MEASUREMENT OF INITIAL PROSTAGLANDIN F METABOLITES IN MEDIUM OF BALB/c 3T3 AND SV3T3 MOUSE FIBROBLAST CULTURES

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Summary

The initial metabolites of prostaglandin $F_{1\alpha}$ and $F_{2\alpha}$ (13,14,dihydro-15-keto-prostaglandin $F_{1\alpha}$, 13,14,dihydro-15-keto-prostaglandin $F_{2\alpha}$, and 9α,11α, 15-trihydroxy prostanoic acid [$F_{0\alpha}$]) have been measured in culture medium of BALB/c 3T3 and SV3T3 mouse fibroblasts employing a newly developed radioimmunoassay for these metabolites. The levels of metabolite detected in culture medium were 5-17 percent of the primary F prostaglandin level, and therefore suggest that the level of prostaglandin F metabolism is low in these cultures as compared with the level of metabolism detected in humans infused with prostaglandin $F_{2\alpha}$. It was further noted that metabolite levels and prostaglandin F levels were proportional for these two cell lines demonstrating that metabolism of prostaglandin F occurs to the same degree in these two cell lines. This result supports the contention that differences in the prostaglandin F concentration of growth medium from 3T3 and SV3T3 cells arise from differences in prostaglandin production or release and are not due to differences in the degree of metabolism which occurs during culture of these two cell lines.

Introduction

Employing "normal" and viral transformed cells as model systems for the study of prostaglandin (PG) levels as a function of differences in cellular growth control, it was noted that the levels of E and F prostaglandins detected in culture medium were higher for polyoma transformed BHK cells than for "normal" BHK cells (1). A similar result was obtained when the concentration of primary prostaglandins in the medium of BALB/c 3T3 cells was compared with the concentration detected in a stable Simian Virus 40 transformant subline, 11A-8 (2). Since work on the infusion of $PGF_{2\alpha}$ in humans has indicated that metabolism occurs rapidly and that the 15-keto metabolite

Abbreviations used: PG = prostaglandin; BHK = baby hamster kidney; BALB/c 3T3 = permanent mouse embryo cell line; SV40 = Simian Virus 40; SV3T3 = a Simian Virus 40 transformed subline of BALB/c 3T3; $PGF_{1\alpha}$ = 9α,11α,15(S)-trihydroxy-13-trans-prostenoic acid $PGF_{2\alpha}$ = 9α,11α,15(S) trihydroxy-5-CIS-13-trans prosatrienoic acid, PGE_1 = 11α,15(S) dihydroxy-9-OXO-13-trans-prostenoic acid, PGF metabolite = the sum of 9α,11α-dihydroxy-15-keto-prostanoic acid, 9α,11α,dihydroxy-15-keto-prost-5-enoic acid, and 9α,11α,15-trihydroxy prostanoic.

of $PGF_{2\alpha}$ may be present in human plasma at concentrations 20 to 30 times greater than the concentration of $PGF_{2\alpha}$ administered (3,4), a newly developed radioimmunoassay for the measurement of initial F prostaglandin metabolites was utilized to assess the relative levels of primary F prostaglandins and initial F metabolites in culture medium of 3T3 and SV3T3 cells. This study was designed to assess the level of PGF metabolism occurring in these cultures, while it provides for a comparison of PGF metabolism by cell lines which differ markedly in their mode of cellular growth control and in the concentration of primary prostaglandin detected in their medium (2). The existance of recently developed prostaglandin F metabolite assays (4,5,6) provides the immunological specificity to measure metabolite levels under normal culture conditions.

Methods and Materials

Maintenance of cell lines. BALB/c 3T3 clone A31, an established line of mouse fibroblasts (7), and 11A-8, a stable SV40 transformant of clone A31 (8) were obtained from Dr. Harvey L. Ozer. Cells were grown on 100 mm plastic dishes (Falcon) at 38.5°C. Growth medium was supplemented with 10% calf serum and conditions for cell growth and passage were maintained as previously described (9).

Collection of growth medium from cell cultures for PGF and PGF metabolite determinations.

BALB/c 3T3 cells and their stable SV40 transformant subline 11A-8 were inoculated at ~7.0 X 10^4 cells/100 mm dish. Two days after cell inoculation, the medium was changed for both cell lines (20 ml per culture). At 4 days and at every 2 day interval up to and including day 16, samples were collected and the growth medium was changed on all cultures. At each time point, the culture medium from 3 cultures was pooled (60 ml) and two 4.0 ml aliquots were pipetted directly into extraction buffer (pH 4.5) and rapidly frozen in a dry ice-methanol bath. Samples were then stored in a freezer at -20°C until all samples were ready for extraction. Medium was incubated in the absence of cells and 2 day collections of incubated medium were stored as controls. Cell number per plate was determined at each collection time as previously described (2).

Preparation of samples for radioimmunoassay.

Samples in an equal volume of extraction buffer (1M acetate buffer, pH 4.5, containing 9% saturated ammonium sulfate) were prepared for extraction by the addition of 1550-1900 DPM of [^{3}H] $PGF_{2\alpha}$ or [^{3}H]9α,11α-dihydroxy-15-keto-prostanoic acid. These labeled compounds were added for determination of recoveries. Samples were extracted with ethyl acetate, evaporated under nitrogen at 40-50°C, and partitioned between 80% methanol and hexane to remove neutral lipids. The details of extraction and neutral lipid separation have been previously described (2). Since anti-F metabolite serum

does not crossreact with any of the primary prostaglandins (5), the methanol fraction of the set of samples for F metabolite determination was evaporated under nitrogen following neutral lipid separation and redissolved in 1.0 ml of radioimmunoassay buffer (0.01M phosphate buffer pH 7.0, 0.15 M sodium chloride, 1.0% normal rabbit serum, 0.05% sodium azide). The set of samples employed for primary F prostaglandin determination was further purified by chromatography on silicic acid columns (2). The PGF column fraction was evaporated under nitrogen and redissolved in 1.0 ml of radioimmunoassay buffer. An aliquot of the samples was counted in a liquid scintillation counter to determine percent recovery for PGF and PGF metabolite (9α,11α-dihydroxy-15-keto-prostanoic acid). The recoveries were ∿80% for both PGF and PGF metabolite.

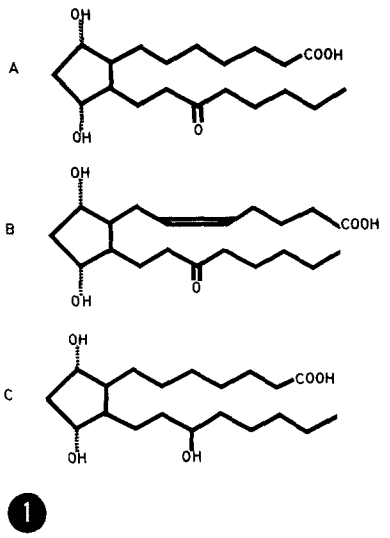
Radioimmunoassay of prostaglandins.

Prostaglandin measurements were conducted as previously described on the PGF column fraction employing an antiserum directed against $PGF_{2\alpha}$ and having 80% crossreactivity with $PGF_{1\alpha}$ (10). This serum provides a good indication of total F prostaglandins present in a sample. The second duplicate of samples which were extracted for PGF metabolite determination were assayed with a newly developed assay for the initial metabolites of the F prostaglandins (5). This antiserum recognizes the initial F metabolites in man as reported by Granström and Samuelsson (11,3). The compounds capable of contributing to the measured F metabolite level are presented in figure 1. Compounds A and B, the 15 keto-13,14-dihydroderivatives of $PGF_{1\alpha}$ and $PGF_{2\alpha}$, are initial PGF metabolites which may arise through the action of 15-hydroxy prostaglandin dehydrogenase and prostaglandin-Δ^{13} reductase (12), while compound C is the result of an additional metabolic step producing 9α,11α,15-trihydroxy prostanoic acid. The measurement of these three compounds is used as an assessment of PGF metabolism.

The accuracy of the PGF metabolite radioimmunoassay has been previously tested with known amounts of 9α,11α,dihydroxy-15 keto-prostanoic acid added to the assay system (5). The assay has a coefficient of correlation of 0.985, 95% confidence limits of 0.914 - 1.02, and a lower boundary on sensitivity of 15 pgm. The prostaglandin F assay has also been assessed for accuracy and found to have a coefficient of correlation of 0.993, 95% confidence limits of 0.945 - 1.09, and a lower boundary on sensitivity of 50 pgm. For both assays, all samples were processed simultaneously and assayed at one time to eliminate any possible intra-assay variation.

Results and Discussion

Growth curves for the 2 cell lines studied are presented in figure 2 as a semi-logarithmic plot of cell number per plate versus time after inoculation of cells into culture. The relative growth curves for these two cell lines make evident the fact that cell growth proceeded logarithmically until both cell lines exhibited a decline in growth rate at day 12 as they reached their saturation density. The normal BALB/c 3T3 (clone A31) cells reached a density of 4.0×10^6 cells/100 mm dish while the transformant subline, 11A-8 reached a density of 5.4×10^7 cells/100 mm dish. The saturation density of the SV40 transformant exceeded that of the parent clone A31 cells by more



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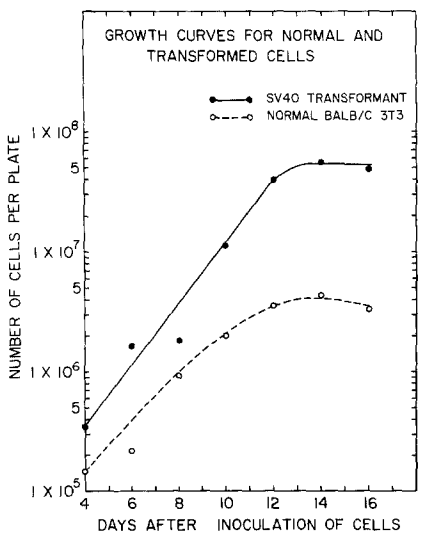

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Figure 1. Initial metabolites of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ measured with anti-F metabolite serum. The three metabolites detected are 9α,11α-dihydroxy-15-keto-prostanoic acid (A), 9α,11α-dihydroxy-15-keto-prost-5-enoic acid (B), and 9α,11α,15-trihydroxy prostanoic acid (C).

Figure 2. Comparative cellular growth curves for BALB/c 3T3 and SV3T3 cells from day 4 to day 16. Each two day point represents a time of medium harvest for prostaglandin determination. Open circles (o——o) represent normal cells and closed circles (•——•) represent transformed cells.

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than 10 fold. This ability of viral transformed cells to grow multilayered in culture reaching higher cell saturation densities is a characteristic of stable viral transformants (13) which clearly demonstrates its altered control of cell growth.

The 2 day medium collections for 3T3 cells, SV3T3 cells, and plates containing medium in the absence of cells were obtained at each time point presented in figure 2 and assayed to determine the level of primary F prostaglandins and initial F metabolites at each point in the growth of these two cell lines. The prostaglandin levels are presented in table 1. Variation from culture to culture was minimized by pooling the medium of 3 cultures at each time point. Deviation from the mean for 4 sets of duplicate samples showed a range of 0.0 - 3.8%. Therefore, variability can be estimated at <4.0%. Cellular F metabolite levels were very low so only medium

TABLE 1

LEVELS* OF PROSTAGLANDIN F AND INITIAL F METABOLITES

IN CULTURE MEDIUM OF 3T3 and SV3T3 CELLS.

DAYS** AFTER CELL INOCULATION	INCUBATED MEDIUM CONTAINING 10% CALF SERUM		GROWTH MEDIUM FROM BALB/c3T3 CELLS		GROWTH MEDIUM FROM BALB/cSV3T3 CELLS	
	PROSTAGLANDIN F METABOLITES	F PROSTAGLANDINS	PROSTAGLANDIN F METABOLITES	F PROSTAGLANDINS	PROSTAGLANDIN F METABOLITES	F PROSTAGLANDINS
4	0.19	ND	0.19	ND	0.16	ND
6	0.11	ND	0.13	1.95	0.33	3.13
8	0.11	1.86	0.13	2.60	0.21	3.85
10	0.17	1.75	0.19	2.30	0.63	4.75
12	0.11	1.75	0.23	2.35	0.53	3.50
14	0.13	1.60	0.35	2.15	0.75	4.35
16	0.12	ND	0.36	2.35	0.57	5.75

*Levels are expressed as ng/ml/48 hrs.

**Each day represents the last day of a 2 day collection of culture medium. For example, day 4 represents a 2 day collection from day 2 to day 4.

ND = not determined

Table 2

Comparison of PG levels
Primary F prostaglandins versus initial F metabolites

Days After Cell Inoculation	F metabolite level expressed as percent of primary F prostaglandin level		Ratio of SV3T3 level / 3T3 level For	
	3T3 cells	SV3T3 cells	F Metabolites	Prostaglandin
6	6.7	10.5	2.54	1.61
8	5.0	5.5	1.62	1.48
10	8.3	13.3	3.32	2.07
12	9.8	15.1	2.30	1.49
14	16.3	17.2	2.14	2.02
16	15.3	9.9	1.58	2.45
AVG. 6-16			2.25	1.85

levels are presented for comparison. If primary F prostaglandin levels are evaluated at different time points, little difference is noted as a function of the cell density of a culture, but differences between cell lines in terms of concentration detected are evident. The concentration of PGF in medium from SV3T3 cells is ~1.5 - 2.5 times higher than that detected in medium from 3T3 cells. Both cell lines show an increase above the control level of incubated medium. The level of initial F metabolites detected in the medium of transformed cells also exceeds that found for 3T3 cells, but the level of F metabolite is clearly much lower than primary F prostaglandin.

Table 2 is presented for further comparison of metabolite and primary prostaglandin levels. The level of metabolite detected for both cell lines is low and represents 5-17 percent of the F prostaglandin level. Both PG levels are also expressed in table 2 as ratios of the level detected in SV3T3 cells/the level detected in 3T3 cells. The ratios for F metabolites and F prostaglandins are similar having average ratios of 2.25 and 1.85,

respectively. The similarity of ratios demonstrates that the higher level detected for PGF and F metabolites in SV3T3 cell medium represents the same proportional increase over the levels detected in 3T3 cell medium. These results demonstrate that PGF metabolism is occurring to a lesser degree in cultures of mouse fibroblasts than has been demonstrated in humans infused with $PGF_{2\alpha}$ (4). In addition, the fact that metabolite levels and primary F prostaglandin levels were proportional for these two cell lines demonstrates the fact that metabolism of F prostaglandins is not seriously altered in SV40 transformed cells as compared with 3T3 cells. While a noticeable difference was not detected in initial F metabolite levels, it is recognized that extensive metabolism beyond the initial stages could result in the production of compounds which might not be recognized by F metabolite antiserum. This possibility seems unlikely since the addition of $[^3H]PGF_{1\alpha}$ to these cultures also suggests that prostaglandin F metabolism is not extensive (unpublished results).

These results support the hypothesis that differences in the PGF concentration of growth medium from cultures of "normal" and viral transformed cells are the result of differences in prostaglandin production or release from cells and not due to differences in the degree of metabolism which occurs during culture of these two cell lines. Additional studies are in progress to further assess the stability of E and F prostaglandins as a function of time in culture to further clarify the influence of prostaglandin stability on the final level of primary prostaglandin detected by radioimmunoassay.

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