PROSTAGLANDIN PRODUCTION BY MOUSE FIBROSARCOMA CELLS IN CULTURE: INHIBITION BY INDOMETHACIN AND ASPIRIN*

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Received April 18, 1972

Summary -- Five clonal strains of mouse tumor cells ($HSDM_1$) synthesize and secrete large quantities (0.70-2.0 μg/mg cell protein/24 hr) of prostaglandin E_2. Five lines of control cells did not synthesize significant amounts of prostaglandins. $HSDM_1$ cells produce prostaglandin E_2 during both the logarithmic and stationary phases of the cell growth cycle. Prostaglandin production was inhibited by aspirin-like drugs; for example, 50% inhibition was obtained with as little as 3×10^{-9} M indomethacin. We conclude that the $HSDM_1$ cell system will serve as a useful model system to study prostaglandin synthesis and secretion.

Growth of functional cells in tissue culture may provide useful model systems in which to study control of the biosynthesis and secretion of special cell products. The availability of sensitive and relatively specific radioimmunoassay procedures for the measurement of prostaglandins has made it possible to study the synthesis of these cyclic, oxygenated C_{20} fatty acids by cultured cells. We describe here the production of large quantities of prostaglandin by a fibroblast-like line of cells ($HSDM_1$) grown in culture. The $HSDM_1$ line was derived from a transplantable mouse fibrosarcoma known to produce a potent bone resorption-stimulating factor (Goldhaber, 1960; Goldhaber, 1966; Voelkel, Tashjian, and Goldhaber, 1972). The chemical and

* Publication No. 825 from the Graduate Department of Biochemistry, Brandeis University.

biological properties of the bone resorption-stimulating material initially led Voelkel et al. (1972) to suggest that it might be a prostaglandin.

Methods -- Establishment of the $HSDM_1$ line of cells in culture has been described (Voelkel et al., 1972), and the cells have been serially propagated for 13 months in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal calf serum (complete F10). Five clonal strains were derived from $HSDM_1$ cells by single cell plating in microcloning dishes; the clonal strains were designated $HSDM_1C_1$ through $HSDM_1C_5$. Cells were grown in plastic tissue culture dishes (50 X 15 mm, Falcon) containing 3 ml of complete F10 medium at 37° in a humidified atmosphere of 5% CO_2 and 95% air. Medium was changed every 3 or 4 days.

Medium for radioimmunoassay of prostaglandins was removed from plates and stored frozen. For the assay of intracellular material, washed cells were scraped in 2 ml buffer (0.01 M Tris HCl, 0.14 M NaCl, pH 7.6) and treated for 2 min at 1-2° in a Raytheon Model DF101 sonic oscillator. The material treated by sonic oscillation was stored at 4° and assayed within 24 hr. To assay ether extracts of cell culture medium, the pH value of the medium was adjusted to 3.5 with acetic acid and the medium extracted 3 times with equal volumes of diethyl ether. The combined ether layers were evaporated to dryness under nitrogen at 40° and the residue dissolved in buffer for assay. Protein was determined by the method of Lowry et al. (1951).

Radioimmunoassay of prostaglandins was carried out according to Levine, Gutierrez-Cernosek, and Van Vunakis (1971). Where indicated, samples were treated with alkali immediately before assay (pH 11.5-12.0, 100° for 5-10 min) to convert PGE or PGA to PGB.* All assays were carried out with antiserum to PGB_1 (Levine et al., 1971) at a final dilution of 1:130,000. For reduction of PGE to PGF, 0.01 mg of $NaBH_4$ was added to 1 ml of tritiated prostaglandin solutions (0.01 M Tris, 0.14 M NaCl, pH 7.5) or to appropriate serum-free tissue

* Abbreviations: PGE, PGA, and PGB for prostaglandins of the E, A, and B series, respectively.

culture fluids and allowed to stand 30 min. The solutions were then acidified with 2 M citric acid. After the bubbling stopped, the pH was adjusted to neutrality with 1 N NaOH.

Results and Discussion -- Medium from $HSDM_1$ cells was tested for its ability to inhibit the binding of $[^3H]PGB_1$ to anti-PGB_1 serum. As shown in Fig 1 and as previously described by Levine et al. (1971), the antibodies in this antiserum are specific for PGB; i.e., they bind PGB more effectively than PGA

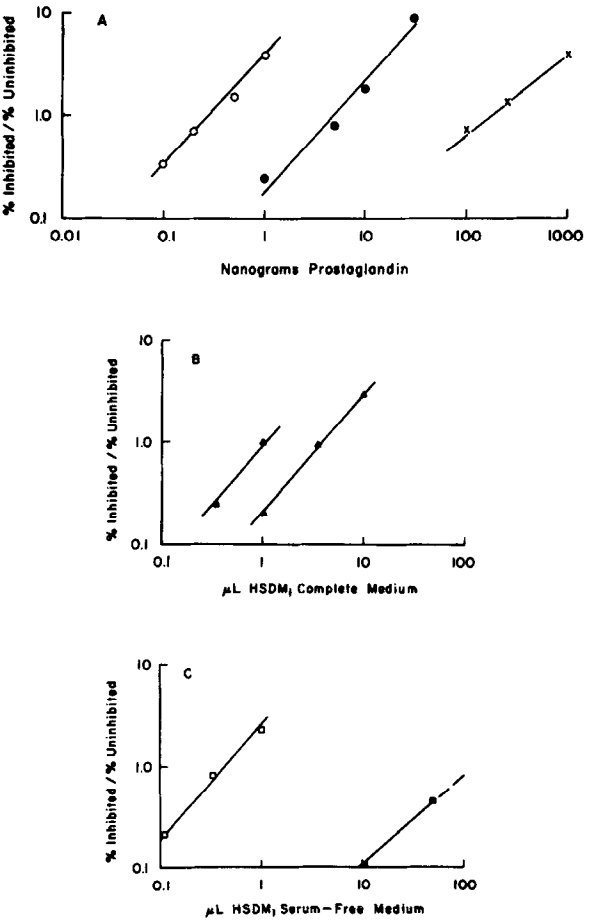

Fig. 1. Inhibition of $[^3H]PGB_1$ anti-PGB_1 binding. A. PGA_2(●), PGB_2 (○), and PGE_2 (X). PGB_2 was obtained by alkaline treatment of PGE_2. B. $HSDM_1$ culture medium before (▲) and after (△) alkaline treatment. Medium (complete F10) was a 3-day collection from $HSDM_1$ cells containing 0.41 mg cell protein. Complete F10 medium alone did not inhibit the binding of $[^3H]PGB_1$ to anti-PGB_1 at the levels used in the assay. C. $HSDM_1$ serum-free culture medium before (■) and after (□) alkaline treatment.

and PGE. Whereas 270 ng of PGB_2 inhibit 50% of the [^{3}H]PGB_1 anti-PGB_1 binding, 5 ng of PGA_2 and 180 ng of PGE_2 are required for equivalent inhibition. Fig. 1B shows that small amounts of $HSDM_1$ cell culture medium inhibit the [^{3}H]PGB_1 anti-PGB_1 binding and that the inhibition produced by the medium is enhanced by treatment with alkali. This serologically active material can be quantitatively extracted into ether from acidified $HSDM_1$ medium (see table). Thus, $HSDM_1$ cells secrete into complete F10 medium relatively large quantities of material that appear to be a mixture of PGB and PGA, as judged by the relative inhibition capacities of the medium before and after treatment with alkali.

If a small amount of PGA_1 is incubated in complete F10 medium under the conditions of these experiments, approximately 30% of the PGA_1 is converted to PGB_1. (Under the same conditions, PGB_1 is quantitatively recovered from complete F10 medium.) This instability of PGA_1 in complete F10 medium suggested that the PGA-like material in the medium of $HSDM_1$ cells was partly isomerized to PGB before assay - findings that would explain the observation that alkaline treatment of $HSDM_1$ culture medium produced a smaller increase in inhibition than alkaline treatment of authentic PGA (Fig. 1, A and B). Thus, the serologically active material in the complete medium from $HSDM_1$ cells appears to be a mixture of PGB and PGA. However, we have found that PGE_1 is not stable in complete F10 medium; some of it is converted to PGA_1 and PGB_1. Therefore, if the prostaglandin being synthesized by $HSDM_1$ cells were PGE, dehydrases present in the complete medium would convert it to PGA; the PGA that was generated could then be partly isomerized to PGB giving the mixture of PGA and PGB found in the complete culture medium.

To test whether $HSDM_1$ cells produce PGE, they were incubated for 3 days in medium lacking serum. Under these conditions PGE was stable. As shown in the table, $HSDM_1$ cells produced smaller but highly significant amounts of prostaglandin in serum-free medium than in medium with serum. The serologically active material in $HSDM_1$ medium lacking serum behaved on treatment with alkali

like PGE and not like PGA. For example, its serological activity was enhanced about 300-fold (Fig. 1C); whereas if it were PGA, it would have been enhanced only 15-fold (Fig. 1A).

PGE can be converted to PGF by treatment with sodium borohydride (Corey, 1971). If PGE were produced by $HSDM_1$ cells in the serum-free medium as indicated by the relative serologic activities before and after alkaline treatment, then $NaBH_4$ catalyzed reduction of PGE to PGF could be measured by analysis with specific anti-PGF. In addition, the relative specificities of anti-$PGF_{1\alpha}$ and anti-$PGF_{2\alpha}$ (Levine et al., 1971) would make it possible to identify the PGE (converted to PGF) as either PGE_1 or PGE_2. Serum-free medium containing the PGE-like material was treated with $NaBH_4$ and assayed with anti-$PGF_{2\alpha}$ and anti-$PGF_{1\alpha}$. The anti-PGFs did not react with the $HSDM_1$ serum-free medium before $NaBH_4$ treatment. (PGE, PGA, and PGB do not react very effectively with these anti-PGFs.) After $NaBH_4$ treatment, however, the serum-free medium inhibited [^{3}H]$PGF_{2\alpha}$ anti-$PGF_{2\alpha}$ binding more effectively than [^{3}H]$PGF_{1\alpha}$ anti-$PGF_{1\alpha}$ binding. Control serum-free medium (before addition to $HSDM_1$ cells), treated with $NaBH_4$, did not react with anti-$PGF_{2\alpha}$ or anti-$PGF_{1\alpha}$. As expected, PGA treated with $NaBH_4$ did not react serologically with anti-PGF. PGE_2, on treatment with $NaBH_4$, is converted to a mixture of $PGF_{2\alpha}$ and $PGF_{2\beta}$, but $PGF_{2\beta}$ cross reacts $< 0.5\%$ with anti-$PGF_{2\alpha}$ (Levine et al., 1971). In our laboratory, after reduction of known quantities of PGE_2 with $NaBH_4$, the yield of serologically active product, when assayed with anti-$PGF_{2\alpha}$, was 50%.

On the basis of these findings, we are tentatively designating the prostaglandin produced by $HSDM_1$ cells as PGE_2. All subsequent assays were carried out on alkaline-treated samples, and calculation of prostaglandin content (by comparison with a PGB_2 calibration curve) was based on the assumption that the serologically active material in base-treated $HSDM_1$ medium and cells was derived from PGE.

In order to test whether the production of PGE_2 is unique to $HSDM_1$ cells, culture medium from several other cell types was assayed for the presence of

PROSTAGLANDIN PRODUCTION BY CELLS IN CULTURE

Cell type	Culture medium	Prostaglandin production* (μg PGE_2/mg cell protein/ 24 hr)
$HSDM_1$	Complete F10	1.12
$HSDM_1$	" " , ether soluble**	1.16
$HSDM_1$	F10, no serum†	0.25
$HSDM_1$	" " " , ether soluble**	0.22
$HSDM_1C_1$	Complete F10	2.04
$HSDM_1C_2$	" "	1.28
$HSDM_1C_3$	" "	2.00
$HSDM_1C_4$	" "	0.80
$HSDM_1C_5$	" "	0.72
Mouse fibroblasts (C1-1D)	" "	< 0.03
Mouse neuroblastoma ($41A_3$)	" "	< 0.01
Rat glial (C_6)	" "	< 0.01
Human HeLa	" "	< 0.01
Mouse adrenal (AT_{10})	" "	< 0.01

* Medium samples were 3-day collections that were assayed for prostaglandin after alkaline treatment.

** Medium was extracted with ether and assayed, as described in the text.

† $HSDM_1$ cells were grown in complete F10 medium. Cells were washed twice with medium lacking serum, and for 3 days F10 medium without serum was added to the dishes.

prostaglandins. As shown in the table, none of the other types of cells tested produced significant quantities of material that reacted with anti-PGB_1. Likewise, none of these cell types contained measurable intracellular levels of prostaglandins. Five clonal lines derived from the $HSDM_1$ cells produced large quantities of PGE_2 (see table). The quantity of PGE_2 produced by several of the clones was sufficiently large so that the amount of PGE_2 produced by a single cell could be measured in this system. Subsequent experiments have been carried out with a single clone, $HSDM_1C_1$.

It was of interest to determine whether large quantities of PGE are

stored in $HSDM_1C_1$ cells. Cells were disrupted by sonic oscillation and the intracellular material examined by immunoassay. The amount of intracellular PGE_2 was at most 300 ng/mg cell protein; this quantity is equivalent to the amount secreted into the culture medium in about 3-1/2 hr. The stage of cell growth does not affect the rate of PGE_2 production by $HSDM_1C_1$ cells. Samples of medium were collected at various times after subculture. As shown in Fig. 2, the rate of PGE_2 production remained approximately the same whether the cells were growing logarithmically or were in the stationary phase.

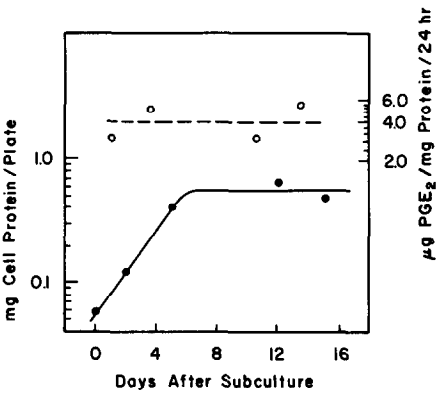

Fig. 2. Production of PGE_2 by $HSDM_1C_1$ cells. $HSDM_1C_1$ cells were innoculated in replicate dishes 12 hr before the start of the experiment. At zero time fresh medium was added to each dish. Every 2 to 4 days the medium was collected and changed. Duplicate dishes were washed twice with saline and frozen for the determination of cell protein. Medium was treated with alkali and assayed for PGE_2, as described in the text.

Since it has been reported that aspirin and related drugs inhibit the synthesis and release of prostaglandins in several systems (Vane, 1971; Ferreira, Moncada, and Vane, 1971; Smith and Willis, 1971), we have examined the effects of aspirin (acetylsalicylic acid), sodium salicylate, and indomethacin on PGE_2 production by $HSDM_1C_1$ cells. Indomethacin was found to be a potent inhibitor of PGE_2 production, causing a 50% reduction in the rate of PGE_2 synthesis at a dose level of 1 ng/ml (3×10^{-9} M); whereas aspirin caused a 50% decrease at a dose level of 10 µg/ml (6×10^{-5} M) (Fig. 3). Sodium salicylate caused < 20% decrease in the rate of PGE_2 synthesis at a dose level of 50 µg/ml. In cells

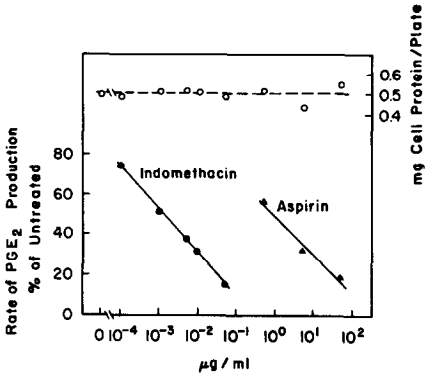

Fig. 3. Inhibition of PGE_2 production by indomethacin and aspirin. Fresh medium containing either no additions or the indicated concentrations of indomethacin or aspirin was added to duplicate dishes of $HSDM_1C_1$ cells. Three days later the medium was collected and frozen for assay. Dishes were washed twice with saline and frozen for the determination of cell protein. Medium was treated with alkali and assayed for PGE_2, as described in the text. Cells from untreated dishes produced 1.94 µg PGE_2/mg cell protein/24 hr. Results for dishes treated with aspirin and indomethacin are expressed as percentages of this control value.

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treated with up to 50 ng/ml indomethacin or 50 µg/ml aspirin, intracellular levels of PGE_2 did not differ from those in untreated cultures. Therefore, the drugs do not act on $HSDM_1C_1$ cells merely by inhibiting the secretion of PGE_2 into the culture medium.

Data obtained with $HSDM_1C_1$ cells support the observations of Vane (1971), Ferreira et al. (1971), and Smith and Willis (1971) that aspirin-like drugs inhibit the synthesis of prostaglandins. Indomethacin is 1000-fold more potent than aspirin in inhibiting PGE_2 synthesis by $HSDM_1C_1$ cells. This difference in potency is considerably greater than that observed in other systems (Vane, 1971; Ferreira et al. (1971), and Smith and Willis (1971).

We conclude that $HSDM_1$ cells should provide a useful and unique system in which to study the biosynthesis and secretion of prostaglandins. A variety of other established cell lines did not appear to produce measurable quantities of prostaglandins (see table). It was interesting in this context to examine whether arachidonic acid is a limiting substrate in PGE_2 synthesis. The addition of arachidonic acid, up to 167 µg/ml, to cultures of $HSDM_1C_1$ cells for 3

days led to no change in the amount of PGE_2 secreted into the medium. Either the supply of arachidonic acid is not limiting or exogenous arachidonic acid does not enter $HSDM_1C_1$ cells.

At the present time there is no direct evidence that the serologically active material produced by $HSDM_1C_1$ cells is the same substance as that which causes stimulation of bone resorption (Voelkel et al., 1972). However, prostaglandins are known to stimulate bone resorption, and the chemical properties of the partially purified $HSDM_1$ bone-resorption stimulating factor are consistent with those of a prostaglandin.

Acknowledgments -- The authors are grateful to Misses N. J. Barowsky and D. J. Jensen for expert assistance and to Dr. Paul Goldhaber, whose discovery and establishment of the transplantable $HSDM_1$ tumor cells made these studies possible. This investigation was supported in part by research grants from the American Cancer Society (1C-10K) and the National Institutes of Health (AM-11011 and DE-02849). L. L. is an American Cancer Society Professor of Biochemistry (Award No. PRP-21) and P. M. H. is a postdoctoral fellow of the American Cancer Society. The authors thank Dr. John Pike of the Upjohn Company, Kalamazoo, Michigan for the prostaglandins. The tritiated prostaglandins were purchased from New England Nuclear Corp., Boston, Massachusetts.

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