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Authors
Bregman, M D
Meyskens, F L, Jr

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Inhibition of Human Malignant Melanoma Colony-forming Cells *in Vitro* by Prostaglandin A_1[1]

Marvin D. Bregman[2] and Frank L. Meyskens, Jr.

Department of Internal Medicine and the Cancer Center, University of Arizona, Tucson, Arizona 85724

ABSTRACT

The direct effect of continuous exposure to prostaglandins on the cloning efficiency and proliferative capacity of human malignant melanoma colony-forming cells in soft agar was evaluated. Prostaglandin A_1 (PGA_1) and prostaglandin E_1 (PGE_1) effected a dose-dependent inhibition of colony formation and proliferative capacity. PGA_1 at a concentration of 5 μg/ml reduced colony formation of cells from human melanoma cell strains C8054, C8130, and C822 by at least 85%. PGA_1 also inhibited colony formation of cells obtained directly from biopsies of melanoma tissues from eight patients by greater than 70% at a concentration of 5 μg/ml. A steep dose-response curve was evident by the little effect of PGA_1 on colony formation at a concentration of 0.5 μg/ml. The mean 50% inhibitory doses for PGA_1 and PGE_1 were 1.25 and 4.25 μg/ml, respectively. Prostaglandin A_2 was much less effective than PGA_1 in inhibiting melanoma colony formation. The related prostaglandins (prostaglandin B_1, prostaglandin $F_{1\alpha}$, and prostaglandin $E_{2\alpha}$) had little or no effect on colony formation. Overall, these results suggested that the presence of a carbonyl group at position 9 of the cyclopentane ring may be required for inhibitory activity as prostaglandins of the A and E series inhibited human melanoma cell growth.

PGA_1 and PGE_1 did not effect a rise in cyclic adenosine 3′:5′-monophosphate levels in C8054 and C8130 cells. However, while α-melanocyte-stimulating hormone and prostaglandin $F_{2\alpha}$ did generate a rise in adenosine 3′:5′-monophosphate levels in C8054 cells, these hormones had no effect on colony formation. These results are consistent with the notion that the PGA_1 and PGE_1 inhibition of melanoma colony-forming cells occurs via a noncyclic nucleotide mechanism.

INTRODUCTION

Human metastatic melanoma is a drug-resistant cancer. Our laboratory is utilizing a soft-agar system to screen for new anticancer agents against metastatic melanoma (11, 15), including biological modifiers such as retinoids (12). One group of compounds which has both decreased the metastatic frequency and inhibited the cellular growth of murine melanoma *in vitro* and *in vivo* was the prostaglandins (2, 6–8, 16, 17). We have shown recently that PGA_1[3] and PGE_1 were potent inhibitors of colony growth of CCL murine melanoma cells in soft agar (1). Consequently, we have examined the effects of prostaglandins on colony formation of human malignant melanoma cells, both from cell lines and from fresh biopsies of melanoma tissue. A range of inhibitory effects was produced by different prostaglandins; PGA_1 was a potent inhibitor of colony formation.

MATERIALS AND METHODS

Prostaglandins. Synthetic PGA_1, PGA_2, PGB_1, PGE_1, $PGF_{1\alpha}$, and $PGF_{2\alpha}$ were obtained from Sigma Chemical Co. (St. Louis, Mo.). The structure of these compounds is presented in Chart 1. PGA_1 was analyzed by glass capillary chromatography using the procedure of Fitzpatrick *et al.* (3). No detectable impurity was found. The prostaglandins were stored in absolute ethanol at a concentration of 10 mg/ml at −20°. These stocks were used within 30 days. All experiments were performed with the prostaglandins in continuous contact with the cells. α-MSH was obtained from Calbiochem-Behring Corp. (La Jolla, Calif.).

Preparation and Culture of Cells from Biopsies of Melanoma Tissue. Biopsies were obtained from patients with metastatic malignant melanoma in accord with a protocol approved by the University of Arizona Committee on Human Subjects. Clinical information about these patients is summarized in Table 1. The pathological diagnosis was histologically confirmed in all instances. Single-cell suspensions were prepared and cultured as described previously (11, 13). Several samples were plated fresh, but generally, we stored the cells in 10% dimethyl sulfoxide (Aldrich Chemical Co., Milwaukee, Wis.) under the vapor phase of liquid nitrogen. The cells were thawed and checked for their colony-forming ability, and those samples that yielded more than 100 colonies per 35-sq cm plate were chosen for study. The single cells were suspended in 0.3% agar in Ham's F-10 medium (Flow Laboratories, Santa Clara, Calif.) containing 10% heat-inactivated fetal calf serum (Grand Island Biological Co., Santa Clara, Calif.) to yield a final concentration of 0.5×10^6 cells per ml. One ml of this mixture was plated onto 1 ml of 0.5% agar, which also contained F-10 medium with 10% fetal calf serum and these additives: insulin (2 units); glutamine (0.8 μg); sodium pyruvate (0.4 μg); and β-mercaptoethanol (0.77 mM). The Petri dishes were incubated at 37° in a humidified atmosphere containing 5% CO_2 for 14 to 21 days. Plates were routinely monitored after plating for single-cell dispersion, and assays were performed in quadruplicate. Melanoma colonies from most of these patients expressed melanin pigmentation, which served as a marker of neoplastic origin of the colony-forming cell (10, 13). We routinely measured both the number of colonies (cloning efficiency) and the size of the colonies (proliferative capacity). The colonies were counted and grouped into size classes based on colony diameter utilizing an optical image analyzer (Omnicon Fas-II; Bausch and Lomb, New York, N. Y.) (9). Size classes of colonies were divided into the following groups: 60 to 104 μm; 104 to 149 μm; and greater than 149 μm (which contained 25 to 65 cells, 65 to 200 cells, and greater than 200 cells per colony, respectively).

Establishment and Culture of Melanoma Cell Lines. The single-cell suspensions obtained from the biopsies were plated for culture in agar for 14 days as described above. The agar was minced lightly and suspended in 100-sq cm Petri dishes containing 10 ml of Ham's F-10 with 10% fetal calf serum. After 2 additional weeks of culture, a proportion of the colonies grew out of the agar and were floating free as spheroids. Some of these spheroids obtained a diameter of 1000 μm. These spheroids were removed with a Pasteur pipet, broken into a

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: PGA_1, prostaglandin A_1; PGE_1, prostaglandin E_1; PGA_2, prostaglandin A_2; PGB_1, prostaglandin B_1; $PGF_{1\alpha}$, prostaglandin $F_{1\alpha}$; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; α-MSH, α-melanocyte-stimulating hormone; cAMP, cyclic adenosine 3′:5′-monophosphate; cGMP, cyclic guanosine 3′:5′-monophosphate; MTCFU, melanoma tumor colony-forming units; ID_{50}, 50% inhibitory dose; PGA, prostaglandin A; PGE, prostaglandin E; PGD_2, prostaglandin D_2; PGB, prostaglandin B; PGD, prostaglandin D.

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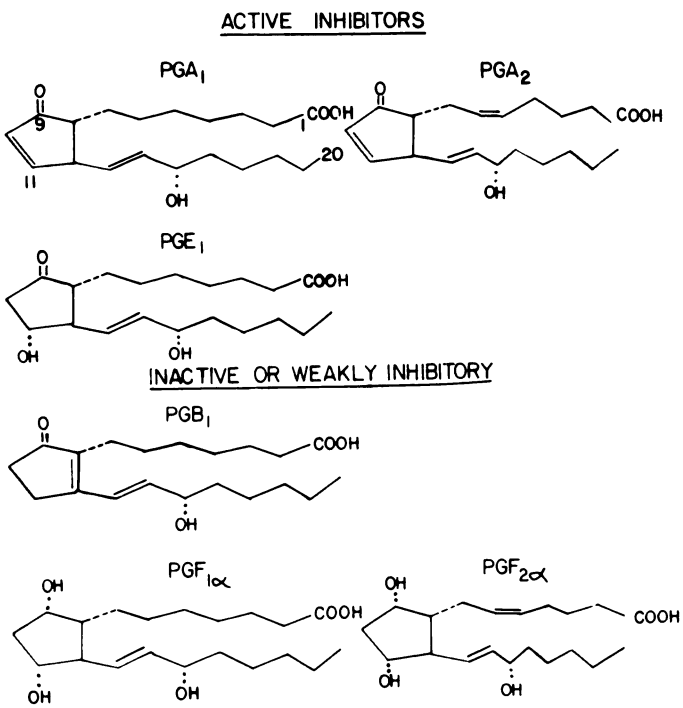

Chart 1. Structure of prostaglandins.

Table 1

Clinical data on human melanoma biopsies and cell lines

Patient	Source of tissue	Treatment prior to biopsy	Sex
Fresh cells			
81-13	s.c. nodule	DTIC[a]	M
81-26	s.c. nodule	DTIC, actinomycin D, procarbazine, bleomycin, *cis*-P, BCG, β-*trans*-retinol	M
81-30	Lymph node	Tamoxifen	F
81-32	Right arm Nodule	Actinomycin D, vinblastine Sulfate, tamoxifen, 13-*cis*-retinoic acid, DTIC	F
81-38	Chest wall	BIS	M
81-46c	s.c. nodules	Radiation + DTIC	F
81-56	Thigh nodule	BCG, β-*trans*-retinol	M
81-57	Supraclavicular node	No Rx	M
Cell strains			
C8146c[b]	Patient 81-46c		
C8130[b]	Patient 81-30		
C8054[b]	Axillary node	No Rx	M
C822	Axillary node	DTIC, BCNU	M

[a] DTIC, 5-(3,3-dimethyl)-1-triazeno)-2-methylimidazole-4-carboxamide; *cis*-P, *cis*-diamminedichloroplatinum(II); BCG, *Bacillus Calmette-Guérin*; BIS, 9,10-anthracened:carboxaldehyde-bis(4,5-dihydro-1H-imidazole-2-yl)dihydrochloride; Rx, treatment; BCNU, 1,3-bis-chloro-(2-chloroethyl)-1-nitrosourea.

[b] Tyrosinase activity present. Tyrosinase was measured as described previously (4, 5).

single-cell suspension, and then transferred to a flask for culturing. The cells were subcultured 4 times, and fractions were frozen for future use. The cells were used for a maximum of 10 subcultures. Cells from these strains were grown in F-10 medium containing 10% fetal calf serum. The soft agar assay procedure was the same as described above for cells from biopsies except that only 5,000 to 15,000 cells were plated, and no additional supplements other than 10% fetal calf serum were required for growth in agar. Colony formation was linear over this range of cell concentrations; cloning efficiency in agar of the cells from different melanoma strains ranged from 1.5 to 12%.

Cyclic Nucleotide Assay. The cAMP and cGMP levels were measured by incubating 100,000 single cells in 0.5 ml for 30 min at 37°. The samples were sonicated for 30 sec at 4° with a Wave Energy Systems Ultra Tip Probe (Wave Energy Systems, Newtown, Pa.) with a MC microtip attachment. Immediately, 250 μl of chilled 0.15 M sodium acetate buffer, pH 6.2, containing 3 mM theophylline were added. Aliquots (100 μl) were removed and assayed for cAMP and cGMP using radioimmunoassay kits from New England Nuclear (Boston, Mass.).

RESULTS AND DISCUSSION

The effect of continuous exposure to prostaglandins on human melanoma colony formation in soft agar was evaluated. Our initial experiments utilized cells from human melanoma cell strains. The effect of PGA_1, PGE_1, and $PGF_{2\alpha}$ on the cloning efficiency of cells from human melanoma strain C8130 is shown in Chart 2. PGA_1 inhibited colony formation in a dose-dependent manner. At a concentration of 5 μg/ml, colony formation was reduced to less than 1% of control. This effect was not secondary to cell death as 100% of the cells in colonies excluded trypan blue after 14 days in culture. We have tested previously the effects of PGE_1 on MTCFU of a murine melanoma cell line (1) and found that it was more potent than PGA_1 in inhibiting colony formation. However, PGE_1 was a much less effective inhibitor than PGA_1 against human MTCFU from this cell strain (Chart 2). PGA_1 at a concentration of 1.5 μg/ml produced an ID_{50} of MTCFU from human melanoma strain C8130. PGE_1 was 3-fold less potent in its inhibitory effect on these cells. Both $PGF_{1\alpha}$ and $PGF_{2\alpha}$ had no effect on MTCFU, suggesting that the inhibition by prostaglandins was highly specific. Similar results were also obtained with another human melanoma cell strain (C8054).

We also measured the effects of continuous exposure to prostaglandins on the proliferative capacity of MTCFU. The effect of PGA_1, PGE_1, and $PGF_{2\alpha}$ on colony sizes from cells of the C8130 cell strain is presented in Table 2. The inhibitory effect of PGA_1 and PGE_1 on colony size followed the same dose-response curve as that recorded for cloning efficiency. Only a few small colonies were formed in the presence of the higher concentrations of PGA_1 and PGE_1. These prostaglandins not only prevented the formation of colonies, but they also decreased the proliferative capacity of the few MTCFU that retained the ability to form colonies. The action of these prostaglandins may be cell cycle specific. Honn *et al.* (7, 8) found that murine melanoma DNA synthesis was rapidly inhibited by the addition of PGA_1 and PGE. These results are all consistent with the notion that PGA and PGE can effect a permanent block on the ability of melanoma

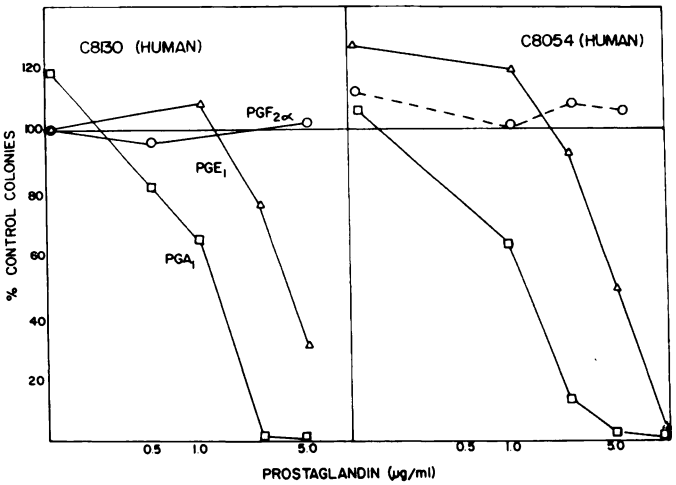

Chart 2. The effect of PGA_1, PGE_1, and $PGF_{2\alpha}$ on colony formation in soft agar of cells from human melanoma cell strains. Five thousand cells were plated in the bilayer soft agar system, and colonies >60 μm were counted on days 14 and 21. Day 14 controls had 636 ± 62 colonies/plate for C8054 and 442 ± 30 for C8130.

Table 2

Effect of continuous exposure to prostaglandins on colony formation from cells of human melanoma strain C8130

Five thousand cells were added with the plating layer to each dish. The plates were incubated at 37° in a humidified atmosphere containing 5% CO_2 for 20 days, and the colonies were quantitated and sized using an image analyzer (Omnicon Fas-II). Control plates yielded 422 colonies (60 μm), giving a plating efficiency of 8.4%. The distribution of colonies by size in the control plates was: colonies >60 to 104 μm, 162; >104 to 149 μm, 76; and >149 μm, 186. The experiment was repeated once with similar results.

Prostaglandin (μg/ml)	% of control colonies with colony diameter (μm) of			Total
	60–104	104–149	>149	
PGA				
5.0	1[a]	0	0	1
2.5	10	0	0	3
1.0	66	61	68	66
0.1	91	96	156	121
PGE_1				
5.0	50	48	14	31
2.5	50	113	59	76
1.0	83	89	138	109
0.1	68	77	135	100
$PGF_{2\alpha}$				
5.0	98	126	101	103
0.1	88	74	126	101

[a] Mean of 3 plates/experiment with S.D.s within ±15%.

Table 3

Effect of prostaglandins (10 μg/ml) on cyclic nucleotides of melanoma cells

Cell line	Prostaglandin	% of control	
		cAMP	cGMP
C8054	PGA_1	111[a]	106
	PGE_1	110	129
	$PGF_{2\alpha}$	147	106
	α-MSH	340	120
C8130	PGA_1	100	
	PGE_1	100	

[a] S.D., <7%.

cells to cycle. However, at lower concentrations (0.1 μg/ml), PGA_1 and PGE_1 produced a 56 and 36% increase, respectively, in the formation of colonies greater than 149 μm (Table 2), despite having no effect on the cloning efficiency. This result is not uncommon, as we have frequently observed that anticancer drugs at sublethal doses produce an increase in the proliferative capacity of MTCFU.[4] This suggests that obtaining a high concentration of PGA_1 at the tumor site *in vivo* would be an important parameter for the successful inhibition of growth *in vivo*.

The ability of prostaglandins to raise cyclic nucleotide levels in these cells was also evaluated. At a concentration (10 μg/ml) at which cloning efficiency of MTCFU was completely inhibited, PGA_1 and PGE_1 had no significant effect on cyclic nucleotide levels on cells from 2 human melanoma cell strains (Table 3). We have measured previously the cyclic nucleotide levels in murine melanoma and found that PGA_1, PGE_1, $PGF_{2\alpha}$, and α-MSH significantly raised cAMP levels within 30 min after the addition of hormone (1). The inability of PGA_1 and PGE_1 to produce an increase in cAMP levels indicates that these compounds inhibited human melanoma cloning efficiency and MTCFU proliferation through a noncyclic nucleotide mechanism. Similar conclusions have also been reached previously by workers evaluating the inhibitory effect of PGA_1 on DNA synthesis of murine melanoma cells (8). This conclusion is further supported by the observation that both α-MSH and $PGF_{2\alpha}$ raised cAMP levels in human C8054 cells (Table 3) without inhibiting colony formation.

A summary of the maximal inhibitory effect and ID_{50} of various prostaglandins on the cloning efficiency of cells from several human melanoma strains is presented in Table 4. PGA_1 was the most effective prostaglandin, having a mean ID_{50} of 1.36 μg/ml and inhibiting colony formation greater than 85% in all 5 cell strains tested. PGA_2 was much less inhibitory than PGA_1. PGA_2 has an additional double bond, and the reduced flexibility in position 5,6 may account for the large reduction in potency. PGE_1 was a slightly better inhibitor of human melanoma growth than PGA_2 but was 3-fold less potent than PGA_1. PGE_1 has a hydroxyl group in the cyclopentane ring in which PGA_1 has a double bond. In one cell strain (C8146c), PGB_1 inhibited cloning efficiency to a similar degree as PGA_2. However, in the other melanomas tested, PGB_1 had no effect. PGB_1 is similar in structure to PGA_1, except the double bond is at position 8,12 in the cyclopentane ring. These comparative studies suggest that the position of the double bond in the cyclopentane ring is an important determinant for both potency and activity. This study utilizing human melanoma cells supports the concept put forth by Honn and coworkers (8, 18) that the antitumor activity of the A series prostaglandin is due to the peculiar structure of the cyclopentane ring. Turner *et al.* (18) suggested recently the anticancer activity of PGA may be related to the presence of the α,β-unsaturated aldehyde group since the carcinostatic activity of citral, acrolein, and crotonaldehyde has been attributed to this group (14). However, this does not explain the anticancer activity of PGD_2 or PGE_1, which do not contain an unsaturated aldehyde group. PGE can be converted to PGA, and further study will be needed to ascertain if melanoma cells have the capability to dehydrate the hydroxyl group. $PGF_{1\alpha}$, which is similar in structure to PGE_1 except for a hydroxyl group at position 9, had no inhibitory effect on cloning efficiency. Overall, these results also suggest that the presence of a carbonyl group at position 9 of the cyclopentane ring along with a further modification at position 11 may be required for inhibitory activity against MTCFU. This study has focused on such prostaglandins as PGA, PGB, and PGE which contain a carbonyl group at position 9 and related the minor differences in structure to observed activity. We are currently studying those prostaglandins with a carbonyl group at position 11 and other modifications at position 9. In our initial experiments (Table 4), PGD_2 was found to be the most potent prostaglandin tested. We are currently testing both PGD_2 and synthetic derivatives of PGA and PGD on several human melanoma cell lines.

Cells in established cultures represent a selected subclone of

Table 4

Effect of continuous exposure to prostaglandins on the cloning efficiency of cells from human melanoma strains in soft agar

Reduction in colony formation was observed with 5 μg of the various prostaglandins per ml.

Prostaglandin	% of maximum inhibition				Mean (μg/ml)
	C8054	C8146c	C822	C8130	
PGA_1	99 (1.2)[a]	92 (1, 2)	88	99 (1.7)	1.36
PGA_2	26	85 (3.1)			
PGB_1		35 (7.0)	0	15	
PGD_2		99 (0.7)			
PGE_1	55 (4.0)			50 (4.5)	4.25
$PGF_{1\alpha}$	0	0		0	
$PGF_{2\alpha}$	0	0		0	

[a] Numbers in parentheses, ID_{50} (μg/ml).

[4] F. Meyskens and M. Bregman, unpublished observations.

cells of the original tumor. We therefore tested the effect of continuous exposure to prostaglandins on MTCFU obtained from biopsies of melanoma tissue from 8 patients. The effect of a continuous exposure to PGA_1 or $PGF_{2\alpha}$ on colony formation in agar is presented in Table 5. PGA_1 inhibited the growth of MTCFU from all 8 biopsies by greater than 70% at a concentration of 5 μg/ml (Table 5). At a concentration of 0.5 μg/ml, little effect was noted, suggesting a steep dose-response effect for PGA_1 on MTCFU growth. These results were similar to those obtained with cells from established cultures.

Our investigations suggest that PGA_1 may be an effective inhibitor of human malignant melanoma cell growth at high concentrations and that this inhibition occurs through a common and specific mechanism. The effect of increasing concentrations of PGA_1, PGA_2, and $PGF_{2\alpha}$ on MTCFU from Patient 81-30 is shown in Chart 3. The structure function relationships we had observed with cells from human melanoma cell strains were also noted for MTCFU from this tumor sample. PGA_1 was more potent than PGA_2, and $PGF_{1\alpha}$ was without inhibitory effect. Compared to the MTCFU of the M8146c cell line, the MTCFU of Patient 81-30 was very insensitive to inhibition by PGA_2, suggesting heterogeneity in the response of human MTCFU to PGA_2. However, the number of melanomas tested to date with PGA_2 is too small to derive a definite conclusion.

PGA_1 was strongly inhibitory to MTCFU growth in all human melanomas tested. We and others have demonstrated previously that PGA_1 also strongly inhibited the growth of murine melanoma cell lines (1, 7). The data presented in this paper suggest that further exploration of PGA_1 and related analogues as anticancer agents should be fruitful. Additionally, the effect of prostaglandins on other tumor types should be measured.

Table 5

Effect of continuous exposure to PGA_1 on colony formation in soft agar of MTCFU from fresh biopsies of melanoma tissue

The results are the average of quadruplicate determinations. Most of the patient studies were repeated and yielded similar results. The amount of colonies produced by the individual patient samples ranged from 55 to 880 colonies per plate. The average control experiment yielded 245 colonies per plate with an average S.E. of 8.5%.

	% of control colonies		
	PGA_1		
Patient	5 μg/ml	0.5 μg/ml	$PGF_{2\alpha}$ (5 μg/ml)
Fresh			
81-30	5 ± 0.5[a]		70 ± 7
81-32	4 ± 0.5		72 ± 10
Cryopreserved			
81-30	15 ± 6	112 ± 20	128 ± 4[b]
80-56	1 ± 0.5	65 ± 5	135 ± 5
81-13	5 ± 2	95 ± 3	112 ± 11
81-26	13 ± 2	75 ± 9	98 ± 1
81-46c	30 ± 10		70 ± 5
81-38	30 ± 2		98 ± 7[c]
81-57	1 ± 0.5	52 ± 2	68 ± 3[c]

[a] Mean ± S.E.
[b] $PGF_{1\alpha}$ and $PGF_{2\alpha}$ gave the same response.
[c] $PGF_{1\alpha}$ was used with these patients.

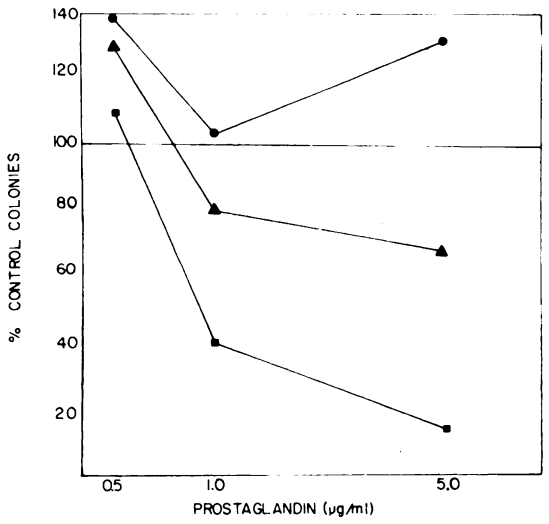

Chart 3. The effect of PGA_1 (■), PGA_2 (▲), and $PGF_{1\alpha}$ (●) on MCTFU of Patient 81-30. Cells (500,000) were plated in the bilayer soft agar system, and colonies >60 μm were counted on Day 14. Control had 416 ± 32 colonies >60 μm.

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