[CANCER RESEARCH 46, 3364–3366, July 1986]

Inhibition of Human Ovarian Cancer Cell Growth *in Vitro* and in Nude Mice by Prostaglandin D_2[1]

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ABSTRACT

***In vitro* and *in vivo* effects of prostaglandin D_2 on human ovarian tumor growth were examined by using a cell line, designated HR, derived from ascites of a patient with serous cystadenocarcinoma of the ovary. The HR cell proliferation *in vitro* was dose dependently inhibited between concentrations of 0.1 and 4.0 μg of prostaglandin D_2 per ml. From results of ^{51}Cr release assays and trypan blue dye exclusion tests the inhibitory effect seemed to result from a direct cytotoxic effect by prostaglandin D_2. All DNA, RNA, and protein syntheses by the HR cells were also inhibited in a dose-dependent manner with exposure time of 48 h to prostaglandin D_2. When 5×10^5 HR cells were inoculated to nude mice, the 50% survival time of them in untreated groups was 52 days after inoculation. Although 4 mg of prostaglandin D_2 per kg caused inhibition of the tumor growth, a significant prolongation of the survival time was not observed. On the other hand, the 50% survival time of nude mice treated with 12 mg of prostaglandin D_2 per kg was significantly ($P < 0.05$) prolonged to 67 days, in addition to a significant inhibition of the tumor growth.**

INTRODUCTION

Since 1972, PGs[3] of E and A series are known to inhibit the growth of many lines of tumor cells both *in vitro* and *in vivo* (1, 2). PGD_2, an analogue of PGE_2, has been demonstrated to be directly involved in prevention of tumor metastasis, possibly through its influence on the formation of platelet tumor emboli (3, 4). Recent studies have revealed that PGD_2 might have a potent cytotoxic activity on several lines of tumor and subsequently inhibit the tumor growth (5–9). Accordingly, we attempted to examine effects of PGD_2 on the growth of a human ovarian cancer cell line. In the present study, we report that PGD_2 inhibits in a dose-dependent manner the human ovarian cancer cell growth not only *in vitro* but also *in vivo*, subsequently prolonging the survival time of nude mice with the tumor.

MATERIALS AND METHODS

Chemical Compounds. PGD_2 was purchased from Funakoshi Pharmaceutical Co., Ltd., Tokyo, Japan. The purity of PGD_2 was over 96.9% as judged by high-pressure liquid chromatography. The PGD_2 was dissolved in absolute ethanol prior to use and diluted in RPMI 1640 to desired concentrations. [6-^{3}H]Thymidine (6.7 Ci/mmol), [6-^{3}H]uridine (24.3 Ci/mmol), and L-[3,4-^{3}H]valine (58.1 Ci/mmol) were obtained from New England Nuclear Corp., Boston, MA.

Cell Culture. The HR cell line was established from ascites of a patient with serous cystadenocarcinoma of the ovary on January 1, 1983 (10), and the passage number is about 106. Tumorigenicity of the HR cells was 100% when 10^5 cells were inoculated s.c. to nude mice. The cells were culitvated in RPMI 1640 containing penicillin, 100 units/ml, Fungizone, 0.25 μg/ml, streptomycin, 100 μg/ml, 10% heat-inactivated fetal calf serum (Gibco Laboratories, Grand Island, NY), and 2 mM glutamine in a 5% CO_2 atmosphere at 37 °C. The medium was changed every 3 days, and the cells were passed when confluency was achieved. The cells were repeatedly tested for *Mycoplasma* contamination by staining with Hoechst 33258 (11).

***In Vitro* Treatment with PGD_2.** For determining the effect of PGD_2 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, various concentrations of PGD_2 were added to the medium. Cells in each well were harvested after 24 h, 48 h, and 72 h of additional culture and counted using a hematocytometer. In all experiments the final ethanol concentration was less than 0.1% (v/v). All counts were done in quadruplicate, and the viability was assessed by trypan blue dye exclusion. In order to examine the direct effect of PGD_2 on the HR cells, aliquots containing 10^6 HR 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 pipeted into microtiter plates. The 10^4 chromium-labeled HR cells were incubated with various concentrations of PGD_2 for 48 h in a 5% CO_2 atmosphere at 37 °C. Supernatants were counted for release of chromium from dead cells. Untreated cells and cells treated with 1 N HCl were used for measurement of spontaneous and maximum releases. The percentage of specific ^{51}Cr release was calculated as follows.

$$\%\ \text{of}\ ^{51}\text{Cr release} = \frac{\text{cpm test release} - \text{cpm spontaneous release}}{\text{cpm maximum release} - \text{cpm spontaneous release}} \times 100$$

To determine the effect of PGD_2 on syntheses of DNA, RNA, and protein of the HR cells, 2×10^4 cells were seeded into microtiter plates (Linbro Scientific, Inc., Hamden, CT). After 24 h of culture, various concentrations of PGD_2 were added into the medium. The DNA, RNA, and protein syntheses by the tumor cells were assayed by adding 1.0 μCi of [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]valine to each well during the last 4 h of the additional 48-h incubation period. Cultures were harvested by aspiration of cells into glass-fiber filters utilizing a semiautomated multiple-sample harvester. Dried filters were placed in scintillation fluid, and radioactivity was measured by a liquid scintillation counter. Assays were done in quadruplicate.

Nude Mice. Six-wk-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.

***In Vivo* Treatment with PGD_2.** To study the *in vivo* effect of PGD_2 on the HR tumor growth, 5×10^5 HR cells were inoculated s.c. into the right flank of nude mice. Injection i.p. of PGD_2 (4 or 12 mg/kg) was initiated on the day of tumor inoculation and performed twice a week for 4 wk. The mice were inspected daily, and the tumor growth was determined by the measurement of diameters in two dimensions of the tumor nodule with a caliper once a week. The untreated group consisted of ten mice. PGD_2-treated groups (4 mg/kg and 12 mg/kg) contained eight mice, respectively. 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.

Statistical Analysis. Results are presented as mean ± SD. The results were analyzed by nonparametric methods.

RESULTS

Effect of PGD_2 on the HR Cell Proliferation *in Vitro*. As shown in Fig. 1, the HR cell proliferation was inhibited in a

Received 11/11/85; revised 2/20/86; accepted 4/1/86.

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[1] Supported in part by a grant from the Special Scientific Research Program of the Defense Agency in Japan.

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[3] The abbreviation used is: PG, prostaglandin.

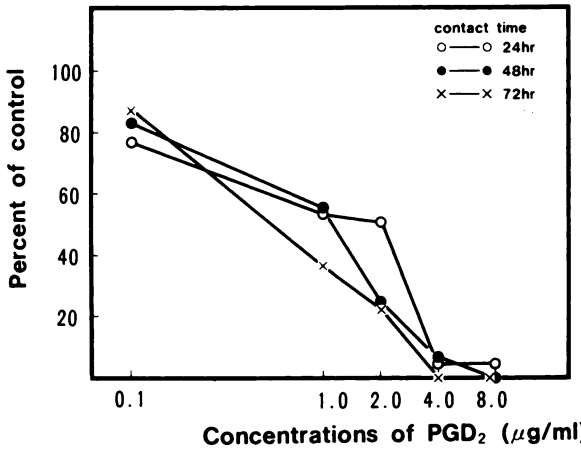

Fig. 1. Inhibitory effects of PGD_2 on the HR cell proliferation *in vitro*. Viable 10^4 HR cells were seeded into 24-well Nunc multidishes, and after 24-h incubation, various concentrations of PGD_2 were added to the medium. After incubation of additional 24-h, 48-h, and 72-h cultures, cells in each well were harvested and counted using a hematocytometer. Values were presented as percentage of cell number in untreated wells. Each *point* shows the mean value in quadruplicate cultures. Concentrations of PGD_2 required for 50% inhibition of cell proliferation were 2.0 μg/ml for 24 h, 1.1 μg/ml for 48 h, and 0.55 μg/ml for 72 h, respectively.

Table 1 *Cytotoxic effect of PGD_2 on HR cell proliferation in vitro*

^{51}Cr release assay was performed as described in "Materials and Methods." The maximum and spontaneous releases were 1240 cpm and 116 cpm, respectively.

PGD_2 (μg/ml)	^{51}Cr release (%)[a]
0.1	18.3 ± 2.5[b]
1.0	31.6 ± 4.9
2.0	46.1 ± 3.3
4.0	58.8 ± 4.5
8.0	79.4 ± 4.7

[a] Percentage of specific ^{51}Cr release.
[b] Mean ± SD from quadruplicate cultures.

dose-dependent fashion between concentrations of 0.1 and 4.0 μg of PGD_2 per ml. Concentrations of PGD_2 required for 50% inhibition of the cell proliferation were 2.0, 1.1, and 0.55 μg/ml with 24, 48, and 72 h of contact time, respectively.

Effect of PGD_2 on the ^{51}Cr Release by HR Cells. To examine any direct effect of PGD_2 on the HR cells, chromium-labeled HR cells were incubated in the presence of various concentrations of PGD_2 for 48 h, and ^{51}Cr release by the dead cells was measured. The percentage of ^{51}Cr release increased in proportion to concentrations of PGD_2, suggesting a cytotoxic effect of PGD_2 on the HR cells (Table 1). This evidence was also confirmed by the trypan blue dye exclusion test.

Effect of PGD_2 on the Syntheses of DNA, RNA, and Protein in the HR Cells. In order to investigate the inhibition mechanism of PGD_2 on the HR cell proliferation, the effect on [^{3}H]-thymidine, [^{3}H]uridine, and [^{3}H]valine uptakes by the cells was examined. The incorporation of all radiolabeled precursors used in this study was inhibited in a dose-dependent fashion as observed in experiments regarding the effect of PGD_2 on the HR cell proliferation *in vitro* (Table 2).

Effects of PGD_2 on the Tumor Growth of HR Cells Inoculated into Nude Mice. Next, we attempted to determine whether the inhibitory effect of PGD_2 on tumor growth *in vitro* can be observed in tumors grown in nude mice. In a group treated with 4 mg of PGD_2 per kg twice a week for 4 wk after the day of tumor inoculation, the tumor volume on 28 days was significantly smaller than that in the untreated group. The tumor volumes in a group treated with 12 mg of PGD_2 per kg were significantly smaller on Days 21, 28, and 35 than those in the untreated group. However, during the experimental period after Day 35, the tumor volumes in PGD_2-treated groups tended to be smaller (but not significant) than those in the untreated group (Table 3). As shown in Fig. 2, the 50% survival time of mice in the untreated group was 52 days, and all mice were dead 62 days after tumor inoculation. The 50% survival time of mice in a group treated with 4 mg of PGD_2 per kg was 55 days. In contrast, when the tumor-bearing nude mice were treated with 12 mg of PGD_2 per kg, the 50% survival time was prolonged to 67 days.

DISCUSSION

In the present study, we demonstrated that PGD_2 inhibited dose dependently the proliferation of HR cells derived from human ovarian cancer. From the results of a ^{51}Cr release assay, the inhibitory effect was considered to result from direct cytotoxic action of PGD_2 on the HR cells. Similar cytotoxic actions of PGD_2 on the other human tumor cells have also been observed (5, 8). PGD_2 has been identified as a major PG in the brain, and the synthesis and degradation of PGD_2 have been reported in detail (12–15). This PG has also been reported to control pulmonary metastasis of malignant melanoma cells (3, 4). Syntheses of DNA, RNA, and protein in the HR cells were also dose dependently inhibited by 48-h exposure with PGD_2. However, we cannot clearly explain the mechanism of cytotoxicity of PGD_2 by this evidence only. Further studies are necessary to elucidate the mechanism of the cytotoxic action of PGD_2. Similar observations regarding the inhibition of DNA, RNA, and protein syntheses by PGD_2 have also been made by several investigators (8, 9, 16). The *in vivo* effects of PGD_2 on tumor growth were also examined by using the HR cells. Injections i.p. of 4 mg of PGD_2 per kg significantly inhibited the tumor growth of HR cells heterotransplanted to nude mice to 62.7% of tumor volume in untreated mice on only 28 days after tumor inoculation. Injections i.p. of 12 mg of PGD_2 per kg resulted in a significant inhibition to 60.0, 50.9, and 65.2% on 21, 28, and 35 days after tumor inoculation, respectively. Although the degree of inhibition of tumor growth *in vivo* was less than that of tumor growth *in vitro*, i.p. injections of 12 mg (but not 4 mg/kg) of PGD_2 per kg prolonged significantly the

Table 2 *Effects of PGD_2 on uptakes of [^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]valine by HR cells*

PGD_2[a] (μg/ml)	[^{3}H]Thymidine (cpm)	[^{3}H]Uridine (cpm)	[^{3}H]Valine (cpm)
Untreated	19,124 ± 2,211[b]	11,519 ± 219	13,599 ± 436
0.1	18,307 ± 1,384 (95.7)[c]	11,408 ± 1,377 (99.0)	12,093 ± 3,483 (88.9)
1.0	19,181 ± 1,184 (100.3)	8,785 ± 1,059[d] (76.3)	9,559 ± 5,640 (70.3)
2.0	15,320 ± 1,988[e] (80.1)	8,652 ± 1,308[d] (75.1)	8,328 ± 2,042[e] (61.2)
4.0	10,029 ± 4,255[e] (52.4)	7,456 ± 1,512[d] (64.7)	6,883 ± 1,642[d] (50.6)
8.0	7,350 ± 1,808[d] (38.4)	6,169 ± 1,211[d] (53.6)	6,158 ± 1,393[d] (45.3)

[a] The contact time with PGD_2, 48 h. The pulse time with radiolabeled compounds, 4 h.
[b] Mean ± SD from quadruplicate cultures.
[c] Numbers in parentheses, percentage of untreated control.
[d] $P < 0.01$.
[e] $P < 0.05$ (Scheffe's test), compared to untreated control.

Table 3 *Effect of PGD_2 on the tumor growth of HR cells inoculated into nude mice*

HR cells (5×10^5) were inoculated s.c. into the right flank of nude mice. In the PGD_2-treated groups, 4 or 12 mg of PGD_2 per kg were administered i.p. twice a week for 4 wk from the day of inoculation. Tumor diameters in two dimensions were measured with a caliper, and the tumor volume was calculated as described in "Materials and Methods."

Days after inoculation	Untreated (10 mice)	PGD_2 (4 mg/kg) (8 mice)	PGD_2 (12 mg/kg) (8 mice)
7	Not palpable	Not palpable	Not palpable
14	0.024 ± 0.025[a] (10)[b]	0.025 ± 0.015 (8)	0.018 ± 0.011 (8)
21	0.35 ± 0.15 (10)	0.23 ± 0.14 (8)	0.21 ± 0.13[c] (8)
28	1.10 ± 0.25 (10)	0.69 ± 0.28[d] (8)	0.56 ± 0.29[d] (8)
35	2.59 ± 0.56 (10)	1.72 ± 1.03 (8)	1.63 ± 0.46[d] (8)
42	3.22 ± 0.76 (9)	2.44 ± 1.30 (5)	2.19 ± 1.58 (5)
50	6.37 ± 1.80 (8)	4.23 ± 2.66 (5)	3.74 ± 2.20 (5)

[a] Mean ± SD of tumor volume (cm^3).
[b] Numbers in parentheses, number of mice that survived.
[c] $P < 0.05$ (Mann-Whitney U test), compared to untreated group.
[d] $P < 0.01$.

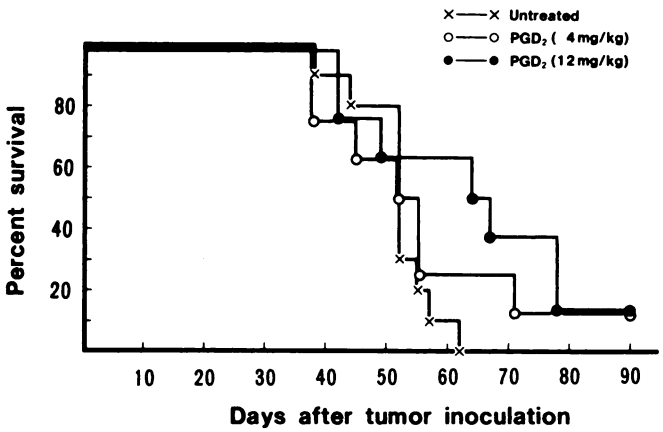

Fig. 2. Effects of PGD_2 on the survival of nude mice bearing human ovarian carcinoma. HR cells (5×10^5) were inoculated s.c. into right flank of nude mice. These nude mice were divided into the untreated group, 4 mg/kg PGD_2-treated group, and 12 mg/kg PGD_2-treated group. The untreated group consisted of ten mice. PGD_2-treated groups consisted of eight mice each. In PGD_2-treated groups, 4 or 12 mg of PGD_2 per kg were administered i.p. twice a week for 4 wk from the day of inoculation. The 50% survival time was 52 days in the untreated group, 55 days in the 4 mg/kg PGD_2-treated group, and 67 days in the 12 mg/kg PGD_2-treated group, respectively. The survival time in the 12 mg/kg (but not 4 mg/kg) PGD_2-treated group was significantly (Cox-Mantel test, $P < 0.05$) longer than that in the untreated group.

survival time. Higashida *et al.* (17) reported that daily i.p. injections of 0.5 to 1.0 mg of PGD_2 per kg resulted in slight regression of tumor weight by using mouse neuroblastoma. In addition, injection of 0.5 mg of PGD_2 per kg into the tumor was demonstrated to be more effective than the same concentration given by i.p. injection, suggesting a direct cytotoxic effect of PGD_2 as was observed in the *in vitro* experiments (7). Recently, a dehydrated derivative of PGD_2 has been reported to have more marked cytotoxic action (18). Although the precise mechanism of PGD_2 action was not elucidated in the present study, it is possible that a metabolite of PGD_2 has such direct inhibitory effects on the tumor growth.

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