Inhibition of B-16 Melanoma Growth in Vivo by a Synthetic Analog of Prostaglandin E₂

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SUMMARY

The effect of systemic administration of 16,16-dimethyl prostaglandin E₆-methyl ester (di-M-PGE₂) on the growth of B-16 melanoma tumors has been studied in C57BL/6J mice. Daily i.p. injection of 5 µg of di-M-PGE₂ commencing on the day of tumor inoculation with 10⁶ and 10⁷ viable cells delayed appearance of tumors; for the smaller tumor inoculum, it also increased median survival among treated mice from 23 to 33 days. Di-M-PGE₂ treatment of mice with established tumors caused significant inhibition of tumor growth, as measured by a number of parameters including tumor diameters and volumes. At the time of sacrifice, di-M-PGE₂-treated mice had tumors that were an average of 32% smaller (by weight), contained 60% fewer melanoma cells, and had higher concentrations of cyclic adenosine 3':5'-monophosphate (+225% and +100%, respectively).

INTRODUCTION

In previous experiments, we demonstrated that PGE's inhibit the growth rates of a number of tumor cell lines in vitro (11, 34). In addition, we recently correlated the effects of PGE's on the growth rates of B-16 mouse melanoma in vitro and in vivo (26). In these latter mouse studies, intratumor administration of di-M-PGE₂, a long-acting synthetic analog of PGE₂, resulted in profound inhibition of tumor growth. The current experiments were undertaken to evaluate the effectiveness of systemic di-M-PGE₂ in suppressing tumor growth and improving survival of mice inoculated with B-16 melanoma.

MATERIALS AND METHODS

Female C57BL/6J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine; di-M-PGE₂ was a generous gift from The Upjohn Co., Kalamazoo, Mich. Modified McCoy's Medium 5A with glutamine (219.2 µg/ml), penicillin, streptomycin, Hanks' balanced salt solution (100 units/ml), 0.2% streptomycin (0.1 mg/ml), and 0.1% Mycostatin (100 units/ml); and placed in modified McCoy's Medium 5A containing 15% heat-inactivated fetal calf serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). The washed tumors were finely minced, and cells were separated by passage through a 120 mesh stainless steel grid. The cell suspensions were washed twice with McCoy's medium containing penicillin and streptomycin, but not fetal calf serum. Under light ether anesthesia, 78 8-week-old female C57BL/6J mice were given s.c. injections in the left flank of 0.2 ml of dig-M-PGE₂ i.p. in a total of 0.1 ml of solvent. Control diluents were absolute ethanol (without prostaglandin) prepared identically.

Administration of Prostaglandins. Di-M-PGE₂ was dissolved in absolute ethanol (200 µg/ml) and maintained as a stock solution at 4°. The final concentration of prostaglandin for injection was made daily by diluting the ethanolic solution 1:4 in sterile 0.9% NaCl solution. Each mouse received 5 µg of di-M-PGE₂ i.p. in a total of 0.1 ml of solvent. Control diluents were absolute ethanol (without prostaglandin) prepared identically.

Measurement of Tumor Size. Each mouse was examined daily by palpation, and the day of appearance of the tumors was noted. When tumors became visible, they were measured in at least 2 dimensions with calipers. Tumor volumes were calculated as spheres if the major and minor diameters did not vary by more than 40%; if they did, volumes were calculated as cylinders (40% of the mice in both groups). Neither ulcerated nor necrotic tumors were utilized for any

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measurements of volume nor for subsequent determinations of weight and cell counting.

Cell Counting. At the completion of the experiments, the mice were weighed and then killed. The tumors were removed and weighed. Randomly chosen portions of the tumors (approximately 500 mg) used to count tumor cells were washed in McCoy's medium and minced; cells were separated and diluted as described above. Total cell numbers were determined by counting the cells in a hemocytometer. For each determination, duplicate counts of 5 different samples were performed and the means were determined; counts varied within a range of less than 10%. Histological examination (by Dr. J. Meyer, Department of Pathology, Washington University School of Medicine, St. Louis, Mo.) of the tumors from di-M-PGE2-treated and control mice revealed no significant differences in the number of inflammatory cells (27). Thus, in the current experiments, differences in total cell number were considered as valid (although imperfect) estimations of differences in tumor cell numbers.

Measurement of Concentrations of Cyclic Nucleotides. The remaining portions of the tumors harvested (the portions not used for cell counting) were frozen at −70°C. Approximately 300-mg random samples of each of 24 tumors (12 from di-M-PGE2-treated and 12 from control mice) were homogenized at 4°C in 1.5 ml of 5% trichloroacetic acid. Homogenates were centrifuged at 4000 rpm for 15 min at 4°C, and the aspirated supernatants were extracted 4 times with water-saturated diethyl ether (4:1, v/v). The aqueous phases were dried in air at 60°C, and the residues were dissolved in 0.05 M acetate buffer, pH 6.2, for radioimmunoanalysis. Concentrations of cAMP and cGMP were performed (by Dr. Charles Parker, Department of Immunology, Washington University School of Medicine, St. Louis, Mo.) with a well-characterized radioimmunoassay technique (30). Concentrations of cyclic nucleotides were expressed as pmoles/g of tumor and as pmoles/10⁶ cells.

Statistics. Statistical comparisons were performed with the use of a t test for unpaired data; p values of less than 0.05 were considered significant. Curves of rates of appearance of tumors were compared with the use of the Sign test (8), and a values of less than 0.05 were considered significant. Data are presented as means ± S.E.

RESULTS

The Rate of Appearance of Tumors. Nineteen mice received 1 × 10⁷ viable B-16 cells s.c. as described above and were randomized into control and di-M-PGE2 test groups (9 mice in the test and 10 mice in the control). Within 5 min after the inoculation with tumor cells, the test group received 5 µg of di-M-PGE2 i.p.; this systemic administration was identicaly repeated every day for up to 35 days, at which time all mice were either dead or had ulcerated tumors. The control mice received PGE-free diluent on an identical injection schedule. In the control group, the 1st tumor became palpable at 9 days after tumor injection, and by the 13th day all 10 of the control mice had developed palpable tumors (Chart 1, top). Four di-M-PGE2-treated mice developed tumors on the 11th postinjection day, and all mice had palpable tumors by the 17th day. At each time point, a higher percentage of control mice had tumors than did the treated animals. Although the differences were relatively small, with the use of the Sign test the rate of appearance of tumors in di-M-PGE2-treated mice was significantly (a < 0.004) slower than was the rate in control mice. A similar study (Chart 1, bottom) was performed with a tumor inoculum of 1 × 10⁷ viable B-16 cells. Daily injections of 5 µg of di-M-PGE2 (n = 10) and control diluent (n = 10) were continued until 35 days, at which time all the mice were either dead (80%) or had ulcerated tumors. Tumors were palpable in 4 control and 2 di-M-PGE2-treated mice on the 4th day after injection of tumor cells. By the 13th day, all control mice had palpable tumors, whereas it took 16 days for all di-M-PGE2-treated mice to develop tumors. As with the smaller inoculum, the rate of tumor appearance was slightly, but highly significantly, slower in the mice treated with di-M-PGE2 than in the controls (a < 0.001).

Survival. The 39 mice described above were followed until they either died or had ulcerated tumors. Survival rates plotted versus time are illustrated in Chart 2. The median survival times for the control animals that were inoculated with 10⁷ B-16 cells was 23 days; in the low-tumor-inoculum group, di-M-PGE2 treatment resulted in a prolongation of median survival to 33 days (a < 0.002).
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One-third of the di-M-PGE₂ treated mice survived for more than 39 days. In the mice that received 10⁸ B-16 cells, there was no significant difference between the animals that received di-M-PGE₂ (median survival, 23 days) and those that received control diluent (median survival, 18 days). By the 32nd day 80% of both groups of mice were dead, and the remaining 20% had ulcerated tumors.

Inhibition of Tumor Growth. For evaluation of the role of di-M-PGE₂ in inhibiting the growth rates of established tumors, an additional experiment was performed in which 39 mice were given injections of B-16 cells as above (19 with 10⁸ cells and 20 with 10⁹ cells). These 39 mice received no further injections until the 14th day, when they were randomly allocated to control and di-M-PGE₂ groups. Sixteen of the 19 mice that received 10⁸ B-16 cells had palpable tumors by Day 14, 9 of 10 allocated to the control group and 7 of 9 in the di-M-PGE₂ group. Mean tumor volumes were 71 ± 22 cu mm in the group allocated to di-M-PGE₂ and 87 ± 31 cu mm in the control group (p > 0.05). All 20 mice inoculated with 10⁹ B-16 cells had palpable tumors by Day 14. There was no significant difference in mean tumor volume among those randomized to the di-M-PGE₂ and control groups (336 ± 30 and 406 ± 72 cu mm, respectively; p > 0.05). Treated mice received 5 μg of di-M-PGE₂ i.p. daily, whereas the controls received an identical volume of diluent. Daily measurements of tumor diameters (and calculations of tumor volumes) were performed. Since under these experimental conditions considerable variation in measurements of tumor diameters and volumes can be expected, these experiments were designed to permit assessment of a number of other parameters of tumor growth, including tumor weights, cell densities, and total cell numbers.

Among mice inoculated with 10⁸ B-16 cells, at each daily time point the control group had a larger mean tumor size. However, no statistically significant differences in either tumor volumes or diameters were documented from Days 15 to 24, largely due to the difficulty in making accurate measurements of tumors of such small size. Seven of the 10 control mice survived for 25 days, as compared to only 20% shown in Chart 2 (top). This difference is consistent with experimental variation, particularly since Day 25 is at the rapidly changing portion of the survival curve, but another possible explanation is the fact that the animals were subjected to less mechanical manipulation, as they received no injections of solvent for the 1st 14 days. At Day 25, tumors in control mice had significantly larger volumes (1023 ± 140 cu mm) than did their di-M-PGE₂ treated counterparts (545 ± 123 cu mm; Table 1). On Day 25, all 14 surviving mice were weighed and killed, and their tumors were harvested. The control mice weighed an average of 18.5 ± 0.5 g; the di-M-PGE₂ treated mice weighed 21.0 ± 1.4 g, p > 0.05. Tumors from di-M-PGE₂-treated mice weighed significantly less (mean, 0.68 ± 0.13 g) than did those from control mice (0.86 ± 0.14; p < 0.05). When considered either as cells/g of tumor or as total cell numbers, cell counts of tumors from di-M-PGE₂ treated mice were significantly (60%) lower than those from controls.

Similar observations were recorded in the mice that received 10⁹ cells. At each time point after initiation of di-M-PGE₂ treatment, mean tumor volumes in the di-M-PGE₂ treated mice were significantly smaller than those from control animals (shown as volumes in Chart 3; data for diameters not shown). This difference persisted through Day 21, at which time the 12 mice were killed and the tumors were processed as above. There was no difference in the average tumor-free weights of the mice (control, 18.4 ± 1.7 g; di-M-PGE₂ treated, 17.1 ± 0.4 g; p > 0.05). As shown in Table 2, mean tumor volumes, mean diameters, numbers of cells per g of tumor, and total cell number were all significantly lower in the di-M-PGE₂-treated mice.

<table>
<thead>
<tr>
<th>Tumor parameters at 25 days after inoculation with 10⁸ B-16 cells</th>
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<tbody>
<tr>
<td>Each number represents the mean of 7 determinations. Test mice received di-M-PGE₂, 5 μg/day/mouse i.p., starting on the 14th day after tumor inoculation.</td>
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<tr>
<td>Tumor diam-&lt;ref&gt;er (mm)</td>
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<tr>
<td>-----------------</td>
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<tr>
<td>Control (n = 7)</td>
</tr>
<tr>
<td>Di-M-PGE₂ (n = 7)</td>
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<td>p</td>
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* Mean ± S.E.
Tumor parameters at 21 days after inoculation with $10^8$-16 cells. Each number represents the mean of 6 determinations. Test mice received di-M-PGE2 i.p., starting on the 14th day after tumor inoculation.

<table>
<thead>
<tr>
<th>Control (n = 6)</th>
<th>Di-M-PGE2 (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>Tumor diameter (mm)</td>
<td>14.22 ± 0.81</td>
</tr>
<tr>
<td>Tumor volume (cu mm)</td>
<td>1639 ± 342</td>
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<tr>
<td>Tumor wt (g)</td>
<td>1.62 ± 0.46</td>
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<tr>
<td>Cells/g of tumor (× 10^6)</td>
<td>230.6 ± 7.5</td>
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<tr>
<td>Total cell no. (× 10^6)</td>
<td>374.2 ± 105.4</td>
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$p < 0.01 < 0.05 > 0.05 < 0.05$

Concentrations of Cyclic Nucleotides. Concentrations of cAMP and cGMP were determined in 12 tumors from the control group, as well as in 12 from the di-M-PGE2-treated group. Since the cyclic nucleotide determination data for tumors derived from mice inoculated with $10^8$ and $10^6$ B-16 cells (6 pairs each) were similar, they were pooled.

Systemic administration of di-M-PGE2 resulted in increases in tumor concentrations of cAMP (Table 3). This difference was much more pronounced when the data were plotted as pmoles of cAMP per $10^6$ cells. The data for cGMP were more difficult to interpret; there was no difference in cGMP concentration per g of tissue, whereas di-M-PGE2 treatment significantly increased the cell content of cGMP (pmoles/10^6 cells). Systemic administration of di-M-PGE2 increased the mean ratio of cAMP to cGMP (control, 9.87 ± 1.67; di-M-PGE2 treated, 12.83 ± 1.29), but this difference was not statistically significant ($p > 0.05$).

**DISCUSSION**

These studies have documented the fact that systemic administration of a long-acting analog of PGE2 (di-M-PGE2) results in a slight but definite inhibition of B-16 melanoma growth in vivo. Since each of the parameters of tumor growth measured is subject to some variation, this conclusion is based on consideration of the consistency of the observations in 2 separate experiments, each of which involved 2 different doses of B-16 cells in the inocula; regardless of the parameter evaluated (time of onset of tumor, length of survival, tumor size, tumor weight, or cell count), di-M-PGE2 treatment was inhibitory. There is no reason to think that di-M-PGE2 induced changes only in the fluid content of the tumors; in the experiment initiated with $10^6$ B-16 cells, no change in the mean tumor density (mg/cu mm) was induced by di-M-PGE2, and yet this agent significantly inhibited several parameters of tumor growth, including tumor diameters, tumor volumes, cell densities, and total cell numbers. Finally, since tumors from both treated and control mice contained the same relative number of inflammatory cells and had similar histological ap-
pearances, the changes induced by di-M-PGE₂ probably represent changes primarily in tumor cell numbers. Since recent experiments have demonstrated that di-M-PGE₂ induces a significant decrease in mitotic index (27), we believe that the analog produces significant inhibition of B-16 growth and tumor cell proliferation in vivo. In evaluation of the mechanisms of this action, 3 possibilities must be considered, a direct effect, mediation by cyclic nucleotides, and the immune response.

A number of investigators have demonstrated that PGE compounds inhibit tumor cell proliferation in vitro (1, 18, 37). With a number of cell lines, including HeLa, Hep-2, L, B-16, and HT-29, our previous studies (11, 34) have documented significant inhibition of proliferation by exogenous PGE, at 1 μg/ml; liberation of endogenous prostaglandins by addition to the medium of dibutyryl cAMP (1 mM) had similar effects. Suppression of prostaglandin biosynthesis by 10⁻⁶ M indomethacin has consistently resulted in at least a 20% stimulation of cell replication, and this effect was reversed by addition to the medium of very small amounts of PGE₁ (10 ng/ml). These studies suggested that PGE compounds may have a direct effect on tumor cell proliferation. Our recent experiments have extended these observations by demonstrating an inhibitory effect on cell proliferation in vivo (26). Several investigators have reported the effects of systemically administered indomethacin on tumor size in tumor-bearing animals; the results of these experiments have been inconsistent. Humes et al. (13) reported that daily administration of indomethacin to BALB/c mice inoculated with Moloney sarcoma resulted in delayed onset of palpable tumors and inhibition of tumor size. Similar observations were reported by Tashjian and colleagues (33) in HSDMI, and by Plescia et al. (22) in MC 16 tumor-bearing mice. Sykes and Maddox (32) and Galasko and Bennett (9) reported no effect of indomethacin on the growth of BP8/P, and Walker sarcoma, respectively. Only Powles and associates (23) have described indomethacin-induced stimulation of tumor size, studying Walker sarcoma in Wistar rats. It is impossible to evaluate these data, since in all of the studies tumor size was measured, rather than numbers of cells being counted. Since indomethacin has such a potent antiinflammatory action, the smaller tumor sizes reported probably reflect only lesser degrees of inflammatory reaction and do not represent any influence on tumor cell proliferation in vivo.

cAMP is an important determinant of the rate of tumor cell proliferation. In our in vitro studies (11, 34), as well as in those of others (14, 36, 38), addition of dibutyryl-cAMP to media has caused significant slowing of cell replication. Furthermore, addition of PGE; stimulates adenylyl cyclase and thereby increases tumor cell concentrations of cAMP in Ehrlich ascites cells (4), chemically and virally transformed fibroblasts (20), L-929 cells (19), and neuroblastomas (24). Exogenous dibutyryl-cAMP also inhibits or even actually arrests tumor cell growth in vivo (5-7, 10, 16, 28). It is thus possible that the observed effects of di-M-PGE₂ on tumor growth were mediated by cyclic nucleotides. In accordance with the findings of Matsuzawa and Nirenberg (17), concentrations of both cAMP and cGMP were increased by administration of PGE, but the ratios of cAMP to cGMP tended to increase slightly.

A number of studies have demonstrated that PGE compounds are proinflammatory (3, 12, 35). In addition, PGE has been shown to be immunosuppressive (22, 25, 29, 31) and to prolong the survival of mouse skin allografts (2) and hamster-to-rat heart xenografts (15). Although the immune and inflammatory responses may be involved in the inhibition of tumor cell growth, the above observations suggest that they are not. Further work is necessary to evaluate the possible importance of these mechanisms.

REFERENCES

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Inhibition of B-16 Melanoma Growth \textit{in Vivo} by a Synthetic Analog of Prostaglandin E$_2$

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