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### Authors

Bregman, M D
Sander, D
Meyskens, F L, Jr



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# PROSTAGLANDIN $A_1$ AND $E_1$ INHIBIT THE PLATING EFFICIENCY AND PROLIFERATION OF MURINE MELANOMA CELLS (CLOUDMAN S-91) IN SOFT AGAR

Marvin D. Bregman[1], Diane Sander and
Frank L. Meyskens, Jr.

Department of Internal Medicine and the Cancer Center
University of Arizona 85721

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SUMMARY: $PGA_1$ and $PGE_1$ reduced the plating efficiency and inhibited proliferation of Cloudman S-91 murine melanoma cells in a dose dependent manner, as assessed by their effects on colony formation in soft agar. $PGF_{2\alpha}$ did not reduce plating efficiency but was as effective as $PGA_1$ in raising cAMP and cGMP levels. This data suggests that the inhibition of Cloudman S-91 murine melanoma cell growth occurs via a non-cyclic nucleotide mechanism.

Melanocyte stimulating hormone (α-MSH) is an established target hormone for murine melanoma cells (1). Extensive studies using a Cloudman S-91 melanoma cell line have shown that α-MSH acts directly on adenylate cyclase (2,3) to effect an increase in tyrosinase activity (4). This stimulation of melanogenesis is associated with an inhibition in the growth of melanoma cells in monolayer culture (5). However, this relationship does not hold *in vivo*. While α-MSH does act on the transplanted tumor to cause an increase in melanogenesis, there is no significant inhibition of tumor growth (1,6). This suggests that results obtained in monolayer culture may not be a good predictor of the *in vivo* response. Recently, prostaglandins (PGs) have been shown to modulate cellular proliferation; studies in the murine B-16 melanoma cell lines have shown that $PGA_1$ and $PGE_1$ are potent inhibitors of DNA synthesis in these cells (7).

In this investigation we have evaluated the effect of these hormones on melanoma colony forming units (MCFU) in soft agar and found that α-MSH mimics the *in vivo* response and produces increased pigmentation of the colony without affecting colony formation. In this system $PGA_1$ and $PGE_1$ completely inhibited

[1]To whom correspondence and reprint requests should be sent.

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colony formation while $PGF_{2\alpha}$ had no effect and yet both $PGA_1$ and $PGF_{2\alpha}$ raised cGMP and cAMP levels to a similar degree.

## MATERIALS AND METHODS

Materials: α-MSH was synthesized in the laboratory of Dr. Victor Hruby, Department of Chemistry, University of Arizona as previously described (8). $PGA_1$, $PGE_1$, $PGF_{2\alpha}$, and theophylline were obtained from Sigma Chemical Company. [$^3$H] Tyrosine (sp. act. 48 Ci/mol) and radioimmune assay kits for cAMP and cGMP were obtained from New England Nuclear. Ham's F-10 nutrient medium was obtained from Flow Laboratories and fetal calf and horse serum were purchased from GIBCO.

Cloudman S-91 3960 (CCL 53.1) melanoma cells were cultured and assayed for tyrosinase activity as previously described (9). The bilayer soft agar system has been used to grow tumor cells from biopsies of melanoma (10) and has been used in this laboratory (11) to measure the effect of retinoids (12) and chemotherapeutic agents (13) on MCFU. The protocols for this assay can be found in references 10 to 13. We routinely measure both the number of colonies (cloning efficiency) and size of colonies (proliferative capacity) in this assay. The colonies were counted and grouped into size classes based on colony diameter utilizing the Omnicon FAS-II (Bausch and Lomb, New York), an optical image analyzer (14). The cAMP and cGMP levels were measured by incubating 100,000 cells/0.5 ml in fresh serum-free media for 20 to 30 minutes at 37°C. The samples were sonicated at 4°C with a Wave Energy Systems Ultra Tip Probe with a MC Microtip attachment for 30 seconds. Immediately 250 µl of chilled 0.15M NaAc buffer, pH 6.2, containing 3M theophylline was added. 100 µl aliquots were removed and assayed for cAMP and cGMP using radioimmune assay kits from New England Nuclear.

## RESULTS AND DISCUSSION

DNA synthesis in B-16 melanoma cells was previously reported to be inhibited by $PGA_1$ and $PGE_1$ (7, 15). We evaluated the effect of these prostaglandins (PGs) and $PGF_{2\alpha}$ on the *in vitro* growth of Cloudman S-91 melanoma. $PGF_{2\alpha}$ did not inhibit either the number or size distribution of the MCFU over the entire concentration range (Fig. 1). In contrast, $PGA_1$ and $PGE_1$ inhibited melanoma colony formation in a dose dependent manner at concentrations as low as 0.5 µg/ml. Thus, PG reduction of cloning efficiency displayed a high degree of specificity as the differences in molecular structure among $PGA_1$, $PGE_1$ and $PGF_{2\alpha}$ are very small. The inhibition of colony formation was not dependent on the time of the cells in agar as similar colony survival on days 10, 16 and 21 was observed (data not shown). However, while the number of colonies on the control plates did not significantly increase with time in agar, the size of the colony did, a measure of cellular proliferative capacity. This indicates that the cells continually grew during the course of the experiment.

In addition to the effect on the cloning efficiency these two PGs altered the size distribution of the MCFU (Table 1). For example, at a concentration of

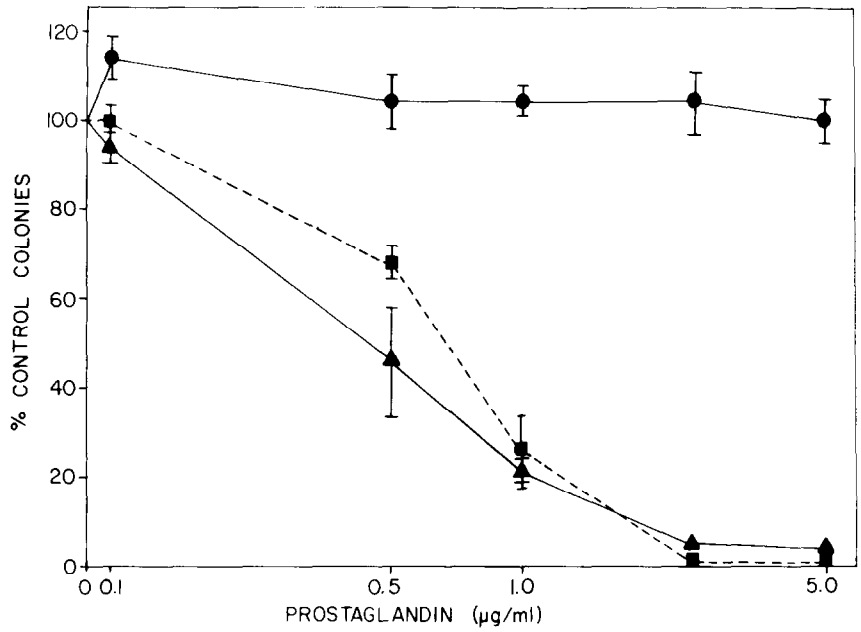


Figure 1. The effect of $PGA_1$ (■ - - - ■), $PGE_1$ (▲——▲) and $PGF_{2\alpha}$ (●——●) on Cloudman S-91 colony formation in agar. 5000 cells were plated in the bilayer soft agar system and colonies > 60 µm were counted on day 10. Controls had 2575 ± 250 colonies/plate.

0.5 µg/ml, $PGA_1$ caused a 30% reduction in the number of MCFU (Fig. 1). If the hormone had no effect on the proliferative capacity of these colony forming cells, then one would have expected to obtain the same proportionate size distribution of colonies. This was not the case. For example, plates treated with 0.5 µg/ml of PGA contained only 60% of the expected number of colonies greater than 104 microns in diameter. Thus, PGs altered not only the ability of these cells to form colonies, but also decreased the proliferative capacity of the colony forming cells.

The effect of PGs on cAMP and cGMP levels was evaluated. $PGE_1$ was a very potent stimulator of both cAMP and cGMP levels (Table II). $PGF_{2\alpha}$ and $PGA_1$ were much less effective than $PGA_1$ in raising cAMP and cGMP levels. It is important to note that even though $PGE_1$ generated a 70-fold increase in cAMP levels it was only slightly more inhibitory than $PGA_1$ on colony formation. Also, even though $PGF_{2\alpha}$ was more effective than $PGA_1$ in raising the levels of cAMP and cGMP, it had no inhibitory effect on the in vitro growth of melanoma in soft agar. These results suggest that $PGA_1$ and $PGE_1$ inhibited S-91 melanoma colony growth via a non-cyclic

Table I. Effect of Prostaglandins on Proliferation of Cells in Colonies[a]

| Compound | Colony Diameter (microns) | | |
|---|---|---|---|
| | 60 to 104 | 104 to 149 | >149 |
| | % Control | | |
| $PGA_1$ µg/ml | | | |
| 5.0 | 0 | 0 | 0 |
| 2.5 | 0 | 0 | 0 |
| 1.0 | 41 | 3 | 0 |
| 0.5 | 84 | 46 | 34 |
| $PGE_1$ µg/ml | | | |
| 5.0 | 2 | 0 | 0 |
| 2.5 | 4 | 0 | 0 |
| 1.0 | 35 | 2 | 0 |
| 0.5 | 68 | 10 | 0 |
| $PGF_{2\alpha}$ µg/ml[b] | | | |
| 0.1 to 5.0 | 109 | 110 | 100 |
| α-MSH | | | |
| $10^{-7}$M | 162 | 55 | 50 |
| Indomethacin | | | |
| 1 µM | 99 | 93 | 117 |

[a]Five thousand S-91 cells were added with the plating layer to each plate. Control plates yielded 2575 colonies > 60 µm, yielding a plating efficiency of 51%. The distribution of colonies by size in control plates was: colonies > 60 < 104 µm, 1582; > 104 < 149 µm, 940; and >149µm, 59. The results represent the mean of 3 plates per experiment and the S.D. were within ± 10%. The plates were incubated at 37°C in a humidified atmosphere containing 6% $CO_2$ for 10 days and the colonies quantitated and sized using an Omnicon FAS-II.

[b]There was no significant difference in the number and size of the colonies formed in the presence of $PGF_{2\alpha}$ over this concentration range.

nucleotide mechanism. $PGA_1$ and $PGE_1$ at a concentration of 5 µg/ml effected an 80% reduction in the growth of these cells in monolayer tissue culture (data not shown). $PGF_{2\alpha}$ had no effect. This inhibition was not due to cytotoxicity since 90% of the cells were viable as determined by the ability to exclude trypan blue.

We compared the effects of PGs to MSH, a polypeptide hormone known to stimulate melanogenesis via a cAMP mediated mechanism (1). α-MSH had no effect on the number of MCFU. Wick (16) has also found that α-MSH inhibited the growth of S-91B melanoma (a cell line that does not produce pigment in the presence of MSH) on plastic but had no significant effect on the number of colonies formed in

Table II. Effect of Prostaglandins on Cyclic Nucleotide Levels

| | | Percentage of Cyclic Nucleotide Compared to Control | |
|---|---|---|---|
| | | cAMP | cGMP |
| $PGA_1$ | 10μg/ml | 200% | 113% |
| $PGE_1$ | 10μg/ml | 7000% | 1067% |
| $PGF_{2\alpha}$ | 10μg/ml | 575% | 191% |
| α-MSH | $10^{-7}$M | 250% | ---- |

Each value represents the mean of triplicate experiments with a S.D. ± 15%. The basal level for cAMP was 6.0 pmol/$10^6$ cells and for cGMP it was 8.0 pmol/$10^6$ cells.

agar. We have extended these observations by measuring the effects of MSH on the proliferative capacity of the colony forming cells and noted changes in melanogenesis. In the present study α-MSH produced an increase in melanogenesis as was assessed by the dark brown appearance of the colonies versus the light brown colonies in the control plates. α-MSH also had an effect on the proliferative capacity of these colony forming cells. There was a decrease in the number of large colonies formed with a concomitant increase in the number of small colonies (Table I). Thus, unlike PGs, α-MSH had no effect on cloning efficiency of these melanoma cells in agar and the decrease in the proliferative capacity was less than that obtained with either $PGA_1$ or $PGE_1$ (Table I).

The mechanism by which $PGA_1$ and $PGE_1$ inhibit melanoma proliferation is not known. Honn et al. (7) found that $PGA_1$ inhibition of B-16 melanoma DNA synthesis was not associated with any effect on cAMP levels. Our results show that elevation of cyclic nucleotide levels does not correlate with the inhibitory effects of these PGs on colony formation. An alternative explanation for these results is that $PGA_1$ and $PGE_1$ enter the cell and directly influence genetic expression. This suggestion is supported by the observations of Bito (17,18) that some tissues selectively and actively transport PGs across membranes. It is not known whether PGs are actively or passively transported into melanoma cells. The possibility that PGs inhibit cell growth by non-specifically incorporating

into the plasma membrane seems unlikely as $PGF_{2\alpha}$ does not affect melanoma proliferation even at high concentrations.

Our investigation indicates that $PGA_1$ and $PGE_1$ are potent inhibitors of murine melanoma cell growth, inhibiting both plating efficiency and cellular proliferation in agar as well as cell growth in monolayer culture. Complete inhibition of colony growth in agar can be achieved by exposing these cells to 2.5 µg/ml of $PGA_1$ or $PGE_1$. We are presently investigating the minimum exposure time needed to bring about this inhibitory response. Favalli et al. (19) recently reported that when mice were given a single injection (10 µg/kg) of $PGA_1$ per day, there was a significant reduction in tumor size. These results suggest that $PGE_1$, and especially $PGA_1$ because of its lower toxicity should be tested against human melanoma cells. We have recently developed a soft agar assay system which will allow us to determine potential chemotherapeutic potential of these compounds (11,20).

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