Growth-inhibitory effect of prostaglandin D₂ on mouse glioma cells

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The effects of prostaglandin D₂ (PGD₂) on the growth of mouse malignant glioma cells were studied in vitro and in vivo. The in vitro studies consisted of various concentrations of prostaglandins (PG's) being added to cultures of mouse glioma cells. At concentrations above 2.5 μg/ml, PGD₂ strongly inhibited the proliferation of glioma cells, whereas PGE₂ had no effect at the same value. Exposure to 5.0 μg/ml PGD₂ for more than 2 hours resulted in inhibition of glioma cell proliferation. This growth-inhibitory effect of PGD₂ was related to the inhibition of DNA synthesis of the cells. The in vivo studies were performed with a subcutaneously transplanted mouse glioma model. Injection of 0.5 mg/kg PGD₂ into the tumor was more effective than the same concentration given by intraperitoneal injection. In mice with intracranially transplanted glioma, daily intraperitoneal injection of 0.5 mg/kg PGD₂ had no significant effect on survival time.

KEY WORDS ▪ prostaglandin ▪ prostaglandin D₂ ▪ brain tumor ▪ mouse glioma ▪ tumor model ▪ glioma

Prostaglandins (PG's) consist of a large group of cyclic derivatives of C20 oxygenated unsaturated fatty acids and have been detected in virtually all cells and tissues of animals. The PG’s are known to have various physiological and pharmacological activities, and recently have been reported to affect the properties of cells, such as cell proliferation, carcinogenesis, and tumor growth.2-7,9 One of the major PG’s found in the central nervous system,1,8 PGD₂, has been found to inhibit deoxyribonucleic acid (DNA) synthesis in human and murine leukemia,5 mouse mastocytoma,11 mouse neuroblastoma,6 and human neuroblastoma cells.15

We report in this paper the effects of PGD₂ on proliferation of cultured mouse glioma cells in vitro. The in vivo effects of PGD₂ was also investigated using an experimental model involving subcutaneous or intracranial transplantation of glioma in mice.

Materials and Methods

Materials

The purity of PGD₂ and PGE₂* was over 96.9% and 99.9%, respectively, as judged by high-pressure liquid chromatography. Stability of PGD₂ in an aqueous medium was determined by incubation in phosphate buffer, pH 7.0, at 37°C. As judged by high-pressure liquid chromatographic profile, the half-life of PGD₂ was 8.6 hours.11

Cells and Cell Cultures

Methylcholanthrene-induced mouse glioma cells (203-glioma),† of C57BL/6 origin were maintained in our laboratory since 1978. Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). Ethylnitrosourea (ENU)-induced rat neurinoma T1, originally established in our laboratory in 197714 of Wistar/Fib. rat origin, was maintained in tissue culture. The T1 cells were cultured in Eagle’s minimal essential medium (MEM) supplemented with 10% FCS.

The single tumor-cell suspensions were prepared by trypsinization with 0.1% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline for 5 minutes. After the cells were washed three times with Hanks’ balanced salt solution (HBSS), the viable cells were counted with a hemocytometer by the

* PGD₂ and PGE₂ were gifts of Ono Pharmaceutical Co., Higashi-ku, Osaka, Japan.

† Cell line originally established by Dr. Ishida, Gunma University, Japan.
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FIG. 1. Comparison of the effect of PGD2 and PGE2 on 203-glioma cell proliferation. First, 203-glioma cells (1 x 10^5/well) were cultured with various concentrations of each prostaglandin for 96 hours. The viable cells were then counted every 24 hours using the trypan blue dye exclusion method. Control medium contained 0.1% ethanol only. Left: Effect of PGD2 administration. Each point represents the mean results of three wells from three independent experiments. Right: Effect of PGE2 administration. Each point represents the mean results of three wells from two independent experiments.

trypan blue dye exclusion method. The PG's were dissolved in 99.5% ethanol and diluted in culture medium immediately before use with a final concentration of 0.1% ethanol. Culture media containing PG's were sterilized by Millipore filtration. Control medium contained 0.1% ethanol. The 203-glioma and T1 cells were plated at a density of 1 x 10^5 cells/well and 5 x 10^4 cells/well, respectively, and grown in culture plates (24 wells) with 2 ml of medium at 37°C under the humidified atmosphere of 5% CO2 in air.

Measurement of DNA Synthesis

Tumor cells were plated at a density of 1 x 10^4 cells/0.2 ml/well in microculture plates (96 wells) in the medium containing PGD2. The DNA synthesis of tumor cells was assayed by adding 1.0 μCi of hydrogen-3 (3H)-thymidine† to each well during the last 6 hours of the three-day incubation period. Cultures were harvested by aspiration of cells onto glass-fiber filters utilizing a semiautomated multiple-sample harvester.§ Dried filters were placed in scintillating fluid, and radioactivity was measured by a liquid scintillation spectrophotometer.|| Data were expressed as percentage uptake of 3H-thymidine compared with the count in control cultures.

Animals and Tumor Model

Female mice, aged between 6 and 8 weeks, from a highly inbred C57BL/6 strain, were maintained in the Facilities of Experimental Animals, Faculty of Medi-

†3H-thymidine obtained from Amersham/Searle, Arlington Heights, Illinois.
‡Mark II sample harvester manufactured by Wakenkayaku Co., Ltd., Kyoto, Japan.

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cine, Kyoto University. Cells harvested from the cultures were suspended in sterile HBSS, and were transplanted, as a 0.1-ml suspension containing 1 x 10^6 cells, subcutaneously in the back of C57BL/6 mice. Tumor size was measured every 2nd and 3rd day, and recorded as a mean tumor size of two perpendicular diameters.

By intracranial inoculation, 1 x 10^5 203-glioma cells suspended in a volume of 0.01 ml of HBSS were injected under sterile conditions through the right frontal cranial bone to a depth of 2 mm by means of a 0.05-ml glass microsyringe and a No. 27 Yaoi needle. The mortality rate due to intracerebral inoculation was less than 0.5%.

Administration of PGD2 in Glioma-Transplanted Mice

A volume of 0.2 ml PGD2, suspended in HBSS with a final concentration of 0.5% ethanol, was administered daily by intraperitoneal or intratumoral injection, starting on Day 7 after glioma transplantation and continuing over a 14-day period. Control mice received HBSS containing 0.5% ethanol in the same manner.

Statistical Analysis

Data are presented as means ± standard deviations. Differences were analyzed by the Student t-test, and p values of 0.05 or less were taken to indicate statistical significance.

Results

Effects of PGD2 on Proliferation of Tumor Cells

Various concentrations of PGD2 and PGE2 were added to cultures of 203-glioma cells to investigate their effects on cell growth as described above. As shown in Fig. 1 left, PGD2 did not have any effect at concentra-
FIG. 2. Effect of PGD<sub>2</sub> on rat neurinoma T1 cell growth. The T1 cells (5 x 10<sup>3</sup>/well) were cultured with various concentrations of PGD<sub>2</sub> for 96 hours. The viable cells were counted every 24 hours using the trypan blue dye exclusion method. Control medium contained 0.1% ethanol only. Each point represents the mean results of three wells from two independent experiments.

FIG. 3. Relationship between exposure time to PGD<sub>2</sub> and growth of 203-glioma cells. The 203-glioma cells were exposed to 5.0 μg/ml PGD<sub>2</sub> for the indicated time at 37°C in 5% CO<sub>2</sub>. The cells were washed three times in HBSS and then resuspended in DMEM containing 10% fetal calf serum for 96 hours at 37°C in 5% CO<sub>2</sub>. The viable cells were counted every 24 hours using trypan blue dye. Each point represents the mean results of two wells from two independent experiments.

FIG. 4. Effect of PGD<sub>2</sub> on DNA syntheses of 203-glioma and T1 cells. Tumor cells were placed at a density of 1 x 10<sup>4</sup>/0.2 ml in the microculture plates in the medium containing various concentrations of PGD<sub>2</sub>. The plates were incubated for 66 hours at 37°C with 1.0 μCi <sup>3</sup>H-thymidine (TdR). The cells were harvested onto filter paper strips with a cell harvester and counted in a liquid scintillation counter. The percentage uptake of <sup>3</sup>H-TdR was calculated determining the count of PGD<sub>2</sub>-free culture as 100%.

Effect of PGD<sub>2</sub> on DNA Synthesis of 203-Glioma Cells

In order to investigate the inhibition mechanism of PGD<sub>2</sub> on proliferation of both 203-glioma and T1 cells, the effect on <sup>3</sup>H-thymidine uptake was examined. Figure 4 shows the dose-dependent suppressive effects of PGD<sub>2</sub> on DNA synthesis of tumor cells. The suppression was seen in both 203-glioma and T1 at concentrations of PGD<sub>2</sub> above 2.5 μg/ml. On the other hand, the suppressive effects of PGE<sub>2</sub> on DNA synthesis were demonstrated only when 203-glioma cells were cultured at a concentration of 10.0 μg/ml (data not shown).
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Effect of PGD2 In Vivo

To study the in vivo effect of PGD2 on 203-glioma, the mouse glioma was transplanted as described above. Subcutaneous inoculation of 203-glioma cells in syngeneic hosts resulted in a palpable and measurable mass by Day 7. The tumor-bearing mice were then separated into four groups: an untreated group, and groups treated intraperitoneally with HBSS containing 0.5% ethanol, 0.2 mg/kg PGD2, and 0.5 mg/kg PGD2. The results are shown in Fig. 5 left. The 0.5% ethanol had no effect on glioma growth and the effect of intraperitoneal injection of 0.2 mg/kg PGD2 was not statistically significant. On the other hand, daily intraperitoneal injection of 0.5 mg/kg PGD2 resulted in significant inhibition of subcutaneous glioma growth (p < 0.05). No side effects were observed during daily intraperitoneal injection of PGD2.

Local administration of PGD2 into the tumor was performed using the same protocol. One group received HBSS containing 0.5% ethanol, and the others received PGD2 at concentrations of 0.2 or 0.5 mg/kg, injected intratumorally over a 14-day period starting on Day 7. As seen in Fig. 5 right, intratumoral injection of PGD2 at both concentrations (0.2 and 0.5 mg/kg) caused a significant reduction of the tumor size, whereas the injection of 0.5% ethanol resulted in no significant effect on tumor growth. According to these results, the in vivo growth-inhibitory effect of PGD2 on 203-glioma seemed to be more evident when PGD2 was administered intratumorally.

In order to investigate the effect of PGD2 on the intracranially transplanted glioma in mice, 0.2 or 0.5 mg/kg PGD2 was administered intraperitoneally. The effect of PGD2 was evaluated as percent survival and mean survival time. As seen in Fig. 6, no significant effect was seen, even when the mice received daily intraperitoneal injections of 0.5 mg/kg PGD2 (p > 0.05).

Discussion

The results of the present study demonstrate that PGD2 had a growth-inhibitory effect on mouse glioma cells both in vitro and in vivo. Although a large number of studies on the action of PG's on a variety of tissues and cells have indicated that PG's usually alter intracellular concentrations of cyclic adenosine monophosphate (AMP), their precise mechanism of action is still unclear. On the other hand, PG's have been reported to affect the properties of cells, such as cell...
proliferation and tumor growth.\textsuperscript{7} Interpretation of their effect on cell proliferation is complicated by the observation that different types of PG's have opposite effects on the same systems, and that different concentrations of the same PG's may have opposite effects on a single system. Therefore, the state of knowledge of the role of PG's in cell proliferation and tumor growth is ambiguous.\textsuperscript{10}

Prostaglandin D\textsubscript{2} has been identified as a major PG in the brain, and the synthesis and degradation of PGD\textsubscript{2} have been investigated in detail.\textsuperscript{1,12,18,19,21-23} This PG has also been reported to control pulmonary metastasis of malignant melanoma cells, possibly through its influence on the formation of platelet-tumor emboli.\textsuperscript{3,20} Recently, PGD\textsubscript{2} has been found to inhibit DNA synthesis in human and murine leukemia,\textsuperscript{5} mouse mastocytoma,\textsuperscript{11} and human neuroblastoma.\textsuperscript{15} In this paper we also show that PGD\textsubscript{2} strongly inhibits DNA synthesis and cell proliferation of mouse glioma cells in vitro.

A large concentration of PGD\textsubscript{2} (more than 10 \textmu g/ml) caused the immediate death of glioma cells (Fig. 1 left). The same results were also obtained in T1 rat neurinoma cells (Fig. 2). On the other hand, 10.0 \textmu g/ml PGE\textsubscript{2} had no effect on cell growth (Fig. 1 right). These results are consistent with the findings of previous reports.\textsuperscript{4,6,11} Higashida, et al.\textsuperscript{6} reported that growth inhibition of mouse neuroblastoma cells by PGD\textsubscript{2} was almost equivalent to that shown by PGE\textsubscript{1}. Fukushima, et al.,\textsuperscript{5} demonstrated that the IC\textsubscript{50} (concentration needed for 50\% growth inhibition) of PGE\textsubscript{2} was almost equal to that of PGD\textsubscript{2}. Kawamura, et al.,\textsuperscript{11} reported the effects of PGD\textsubscript{2}, PGE\textsubscript{2}, and PGF\textsubscript{2a} on the growth of murine mastocytoma cells. They demonstrated that PGD\textsubscript{2} was the strongest inhibitor of mastocytoma cell growth, and that PGF\textsubscript{2a} had no effect on cell growth, even at concentrations above 50 \textmu g/ml.

We have found that treatment of glioma cells for more than 4 hours resulted in irreversible changes (Fig. 3). Glioma cells treated with 5.0 \textmu g/ml PGD\textsubscript{2} for more than 4 hours were not able to attach themselves to the plastic surface of culture plates, and floated free. Higashida, et al.,\textsuperscript{6} in a scanning and transmission electron microscopic examination, reported that treatment with PGD\textsubscript{2} resulted in the appearance of numerous blebs of various sizes along the cell surface and also in the destruction of surface membrane and of cytoplasmic organelles.

The in vivo effect of PGD\textsubscript{2} on glioma cells has not previously been reported. In our in vivo experiments, the choice of PGD\textsubscript{2} dose administered through intraperitoneal or subcutaneous injection was decided by referring to previous reports,\textsuperscript{4,6} in order to compare the glioma with other kinds of tumor cells. Higashida, et al.,\textsuperscript{6} showed that the tumor weight of N18TG-2 neuroblastoma inoculated subcutaneously on the backs of A/J mice was about 35\% to 70\% less than that of controls after 14 days of single daily intraperitoneal or subcutaneous injections of 0.5 to 1.0 mg/kg of PGD\textsubscript{2}. We have also demonstrated in this paper that the growth of established murine glioma inoculated subcutaneously in a syngeneic host was significantly inhibited by a single daily intraperitoneal injection of 0.5 mg/kg of PGD\textsubscript{2}, and that the growth-inhibitory effect became more dramatic after intratumoral injection of PGD\textsubscript{2} (Fig. 5). According to these results, the in vivo effect of PGD\textsubscript{2} seemed to be a direct cytotoxic action against tumor cells. Santoro, et al.,\textsuperscript{17} reported that subcutaneous administration of 5.0 \mu g of 16,16-dimethyl-PGE\textsubscript{2}-methyl ester significantly inhibited the growth of B16 mouse melanoma in vivo; however, we found that daily intraperitoneal or local injection of 0.5 mg/kg PGE\textsubscript{2} had no effect on the growth of glioma in our experimental system (unpublished data).

In our present investigation, intraperitoneal administration of PGD\textsubscript{2} failed to prolong the survival times of mice with intracranially transplanted glioma (Fig. 6). The reasons for this failure are not well understood. Although we speculate that PGD\textsubscript{2} might not permeate through the blood-brain barrier or that the intraperitoneal dose of PGD\textsubscript{2} might be insufficient, these problems have not been quantified. Another problem to be solved is the relationship to the endogenous PGD\textsubscript{2}. The physiological quantity of the endogenous PGD\textsubscript{2} produced by normal cells is thought to be less than 1 \textmu g/gm tissue. Therefore, the concentration of PGD\textsubscript{2} in our present study or in previous reports\textsuperscript{4,6,11,15} might not necessarily reflect the in vivo values. Although local administration of PGD\textsubscript{2} to mice with intracranially glioma transplantation is expected to be more effective, it is not feasible to administer PGD\textsubscript{2} intracranially every day because of the high risk of intracranial infection.

Fukushima, et al.,\textsuperscript{4} reported recently that a dehydrated derivative of PGD\textsubscript{2} had a growth-inhibitory effect on L1210 leukemia cells that was three times stronger than that of PGD\textsubscript{2}. This dehydrated compound will be investigated further in the future. Some recent reports have shown that the effect of PGD\textsubscript{2} is less cytotoxic on normal cells than on tumor cells;\textsuperscript{13} however, the precise mechanism of PGD\textsubscript{2} action is presently unknown. Further studies are required to clarify the different response mechanisms between benign and malignant cells.

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