Combination of N-Methylformamide with cis-Diaminedichloroplatinum(II) in Murine Mammary Carcinoma: Importance of Timing

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ABSTRACT

The maturational agent N-methylformamide (NMF) is an antitumor agent that also enhances the response of tumor cells in vitro to chemotherapeutic agents. Here, we tested whether NMF can improve therapy of the murine MCA-K mammary carcinoma with cis-diaminedichloroplatinum(II) (cis-DDP). Although the in vitro cell cultures of MCA-K tumor cells exhibited increased sensitivity to cis-DDP cytotoxicity when they were first treated with NMF, administration of NMF to mice bearing MCA-K tumors did not enhance cis-DDP-induced tumor growth delay. However, when NMF treatment was begun after cis-DDP administration, the growth delays were significantly greater than those induced by the individual treatment, with an increase in temporary tumor regression and a small proportion of cures. These results indicate that therapeutic benefit can be achieved in this experimental tumor system when NMF is administered after cis-DDP. In addition, they demonstrate the significance of the timing of administration in combined protocols involving NMF.

INTRODUCTION

NMF,4 a maturational agent in the class of polar solvents, exhibits antitumor activity against both primary tumors (1-4) and metastases (4, 5). Its antitumor efficacy, however, depends on tumor type and the size of tumors at the time of treatment (4, 5). Smaller tumors respond to the NMF treatment better than larger tumors (4, 5).

To treat experimental malignant tumors, NMF has also been used in combination with local tumor irradiation (2, 3, 6) and chemotherapeutic agents (7-9). The ability of NMF to render tumor cells more sensitive to ionizing radiation (10-12) and chemotherapeutic drugs (10-13) combined with its lack of significant toxicity to normal tissues provides a strong rationale for combining it with cytotoxic agents. NMF increases the radioresponse of experimental tumors, but only under certain experimental conditions (3, 6). Similarly, combinations of NMF with chemotherapeutic agents were more effective than the individual treatments (7-9). However, these studies have been limited and have not fully addressed settings in which the therapeutic potential of NMF, we have examined the importance of the timing of the NMF injection in relation to the administration of the chemotherapeutic drug cis-DDP. Specifically, we tested antitumor efficacy of NMF when given either before or after cis-DDP.

MATERIALS AND METHODS

Mice. Male inbred C3Hf/Kam mice, maintained in our own specific-pathogen-free mouse colony, were used. They were 12 to 14 wk old at the beginning of the experiments. Within each experiment, mice of the same sex were housed 4 to 6 per cage.

Drugs. NMF (Aldrich Chemical Co., Milwaukee, WI) was dissolved in phosphate-buffered saline and injected i.p. in a volume equal to 0.01 ml/g of body weight. NMF was administered at 300 mg/kg of body weight daily for 6 or 12 consecutive days. cis-DDP (PlatinoI; Bristol-Myers Co., Syracuse, NY) was dissolved in sterile water and administered i.p. at a dose of 4, 7, or 10 mg/kg.

Tumors. Experiments were performed using a spontaneous mammary carcinoma, designated MCA-K, syngeneic to C3H/Kam mice. This tumor is highly metastatic and moderately immunogenic (14). Single cell suspensions from the tumor were prepared using a mechanical method (14); approximately 60% of cells were viable.

Tumor Growth Delay Assay. Tumors were generated by injecting 5 x 105 viable MCA-K cells into the right thighs of mice. For single-drug treatments, mice received a single injection of cis-DDP or NMF daily for 6 or 12 days once tumors reached 6 mm in diameter. The combined treatment of NMF and cis-DDP was administered according to two basic schedules: (a) at a tumor diameter of 6 mm, NMF treatment was initiated as 300 mg/kg injected daily for 6 or 12 days; 1 day after the last NMF injection, mice received a single injection of cis-DDP (4, 7, or 10 mg/kg); and (b) when tumors reached 6 mm in diameter, a single injection of cis-DDP (4, 7, or 10 mg/kg) was administered, and the NMF treatment (300 mg/kg injected daily for 6 days) was initiated either 2, 5, or 7 days later. To obtain tumor growth curves, three mutually orthogonal diameters of tumors were measured 3 times a wk with a vernier caliper, and the mean values were calculated. Tumor growth was measured until the size of tumors reached 16 to 18 mm in diameter, at which time the mice were killed. In the case of the tumor regression study, the animals were observed for 150 days from the time of tumor cell injection. If by this time tumors did not appear, the animals were considered to be cured.

Cell Culture. MCA-K cells were cultured in 75-cm2 tissue culture flasks using Hsu's medium containing 20% fetal calf serum at 37°C in a humidified 5% CO2-95% air atmosphere. To determine the effects of NMF on cis-DDP-induced cell killing, monolayer cultures (exponentially growing or plateau phase) were exposed to NMF (1%) for 4 days before or after cis-DDP. Specifically, we tested antitumor efficacy of NMF when given either before or after cis-DDP.

RESULTS

In Vitro Augmentation of cis-DDP Cytotoxicity. We previously showed that the MCA-K tumor is highly responsive to NMF treatment (4), and that cells derived from this tumor had impaired clonogenicity after being cultured in vitro in the presence of NMF (4). Here we tested whether NMF-treated MCA-K cells become more sensitive to cis-DDP. After being cultured...
for 4 days in the presence of 1% NMF, exponentially growing MCA-K cells were exposed to graded doses of cis-DDP (1 h), and a clonogenic cell survival assay was performed. The dose-response curves plotted in Fig. 1 (circles) show that NMF-treated cells were more susceptible to cis-DDP killing than cells not exposed to NMF. The MF, defined as the cis-DDP dose required to reduce cell survival to 0.5 in cells not exposed to NMF, was 3.3. To determine the influence of cell growth state on the NMF-mediated enhancement of cis-DDP cytotoxicity, experiments were also performed using plateau phase cells. MCA-K cell cultures were grown to confluency, medium containing NMF (1%) was added, and the cis-DDP cell survival assay was performed 4 days later (Fig. 1, squares). For cells not exposed to NMF, plateau phase MCA-K cells were less sensitive to cis-DDP than exponentially growing cells (open symbols). As for exponentially growing cells, however, NMF enhanced cis-DDP-induced cell killing in plateau phase cells (MF = 2.4).

Effect of NMF plus cis-DDP on MCA-K Tumor: NMF Given before cis-DDP. Treatment of mice with NMF was initiated when tumors grew to 6 mm and was given daily for 6 or 12 consecutive days. This treatment inhibited tumor growth such that tumors remained at the pretreatment size throughout the period of NMF administration. Tumors began to grow soon after the treatment was discontinued. One day after the treatment with NMF was completed, the mice were given i.p. injections of 4, 7, or 10 mg/kg of cis-DDP. For mice treated with cis-DDP only, tumors were also 6 mm in diameter at the time of cis-DDP administration. The antitumor effects of these treatments were assessed by the ability of the drugs to delay tumor growth or to cure the mice. cis-DDP caused a dose-dependent tumor growth delay, but caused no permanent tumor regressions (Table 1). Tumor growth in mice that received both NMF and cis-DDP was similar to that in mice that received cis-DDP only (Table 1; Fig. 2A). Thus, the effect of NMF followed by cis-DDP was less than the additive effects of the individual treatments. To better illustrate the effects of NMF pretreatment on tumor response to cis-DDP, tumor growth delay as function of cis-DDP dose is shown in Fig. 3. In this figure the tumor growth delay for the groups receiving NMF and cis-DDP was compared to NMF treatment only in order to calculate any additional delay induced by the combination treatment. In both NMF treatment groups (6 and 12 day), however, the tumor growth delay induced by cis-DDP was significantly less than the cis-DDP-induced growth delay detected in mice not pretreated with NMF. NMF pretreatment reduced the antitumor efficacy of cis-DDP by a factor of more than 2.5. The 12-day treatment with NMF was more inhibitory than the 6-day treatment. Findings similar to these were also seen when the treatments with NMF had been completed 2 or 4 days before the treatment with cis-DDP (data not shown). These results suggest that NMF pretreatment reduces the antitumor actions of cis-DDP, perhaps by inhibiting cis-DDP actions or because the two drugs have overlapping tumor cell subpopulations against which they are both active.

Table 1 Treatment of MCA-K tumors with NMF plus cis-DDP: NMF treatment preceded that with cis-DDP

<table>
<thead>
<tr>
<th>Days for tumors to double in diameter</th>
<th>No NMF</th>
<th>NMF (6 days)</th>
<th>NMF (12 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cis-DDP</td>
<td>8.6 ± 0.4*</td>
<td>17.1 ± 0.6</td>
<td>25.6 ± 1.0</td>
</tr>
<tr>
<td>cis-DDP (4 mg/kg)</td>
<td>18.7 ± 1.0</td>
<td>21.1 ± 0.6</td>
<td>26.7 ± 0.8</td>
</tr>
<tr>
<td>cis-DDP (7 mg/kg)</td>
<td>28.2 ± 3.5</td>
<td>23.4 ± 0.8</td>
<td>27.9 ± 0.4</td>
</tr>
<tr>
<td>cis-DDP (10 mg/kg)</td>
<td>30.0 ± 1.2</td>
<td>26.4 ± 1.1</td>
<td>28.5 ± 0.6</td>
</tr>
</tbody>
</table>

*Mean ± SE of days needed by tumors to grow from 6 to 12 mm.

Fig. 1. Effect of in vitro exposure to NMF on MCA-K cell sensitivity to cis-DDP. Exponentially growing MCA-K cells were exposed in vitro to medium containing NMF (1%, v/v) for 4 days (circles), or medium containing 1% NMF was added to plateau-phase cultures 4 days before cis-DDP treatment (squares). After 4 days of NMF exposure, cells were treated with 1 h with graded concentrations of cis-DDP and trypsinized, and the colony-forming efficiency assay was performed. Values represent the mean of 3 independent experiments for exponentially growing cells and 2 experiments for plateau-phase cells. □ and ○, cis-DDP only; ■ and ●, NMF plus cis-DDP. Error bars have been omitted for clarity.
with a single injection of 7 mg/kg of cis-DDP or with NMF (300 mg/kg) given daily for 6 days. When these treatments were combined, NMF treatment was initiated 2, 5, or 7 days after cis-DDP. Results presented in Table 2 and Fig. 2B show that the combined treatment was significantly (P < 0.05) more effective than the individual treatments given separately. The combined treatment resulted in a significant increase in the percentage of transient tumor regressions and was even curative for a small proportion of the mice.

**Flow Cytometric Analysis of MCA-K Tumor**

Flow cytometric analysis of MCA-K tumors was performed 1 day after the treatment with NMF was stopped. Compared to the same size tumors from untreated mice, these tumors exhibited a decrease in the percentage of cells in the S and G2 + M cell cycle phases and an increase in the percentage of cells in the G1 cell cycle phase (Fig. 4). The values for control mice were: 57.0 ± 4.1% G1-phase cells; 29.3 ± 4.7% S-phase cells; and 13.8 ± 1.5% G2 + M-phase cells. The values for NMF-treated mice were: 79.6 ± 3.7% G1-phase cells; 11.1 ± 1.8% S-phase cells; and 9.3 ± 4.8% G2 + M-phase cells.

**DISCUSSION**

Both in vitro and in vivo experimental settings were used to test the feasibility of combining NMF with cis-DDP in tumor treatment. MCA-K tumor cells growing in vitro in the presence of NMF for 4 days became more sensitive to the cytotoxic action of cis-DDP than MCA-K cells not exposed to NMF. This observation is in general accord with findings reported earlier (10, 13) using tumor cell cultures other than MCA-K, implying that increasing the sensitivity of in vitro tumor cell cultures to chemotherapy agents might be a common property of NMF. Earlier observations by us using another cell line showed that 72 h of NMF exposure are required in order to enhance cis-DDP sensitivity and that, once NMF is removed from the culture medium, the enhanced sensitivity is retained for at least 24 h (13). These results indicated that the increase in cell sensitivity to cis-DDP is not a result of a direct interaction between NMF and cis-DDP, but the result of some type of metabolic change induced by NMF exposure. In addition, the cis-DDP sensitization of plateau-phase MCA-K cells by NMF (Fig. 1) suggests that DNA synthesis in the presence of NMF is not required for the enhancement of cis-DDP sensitivity. The molecular events responsible for the observed increase in chemosensitivity, however, have not been established. Among the various phenotypic changes induced by NMF are alterations in membrane characteristics (16) and chromatin structure (12), both of which can influence cellular cis-DDP sensitivity. For a number of tumor cell lines, drug uptake has been shown to be a factor contributing to the cells' response to cis-DDP (17). In addition, because cis-DDP-induced cell lethality is dependent on a divalent interaction with DNA or DNA and protein, chromatin structure has also been implicated as a determinant in cis-DDP sensitivity. Whether the membrane or chromatin changes induced by NMF are responsible for the enhanced sensitivity of MCA-K cells in vitro to cis-DDP will be the subject of future investigations.

The ability of NMF to sensitize tumor cells in vitro to chemotherapy agents is a major rationale for combining NMF with chemotherapy in tumor treatment, on the premise that NMF treatment would make tumors more responsive in vivo to chemotherapeutic agents. The data presented here, however, show that this is not the case in the treatment of MCA-K.
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tumors with NMF followed by cis-DDP. While NMF and cis-DDP given as separate treatments were highly effective in slowing tumor growth, the combined treatment, in which the treatment with NMF preceded that with cis-DDP, resulted in growth delay smaller than the additive effect of the individual treatments. As shown in Fig. 3, pretreatment with NMF in fact reduced the response of MCA-K tumors to cis-DDP: as the duration of treatment with NMF increased, so did the reduction in tumor response.

A number of possibilities could account for the reduced antitumor effect of cis-DDP in NMF-treated mice. NMF-induced perturbation in the cell proliferation state should be considered a major factor. NMF caused cessation in MCA-K tumor growth that lasted throughout the NMF treatment period and a few days after that. Flow cytometric analysis of these tumors showed inhibition of cell proliferation and accumulation of cells in G0 + G1 phases (Fig. 4). In addition, the proportion of normal cells was increased in the treated tumors (Fig. 4). We have earlier reported the histological analysis of MCA-K tumors in mice treated with NMF (4). Histology revealed tumor cell depopulation, almost complete disappearance of mitotic figures, and increased prominence of stroma. All this evidence clearly demonstrates that treatment with NMF inhibits proliferation of tumor cells. Although cis-DDP has been shown to have some cell cycle dependency in vitro killing more cells in G1 than in S and G2 (18), it has also been shown that nonproliferating cells are less sensitive to cis-DDP than proliferating cells by a factor of 2 or more (19). Therefore, NMF inhibition of cell proliferation of MCA-K tumors is likely to be responsible for the reduction of MCA-K tumors' responsiveness to cis-DDP. However, other factors could also be responsible, such as alterations in pharmacokinetics of cis-DDP and reversion of cells to more benign phenotype.

However, when NMF followed treatment with cis-DDP an additive, and in the case when NMF was given 2 days after cis-DDP even slightly supraadditive, effect was measured by the tumor growth delay assay. Combined treatment was even curative for some mice. The effect of this protocol might also be explained by tumor cell kinetics. It is possible that the NMF inhibition of MCA-K cell proliferation reduces tumor repopulation after cis-DDP treatment. At this time, however, this remains speculation. Thus, although some supraadditive action might have existed, the overall results show that the increased therapeutic benefit of the combined treatment was likely the result of the independent antitumor actions of the two agents and not the result of the interaction between them.

Our results, therefore, clearly show that the timing of NMF treatment in relation to chemotherapy is crucial when the two treatments are combined. Administration of NMF before chemotherapy is unlikely to be beneficial. In fact, it could reduce the antitumor efficacy of a chemotherapeutic agent for the reasons discussed above. Thus, to increase therapeutic benefit of the combined treatment, NMF should be given after chemotherapy. In this way the bulk of the tumor would be destroyed by chemotherapeutic agents, leaving the NMF to arrest or retard the growth of the remaining small tumor cells. As reported earlier, NMF treatment is strongly effective against a small tumor mass, exhibiting its effect mainly through its cell maturation actions (4).

REFERENCES

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