Inhibition by 5-Fluorouracil of cis-Diaminedichloroplatinum(II)-induced DNA Interstrand Cross-Link Removal in a HST-1 Human Squamous Carcinoma Cell Line

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ABSTRACT

To elucidate the mechanism of the synergistic cytotoxicity of 5-fluorouracil (5-FU) and cis-diaminedichloroplatinum(II) (CDDP), we studied the interaction of these agents using a human squamous carcinoma cell line (HST-1). Exposure to 5-FU for 24 h and to CDDP for 1 h produced a 50% inhibitory concentration of 1.0 µg/ml (7.7 µM) and 2.5 µg/ml (8.3 µM), respectively. The cytotoxic action of CDDP was augmented, and a greater than additive effect was observed when the cells were exposed to 5-FU (1.0 µg/ml; 7.7 µM) for 24 h before the CDDP treatment. This synergistic activity was maximal when the interval between 5-FU and CDDP exceeded 24 h. In contrast, the cytotoxicity of CDDP was attenuated when it preceded the exposure to 5-FU. Thymidine did not alter the 5-FU-CDDP interaction. Evaluation of the kinetics of the removal of DNA interstrand cross-links, measured by alkaline elution, showed a significant reduction of this removal in the cells exposed to 5-FU followed by CDDP with a drug-free interval of 48 h, as compared with cells exposed to CDDP alone, or to 5-FU immediately followed by CDDP, although no differences were found in the formation of DNA interstrand cross-links by CDDP among these cells. No significant differences in the accumulation of intracellular platinum were detected by atomic absorption spectrophotometry. These findings suggest that 5-FU modulates the repair of platinum-DNA adducts, thereby potentiating the antitumor activity of CDDP.

INTRODUCTION

CDDP 2 and 5-FU are commonly used antineoplastic agents for solid tumors. Each has a broad spectrum of activity and has shown marked therapeutic efficacy in several human malignancies that are relatively refractory to chemotherapy, such as head and neck cancer (1, 2) and, more recently, colorectal carcinoma (3, 4), lung cancer (5), and skin tumor (6). The combination exhibits synergistic cytotoxicity both in vitro (7) and in tumor-bearing animals (8, 9). However, the optimal administration of these agents has yet to be determined, and the biochemical mechanism of this interaction remains unclear. Scanlon et al. (7) showed that the combination of CDDP followed by 5-FU is more cytotoxic than the reverse sequence. The enhanced antitumor activity was ascribed to the CDDP-induced increase in the intracellular levels of reduced folates, which potentiate the action of 5-fluoroacytidine monophosphate by forming a covalent ternary complex with thymidylate synthase. In contrast, several in vivo studies including our investigation of human tumor xenografts in nude mice demonstrated that the sequence of 5-FU followed by CDDP was more active than the reverse sequence (9–12).

The discrepancy between these in vivo and in vitro results prompted us to further study the biochemical mechanism of the sequence-dependent interaction of 5-FU and CDDP using a HST-1 human squamous carcinoma cell line. The cytotoxicity of CDDP is mediated by the formation of DNA cross-links (13). Although most of these lesions are intranuclear cross-links occurring between adjacent guanine nucleotides (14), DNA interstrand cross-links, which constitute only about 1% of platination, have been correlated quantitatively with cytotoxicity (15–19). We therefore analyzed the formation and removal of DNA interstrand cross-links by alkaline elution to characterize the mechanism underlying the sequence-dependent antitumor activity of 5-FU and CDDP. We have found that synergistic cytotoxicity of the 5-FU and CDDP combination was exhibited only when 5-FU precedes CDDP exposure and that this enhancement by 5-FU of CDDP cytotoxicity appears to be caused by the modulation of the repair of CDDP-induced DNA interstrand cross-links.

MATERIALS AND METHODS

Cell Cultures and Chemicals. A clonally isolated subline of HST-1 human tongue squamous carcinoma cells (20) was maintained in DMEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Gibco Lab., Life Technologies, Inc., Grand Island, NY), 100 IU/ml of penicillin, and 100 µg/ml of streptomycin (Gibco Lab.) at 37°C in a humidified atmosphere of 5% CO2 and 95% air. The cloning efficiency of this cell line was approximately 10%. 5-FU and CDDP were kindly provided by Kyowa Hakko Co., Tokyo, and Nihon Kayaku Co., Tokyo, Japan, respectively. Immediately before their use, 5-FU and CDDP were dissolved in a culture medium.

L1210 mouse leukemia cells were provided by the Japanese Cancer Research Resources Bank, Tokyo, Japan, and maintained in suspension culture in DMEM supplemented with 10% heat-inactivated fetal bovine serum, with daily passage to keep the cells in log-growth phase.

Cell Survival Assay. To avoid the density-dependent inhibition of cell growth, an appropriate number of cells was seeded into 60-mm culture dishes (Falcon 3002) such that control cultures did not reach confluency at the time of cell counting. The cells were allowed to attach to plastic overnight. To study the effect of 5-FU on the cytotoxicity of CDDP, the cultures treated with various concentrations of CDDP for 1 h were pre- or postexposed to 1.0 µg/ml of 5-FU (7.7 µM) for 24 h. To study the influence of CDDP on the cytotoxicity of 5-FU, the cultures treated with various concentrations of 5-FU for 24 h were pre- or postexposed to 2.5 µg/ml of CDDP (8.3 µM) for 1 h. After drug treatment, the medium was replaced with fresh medium, and the cells were cultured for 7 days. The cell number was counted using a Coulter Counter (Model ID). The percentage of survival was calculated by dividing the number of cells in the drug-treated culture by the number of cells in the culture not exposed to drug.

Alkaline Elution Assays for DNA Interstrand Cross-Linking. The alkaline elution technique used is a modification of the method of Kohn et al. (21, 22). Approximately 2 × 106 cells were seeded in a 25-cm2 culture flask (Corning 25100). After overnight incubation, the DNA of these cells was labeled by growing the cells for 48 h in 0.02 µCi/ml of

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2 The abbreviations used are: CDDP, cis-diaminedichloroplatinum(II); 5-FU, 5-fluorouracil; DMEM, Dulbecco's modified minimal essential medium; IC50, drug concentration that inhibits cell growth by 50%; CI, cross-linking index.
The cell pellets were later "wet-ashed" according to the method of Coulter Counter. The cell pellets were stored at -20°C until analysis.

Drugs were dissolved by incubation with nitric acid at 150°C for 6 h. The resulting cellular extracts were then analyzed for platinum on a Hitachi Model 180-70 polarized Zeeman atomic absorption spectrophotometer (Hitachi, Ltd., Tokyo, Japan). The platinum content of the samples was determined by comparison with an external standard prepared by dilution with 0.1 N HCl.

Statistical Analysis. The data of DNA interstrand cross-link indexes were analyzed for significance by the Mann-Whitney U test.

RESULTS

Sequence-dependent Cytotoxicity of the 5-FU and CDDP Combination. Exposure to 5-FU for 24 h and exposure to CDDP for 1 h as single agents each caused an IC50 at the concentrations of 1.0 μg/ml (7.7 μM) and 2.5 μg/ml (8.3 μM), respectively. Fig. 1 shows the survival curves of CDDP, as measured by the percentage of cell growth, in the cells treated with or without 1.0 μg/ml of 5-FU (7.7 μM) for 24 h. The survival of CDDP was corrected for the cytotoxicity attributable to 5-FU treatment. The survival curve of CDDP for cells immediately treated with CDDP after exposure to 5-FU was almost identical with that for cells treated with CDDP alone,

[14C]thymidine (Amersham Japan; specific activity, 57 mCi/mm). After removing the medium, the cells were chased with a nonradioactive medium for an additional 24 h before drug treatment. L1210 mouse leukemia cells, used as internal standards in all alkaline elution analyses, were labeled by growing them overnight in a medium with 0.05 μCi of [3H]thymidine (Amersham Japan; specific activity, 25 Ci/mm).

Drug-treated cells were subjected to 3 Gy of γ irradiation (Gamma-cell 40; Atomic Energy of Canada, Limited, Commercial Products) in an ice-cold, fully supplemented medium. Approximately 5 × 10⁵ cells (50% [14C]-labeled cells, 50% [3H]-labeled L1210 cells that had received 3 Gy of γ irradiation) were collected onto a 25-mm-diameter, 0.8-μm pore size polycarbonate filter (Nucleopore Co., Cambridge, MA) and lysed for 1 h with 4 ml of a solution containing 2% sodium dodecyl sulfate in 0.05 M Tris aminomethane (pH 9.6) and 0.5 mg of proteinase K (Wako Pure Chemical Industries, Ltd., Osaka, Japan) per ml. After lysis solution had flowed through the filters at unit gravity, the filters were washed with 5 ml of 0.02 M disodium EDTA (pH 10.0), and DNA was eluted in the dark with 33 ml of a solution consisting of 3.6% tetrapropylammonium hydroxide, 0.1% sodium dodecyl sulfate, and 0.04 M tetrahydro-EDTA (pH 12.1). The output tube was passed through a peristaltic pump to a fraction collector. The pumping rate was 0.05 ml/min. Ten fractions were collected at 1-h intervals. The [14C]DNA and [3H]DNA reactivities remaining on the filters and in the eluted fractions were determined by counting in a Beckman LSC-703A liquid scintillation counter, and the retention of labeled DNA on the filter as a function of time of elution was calculated. DNA interstrand cross-link frequency, expressed as a cross-link index (CI), was calculated as

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CI = [(1 - \gamma_0)/(1 - \gamma)]^{1/2} - 1 \quad (18)
\]

where \( \gamma \) and \( \gamma_0 \) are the fractions of [14C]-labeled DNA of the treated and control cells remaining on the filter when 25% of all-labeled DNA is retained on the filter.

Intracellular Accumulation Studies. HST-1 cells with or without 24-h treatment with 5-FU were exposed to CDDP at doses of 10, 20, and 40 μg/ml (33.3, 66.7, and 133.3 μM) for 1 h; cells were immediately harvested, and the cell number of trypsinized cells was counted using a Coulter Counter. The cell pellets were stored at -20°C until analysis. The cell pellets were later "wet-ashed" according to the method of McGahan and Tyczkowski (23). Briefly, materials were dissolved by incubation with nitric acid at 150°C for 6 h. The resulting cellular growth was then assessed.

Fig. 1. Representative survival curves for CDDP pre- or posttreated with 5-FU: 1-h CDDP only (•); CDDP followed by 1 μg/ml of 5-FU for 24 h (△); and CDDP with drug-free interval 0 (○), 24 (▲), and 48 h (□) after exposure to 1 μg/ml of 5-FU at varying doses of CDDP. Points, mean of duplicates.

Fig. 2. Representative data on inhibition of cell growth by 5-FU followed by CDDP separated by drug-free intervals of 0 to 48 h. A fixed number of cells (6 × 10⁴) were seeded and exposed to 2.0 μg/ml of CDDP (○) and 5.0 μg/ml of CDDP (□) for 1 h following 1 μg/ml of 5-FU for 24 h. The percentage of control cell growth was then assessed. Points, mean of duplicate.

Fig. 3. Representative survival curves for CDDP following exposure to 5-FU with or without thymidine: 1-h CDDP only (•); immediately treated with CDDP after exposure to 1 μg/ml of 5-FU for 24 h with (●) or without (▲) 10 μM thymidine; and 5-FU with (●) or without (▲) thymidine followed by CDDP with a 48-h drug-free interval at varying doses of CDDP. Points, mean of duplicates.
indicating an additive effect. However, pretreatment with 5-FU augmented the cytotoxicity of CDDP by nearly 2-fold, when CDDP was given in a sequential manner separated by a 24- or 48-h drug-free interval (IC₅₀, 1.3 μg/ml; 4.3 μM) as compared to CDDP alone (IC₅₀, 2.6 μg/ml; 8.7 μM). The observed significant separation between this corrected curve and the CDDP-only curve indicates the degree of cytotoxic synergy. In contrast, the cytotoxicity of CDDP was attenuated when CDDP preceded the exposure to 5-FU.

This sequence-dependent synergy was also examined in experiments that compared the total number of cells yielded after the same number of cells had been treated with a sequential combination of 5-FU and CDDP, separated by drug-free intervals of 0 to 48 h (Fig. 2). The total yield of cells thus obtained after 10 days decreased in a sequence-dependent manner and was maximal after 36 to 48 h, consistent with survival data. Moreover, the synergistic activity exhibited by the sequence 5-FU followed by CDDP was observed even in the presence of 10 μM thymidine, which compensates for the inhibition of de novo DNA synthesis by 5-FU (Fig. 3). Unlike 5-FU, which potentiates CDDP cytotoxicity, pre- or posttreatment with 2.5 μg/ml of CDDP (8.3 μM) did not influence the cytotoxicity of 5-FU (Fig. 4).

DNA Interstrand Cross-Link Assay. To elucidate the mechanism by which 5-FU pretreatment enhances the cytotoxicity of CDDP, the effect of 5-FU on the formation and removal of platinum-induced DNA interstrand cross-linking was explored using the DNA alkaline elution technique. Cells were incubated following drug removal, and assays were performed at 0, 6, 24, and 48 h following removal of CDDP. Treatment schedules included CDDP alone, CDDP immediately after 5-FU, and 5-FU followed by CDDP in a sequential manner separated by a 48-h interval. Fig. 5 shows the representative elution curves for the 6-, 24-, and 48-h time points following CDDP removal for each schedule. Increased cross-linking levels were observed at 24 and 48 h after CDDP exposure, relative to CDDP only, when CDDP was preceded by 5-FU with a drug-free interval of 48 h. No increase in cross-linking level was seen when cells were treated with CDDP immediately after 5-FU treatment as compared to CDDP alone (data not shown).

We also analyzed the relative rate of formation and removal of DNA interstrand cross-links by calculating the CI from three or more independent experiments (Fig. 6). The CI was determined for each elution curve over the course of 48 h. To determine the CI for combinations of 5-FU and CDDP, cells that had received the same dose and schedules of 5-FU exposure and no CDDP treatment were used as irradiated controls. Therefore, the CI calculation compensated for any accumulation of DNA strand break due to 5-FU. The presence of 5-FU-induced
treated with 2.5 μg/ml of CDDP after exposure to 1.0 μg/ml of 5-FU for 24 h (l); with CDDP for 1 h. A, 1-h exposure to 2.5 μg/ml of CDDP alone (●); immediately after 24-h exposure to 1.0 μg/ml of 5-FU with 48-h drug-free interval (•). B, 1-h exposure to 5.0 μg/ml of CDDP alone (●); immediately treated with 5.0 μg/ml of CDDP after exposure to 1.0 μg/ml of 5-FU for 24 h (■); 5.0 μg/ml after 24-h exposure to 1.0 μg/ml of 5-FU with 48-h drug-free interval (▲). Points, mean of three or more independent experiments; bars, SE; *, P < 0.05 by the Mann-Whitney U test.

Fig. 6. DNA interstrand cross-link index as a function of time after treatment with CDDP for 1 h. A, 1-h exposure to 2.5 μg/ml of CDDP alone (●); immediately treated with 2.5 μg/ml of CDDP after exposure to 1.0 μg/ml of 5-FU for 24 h (■); 2.5 μg/ml of CDDP after 24-h exposure to 1.0 μg/ml of 5-FU with 48-h drug-free interval (▲). B, 1-h exposure to 5.0 μg/ml of CDDP alone (●); immediately treated with 5.0 μg/ml of CDDP after exposure to 1.0 μg/ml of 5-FU for 24 h (■); 5.0 μg/ml after 24-h exposure to 1.0 μg/ml of 5-FU with 48-h drug-free interval (▲). Points, mean of three or more independent experiments; bars, SE; *, P < 0.05 by the Mann-Whitney U test.

DNA strand breaks was not apparent since the slope of the 5-FU-treated, irradiated cells was virtually identical with that of untreated, irradiated cells. Peak levels of interstrand cross-links were formed 6 h postincubation. At this point, the formation of DNA interstrand cross-links was not significantly different than that seen in the combination-treated cells. The relative rates of formation and removal of these cross-links were comparable between CDDP used alone and the combination of 5-FU followed immediately by CDDP. However, the rates of their removal were delayed over the 48-h postincubation period for cells exposed to 5-FU followed by CDDP with a 48-h drug-free interval as compared with CDDP alone. Differences in CI between cells exposed to CDDP alone or 5-FU followed by CDDP were not significant at 0 h and 6 h, whereas the differences at 24 h and 48 h were significant (p < 0.05), indicating an inhibition of the removal of these cross-links.

Accumulation of CDDP. To determine whether the difference in intracellular accumulations of CDDP between the drug treatment schedules would produce a difference in the formation of interstrand cross-links, we assayed the intracellular platinum content by atomic absorption spectrophotometry. The amount of intracellular platinum per 10⁶ cells after 1-h exposure to CDDP was found to be directly proportional to the concentration of CDDP in each group (Fig. 7). The intracellular platinum content was virtually identical between CDDP only and 5-FU followed by CDDP with a drug-free interval of 48 h.

DISCUSSION

Using xenografts of the HST-1 human squamous carcinoma cell line, we recently demonstrated that the sequential combination of 5-FU followed by CDDP was more effective than the reverse sequence (12). In the present in vitro study, we showed that the pretreatment of HST-1 cells with an IC₅₀ concentration of 5-FU potentiates the cytotoxicity of CDDP, whereas posttreatment 5-FU administration attenuated CDDP cytotoxicity, consistent with previous in vivo results. The synergistic cytotoxicity was maximal when the drugs were administered in a sequence separated by a 24- to 48-h drug-free interval. In contrast, neither pre- nor posttreatment with the IC₅₀ concentration of CDDP influenced the cytotoxicity of 5-FU, indicating an additive effect only.

Our findings contrast with those of Scanlon et al. (7) who reported that giving a sublethal dose of CDDP before 5-FU enhanced the 5-FU cytotoxicity by increasing the intracellular levels of reduced folates, which are synthesized de novo as a result of the CDDP-induced blockade of methionine uptake into tumor cells. Although it is difficult to directly compare their studies with IC₅₀ concentration doses of CDDP and 5-FU in this study, the synergistic activity of the 5-FU and CDDP combination seen in the present study cannot be explained by this mechanism, since the synergy observed in the sequence 5-FU followed by CDDP was exhibited even in the presence of thymidine, which compensates for the 5-FU-induced inhibition of thymidylate synthase, via a salvage pathway. Therefore, another major mechanism underlying the action of 5-FU, specifically, the damage to RNA induced by 5-FU (24), may be responsible for the increased sensitivity to CDDP.

To further investigate the biochemical mechanism for the interaction of 5-FU and CDDP, we determined whether 5-FU inhibited the repair of the DNA interstrand cross-links formed by CDDP. Evaluation of the kinetics of the excision of interstrand cross-links showed a significant reduction of the removal

![Fig. 7. Representative data on intracellular accumulation of platinum after exposure to 10 μg/ml, 20 μg/ml, and 40 μg/ml of CDDP for 1 h. ●, 1-h exposure to CDDP alone; ▲, 24-h exposure to 1.0 μg/ml of 5-FU followed by CDDP with 48-h drug-free interval. Points, mean of duplicates.](cancerres.aacrjournals.org)
of these lesions in cells exposed to 5-FU followed by CDDP with a drug-free interval of 48 h. The close association between a decrease in repair kinetics and the increase in cytotoxicity indicates that pretreatment with 5-FU may inhibit the CDDP-induced DNA adduct repair. The increased CDDP sensitivity could not be explained by a decrease in detoxification enzymes which reduce the platination such as glutathione, glutathione S-transferase, or metallothionein (25–29), nor by an increase in intracellular accumulation of drug, since there were no differences in the peak level of interstrand cross-links formed by CDDP or in the intracellular content of platinum.

Drugs that interfere with excision repair have recently been reported to potentiate the cytotoxicity of CDDP. Aphidicolin can inhibit the repair of CDDP-induced intranuclear DNA cross-links (30, 31), and arabinofuranosycytosine and hydroxyurea inhibit the repair of platinum-induced interstrand cross-links (32–34). In addition, the enhanced repair of cross-links has been correlated with resistance to CDDP (35, 36). These data indicate an inverse relationship between the DNA repair capacity and the susceptibility to CDDP. Although the mechanism of the interaction of 5-FU and CDDP remains unclear, the present observations indicate that 5-FU may interfere with the repair of CDDP-induced DNA damage, thereby potentiating the cytotoxicity of CDDP. 5-FU has been reported to induce a DNA lesion indirectly by inhibiting the repair of DNA via a mechanism that does not involve drug incorporation into DNA (37, 38). This suggests that some inhibitory mechanism against DNA repair is operant after 5-FU treatment. Since a misincorporation of 5-FU into RNA could be associated with a block in processing and/or in nuclear cytoplasmic transport, such alterations may interfere with the maturation of nuclear RNA, leading to a depletion of enzymes critical to the repair of CDDP-induced DNA damage, such as ERCC-1 (39, 40). The substantial delay between the 5-FU and CDDP treatments required for the maximal cytotoxicity of CDDP supports this hypothesis. In summary, our findings suggest that 5-FU modulates the repair of platinum-DNA adducts, thereby potentiating the antitumor activity of CDDP, and that the sequence and timing of administration of these drugs are important in determining the extent of therapeutic synergy.

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