

Cisplatin induced apoptosis of ovarian cancer A2780s cells by activation of ERK/p53/PUMA signals

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Summary. Cisplatin (CDDP) is one of the most effective anticancer agents widely used in the treatment of solid tumors, including ovarian cancer. It is generally considered as a cytotoxic drug which kills cancer cells by causing DNA damage, and subsequently inducing apoptosis in cancer cells. However, the underlying mechanisms leading to cell apoptosis remain obscure. In this study, the signaling pathways involved in CDDP-induced apoptosis were examined using CDDP-sensitive ovarian cancer A2780s cells. A2780s cells were treated with CDDP (1.5-3 µg/ml) for 6h, 12h and 24h. Using siRNA targeting P53 and PUMA, and a selective MEK inhibitor, PD98059 to examine the relation between ERK1/2 activation, p53 and PUMA expression after exposure to CDDP, and the effect on CDDP-induced apoptosis. The results shown that treatment of A2780s cells with CDDP (3 µg/ml) for 6-24h induced apoptosis, resulting in the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and accumulation of p53 and PUMA (p53 upregulated modulator of apoptosis) protein. Knockdown of P53 or PUMA by siRNA transfection blocked CDDP-induced apoptosis. Inhibition of ERK1/2 using PD98059, a selective MEK inhibitor, blocked the apoptotic cell death but prevented CDDP-induced accumulation of p53 and PUMA. Knockdown of P53 by siRNA transfection also blocked CDDP-induced accumulation of PUMA. We therefore

concluded that CDDP activated ERK1/2 and induced-p53-dependent PUMA upregulation, resulting in triggering apoptosis in A2780s cells. Our study clearly demonstrates that the ERK1/2/p53/PUMA axis is related to CDDP-induced cell death in A2780s cells.

Key words: Ovarian cancer, Cisplatin, Apoptosis, ERK1/2, P53, p53 upregulated modulator of apoptosis

Introduction

Cisplatin (CDDP) is a potent inducer of apoptosis in different cell types and is one of the most effective and widely used chemotherapeutic agents in the treatment of human cancers, including ovarian cancer (Meng et al., 2013). Although this treatment regime is effective as the first-line treatment, recurrence occurs in up to 75% of ovarian cancer patients. Patients with recurrent ovarian cancer ultimately develop resistance to chemotherapy and eventually succumb to the disease (Agarwal and Kaye, 2003). CDDP derivatives are used as the mainline treatment of ovarian cancer, despite their severe side effects and development of resistance. In general, CDDP and other platinum-based compounds are considered as cytotoxic drugs which kill cancer cells by damaging DNA, inhibiting DNA synthesis and mitosis, and inducing apoptotic cell death (Wyllie et al., 1980). However, the underlying mechanisms leading to cell apoptosis remain obscure.

p53 is a short lived protein, which is activated (phosphorylation) by DNA damage signal. The activated

p53 in turn activates its downstream signals, and regulates cell cycle progression, DNA repair and apoptosis (Basu and Krishnamurthy, 2010).

Mitogen activated protein kinases are a family of structurally related serine/threonine protein kinases that coordinate various extra cellular signals to regulate cell growth and survival (Johnson and Lapadat, 2002). CDDP has been shown to cause activation of ERK in several cell types although there are controversies whether activation of ERK prevents or contributes to CDDP induced cell death (Hayakawa et al., 1999). CDDP-induced extra-cellular-signal-regulated kinase (ERK) activation precedes p53-mediated DNA damage response since ERK directly phosphorylates p53 causing up regulation of p21, 45 kd-growth arrest and DNA damage (GADD45), and mouse double minute 2 homolog (Mdm2) (DeHaan et al., 2001). Thus, activation of ERK may cause cell cycle arrest allowing time for the repair of CDDP-induced DNA damage via p53.

PUMA (p53 upregulated modulator of apoptosis), a BH3-only Bcl-2 family protein, plays an essential role in p53-dependent and p53-independent pathway induced apoptosis by various stimuli (Yu et al., 2001; Wu et al., 2005). CDDP could activate p53 dependent PUMA upregulation in ovarian cancer cells and act on the mitochondria and cause the release of mitochondrial death proteins (Maurmann et al., 2015; Gu et al., 2014; Fraser et al., 2014).

In the present study, we sought to define the activity of ERK1/2/p53/PUMA-dependent pathway involved in the CDDP-induced apoptosis of human A2780s cells.

Materials and methods

Ethics statement

All studies involving mice were approved by the Institutional Animal Care and Treatment Committee of the affiliated hospital of Qingdao University.

Cell lines and culture

The human ovarian cancer A2780s cell lines (CDDP-sensitive cell) were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) in 5% CO₂/95% air at 37°C. Then, they were plated subconfluent in 75 mm² flasks and cultured in serum-free medium 1 day prior to treatment.

siRNA transfection

Lipofectamine 2000 (Invitrogen) kits were used for transfections of siRNAs, according to the manufacturer's instructions. Briefly, A2780s cells were seeded in six-well plates (density 2.5×10⁵cells/well) in antibiotic free media, and transfected with 2μM p53 siRNAs or PUMA siRNAs or scramble siRNA in serum-free medium for 5-

8 h. The culture medium was then replaced with fresh medium for 24 h. Cells were then treated accordingly.

Cisplatin treatment

For the induction of apoptosis, the A2780s cells were incubated for 2 h with different concentrations of CDDP (1.5 μg/ml and 3 μg/ml). After washing, cells were incubated in culture medium for a further 6, 12 or 24 h. To determine the effect of p53 and PUMA on CDDP-induced apoptosis, A2780s cells were transfected with siRNA targeting PUMA or p53 24 h before CDDP treatment, or treated with PD98059 (20 μM) 6 h before exposure to CDDP, then the cells were incubated in culture medium for further 6, 12 or 24 h.

Apoptosis assay

Apoptosis induction was quantified by Annexin V/PI double staining followed by flow cytometry. Annexin V/PI double staining was performed using an apoptosis detection kit (Biovision, Mountain view, CA) following the manufacturer's instruction. Briefly, after exposure to various treatments, cells were gently detached by brief trypsinization, and then washed with ice cold PBS. After another wash with binding buffer, cells were suspended in 300 μL binding buffer containing Annexin V and propidium iodide, and incubated for 5 min at room temperature. Early apoptotic cells were identified as Annexin V positive/PI negative cells, while late apoptotic/necrotic cells were identified as Annexin V positive/PI positive cells using a BD LSR II cell analyzer.

TUNNEL assay

DeadEnd™ Fluorometric TUNEL System (Promega Corp., Madison, WI) was utilized for apoptosis detection. Briefly, 4×10⁴ A2780s cells were seeded on cover glass slips inside 6 well plates. Cells were allowed to attach overnight. To determine the effect of p53 and PUMA on CDDP-induced apoptosis, A2780s cells were transfected with PUMA siRNA or p53 siRNA 24 h before CDDP treatment, or treated with PD98059 (20 μM) 6h before CDDP treatment, then the cells above (4×10⁴) were seeded on cover glass slips inside 6 well plates. Next day, cells were treated with 3 μg/ml CDDP for 6-24 h. At indicated time points, cells were washed 3X in PBS, fixed in 4% methanol-free formaldehyde for 25 min at 4°C, washed again, then permeabilized in 0.2% Triton X-100 in PBS for 5 min. After permeabilization, cells were equilibrated in equilibration buffer for 5 min at room temperature and then incubated with a buffer containing nucleotide mix and rTdT enzyme for 1hr. Cell nuclei were stained with DAPI. Fluorescence microscopy was performed using olympus IX81-DSU Spinning disk confocal microscope and images were taken with Hamamatsu ORCA II ER camera.

Cisplatin and apoptotic signals

Western blotting

Cell lysates were homogenized by sample buffer (100 mM Tris-HCl (pH 6.8), 2% Sodium Dodecyl Sulfate (SDS), 0.002% bromophenol blue, 20% glycerol, 10% β -mercaptoethanol (all from Sigma-Aldrich)). Those were subjected to SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences). The following primary antibodies were used for immunodetection: anti- β -actin, pERK1/2.ERK1/2, P53 and PUMA and the Western Blot Substrate kit (Pierce) were used to detect chemiluminescence.

Statistics

Student's t-test (two-tailed) was performed to analyze the data. A P-value of <0.05 was considered to be statistically significant.

Results

CDDP induces apoptosis of A2780s cells in a dose- and time-dependent manner

A2780s treated with 1.5 μ g/ml CDDP for 6h, 12h and 24h revealed that the apoptotic rate of A2780s cells increased from 23.5 \pm 1.6% at 6 h to 45.4 \pm 5.2% at 12h and 68.4 \pm 6.5% at 24h after drug application (Fig. 1A). A2780s treated with 3 μ g/ml CDDP revealed that the apoptotic rate of A2780s cells increased from 38.7 \pm 2.3%

at 6h to 63.4 \pm 6.3% at 12h and 82.3 \pm 7.2% at 24h after drug application (Fig. 1A). We used TUNEL staining to assess the apoptotic rate in A2780s cells treated with 3 μ g/ml CDDP for 6h, 12h and 24h, which elicits apoptosis in 6.6 \pm 0.8%, 14.3 \pm 1.2% and 23.3 \pm 2.1% (Fig. 1B), respectively. It was indicated that CDDP induces apoptosis of A2780s cells in a dose- and time-dependent manner.

Activation of ERK1/2 by CDDP correlates with apoptosis induction in A2780s cells

As shown in Fig. 2A, ERK1/2 was activated at 6h after 3 μ g/ml CDDP treatment in the A2780s cells, and reached a peak at 24 h. After ERK1/2 activity was inhibited by PD98059 (20 μ M), CDDP-induced ERK1/2 activity was inhibited, and CDDP-induced apoptosis was blocked (Fig. 2B). TUNEL staining has the same results as FCM (Fig. 2C). It was indicated that CDDP induces apoptosis of A2780s cells by ERK-dependent pathway.

CDDP activates ERK1/2-dependent p53 and PUMA expression

Western blot assay showed that expression of p53 and PUMA was increased at 6h after 3 μ g/ml CDDP treatment in the A2780s cells, and reached a peak at 24 h (Fig. 2A). After ERK1/2 activity was inhibited by PD98059 (20 μ M), CDDP-induced p53 and PUMA expression was suppressed significantly, indicating that

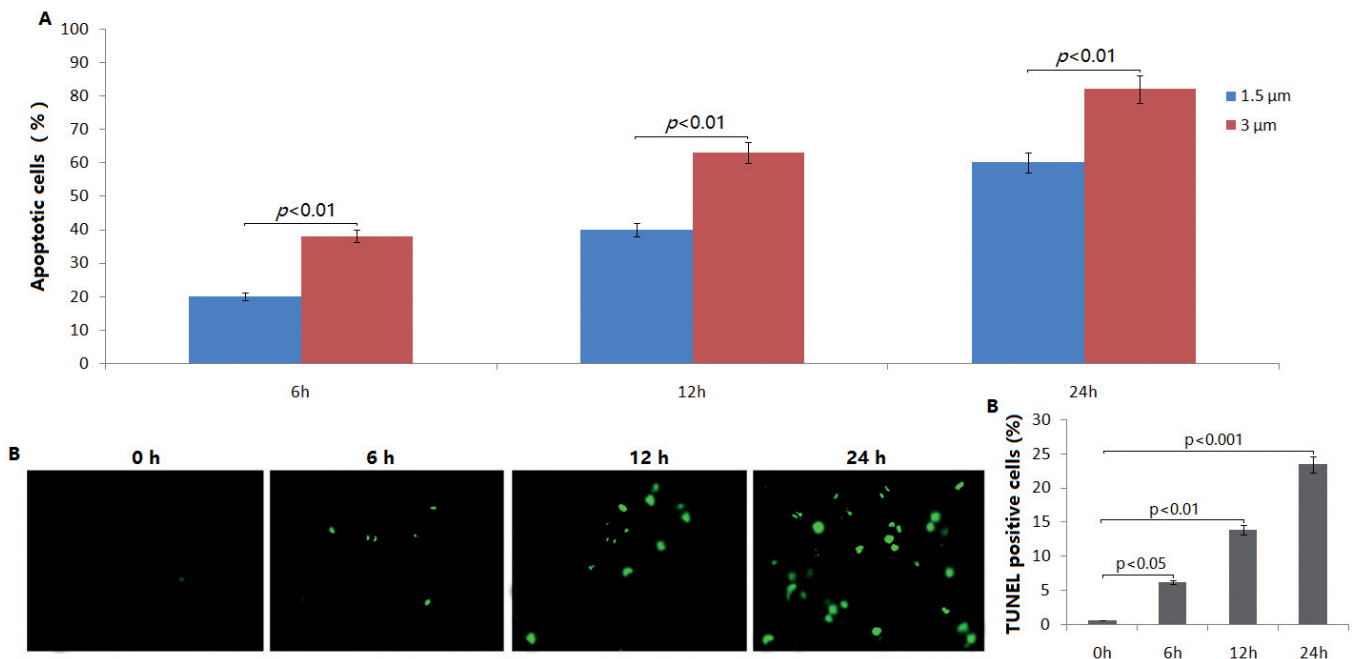


Fig. 1. Dose- and time-dependent effects of CDDP on A2780s cells. **A.** A2780s cells were treated with different CDDP concentrations. After 6, 12 and 24h, the apoptotic rate of A2780s cells was assessed by Annexin V/PI double staining followed by flow cytometry. **B.** Representative photomicrographs of TUNEL staining in untreated (control) and drug-treated A2780s cells illustrate increased apoptosis of tumour cells treated with 3 μ g/ml CDDP for 6-24 h when compared to the control. Data presented is the mean of triplicates; similar results were obtained in three separate experiments.

Cisplatin and apoptotic signals

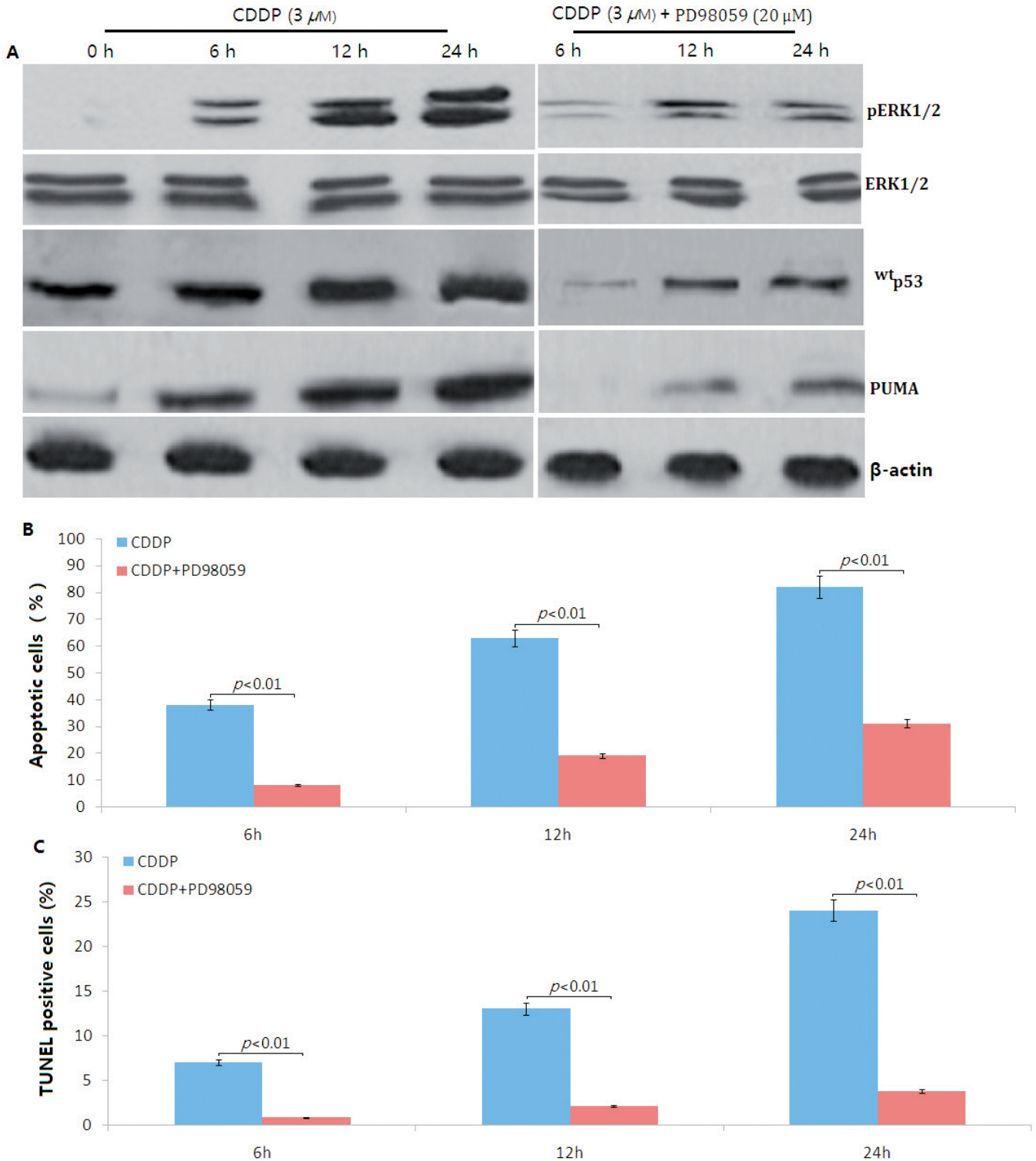


Fig. 2. Effect of CDDP on ERK1/2-dependent p53 and PUMA expression and apoptosis regulation. A2780s cells were treated with PD98059 (20 μ M) for 6 h, then treated with 3 μ g/ml CDDP for 6h,12h and 24h, respectively. **A.** ERK1/2, p53 and PUMA expression was detected by Western blot assay. **B.** the apoptotic rate of A2780s cells was assessed by Annexin V/PI double staining followed by flow cytometry. **C.** TUNEL was used to detect cell apoptosis. Data presented is the mean of triplicates; similar results were obtained in three separate experiments.

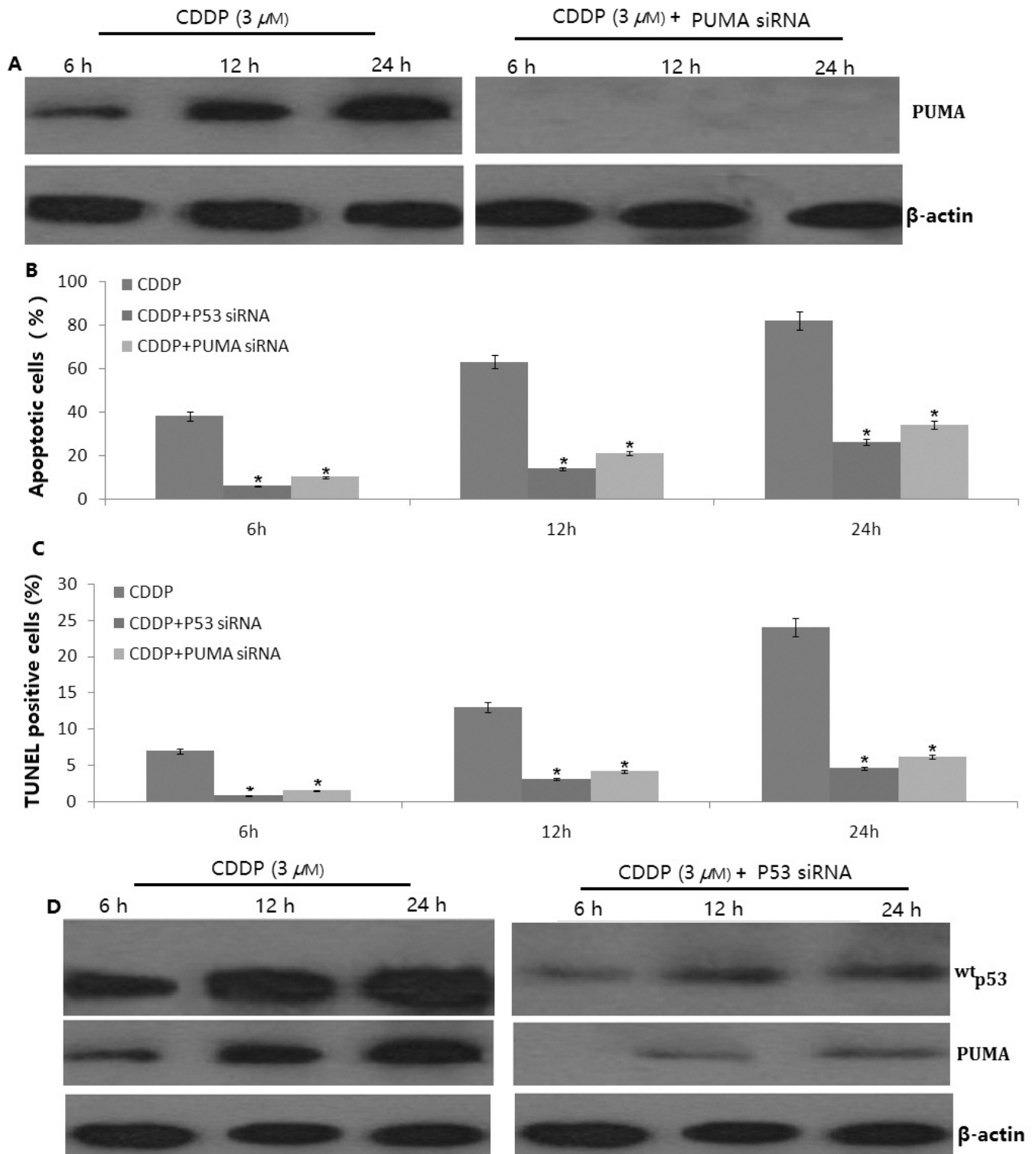


Fig. 3. CDDP induced apoptosis by Activation of ERK1/2/ p53/PUMA pathway. **A.** A2780s cells were transfected with PUMA siRNA, then treated with 3 μ g/ml CDDP for 6h,12h and 24h. PUMA protein was detected in A2780s cells by western blot assay. **B.** A2780s cells were transfected with PUMA siRNA or p53 siRNA, then treated with 3 μ g/ml CDDP for 6h, 12h and 24h. The apoptotic rate of A2780s cells was assessed by Annexin V/PI double staining followed by flow cytometry. **C.** TUNEL was used to detect cell apoptosis. Data presented is the mean of triplicates; similar results were obtained in three separate experiments.* $p < 0.01$. **D.** A2780s cells were transfected with p53 siRNA, then treated with 3 μ g/ml CDDP for 6h, 12h and 24h. p53 and PUMA protein was detected in A2780s cells by western blot assay.

CDDP activates p53 and PUMA by ERK-dependent pathway.

PUMA is necessary for CDDP-induced apoptosis

Treatment with 3 µg/ml CDDP for 6h, 12h and 24h strongly induced PUMA protein expression in a time-dependent manner (Fig. 2A). Followed by increased cell apoptosis of A2780s cells in a time-dependent manner (Fig. 1). However, after PUMA knockdown by siRNA transfection, CDDP-induced PUMA upregulation was inhibited (Fig. 3A), and CDDP-induced cell apoptosis was blocked (Fig. 3B,C).

PUMA activation by CDDP is mediated by p53

The induction of p53 and PUMA by CDDP was found in the A2780s cells (Fig. 2A). Knockdown of p53 by siRNA blocked CDDP-induced PUMA upregulation (Fig. 3D) and CDDP-induced cell apoptosis (Fig. 3B,C).

Discussion

Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase super family that can mediate cell proliferation and apoptosis (Yamamoto et al., 2006). Although ERK activation has generally been associated with cell survival and proliferation, a number of studies show that depending on the stimuli and cell types involved, activation of ERK can mediate cell death (Zhou and Elledge, 2000). Some studies suggested that the balance among the intensity and duration of pro-versus anti-apoptotic signals transmitted by ERK1/2 determines whether a cell proliferates or undergoes apoptosis (Pearson et al., 2001).

In the present study, A2780s cells treated with 1.5-3 µg/ml CDDP for 6h, 12h and 24h showed a dose- and time-dependent apoptosis, followed by ERK1/2 activation at 6h after CDDP exposure, and reached the peak at 24h. To clarify the importance of the ERK1/2 activation for the apoptosis of CDDP-treated A2780s cells, we inhibited ERK1/2 activity using a MEK inhibitor, PD98059. Experiments demonstrated that PD98059 not only downregulated the level of ERK phosphorylation but also reduced the apoptotic rate of CDDP-treated A2780s cells. Results from Western blot analyses suggested that the CDDP-induced apoptosis in A2780s cells depended on phosphorylation of ERK1/2. However, the molecular mechanisms that define the conditions for ERK1/2-mediated cell death remain poorly understood.

The p53 tumor repressor protein is related to the sensitivity to CDDP in cancer (Park et al., 2001; Karger et al., 2005). CDDP-inducible p53 gene therapy might provide a control of transgene expression while enhancing the effectiveness of commonly used chemotherapeutic agents. In our study, treatment of A2780s cells with CDDP activated p53, and induced cell

apoptosis. With p53 knockdown by siRNA, CDDP induced p53 activation was blocked and apoptosis was inhibited. It was indicated that activation of p53 contributed to CDDP-induced apoptosis.

PUMA, a p53 up-regulated modulator of apoptosis is a potent proapoptotic molecule that is rapidly induced in cells following DNA damage and is required for p53-induced apoptosis (Yu et al., 2001). In our study, treatment of A2780s cells with CDDP increased PUMA expression, and induced cell apoptosis. When PUMA was knockdown by siRNA, CDDP-induced PUMA was blocked and apoptosis was inhibited. In addition, knockdown of p53 blocked CDDP-induced PUMA upregulation and cell apoptosis. It was indicated that activation of p53-dependent PUMA upregulation contributed to CDDP-induced apoptosis.

Previous studies reported that DNA damage-induced ERK activation causes apoptosis by activation of p53 phosphorylation (Tang et al., 2002). In addition, inhibition of ERK improves cell survival by inhibiting p53-induced apoptosis in renal cell lines and primary cultures of renal proximal tubular cells (Kim et al., 2005; Wang et al., 2000).

In this study, we found that inhibition of ERK1/2 activity blocked both p53 and PUMA expression, follow by decreased A2780s cell apoptosis, suggesting that ERK1/2 activation may be mediating signaling pathways downstream of p53-dependent PUMA activation.

Conclusion

In conclusion, the data suggested that CDDP mediates its ERK1/2-dependent apoptosis-inducing effect on the CDDP sensitive A2780s cells through activation of p53-dependent-PUMA signals. The data would also suggest that an antitumour therapy with CDDP should be combined with ERK activation agents because such a combination might activate PUMA and be of benefit for the outcome of patients with ovarian cancer.

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Cisplatin and apoptotic signals

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