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Effects of Interleukin-1α on DNA Repair in Human Ovarian Carcinoma (NIH:OVCAR-3) Cells: Implications in the Mechanism of Sensitization of *Cis*-Diamminedichloroplatinum(II)

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SUMMARY

The cytokine interleukin-1α (IL-1α) showed a cytostatic effect on human ovarian carcinoma cells and significantly enhanced the antiproliferative activity of *cis*-diamminedichloroplatinum(II) (cisplatin) toward the NIH:OVCAR-3 tumor cell line in culture. The factor of sensitization was 15–20-fold. The maximum levels of sensitization were observed both with simultaneous exposure to cisplatin and IL-1α and with 24-hr pretreatment with IL-1α. Synergy between these agents was diminished when cells were pretreated with an IL-1α-specific receptor antagonist, indicating that synergistic interaction was receptor mediated. Using atomic absorption spectroscopy, we evaluated the cellular accumulation of cisplatin and the DNA platination; the results showed that IL-1α increased cellular accumulation of cisplatin and DNA platination. Cisplatin did not affect IL-1α accumulation in NIH:OVCAR-3 cells. Further studies showed that IL-1α reduced the removal of platinum from DNA. These results strongly suggest that IL-1α inhibits DNA repair, and this decrease in DNA repair may explain, in part, the strong synergistic interaction between IL-1α and cisplatin in NIH:OVCAR-3 cells.

CDDP is one of the most commonly used agents for the treatment of a wide variety of human cancers and is particularly useful in the treatment of testicular and ovarian carcinomas (1–3). Although a large number of papers have been published regarding mechanisms of CDDP cytotoxicity in tumor cell lines *in vitro*, the primary lesions ultimately responsible for cell death are not known. CDDP has been reported to react with DNA to produce interstrand DNA-DNA cross-links, and the major adduct of the platinum bound to DNA has been identified as an intrastrand N^7d(GpG)-diammineplatinum adduct, in addition to intrastrand d(ApG)- and d(GpXpG)-platinum adducts (4–6). DNA-protein adducts have also been detected from the reaction of CDDP with cellular macromolecules (4–6).

Despite the potency of CDDP, the frequent development of CDDP resistance by tumors is a major problem preventing curative therapy (7, 8). Furthermore, administration of higher doses of CDDP has not been successful, because of the severe toxicity associated with CDDP therapy. Although several mechanisms of resistance to CDDP have been identified, including an increase in total cellular GSH content, reduction of CDDP uptake is one of the important mechanisms contributing to CDDP resistance (9–16). Furthermore, DNA repair-defective cells are reported to be hypersensitive to CDDP, and enhanced DNA repair has been implicated in the resistant CDDP phenotype (14).

IL-1α and IL-1β are produced by activated monocytes and macrophages. IL-1 has been shown to possess a wide spectrum of biological activity, including modulation of T and B cell function, stimulation of mouse fibroblast proliferation, induction and release of prostaglandin E_2, and stimulation of the synthesis of other cytokines, e.g., IL-6 or tumor necrosis factor (17–19). Moreover, IL-1 possesses antitumor activities both *in vitro* and *in vivo* (20–22). Work from our laboratory has focused on defining the mechanism of the antitumor activity of IL-1α in several tumor cell lines, including human melanoma and ovarian carcinoma (23–26). At this time, neither the biochemical mechanisms nor the cellular targets involved in IL-1α-induced tumor cell kill are clearly defined; however, the binding of IL-1α to the IL-1 receptor appears to be essential for the biological and pharmacological activities of IL-1α (17–19).

The extreme toxicities of IL-1α have limited its potential clinical use for the management of human tumors. Thus, it is possible to combine IL-1α with clinically active anticancer drugs, with the aim of increasing the chemotherapeutic efficacy and simultaneously reducing host toxicity. In this re-

ABBREVIATIONS: CDDP, *cis*-diamminedichloroplatinum(II); IL-1α, interleukin-1α; IL-1RA, interleukin-1 receptor antagonist; VP-16, etoposide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; GSH, glutathione; PBS, phosphate-buffered saline; AAS, atomic absorption spectroscopy.

gard, we have shown that combinations of VP-16 or doxorubicin with IL-1α are highly effective against human melanoma A-375 cells and ovarian NIH:OVCAR-3 tumor cells *in vitro*, with dose modification factors of 2–4-fold for IL-1α and 8–10-fold for VP-16 and doxorubicin (23–26). Recently, we also reported that IL-1α was synergistic with CDDP, carboplatin, and camptothecin in NIH:OVCAR-3 cells (27, 28). In this report, we show that the combination of IL-1α with CDDP results in a 15–20-fold dose modification factor for CDDP against a human ovarian carcinoma cell line *in vitro*. Furthermore, this increase in cell killing by CDDP when combined with IL-1α appears to result from increased CDDP uptake and decreased removal of platinum from cellular DNA by inhibition of DNA repair.

Materials and Methods

Chemicals. Recombinant human IL-1α (specific activity, 3×10^8 units/mg) was kindly provided by Dr. P. Lomedico (Hoffmann-La Roche, Nutley, NJ). Recombinant human IL-1RA was purchased from R & D Systems (Minneapolis, MN). MTT and dimethylsulfoxide were purchased from Sigma Chemical Co. (St. Louis, MO). ^{125}I-IL-1α (specific activity, 17.2 mCi/μg) was obtained from Amersham (Arlington Heights, IL). CDDP was obtained from the Drug Development Branch, National Cancer Institute, National Institutes of Health (Bethesda, MD).

Cell culture. Human ovarian carcinoma (NIH:OVCAR-3) cells (HTB 161; American Type Culture Collection, Rockville, MD) were maintained in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with antibiotic mixture (5 mg/ml penicillin, 5 mg/ml streptomycin, and 10 mg/ml neomycin; GIBCO) and 10% fetal bovine serum (GIBCO), under standard culture conditions at 37° in a humidified CO_2 atmosphere.

Cytotoxicity assay. The cytotoxicities of IL-1α, CDDP, and the combinations were measured by the MTT assay, as described previously (29). Briefly, cells were harvested by trypsinization from cultures in the exponential growth phase, and cells were suspended in fresh medium at 5000 cells/well, in 96-well microtiter plates (Costar, Cambridge, MA). After 24 hr of incubation, IL-1α and/or CDDP were added and then the cells were further incubated for 120 hr. MTT (2 mg/ml in PBS) was added (50 μl/well), and cells were incubated for 3 hr. Cells were centrifuged for 5 min at 2000 rpm, and the medium was removed. Dimethylsulfoxide was then added to each well, the plates were shaken for 30 min, and the absorbance at 570 nm was read with a kinetic microplate reader (Molecular Devices, Menlo Park, CA). Data were collected as replicates of six wells. The effect of IL-RA was also evaluated by the MTT assay.

The analysis for synergy was performed by the combination index/median-dose effect methods of Chou and Talalay (30). Median dose values were determined from median effect plots, by $\log(\text{fraction}_{\text{affected}}/\text{fraction}_{\text{unaffected}}) = \log(\text{dose}/\text{dose}_{\text{median}})^m$, where m is the Hill-type coefficient of sigmoidicity as described by Chou and Talalay (30).

Effects of IL-1α on cellular CDDP accumulation. The NIH:OVCAR-3 cells grown in T-225 flasks were treated with CDDP (50 μM) and cells were harvested by scraping at the indicated times after drug treatment. Cells were immediately counted (Coulter ZM counter; Coultronix) and "wet-ashed" according to the method of McGahan and Tyczkowska (31). Briefly, the cell pellets were treated with nitric acid (1 volume) at room temperature overnight, heated to 90° for 5 min, cooled in ice (10 min), and boiled in the presence of hydrogen peroxide (1 volume of a 30% solution). The platinum concentration in the sample was determined by AAS, with the Zeeman correction (32, 33). Total cellular accumulation is expressed both as picograms of platinum/10^6 cells and as the percentage of maximal drug accumulation.

Measurement of platinum in cellular DNA. The formation and removal of cisplatin-DNA adducts in cellular DNA after 1-hr CDDP exposures were examined in the presence and absence of IL-1α. Cells were grown in T-225 flasks (Costar) to 50–60% confluency, labeled with [^{3}H]thymidine (0.1 μCi/ml) for 24 hr, washed twice with PBS, supplemented with fresh medium with or without IL-1α, and incubated for an additional 24 hr. In some experiments, different concentrations of IL-1α were used and incubation times were varied.

For platinum removal studies, after a 1-hr CDDP exposure cells were washed twice with ice-cold PBS and harvested at the following time points: 0 hr (immediately at the end of cisplatin exposure), 6 hr, and 24 hr. Cells were frozen immediately at −80° until DNA isolation. Total cellular DNA was isolated on cesium chloride density gradients (34), and the DNA was dialyzed against distilled water, with three or four changes over 36–48 hr. DNA was quantitated by absorbance at 260 nm. Total platinum present in DNA was measured by AAS, as described previously (32, 33). [^{3}H]Thymidine content was determined by liquid scintillation counting. A decrease in the specific radioactivity of DNA (dpm/micrograms of DNA) at each time point, compared with that obtained at 0 hr, represents DNA replication. This ratio was used to determine the platinum content of nonreplicated DNA.

Results

Cytotoxicity studies. Although some synergy was observed when cells were pretreated with IL-1α for 12 hr, a much greater synergy was observed when cells were treated with IL-1α for 24 hr, followed by CDDP treatment for 120 hr. As shown in Fig. 1A, the pretreatment with IL-1α for 24 hr reduced the concentration of CDDP required for 50% cell kill by 20-fold, to 0.02 μM from 0.4 μM. Analysis for synergy using the method of Chou and Talalay (30) showed a strong synergy (Fig. 1B) between CDDP and IL-1α. In contrast, very

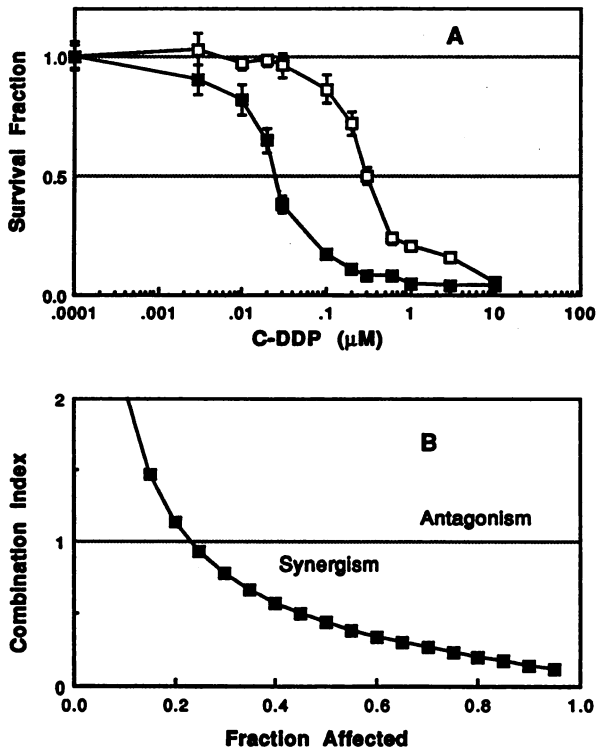

Fig. 1. Effects of IL-1α on CDDP cytotoxicity against NIH:OVCAR-3 cells *in vitro* during simultaneous exposures for 120 hr. A, CDDP was used alone (□) or in combination with IL-1α (■). Cells were pretreated with IL-1α for 24 hr. B, The combination index was calculated according to the method of Chou and Talalay (30) and the molar ratio between IL-1α and CDDP was fixed at 1:100,000. Values are the mean ± standard deviation of six replications.

little synergy was observed when these agents were used in a sequential manner, where cells were treated with CDDP first, followed by IL-1α. Pretreatment of cells with IL-1α for 24 hr followed by 1-hr CDDP exposure also resulted in a significant enhancement of CDDP cytotoxicity (Table 1), indicating that binding and internalization of IL-1α were necessary for the enhancement of CDDP activity in NIH:OVCAR-3 cells.

Effects of IL-1RA on cytotoxicity. To further evaluate the role of IL-1 receptors in the cytotoxicity of the combinations, we used the IL-1RA. As shown in Fig. 2A, the IL-1RA significantly decreased the cytotoxicity of IL-1α; however, it had no effect on the cytotoxicity of CDDP in NIH:OVCAR-3 cells. Furthermore, the IL-1RA also reduced the cytotoxic effects of the combination of IL-1α with CDDP (Fig. 2B); however, the combination was still synergistic (Fig. 2C). These results suggest that the synergistic cytotoxic effects of IL-1α and CDDP are IL-1 receptor mediated.

Effects of IL-1α on CDDP accumulation. The mechanism of the synergy between these agents might involve modulation by IL-1α of the cellular accumulation of CDDP. We examined this possibility, and our data (Fig. 3A) show that the pretreatment of NIH:OVCAR-3 cells with IL-1α for 24 hr slightly increased (1.5-fold) CDDP accumulation during a 1-hr CDDP exposure. This increased accumulation of CDDP was dependent upon the concentration of IL-1α used. It should be noted that this increase in CDDP accumulation was observed with as little as 10 pM IL-1α, and the maximum increase was observed when cells were pretreated with 0.1–1 nM IL-1α (Fig. 3B).

Although the absolute amounts of cellular platinum were higher under these conditions, the rates of CDDP efflux were similar in both groups, indicating that IL-1α did not inhibit CDDP efflux (Fig. 3B). As depicted in Fig. 3C, this increase in CDDP accumulation was also dependent upon the time of the pretreatment of NIH:OVCAR-3 cells with IL-1α. Although 12-hr pretreatment of the cells with 1 nM IL-1α increased CDDP accumulation, the maximum accumulation was observed after 24 hr of pretreatment (Fig. 3C).

Effects of IL-1α on platinum-DNA adduct formation and removal. Because IL-1α pretreatment increased CDDP cellular accumulation in a dose-dependent fashion, it was possible that IL-1α also increased platinum-DNA adduct formation, which might result in increased tumor cell kill. We examined this platinum-DNA adduct formation using AAS. Our results, depicted in Fig. 4, show that pretreatment of NIH:OVCAR-3 cells with IL-1α significantly increased platinum-DNA adduct formation, compared with the non-IL-1α-treated cells (Table 2). Moreover, IL-1α treatment significantly decreased platinum removal from cellular DNA. As little as 10 pM IL-1α completely inhibited platinum-DNA adduct removal (Fig. 4B; Table 2). Furthermore, this inhibition of platinum removal from the DNA of cells treated with IL-1α was observed to persist for up to 24 hr (Fig. 5). Thus, about 3–4-fold more DNA-platinum adducts were present in the IL-1α-treated cells, compared with the untreated cells, 24 hr after a 1-hr exposure to 50 μM CDDP.

Effects of IL-1α on GSH and GSH transferase. The cytotoxicity of CDDP to certain tumor cells has been reported to be modulated by GSH and GSH-dependent transferase, most likely by prevention of platinum-DNA adduct formation and/or enhancement of platinum removal (35, 36). It was possible that IL-1α affected the GSH status of NIH:OVCAR-3 cells and decreased platinum removal from cellular DNA. Our results, however, indicated that neither CDDP, IL-1α, nor the combinations significantly affected either GSH or GSH transferase activity under our experimental conditions (Table 3). This suggests that the synergistic interactions were not due to effects on GSH/GSH transferase in NIH:OVCAR-3 cells.

Discussion

Recently, cytokines and lymphokines have been recognized as important biochemical modulators that could either sensitize tumor cells to the effects of other anticancer drugs or exert direct cytotoxic effects on tumor cells. Although the mechanism of the antitumor activity of IL-1α is not known, it is now well established that IL-1α has direct antitumor properties both *in vitro* and *in vivo* (20–22). Kilian *et al.* (22) showed that IL-1α inhibited the proliferation of NIH:OVCAR-3 tumor cells *in vitro*. The human ovarian NIH:OVCAR-3 cells were established in culture from a patient who had failed therapy with both doxorubicin and cisplatin (37). Our previous studies (26) showed that IL-1α signifi-

TABLE 1

Effects of IL-1α on cytotoxicity of CDDP in NIH:OVCAR-3 cells *in vitro*

IC_{50} values represent the average of three independent experiments.

Conditions	IC_{50}			Sensitization factor (IC_{50} ratio)
	Single drug		Combination, CDDP	
	IL-1α	CDDP		
	pM	μM	μM	
Simultaneous exposure[a]	0.94 ± 0.14	0.37 ± 0.04	0.020 ± 0.003[b]	18.5
Sequential exposure				
IL-1α 6 hr before CDDP[c]	>100	0.40 ± 0.05	0.370 ± 0.045	1.0
IL-1α 12 hr before CDDP[c]	>100	0.37 ± 0.04	0.180 ± 0.026[d]	2.0
IL-1α 24 hr before CDDP[c]	58.6 ± 7.15	0.39 ± 0.05	0.036 ± 0.005[b]	10.8
IL-1α 24 hr before CDDP[e]	58.6 ± 7.15	1.30 ± 0.20	0.203 ± 0.028[b]	6.4
CDDP 24 hr before IL-1α[f]	2.63 ± 0.35	0.39 ± 0.04	0.400 ± 0.046	1.0

[a] Values obtained for 5-day exposure.
[b] Significantly different ($p < 0.001$) from the control (drug alone).
[c] At 6, 12, and 24 hr, IL-1α was removed and CDDP was added for 5 days.
[d] Significantly different ($p < 0.01$) from the control (drug alone).
[e] After 1-hr CDDP treatment, the drugs were removed and cells were grown for 5 days.
[f] At 24 hr, CDDP was not removed when IL-1α was added.

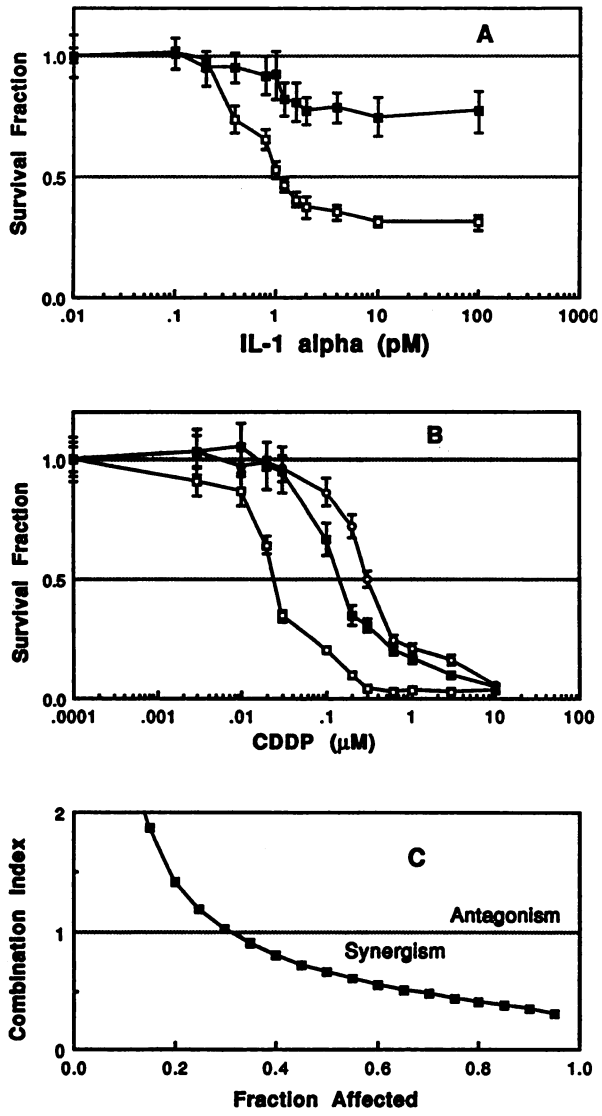

Fig. 2. Effects of IL-1RA on the antiproliferative effects of IL-1α alone (□) or in the presence of a 200-fold excess of IL-1RA (■) (A) or CDDP alone (○), in combination with IL-1α (□), or in the presence of IL-1RA (■) (B), during simultaneous exposures for 120 hr. IL-1RA was added (at 200-fold excess) 2 hr before the addition of other drugs. Values are the mean ± standard deviation of six replications.

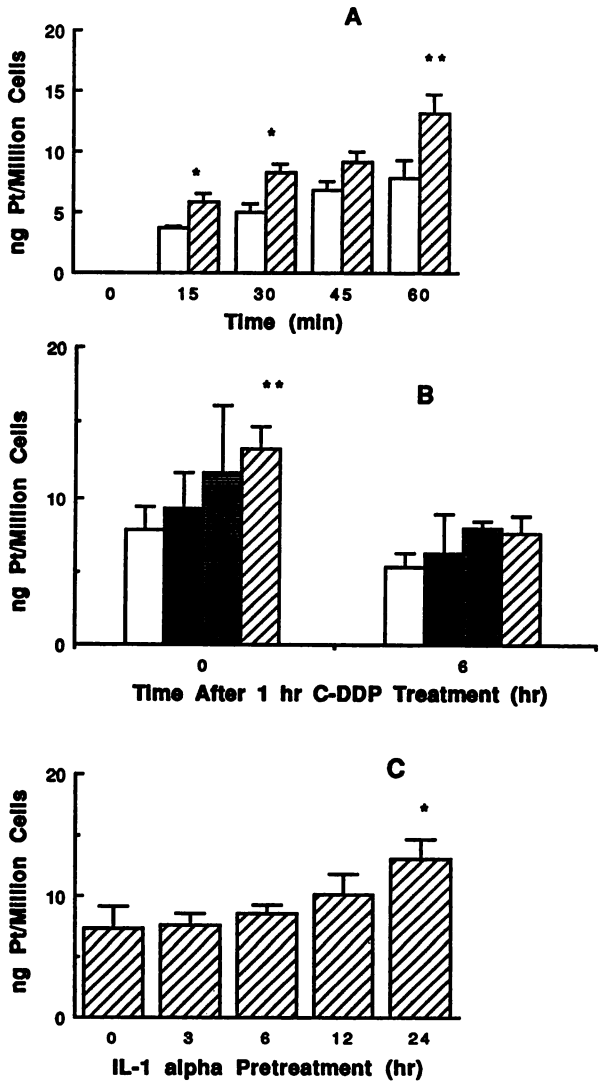

Fig. 3. Time- and dose-dependent effects of IL-1α on cellular accumulation and efflux of CDDP in NIH:OVCAR-3 cells. A, Cells were treated with 50 μM CDDP for the indicated times, in the presence (▨) or absence (□) of 1 nM IL-1α. IL-1α was present for 24 hr before CDDP treatment. B, In the presence of IL-1α (□, 0; ■, 0.01; ■, 0.1; ▨, 1 nM), cells were pretreated with IL-1α for 24 hr. C, Accumulation of CDDP after pretreatment of cells with IL-1α (1 nM) for the indicated times was determined. Accumulation and efflux were carried out as described in Materials and Methods. Values are mean ± standard deviation for at least three independent experiments. *, Significantly different ($p < 0.01$) from CDDP alone; **, significantly different ($p < 0.001$) from CDDP alone.

cantly potentiated the antiproliferative activity of doxorubicin in this cell line, indicating that combinations of IL-1α with other anticancer drugs may potentially be clinically effective against drug-resistant tumors.

In this report, we examined the interactions of IL-1α with cisplatin in human ovarian NIH:OVCAR-3 cells as a potentially important drug combination for the treatment of ovarian carcinoma. Our results clearly showed that the combination of these agents was highly synergistic against this cell line *in vitro*, with both simultaneous and sequential exposures. Interestingly, the presence of IL-1α was necessary for the synergistic interactions, because treatment of cells with cisplatin followed by IL-1α was not synergistic. Moreover, when cells were treated with the cytokine for 12 hr before cisplatin, some synergy was observed; however, a greater synergy was observed when IL-1α was present for 24 hr before cisplatin. These observations confirm previous findings that the binding of IL-1α to its membrane receptor and IL-1α internalization may be necessary for IL-1α-mediated antitumor activity and also for the potentiation of CDDP cytotoxicity.

This interpretation was further supported by the observation that inclusion of the IL-1RA inhibited cytostatic effects of IL-1α and concomitantly decreased the potentiation of CDDP cytotoxicity. However, this combination was synergistic in spite of the presence of the IL-1RA. We (26) and Kilian *et al.* (22) showed that NIH:OVCAR-3 cells express 3000 receptors/cell and that a 200-fold excess of IL-1RA inhibited the binding of IL-1α by 75%. Under these conditions, about 700 receptors/cell are still available for IL-1α binding and biological activity. It is interesting to note that CDDP did not increase IL-1α receptor numbers or binding. This finding is

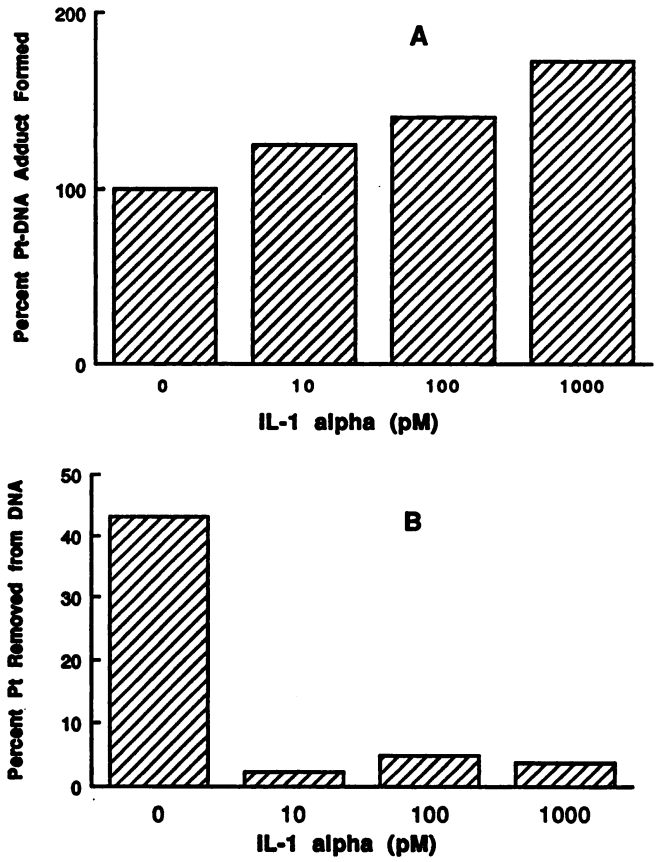

Fig. 4. Effects of IL-1α on platinum-DNA adduct formation (A) and removal at 6 hr (B). Cells were treated with 50 μM CDDP for 1 hr, with or without 24-hr pretreatment with IL-1α.

TABLE 2

Dose-dependent effects of IL-1α on platinum-DNA adduct formation and removal in NIH:OVCAR-3 cells

Platinum-DNA adduct formation and removal were carried out as described in Materials and Methods. Cells were pretreated with IL-1α for 24 hr before the addition of 50 μM CDDP for 1 hr.

IL-1α	CDDP	Amount of adduct formed	Amount of adduct removed	
			At 6 hr	At 24 hr
pM	*μM*	*pg of platinum/μg of DNA*	*pg of platinum/μg of DNA*	
0	50	5.97 ± 1.3	2.56 ± 0.21[a]	3.2 ± 0.32[a]
10	50	7.46 ± 1.7	0.17 ± 0.04	
100	50	8.34 ± 2.9	0.41 ± 0.03	0.63 ± 0.08
1000	50	10.3 ± 1.8[a]	0.38 ± 0.12	0.8 ± 0.14

[a] Significantly different ($p < 0.001$) from control (no IL-1α).

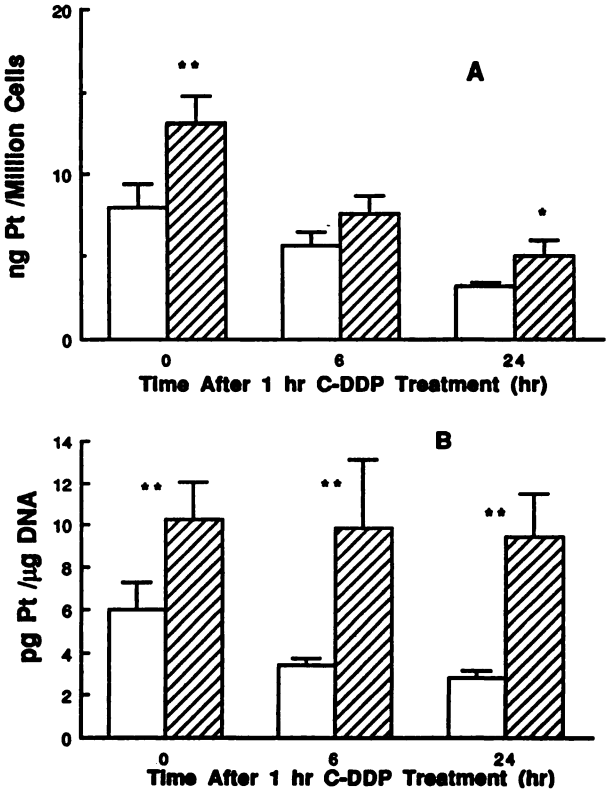

Fig. 5. Effects of IL-1α on cellular accumulation/efflux of CDDP (A) and platinum-DNA adduct formation and removal (B) with time. Cells were pretreated (▨) or not treated (□) with 1 nM IL-1α for 24 hr before treatment with 50 μM CDDP for 1 hr. The platinum-DNA adduct formation and the removal of platinum from DNA were carried out as described in Materials and Methods. Values are mean ± standard deviation for at least three independent experiments. *, Significantly different ($p < 0.01$) from CDDP alone; **, significantly different ($p < 0.001$) from CDDP alone.

TABLE 3

Concentration-dependent effects of IL-1α and cisplatin on GSH and GSH *S*-transferase in NIH:OVCAR-3 cells

NIH:OVCAR-3 cells were pretreated with IL-1α for 24 hr before CDDP exposure for 1 hr. GSH and GSH *S*-transferase were determined according to the methods of Tietze (43) and Habig *et al.* (44), respectively.

IL-1α	CDDP	GSH	GSH *S*-transferase
nM	*μM*	*nmol/mg of protein*	*nmol/mg of protein/min*
0	0	9.57 ± 3.12	96.2 ± 23.0
1	0	10.0 ± 3.49	140.0 ± 43.6
0	50	8.10 ± 3.28	118.0 ± 28.3
1	50	7.86 ± 2.71	126.0 ± 32.2

in contrast to those with VP-16 and doxorubicin, where a significant increase in IL-1α binding was observed (24, 25).

The molecular and cellular targets of IL-1α for tumor cell kill are not known. We recently showed that IL-1α did not directly induce topoisomerase-dependent DNA damage and, likewise, IL-1α did not increase VP-16-induced DNA damage (38). Moreover, IL-1α did not affect VP-16-dependent DNA repair in human melanoma cells (38). However, studies to define the cellular and molecular mechanisms of synergy between CDDP and IL-1α in NIH:OVCAR-3 cells indicated that, whereas CDDP had no effect on IL-1α accumulation in cells, IL-1α had profound effects on the cellular and subcellular pharmacokinetics of cisplatin. First, pretreatment of cells with IL-1α increased CDDP uptake in a dose-dependent manner, which was observed with as little as 10 pM IL-1α (Fig. 3). However, the efflux of CDDP was not affected (Fig. 5A). IL-1α significantly enhanced platinum-DNA adduct formation in IL-1-treated NIH:OVCAR-3 cells, compared with untreated cells. More importantly, inhibition of DNA repair induced by IL-1α, at concentrations that are easily achieved *in vivo*, was observed for up to 24 hr after CDDP treatment (Fig. 5B).

These observations indicate that IL-1α inhibits platinum-DNA adduct repair in ovarian cancer cells *in vitro*. Because the cytotoxicity of CDDP may result from formation of DNA intrastrand cross-links in tumor cells (4–6), decreased removal, induced by the cytokine, of these lethal lesions may be one of the reasons for the observed synergy between IL-1α and CDDP. IL-1α has been reported to decrease high energy phosphate in

tumors *in vivo* (39, 40). It has been postulated that this may lead to disruption of the activities of energy-dependent repair enzymes, as well as the synthesis of ATP (39–41). Because the repair of cisplatin-DNA adducts has been reported to be carried out by exonucleases, which are ATP-dependent enzymes (42), it is quite possible that IL-1α-induced inhibition and/or depletion of ATP synthesis may be involved in this inhibition of repair and hence the increased sensitization to CDDP-induced cytotoxicity. We are currently examining this hypothesis, as well as the exact nature of the repair enzymes affected by IL-1α in this ovarian cancer cell line.

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