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Mechanism of vascular endothelial growth factor expression mediated by cisplatin in human ovarian cancer cells

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Abstract

Cisplatin (CDDP) and its analogues are widely used for the treatment of a variety of human solid tumors. However, the molecular mechanism of its action remains to be understood. Vascular endothelial growth factor (VEGF) is a potent inducer of angiogenesis and is upregulated in many human cancers. In this study we demonstrated that CDDP-inhibited VEGF expression in human ovarian cancer cells. We found that CDDP inhibited the VEGF reporter activity in a dose-dependent manner, indicating that CDDP-inhibited transcriptional activation of VEGF. We also found that: (1) luciferase activity mediated by the VEGF reporter containing a mutation of the HIF-1 binding site was much lower than that of the reporter containing a wild-type HIF-1 binding site in ovarian cancer cells, thus confirming that HIF-1 is a major transcriptional regulator of VEGF expression; and that (2) CDDP greatly inhibited VEGF reporter activity containing the wild-type but not the mutant HIF-1 binding site. This result indicates that CDDP-inhibited VEGF transcriptional activation specifically by decreasing HIF-1 activity. Co-transfection of a dominant negative construct of HIF-1 inhibited VEGF reporter activity in ovarian cancer cells. CDDP-inhibited VEGF transcriptional activation specifically through the expression of HIF-1α, but not HIF-1β. We demonstrated that VEGF receptor KDR was expressed in ovarian cancer cells, and that CDDP-inhibited VEGF expression was linked with cellular apoptosis, which was rescued by VEGF treatment. These results suggest a novel mechanism of CDDP's anti-tumor activity in ovarian cancer cells via HIF-1 expression and VEGF transcriptional activation.

Keywords: Cisplatin; Ovarian cancer; HIF-1; VEGF; Angiogenesis

CDDP and its analogues are useful particularly in the treatment of late stage ovarian cancer and are part of the standard chemotherapy treatment for this disease [1]. The primary mechanism responsible for the anti-tumor activity of CDDP is thought to be the cross-linking of CDDP with genomic DNA and the induction of apoptosis [2–4]. However, the cellular response to DNA damage is complex and the specific mechanism that causes cell death remains to be elucidated [5].

Vascular endothelial growth factor (VEGF) is a potent inducer of angiogenesis both *in vivo* and *in vitro* [6–8]. Mutations of one or both alleles of the VEGF gene result in an embryonic lethal phenotype in mice due to inadequate vascularization, suggesting that VEGF is required for the early stages of vascular development [9,10]. VEGF expression is upregulated in a variety of human tumors, including ovarian tumors [11], and VEGF-stimulated angiogenesis seems to be required for this type of tumor [12,13]. The inhibition of VEGF expression in turn inhibited tumor angiogenesis *in vivo* and decreased tumor size in nude mice

Abbreviations: CDDP, cisplatin; VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor 1; KDR, VEGF receptor 2.

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[14–16]. VEGF expression is also upregulated by the hypoxic conditions in tumor cells and the activation of oncogenes such as v-Src and Ras [17–19].

Hypoxia inducible factor 1 (HIF-1) is a heterodimeric helix–loop–helix transcription factor that consists of two subunits: HIF-1α and HIF-1β [20,21]. HIF-1β is constitutively expressed in cells, while HIF-1α stability is stimulated by hypoxia, growth factors, and several oncogenes [22]. HIF-1 has a number of transcriptional targets including VEGF [23–26].

Materials and methods

Reagents and cell culture. Cisplatin (CDDP) was obtained from Sigma (St. Louis, MO). The human ovarian cancer cell lines OVCAR-3 and A2780/CP70 were maintained in RPMI 1640 media supplemented with 10% fetal calf serum. All cells were cultured at 37 °C in a 5% CO_2 incubator.

DNA constructs. The dominant negative form of HIF-1, HIF-1α DN, was subcloned into pCEP4 vector (Invitrogen). The human VEGF reporter construct was constructed by inserting a 1.9 kb of the human VEGF gene promoter into the pGL2-basic vector. The pMAP11 WT VEGF reporter was constructed by PCR amplification of a fragment of the VEGF promoter from –985 to –939; the pMAP11 mutant was constructed by a three base pair substitution in the HIF-1 binding sequence in the pMAP11 WT VEGF reporter.

Immunoblot analysis. Aliquots of protein extracts were resolved in SDS/polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and analyzed using antibodies against HIF-1α and HIF-1β (Transduction Laboratories, Lexington, KY).

Preparation of RNA and Northern blots. Total cellular RNAs were isolated using RNA STAT-60 (Tel-Test Inc., Friendswood, TX), aliquots of 10 μg total RNAs were separated by electrophoresis in 2.2 M formaldehyde/0.9% agarose gel, transferred to a nylon membrane, and analyzed by Northern blots with [^{32}P]dATP-labeled human VEGF cDNA and actin cDNA.

Transient transfection and luciferase assays. The cells were transfected with the plasmid DNAs using LipofectAMINE (Invitrogen, Carlsbad, CA). After the transfection, the cells were cultured. The cells were lysed,

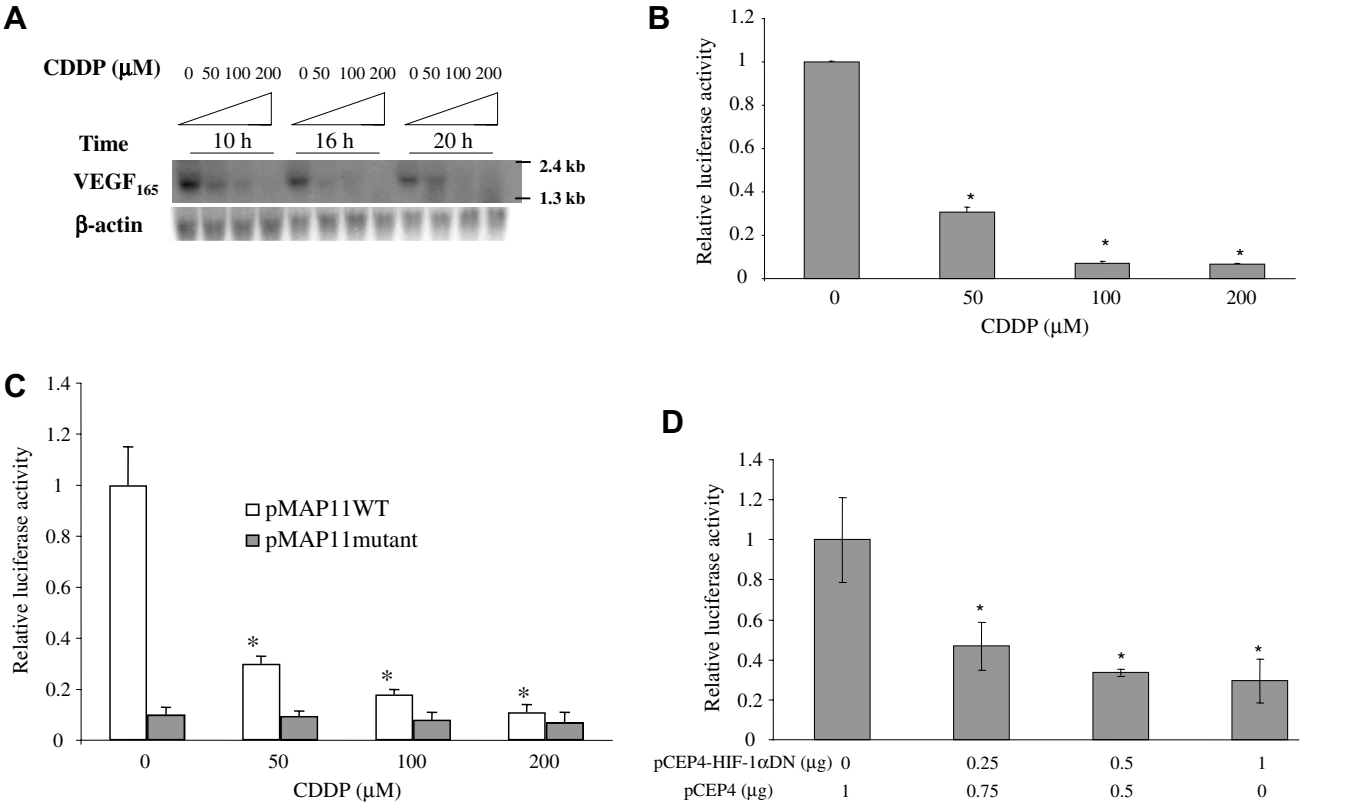

Fig. 1. Effects of CDDP on VEGF mRNA expression and transcriptional activation through the HIF-1 DNA binding site in the promoter. (A) A2780/CP70 cells were treated in the absence or presence of CDDP (50, 100 or 200 μM) for 10, 16, or 20 h as indicated. Total cellular RNAs were prepared, and used for Northern blot analysis. (B) A2780/CP70 cells were co-transfected with pCMV-β-gal plasmid and a human VEGF reporter, pVEGF-Luc, containing a 1.9 kb human VEGF promoter fragment inserted upstream of firefly luciferase. After the transfection, the cells were cultured for 12 h, followed by the treatment of CDDP at the concentrations indicated for 24 h. Relative luciferase activity was determined by the ratio of luciferase to β-gal activity, and normalized to the value obtained from A2780/CP70 cells in the absence of CDDP. The figure shows mean ± SD of relative luciferase activities for each group. *indicates that the relative luciferase activity was significantly different when compared to that of the control ($p < 0.01$). (C) A2780/CP70 cells were co-transfected with pCMV-β-gal and a VEGF promoter containing a 46 bp functional human VEGF promoter with the HIF-1 binding site, pMAP11WT reporter, or the pMAP11mutant which contained 3-bp mutation of the HIF-1 binding site. Relative luciferase activity was obtained and normalized to that from the cells transfected with the pMAP11WT and treated with vehicle only. *indicates that the relative luciferase activity was significantly different when compared to that of the pMAP11WT control in the absence of CDDP ($p < 0.01$). (D) Effect of HIF-1α on the VEGF reporter activity. A2780/CP70 cells were co-transfected with 0.5 μg pMAP11WT reporter and a HIF-1α dominant negative construct in pCEP4 vector at 0, 0.25, 0.5, and 1 μg. Relative luciferase activity was normalized to that from the parental expression vector pCEP4 only. The figure shows mean ± SD of relative luciferase activities for each group. *indicates that the relative luciferase activity was significantly different when compared to that of the control ($p < 0.01$).

and relative luc activity (mean ± SD) was calculated as luc (RLU)/β-gal, and normalized to that of the control.

Immunofluorescence staining. Cells were seeded onto coverslips in complete medium and cultured overnight. Cells on the coverslips were analyzed with the mouse monoclonal antibody against FlK-1 (Santa Cruz Biotechnology, Santa Cruz, CA) and detected by confocal microscopy using a Zeiss 510 LSM microscope.

Annexin-V staining and flow cytometry assay. The cells were stained using an Annex-V-FLUOS Staining Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and analyzed for cellular apoptosis by flow cytometry.

DNA fragmentation assay. Both floating and adherent cells were collected. Cells were lysed and genomic DNA was prepared using the Wizard® Genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions.

Results

Cisplatin (CDDP) led to a concentration-dependent decrease in VEGF mRNA levels

Angiogenesis is essential for ovarian tumor growth and metastasis. High levels of VEGF expression and microvessel density have been shown to be associated with poor survival in patients with advanced stage ovarian cancer [12]. To determine whether CDDP treatment affects VEGF expression, A2780/CP70 ovarian cancer cells were incubated in the absence or presence of different concentrations of CDDP at different times. Levels of VEGF mRNA were inhibited by CDDP at all treatments in a dose-dependent manner (Fig. 1A). Actin levels were not affected by CDDP treatments (Fig. 1A). This result indicated that CDDP decreased the VEGF mRNAs in the ovarian cancer cells.

CDDP-inhibited VEGF transcriptional activation

To determine whether inhibition of VEGF mRNA levels was due to a decrease in VEGF transcriptional activation, we tested the effect of CDDP treatment on human VEGF reporter activity. A2780/CP70 cells were co-transfected with pCMV-β-gal plasmid and a VEGF reporter that contains the 1.9 kb human VEGF promoter element inserted upstream of firefly luciferase in a pGL2 vector. After transfection, the cells were cultured overnight, followed by incubation with CDDP at the concentrations indicated for 24 h (Fig. 1B). VEGF transcriptional activation was indicated by the relative luciferase activity in the cells. When compared to the solvent control, VEGF reporter activity was decreased to 30% of the control in the cells treated with 50 μM CDDP, and to 10% of the control in the cells treated with 100 or 200 μM CDDP (Fig. 1B). These data indicated that CDDP indeed inhibited VEGF transcriptional activation in a dose-dependent manner, and this inhibition reached a plateau at 100 μM CDDP. The effect of CDDP on VEGF transcriptional activation correlated with the levels of VEGF mRNA as shown in Fig. 1A.

Inhibition of VEGF transcriptional activation by CDDP required the HIF-1 DNA binding site

To test whether CDDP inhibits VEGF transcriptional activation through the HIF-1 DNA binding site in the

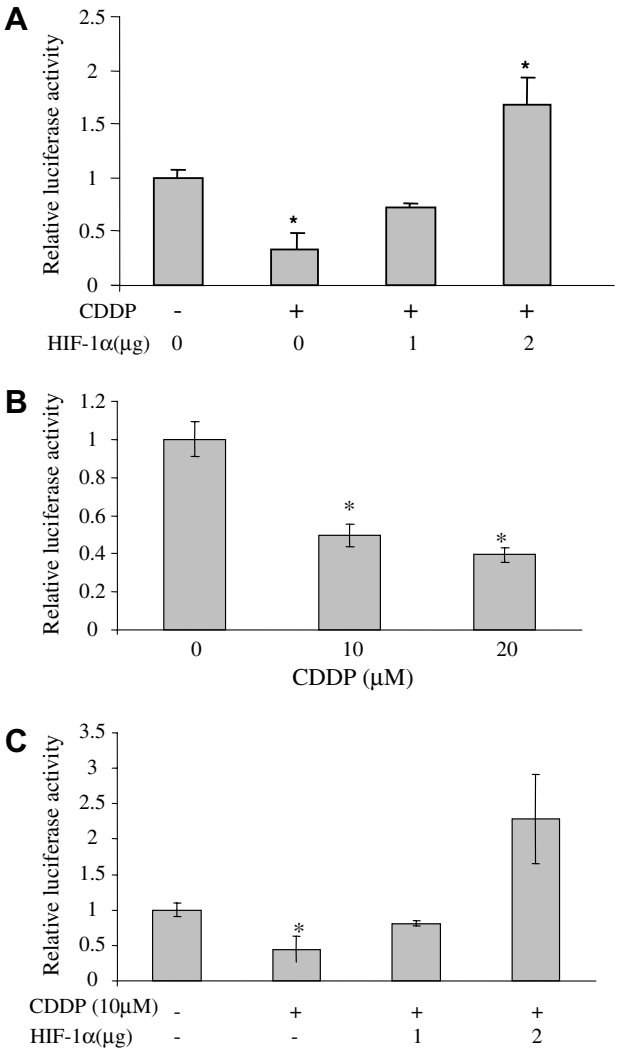

Fig. 2. HIF-1 expression restored CDDP-inhibited transcriptional activation of VEGF. (A) A2780/CP70 cells were co-transfected with 1 or 2 μg of wild-type HIF-1α plasmid and the human VEGF reporter pVEGF-Luc. Relative luciferase activity was determined and normalized to the value obtained from A2780/CP70 cells in the absence of CDDP as described in Fig. 1. The figure shows mean ± SD of relative luciferase activities for each group. *indicates that the relative luciferase activity was significantly different when compared to that of the control ($p < 0.01$). (B) A2780 cells were co-transfected with pCMV-β-gal plasmid and the human VEGF reporter, pVEGF-Luc. Luciferase and β-gal activities were measured. Relative luciferase activity was determined by the ratio of luciferase to β-gal activity, and normalized to the value obtained from A2780 cells in the absence of CDDP. The figure shows mean ± SD of relative luciferase activities for each group. (C) A2780 cells were co-transfected with 1 or 2 μg of wild-type HIF-1α plasmid and the human VEGF reporter, pVEGF-Luc. Relative luciferase activity was determined and normalized to the value obtained from A2780 cells in the absence of CDDP. The figure shows mean ± SD of relative luciferase activities for each group. *indicates that the relative luciferase activity was significantly different when compared to that of the control ($p < 0.01$).

promoter, a fragment of functional human VEGF promoter containing the HIF-1 DNA binding site was inserted into pGL2 vector as pMAP11WT. A2780/CP70 cells were co-transfected with pMAP11WT reporter and pCMV-β-gal plasmids. As shown in Fig. 1C, CDDP treatment greatly inhibited the pMAP11WT reporter activity in a dose-dependent manner. To determine if the observed inhibition required the HIF-1 DNA binding site in the promoter, we made a mutant reporter with three amino acid substitutions (pMAP11mutant). A2780/CP70 cells were transfected with pMAP11mutant and tested for the effect of CDDP on pMAP11mutant reporter activity. When compared to the wild-type reporter, the relative luciferase activity mediated by the mutant reporter containing the mutation of the HIF-1 binding site was much lower in ovarian cancer cells (Fig. 1C). This result confirms that HIF-1 is a major transcriptional regulator for VEGF transcriptional activation. CDDP treatment did not decrease the reporter activity containing the mutation at the HIF-1 binding site, while it greatly inhibited the VEGF reporter activity containing the wild-type HIF-1 binding site (Fig. 1C). This result demonstrated that CDDP-inhibited VEGF transcriptional activation specifically through the HIF-1 DNA binding site. These results suggested that the HIF-1 DNA binding site is required for the CDDP-mediated inhibition of VEGF transcriptional activation. To further confirm the role of HIF-1 in VEGF transcriptional activation, the cells were co-transfected with pMAP11WT and a HIF-1 dominant negative construct pCEP4/HIF-1α DN. VEGF reporter activity was inhibited by the HIF-1α DN construct in a dose-dependent manner (Fig. 1D), thus confirming that HIF-1 DNA binding activity is necessary for CDDP to inhibit VEGF transcriptional activation in ovarian cancer cells.

HIF-1α expression restored the CDDP-inhibited transcriptional activation of VEGF

To determine whether the expression of HIF-1α was sufficient to restore the CDDP-inhibited VEGF reporter activity, A2780/CP70 cells were co-transfected with HIF-1α expression plasmid and the VEGF reporter that contains the 1.9 kb VEGF promoter element as we described previously [20]. After transfection, the cells were cultured overnight, followed by incubation for 24 h with or without 50 μM CDDP. VEGF transcriptional activation was normalized to the solvent control. CDDP treatment inhibited VEGF reporter activity to 30% of the control (Fig. 2A). Co-transfection of HIF-1α expression plasmid restored VEGF transcriptional activation in a dose-dependent manner (Fig. 2A). This result suggests that HIF-1α expression is sufficient to restore CDDP-inhibited VEGF transcriptional activation. To determine whether CDDP also inhibits VEGF transcriptional activation in its CDDP-sensitive parental A2780 cells, A2780 cells were co-transfected with pCMV-β-gal plasmid and the human VEGF reporter,

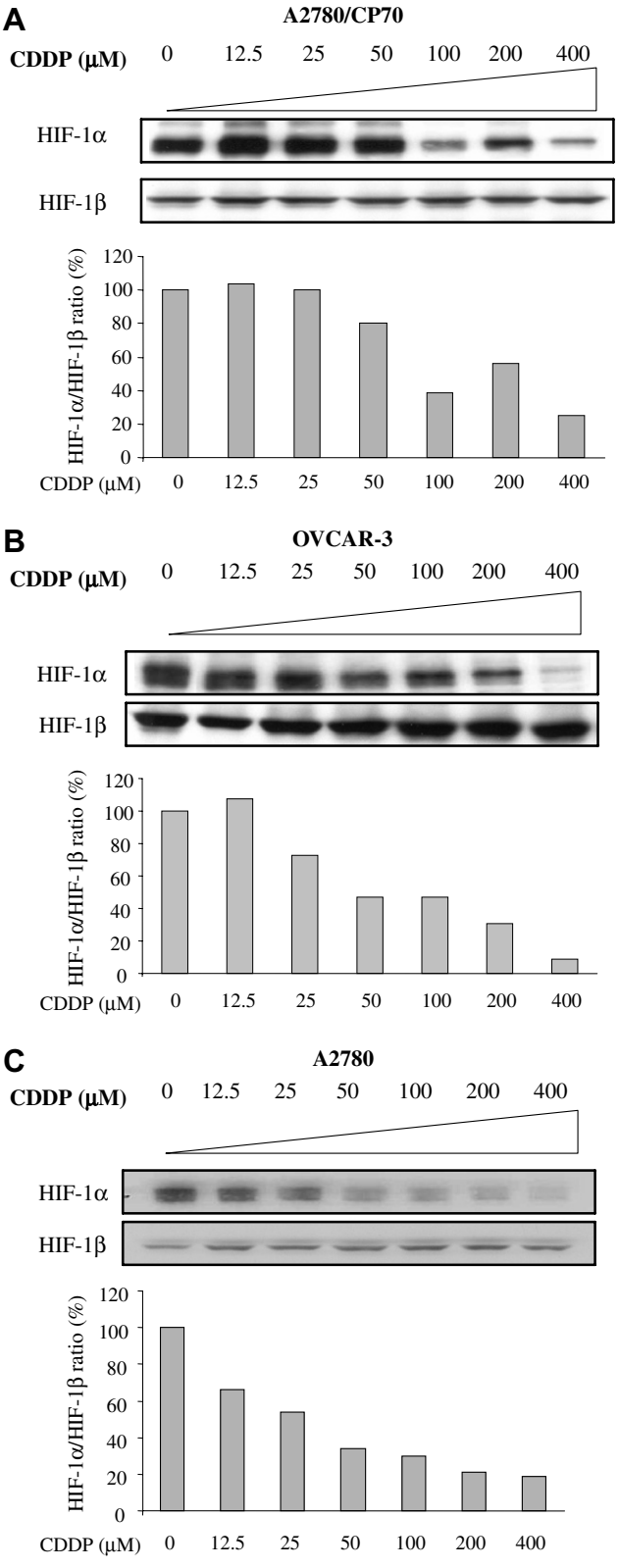

Fig. 3. Effects of CDDP treatment on HIF-1 expression. A2780/CP70 (A), OVCAR-3 (B), and A2780 cells (C) were cultured and treated in the absence or presence of CDDP (12.5, 25, 50, 100, 200, or 400 μM) for 6 h as indicated. Total cellular protein extracts were prepared and 25 μg of protein was resolved by SDS–PAGE and analyzed by Western blot using antibodies against HIF-1α and HIF-1β proteins. The signal intensity of HIF-1α was divided by HIF-β, and normalized to the untreated control.

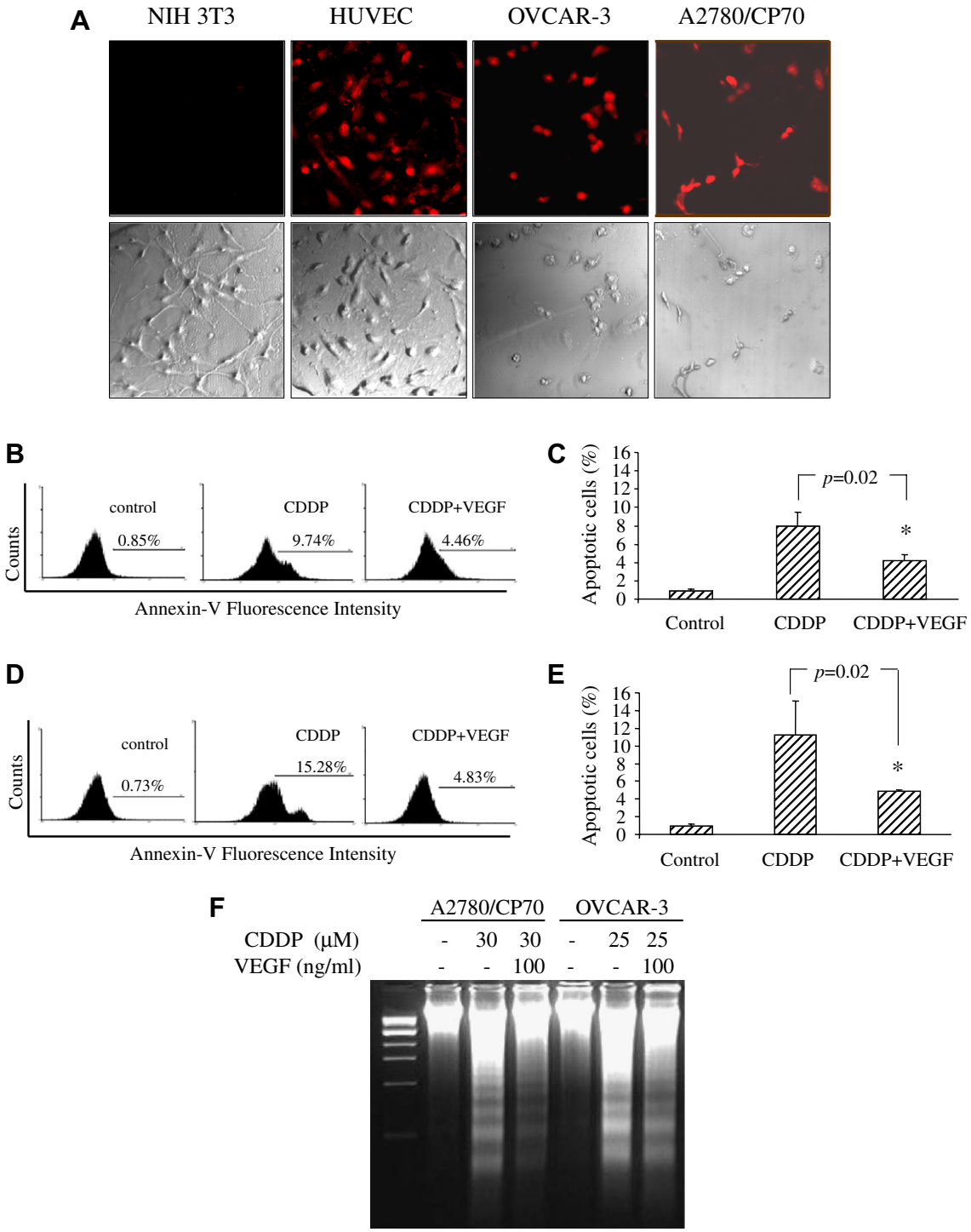

Fig. 4. Expression of VEGF receptor KDR in OVCAR-3 and A2780/CP70 cell lines, and its effects in VEGF-mediating cellular survival. (A) VEGF receptor KDR expression was detected by immunofluorescence staining. Mouse fibroblast NIH 3T3 cells were used as a negative control. Human umbilical vein endothelial cells (HUVEC) were used as a positive control for VEGF receptor expression. The upper panels show the fluorescence signals (red) indicating expression of the VEGF receptor. The lower panels show the phase contrast images of the same areas as the upper panels. Original magnification, 100×. (B) Subconfluent A2780/CP70 cells were cultured in serum-free and insulin-free medium overnight, followed by treatment with solvent alone, 30 μM cisplatin, or 30 μM cisplatin plus 100 nM VEGF for 24 h. Apoptosis was assayed by flow cytometry analysis of annexin-V staining. (C) Mean ± SD of apoptotic A2780/CP70 cell percentage was obtained from three replicate experiments performed as described above. (D) Subconfluent OVCAR-3 cells were cultured in serum-free and insulin-free medium overnight, followed by treatment with 25 μM cisplatin or 25 μM cisplatin plus 100 nM VEGF for 24 h. The cells were stained by annexin-V, and the percentage of apoptotic cells was assayed by flow cytometry analysis. (E) Mean ± SD of apoptotic OVCAR-3 cell percentage was obtained from three replicate experiments. (F) VEGF treatment inhibits cisplatin-induced DNA fragmentation. A2780/CP70 and OVCAR-3 cells were treated as described above. DNA fragmentation assay was performed as described in the Materials and methods. Lane 1, 1 kb DNA ladder. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

pVEGF-Luc. After the transfection, the cells were cultured for 12 h, followed by the treatment of CDDP at 10 and 20 μM for 24 h. VEGF reporter activity was normalized to the solvent control. CDDP treatment inhibited VEGF reporter activity in a dose-dependent manner (Fig. 2B). To test whether HIF-1α expression is sufficient to restore CDDP-inhibited VEGF transcriptional activation, A2780 cells were co-transfected with wild-type HIF-1α plasmid and the human VEGF reporter pVEGF-Luc. After transfection, the cells were cultured for 12 h, followed by treatment with 10 μM CDDP for 24 h. CDDP-inhibited VEGF reporter activity, but this inhibition was reversed by co-transfection of HIF-1α plasmid (Fig. 2C). The results suggest that HIF-1α expression is sufficient to restore CDDP-inhibited VEGF transcriptional activation in the CDDP-sensitive ovarian cancer cells.

CDDP decreased levels of HIF-1α protein expression

The results above showed that HIF-1 DNA binding activity is important for CDDP-inhibited VEGF expression. To determine whether CDDP had any effects on the amount of HIF-1α protein level in the cell, A2780/CP70, OVCAR-3, and A2780 ovarian cancer cells were cultured in the presence or absence of CDDP. As shown in Fig. 3A, CDDP treatment at 12.5 and 25 μM did not inhibit HIF-1α protein expression, and CDDP at 50 μM and higher concentrations inhibited HIF-1α but not HIF-1β expression in A2780/CP70 cells. In contrast, CDDP treatment at 25 μM inhibited HIF-1α expression in both OVCAR-3 and A2780 cells, which are CDDP-sensitive cells (Figs. 3B and C). These results indicated that CDDP treatment inhibited specifically HIF-1α but not HIF-1β expression in ovarian cancer cells, which are correlated with the CDDP resistance in the cells.

Expression of the VEGF receptor KDR in ovarian cancer cells

To determine whether the inhibition of HIF-1 and VEGF expression by CDDP in ovarian cancer cells have any functional link, we first determined whether the VEGF receptor KDR was expressed in the ovarian cancer cells used. The cells were stained by immunofluorescence using antibodies against Flk1/KDR protein. Both OVCAR-3 and A2780/CP70 cells were strongly stained with the antibodies, indicating that the VEGF receptor is expressed in the ovarian cancer cells (Fig. 4A).

VEGF treatment inhibited CDDP-induced cellular apoptosis

To determine the role of VEGF expression in mediating CDDP-induced apoptosis in the ovarian cancer cells, A2780/CP70 and OVCAR-3 cells were treated by CDDP in the absence or presence of VEGF. Cellular apoptosis was analyzed 24 h after the treatment by annexin-V staining and flow cytometry analysis. CDDP treatment induced the cellular apoptosis in both cell lines, and the addition of VEGF significantly inhibited CDDP-induced apoptosis ($p = 0.02$) (Figs. 4B–E). Similarly, apoptosis was assayed by DNA fragmentation analysis 24 h after the treatment. VEGF treatment inhibited CDDP-induced DNA fragmentation in both A2780/CP70 and OVCAR-3 cells (Fig. 4F). These data demonstrated that the inhibition of VEGF expression by CDDP was associated with cellular apoptosis, which represents a new mechanism of CDDP in inhibiting VEGF expression. VEGF in turn regulates cellular apoptosis, tumor growth, and angiogenesis.

Discussion

Although the ability of CDDP to cross-link genomic DNA has been known for some time, the sequence of events investigated by the cross-linking that culminates in anti-tumor activity remains unclear [2]. With the emergence of CDDP as the primary therapy for ovarian cancer, it is important to obtain a better understanding of its mechanism of action. VEGF is an endothelial cell-specific potent inducer of angiogenesis. Expression of VEGF was greatly increased in ovarian cancer cells [12]. However, the effect of CDDP on VEGF expression in ovarian cancer cells is not known. In this study we demonstrated that CDDP treatment decreased VEGF mRNA levels through transcriptional activation in the ovarian cancer cell lines A2780/CP70, OVCAR-3, and A2780. Although our reporter assay results may be obtained only in living cells, there is a possibility that dead cells or other unknown CDDP-inhibited effectors could have partial effect. Our study indicates a possible link between CDDP chemotherapy and VEGF expression levels in ovarian cancer. VEGF is a central regulator of tumor growth and angiogenesis. The inhibition of VEGF by CDDP may represent a novel mechanism of CDDP action in inducing cellular apoptosis and in inhibiting tumor angiogenesis in the ovarian tumorigenesis. Another interesting finding in this study is that CDDP specifically decreased the expression of HIF-1α, but not of HIF-1β. The inhibition of HIF-1 by CDDP could have synergetic effects at multiple levels to inhibit ovarian tumor growth and angiogenesis. Taken together, this study demonstrated that CDDP-inhibited VEGF transcriptional activation and HIF-1α expression. This result may provide a new mechanism of CDDP action in human ovarian cancer cells.

Acknowledgments

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