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REQUIREMENT OF A REACTIVE α,β-UNSATURATED CARBONYL FOR INHIBITION OF TUMOR GROWTH AND INDUCTION OF DIFFERENTIATION BY "A" SERIES PROSTAGLANDINS

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SUMMARY. Prostaglandins of the A series have been reported to inhibit tumor cell growth and induce tumor cell differentiation by a yet unknown mechanism. We propose that these effects are due to the presence of a reactive α,β-unsaturated carbonyl group ($\Delta^{10,11}$) in the cyclopentane ring of the PGA molecule. PGA was effective whereas PGB (sterically hindered α,β-unsaturated carbonyl at $\Delta^{8,12}$) and PGA conjugated to glutathione were ineffective. 15-Epi-PGA_2 was as effective as PGA_2 suggesting that the S absolute configuration of the 15-hydroxyl group is not essential. There was no correlation between generation of cAMP and inhibition of cell proliferation or induction of differentiation by various prostaglandins. The data suggest that PGA's and PGA-like compounds inhibit tumor cell growth and induce differentation because of the chemical reactivity of the α,β-unsaturated carbonyl rather than hormonal activity of the prostanoid nucleus.

Prostaglandins of the A series (PGA's) were first shown to inhibit tumor growth in 1974 (1,2) and to induce tumor cell differentiation in 1979 (3). Subsequent studies have confirmed their antiproliferative effects (4,5). Although they compare favorably with standard cytotoxic chemotherapeutic agents in inhibition of tumor cell DNA synthesis (5) the mechanism(s) by which PGA's inhibit proliferation and induce differentiation is unknown.

A number of compounds containing α,β-unsaturated carbonyl groups exhibit antibacterial, antiviral, antifungal, and antitumor activity by virtue of their ability to react with cellular nucleophiles, e.g., sulfhydryl enzymes (7). PGA's contain this functionality, which may account for their antitumor activity (4). On the other hand, PGA's may act, via a receptor mediated mechanism, as hormonal agents to stimulate adenylate cyclase and regulate growth and differentiation. In the present study, we report that the antitumor effect of PGA's requires the presence of the α,β-unsaturated carbonyl function,

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is independent of the stereochemical demands of typical prostaglandin receptors, and is not mediated by cyclic adenosine monophosphate (cAMP).

MATERIALS AND METHODS

The B16 amelanotic melanoma (B16a) was originally obtained from the DCT Animal and Human Tumor Bank. Subcutaneous tumors were maintained in male syngeneic C57Bl/6J mice (Jackson Laboratories, Bar Harbor, ME). For studies of DNA synthesis (^{3}H-thymidine incorporation) subcutaneous B16a tumors were dispersed and tumor cells isolated by centrifugal elutriation as previously described (8). Elutriated tumor cells were > 95% viable (trypan blue dye exclusion) and contained < 3% host cell contamination. The effects of various prostaglandins on ^{3}H-thymidine incorporation during a four hour incubation were evaluated as described (5). The specific activity of the ^{3}H-thymidine (New England Nuclear, Boston, MA) was 80 Ci/mmol (1 Ci = 3.7 x 10^{10} Bq). For proliferation studies B16a cells were adapted for culture in Eagle's minimal essential medium (MEM) with 10% (v/v) fetal calf serum as previously described (9). Secondary cultures were plated (day 0) at a density of 4 x 10^4 in T-75 flasks. Culture media was changed daily from day 1 - day 4. Each day prostaglandins (dissolved in ethanol at a stock concentration of 10 mg/ml) or ethanol diluent (final concentration = 0.01%) were added to the cultures. On day 5 cells were removed with 0.25% (w/v) trypsin + 3 mM EDTA in Ca^{2+}, Mg^{2+} free MEM and counted with a Coulter counter (Model ZBI). For inactivation of the reactive α,β-unsaturated carbonyl group, PGA_2 or PGB_2 was incubated with reduced glutathione for 30 minutes as described by Cagen and Pisano (10). The PGA_2 or PGB_2 plus glutathione were then added to elutriated B16a cells in the presence of ^{3}H-thymidine. DNA synthesis was determined as described above. The effects of various prostaglandins on generation of cAMP by B16a cells was determined as previously described (9). Antisera to cAMP and ^{125}I-labeled cAMP was purchased from Collaborative Research (Waltham, MA).

The NBP_2 clone of a murine neuroblastoma line was obtained through the courtesy of Dr. Kedar Prasad, University of Colorado Medical Center, Denver, CO. The cells were cultured in Eagle's minimal essential medium with 10% (v/v) fetal calf serum. For differentiation studies, cells were plated (day 0) at 6.5 x 10^4 in T-75 flasks. Culture media was changed daily from day 1 - day 2. Each day prostaglandins (dissolved in ethanol at 10 mg/ml) or ethanol diluent (final concentration = 0.01%) were added to the flasks. On day 3 the cultures (200 cells/flask and 10 replicate flasks) were evaluated with a calibrated ocular micrometer. The criterion for differentiation was an axon length ≥ 10 μm.

Prostaglandins A_1, A_2, 15-epi-A_2, B_1, B_2, D_2, E_1, dimethyl E_1 and dimethyl E_2 were obtained through the courtesy of Dr. John Pike, The Upjohn Company, Kalamazoo, MI.

Each experiment was repeated at least three times with comparable results. Data was analyzed by Student's t-test, analysis of variance and the Kruskall-Wallis test.

RESULTS AND DISCUSSION

Prostaglandins of the A and B series possess α,β-unsaturated carbonyl groups in the cyclopentane ring. The structures of PGA_2 and PGB_2 are shown in Figure 1; PGA_1 and PGB_1 are similar in structure, lacking only the 5,6 double bond. The reactivity of the unsaturated carbonyl in the 10,11 position of PGA

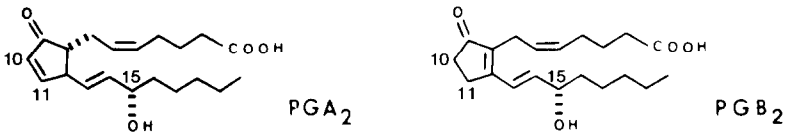

Figure 1. Molecular Structures of PGA_2 and PGB_2.

is greater than in the 8,12 position of PGB because of steric hindrance in the latter. Both PGA_1 and PGA_2 produced a dose dependent inhibition of B16a DNA synthesis over a dose range of 1-10 μg/ml, whereas PGB_1 and PGB_2 were essentially ineffective in the inhibition of DNA synthesis over the same dose range (Table 1). The α,β-unsaturated carbonyl function of PGA can be conjugated with reduced glutathione (10). We found that reaction of 25 μg/ml PGA_2 with glutathione in increasing concentrations (5-100 mM) progressively reduced the ability of PGA_2 to inhibit DNA synthesis by B16a cells (Figure 2). Incubation of glutathione (100 mM) with 25 μg/ml PGB_2 had no effect on the response to PGB_2 (Figure 2). These results confirm the importance of a reactive α,β-unsaturated carbonyl group for inhibition of B16a DNA synthesis by PGA's.

Jaffe and coworkers (11,12) reported inhibition of the growth *in vitro* and *in vivo* of B16 melanoma by the long-lived PGE_2 analogue, 16,16-dimethyl-PGE_2. They suggested that the inhibitory activities of this compound were due to its ability to stimulate tumor cell cAMP. Cyclic AMP has been proposed to inhibit proliferation of several tumor cell lines *in vitro* (13,14). PGA_1 and PGE_1 have

Table 1. Effects of A and B Series Prostaglandins on DNA Synthesis by B16 Amelanotic Melanoma Cells *In Vitro*

Concentration	PGA_1	PGA_2	PGB_1	PGB_2
1 μg/ml	91 ± 2[a]	84 ± 2	106 ± 3	104 ± 4
3 μg/ml	63 ± 2	50 ± 2	99 ± 2	102 ± 4
5 μg/ml	45 ± 1	20 ± 1	89 ± 2	94 ± 2
10 μg/ml	28 ± 1	9 ± 1	96 ± 1	89 ± 2

[a]Data expressed as percent of control ^{3}H-thymidine incorporation; Mean ± SEM, n = 4.

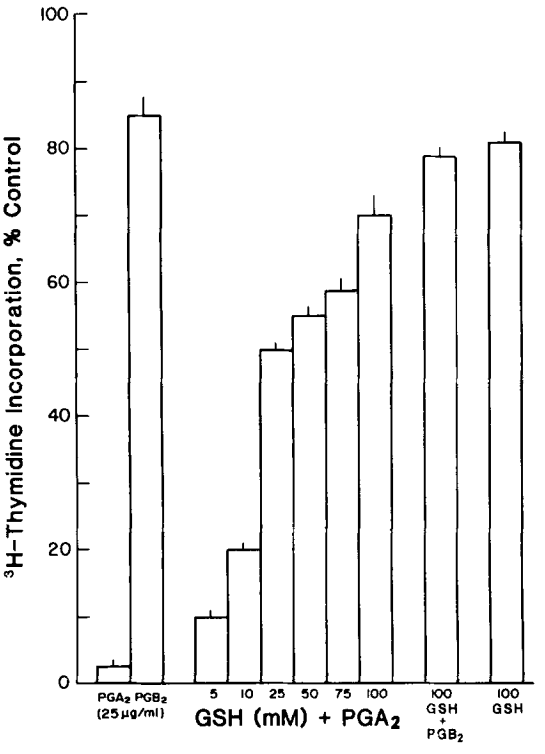

Figure 2. Inhibition of B16a Cell DNA Synthesis by PGA_2 and PGB_2 Following Preincubation with Reduced Glutathione.

been reported to have either stimulatory (15) or no effect (6) on tumor cell cAMP generation. We examined the effects in vitro of various prostaglandins on tumor cell proliferation and cAMP generation. Dimethyl PGE_1 and dimethyl PGE_2 at 5 µg/ml inhibited B16a cell proliferation by 80% and 75%, respectively, while increasing cAMP generation by 9000% and 1200%, respectively (Figure 3). In contrast, PGA_1 and PGA_2 at 5 µg/ml inhibited B16a cell proliferation by 88% and 92%, respectively, while only minimally affecting generation of cAMP relative to dimethyl PGE_1 and dimethyl PGE_2. PGB_2 at 2.5 and 5.0 µg/ml had no effect on B16a cell proliferation and a minimal effect on cAMP generation. PGD_2 has been shown to inhibit proliferation in vitro of murine and human tumor cells (16,17). PGD_2 at 5 µg/ml inhibited B16a cell proliferation by 70%, comparable to that by dimethyl PGE_2. However, PGD_2's stimulation of cAMP generation was only 1/3 that by dimethyl PGE_2. Thus in our study there does not appear to be any correlation, either qualitatively or quantitatively, between inhibition of tumor cell proliferation and stimulation of cAMP.

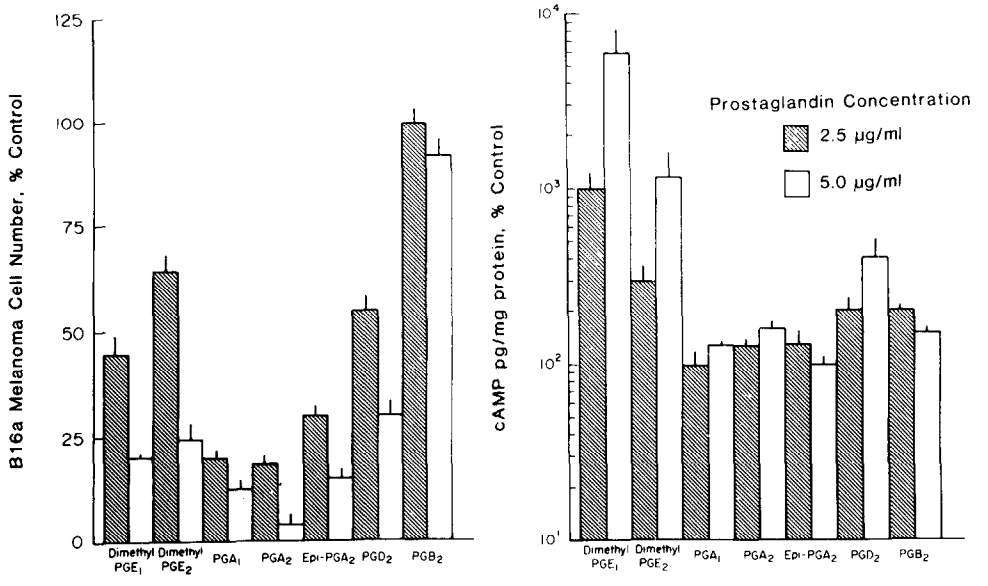

Figure 3. Comparison of the Effects of Various Prostaglandins on Cell Proliferation and the Generation of cAMP by B16a Cells.

To further substantiate the independence of PGA's inhibition of B16a cell proliferation and the generation of cAMP, we compared PGA_2 and 15-epi-PGA_2. The generation of cAMP by prostaglandins is believed to be receptor-mediated (18). Receptor binding requires the 15-hydroxyl group to be in the S position. 15-epi-PGA_2, in contrast to PGA_2, has the 15-hydroxyl group in the R position. 15-epi-PGA_2 (5 µg/ml) was ineffective in stimulating cAMP biosynthesis by B16a cells (Figure 3). However, 15-epi-PGA_2 (5 µg/ml) was only slightly less effective in inhibition of B16a cell proliferation (85% vs 92%). The above results demonstrate: 1. the importance of a reactive α,β-unsaturated carbonyl in the PGA molecule for inhibition of B16a cell proliferation, 2. the lack of a correlation between inhibition of B16a cell proliferation and generation of cAMP and 3. that stereospecificity of the 15-hydroxyl group in the PGA molecule is not required for inhibition of B16a cell proliferation.

PGE_1 has been demonstrated to induce differentiation of a murine neuroblastoma cell line, presumably through the generation of cAMP (19). We compared the effects of various prostaglandins on the induction of differentiation in the NBP_2 neuroblastoma clone. PGA_1, PGA_2 and 15-epi-PGA_2

Table 2. Effects of Prostaglandins on Neuroblastoma Cell Differentiation

Additive	(%)[a] Differentiated Cells
Solvent Control	6 ± 1[b]
PGA_1	74 ± 5
PGA_2	80 ± 8
Epi-PGA_2	73 ± 10
PGE_1	43 ± 7
PGB_1	6 ± 3
PGB_2	8 ± 1

[a]A total of 2 x 10^3 cells were measured for axon length (see Materials and Methods).

[b]Mean ± SEM.

were equipotent in the induction of axon formation (cellular differentiation) and approximately twice the potency of PGE_1 (Table 2). PGB_1 and PGB_2 did not stimulate cellular differentiation above the level found to occur spontaneously (Table 2). PGE_1 was a potent stimulator of NBP_2 cAMP levels, whereas PGA_1, A_2, 15-epi-A_2, B_1 and B_2 did not affect cAMP levels in this cell line (data not shown). Thus, in parallel with the proliferation studies these results suggest the importance of the reactive α,β-unsaturated carbonyl group for induction of differentiation by PGA's.

Recent reports have demonstrated that a metabolite (9-deoxy-$\Delta^{9,12}$ PGD_2) of PGD_2 is three times more potent then PGD_2 for inhibition of L-1210 cell proliferation (20). In fact, Fitzpatrick and Wynalda (21) have demonstrated that albumin can catalyze the dehydration of PGD_2 to three compounds, including 9-deoxy-$\Delta^{9,12}$ PGD_2, with conjugated α,β-unsaturated carbonyls. The present studies support our hypothesis (4) that the inhibition of tumor cell proliferation and induction of differentiation by A series prostaglandins is mediated by their reactive α,β-unsaturated carbonyl group. We further suggest that the antiproliferative effects of 9-deoxy-$\Delta^{9,12}$ PGD_2 are due to the presence of conjugated α,β-unsaturated carbonyls in this molecule.

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