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Adjuvant Effects of Prostaglandin D_2 to Cisplatin on Human Ovarian Cancer Cell Growth in Nude Mice*

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Abstract—*Adjuvant effects of prostaglandin D_2 to cisplatin on tumor growth were studied by using nude mice bearing HR cells derived from human ovarian carcinoma. Combinations of* 0.2 *or* 0.4 μg/ml *cisplatin and* 0.05 *or* 0.1 μg/ml *prostaglandin D_2, which did not affect the HR cell proliferation alone, resulted in a significant inhibition of cell proliferation. In addition, tumor take of HR cells by nude mice in groups treated with a combination of cisplatin and prostaglandin D_2 was inhibited. Although there was no significant difference between tumor volumes in mice treated with prostaglandin D_2 alone or cisplatin alone and untreated mice, when cisplatin was administered with* 1 mg/kg *prostaglandin D_2 the tumor volume was significantly smaller on days* 21 *and* 35, *compared to that of untreated mice. When cisplatin and* 2 *or* 4 mg/kg *prostaglandin D_2 were combined, the tumor growth was significantly inhibited after day* 21, *compared not only to that of untreated mice but also of mice treated with cisplatin alone or prostaglandin D_2 alone. Such a combination therapy by cisplatin and prostaglandin D_2 seemed to result in prevention by prostaglandin D_2 of immunological suppression which may be induced by cisplatin. Thus, such a combination therapy brought about a significant prolongation to the survival time.*

INTRODUCTION

MOST PATIENTS with advanced ovarian carcinoma have been treated with cisplatin-based combination chemotherapy after surgery. It was recently reported that such combination chemotherapy has improved survival from advanced ovarian carcinoma [1–3]. However, most patients who respond to such therapy subsequently relapse, as is commonly the case with patients with ovarian carcinoma as well as patients with other cancers. They become resistant to drug therapy [4]. Accordingly, in such cases it is of great importance to devise a strategy for the enhancement of the antitumor effect of cisplatin.

It is well known that prostaglandin D_2 (PGD_2) inhibits proliferation of various malignant cell lines *in vitro* [5–7]. We also previously reported that PGD_2 inhibited in a dose-dependent manner human ovarian cancer cell growth not only *in vitro* but also *in vivo*, subsequently prolonging the survival time of nude mice with the tumor [8].

In the present study, using nude mice bearing a human ovarian cancer cell line, we attempted to use PGD_2 to enhance the antitumor activity of cisplatin against drug-sensitive ovarian cancer cells so that such cells can be successfully destroyed before they become resistant.

MATERIALS AND METHODS

Agent

PGD_2 was purchased from Funakoshi Pharmaceutical Co., Ltd., Tokyo, Japan. The PGD_2 was dissolved in absolute ethanol prior to use and diluted in RPMI 1640 to desired concentrations.

Cells

HR cells derived from ascites of a patient with serous cystadenocarcinoma of the ovary were exclusively used in this study. The passage number is about 138. Tumorigenicity of the HR cells in the nude mice was 100%. The cells were cultured as described previously [8].

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Nude mice

About 8-week-old female BALB/c nude mice were obtained from Japan Clea Laboratories, Tokyo, Japan, and maintained in a pathogen-free environment. The animals were inspected daily, and tumor growth was determined with a caliper. When necessary, the animals were killed and dissected. The tumor tissues were fixed in formalin for histological examination. Although the tumor formed in nude mice showed solid growth, the large tumor contained a larger necrotic area in the center. Distant metastases were not observed during the experimental period.

In vitro *treatment*

To determine adjuvant effects of PGD_2 to cisplatin on the HR cell proliferation, 10^4 cells were seeded in 24-well Nunc multidishes (Nunc, Roskilde, Denmark), and they were incubated in a humidified atmosphere of 5% CO_2 at 37°C. After 24 h of culture, we added 0.05 or 0.1 μg/ml PGD_2, which did not seem to affect significantly the cell proliferation with alone [8], to the medium containing various concentrations of cisplatin; after an additional 3 days of culture, the cells were counted, and their number was compared to the cell number in the well treated with cisplatin alone. All counts were done in triplicate, and the viability was assessed by the trypan blue dye exclusion test.

In vivo *treatment*

To determine the adjuvant effects of PGD_2 to cisplatin on HR tumor growth, 5×10^5 HR cells were inoculated s.c. into the right flank of nude mice. After 7 days of tumor inoculation, 1 mg/kg cisplatin, 1 mg/kg PGD_2, 2 mg/kg PGD_2 or 4 mg/kg PGD_2 was administered i.p. once a week for 5 weeks. The mice were divided into the following groups; 10 mice treated with 0.1 ml medium alone containing 10% ethanol, 10 mice treated with 1 mg/kg PGD_2 alone, 10 mice treated with 2 mg/kg PGD_2 alone, 10 mice treated with 4 mg/kg PGD_2 alone, 10 mice treated with 1 mg/kg cisplatin alone, 10 mice treated with 1 mg/kg cisplatin and 1 mg/kg PGD_2, 10 mice treated with 1 mg cisplatin and 4 mg/kg PGD_2, and 10 mice treated with 1 mg/kg cisplatin and 4 mg/kg PGD_2. The tumor growth was determined by the measurement of diameters in two dimensions of the tumor nodule with a caliper once a week. Tumor volume (cm^3) was calculated according to the following formula $4\pi/3 \times (r_1 + r_2)^3/8$, where r_1 is longitudinal radius and r_2 is transverse radius. Blood from a tail vein was collected into hematocrit tubes every week and hematocrit values and body weight were recorded for monitoring the side-effects of drugs.

Measurement of lytic activity

Spleen cells of nude mice bearing HR cells or non-tumor bearing nude mice were used as effector cells. The spleen cells were prepared as described previously [9]. HR cells were used as target cells. Aliquots containing 10^6 target cells were labeled with 100 μCi of sodium chromate-51 solution (New England Nuclear Corp., Boston, MA) for 1 h in 1 ml of medium. After three washings, 10^4 cells in 0.1 ml of medium were pipetted into micro-titer plates (Limbro Scientific Inc., Hamden, CT). Various concentrations of effector cells in 0.1 ml of medium were added in triplicate to give effector-to-target cell ratios of 100:1 and 50:1, respectively. After incubation for 18 h at 37°C in a humidifed atmosphere of 5% CO_2 in air, supernatants were collected with a Titertek collection system (Flow Laboratories, Inc., Rockville, MD) and counted in a gamma counter. The percentage specific ^{51}Cr-release was calculated as follows:

$$\frac{\text{cpm test release} - \text{cpm spontaneous release}}{\text{cpm maximum relase} - \text{cpm spontaneous release}} \times 100$$

Spontaneous and maximum releases are cpm releases from target cells incubated in medium and in medium to which 1 N HCl was added, respectively.

Statistical analysis

Results are presented as the mean ± S.D. The results were analyzed by the Mann–Whitney U test.

RESULTS

In the *in vitro* experiments, we demonstrated that co-administration of 0.2 or 0.4 μg/ml cisplatin and 0.05 or 0.1 μg/ml PGD_2, which did not affect the HR cell proliferation alone, significantly inhibited cell proliferation, compared to cell number in wells treated with cisplatin alone (Fig. 1).

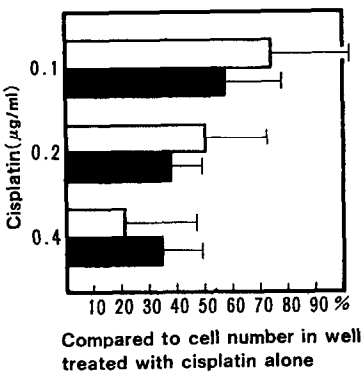

Fig. 1. Adjuvant effects of PGD_2 to cisplatin on the inhibition of HR cell proliferation in vitro. *Each point was presented as the percentage of the cell number in the well treated with cisplatin alone. Bars show the mean ± S.D.* 0.1 μg/ml *PGD_2*, □; 0.05 μg/ml *PGD_2*, ■.

In addition, we examined whether such adjuvant effects can be obtained in *in vivo* experiments. When 5×10^5 HR cells were inoculated s.c. to the right flank of nude mice, they developed palpable tumors on day 21 unless any treatment was performed. As shown in Table 1, in the cisplatin (1 mg/kg) alone treated and PGD_2 (2 mg/kg) alone treated groups all mice had palpable tumor on day 35, in the PGD_2 (1 mg/kg) alone treated group on day 28 and in the PGD_2 (4 mg/kg) alone treated group on day 49, respectively. On the other hand, in mice treated with a combination of cisplatin and PGD_2 the tumor take was significantly inhibited. One of 10 mice treated with 1 mg/kg cisplatin and 1 mg/kg PGD_2, 5 of 10 mice treated with 1 mg/kg cisplatin and 2 mg/kg PGD_2 and 4 of 10 mice treated with 1 mg/kg cisplatin and 4 mg/kg PGD_2 did not have any palpable tumor during the experimental period. When 1 mg/kg cisplatin and 1 mg/kg PGD_2 were combined the tumor volume was significantly smaller than that of untreated mice on days 21 and 35, while the tumor growth of mice treated with cisplatin alone or PGD_2 alone was not significantly inhibited. When 1 mg/kg cisplatin was administered with 2 or 4 mg/kg PGD_2, the tumor volume was significantly smaller than that not only of untreated mice but also of cisplatin or PGD_2 alone treated mice after 21 days of tumor inoculation (Fig. 2). The effects of combination therapy by cisplatin and PGD_2 on the survival time of tumor bearing mice are presented in Fig. 3. Although only 1 of 10 mice treated with 4 mg/kg PGD_2 alone survived during the experimental period, the survival time in cisplatin alone treated mice as well as PGD_2 (1 or 2 mg/kg) alone treated mice did not show any significant difference, compared to that in untreated mice. A combination of 1 mg/kg cisplatin and 1 mg/kg PGD_2 resulted in a significant prolongation of the survival time, compared to that in untreated mice. In addition, the survival time in mice treated with 1 mg/kg cisplatin and 2 or 4 mg/kg PGD_2 was significantly longer than that not only in untreated mice but also in cisplatin alone or PGD_2 alone treated mice.

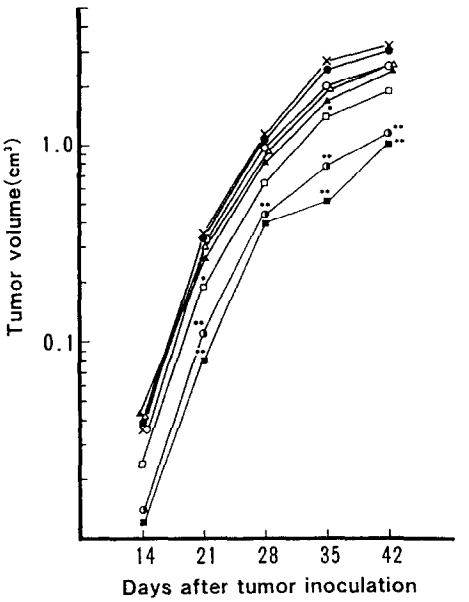

*Fig. 2. Adjuvant effects of PGD_2 to cisplatin on HR cell tumor growth in nude mice. HR cells (5×10^5) were inoculated s.c. into the right flank of nude mice. These mice were divided into untreated group (×—×), 1 mg/kg cispatin alone treated group (○—○), 1 mg/kg PGD_2 alone treated group (●—●), 2 mg/kg PGD_2 alone treated group (△—△), 4 mg/kg PGD_2 alone treated group (▲—▲), 1 mg/kg cisplatin plus 1 mg/kg PGD_2 treated group (□—□), 1 mg/kg cisplatin plus 2 mg/kg PGD_2 treated group (■—■), and 1 mg/kg cisplatin plus 4 mg/kg PGD_2 treated group (◑—◑). Each group consisted of 10 mice. PGD_2 and cisplatin were administered i.p. once a week for 5 weeks from 7 days after tumor inoculation. Each point shows the mean value. *$P < 0.05$, compared to cisplatin alone treated group. **$P < 0.05$, compared to cisplatin alone and PGD_2 alone treated groups.*

Changes of lytic activities to HR cells of spleen cells in each treated group during the treatment course are shown in Table 2. Although spleen cells

Table 1. Effect of treatment on tumor take after HR cell inoculation

Treatment	Days after tumor inoculation											
	14		21		28		35		42		49	
Untreated	2/10*	(20%)	10/10	(100%)	10/10	(100%)	9/9	(100%)	8/8	(100%)	6/6	(100%)
Cisplatin alone†	1/10	(10%)	9/10	(90%)	9/10	(90%)	10/10	(100%)	10/10	(100%)	8/8	(100%)
1 mg/kg PGD_2 alone	0/10	(0%)	8/10	(80%)	10/10	(100%)	10/10	(100%)	10/10	(100%)	8/8	(100%)
2 mg/kg PGD_2 alone	2/10	(20%)	8/10	(80%)	8/10	(80%)	9/9	(100%)	9/9	(100%)	7/7	(100%)
4 mg/kg PGD_2 alone	0/10	(0%)	5/10	(50%)	7/10	(70%)	8/10	(80%)	8/9	(89%)	9/9	(100%)
Cisplatin plus 1 mg/kg PGD_2	1/10	(10%)	6/10	(60%)	7/10	(70%)	8/9	(89%)	7/8	(88%)	6/7	(86%)
Cisplatin plus 2 mg/kg PGD_2	1/10	(10%)	4/10	(40%)	4/10	(40%)	5/10	(50%)	5/10	(50%)	5/10	(50%)
Cisplatin plus 4 mg/kg PGD_2	0/10	(0%)	4/10	(40%)	4/10	(40%)	5/9	(56%)	6/9	(67%)	6/9	(67%)

*Number of mice with palpable tumor/number of survived mice (%).
†1 mg/kg cisplatin alone.

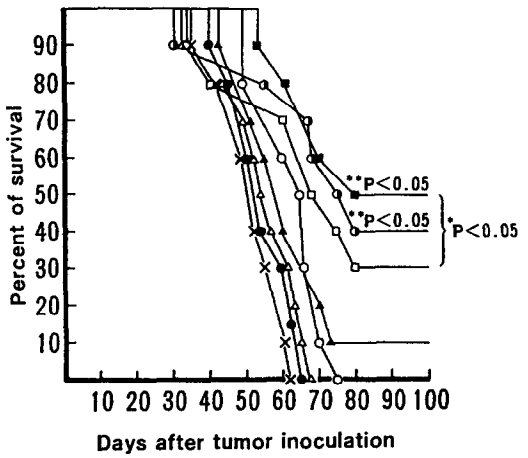

*Fig. 3. Adjuvant effects of PGD_2 to cisplatin on the survival time of nude mice bearing HR cell tumor. HR cells (5×10^5) were inoculated s.c. into the right flank of nude mice. These mice were divided as shown in Fig. 1. ×, Untreated group; ○, 1 mg/kg cisplatin alone treated group; ●, 1 mg/kg PGD_2 alone treated group; △, 2 mg/kg PGD_2 alone treated group; ▲, 4 mg/kg PGD_2 alone treated group; □, 1 mg/kg cisplatin plus 1 mg/kg PGD_2 treated group; ■, 1 mg/kg cisplatin plus 2 mg/kg PGD_2 treated group; ◑, 1 mg/kg cisplatin plus 4 mg/kg PGD_2 treated group. *P < 0.05, compared to untreated group. **P < 0.05, compared to cisplatin alone and PGD_2 alone treated groups.*

in intact nude mice before tumor inoculation did not have any significant lytic activity to HR cells, spleen cells in untreated mice showed a significant lytic activity 3 weeks after tumor inoculation and those further elevated to 41.0% 6 weeks after tumor inoculation. Changes of lytic activities in a PGD_2 alone treated group were similar to those in an untreated group. On the other hand, lytic activities in a cisplatin alone treated group decreased to 9.7% 6 weeks after tumor inoculation. The inhibition of lytic activity by cisplatin could be reversed with administration of PGD_2.

Table 2. Effect of PGD_2 and cisplatin on lytic activity to HR cells of spleen cells in nude mice bearing HR tumors

Spleen cells	Percentage cytotoxicity*
Before inoculation	1.7 ± 3.1
3 weeks after inoculation	
Untreated group	16.9 ± 3.1†
PGD_2-treated group	17.5 ± 2.8
Cisplatin-treated group§	18.0 ± 3.4
Cisplatin plus PGD_2 treated group	19.1 ± 4.2
6 weeks after inoculation	
Untreated group	41.0 ± 5.2‡
PGD_2-treated group	35.4 ± 10.3‡
Cisplatin-treated group	12.6 ± 5.6¶
Cisplatin plus PGD_2 treated group	44.0 ± 13.8‡

*Effector:target cell ratio: 100:1.
†The mean ± S.D.
‡Mice treated with 2 mg/kg PGD_2 alone.
§Mice treated with 1 mg/kg cisplatin alone.
‖ $P < 0.01$, compared to 3 weeks after inoculation.
¶ $P < 0.001$, compared to the other groups.

DISCUSSION

We have already reported that 12 mg/kg (but not 4 mg/kg) PGD_2 inhibited ovarian cancer growth not only *in vitro* but also *in vivo* [8]. In the present study, we confirmed that a combination of cisplatin and PGD_2 also inhibited significantly the HR cell proliferation *in vitro*. It has been reported that PGD_2 blocked cells in G_1 [10]. Accordingly, it is possible that exposure of such synchronized cells in G_1 to cisplatin results in enhancement of cell kill. Recently, it has also been reported that PGD_2 was converted to 9-deoxy-$\Delta^{9,12}$-13,14-dihydro-PGD_2 (Δ^{12}-PGJ_2) by enzymatic dehydration and the dehydration product caused the tumor cell growth inhibition [11]. In addition, Narumiya *et al.* [12, 13] demonstrated that Δ^{12}-PGJ_2 is transported into cells to elicit a growth-inhibitory action and most of the nuclear Δ^{12}-PGJ_2 is bound covalently to protein(s) of chromatin and nuclear matrix, thus growth inhibition by Δ^{12}-PGJ_2 is irreversible and cytotoxic. Similarly, it is well known that cisplatin exerts its cytotoxic and antitumor effect through interaction with DNA. Therefore, it is conceivable that binding to different sites of DNA by Δ^{12}-PGJ_2 and cisplatin brings about adjuvant effects with respect to tumor growth inhibition.

In addition, we attempted to enhance the antitumor effects of cisplatin by using concentrations of PGD_2 which did not seem to affect the tumor growth *in vivo* alone. We have demonstrated that a combination of PGD_2 with cisplatin resulted in an enhancement of its antitumor effects. The degree of enhancement of antitumor effects by PGD_2 was most marked when 2 mg/kg PGD_2 was administered with cisplatin, with regard to the inhibition of tumor take and tumor growth and prolongation of the survival time. In addition to direct mechanisms observed *in vitro*, prevention by PGD_2 of inhibition of lytic activities which may be caused by cisplatin was also considered to potentiate the antitumor activity of cisplatin *in vivo*. An increase of histamine content in neoplastic tissues has been previously described [14]. A growing body of evidence suggests that suppressor T cells carrying histamine type-2 receptors may play an important immunoregulatory role in the execution of the normal immune response [15–18]. It is possible that such suppressor cells are stimulated by the increased histamine in blood of tumor-bearing mice. In the present study, we demonstrated that a significant lytic activity in nude mice challenged with HR cells was induced 3 weeks after tumor inoculation and elevated to 41.0% 6 weeks after tumor inoculation. There was no

difference between lytic activities in other groups 3 weeks after tumor inoculation. However, 6 weeks after tumor inoculation the activities in untreated mice, PGD_2 alone treated mice and cisplatin plus PGD_2 treated mice were almost the same while those in cisplatin alone treated mice decreased to about 1/3 of those in the other treated groups. These results suggest that histamine released from mast cells within tumor tissues activates suppressor T cells carrying histamine type-2 receptors, subsequently lowering the killer activity. In addition, it has been reported that PGD_2 inhibits histamine release [19, 20]. Since the effector cells used in this study were nonadherent splenic lymphocytes, the effector cells which are believed to exert tumor cell kill activity seemed to be killer T cells.

In the present study, we suggest three different possible mechanisms to explain potentiating effects of PGD_2. The three different possible suggested mechanisms are: (a) PGD_2 blocks cells in G_1 phase so that exposure of synchronized cells to cisplatin results in enhanced cell kill; (b) binding of the active metabolite Δ^{12}-PGJ_2 to proteins of chromatin in nuclear matrix; (c) PGD_2 prevents inhibition of splenic lytic activity produced by cisplatin. All these three different mechanisms may be operative *in vivo*. We can speculate that PGD_2 initially enhances tumor cell kill of cisplatin by mechanisms (a) and (b), and thereafter stimulates killer T-cell activity by inhibiting suppressor T-cell activity (c).

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