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CISPLATIN INDUCES PROGRAMMED DEATH-1-LIGAND 1(PD-L1) OVER-EXPRESSION IN HEPATOMA H22 CELLS VIA ERK /MAPK SIGNALING PATHWAY

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Abstract – Cisplatin has been widely used in cancer treatment. However, the prognosis of the cancer patients following chemotherapy has not been substantially improved and several different mechanisms could be involved. Clinically alternative strategies such as immunotherapy and their combinations with chemotherapy have being used. Cancer immunoresistance and immune escape are major obstacles in chemotherapy. However, the effects of cisplatin on the immune responses of cancer cells are not clear. In the present studies, we investigate the expression of immunoresistance moleculor PD-L1 (the negative regulator programmed death-1-ligand 1) on cisplatin-induced hepatoma H22 cells, which can interact with PD-1 on T cells to mediate cancer immunoresistance. Hepatoma H22 cells were treated with cisplatin in vivo or in vitro to analysis the expression of PD-L1 by flow cytometry (FACS). Erk1/Erk2 phosphorylation expressions were examined by western blotting. We demonstrated that cisplatin was able to induce H22 cell apoptosis and when the concentration less than IC50 cisplatin could up-regulate PD-L1 expression in hepatoma H22 cells. The optimal concentration of cisplatin for the highest expression of PD-L1 was 0.5 μg/ml in vitro. Meanwhile, cisplatin could induce the phosphorylation of Erk1/2.The lack of effect during treatment with a specific MAPK pathway inhibitor PD98059, demonstrated that cisplatin-induced PD-L1 expression is dependent of Erk1/2 phosphorylation. Our studies reveal a potential link between chemotherapy and cancer immunoresistance. PD-L1 and its signaling pathway appear to be a potential therapeutic target for the cisplatin treatment of hepatoma.

Key words: Cisplatin;programmed death-1-ligand 1(PD-L1);Hepatoma H22 cells; Erk phosphorylation.

INTRODUCTION

Cisplatin (cisplatinum or cis-diamminedichloroplatinum (II) (CDDP)) has been used chemotherapeutically for many years and is one of the most widely used cytotoxic drugs. However, resistance to cisplatin leads to poor response to chemotherapy and treatment failure (2,13,19). Multiple mechanisms have been suggested in the development of cisplatin resistance including reduction of intracellular drug accumulation, overexpression of thiol-containing molecules, increased DNA damage repair, upregulation of antiapoptotic genes as well as immunoresistance related genes (14,22).

Abbreviations: CDDP: Cisplatin,cisplatinum or cis-diamminedichloroplatinum(II); **ECL**: Chemiluminescence; **FACS**:Flow cytometry; **IR**:Inhibition rate of cisplatin; **PD-1**:Program death receptor 1; **PD-L1**:Programmed death-1-ligand 1; **PVDF**:Polyvinylidene difluoride membranes; **SD**:Standard deviation; **SDS-PAGE**:Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Thus, a better understanding of how cisplatin affects the immune responses of cancer cells, in particular,the anti-tumor immunity or immune evasion, is necessary for the improvement of the efficacy of cisplatin chemotherapy (4,10,20).

In the current studies, some chemopreventive agents were able to induce Programmed death-1-ligand 1(PD-L1) surface expression in some cancer cells, which then promoted PD-L1-mediated T cell apoptosis (3,11,24), and the resultant decrease of tumor cell-specific T cell activity may promote the development of immunoresistance. PD-L1, also denoted as B7-H1, is a cell surface protein of B7 family (3). PD-L1 is one of the two ligands for program death receptor 1 (PD-1), a co-stimulatory molecule that negatively regulates T cell activities. It is now well accepted that PD-L1 expression in cancer cells contributes to immunoresistance (8,9).Thus, increased PD-L1 expression on cancer cells could be an important escape mechanism from the host T cell

immunity. It's a potential link between chemotherapy and cancer immunoresistance. The aim of this study was to examine the effects of cisplatin on PD-L1 expression in hepatoma H22 cells and investigate whether MEK/Erk pathway was involved in this process.

MATERIALS AND METHODS

Cell culture

The Mouse hepatoma H22 cells (BalB/c background),was kept by Institute of Molecular Biology of Three Gorges University (Yichang, P.R.China) and cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum(Invitrogen Life Technologies), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 μM L-glutamine(Sigma). The cells were maintained in an incubator at 37℃ in 5% CO_2 and 95% air.The cells were subcultured in BALB/c mice. The viability of H22 cells, stained with trypan blue, was above 97%.

Mice, antibodies and reagents

Balb/c mice weighing 20-22 g were obtained from the Hubei Province Experiment Animal Center (Wuhan, Hubei, China). All studies involving mice were approved by the animal care and use committee of the University. Anti-mouse Foxp3(PE-labeled), Anti-mouse PD-L1 (CD274,B7-H1) (FITC-labeled), FITC-anti-mouse IgG2a isotype control antibodies were obtained from eBioscience (San Diego, CA); Antibodies against p-ERK1/2, ERK1/2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). HRP-conjugated anti-rabbit mouse antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cisplatin was from Sigma-Aldrich (St. Louis, MO). AlamarBlue was from Biosource(USA).PD98059 (MAPK kinase inhibitor that inhibits ERK1/2 activation) was supplied by Cayman Chemical(Michigan,USA). Protease inhibitor cocktail (complete, EDTA-free) was from Roche Applied Science (Mannheim, Germany).

Cell growth curve by alamarBlue assay

For the analysis of cell growth rate, H22 cells were plated in 96-well plates at a density of $1x10^4$ cells/well, cisplatin (10 μg/ml, 5 μg/ml, 2.5 μg/ml,1.25 μg/ml,0.625 μg/ml, or 0.3125 μg/ml)were added to the serial dilutions, and the plates were incubated for 24 h,48h and 72h at 37°C in 5% CO2. H22 cells without cisplatin as a control. AlamarBlue assay was according to the manufacturer's instructions. AlamarBlue was added at 10% of the well volume. Because the conversion of oxidized alamarBlue to its reduced form was used to determine the cellular metabolic activity, absorbance was measured at a wavelength of 530 nm (oxidized alamarBlue) and 590 nm(reduced alamarBlue) daily during 3 days. Inhibition rate of cisplatin(IR) was calculated as following: $IR(\%)=(D_{control}-D_{cisplatin})/D_{control}\times 100\%$ Assays were performed in triplicate and were repeated three times.

Flow cytometry(FACS)

PD-L1 surface expression was analyzed by flow cytometry. Briefly, Mouse hepatoma H22 cells were harvested and washed with FACS buffer (PBS with 5% FBS) for twice and then incubated with anti-mouse PD-L1(FITC-labeled) or isotype control antibodies for 30 min at 4°. Cells were washed with FACS buffer for 3 times. Cells were then washed and analyzed on flow cytometer with CellQuest sortware (Beckman Coulter, EPICS XL-4).

Western blot analysis

Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 10 mM $MgCl_2$, 1% Triton X-100 and 0.1% SDS) supplemented with 1% protease inhibitor cocktail. Lysates were centrifuged at 15,000×g for 30 min at 4°C.After protein concentrations were determined, proteins (50 μg) were then subjected to 12% standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to immunoblot polyvinylidene difluoride membranes(PVDF) (Bio-Rad,Hercules,CA).Membranes were blocked in 5% non-fat milk in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% (w/v) Tween-20 for 1 h.The membranes were first incubated with antibodies against phospho-specific and total ERK1/2,respectively, overnight at 4°C, washed three times in Tris-buffered saline containing 0.05%Tween 20 and incubation with HRP-conjugated secondary antibodies for 1 h at room temperature. After three additional washes,the immune complexes were visualized by the enhanced chemiluminescence(ECL) detection system (Amersham, Piscataway, NJ).

Statistical analysis

Results were expressed as mean ± SD and interpreted by ANOVA repeated measures test and Kaplan-Meier analysis. Differences were considered to be statistically significant when *p<0.05，**p<0.01.

RESULTS

CDDP induces H22 cells death

CDDP have been used in chemotherapy including hepatic carcinoma treatment. We first assessed the effects of CDDP on H22 hepatoma cells death in vitro with different concentrations at different time points.CDDP was found to be significantly susceptible to H22 cells at all concentrations used up to 10μg/ml. CDDP induced over 20% cell death at 72 h after treatment at the concentration of 0.625μg/ml (Fig.1), which prohibited us from examining PD-L1 surface expression at longer time. It's useful for the next set of the experiments.

CDDP induces PD-L1 surface expression in hepatoma H22 cells in vitro or in vivo

To examine whether CDDP induces PD-L1 surface expression in hepatoma H22 cells in vitro. We examined the dose and time responses of CDDP-induced PD-L1 surface expression. Hepatoma H22 cells were treated with different concentrations of CDDP (0.125μg/ml -2μg/ml). PD-L1 surface expression was analyzed by flow cytometry for 24 h and 48 h. In the time course study, an increase of PD-L1 surface expression was observed as early as 24 h after the treatment, and reached higher level at 48h after the

treatment. After 48h treatment of CDDP, PD-L1 expression was the highest (42.5%±5.5%) at 0.5μg/ml. (Fig.2A,B).

Whether CDDP can also induce PD-L1 surface expression in hepatoma H22 cells in vivo? Hepatoma H22 cells (1.0×10^7/each) were injected i.p. into BalB/c mice. Once tumors were palpable, mice (n=8) were given i.p. with CDDP at a dose of 10 mg/kg every 3 days for 3 times. At the time of day 5 and 10, H22 cells were taken out for PD-L1 surface expression detection by flow cytometry. As shown in Fig.2C, CDDP significantly induced the expression of PD-L1 in hepatoma H22 cells in vivo when compared to control mice receiving H22 cells alone (None) ($p<0.05$).This result corroborates our previous result demonstrating upregulation of PD-L1 in hepatoma H22 cells by CDDP in vitro.

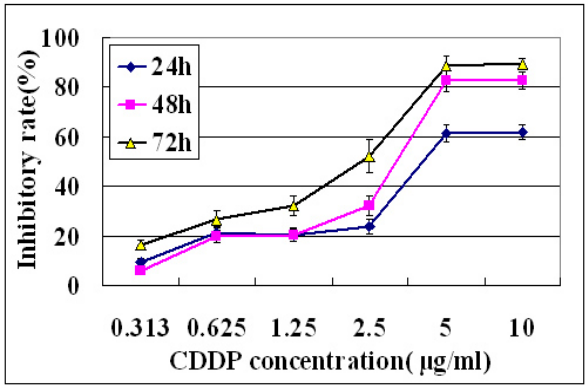

Figure 1. Inhibitory effects of CDDP on growth of hepatoma cells in vitro. The hepatoma H22 cells were treated with CDDP(0.3125-10 μg/ml) for 24h,48h and 72h as described in the Material and methods section. Cell viability was measured by alamarBlue assay and the inhibition rate of CDDP (IR) was calculated as following: IR (%)=($D_{control}-D_{cisplatin}$)/$D_{control}\times100\%$The results showed that CDDP inhibited H22 cell growth in a dose-dependent and a time-dependent manner.

Induction of PD-L1 in hepatoma H22 cells treated with CDDP via ERK/ MAPK

The previous report demonstrated that MAPKs mediated cell survival and death and member of the MAPKs such as ERK was activated after CDDP treatment both in vivo and in vitro[14,15].Here, we want to know whether CDDP can activate ERK/MAPK in hepatoma H22 cells. If so,whether CDDP induces PD-L1 exppression of H22 cells through ERK/MAPK signal transduction.

To assess the effect of CDDP in hepatoma H22 cells on ERK activation, hepatoma H22 cell lysates,which were treated or untreated with CDDP(0.5 μg/ml) for the time indicated, were resolved on SDS-PAGE and transferred to PVDF membrane. The blot was detected with anti-p-ERK1/2 antibodies. p-ERK1/2 was demonstrated higher density in hepatoma H22 cells at different time points with CDDP treatment than in hepatoma H22 cells alone. Protein loading in each lane was determined to be equal by reprobing the membrane with anti-ERK1/2 antibody. P-ERK1/2 of H22 cells was increasing in a time-dependent manner. Meanwhile, at the same time point, CDDP can increased p-ERK1/2 compared to control cells (Fig.3A).

Signaling pathway inhibitors have been widely used in signal transduction research, such as the MAPK kinase inhibitor PD98059. We next determined whether CDDP could activate PD-L1 through ERK/MAPK. P-ERK1/2 protein levels significantly down-regulated in a dose-dependent manner when the inhibitor PD98059 (10-40μM) was added in the CDDP-treated H22 cells for 24h (Fig.3B). Importantly, the inhibitor PD98059 down-regulated p-ERK1/2 significantly reduced PD-L1 expression detected by flow cytometry in the same CDDP-treated cells (Fig.3C, D). FCM results showed that the percentage of PD-L1 positive cells in H22 cells with or without (w/o) CDDP was 40.8±5.1% and 8.2±1.2% respectively ($P<0.01$). When PD98059 (40 μM) added, the percentage of PD-L1 positive cells in H22 cells treated with CDDP and control was 11.8±1.5% and 8.3±1.2% respectively ($P>0.05$).Thus, ERK/MAPK, is an upstream regulator of PD-L1 in hepatoma H22 cells, treated by CDDP.

DISCUSSION

Chemotherapy has been widely used to inhibit cancer cell proliferation and induce cancer cell apoptosis. However,the prognosis of the cancer patients following chemotherapy has not been substantially improved (16). Many chemopreventive agents such as paclitaxel, etoposide and 5-fluorouracil can induce PD-L1 surface expression in human breast cancer cells, which mediates PD-L1-specific T cell apoptosis and immunoresistance (24). In this study, we demonstrated that the CDDP could inhibit H22 cell proliferation and induce cancer cell apoptosis in vitro (Fig.1). However, it also can induce PD-L1 surface expression in hepatoma H22 cells in vivo and in vitro (Fig.2), suggesting that it may play an important role in the CDDP-induced cancer immunoresistance.

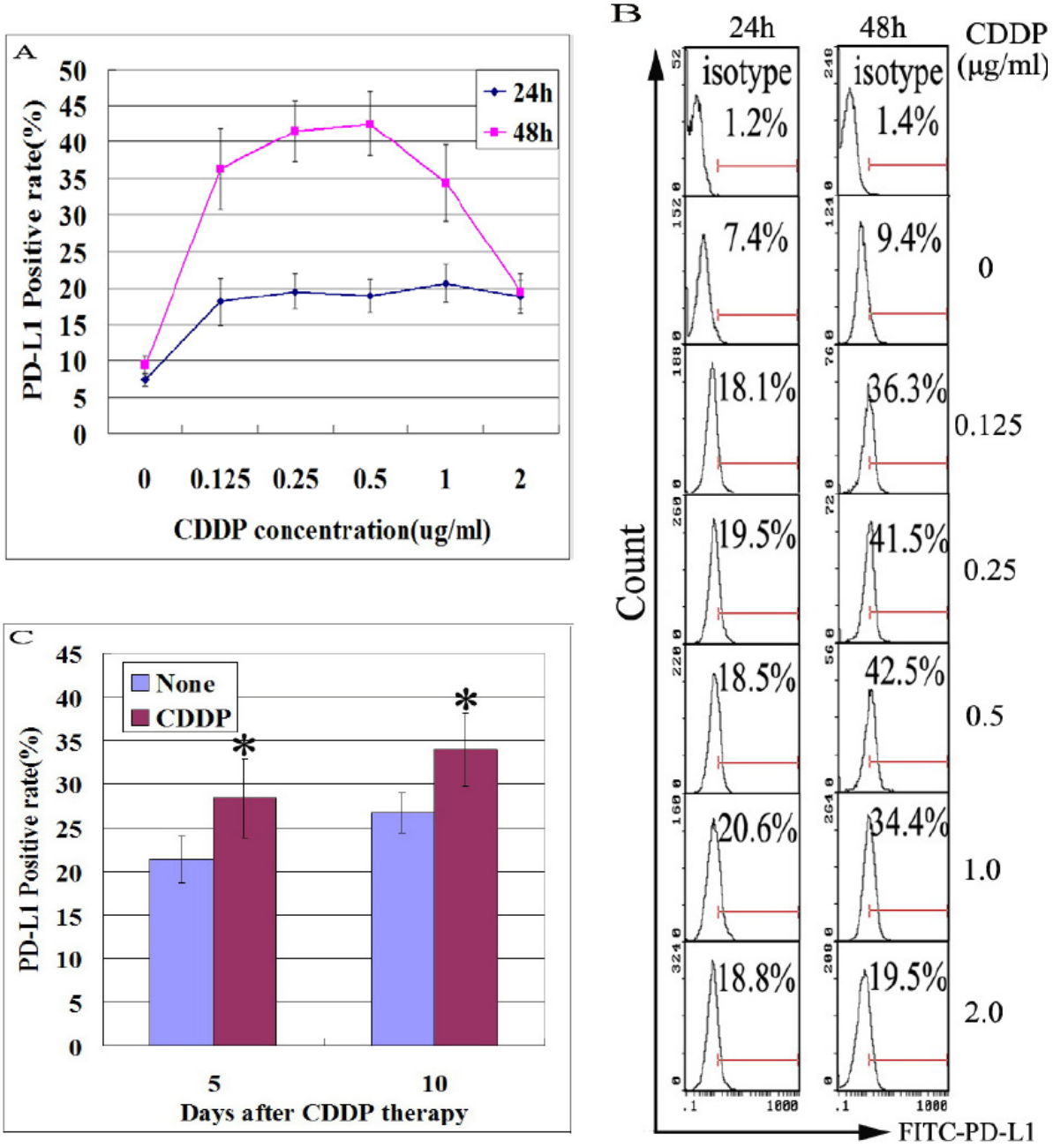

Figure 2. Effect of CDDP on the expression of PD-L1. PD-L1 surface expression in hepatoma H22 cells in vitro (A and B) or in vivo(C) was analyzed by flow cytometry (FCM).(B) PD-L1 expression in hepatoma H22 cells were detected by FCM when the cells were treated with different concentrations of CDDP for 24h and 48h. (A) Quantitation of PD-L1 expression from independent experiments depicted in B. (C) Hepatoma H22 cells taken from BalB/c mice which were treated or untreated CDDP(10 mg/Kg) were analyzed for PD-L1 surface expression. Asterisk (*) indicates a value significantly different from that of untreated control; $P<0.05$.

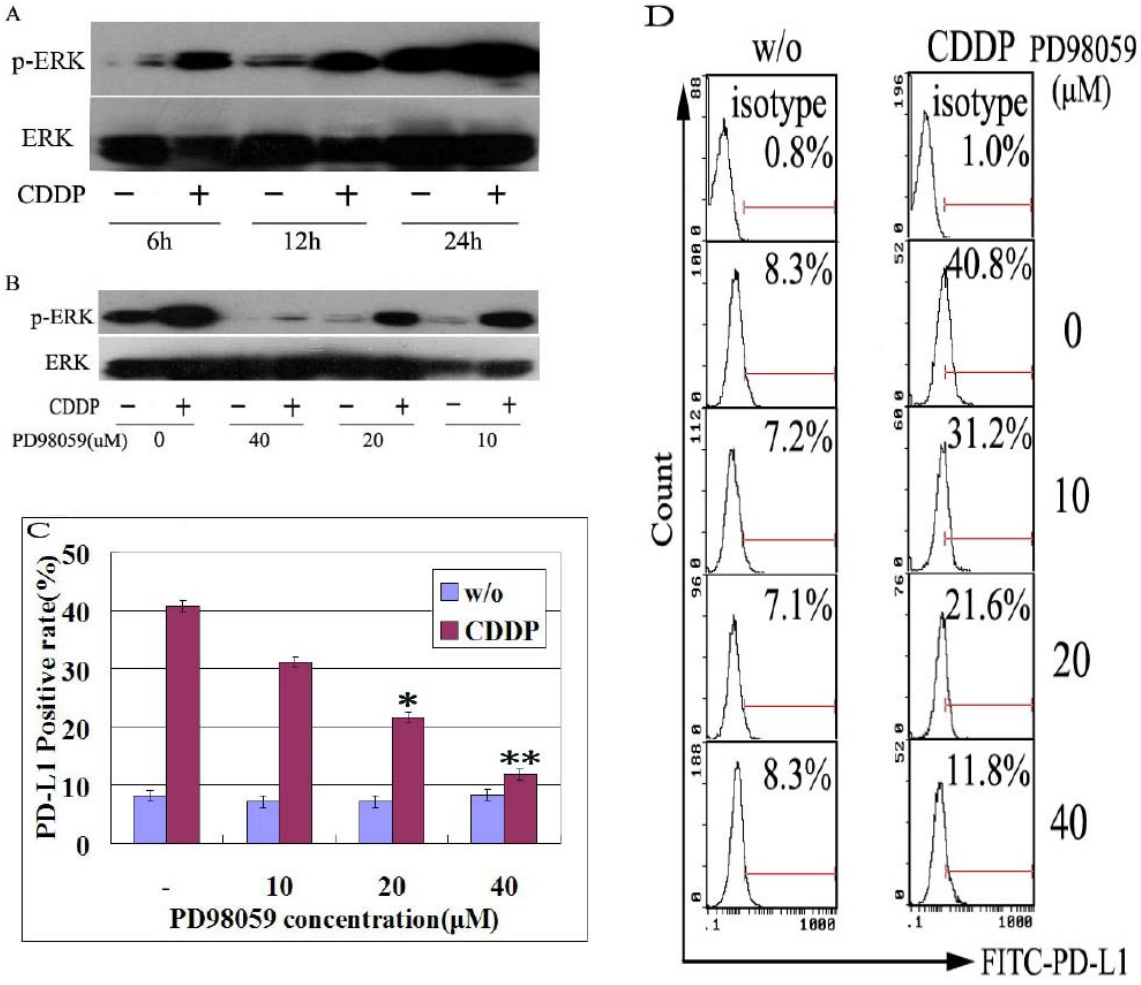

Figure 3. CDDP up-regulate PD-L1 expression through ERK/MAPK pathway. (A) p-ERK1/2 expression was detected by Western blotting when H22 cells were treated with CDDP (0.5μg/ml) for 6h, 12h or 24h. Immunoblots of internal control show equivalent levels of total ERK1/2 protein in each of the cell extracts. (B) p-ERK1/2 expression was analized by Western blotting after exposure to an MEK1/2 inhibitor PD98059 (10-40 μM) for 24h. (C) H22 cells were treated as same as (B), and PD-L1 expressions on cell surface were detected by FCM. Staining of the cell surface molecules was performed using monoclonal antibodies FITC-anti-mouse PD-L1, FITC-anti mouse IgG1 as isotype control antibody. Representative histograms of relative cell number versus FITC fluorescence intensity for the PD-L1-positive cells; the percentage of positive cells is indicated (D). The percentage of PD-L1-positive cells from independent experiments depicted in (C). Data are expressed as the mean ± SEM (n=3).

PD-L1 is expressed on both hematopoietic and non-hematopoietic cells. PD-L1 surface expression can be found on almost all tumor entities. It has been detected by immunohistochemistry,for example,on the surface of human cancers of larynx, lung, stomach, colon, breast,cervix,ovary,renal cell,bladder,liver,glioma and melanoma (24). PD-L1 expression on carcinomas is correlated with poor clinical prognosis. In vitro experiments indicate that many tumor cell lines also express PD-L1 and/or up-regulate PD-L1 surface expression upon exposure to IFN-γ (15).This expression is in strong contrast to the expression to B7-1 and B7-2, which are seldom found on tumors. PD-L1 on cancer cells can bind to PD-1 receptor on activated T cells, inhibit the proliferation of tumor-reactive cytotoxic T cells, and induce Treg phenotype, which could be play a crucial mechanism in suppressing the overall T-cell responses and the development of cancer immunoresistance (15). Over-expression of human PD-L1 or interference using anti-PD-L1 blocking antibodies support the notion of tumor-expressed PD-L1 being capable of suppressing T-cell immune functions and mediating immunoresistance (6,7,12,21).

The regulation of PD-L1 expression is only to be understood recently. IFN-γ is so far the best-known regulator of PD-L1 expression (15,17,23). It has been shown that both the JAK/STAT and MAP kinase signaling pathways are involved in IFN-γ induced PD-L1 expression (15). The importance of MEK/ERK pathway in regulating apoptosis after CDDP treatment has been suggested. A recent study suggested that cisplatin could induce a longer-lasting ERK activation in various systems (5,18) and mediates cisplatin-induced cell death. Our data showed that inhibition of MAPK by the inhibitor PD98059 also decreased cisplatin-associated PD-L1 expression (Fig.3C, D) and inhibited cisplatin-induced p-ERK activation (Fig.3B).Thus CDDP-induced PD-L1 over-expression,is MEK/Erk dependent.

In conclusion,we have found that CDDP can induce PD-L1 surface expression in hepatoma H22 cells via Erk /Mapk signaling pathway. Our observations from this study suggest that targeting Erk /Mapk signaling pathway,as an important target point,could prove to be a novel therapy in chemotherapy combining with immune remedy.

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