by UV irradiation (6 J/m²/minute, 1 minute) was run in parallel as a positive control (4).

Assay for caspase activation. Cells were washed with PBS and lysed in lysis solution (MBL). After standing for 10 minutes on ice and centrifugation for 10 minutes at 15,000xg, the supernatant was collected. Lysate (50 μ L, equivalent to 100 μ g protein) was mixed with 50 μ L 2 x reaction buffer (MBL) containing substrates for caspase 3 (DEVD-pNA (*p*-nitroanilide)), caspase 8 (IETD-pNA) or caspase 9 (LEHD-pNA). After incubation for 4 hours at 37 °C, the absorbance at 405 nm of the liberated chromophore pNA was measured by microplate reader. Comparison of the absorbance of pNA from an apoptotic sample with an uninduced control allows the determination of the relative caspase activity (expressed as % of control), according to the manufacturer's instruction (MBL) (3).

Results

Cytotoxic activity of various agents. Doxorubicin showed the highest cytotoxic activity against HepG2 cells (CC₅₀=9 μ M), followed by CDDP (CC₅₀=12 μ M) > etoposide (CC₅₀=120 μ M) > gallic acid (CC₅₀=670 μ M) > EGCG (CC₅₀=780 μ M) > 5-FU (CC₅₀=970 μ M) > H₂O₂ (CC₅₀=1,820 μ M) > dopamine (CC₅₀=1,970 μ M) > sodium ascorbate (CC₅₀=3,900 μ M) (black square in Figure 1). The CC₅₀ values of methotrexate and peplomycin sulfate could not be determined due to their much lower cytotoxic activity (CC₅₀ > 14,200 μ M, CC₅₀ > 500 μ M, respectively).

HSC-2 cells were more sensitive to these agents except for 5-FU and methotrexate. Doxorubicin again showed the greatest cytotoxic activity (CC₅₀=3.6 μ M) against HSC-2 cells, followed by etoposide (CC₅₀=6.0 μ M) > peplomycin sulfate (CC₅₀=8.5 μ M) > CDDP (CC₅₀=19 μ M) > dopamine (CC₅₀=130 μ M) > gallic acid (CC₅₀=180 μ M) > EGCG (CC₅₀=280 μ M) > H₂O₂ (CC₅₀=590 μ M) > sodium ascorbate (CC₅₀=980 μ M) (open squares in Figure 1). The CC₅₀ values of methotrexate and 5-FU could not be determined due to their much lower cytotoxic activity (CC₅₀ > 14,200 μ M, CC₅₀ > 5,000 μ M, respectively). These data demonstrate that only CDDP among these 11 compounds was comparably cytostatic against both HSC-2 and HepG2 cells, at relatively lower concentrations. We next investigated the apoptosis-inducing activity of CDDP against HepG2 and HSC-2 cells.

Induction of DNA fragmentation by cisplatin. Figure 2A shows that CDDP (10 μ M) induced internucleosomal DNA fragmentation after 36 hours in HepG2 cells. When the concentration of cisplatin was elevated to 25 μ M, the DNA fragmentation was detected as early as 16 hours, reached a maximum level at 24 hours and declined after 36 hours, falling to undetectable levels at 48 hours.

In contrast, CDDP (10 μ M) only produced large DNA fragments (indicated by an arrow in Figure 2B), without induction of internucleosomal DNA fragmentation during 48 hours in HSC-2 cells.

Activation of caspase. When HepG2 cells were incubated with CDDP (10 μ M), caspase 3 was time-dependently activated (Figure 3A). At 36 hours, the caspase activity was increased by 11-fold and thereafter decreased. CDDP (10 μ M) also activated the caspase 8 and caspase 9, but to much lesser extents (by 2.8- and 2.0- fold, respectively at 36 hours) (Figure 3A).

CDDP (10 μ M) also activated caspase 3 in HSC-2 cells, but only by 2.2-fold after 36 ~ 48 hours (Figure 3B). CDDP (10 μ M) only marginally, but not significantly, activated caspase 8 and caspase 9 in HSC-2 cells (Figure 3B).

Combination treatment. Combination treatment with CDDP (10 μ M) and 5-FU (100 μ M) produced synergistic activation of caspase 3 (8.2- fold increase) in HepG2 cells, exceeding that of the sum of single treatment by CDDP (5.0- fold) and 5-FU (2.2- fold) (Figure 4A). Combination treatment also produced synergistic activation of caspase 8 and caspase 9 in HepG2 cells, but to a much lesser extent (Figure 4A).

When HepG2 cells were first treated for 24 hours with 5-FU, followed by CDDP treatment for the next 24 hours, the extent of caspase activation was only half of that observed by simultaneous treatment with 5-FU and CDDP. These data suggest that both cisplatin and 5-FU should be present at the same time for the induction of maximal caspase activation (Figure 4A).

In contrast, combination treatment of HSC-2 cells with cisplatin and 5-FU activated caspase 3 by only 3- fold, a similar extent of activation achieved by 5-FU alone (2.9- fold increase) (Figure 4B). Similar results were obtained for caspase 8 and caspase 9 (Figure 4B).

Discussion

Caspases in unstimulated cells are present in the form of inactive proenzymes. Upon treatment with anticancer drugs, cellular caspases are activated. There are at least two regulatory pathways of the caspase activation cascades. The first extra-mitochondrial pathway (so-called extrinsic pathway) is mediated by death receptor, such as Fas and caspase 8 (5). The other intra-mitochondrial pathway (so-called intrinsic pathway) is mediated by cytochrome C and caspase 9 (6). It has been postulated that the activation of the Fas/FasL system is involved in the regulation of anticancer drug-induced cell death (7-9).

We found that CDDP (10 μ M) induced DNA fragmentation after 36 hours, when the activation of caspase 3 became maximal in HepG2 cells. We found that the caspase 3

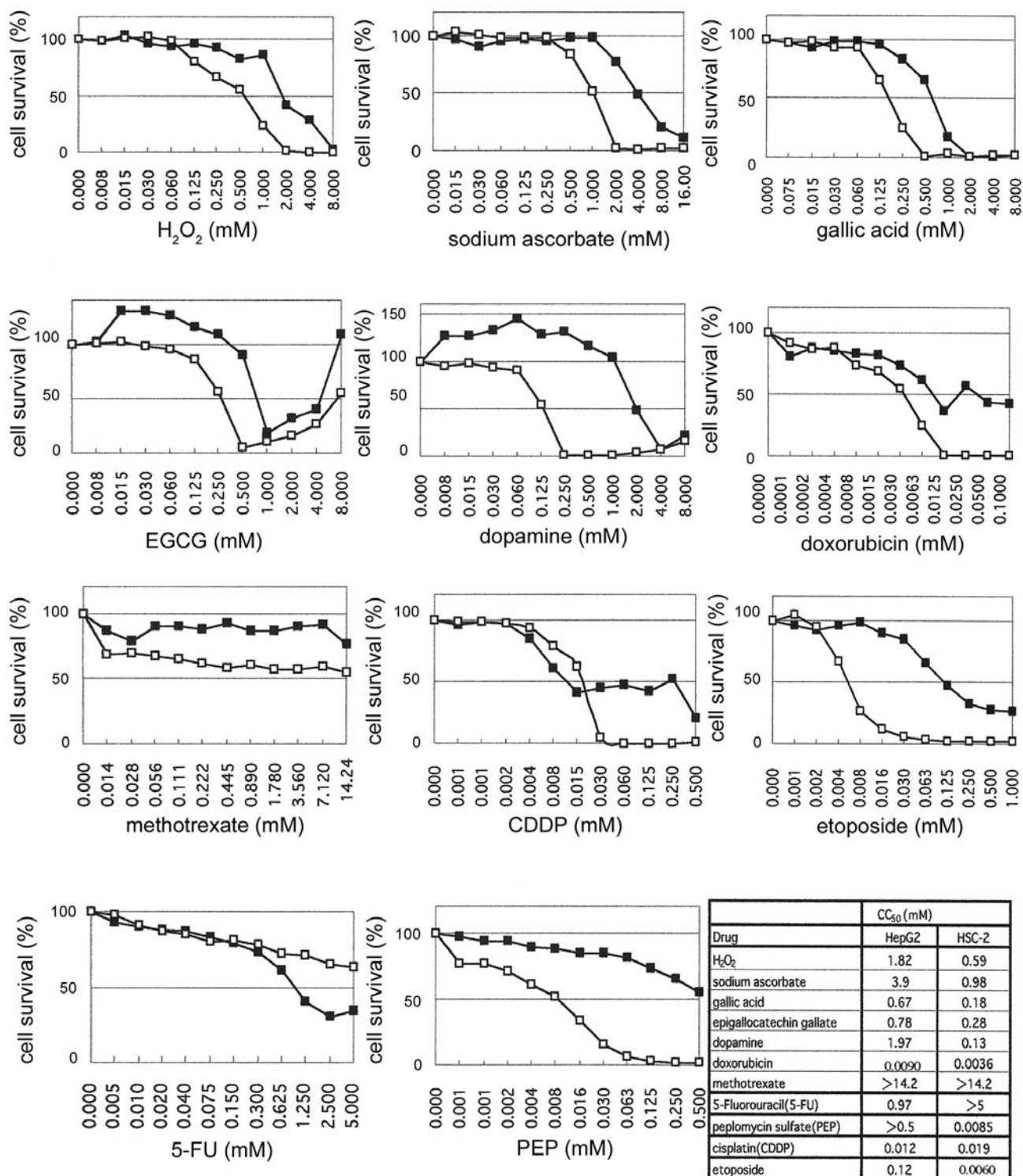


Figure 1. Cytotoxic activity of oxidant, antioxidants and anticancer drugs against two human cancer cells. Near confluent HepG2 (■) or HSC-2 (□) cell lines were incubated for 24 hours with the indicated concentrations of each drug and the relative viable cell number was determined by MTT method. Each value represents the mean from 4 determinations.

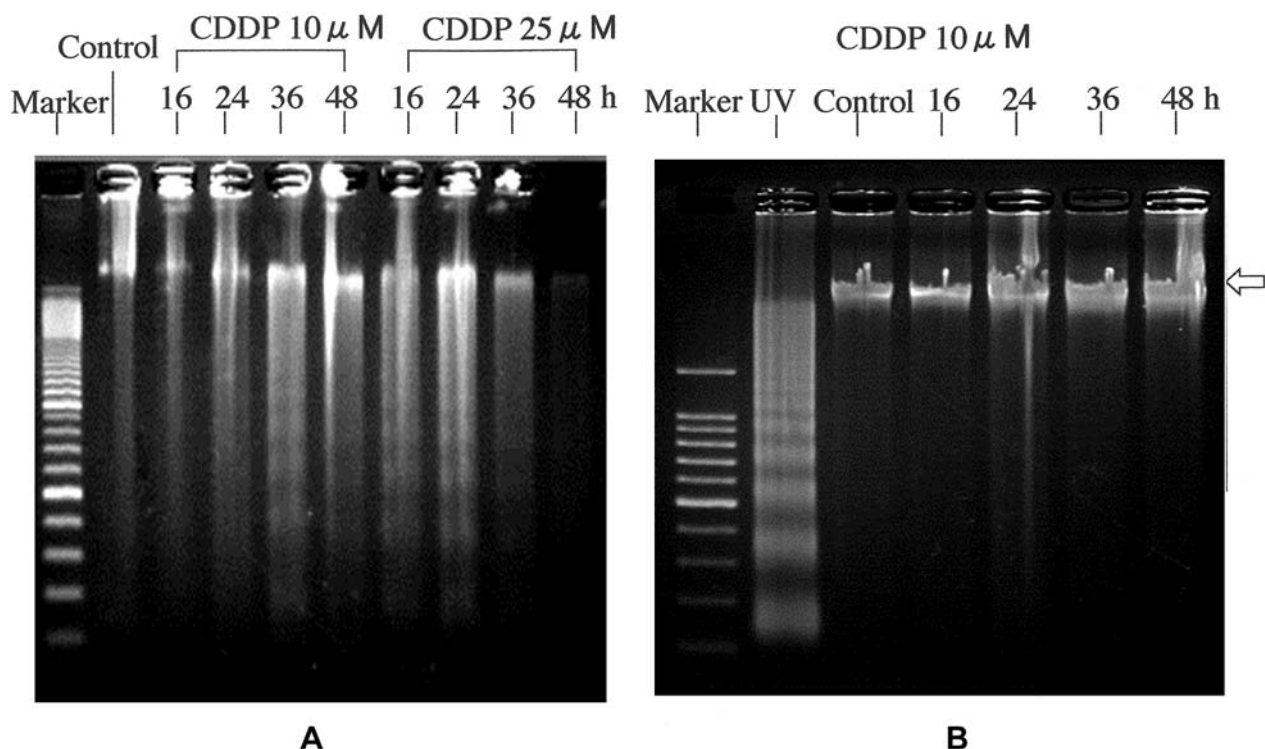


Figure 2. Induction of DNA fragmentation by CDDP. Near confluent HepG2 (A) and HSC-2 (B) cells were incubated for the indicated times with 10 or 25 μ M CDDP and assayed for DNA fragmentation. UV, DNA from apoptotic HL-60 cells induced by UV-irradiation (4). Similar results were obtained in another 2 independent experiments. Arrow indicates large DNA fragment.

activation slightly preceded that of DNA fragmentation, suggesting that caspase 3 may be an effector molecule. A time course study showed that both caspase 8 (involved in the extrinsic pathway) and caspase 9 (involved in the intrinsic pathway) were activated in concert with caspase 3, suggesting that CDDP triggered both of these two pathways in HepG2 cells. Recently, it has been reported that caspase-9 - dependent apoptosis plays an important role in CDDP-induced human head and neck squamous cell carcinoma apoptosis (10, 11). In contrast, we did not observe such an activation of caspase 9, nor internucleosomal DNA fragmentation, although only a 2-fold increase of caspase 3 and marginal activation of caspase 8 were observed in HSC-2 cells.

However, CDDP activated caspase 9 by 2-fold in HepG2 cells (Figure 4B). This suggests that whether CDDP activates caspase 8 or caspase 9 may depend on the type of the target cells. CDDP induced a 11-fold increase in the caspase 3 activity and typical internucleosomal DNA fragmentation in HepG2 cells, whereas it induced only a 2-fold increase of caspase 3 without clear-cut DNA fragmentation in HSC-2 cells. This suggests that the extent of caspase 3 activation may relate to that of induction of internucleosomal DNA fragmentation. It is interesting to note that treatment of HSC-

2 with CDDP induced few or no apoptosis-associated properties (such as caspase activation and DNA fragmentation), although HSC-2 cells are much more sensitive to all anticancer agents than HepG2 cells. This suggests that the extent of expression of apoptosis-associated markers is not always related to that of drug sensitivity.

Kin *et al.* have reported that treatment of MKN45 gastric cancer cells with low concentrations of CDDP and 5-FU elevated the intracellular concentrations of Bax, cytochrome c, Fas /and caspase 3 proteins, but not that of caspase 8 (12,13). We found that CDDP exerted a similar magnitude of cytostatic effects on both HSC-2 and HepG2 cells and that the treatment with low concentrations of CDDP and 5-FU synergistically activated caspases 3, 8 and 9 in relatively resistant HepG2 cells. This suggests that the combination of 2 different popular anticancer drugs (such as CDDP and 5-FU) may be applicable for the chemotherapy of hepatoma.

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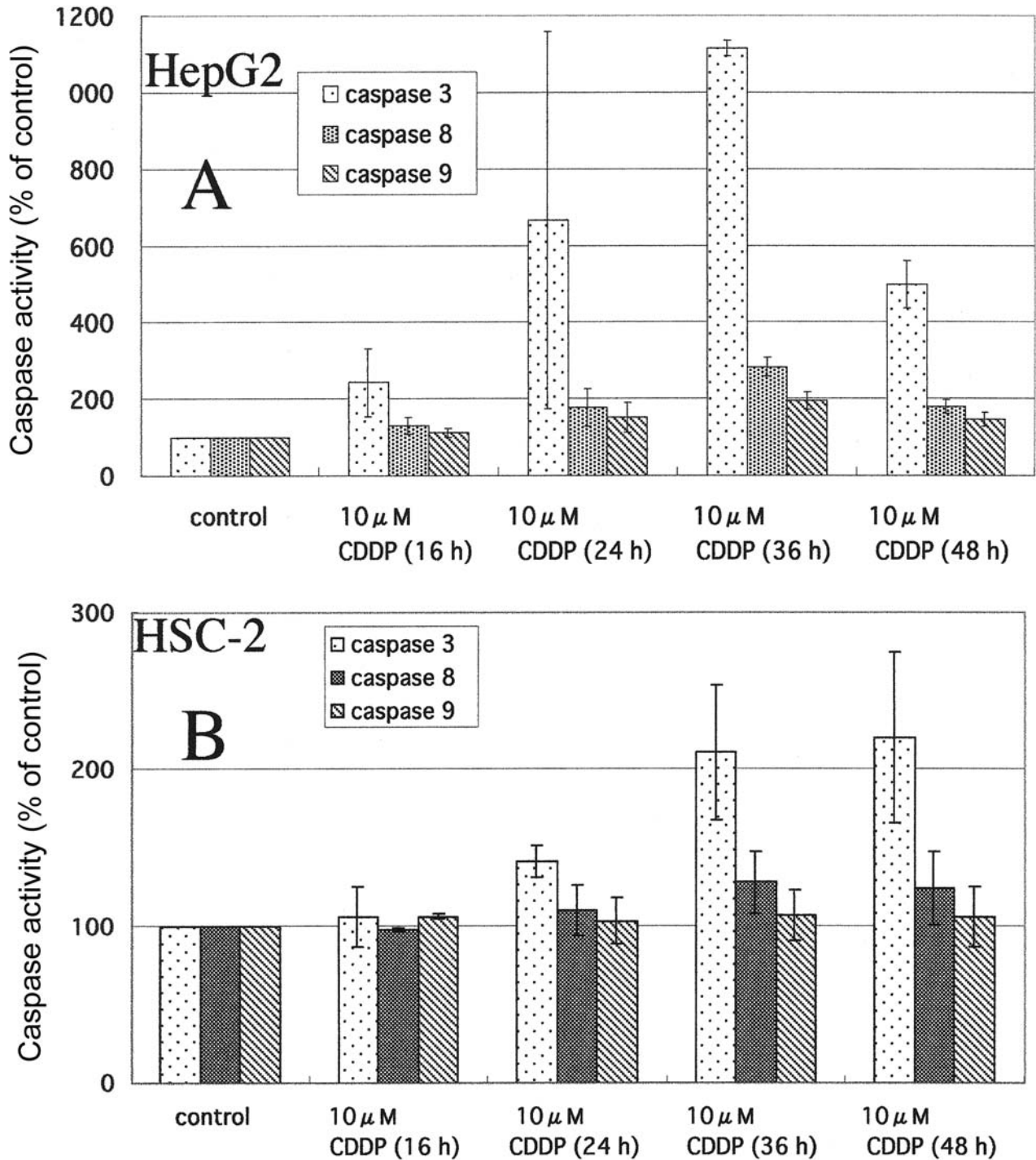


Figure 3. Activation of caspases 3, 8 and 9 by CDDP. Near confluent HepG2 (A) and HSC-2 (B) cells were incubated for 16, 24, 36 or 48 hours without (control) or with 10 μM CDDP and caspase activity was assayed and expressed as % of control. Each value represents the mean ± S.D. from 3 independent experiments.

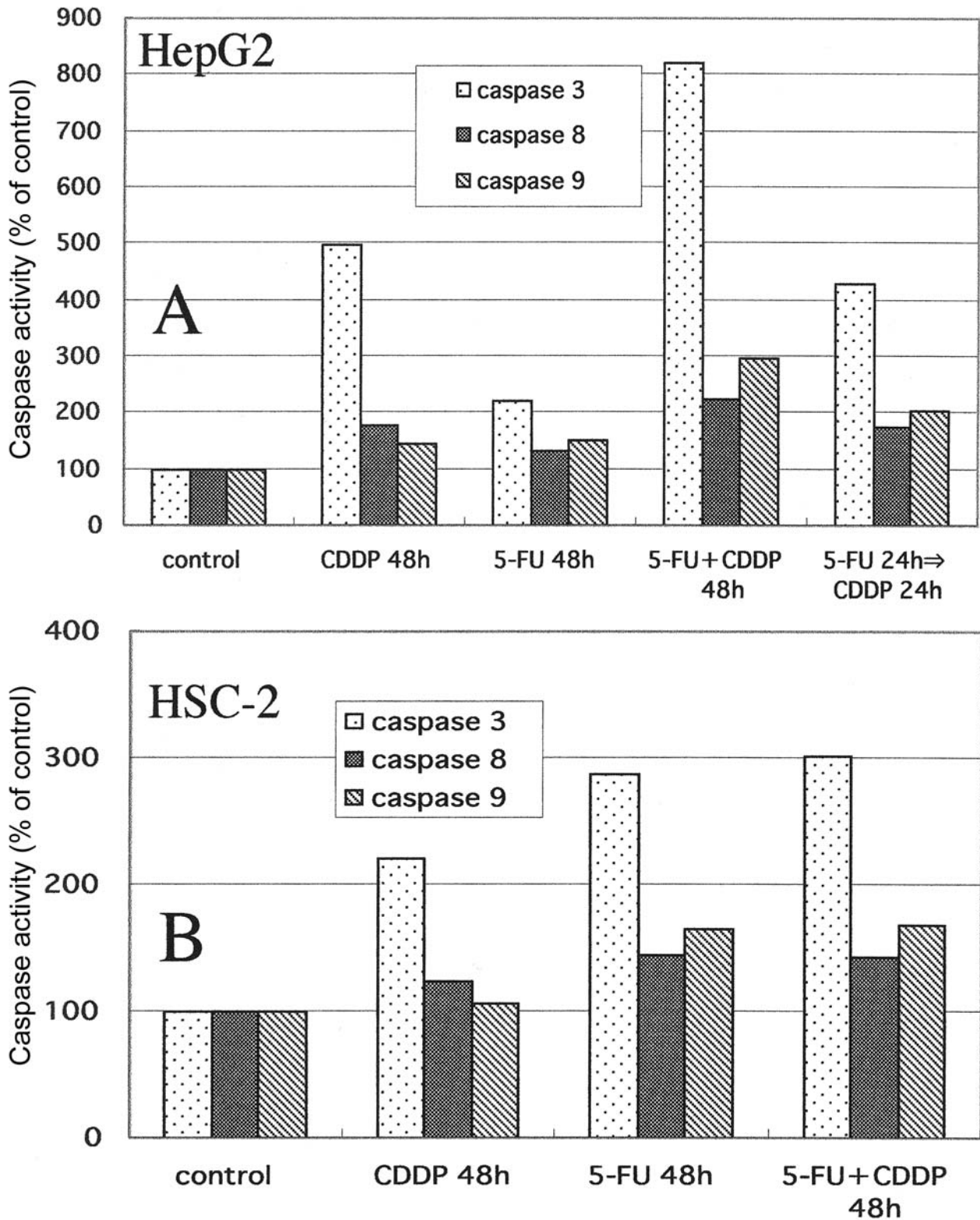


Figure 4. Effect of combination treatment of CDDP and 5-FU on caspase activity. Near confluent HepG2 (A) and HSC-2 (B) cells were incubated for the indicated times without (control) or with CDDP or 5-FU or in combination of both, or with 5-FU followed by CDDP. Caspase activity was then assayed and expressed as % of control.

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