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PROSTAGLANDIN PRODUCTION IN HUMAN CANCER: CELLULAR ORIGIN AND TUMOR CELL CLONOGENICITY

The University of Arizona PH.D. 1982

PROSTAGLANDIN PRODUCTION IN HUMAN CANCER: CELLULAR ORIGIN AND TUMOR CELL CLONOGENICITY

by

Michael Edward Berens

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF GENERAL BIOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Michael Edward Berens

entitled PROSTAGLANDIN PRODUCTION IN HUMAN CANCER: CELLULAR ORIGIN AND TUMOR CELL CLONOGENICITY

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

Ray B. Nagle	6-10-82
	Date
Wm. J. McCauley	6-10-82
	Date
	June 10-82
	Date
	June 10, 1982
	Date
	6/10/82
	Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

	7/7/82
Dissertation Director	Date

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SIGNED: Michael E. Brems

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Profound gratitude and respect are held for the research staff in the Hematology-Oncology research labs. I regret that I cannot give adequate space to my feelings.

Most importantly, I acknowledge the support and love which my family has given to me. My precious wife, Patty, has truly been my lifeline through graduate school. "A faithful wife, who can find", Proverbs 31:10. Thank you, precious.

ad mejorum Dei glorium.

In Memorium

Dr. Ivan M. Lytle passed away on October 21, 1980 while acting as chairman of my graduate committee. Dr. Lytle was faithful in challenging me to set long term goals for myself and in providing counsel for their achievement. I dedicate this dissertation in his memory.

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ABSTRACT

The cellular origin of prostaglandins in human tumors was investigated using cell fractionation procedures and high resolution gas chromatography. Additionally, the role of macrophages and prostaglandins on human tumor cloning *in vitro* was investigated. Spontaneous human tumors were prepared as single cell suspensions which were subsequently manipulated to yield macrophage-enriched, and tumor cell enriched (macrophage-depleted) subpopulations of cells. A fused silica capillary gas chromatographic analysis with electron capture detection was developed to measure derivatized prostaglandins in the supernatant of the cell subpopulation incubations. The derivative used for the analysis was the pentafluorobenzyl ester-methoxime-trimethylsilyl ether. The assay showed a detection limit of 25 picograms of prostaglandins E_1, E_2, $F_{2\alpha}$, and I_2 (which was detected as 6-Keto-$PGF_{1\alpha}$). Analysis of cell subpopulations of seventeen tumor samples showed that the macrophage-enriched cells were responsible for the large majority of prostaglandin production *in vitro* ($p \leq 0.02$). It was found that the major prostaglandins were PGE_2 and PGI_2. The range of values measured for macrophage produced prostaglandin E_2 was 1.1 to 704.8 ng/ml after a 24 hour incubation of 10^6 cells. Prostaglandin I_2 was also produced by the macrophage-enriched cell subpopulation with values ranging from 1.2 to 334.3 ng/ml. This is the first report of prostaglandin I_2 production by host macrophages infiltrating human

tumors. Studies of the effect of macrophage depletion and reconstitution on the ability of tumor cells to form colonies in vitro were performed. A two layer soft agar assay was used to evaluate tumor cell clonogenicity. The results demonstrated that macrophages infiltrating human carcinoma samples function in a supportive role for tumor cell colony formation in vitro. Using a prostaglandin synthesis inhibitor, flurbiprofen, it was shown that this support was not the result of a direct effect of prostaglandins on the tumor cells. Possible roles for macrophage produced prostaglandins in cancer are discussed.

CHAPTER 1

INTRODUCTION

This dissertation was undertaken to explore the cellular origin and role of prostaglandins on tumor cell clonogenicity. The investigation has taken two broad directions: first, to explore which cell types within tumor biopsies are responsible for prostaglandin biosynthesis; second, to investigate the role of cell-to-cell interactions and prostaglandins on the clonogenicity of human tumors in a soft agar clonogenic assay system.

In a preliminary study from this laboratory (Buick, Fry and Salmon, 1980), macrophages within human carcinomas were suggested to be the source of the large majority of prostaglandin E_2. I have evaluated prostaglandin production by the development of a high resolution gas chromatography assay capable of qualitative and quantitative measurements of the major prostaglandins of biological importance. The developed analytical method is capable of simultaneously detecting a number of prostaglandins at extremely low concentrations (10^{-10} molar) in biological fluids and culture media. Human tumor specimens can be manipulated to enrich subpopulations of distinct cell types, which can then be incubated in liquid culture and assayed for prostaglandin production.

In studies of tumor clonogenicity, biopsies of human carcinomas were processed to single cell suspensions and subsequently plated in a

nutrient supplemented soft agar media which supports the proliferation of tumor cells (Hamburger and Salmon, 1977). This assay has been utilized for studies of drug sensitivity to anti-neoplastic agents (Alberts, Chen, and Salmon, 1980) and for studies of basic biology of tumor clonogenic cells (Buick, 1982). For my studies, primary and metastatic carcinomas were plated in the soft agar clonogenic assay. The cells were plated after depletion of macrophages and also with reconstitution of the macrophages. Addition of exogenous prostaglandins, and prostaglandin synthesis inhibitors was also tested in the clonogenic assay.

These investigative studies of prostaglandin production and tumor clonogenicity offer insight into the biology of human cancers. Study of prostaglandin synthesis by enriched cell populations yields information about cell-to-cell communication among the various cell types within a tumor. The clonogenic studies explore the role of prostaglandins on human tumor cell clonogenicity.

The two research areas will be presented separately to clearly delineate the results in each area. A brief discussion will conclude each chapter. The last chapter is intended to provide an overview and summary-discussion of the data and present thoughts on the possible in vivo significance of the findings.

CHAPTER 2

CHEMICAL CHARACTERIZATION OF PROSTAGLANDINS IN HUMAN TUMORS

Introduction

This section will briefly review prostaglandin biochemistry and physiology. In addition this section will describe the development of a high resolution gas chromatography assay for the measurement of the major prostaglandins of biological importance. Also, a commercially available radioimmunoassay method is described for quantitation of PGE. The methods were employed to measure the prostaglandins of discreet cell subpopulations which were isolated from biopsied human tumors.

Prostaglandin Biochemistry

The prostaglandins are a class of lipid-soluble, unsaturated fatty acid derivatives which contain twenty carbon atoms and a cyclopentane ring. Nine types of prostaglandins (designated A through I) are discernable based on the placement and type of functional groups on the pentane ring (see Figure 1). These are determined by the various endoperoxide synthetase and isomerase enzymes which synthesize the prostaglandins. Additionally the prostaglandins are grouped into three series (designated 1 through 3) determined by the number of unsaturated bonds in the two carbon side chains on the pentane ring. The precursor fatty acid determines which series of prostaglandin will be synthesized (see Figure 2). Prostaglandins are synthesized from eicosatrienoic or

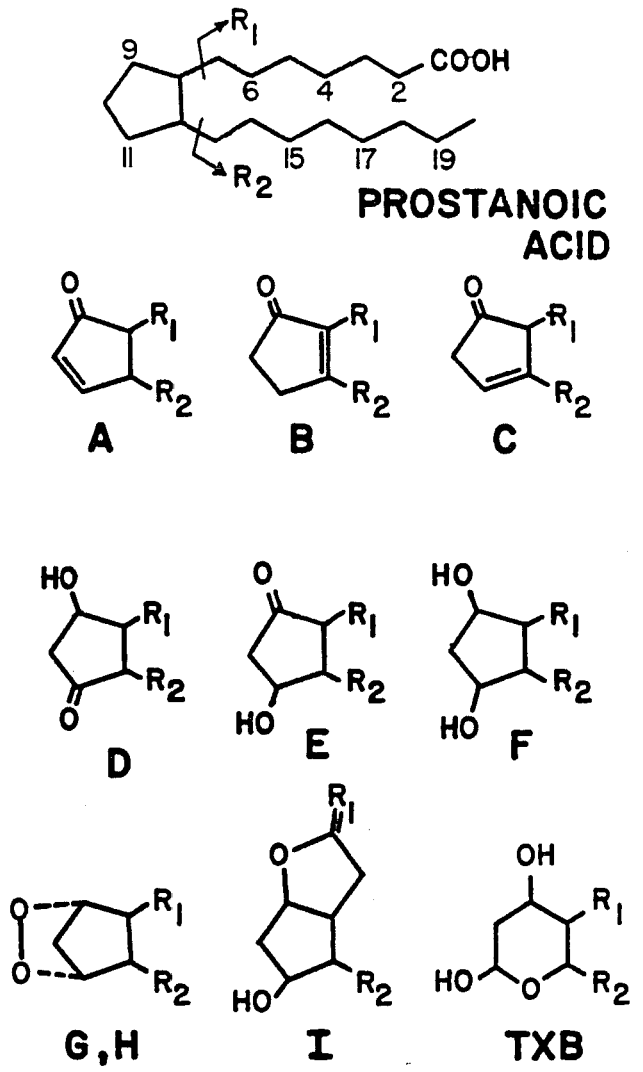

Figure 1. Structure and nomenclature of prostaglandins.

TXB = Thromboxane B.

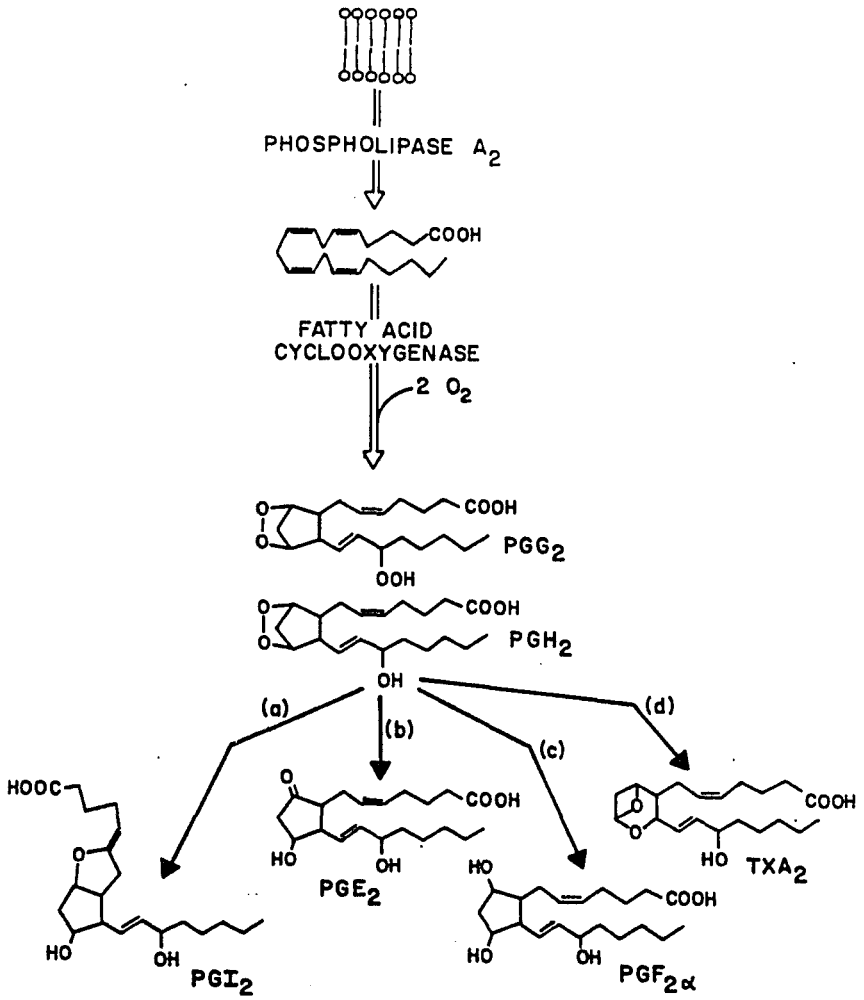

Figure 2. Metabolic pathway of prostaglandin biosynthesis.

Membrane phospholipids are hydrolyzed by phospholipase A_2 to arachidonic acid (or other precursors). Fatty acid cyclooxygenase converts fatty acid to unstable intermediates (PGG_2, PGH_2). Isomerase enzymes determine the final product. (a) Prostacyclin isomerase, (b) PGE_2 isomerase, (c) $PGF_{2\alpha}$ reductase, (d) Thromboxane A_2 isomerase.

dihomo-γ limolenic acid (Series I), eicosatetraeonic or arachidonic acid (Series 2), or eicosapentanoic acid (Series 3) (Moncada, Flower, and Vane, 1981).

The actual source of substrate for prostaglandin synthesizing enzymes is the phospholipid pool of the cell membrane (Moncada et al., 1981). The fatty acids are released from the membrane by the action of phospholipase A_2 (Flower and Blackwell, 1976). This liberation of fatty acid substrate is believed to be the rate-limiting step in prostaglandin biosynthesis (Flower and Blackwell, 1976). Additionally, there is evidence that the prostaglandins synthesized in vivo are derived from membrane phospholipid rather than from free substrate pools (Flower, 1978; Marcos, 1978).

Figure 2 summarizes the biosynthetic "cascade" of prostaglandin formation. Little prostaglandin is stored by cells which synthesize them, but rather they are quickly released into the extra-cellular milieu (Samuelsson, Goldyne, and Gramstrom, 1978).

Prostaglandins are almost ubiquitous throughout the body and body fluids (Moncada et al., 1981). The scope of responses to the prostaglandins is also quite diverse. Significant physiological properties are evident in the cardiovascular, gastrointestinal, renal, hemopoietic, reproductive, immunological, respiratory, and central nervous systems (Moncada et al., 1981).

The elucidation of the biological role of the prostaglandins was hampered by the prevalence of the synthesizing enzymes throughout the body (Piper, 1977). Only as the brief half-life of the

prostaglandins became evident, was the biological behavior of this class of compounds realized. The transient existence of the prostaglandins suggested their physiological role as a "local" hormone; that is, they exert their influence in or upon the tissue in which they are synthesized and secreted.

The effect of prostaglandins on the immune system is of particular interest. Physiological concentrations of PGE_2 are able to abrogate most normal T-lymphocyte activities in vitro (Goodwin, 1981). These functions include mitogen responsiveness (Goodwin, Messner, and Peake, 1974), antibody dependent cytotoxicity against tumor cells (Droller et al., 1979), lymphokine production (Gordon, Bray, and Morley, 1976), T-cell cytotoxicity (Plaut, 1979), and natural killer cytotoxicity against tumor cells (Roder and Klein, 1979). These activities may be important in the "escape" from immune detection of the tumor cells. Additionally, the effect of prostaglandins on tumor growth has been explored (Karmali, 1980). This realm will be extensively reviewed in the historical perspective.

Prostaglandin Metabolism

The rapid degradation of the prostaglandins occurs via oxidation of the hydroxyl group at the C-15 position (Anggard and Samuelsson, 1964; Hamberg and Samuelsson, 1971)(see Figure 3). This step almost totally eliminates the biological potency of the prostaglandins (Samuelsson et al., 1978). Prostaglandin Δ^{13} reductase reduces the 15-keto derivative to the 13,14-dihydro form. These first two steps in the degradative pathway occur rapidly, with subsequent catabolic

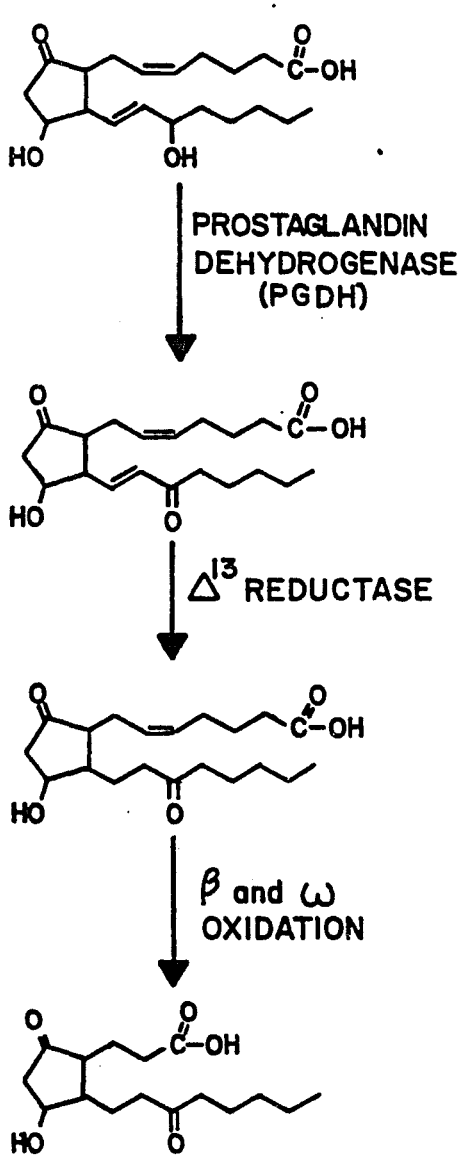

Figure 3. Metabolic pathway of prostaglandin E_2 degradation.

steps progressing relatively slowly as per normal β and ω oxidation of most fatty acids (Flower, 1978). The degradative enzymes are found in the liver, lung, kidney and intestine (Flower, 1978).

Methods of Prostaglandin Analysis

The analysis of prostaglandins from biological materials has employed various analytical tools. This section will briefly review these methods and promote the rationale for the development of a routine, high-resolution gas chromatographic assay for prostaglandins.

The observation that isolates from prostate gland secretions cause contraction of intestinal smooth muscle acted as the initial stimulus for research into prostaglandins (Samuelsson, 1963). These bioassays relied on changes in the force or rhythmicity of smooth muscle contractions following the addition of various drugs, chemicals or tissue extracts. The organs utilized in various prostaglandin bioassays include rabbit aorta, rabbit coeliac artery, rat colon, bovine coronary arteries, platelet aggregation reactions, as well as many others (Moncada et al., 1978). This spectrum of target tissue can be used to partially differentiate among the various cyclooxygenase metabolites or arachidonic acid (Moncada et al., 1978). Bioassay procedures are at best sensitive to prostaglandins in the range of 1-20 ng/ml. The bioassay is also the tool which only recently instigated the discovery of prostacyclin (PGI_2)(Moncada et al., 1976).

However, with the advent of sensitive analytical means of detection, in addition to antisera raised against prostaglandins, the bioassay is seldom used. Its nonspecificity and limitations on

sensitivity limit it from being as useful an analytical method as others now available.

The introduction of a radioimmunoassay for PGE_1 and $PGF_{2\alpha}$ (Levine and Van Vanakis, 1970) stimulated a large number of investigations of the prostaglandins. Radioimmunoassay (RIA) is a quantitative procedure based on competition between a radioactively-labeled molecule and an unlabeled molecule for specific binding sites on an antibody directed against the labeled molecule. The assay operates using controlled constant amounts of antibody and labeled molecule, to which quantitative standards or biological samples are then added which compete with the labeled molecule for limited binding sites on the antibody. The antibody-bound molecules are then separated from the free molecules, and appropriate radioactive counting methods are used to quantitate either the bound or free portion of the reaction. Comparison between known standards and the biological samples produces a quantitative measure of the molecule of interest. The qualitative aspect of a radioimmunoassay resides in the specificity with which the antibody molecule discriminates for its target. As previously described, the prostaglandins are grouped into nine categories based on functional groups on the cyclopentane ring; additionally three series are described within each group as determined by the degree of unsaturation in the carbon chains extending from the ring. These permutations of structure result in a range of physically distinguishable differences between the prostaglandins, spanning from very slight to clearly distinct characteristics. The near-homology within some

groups, however, causes difficulty in discrimination between similar prostaglandins and consequently impairs the qualitative aspect of the assay. Whereas, bioassay protocols may detect both known and unknown materials present in the samples, radioimmunoassay techniques are only potentially able to measure compounds of which a radioactive standard is available (or synthesizable).

Furthermore, the possibility exists that non-prostaglandin molecules may bind to the antibody, causing artifactually elevated quantitative results. Nonetheless, radioimmunoassay is a reasonably accurate and quick method for evaluating several of the stable arachidonic acid metabolites (Granstrom and Kindahl, 1978).

Some of these limitations were circumvented in the RIA developed by Dray et al. (1975). His group processed 10 ml sample of peripheral blood by first removing cells and platelets, followed by organic solvent extraction of the prostaglandins. As a final step prior to RIA, the prostaglandins were separated on a silicic acid column yielding three elutions, with PGA and PGB in the first, a second fraction containing PGE_2 and a third fraction containing $PGF_{2\alpha}$. The assay was capable of detecting these prostaglandins in the low picogram range.

Radioimmunoassay continues to be used for prostaglandin measurements. The problem of cross-reactivity can be solved by chromatographic resolution of the compounds of interest, and the dilemma of non-specific binding is addressed by analyzing media blanks as controls (Taffet, Pace, and Russell, 1981).

In addition to RIA, two very powerful analytical chemistry techniques are amenable to qualitative and quantitative analysis of prostaglandins: high pressure liquid chromatography (HPLC) and gas-liquid chromatography (GLC or GC).

The majority of HPLC techniques developed for analysis of prostaglandins utilize ^{14}C-arachidonic acid as the precursor substrate for the synthesis and subsequent detection of radioactive prostaglandins (Powell, 1981; Van Rollins et al., 1980; Eling et al., 1980). The recent method developed by Powell (Powell, 1981) is a versatile procedure capable of isolating individually PGE_1, PGE_2, PGD_2, 6-Keto-$PGF_{1\alpha}$, PGF_{1_α}, and $PGF_{2\alpha}$ in a single fifty minute run. Using a 0.46x35cm RSil CAT (5μm) argentation HPLC column, this protocol was used to monitor the arachidonic acid metabolic profile from rabbit kidney medullary cells. Since the final detection apparatus was a radiocolumn monitor, the units of measure reported were simply "radioactivity." This recording mechanism does not allow ready comparisons with the quantitative measures of other analytical schemse. However, valuable information is accrued concerning the direction of the arachidonic acid cascade in different cell populations.

The elegant chromatographic technique devised by Terragno (Terragno et al., 1981) is an attempt at low sensitivity ultraviolet detection of the major arachidonic acid metabolites. By using 192 nm wavelength to detect the double bonds in the molecules, he achieved baseline resolution of eleven standard compounds in a sixty minute run, with detection limits of 30 nanograms on column. This work appears as

a possible answer to circumventing other complicated chromatographic procedures. Application of this method to biological specimens, and extension of sensitivity to the picogram range have yet to be reported.

Finally, recent techniques have utilized HPLC as a preparative tool for subsequent gas chromatographic analysis (Carr et al., 1976). Using deuterated and tritiated internal standards, urine, plasma, seminal fluid and renal medullary extracts were passed through a micro-particle silicic acid system in preparation for qualitative and quantitative analysis by GC-mass spectrometry. This method required derivatization of the eluted material prior to final analysis, but detection limits were in the low picogram range for PGE_2, PGA_2, and $PGF_{2\alpha}$. This chromatographic work-up seems somwehat unnecessary when evaluated in relation to other superior gas chromatographic methods which are readily interfaced with mass spectrometers (vide infra).

In summary, bioassays for prostaglandins are limited by non-specificity and poor sensitivity; radioimmunoassay is hampered by difficulty in procuring radioactively labeled standards and by cross-reactivity of the antisera; and although high pressure liquid chromatography is used as a preparative tool or as an analytical method for metabolites of ^{14}C-arachidonic acid, it is far too insensitive for analysis of many routine biological samples. The most promising approach for routine analysis of the arachidonic acid metabolic cascade is the recent development of gas chromatographic techniques for resolution and detection of these compounds (Fitzpatric et al., 1979). These procedures require extraction and derivatization of the

prostaglandins, however measurements of all arachidonic acid metabolites with limits of detection in the low picogram range are possible. Gas chromatographs (GC) can be interfaced to mass spectrometers (Rosello et al., 1977; Tussel and Gelpi, 1980; Rosello et al., 1981) resulting in a highly sensitive instrument for detailed metabolic studies of molecular products of the arachidonic acid cascade.

High resolution fused silica gas chromatography is an effective means of separating the products of arachidonic acid metabolism (Fitzpatrick, 1978); the major variable among different methods resides in the detector employed to monitor the gas effluent from the column. Three detection assemblies are commonly used: flame ionization, mass spectrometer, and electron capture. Each has its own advantage. The flame ionization detector (FID) is the least specific of the three common GC detectors, yet this non-specificity allows facile detector response to most eluted compounds. The FID is unlikely to "omit" detection of chromatographic solutes. The eluted components from the GC column are mixed with hydrogen and air, then the mixture is burned. The decomposition in the flame generates ions which are collected by a charged housing surrounding the flame tip. The incidence of ions is amplified as the chromatographic signal which is then plotted on a strip-chart recorder. The FID is limited in sensitivity to the low nanogram range (Maclouf et al., 1980). This method allowed studies of in vitro incubations with arachidonic acid of platelets, endothelial cells, and peritoneal macrophages. The range of sensitivity limits this method to analysis of artificially stimulated cell populations

(Maclouf et al., 1980). Rosello and colleagues (Rosello et al., 1981) have used glass capillary columns to generate a profile of the prostaglandins produced by mouse peritoneal macrophages. The technique involved isolating macrophages from peritoneal washes by adherence to plastic. The cells were then incubated for 20 minutes with 10 µg arachidonic acid; the supernatant was adjusted to pH 3, and the prostaglandins extracted with ethyl acetate. A four step derivatization procedure rendered the porstaglandins stable and volatile. Prostaglandin $F_{2\beta}$ (40 ng) was used as the internal standard. The mass spectrometer was operated in the selected ion mode, in which the mass filter is programmed to monitor specific ion molecular weights characteristic of the individual prostaglandins at the appropriate elution time from the capillary column. These procedures allowed detection and quantitation of $PGF_{2\alpha}$, PGD_2, PGE_2, TxB_2, and 6-Keto-$PGF1_\alpha$ at 10 picograms. The same method of selected ion monitoring was employed by Tussel and Gelpi (Tussel and Gelpi, 1980) to profile the prostaglandins in semen. Their analytical results were in the microgram range.

Fitzpatrick (1978) used GC-FID for development of capillary chromatography. However, the detector sensitivity was 20 ng per injection, restricting the use of this method from many biological applications. The FID response to prostaglandins was extended to 10 ng, and utilized to measure prostaglandin content of bovine semen (Mai and Kinsella, 1980). Prostaglandin production by platelets, endothelial cells and mouse peritoneal macrophages was profiled by glass capillary

GC with FID. The detection limit was 2 ng for each of the major arachidonic acid metabolites (Maclouf et al., 1980).

A ^{63}Ni-electron capture detector (ECD) was applied to GC analysis of prostaglandins by Wickramasinghe and coworkers (Wickramasinghe et al., 1973) with excellent linearity of detector response to the pentafluorobenzyl ester derivative of $PGF_{2\alpha}$ over a range of 0.03 to 0.84 nanograms. Detection limit was 12.5 picograms on column. This technique was subsequently utilized to measure $PGF_{2\alpha}$ in monkey plasma (Wickramasinghe and Shaw, 1974). The biological fluid was extracted, then prepared as a pentafluorobenzyl ester derivative. The method was compared with GC-mass spectrometry and also with RIA. There was good correlation between the three methods.

Platelet metabolism of PGH_2 was studied using GC-ECD (Fitzpatric et al., 1977). The products PGA_2, PGE_2, PGD_2, PGB_2, and TxB_2 were resolved and quantified to the 30 pg level using this method. The pentafluorobenzyl ester derivative group affords chromatographic stability and good detector response to all the arachidonic acid metabolites. An extensive study was done to optimize derivative options for both chromatographic resolution and ECD response of the prostaglandins (Fitzpatricket al., 1979). Mouse fibroblast cell lines (Balb 3T3 and Balb 3T1w) were incubated with 1.0 µg of PGH_2. The media supernatant was extracted with ether then various derivatization schemes performed. The optimum performance for profile studies was rendered by the pentafluorobenzylester-trimethyl silyl ether derivatives. When signal to noise ratios are allowed at 5:1, a lower limit of

detection of 1 pg was possible. A similar analytical performance was reported (Leffler et al., 1981) using pentafluorobenzyloxime derivatives. This assay yielded adequate analysis of prostaglandins in urine, lung perfusates and serum, with detection limits of 10 pg for each of the major PGH_2 metabolites.

Although the sample work-up is significant in preparing biological fluids for prostaglandin analysis by glass capillary gas chromatography with electron capture detection this analytical technique is the most useful current technology for profiling the arachidonic acid metabolites in the low picogram range of sensitivity.

Historical Perspective: Prostaglandins and Cancer

Interest in the role of prostaglandins in the neoplastic process has continued to increase since the first published report in 1968 of plasma prostaglandin levels from a patient with medullary carcinoma of the thyroid (Williams, Karim, and Sandler, 1968). Recently, the research activity in this field was channeled into an international conference directed at stimulating interactions between tumor biologists and prostaglandin biochemists (Powles, Bookman, and Hann, 1982). In the fifteen year development of this field, progress has been made both in the analytical abilities to measure prostaglandins and in the biological tools employed to evaluate their possible roles in neoplasia.

Williams et al. (1968) used a bioassay to quantitate prostaglandins in the venous drainage of a medullary carcinoma of the thyroid gland. The results showed a marked discrepancy between normal

plasma levels and that of the tumor patient. The results showed carcinoma patient plasma levels of PGE_2 in the range from 0.8-41 ng/ml, and of $PGF_{2\alpha}$ ranging from 0.4-120 ng/ml. The plasma levels in normal controls were below the detection limit of the assay, 0.5 ng/ml (Karim, Sandler, and Williams, 1967). Prostaglandin physiology was still an uncharted domain, and other researchers sought to evaluate the presence of this class of hormone in cancers.

Sarcomas were studied by Bhana and coworkers (Bhana, Hiller, and Karim, 1971) using a colon bioassay to measure prostaglandins. The investigation found that Kaposi's sarcoma tissue contained PGE_2 in a range or 25-234 ng/gm of tissue; $PGF_{2\alpha}$ was also present in a similar concentration. Plasma samples were also evaluated but any prostaglandin-like material present was below the detection limits of their assay. Two experimental tumors were studied by Sykes and Maddox (1972), the $PB8/P_1$ tumor and sarcoma 180. Both were passaged in mice, and tumor tissue was evaluated for prostaglandin content using bioassay. $BP8/P_1$ grows as both an ascites and solid tumor. In the solid tumor PGE_2 content was 2.29 µg/gm tissue; the ascites cells contained 1.98 µg/gm. Verification of the prostaglandins was performed by gas chromatography with mass spectrometer analysis. The report also indicated that by treating the tumor-carrying animals with indomethacin (3 µM), the prostaglandin level was reduced to near zero, but the tumor growth was not reduced by any significant amount. Sarcoma 180 contained 0.63 µg/gm tissue; the growth was not hindered by prostaglandin synthesis inhibitors. The authors state, "It therefore does not

seem that prostaglandins are essential for tumor growth, and may merely reflect inappropriate tissue hormone production." This view was not a majority consent among other workers (vidae infra).

Maloney sarcoma tumors were found to contain 53 times greater PGE content compared with control tissue (Humes and Strausser, 1974). Prostaglandin $F_{2\alpha}$ was present in a seven fold greater amount in the tumor compared to control tissue. Burkitt's lymphoma tissue extracts also contained prostaglandin-like material (Ajayi and Okpako, 1977). These tumors contained a range of Prostaglandin E from 50-440 ng/g tissue. Radioimmunoassay of plasma samples from patients with ovarian tumors showed values of $PGF_{2\alpha}$ from 25 to 97 pg/ml compared to normal levels of 9 to 24 pg/ml (Lee, Sanders, and Jones, 1978). A follow up study later suggested that elevated $PGF_{2\alpha}$ may be a marker for female genital tumor growth (Sanders et al., 1980). Rolland and coworkers (Rolland et al., 1980) found retrospective evidence of elevated prostaglandin E being a marker of metastatic potential in breast cancer patients. When primary tumor biopsies were studied, the finding of elevated prostaglandin E in the homogenized tissue correlated with a likelihood of finding neoplastic cells in tumor lymphatic and blood vessels and in axillary lymph nodes.

Jaffee reported on the levels of prostaglandin E measured in the plasma of patients with medullary carcinoma of the thyroid, carcinoid tumors, phechromocytoma, and neuroblastoma (Jaffe, 1974). There was an overwhelming incidence of elevated plasma PGE in the cancer patient plasma (1 ng/ml) compared with controls; this work also

reported normal plasma PGE to be 385 pg/ml by RIA. Four tumor cell lines, HT-29, HEP-2, L, HeLa, were also investigated for their prostaglandin synthetic activity. The cell lines produced from 0.13 to 5.74 ng PGE/10^6 cells/day. The amount of prostaglandin produced correlated directly with the length of doubling time for the four cell lines studies. At this stage in the development of prostaglandins and cancer studies, investigators had shown that homogenized tumor specimens synthesized prostaglandins (Jaffe, 1974); malignant cell specimens contained more PGE than did comparable normal cell samples (Ritgi and Stylos, 1976), and there was an inverse relationship between prostaglandin production and cell proliferation rate (Thomas et al., 1974). Although no definitive mechanism of action had been forwarded, cyclic AMP was being considered as the intracellular mediator of the effects from prostaglandins (Jaffe, 1974).

Using a bioassay, homogenized human breast tumor biopsies were investigated for prostaglandin synthesis activity. The results were correlated with bone metastases (Bennett et al., 1975; Bennett et al., 1977). These results showed greatly elevated levels of prostaglandin-like material in most of the malignant tumors and only marginally elevated levels in the benign tumors compared to controls. From one hundred and two patient samples analyzed, the results clearly displayed a correlation between elevated prostaglandin-levels and overt malignancy as manifested by metastases. The record of one hundred fourteen patients with various degrees of neoplasia conducted

by Powles and associates (Powles et al., 1977) corroborate these findings.

A natural extension of the relation shown between prostaglandins and metastases was to consider an interaction between prostaglandins and hypercalcemia because of the frequent metastatic lesions in bone.

Seyberth and coworkers (1975) evaluated this relationship in 51 patients, twenty of whom were hypercalcemic in conjunction with various neoplasms. Degradative metabolites of prostaglandin E were extracted from urine specimens then analyzed by gas chromatography-mass spectrometry in the selected ion mode. Parathyroid hormone (PTH) was also analyzed by radioimmunoassay. In the group of 14 patients with solid tumors and expressing hypercalcemia the urinary excretion of PGE metabolites was more than two fold greater than the control group. None showed elevated PTH. Hypercalcemia in non-cancerous patients was not accompanied by elevated PGE-metabolite excretion in their urine. Although only a small sample of solid tumor types was studied, the link between the hypercalcemia of neoplasia seemed to coincide with elevated prostaglandin-levels. A brief study of one patient with bronchial carcinoid tumor with diffuse hepatic metastases also showed elevated plasma prostaglandins (Delmont and Rampol, 1975). Demers and coworkers (1977) executed a study of 79 patients, measuring plasma prostaglandin E and serum calcium. Normal (control) plasma PGE resides in a range of 25-100 pg/ml. Hypercalcemic cancer patients displayed plasma PGE values of 312 pg/ml; the difference from the

normocalcemic population being significant at the $p < 0.001$ level in this study. Clearly the results of this comprehensive investigation suggested strongly that elevated prostaglandins associated with neoplastic disease was at least a modulator of hypercalcemia.

The mechanism by which prostaglandins cause hypercalcemia has yet to be defined (Mundy, 1982). Tashjian and colleagues (1982) describe two plausible mechanisms. The first posits that the tumor cells produce increased prostaglandin-levels which lead to bone resorption. The second hypothesis is that tumors cause production of a peptide factor which stimulates elevated prostaglandin-levels in the bone leading to resorption. (also Bockman, 1982).

Although there has been good correlation between elevated prostaglandin levels and hypercalcemia, a clinical trial designed to utilize prostaglandin synthesis inhibitors to prevent bone metastases in high risk breast cancer patients was disappointingly ineffective (Powles, Muindi, and Coombes, 1982). One possible explanation for the failure to prevent neoplasia-associated bone resorption by blocking prostaglandin synthesis would be that the osteoclastic site did not receive an adequate dose of the PG-synthesis inhibitor. The exact role of tumor related prostaglandin synthesis and bone resorption (whether manifested as bone metastases or hypercalcemia) is yet to be clarified.

Effort has also been expended towards unfolding the role, if any, of prostaglandins on tumor cell proliferation. Jimenez de Asua and coworkers (1975) showed that $PGF_{2\alpha}$ at 300 ng/ml caused initiation of

DNA synthesis and cell division in quiescent mouse 3T3 fibroblasts. When the cells were cultured in media supplemented with 75 ng/ml $PGF_{2\alpha}$ the confluent cell density was 2.5 fold greater.

When prostaglandin E values are measured at timepoints during growth of polyoma virus transformed 3T3 fibroblasts, the concentration rises from 20 ng/ml at 24 hours after plating to 225 ng/ml six days later at confluency (Claesson, Lindgren, and Hammarstrom, 1977). The cyclic AMP levels rise directly along with PGE. The authors conclude that endogenous prostaglandin production may serve to auto-regulate cell density via adenylate cyclase activation. Taylor and Polgar (1977) also forwarded a role for prostaglandins in autoregulation of human diploid fibroblasts density in culture. Addition of exogenous prostaglandin E_2 (10^{-10} to 10^{-8} Molar) showed a dose response inhibition of cell number. Prostaglandins $F_{2\alpha}$ was found to stimulate cell growth in this system.

Jaffe's group had shown that PGE levels in the supernatant of cell lines <u>in vitro</u> correlated directly with cell cycling time (Thomas et al., 1974). These studies were extended to <u>in vivo</u> tests using B16 melanoma growth in mice (Santoro, Philpott, and Jaffe, 1977). Systemic administration of a long lasting analog of PGE_2, 16, 16-dimethyl prostaglandin E_2, delayed the gross appearance of tumor after innoculation. Also PGE_2-treated animals grew smaller tumors and had longer median survival curves compared with untreated controls. Particularly noteworthy was the finding that histological examination

of tumors from both PGE_2-treated and control animals showed the same degree of inflammatory cell infiltration. The authors concluded that the effect of the prostaglandin analog probably represented changes primarily in tumor cell number

In this respect it is of interest to note the findings of Owen and coworkers (1980) which concerned the role of lymphocytes on tumor cell prostaglandin production. Four carcinogen-induced rat tumor cell lines were studied (two bladded transitional epithelial cell lines, one mammary carcinoma cell line, and one embryo fibroblast line). Peripheral blood lymphocytes from syngeneic rats were isolated and co-incubated with each of the cell lines. The study found that carcinogen-induced rat neoplastic cells produce prostaglandins in vitro and that this production is increased when the cells are exposed to purified lymphocytes from peripheral blood of syngeneic rats.

The role of prostaglandins in the immune response has attracted much investigation (Karmali, 1980; Cueppers and Goodwin, 1981; Stenson and Parker 1980). The antibody response to sheep erythrocytes by tumor-bearing mice was shown to be inversely related to the growth of a syngeneic tumor in vivo (Plescia, Smith, and Grinwich, 1975). These studies also revealed that co-culture of tumor cells with mouse spleen cells markedly reduced the development of antibody response to sheep erythrocytes in vitro. This immunosuppressive effect of tumor cells was partially blocked by the addition of either indomethacin or aspirin (both inhibitors of prostaglandin synthesis) to the tumor

cell-host system *in vitro* and *in vivo*. Exogenous PGE_2 effectively blocks the antibody response of mouse spleen cells to sheep erythrocytes. Unfortunately, no measurements were made of the ability of the tumor cell lines tested to synthesize prostaglandins. Also, the role of humoral immunity in host defense against cancer is uncertain, and possibly irrelevant.

Activated macrophages show tumoricidal activity (Hibbs, Lambert, and Remington, 1972) using an *in vitro* system. Prostaglandin E_2 and E_1, but not $PGF_{2\alpha}$, inhibit the tumoricidal function of interferon-activated macrophages in a linear dose response above 10^{-7}M, reaching nearly 100% inhibition at 10^{-5}M (Schultz, Pavlidis, Stylos, Chirigos, 1978). This assay system, however, uses a macrophage to tumor cell ratio of 10:1, which is extraordinarily high in reference to *in vivo* ratios (Evans, 1979).

Evans (1979) reports an elegant study on the role of host cells in developing tumors in mice. In tumors which reappeared after cyclophosphamide therapy, the infiltration of macrophages and T-lymphocytes paralleled the growth of neoplastic cells. Earlier work by Evans (1976) showed that a variety of cells was stimulated to divide by culture medium conditioned by macrophages isolated from solid tumors.

In vitro culture studies of primary human tumors also show that *in situ* macrophages assist the clonal proliferation of a wide variety of tumor cells (Salmon and Hamburger, 1978; Hamburger, Salmon, and Alberts, 1980; Meyskens, 1980; Buick, Fry, and Salmon, 1980; Berens Salmon, 1981). The mechanism of cloning stimulation by *in situ*

macrophages is an area of current research, to which the material of this dissertation attempts to contribute.

Materials and Methods

Sample Preparation

Human carcinomas were prepared for prostaglandin synthesis studies. The tumor samples originated as either solid tumor masses resected during surgery, or as ascitic fluid retrieved by abdominal paracentesis.

Solid tumors were finely minced into 1 mm pieces using forceps, scalpel, and scissors. The specimen was maintained in a wet environment by adding McCoy's 5A culture media (Gibco, Santa Clara, CA) enriched with heat inactivated fetal calf serum 10% (FCS) (Flow Laboratories, Inglewood, CA). The cell-laden media was drawn into a syringe and expelled through a 22 gauge needle to ensure dispersal of clumps into single cell suspension. The retrieved cells were washed by resuspending in McCoy's 5A + 10% FCS, followed by centrifugation at 600 x G for 10 minutes to isolate the cell pellet. For samples showing extensive clumping, the minced material was passed through a double layer of sterile gauze to remove large clumps prior to needle aspiration.

Ascitic fluid tumor specimens existed, as retrieved, in a near single cell suspension. The material was centrifuged at 600 x G for 30 minutes at 4°C to isolate the cells. Peritoneal fluid supernatant was poured off and a small aliquot stored at -80°C for subsequent

prostaglandin analysis. The cells were removed from the bottom of the centrifuge bottle and washed once in McCoy's 5A plus 10% FCS.

The single cell suspensions were counted under microscopy (100x magnification) in a hemocytometer to determine cell content. The number of cells was calculated from the mean of four counts, then appropriate dilution factors were used to determine the cell concentration in the sample cell suspension. The cell concentration was adjusted to 1×10^6 nucleated cells per milliliter. Cell viability was ascertained by scoring the percentage of cells able to exclude trypan blue dye (Gibco, Santa Clara, CA) (Kruse and Patterson, 1973). This was evaluated by microscopy at 100X on a coverslipped drop of cells on a glass slide. A minimum of 200 cells was counted.

The single cell suspension resulting from mincing and washing was labeled the Unfractionated (UF) population. Physical manipulations were performed to yield a tumor-cell enriched population of cells and a macrophage-enriched population of cells.

Macrophage isolation was accomplished by allowing the cells to adhere to plastic tissue culture dishes (Falcon, Scientific Products, McGraw Park, IL) for two hours at 37°C, 5% CO_2, humidified air (Gadberg et al., 1979). Prior to applying the cells for adherence, the tissue culture dishes were pre-coated with fetal calf serum then rinsed twice with phosphate-buffered saline (pH 7.4) to maximize macrophage isolation (Kumagai et al., 1979). After the period of incubation, the non-adherent cells were decanted off the dishes, and the dish surface was rinsed twice with fresh McCoy's 5A + 10% FCS. The rinses were

combined, and washed as previously described. This population of cells represents the non-adherent fraction (NA). The NA cell population has been depleted of adhering macrophages and some lymphocytes (Gadberg et al., 1979). Further depletion of macrophages was effected by incubating the NA cells with carbonyl iron filings using 10 µg per 10^6 cells (Buick, Fry and Salmon, 1980). Phagocytic cells ingest the iron particles after which they are removed from the other cells by passage over a magnet. The resulting cell suspension is decidedly deficient in macrophages, and somewhat enriched for tumor cells (Buick, Fry, and Salmon, 1980). This cell collection is referred to as the non-adherent non-phagocytic (NA-NP) population.

Cells remaining adhered to the plastic tissue culture dishes were gently scraped free with a rubber policeman. These cells were washed once in McCoy's 5A + 10% FCS then adjusted to a cell concentration of 1×10^6 per ml.

These manipulations process a tumor specimen into four categories based upon macrophage and tumor cell content. The unfractionated specimen approximates the collection of cells as found *in situ*. The non-adherent cells are moderately depleted of macrophages; the non-adherent, non-phagocytic cells are largely depleted of macrophages. The adherent cell population (AD) is enriched for the *in situ* macrophage fraction.

Two experimental procedures were performed using the isolated cell fractions. In part, Chapter 3 will present the results of experiments exploring the effects of macrophage depletion and

reconstitution on tumor clonogenicity in soft agar. The other investigation was to evaluate each of the four cell fractions for prostaglandin synthetic activity *in vitro*.

The cell fractions were each adjusted to 1 X 10^6 cells per milliliter in McCoy's 5A + 10% FCS. Tubes containing the cells were incubated at 37°C, 5% CO_2, humidified air for 24 hours. The supernatant was isolated by centrifugation (600 x g, 10 min.) and then pouring the cell free fluid into polypropylene test tubes which were stored at -80°C until the time of prostaglandin assay.

Each isolated fraction of cells was prepared for morphological evaluation. The cells were processed using a cytocentrifuge (Shadron Cytospin, Rancon, Cheshire, England) which applies approximetaly 10^5 cells as a cell button onto a microscope slide. The glass slides were then fixed appropriately for morphological staining.

Three morphological schemes were used to evaluate the differential cell profile of the different populations. Dr. John Davis, Department of Pathology, AHSC, University of Arizona assisted in developing the routine differential criteria for the cell types.

Papanicolaou stain was performed on slides fixed in 95% ethanol (Luna, 1968). After fixation for a minimum of fifteen minutes the slides were transferred through 95% ethanol, 80% ethanol, 70% ethanol, 50% ethanol, then into tap water, and finally distilled water. The slides were then stained in Harris' hematoxylin for 2 to 3 minutes, followed by a gentle rinse in tap water. A brief pass through 0.25% hydrochloric acid in distilled water was used to differentiate the

stain, then subsequently set in running tap water for 5 minutes. Slides were rinsed in distilled water then transferred through 50% alcohol, 70% alcohol, 80% alcohol, and 90% alcohol prior to a two minute stain in orange G6 (Ortho Diagnostics, Raritan, NJ). Three rinses in 95% ethanol were followed by two minutes in eosin-azure 50 (Ortho Diagnostics, Raritan, NJ). This was then rinsed in three changes of 95% ethanol, two changes of 100% ethanol, and four changes of xylene. The slides were then coverslipped using Permount (Fisher Scientific Company, Fair Lawn, NJ) as mounting medium. Macrophages were identified by standard cytological criteria (Gompel, 1978): 15-60 μ diameter with a nuclear diameter between 7-10 μ ; the nucleus is usually irregular, with finely dispersed, dense chromatin; the cytoplasm is palely basophilic, characteristically containing vacuoles and cellular debris. Tumor cells were identified based on the following: an increased nucleocytoplasmic ratio, an abnormal and irregular chromatin distribution, the presence of abnormal and irregular appearing mitosis, multinucleation, and nucleolar hypertrophy.

Functional criteria assisted in evaluation of the macrophage presence in the different cell fractions. A non-specific esterase stain was employed to allow this enzymatic property of macrophages to express itself (Stuart, 1977). The method of Koski (Koski, Poplack, and Blaese, 1976) was modified to use alpha-naphthyl-acetate as indicator reaction (Appendix A). The slides were fixed in cold, buffered formaldehyde for 30 seconds, then rinsed in tap water, and distilled water. Air drying was allowed for 30 minutes followed by

45 minutes incubation in a solution of buffer, pararosaniline and alpha naphthylacetate. The slides were rinsed in distilled water, followed by counterstaining with methyl green. Non-specific esterase containing cells are easily distinguished by the presence of multiple intensely red-stained granules in the cytoplasm as compared with the green counterstain of the esterase-negative cells.

Finally, the phagocytic activity of the macrophages was used to discern their presence in the cell fractions. The method of Pelus (Pelus et al., 1981) was used (Appendix B). Briefly, the cells were incubated in media containing latex beads. Following two washes to remove non-phagocytized beads, the cells are prepared by cyto-centrifuge, fixed in 95% ethanol, air dried, then stained with Wright's stain (Harleco, Gibbstown, NJ). Those cells displaying five or more beads in the cytoplasm are counted as phagocytic.

Radioimmunoassay

Antiserum directed against prostaglandin E_2 (Sigma, St. Louis, MO) was used to quantitate PGE_2 by radioimmunoassay. Lyophylized sera was reconstituted in 10 ml phosphate buffer. The buffer was prepared by dissolving 8.34 gm $K_2HPO_4 \cdot 3H_2O$ and 1.84 gm KH_2PO_4 in 400 ml distilled water. To this was added 4.5 gm NaCl and 0.5 gm chopped gelatin. The buffer was heated mildly until all the components were in solution. After cooling to room temperature, 0.5 gm NaN_3 was added. The volume was increased to 500 ml and the pH adjusted to 7.4.

Tritiated prostaglandin E_2 was purchased from New England Nuclear (Boston, MA). The labeled molecule was 5,6,8,11,12,14,15-^{3}H(N)-prostaglandin E_2, with a specific activity of 130 Ci/mMole. The tracer was made to a concentration of 100 nCi/ml (280 pg/ml) in distilled water, and stored at -20°C.

Prostaglandin E_2 standard (Upjohn Co., Kalamazoo, MI) was prepared in double-distilled water at a concentration of 7.0 ng/ml. For calculation of the standard curve, PGE_2 was further diluted to concentrations of 3.5 ng/ml, 1.75 ng/ml, 0.70 ng/ml, and 0.35 ng/ml in double-distilled water.

Radioimmunoassay of standards and biological samples was done in duplicate without extraction of the prostaglandins from the biological samples. Two blank tubes (no prostaglandin or antiserum) were run in the assay to monitor radioactive background. The mean of the scintillation counts from these tubes was subtracted from all other counts. Two zero standard tubes (no cold prostaglandins) were run to determine the total binding of the radioactive PGE_2 by the antiserum. An equal aliquot of labeled PGE_2 was counted to determine the total radioactivity available for possible antiserum binding.

The assay was executed by adding 100 µl of standard or sample (or 1:10 and 1:100 dilutions of the sample) to borosilicate glass tubes (VWR Scientific Inc., San Francisco, CA). To this was added 100 µl of the tritiated standard and 100 µl of reconstituted antiserum. The tubes were mixed on a vortex mixer, incubated at room temperature for ten minutes, then placed in an ice bath for two hours.

The antiserum-bound labeled PGE_2 was separated from the free PGE_2 by incubation with dextran-coated charcoal, which was prepared by suspending 250 mg Norit A charcoal (Pfanstiehl Laboratories, Inc., Waukegan, IL) and 25 mg Dextran T70 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) in 100 ml phosphate buffer. A magnetic stir-bar was used to promote and maintain the materials in suspension. The dextran-coated charcoal was kept at 4°C. One hundred microliters of the coated charcoal was added to each of the assay tubes, followed immediately by vortex mixing. The assay tubes were incubated for 10 minutes in an ice bath after which they were centrifuged (3000 x G, 5 min) to sediment the charcoal.

Three hundred microliters of the supernatant were quantitatively removed from the assay tube by pipette, and transferred to plastic scintillation vials, to which was added 10 ml scintillation solution. The scintillation solution was prepared by dissolving 5.3 gm PPO (2,5-diphenyloxazole)(Sigma, St. Louis, MO) and 0.666 gm POPOP (1,4-bis-2-(4-methyl-5-phenyloxazoyl)-benzene)(Packard Instrument Co., Downers Grove, IL) in a mixture of 660 ml toluene and 340 ml Triton X-100.

The radioactivity in the supernatant was measured using a scintillation counter (Beckman LS-230, Anaheim, CA). Each tube was counted for 10 minutes, and the radioactivity recorded as counts per minute.

The prostaglandin content of each assay tube was quantitated by the amount of tritiated prostaglandin which was displaced from binding

to the antiserum. Correction was made for free, labeled PGE_2 which did not adsorb to the dextran-coated charcoal by subtracting the counts from the blank tubes from all other counts. The actual calculation of PGE_2 content was based on the calibration curve which was constructed by plotting the percent of total radioactivity bound to the antiserum versus the concentration of known standard PGE_2. As PGE_2 content increased, the relative percentage of labeled ligand bound to the antiserum decreased.

Testing performed by the antiserum supplier reported that the cross-reactivity of the PGE_2-antiserum with other prostaglandins was as follows: PGA_1 0.02%; PGA_2 0.20%; PGB_1 0.01%; PGB_2 0.01%; PGE_1 3.2%; $PGF_{1\alpha}$ 0.01%; and $PGF_{2\alpha}$ 0.06%.

Gas Chromatography

Supernatants from ascitic tumor specimens and from 24 hour liquid culture incubations of various cell populations were analyzed for prostaglandin content. Following removal of the incubated cells by centrifugation (600 x g, 10 minutes) the prostaglandins were extracted from the fluid using a modification of the procedure developed by Powell (Powell, 1980). A 1.0 ml aliquot of the fluid to be extracted was prepared by adjusting the pH to 4.0 using 4% formic acid (v/v) (Goswami et al., 1981; Valenzula and Harper, 1976). The fluid was then applied to a methanol and water conditioned Bond-Elut octadecylsilyl silica column (Analytichem International, Harbor City, CA). The column was rinsed under vacuum with 10 ml distilled water followed

by 10 ml 15% ethanol in water (v/v) to remove polar substances. A 10 ml hexane rinse was used to remove nonpolar lipids and fatty acids. Finally, two 1.0 ml rinses of methyl formate (Eastman Kodak Co., Rochester, NY) were used to elute the prostaglandins which were collected in a silanized test tube. The eluting solvent was removed by evaporation under vacuum by means of a Speed-Vac Concentrator (Savant Instruments Inc., Hicksville, NY).

Tritiated prostaglandin E_2 was used to evaluate the efficiency of extraction from biological fluids. Extraction recovery was determined by comparison of the scintillation counts in a preextracted aliquot with those of an extracted portion. Tritiated prostaglandin E_2 (130 Ci/mMole) was prepared as stock solutions in ethanol at concentration of 280 pg/ml. One hundred microliters were quantitatively aliquoted into three scintillation vials. The alcohol was evaporated under a nitrogen stream at room temperature, then 10 ml scintillation cocktail (see Radioimmunoassay) were added. Equal 100 µl aliquots of ^{3}H-PGE$_2$ were pipetted into borosilicate glass test tubes; the alcohol was dried under a nitrogen stream at room temperature. One milliliter of McCoy's 5A culture media enriched with 10% fetal calf serum was added to each test tube and the biological fluid was extracted as described above. The methy formate elution was collected in a scintillation vial, and the solvent was evaporated under nitrogen at room temperature. Ten milliliters of scintillation cocktail was added to each vial, and radioactivity determined in a scintillation counter (Beckman LS-230, Anaheim, CA). The radioactivity recovered in

the extracted sample (mean of triplicate trials) was divided by the radioactivity of the pre-extracted aliquot to yield an expression of percent recovery.

For gas chromatographic analysis, the extracted prostaglandins were derivatized to impart chromatographic stability and detector responsiveness. Prostaglandin E_1, PGE_2 and 6-Keto-$PGF_{1\alpha}$ (the spontaneous breakdown product of PGI_2) were converted to the pentafluorobenzyl ester-methoxime-trimethylsilyl ether derivatives (PFB-MOX-TMS) to assist stability of the ketone group in the cyclopentane ring; the other prostaglandins were prepared as pentafluorobenzyl ester-trimethylsilyl ether compounds (PFB-TMS).

The pentafluorobenzyl esters of the carboxylic acid moiety were generated according to the method of Wickramasinghe and Shaw (1974). Briefly, the dried extract was dissolved in 10 µl of pentafluorobenzyl bromide (Pierce Chemical Co., Rockford, IL) in acetonitrile (1:2, v/v) to which was added 10 µl of diisopropylethylamine (Aldrich, Milwaukee, WI) in acetonitrile (1:7, v/v). This solution was vortexed and heated at 40°C for five minutes. The reaction solution was dried under a stream of nitrogen at room temperature and the esterification procedure repeated. Excess derivatizing reagents were evaporated under nitrogen at room temperature.

The keto group at the C_9 position of the E-series prostaglandins and at the C_6 position of 6-keto-$PGF_{1\alpha}$ require derivatization to retard keto-enol isomerization during subsequent derivatization steps (Chiabrando et al., 1980). Methoximation proved acceptable for

enhanced chromatographic stability of these compounds (Middleditch and Desiderio, 1972; Fitzpatrick, Wynalda, and Kaiser; Wickramasinghe, 1973). No deleterious effects were seen on the other prostaglandin standards tested. To produce the methoxime derivative of the keto group the dried residue from the pentafluorobenzyl ester step was dissolved in 50 µl of a saturated solution containing methoxylamine HCL (Supelco, Bellefonte, PA) in pyridine (approximately 4%, w/v). The reaction mixture was heated at 60°C for 60 minutes. Excess pyridine was removed by evaporation under a nitrogen stream at room temperature. Any remaining derivatizing reagents were removed by partitioning the reactants twice between hexane (1.0 ml) and water (1.0 ml, pH 3.5) (Fitzpatrick et al., 1979). The combined hexane phases were evaporated under a nitrogen stream in one milliliter reacti-vials.

Trimethylsilyl ether derivatives of the hydroxyl functional groups on the prostaglandins were fashioned by reaction with 100 µl bis-trimethylsilyl-trifluoroacetamide (BSTFA)(Sigma Chemical Co., St. Louis, MO)(Vane and Horning, 1969). The final derivatized product was chromatographed via 1.8 µl injections from a 10 µl Hamilton syringe, model 701N (Pierce Chemical Co., Rockford, IL).

Verification of the derivatization reactions was examined using prostaglandin standards on a Finnigan 3300 gas chromatograph-mass spectrometer (Finnigan, Inc., Cincinnati, OH). Chromatographic conditions were: injector 270°C; column temperature 200 to 300°C at a rate of 10°C/min; helium carrier gas was used at a flow of 30 ml/min through

the 2 meter x 2 mm glass column packed with OV 101 on 100/120 GCQ (Alltech, Deerfield, IL). The mass spectrometer parameters were: ion source, 25 eV; electron multiplier 2200 volts; emission current 5 amps, glass jet separator 300°C.

Routine chromatographic analysis of the biological samples was performed using an HP 5880A gas chromatograph (Hewlett-Packard, Englewood, CO) with a fused silica glass capillary column, 25 meter, 0.2 mm i.e., OV 101 WCOT (Scientific Glass Engineering, Ringwood, Victoria, Australia). A ^{63}Ni electron capture detector was operated at 300°C; the all glass-lined injection port was maintained at 250°C. Column oven temperature programming was used to optimize resolution and sensitivity of the assay, and a purged-splitless injection technique was employed to enhance trace analysis of the prostaglandins. The injector was operated in the "splitless" mode (the entire injected, vaporized sample was applied to the column) for a period of sixty seconds, after which the injector was commanded by a run program to shift to the split mode (approximately 200:1 split). The splitless period of one minute allowed optimum transfer of the analytes from the injector to the column; the split shift served to vent any lingering solvent in the injector away from the column to reduce solvent-tailing in the chromatogram (Freeman, 1981).

The column oven was programmed to operate at a temperature suitable to function as a solvent cold-trap of the injection. Using hexane as the solvent of injection, the oven temperature was 50°C (Freeman, 1981). The column temperature was increased at a rate of

20°C/min to 200°C; a second temperature program increased the column oven at 10°C/min to 250°C where isothermal conditions remained for the duration of the run. These temperature rates were found to be within the reproducible, linear functions of the hardware.

Dilutions of authentic standards were used for determination of minimum detection limits of the prostaglandins. The HP5880A was connected to an HP3390A Integrator (Hewlett-Packard, Englewood, CO) which monitored retention times of the chromatographic peaks, and integrated the signal to generate an output of peak area and peak height.

Repeated injections from the same vial of standards were done to evaluate both the stability of the derivatives and to measure the precision of the assay. Arithmetic dilutions of authentic standards were prepared over an approximate three log range (10 pg to 10 ng). Gas chromatographic analysis of standard preparations were used to verify the linearity of detection. Likewise, dilutions of standards were dissolved in culture media, extracted, derivatized, and analyzed to monitor the linearity of extraction.

Finally, biological specimens were prepared using the same technique described above. Signal responses of chromatographic peaks corresponding to standard retention times were compared with standard curves to quantitate the prostaglandins in the biological fluids.

Results

PGE Radioimmunoassay

Seventeen human carcinoma samples were processed into single cell suspension, then fractionated into the four cell categories (UNF, NA, NA-NP, AD) and studied for prostaglandin synthesis in vitro (Table 1). Twelve samples were analyzed using radioimmunoassay, five by gas chromatography, and one using both methods. The radioimmunoassay was used during the period in which the gas chromatography assay was under development. A representative standard curve from the assay is shown in Figure 4. The sigmoidal curve spans a three log range of PGE_2 concentration with a linear segment covering an approximate one log range of 0.10 to 1.00 picomoles/ml. Each of the data points on the curve represents the mean of duplicate measurements. As can be appreciated from the standard curve, the binding of labeled PGE_2 by antiserum when zero competition exists is approximately 23%. This value fluctuated from 16% to 49% between assays and lots of antisera.

The values for PGE measured by radioimmunoassay in the supernatants of cell populations is shown in Table 2. The range of values is large, from 0.4 to 704 ng/ml/10^6 cells/24 hours. Several observations can be made on the results of PGE production by the cell fractions studied. First, depletion of the adherent and the phagocytic cells greatly diminished the prostaglandin E synthesis of the remaining (NA-NP) cells compared with the unfractionated cells. PGE synthesis by the NA-NP cells was less than the UNF in 10 of 12 cases, and equal in one case. Secondly, the adherent cells (AD) produced more PGE than

Table 1. Patient population analyzed for prostaglandin production.

Patient	Tumor Type	PG. Assay		Adherent Cells
		RIA	GC	%
A	Breast	X		7
B	Breast	X		9
C	Ovarian	X		26
D	Ovarian	X		10
E	Breast	X		12
F	Parotid	X		9
G	Endometrial	X		2
H	Ovarian	X	X	15
I	Breast	X		39
J	Ovarian	X		6
K	Pancreas	X		10
L	Ovarian	X		20
M	Ovarian		X	6
N	Endometrial		X	2
O	Ovarian		X	2
P	Ovarian		X	*
Q	Ovarian		X	*

* = Not evaluated.

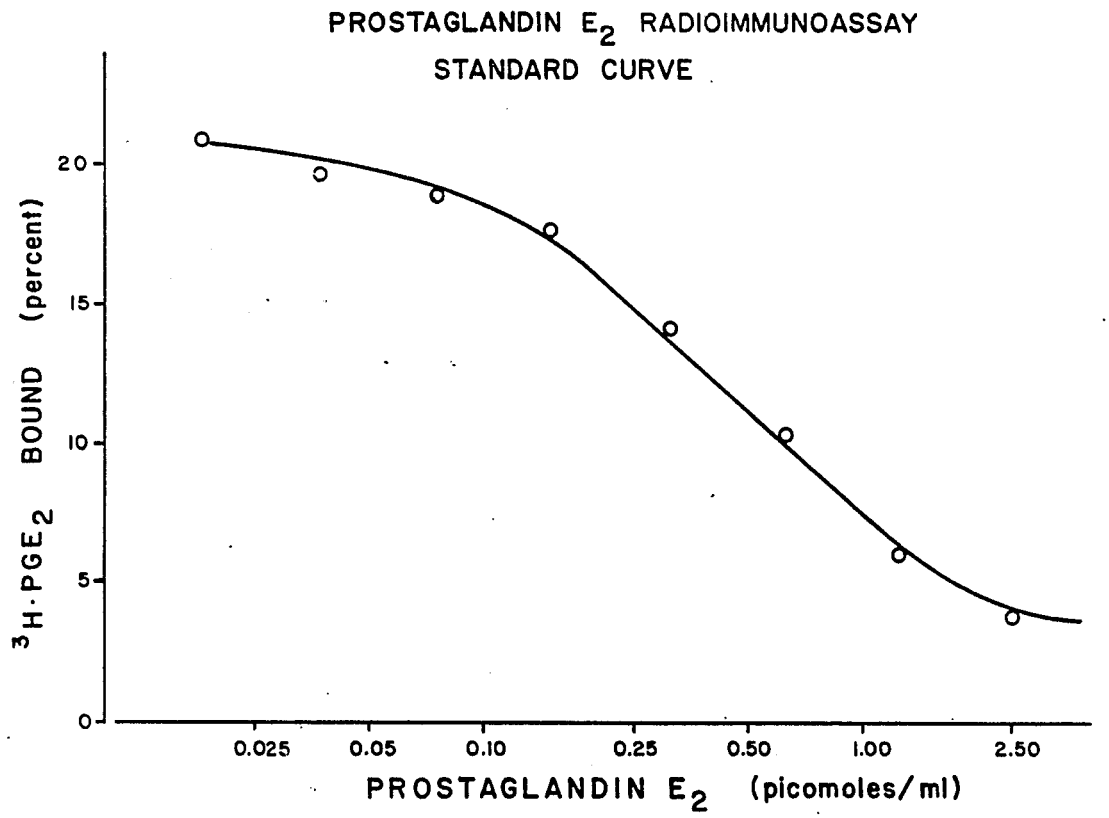

Figure 4. Standard curve for PGE radioimmunoassay.

Each point is the mean of duplicate determinations.

Table 2. Prostaglandin E_2 Production* by Cell Fractions from Human Tumors. -- ($ng/ml/10^6$ cells/24 hours).

Patient	Cell Population			
	UNF	NA	NANP	AD
A	11.0	8.7	0.3	14.3
B	1.9	1.2	0.4	1.1
C	15.5	2.8	0.7	7.5
D	13.5	0.7	2.8	107.1
E	5.3	33.7	3.9	98.7
F	137.4	140.0	8.1	84.6
G	130.4	78.2	45.1	125.1
H	4.3	5.3	1.5	1.8
I		16.2	1.2	105.7
J	1.9	0.9	0.7	0.4
K	253.7	355.9	246.7	704.8
L	13.0	10.9	13.0	15.9

* Determined by radioimmunoassay.

the NA-NP cell in 10 of 12 cases, the same amount in one instance, and less than the NA-NP in only one sample. These observations are amenable to statistical evaluation using the Friedman's 2-way analysis of variance (Table 3). Paired comparisons were done of the rank of value (I, II, III, IV) based on relative amount of PGE in each of the four groups per patient. This analysis showed that the NA-NP group is statistically different from the UNF and the AD fractions with p 0.01. These data provide evidence that the major source of PGE production in vitro from human tumors is usually the adherent macrophage population. More detailed investigation into prostaglandin synthesis by cell fractions from human tumors was done using gas chromatography analysis.

Extraction of Prostaglandins

In order to prepare biological samples for analysis by gas chromatography, the prostaglandins were extracted into organic solvent. The recovery of $^{3}H \cdot PGE_2$ from culture media using octadecylsilyl silica was 95.56 ± 2.77% (Table 4). Standard prostaglandin E_1, E_2, $F_{2\alpha}$ and I_2 were tested for extraction recovery using the same technique (Figure 5, Table 5). The chromatograms of the standard prostaglandins and the extracted standards were clean, with baseline resolution of each compound. The chromatographic analysis of the extracted standards also showed little, if any, interference by contaminants from the culture media. The recovery for PGF_1 , PGE_2 and PGE_1 were acceptable. Prostacyclin (PGI_2) was poorly extracted from the media via the octadecylsilyl silica, however the recovery was sufficient to allow detection to below 100 picograms from extracted material (see below).

Table 3. Statistical analysis.

	UNF	NA	NA-NP	AD
A	11.0 (III)	8.7 (II)	0.3 (I)	14.3 (IV)
B	1.9 (IV)	1.2 (III)	0.4 (I)	1.1 (II)
C	15.5 (IV)	2.8 (II)	0.7 (I)	7.5 (III)
D	13.5 (III)	0.7 (I)	2.8 (II)	107.1 (IV)
E	5.3 (II)	33.7 (III)	3.9 (I)	98.7 (IV)
F	137.4 (III)	140.0 (IV)	8.1 (I)	84.6 (II)
G	130.4 (IV)	78.2 (II)	45.1 (I)	125.1 (III)
H	4.3 (III)	5.3 (IV)	1.5 (I)	1.8 (II)
I	N.A.	16.2 (III)	1.2 (II)	105.7 (IV)
J	1.9 (IV)	0.9 (III)	0.7 (II)	0.4 (I)
K	253.7 (II)	355.9 (III)	246.7 (I)	704.8 (IV)
L	13.0 (III)	10.9 (I)	13.0 (II)	15.9 (IV)
	5(IV) 20	1(IV) 4	0(IV) 0	6(IV) 24
	4(III) 12	6(III) 18	0(III) 0	2(III) 6
	2(II) 4	3(II) 6	4(II) 8	3(II) 6
		2(I) 2	8(I) 8	1(I) 1
	36	30	16	37
	UNF vs NA-NP	$p < 0.01$		
	NA-NP vs AD	$p < 0.01$		

Table 4. ^{3}H-PGE_2 Recovery by Octadecylsilyl Silica Extraction.

		Counts per Minute
Pre-extract		89659.6
		89171.2
	$\bar{x}$	89415.4
Post-extract		81186.0
		83934.6
		89124.8
		86459.8
		85208.6
		86744.4
		85447.2
	$\bar{x}$	85443.6
		± 2474.4

$$\text{Coefficient of variation} = \frac{S.D}{\bar{x}} \times 100 = 2.896\%$$

$$\text{Recovery: } \frac{85443.6}{89415.4} \times 100 = 95.56\%$$

$$\frac{85443.6 \pm 2474.4}{89415.4} \times 100 \mp 95.56 = 2.77\%$$

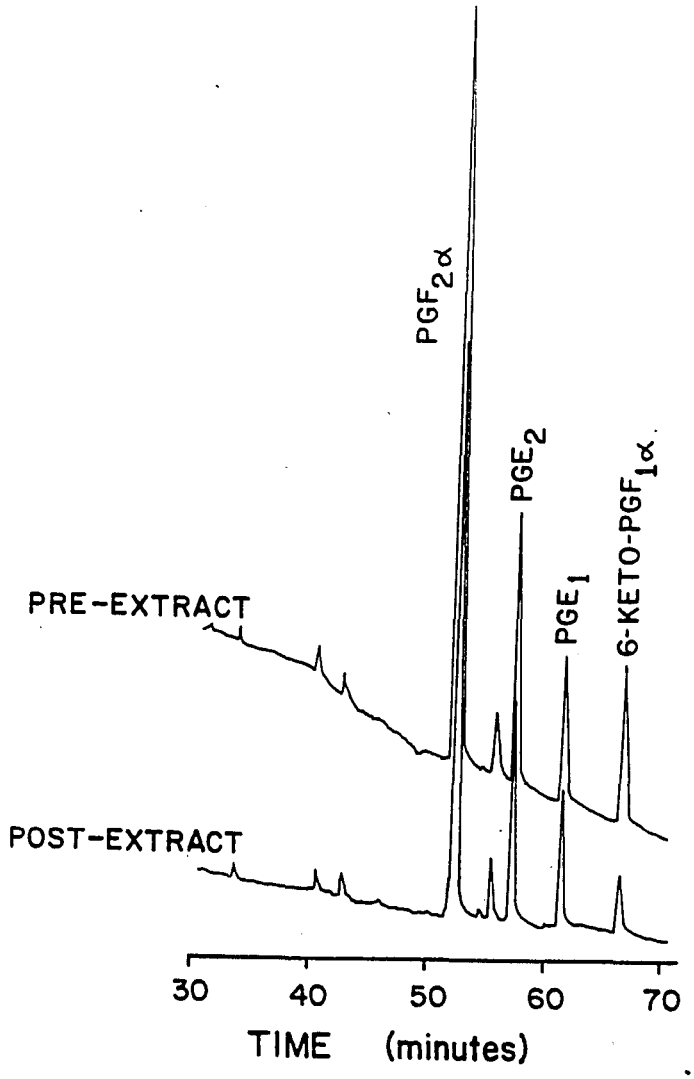

Figure 5. Chromatogram of pre-extracted and extracted prostaglandin E_1, E_2, $F_{2\alpha}$, and I_2, extracted from McCoy's 5A +10% FCS using reverse phase octadecylsilyl silica columns.

Derivatization was as described in Materials and Methods.

Table 5. Recovery of Prostaglandins by C18 Extraction.

Prostaglandin	Peak Area		% Recovery
	Pre-extract	Post-extract	
$F_{2\alpha}$	8362800	6053600	72
E_2	2962100	2410600	81
E_1	1762000	1638200	93
I_2	2399300	881050	37

Gas Chromatography and Mass Spectral Confirmation of Derivatives

The preparation of derivatives of the prostaglandins amenable to analysis by gas chromatography with electron-capture detection was verified by mass spectral analysis (Figures 6, 7, 8, 9). The total ion current from the spectrometer was plotted against scan number, and the major peaks which eluted were then plotted as mass spectra. Figure 6 shows the mass spectrum of PGE_1-pentafluorobenzyl ester-methoxylamine-trimethylsilyl ether (PGE_1-PFB-MOX-TMS). The molecular ion of 707 is below detection abilities of the instrument, however, characteristic fragment ions do appear which confirm the molecule as the standard compound (Table 6). The mass of 676 represents the loss of the methoxyl group from the methoxylamine derivative: 534 arises from the loss of the C_{16}-C_{20} alkyl chain and its accompanying trimethylsilyl ether group; 463 occurs as the molecular ion decomposes, loosing C_{13}-C_{20} with its trimethylsilyl ether in addition to the loss of the methoxylamine group; the ion signal at 237 is characteristic of the fragment arising at the C_1 position with the accompanying pentafluoro-benzyl ester group. Similar fragmentation patterns appear for PGE_2-PFB-MOX-TMS (Fig. 7). Here the molecular ion is detectable at 705; other characteristic peaks are seen at 675, 532, 461, and 237 resulting from the comparable fragment ions described for PGE_1 (Table 6).

The mass spectrum of $PGF_{2\alpha}$-PFB ester-TMS ether is shown in Figure 8. The molecular ion of $PGF_{2\alpha}$ is present at 751. Characteristic fragment ions are: 680, due to the loss of C_{16}-C_{20}; 661, due to the loss of trimethylsilyl ether groupl 645, arises from the loss of both a

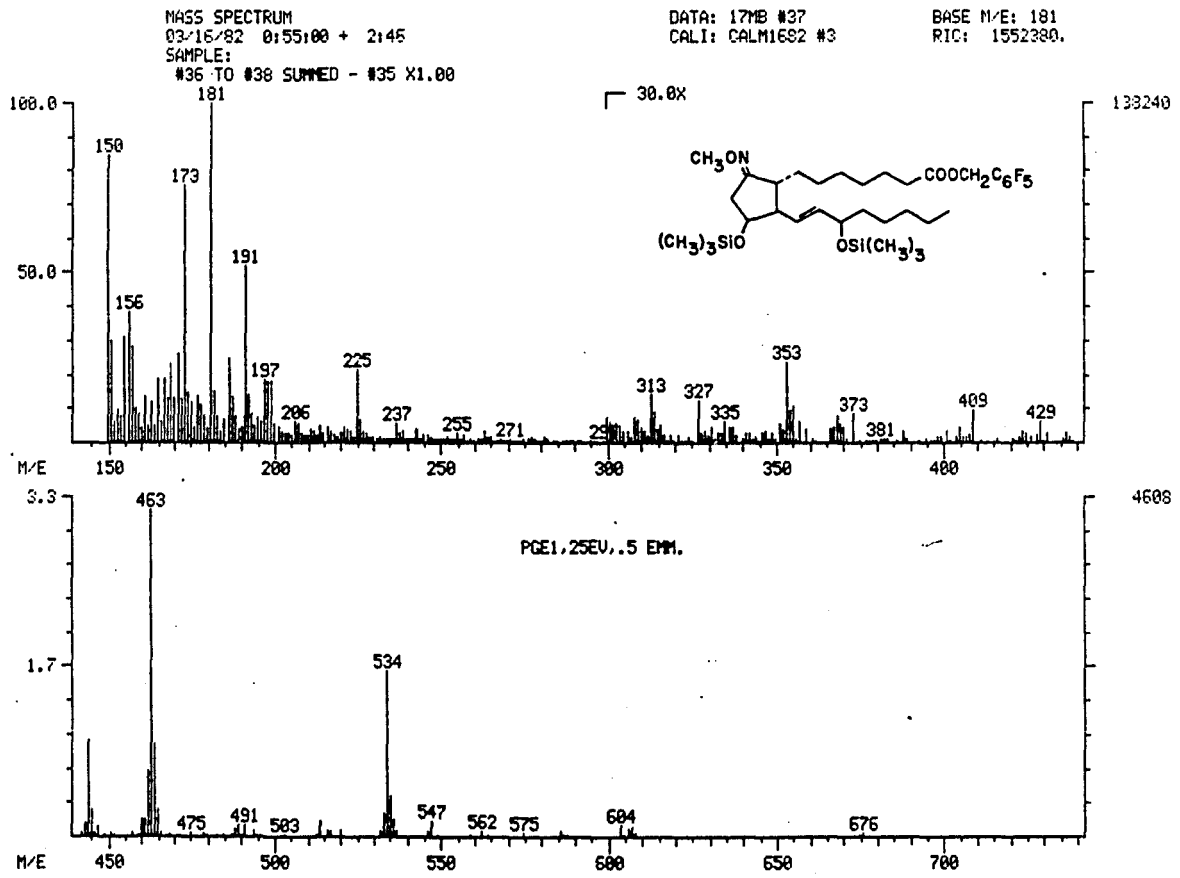

Figure 6. Mass spectrum of PGE_1-pentafluorobenzyl ester-methoxine-trimethylsilyl ether.

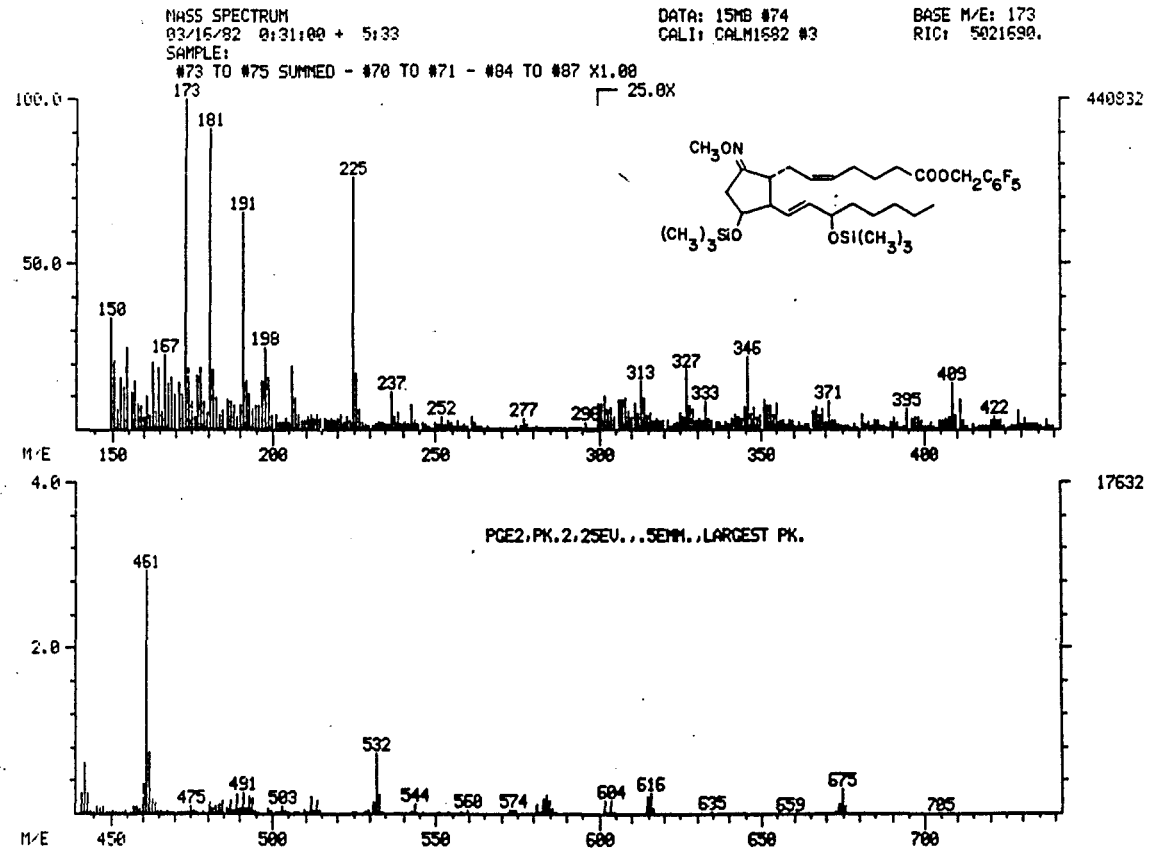

Figure 7. Mass spectrum of PGE_2-pentafluorobenzyl ester-methoxine-trimethylsilyl ether.

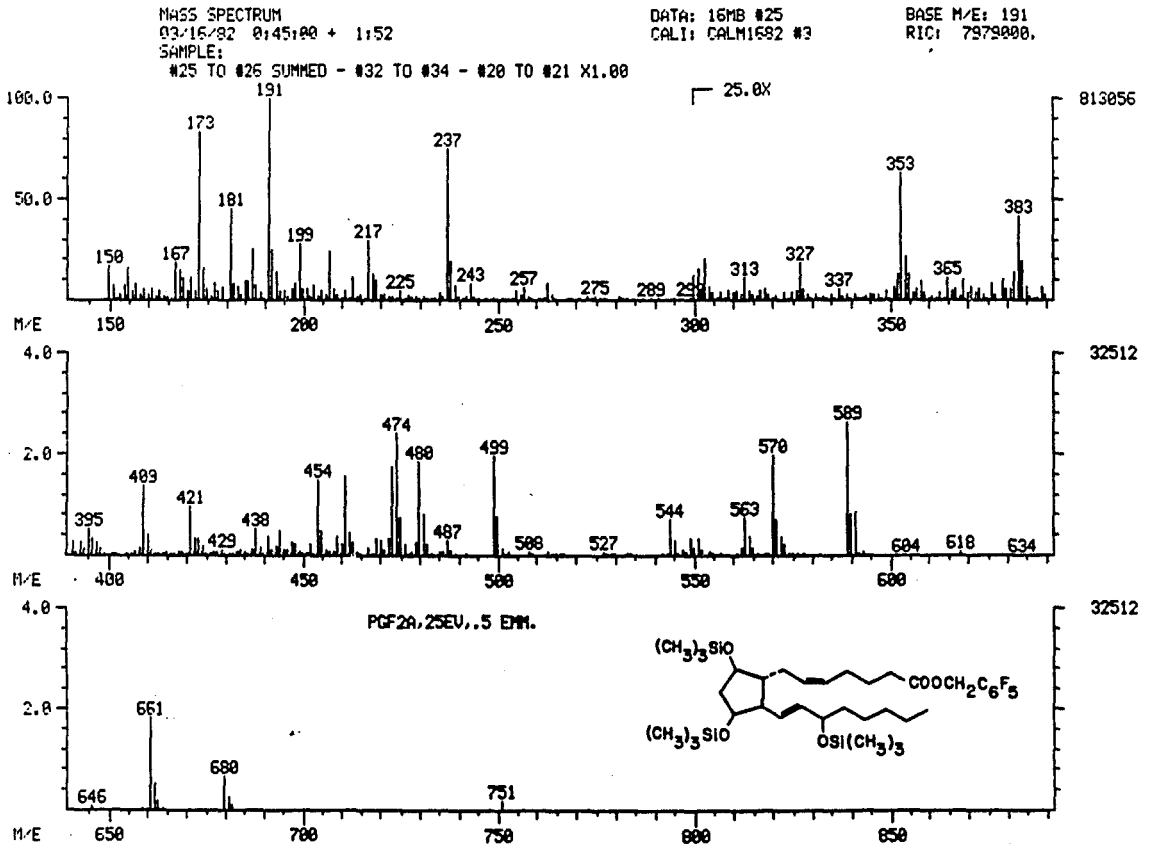

Figure 8. Mass spectrum of $PGF_{2\alpha}$-pentafluorobenzyl ester-trimethylsilyl ether.

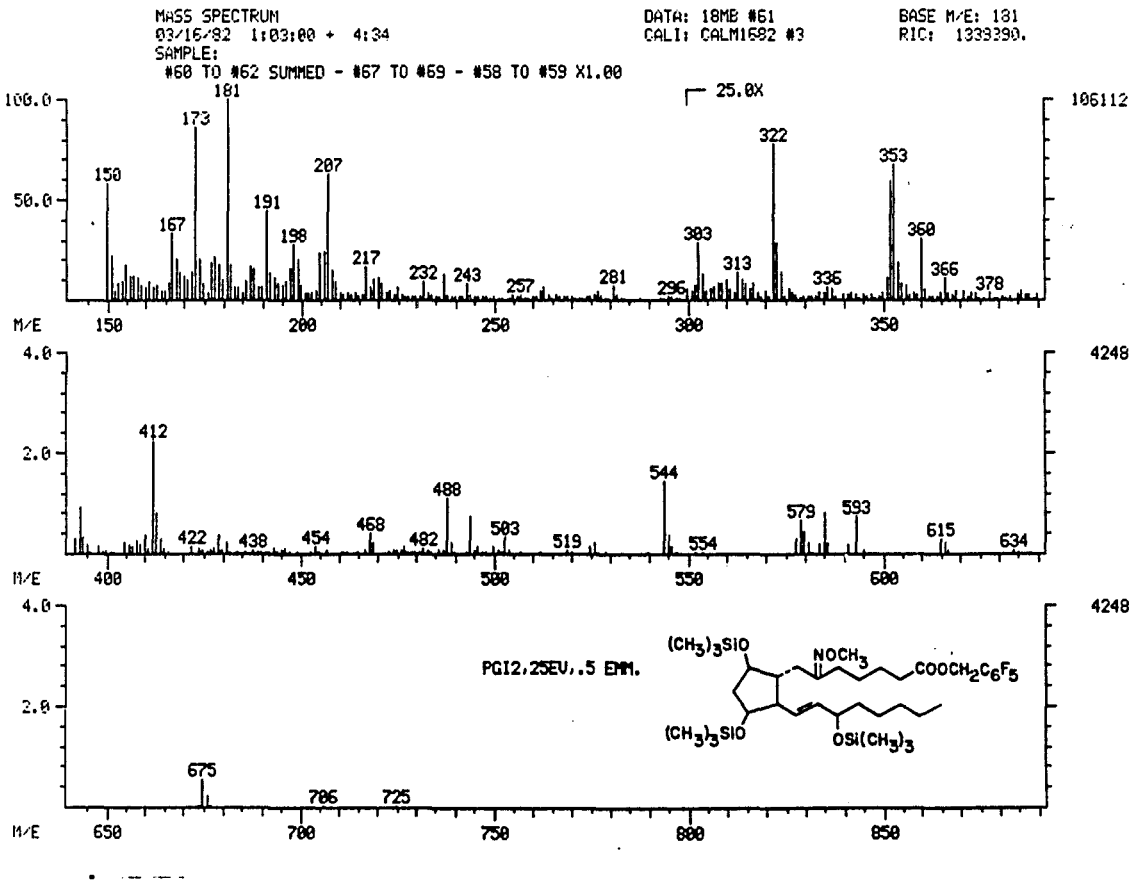

Figure 9. Mass spectrum of 6-Keto-$PGF_{1\alpha}$-pentafluorobenzyl ester-methoxine-trimethylsilyl ether (spontaneous hydrolysis product of PGI_2).

Table 6. Partial Mass Spectra of Various Prostaglandins as Pentafluorobenzyl Ester-(Methoxamine)-Trimethylsilyl Ether Derivatives.

Fragment	PGE_1	PGE_2	$PGF_{2\alpha}$	PGI_2
$[M]^+$	707 (-)	705 (+)	750+1 (+)	795+1 (-)
$[M-15]^+$	692 (-)	690 (-)	736 (-)	781 (-)
$[M-31]^+$	676 (+)	674 (+)	720 (-)	765 (-)
$[M-71]^+$	636 (-)	634 (+)	680 (+)	725 (+)
$[M-90]^+$	617 (-)	615 (+)	661 (+)	706 (+)
$[M-(90 + 15)]^+$	602 (+)	600 (-)	646 (+)	691 (-)
$[M-(90 + 71)]^+$	546 (+)	544 (+)	590 (+)	635 (+)
$[M-173]^+$	534 (+)	532 (+)	578 (-)	623 (-)
$[M-(2x90)]^+$	527 (-)	525 (+)	571 (+)	616 (+)
$[M-(199 + 45)]^+$	463 (+)	461 (+)	N.A.	552 (+)
$[M-((2x90) + 71)]^+$	456 (+) 237 (+)	544 (+) 237 (+)	500 (+) 237 (+)	545 (+) 237 (+)

Fragment origins: M (molecular ion), M-15 (loss of CH_3), M-31 (loss of CH_3O), M-71 (loss of C_{16}-C_{20}), M-90 (loss of TMS-OH), M-(90 + 15) (loss of TMS-OH and CH_3), M-(90 + 71) (Loss of TMS-OH and C_{16}-C_{20}), M-173 (loss of C_{15}-C_{20} with accompanying TMSO), M-(2x90)(loss of 2xTMSO), M-(199 + 45)(loss of C_{13}-C_{20} with accompanying TMSO and CH_3ON), M-((2x90) + 71)(loss of 2xTMSOH and C_{16}-C_{20}), ion 237 arises from the fragment generated by the C_1 plus PFB-ester derivative.

trimethylsilyl ether group and a methyl group from one of the three methyls of a TMS group; 589 occurs by the dual loss of C_{16}-C_{20} and a trimethylsilyl ether; 570 represents the molecular ion less two trimethylsilyl ether groups; 237 corresponds to the C_1 plus its pentafluorobenzyl ester (Table 6).

Figure 9 shows the mass spectrum of 6-keto-PGF_1-PFB-MOX-TMS. The molecular ion at 796 is absent. Mass 725 is the fragment generated by the loss of C_{16}-C_{20}; 706 is the result of the loss of a trimethylsilyl ether group; ion 675 is due to the loss of the methoxy groups and a trimethylsilyl ether group; mass 634 results as C_{16}-C_{20} and a trimethyl group leave the molecular ion; 615 is generated by the loss of two TMS ether groups; and finally, 237 is characteristic of the fragment C_1 plus the pentafluorobenzyl ester group (Table 6).

After verification of the derivatization procedure for the prostaglandins, the chromatographic resolution was evaluated using a 25 meter, 0.2 mm i.d. FSOT, OV-101 capillary column. A mixture containing 250 nanograms each of PGA_1, PGA_2, PGE_1, PGE_2, PGF_2, and PGI_2 was prepared, and derivatized as previously described. The final product was dried under nitrogen then dissolved in 100 µl hexane (final concentration 2.5 ng/ml). Splitless injections of 1.8 µl were done to generate the chromatogram shown in Figure 10. Baseline resolution of the prostaglandins was accomplished in a 70 minute run. This standard mixture was diluted 1:10 and 1:100 with hexane. 1.8 µl injections were chromatographed (Figures 11 and 12, respectively). These represent the analysis of 450 and 45 picograms of each of the

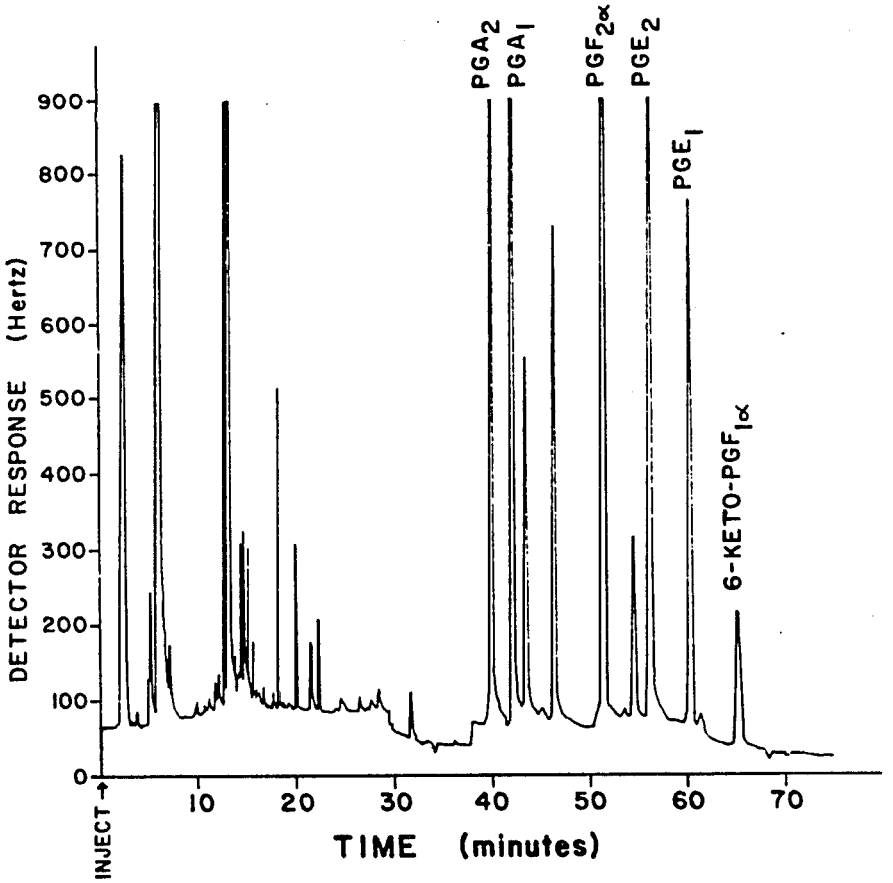

Figure 10. Fused silica capillary gas chromatography of prostaglandins A_1, A_2, E_1, E_2, $F_{2\alpha}$, and I_2.

Derivatization was as described in the text. Chromatography: 25 meter x 0.2 mm i.d. OV-101; Helium carrier gas at 44 cm/sec; make-up gas: Argon:Methane, 95:5 at 18 ml/min; electron capture detector 30°C; injector 25°C; column over 50°C for 60 sec, 20°C/min to 200°C then 10°C/min to 250°C; 4.5 nanograms of each compound injected.

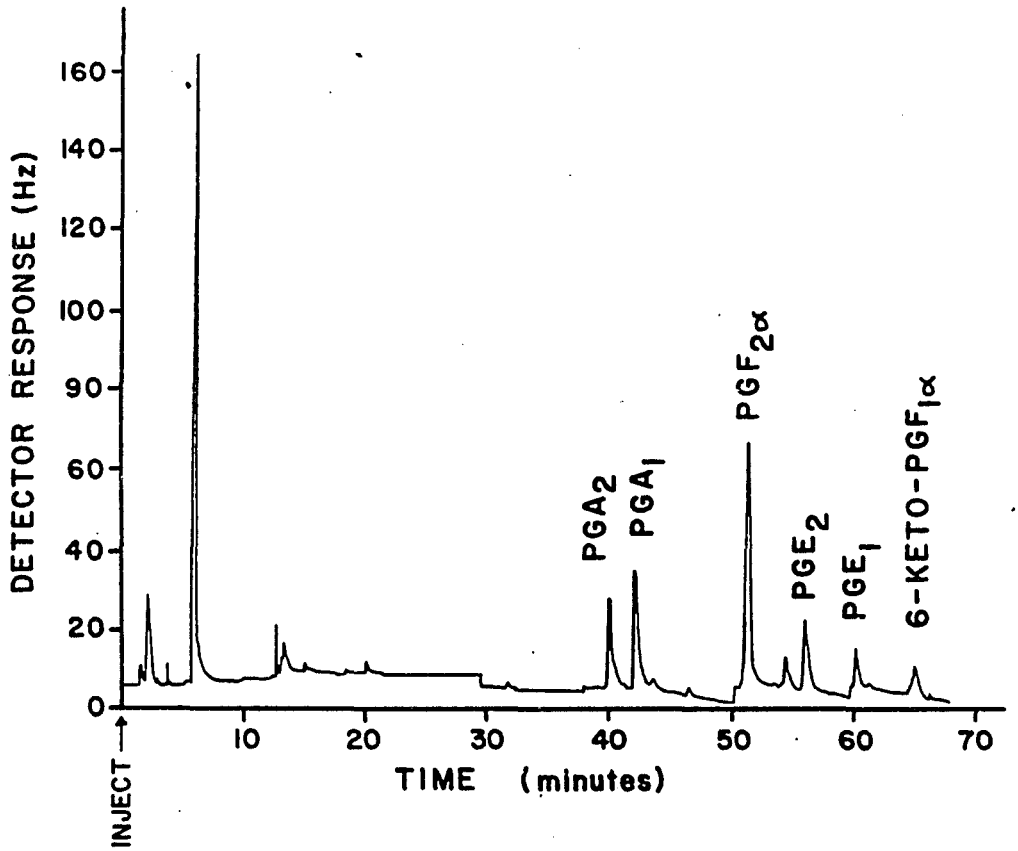

Figure 11. Fused silica capillary gas chromatography of prostaglandin standards.

Conditions as in Figure 10. 450 picograms injected on column.

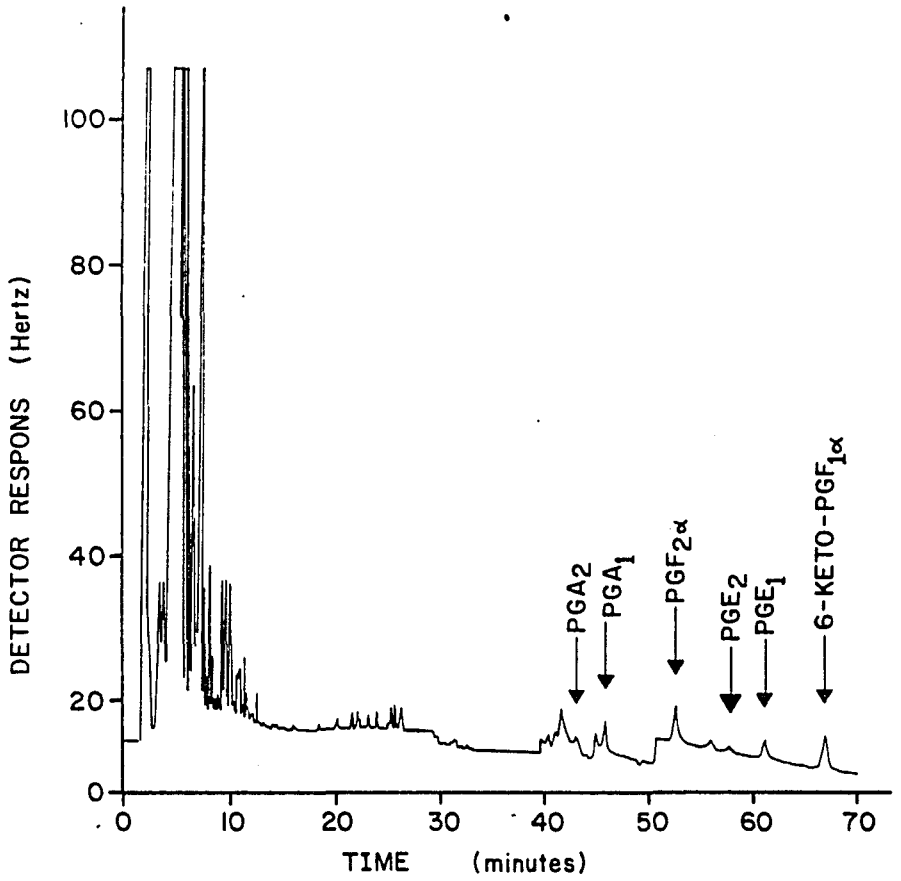

Figure 12. Fused silica capillary gas chromatography of prostaglandin standards.

Conditions are as in Figure 10. 45 picograms injected on column.

analytes on column, respectively. The lower limits of detection were approximately 25 picograms on column with a signal-to-noise ratio of 20:1. A blank derivatization procedure was carried out with the solvent used to dissolve the prostaglandins. The results of chromatography of this material is shown in Figure 13. In the region which corresponded to the elution of the prostaglandins, there were no extraneous peaks attributable to the solvent, derivatization reagents, or other contaminants.

Figure 14 shows the linearity of the electron capture detector response to equivolumetric injections of various amounts of derivatized PGE_2. The resultant line correlates well with the data points, $r = 0.99842$; the slope is 38.96 ($\times 10^4$) area units per picogram PGE_2; the y-intercept is -1.447×10^4 area units. The linearity of response has been tested over a three log range of prostaglandin E_2 with equally acceptable linearity.

Four replicate injections of 450 picograms each of derivatized PGE_1, PGE_2, $PGF_{2\alpha}$, and PGI_2 were done to evaluate the precision of the electron capture detector signal, and to measure the precision of the fused silica capillary column (Table 7). The capillary column performed extremely well in reproducible resolution of the prostaglandin standards. The detector also responded with good reproducibility.

To determine the linearity of extraction, each of the prostaglandins was dissolved in culture media enriched with 10% fetal calf serum, then extracted and derivatized. The analysis of this

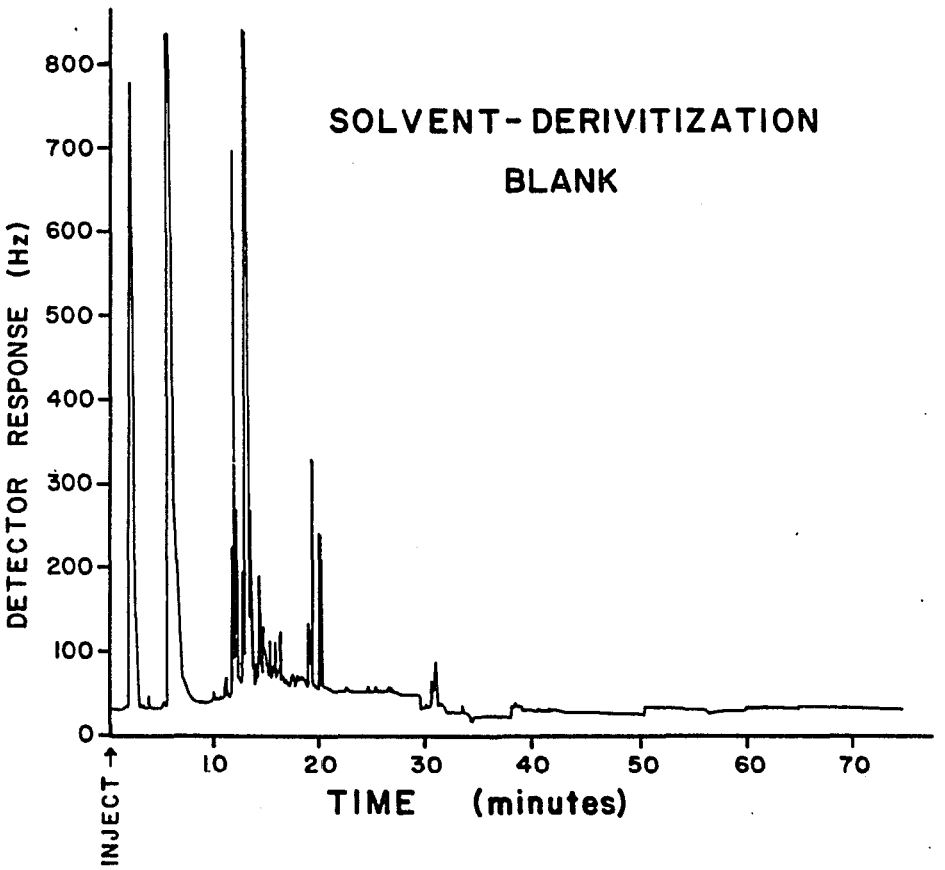

Figure 13. Fused silica capillary gas chromatography of solvent used for prostaglandin standards.

Chromatographic conditions are as in Figure 10.

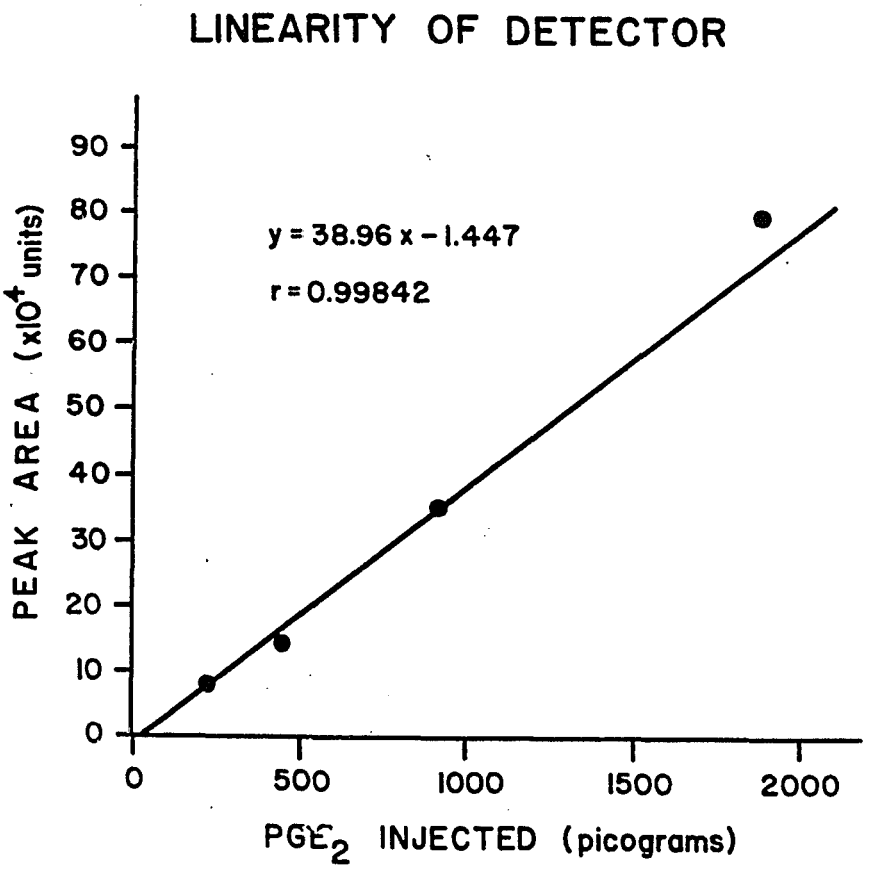

Figure 14. Linearity of electron capture detector response to equivolumetric injections of increasing concentrations of PGE_2-PFB-MOX-TMS.

Chromatographic conditions are as in Figure 10.

Table 7. Precision of Capillary Chromatography and Electron Capture Detection of Prostaglandins.*

	Retention time (min)	Peak height (mm)
$PGF_{2\alpha}$	52.39	73.0
	52.39	67.0
	52.37	73.0
	52.39	75.0
	$\bar{x}$ = 52.385 ± 0.01	$\bar{x}$ = 72.0 ± 3.46
	C.V. = 0.019%	C.V. = 4.81%
PGE_2	57.32	23.0
	57.32	22.0
	57.30	27.5
	57.31	28.5
	$\bar{x}$ = 57.313 ± 0.00957	$\bar{x}$ = 25.25 ± 3.22
	C.V. = 0.017%	C.V. = 12.78%
PGE_1	61.50	29.5
	61.50	29.0
	61.49	32.5
	61.50	39.0
	$\bar{x}$ = 61.4975 ± 0.005	$\bar{x}$ = 32.5 ± 4.60
	C.V. = 0.008%	C.V. = 14.15%
PGI_2	66.52	68.5
	66.53	63.0
	66.50	71.5
	66.49	74.0
	$\bar{x}$ = 66.51 ± 0.01826	$\bar{x}$ = 69.25 ± 4.73
	C.V. = 0.027%	C.V. = 6.84%

* 450 picograms injected.

parameter of the assay is given in Figures 15 through 18. Both peak area and peak height are plotted against the picograms of the various prostaglandins extracted, derivatized, and injected. For each of the prostaglandins studied, the extraction curves show a positive y-intercept, indicating that the media with fetal calf serum contained background prostaglandins. Since the cell incubations were also done in this media, the linearity of extraction plots were used as the reference quantitative standard curves by which to measure prostaglandins in the cell incubation media.

Representative chromatograms of extracted, derivatized supernatants from unfractionated, non-adherent, and adherent cell incubations from patient M are shown in Figures 19, 20 and 21, respectively. The quantitative measurements by fused silica capillary gas chromatography of PGE_2, PGE_1, $PGF_{2\alpha}$ and PGI_2 from the cell fractions of six patient samples are compiled in Table 8. The major prostaglandins produced by each cell fraction of each patient were PGE_2 and PGI_2.

When the NA fraction is compared to the AD fraction with respect to PGE_2 production (Table 9), patients P and Q show greater amounts in the NA fraction; the other four patients show a pattern concordant with the data from the radioimmunoassay: the AD cells produce more PGE_2 than the fraction depleted of adherent and phagocytic cells. Four of these patients had sufficient cells to prepare NA-NP fractions (pts. N, O, Q, and H) (Table 10). Two of these (N and H) showed that the AD cells were responsible for the majority of PGE_2

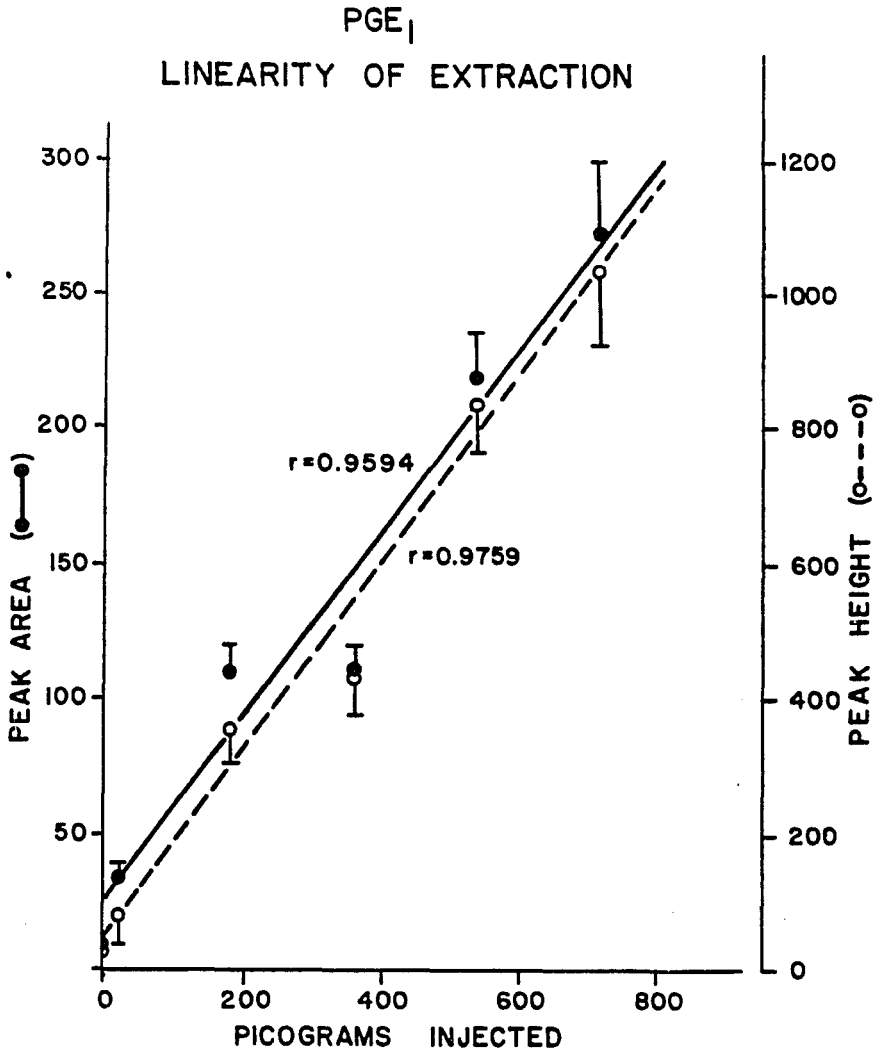

Figure 15. Linearity of extraction of PGE_1 from McCoy's 5A + 10% FCS.

Derivatization was as described in Materials and Methods. Chromatographic conditions were as in Figure 10. In peak area measurements: y-intercept = 27, slope = 0.3366. For peak height determinations: y-intercept = 50, slope = 1.3657.

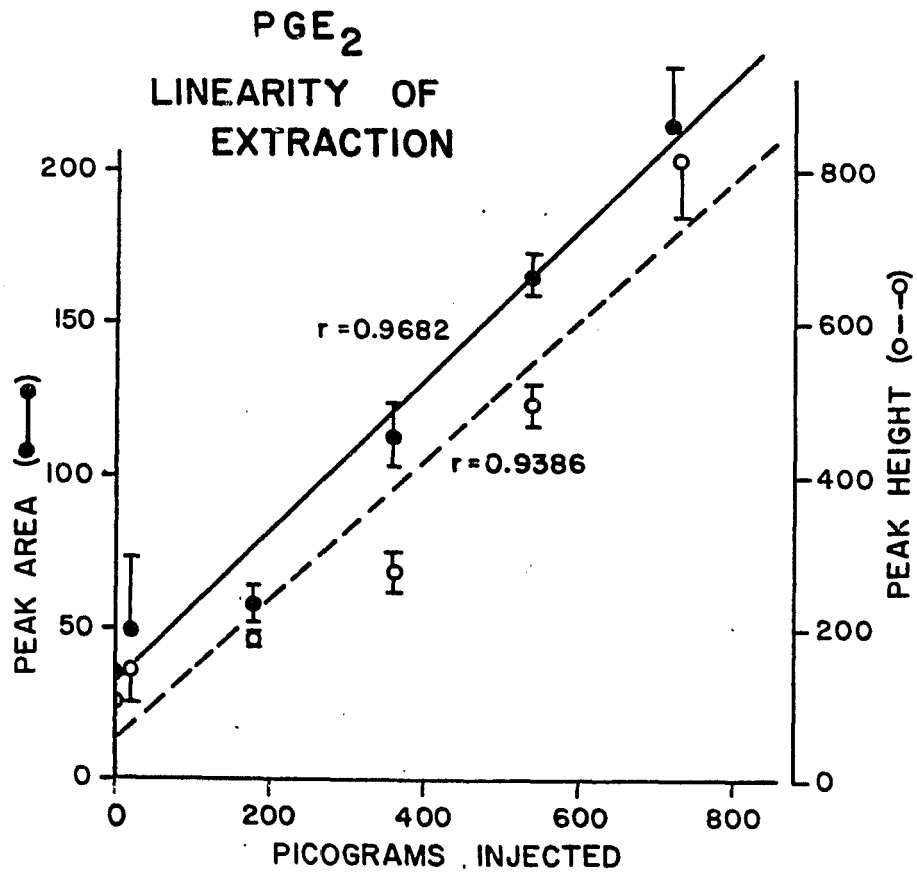

Figure 16. Linearity of extraction of PGE_2 from McCoy's 5A + 10% FCS.

Derivatization was as described in Materials and Methods. Chromatographic conditions were as in Figure 10. For peak area measurements: y-intercept = 32, slope = 0.2481. For peak height determinations: y-intercept = 51, slope = 0.9203.

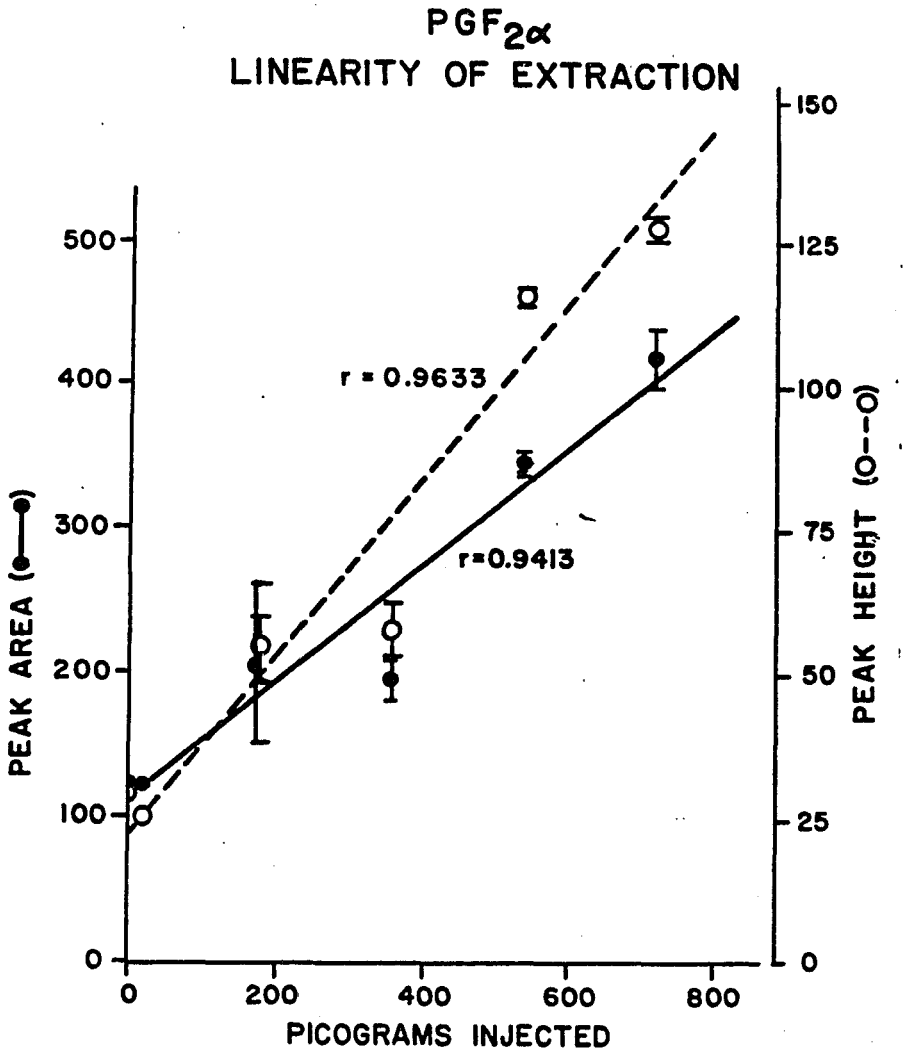

Figure 17. Linearity of extraction of $PGF_{2\alpha}$ from McCoy's 5A + 10% FCS.

Derivatization was as described in Materials and Methods. Chromatographic conditions were as in Figure 10. For peak area measurements: y-intercept = 112, slope = 0.4076. For peak height determinations: y-intercept = 22, slope = 0.1544.

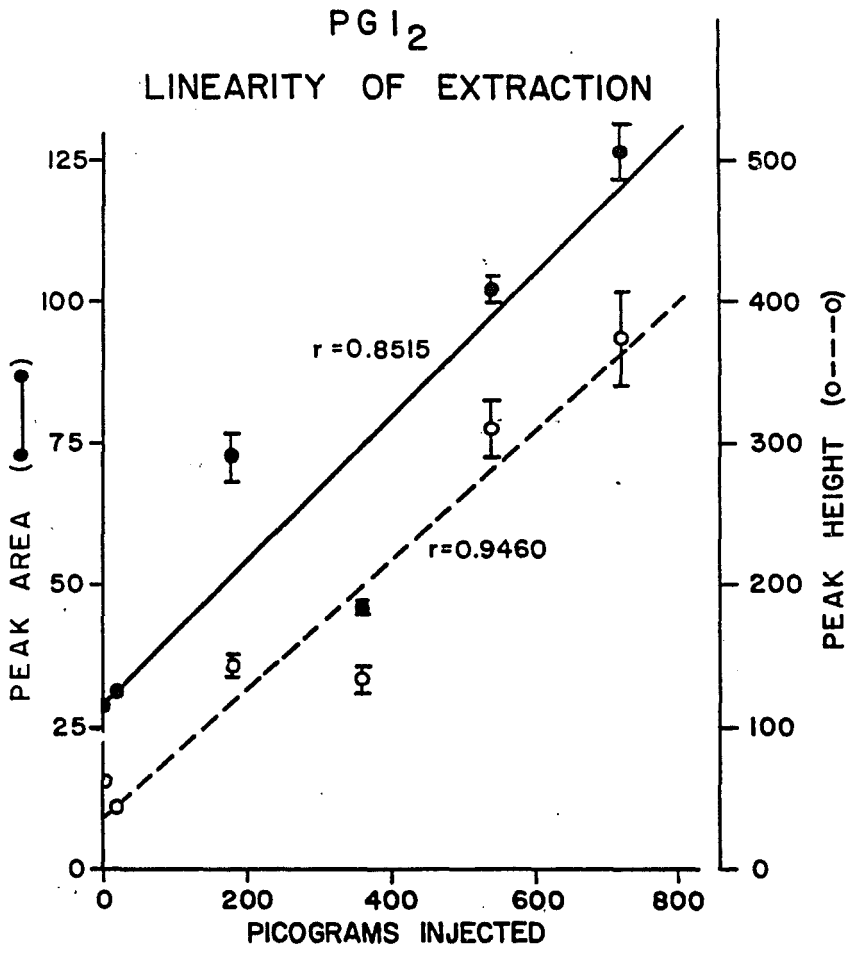

Figure 18. Linearity of extraction of PGI_2 from McCoy's 5A + 10% FCS.

Derivatization was as desceibed in Materials and Methods. Chromatographic conditions were as in Figure 10. For peak area measurements: y-intercept = 28, slope = 0.1284. For peak height determinations: y-intercept = 36, slope = 0.4556.

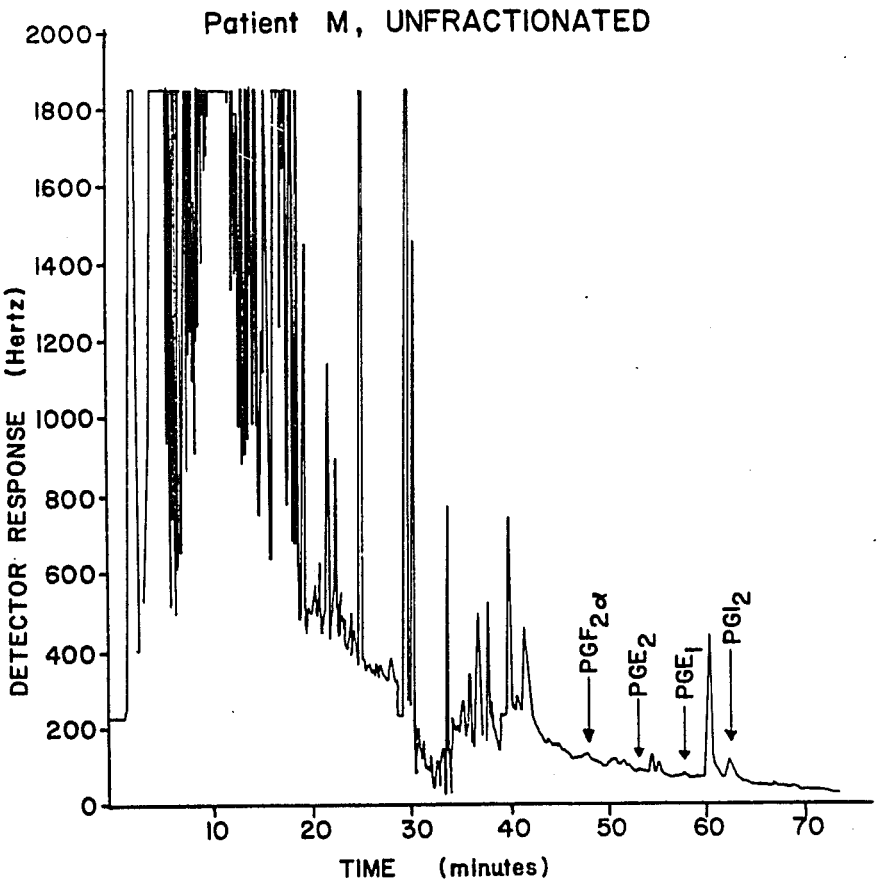

Figure 19. Fused silica capillary gas chromatographic analysis of unfractionated cells from patient M.

Chromatographic conditions as in Figure 10.

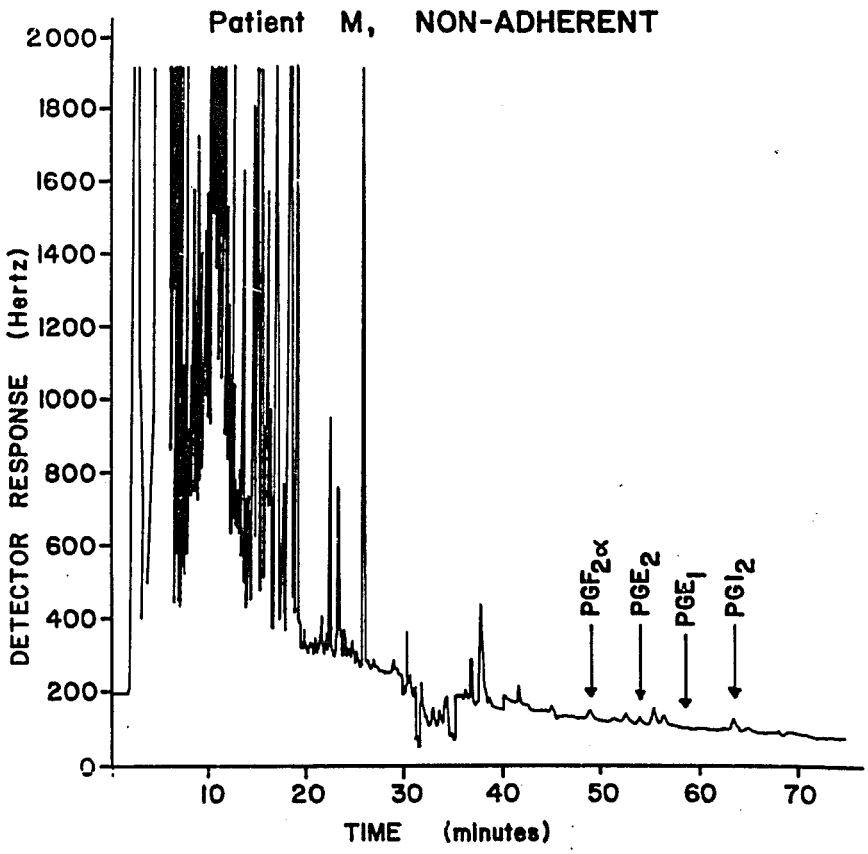

Figure 20. Fused silica capillary gas chromatographic analysis of nonadherent cells from patient M.

Chromatographic conditions as in Figure 10.

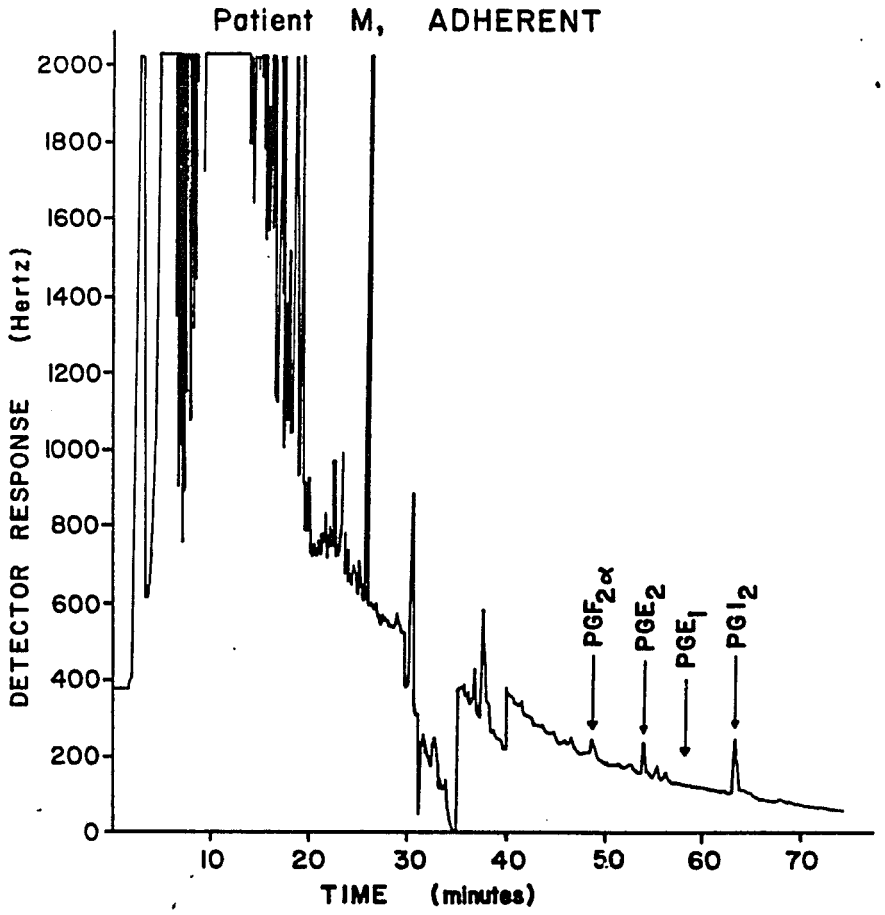

Figure 21. Fused silica capillary gas chromatographic analysis of adherent cells from patient M.

Chromatographic conditions as in Figure 10.

Table 8. Prostaglandin Production by Cell Fractions of Human Tumors.

Patient (tumor type)	Cell fract.	ng/ml/10^6 cells/24 hours			
		PGE_2	PGE_1	$PGF_{2\alpha}$	PGI_2
M	UNF	N.D.*	N.D.	N.D.	75.8
(OVA)	NA	4.0	0.7	4.7	43.9
	AD	33.2	N.D.	6.2	193.0
N	UNF	9.5	N.D.	N.D.	3.0
(ENDO)	NA	4.4	N.D.	N.D.	8.5
	NA-NP	5.4	N.D.	N.D.	3.5
	AD	16.7	N.D.	N.D.	N.D.
O	UNF	13.7	N.D.	N.D.	3.6
(OVA)	NA	10.5	1.1	N.D.	N.D.
	NA-NP	32.2	2.1	N.D.	36.0
	AD	22.5	1.1	N.D.	1.2
P	NA	210.0	14.1	75.8	1963.0
(OVA)	AD	78.8	2.9	50.9	334.3
Q	UNF	235.2	14.6	88.5	247.7
(OVA)	NA	38.1	11.4	134.6	4.9
	NA-NP	51.2	N.D.	N.D.	N.D.
	AD	14.0	60.4	N.D.	34.4
H	UNF	64.3	21.8	10.8	7.5
(OVA)	NA	47.6	18.7	53.8	N.D.
	NA-NP	51.8	N.D.	45.5	N.D.
	AD	240.6	14.5	48.2	N.D.

*N.D. = Below detection limits of assay.

Table 9. PGE_2 Production by Non-adherent and Adherent Cell Populations from Human Tumors

Patient	Tumor Type	PGE_2 ng/ml/10^6 cells/24 hrs NA	AD	NA:AD
M	OVA	4.0	33.2	1:8.2
N	ENDOM	4.4	16.7	1:3.8
O	OVA	10.5	22.5	1:2.1
P	OVA	210.0	78.8	2.6:1
Q	OVA	38.1	14.0	2.7:1
H	OVA	47.6	240.6	1:5.1

Table 10. PGE_2 Production by Non-adherent-Non-phagocytic and Adherent Cell Populations from Human Tumors.

Patient	Tumor Type	PGE_2 ng/ml/10^6 cells/24 hrs NA-NP	AD	NANP:AD
N	ENDOM	5.4	16.7	1:3.1
O	OVA	32.2	22.5	1.4:1
Q	OVA	51.3	14.0	1:4.6
H	OVA	51.8	240.7	3.7:1

produced; the other two patients show the opposite relationship. In the cells from patient Q, while the adherent cells made less PGE_2 than the non-adherent cells, the amount of PGE_1 produced by the AD cells was greater by at least 5 fold (Table 8). However, this shift to exaggerated PGE_1 production rather than PGE_2 synthesis was not seen in the other patient showing greater PGE_2 synthesis by the macrophage-depleted (NA) cells (Patient P)(Table 8).

The pooled data of PGE_2 measurements from both radioimmunoassay and gas chromatography are shown in Table 11. The Friedman's analysis of variance test shows that the nonadherent-nonphagocytic subpopulation of cells is statistically different from the adherent population.

Discussion

Radioimmunoassay and gas chromatography were used to quantitate prostaglandins in culture media from cell populations of human tumors. Fused silica capillary gas chromatography with electron capture detection has excellent reproducibility of retention times and detector response for qualitative and quantitative analysis of this group of compounds.

Using physical manipulations, tumor samples were fractionated into subpopulations of cells which were incubated and analyzed for prostaglandin production.

Human carcinoma specimens studied in this fashion were shown to be producers of PGE_1, PGE_2, $PGF_{2\alpha}$ and also PGI_2. Additionally, it

Table 11. Prostaglandin E_2 Production by Human Tumor Cell Subpopulations.

Patient	Mode of Analysis	PGE (ng/ml/10^6 cells/24 hrs) Cell Population UNF	NA	NA-NP	AD
A	RIA	11.0(III)	8.7(II)	0.3(I)	14.3(IV)
B	RIA	1.9(IV)	1.2(III)	0.4(I)	1.1(II)
C	RIA	15.5(IV)	2.8(II)	0.7(I)	7.5(III)
D	RIA	13.5(III)	0.7(I)	2.8(II)	107.1(IV)
E	RIA	5.3(II)	33.7(III)	3.9(I)	98.7(IV)
F	RIA	137.4(III)	140.0(IV)	8.1(I)	84.6(II)
G	RIA	130.4(IV)	78.2(II)	45.1(I)	125.1(III)
H	RIA	4.3(III)	5.3(IV)	1.5(I)	1.8(II)
H	GC	64.3(III)	47.6(I)	51.8(II)	240.68(IV)
I	RIA	--	16.2(III)	1.2(II)	105.7(IV)
J	RIA	1.9(IV)	0.9(III)	0.7(II)	0.4(I)
K	RIA	253.7(II)	355.9(III)	246.7(II)	704.8(IV)
L	GC	13.0(III)	10.9(I)	13.0(II)	15.9(IV)
M	GC	--	4.04(III)		33.29(IV)
N	GC	9.55(III)	4.43(I)	5.41(II)	16.76(IV)
O	GC	13.73(II)	10.57(I)	32.24(IV)	22.50(III)
P	GC		210.00(IV)		78.80(III)
Q	GC	235.28(IV)	38.14(II	51.28(III)	14.02(I)
		5xIV = 20	3xIV = 12	1xIV = 4	9xIV = 36
		7xIII = 21	6xIII = 18	1xIII = 3	4xIII = 12
		3xII = 6	4xII = 8	7xII = 14	3xII = 6
		0xI = 0	5xI = 5	7xI = 7	2xI = 2
		47	43	28	56

UNF vs NA-NP $p \leq 0.02$

AD vs NA-NP $p \leq 0.04$

was demonstrated that the adherent (macrophage enriched) cell subpopulation was responsible for the majority of prostaglandin E_2 produced. This phenomenon was observed using two independent assays (RIA and GC) for prostaglandin synthesis.

Kurland and Bockman (1978) studied PGE production from monocytes of human peripheral blood and mouse peritoneal macrophages. Using radioimmunoassay, they demonstrated that peripheral blood monocytes and tissue macrophages synthesize and release PGE. When human peripheral blood monocytes were fractionated by discontinuous density gradient centrifugation and studied for prostaglandin production (Goldyne and Stobo, 1979) both PGE_1 and PGE_2 were found using GC-mass spectrometry. There was also demonstrated heterogeneity between different density fractions of monocytes. Although each density fraction appeared equivocal by morphological criteria, 3 fold differences were found in the prostaglandin production between heavy cells and lighter ones. Also, PGE_2 was found to be the major prostaglandin produced by peripheral blood monocytes. PGE_1 was present only in the heavier density cells and in diminutive amounts compared to PGE_2. The production of prostaglandins by mouse peritoneal cells has been studied (Scott et al., 1980). This report found that PGE_2 and PGI_2 were the major synthetic products of arachidonic acid metabolism.

My observations clearly show that cells from human carcinomas produce prostaglandins <u>in vitro</u>. Particularly germain to further studies of tumor biology and prostaglandins is the additional finding that it is the infiltrating macrophage within the tumor which is

largely responsible for the tumor-associated prostaglandins. This origin of prostaglandins also shows great heterogeneity between different patients with similar or different tumor types.

My studies confirm and extend the preliminary investigation by Buick et al. (1980) which also found evidence of PGE_2 production by adherent cells from effusions of two patients with carcinoma. In this current study I found predominant prostaglandin production by adherent cells in 13 of seventeen cases. The gas chromatographic assay described detects PGE_1, PGE_2, $PGF_{2\alpha}$, and PGI_2 in biological fluids in the low picogram range; my results show that PGE_2 and PGI_2 are quantitatively the most abundant prostaglandins produced by macrophages infiltrating human carcinomas. Prostaglandin E_1 and $PGF_{2\alpha}$ are of lesser quantities.

CHAPTER 3

BIOLOGICAL ASPECTS OF PROSTAGLANDINS IN HUMAN TUMOR CLONING *IN VITRO*

Introduction

Human clonogenic cancer cells proliferate to form colonies from a single cell suspension in a nutrient and serum-supplemented semi-solid matrix *in vitro* (Hamburger and Salmon, 1977; Salmon, 1980). This cloning behavior is useful as a bioassay for the influence of various factors and cell-to-cell interactions on tumor growth. The initial cell preparation, originating from a spontaneous human tumor, is comprised of not only neoplastic cells but also stromal cells, vascular elements, and immune cells of the host (Buick et al., 1980; Svennerig and Anderson, 1982). Using various cell enrichment and depletion mechanisms, the cell-to-cell interactions affection clonogenicity can be explored. The clonogenic assay also affords the opportunity to study the effect of exogenous factors and drugs on tumor cell proliferation *in vitro*.

Materials and Methods

Human tumor specimens were processed to single cell suspensions by mechanical means as described in Chapter 2. The cell preparation was subsequently fractionated into adherent, nonadherent, and nonadherent-nonphagocytic subpopulations as previously detailed (Chapter 2). The interactions of these cell subpopulations affecting

tumor cloning were studied using a soft-agar clonogenic assay (Hamburger and Salmon, 1977; Salmon, 1981).

The soft agar clonogenic assay consists of suspending cells in a nutrient-enriched matrix of liquid agar which subsequently gels with cooling. Enriched culture media was prepared according to methods developed by Hamburger and Salmon (1977). Two nutrient media were used, one for a lower layer of agar which functioned as a feeder layer, and the other for the cell suspension broth in the upper layer of agar.

The underlayer media was prepared as a stock solution of 500 ml McCoy's 5A culture media (Gibco, Santa Clara, CA) supplemented with 25 ml horse serum, 50 ml heat-inactivated fetal calf serum, 5 ml sodium pyruvate (2.2% in d·H_2O), 1 ml L-serine (21 mg/ml), 5 ml glutamine (200 mM), and 5 ml penicillin/streptomycin (10,000 units/ml). This solution was stored at 4°C until use, and unused portions discarded after six months. Immediately prior to preparation of the underlayer, the supplemented McCoy's 5A was enriched with 3% tryptic soy broth (1:4, v/v), asparagine (6.6 mg/ml) 1:67, v/v), and DEAE dextran (50 mg/ml)(1:133 v/v). This enriched McCoy's was warmed to 37°C, then mixed with 3% boiled agar (maintained at 50°C) to achieve a final agar concentration of 0.5% (6:1, v/v). The media-enriched agar was aliquoted in 1.0 ml portions into 35 mm petri dishes (Falcon, Scientific Products, McGaw Park, IL) which were allowed to stand at room temperature until the agar gelled (approximately 15 minutes).

Cells were plated in a 0.3% agar overlayer. The overlayer media was prepared from CMRL 1066 (Gibco, Santa Clara, CA) which was enriched (v/v) with 15% horse serum, 1% vitamin C (30 mM), 2% insulin (100 units/ml), 2% glutamine (200 mM), and 1% penicillin/streptomycin (10,000 units/ml). Immediately prior to plating, the CMRL was further supplemented (v/v) with asparagine (1:66, 6.6 mg/ml), DEAE dextran (1:133, 50 mg/ml), and mercaptoethanol (1:100, 0.5 mM).

The role of autologous adherent cells on the clonogenicity of tumor cells in vitro was examined by adherent cell depletion and reconstitution experiments. For depletion studies, nonadherent and nonadherent-nonphagocytic subpopulations were plated at 500,000 cells/ml overlayer. The clonogenicity of these subpopulations was compared with that of an equal number of unfractionated cells. All clonogenic studies were done in triplicate. For reconstitution experiments, isolated adherent cells were plated in the 0.5% agar underlayer. The number of cells plated was adjusted to allow a dose response whenever adequate cells were recovered. Autologous nonadherent-nonphagocytic cells were then plated in the upperlayer at 500,000 cells/ml/dish over the adherent cell-containing, gelled underlayer. The dishes were incubated at 37°C, 7% CO_2, humidified air atmosphere for 10-20 days. Colonies appearing from the single cell suspension were scored by either manual counting by inverted microscopy or by automated image analysis using an Omnicon FAS-II (Bausch and Lomb, Rochester, NY). Visually, a colony was counted for each cell cluster containing 40 cells or more. The FAS-II counted cell clusters and colonies with diameters greater than 60 microns.

Direct studies of the effect of prostaglandins on tumor cell line clonogenicity were undertaken. Human endometrial carcinoma (HEC-1A) was passaged serially in liquid culture using McCoy's 5A + 10% FCS and plated in the soft agar clonogenic assay to establish prostaglandin effects. Prostaglandin E_2, PGE_1 and $PGF_{2\alpha}$ (Upjohn Co., Kalamazoo, MI) were added to the overlayer prior to plating. The prostaglandin standards were made up in 50% methanol, final concentration in agar culture was <0.05%. Prostaglandins were added at a final concentration of 10^{-10}, 10^{-8}, and 10^{-6} M. The number of colonies resulting from these experimental plates was compared with untreated, control colony growth.

Finally, prostaglandin synthesis inhibitors were also studied for their effect on human tumor cell clonogenicity in vitro. Nine human tumor samples were tested for the effect of flurbiprofen [2-(2-fluoro-4-biphenyl)-propionic acid](Boots Pharmaceutical, Sutton, England) on clonogenicity. The drug was tested at concentrations of 10^{-7}, 10^{-6}, and 10^{-5} M, by addition to the upper plating layer. Triplicate tests were done for each concentration; the human tumor cell clonogenicity was then compared with that of untreated, control plates.

Statistical Analysis

All clonogenic experiments were executed in triplicate. The data is reported as mean colonies counted plus or minus the standard deviation from the mean.

Results

Depletion of adherent and phagocytic cells on clonogenicity of human tumor cells is shown in Figure 22. In five of the six tumors studied, clonogenicity declined with the removal of adherent cells; the endometrial tumor (patient C) displayed increased numbers of colonies when the adherent cells were absent. Subsequent removal of phagocytic cells caused different responses. Two samples, patients C and D, grew more colonies under this test condition than the control. Patient E, which failed to grow after adherent cell and phagocytic cell depletion. Tumor cells from patient F showed further reduced clonogenicity with phagocytic cell depletion. Lack of homology in response to cellular manipulation proved to be characteristic of these experiments.

Reconstitution of autologous resident adherent cells in the underlayer was tested in six patients (Figure 23). Four samples yielded sufficient adherent cells to test the reconstitution effect over a range of cell numbers (patients A, C, E, and G). A mild suggestion of dose-responsiveness is seen in the graph. Linear regression analysis of the values resulted in a positive slope of 45%/ log [AD cells] although the fit of the points to the line is weak, $r = 0.4976$. The reconstitution experiments involve adding back a subpopulation of the depleted cells to the tumor cell-enriched population. The reconstitution is not a return to unfractionated conditions since the depletion of phagocytic cells is irretrievible.

When prostaglandins were added to culture dishes of HEC_1A cells, the resulting cloning behavior is recorded in Figure 24.

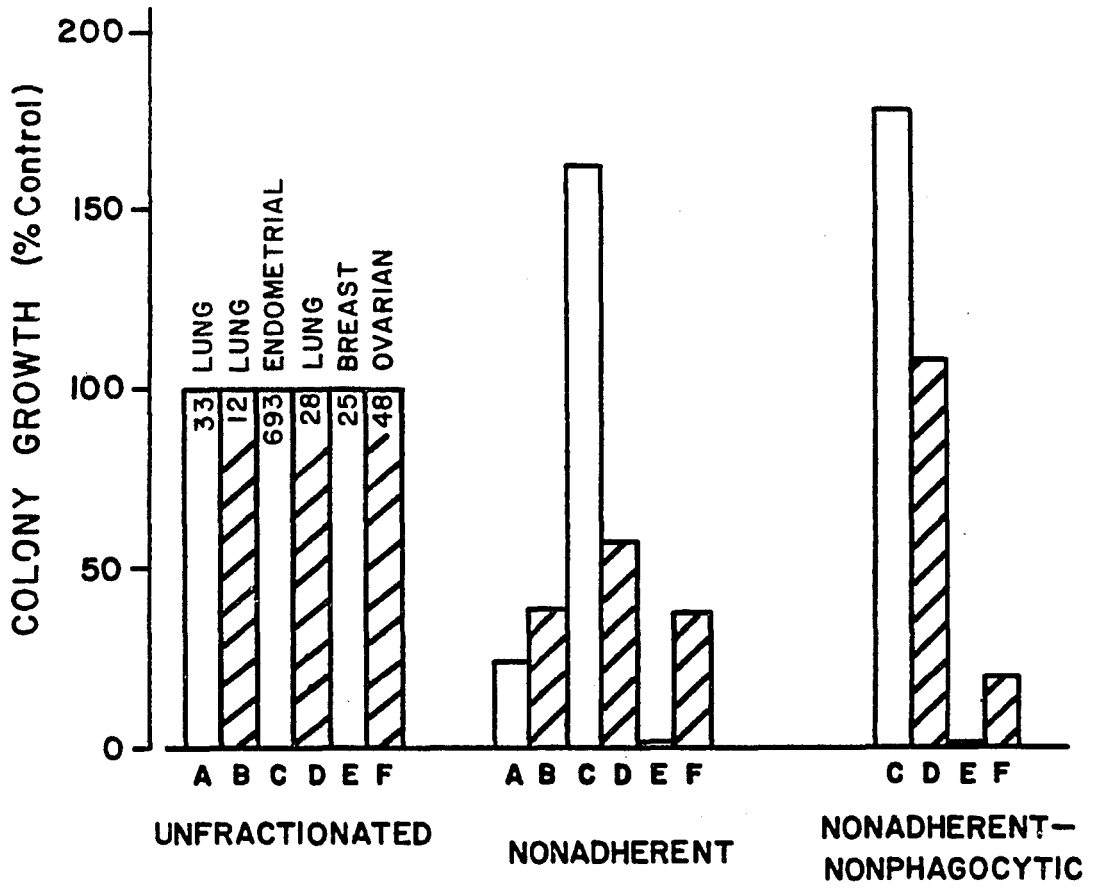

Figure 22. Effect of depletion of adherent and phagocytic cells on human tumor cloning in vitro.

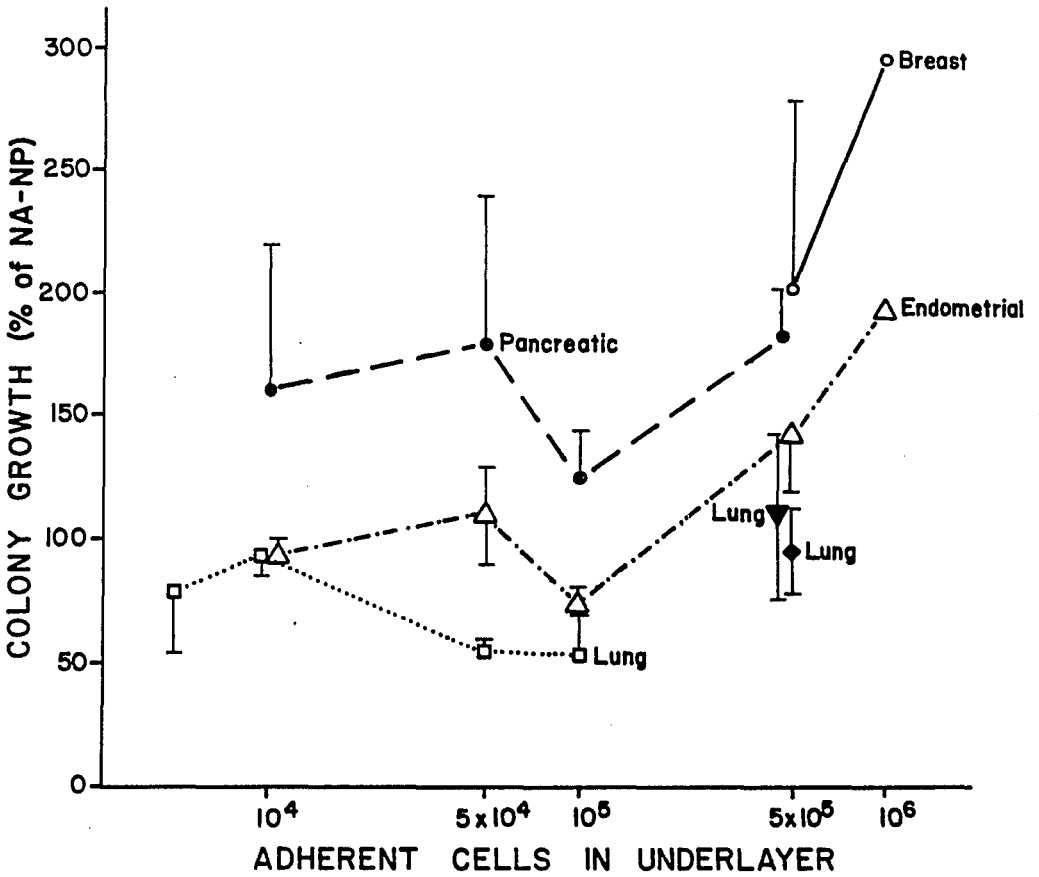

Figure 23. Effect of adherent cell reconstitution on nonadherent-nonphagocytic cell clonogenicity *in vitro*.

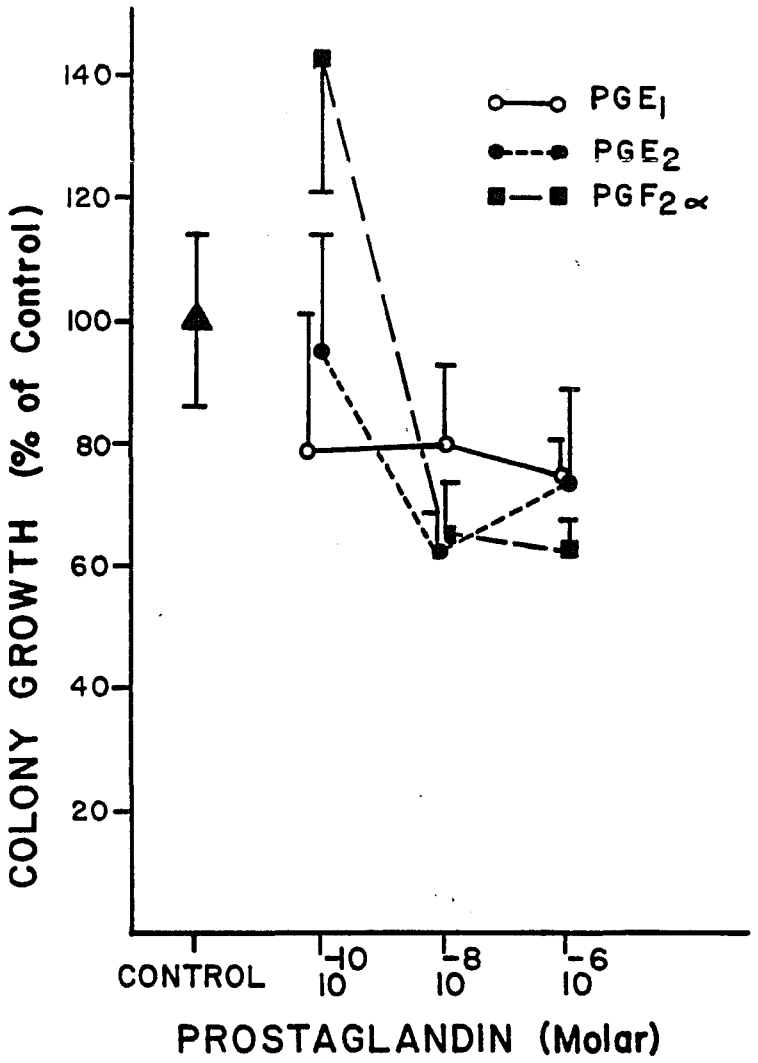

Figure 24. Effect of prostaglandins E_1, E_2, and $F_{2\alpha}$ on HEC 1A carcinoma cell line cloning *in vitro*.

Prostaglandin E_1 caused a mild reduction in colony formation at each dose tested. Prostaglandin E_2 was without effect at 10^{-10} M, but was inhibitory at 10^{-8} and 10^{-6} M. Prostaglandin $F_{2\alpha}$ was stimulatory for the production of colonies in soft agar at a low dose (10^{-10} M), and showed inhibitory effects at the two higher doses tested.

Finally, the effect of flurbiprofen on the clonogenicity of human tumor cells is presented in Table 12. At the low doses (10^{-7} and 10^{-6} M) no significant effects are observed, however, a trend towards stimulation of clonogenic proliferation is evident when fluribiprofen is present at 10^{-5}M.

Discussion

The soft agar assay is a convenient measure of the proliferation of clonogenic tumor cells in human cancers, and an effective tool by which to evaluate modulating factors relevant to tumor biology. The assay allows exploration of host cell-tumor cell interactions using the cells which are actually populating the tumor.

The studies reported in this chapter provide evidence that the adherent cells within a tumor (primarily macrophages) function in a supportive role for tumor cell clonogenicity in soft agar. This support shows a dose response: increasing numbers of adherent cells produce enhanced cloning of tumor cells. These results corroborate those of Buick et al. (1980), Meyskens (1980) and Hamburger and Salmon (1978).

The variability in cloning response in the macrophage depletion and reconstitution experiments raises the concern of macrophage

Table 12. Effect of flurbiprofen on human tumor colony forming units.

Patient	Tumor Type	Colony Formation (% of control)			
		Control	Flurbiprofen (molar)		
			10^{-7}	10^{-6}	10^{-5}
FL 01	Choriocarcinoma	55		105	
FL 04	C.M.L.	97	92	107	133
FL 05	Ovary	80	118	90	153
FL 06	Breast	126	108	117	
FL 10	Breast	33			167
FL 12	Ovary	48		60	71
FL 13	Ovary	33			250
FL 14	Breast	25			90
FL 15	Pancreas	146			10
		$\tilde{x}$ =	106	96	125

heterogeneity in this assay system (Forster and Landy, 1981). This heterogeneity may express itself as a diversity in levels of prostaglandin synthesis (Goldyne, Kennedy and Stobo, 1980), as a myriad tendency towards tumoricidal activation (Hibbs, 1977), as a range in morphological appearances (Stuart, 1977), as a multiplicity of metabolic activity (Stuart, 1977), and as differences in phagocytic activity (Sadler, Jones, and Castro, 1977). Further differences in the apparent role of macrophages in tumor biology may result from the manipulations used to study their effects. Mantovani (1981) found that macrophages infiltrating a murine sarcoma were suppressive of thymidine uptake by the tumor cells in vitro when the macrophage to tumor ratio was more than 20:1. A stimulatory effect on thymidine uptake by the tumor cells resulted when this ratio was below 2:1. Certainly experimental exaggeration of in vivo conditions may help uncover effects of cell to cell interactions, however aggressive perterbation of the relative numbers of different cells may also be greatly misleading. The trend of the response to adherent cell reconstitution over the range tested in the clonogenic experiments of this chapter shows that there is a dose response to macrophage numbers in the in vitro system. The optimum amount of macrophage support for tumor cloning may be a balance between macrophage conditioning of the media with growth factors and macrophage depletion of essential nutrients. Also, the macrophages may play a role in detoxifying the in vitro environment (Buick and Salmon, 1980).

The effects of flurbiprofen on the clonogenicity of human tumor cells is of interest. Bennett et al. (1979) reported that inhibition of prostaglandin synthesis by flurbiprofen administered daily to mice *in vivo* was effective in reducing the growth of a syngeneic mammary tumor, and prolonged the survival of tumor-bearing animals. The use of the syngeneic, spontaneous tumor (non-immunogenic) for these tests encouraged interpretation of the data in the direction away from immunomodulation by perterbations in prostaglandin-synthesis in favor of a direct influence on the tumor cells. Bennet et al. (1979) also note the paucity of immunologic cells infiltrating the tumors. The host-sparing effect of flurbiprofen, however, was not evident when the study was done using B16 melanoma cells in mice. There were also no positive tumor control effects as a result of flurbiprofen in B16 melanoma and Lewis lung carcinoma tested *in vivo* and *in vitro* (Hellman and Pym, 1982). Whether these differences reflect the *in vivo*/*in vitro* differences, or the *in vivo* study, or only a single responsive tumor type remains to be determined.

The effect on clonogenicity of direct application of prostaglandins to the *in vitro* soft agar assay was marginal, even at the high concentration (10^{-5} M). This is not the only report of such non-response. Sykes and Maddox (1972), Favulli et al. (1980) and Hofer et al. (1980) all report that addition of exogenous prostaglandins showed little to no effect on tumor growth *in vitro* or *in vivo*.

The adherent cells present within human carcinomas enhance tumor cell clonogenic growth *in vitro*. The exact nature of this stimulation remains to be clarified.

CHAPTER 4

DISCUSSION AND SUMMARY

The investigative steps between noting the presence of a biological compound in a tissue and determining its role there is a tortuous one and not uncommonly multidirectional. Such is the status of determining the role of prostaglandins in cancer.

Cancer biology is a multifaceted arena of research, and it appears that the prostaglandins have attracted the attention of workers from most of these facets. This discussion will focus on the findings of the experiments of this dissertation and attempt to orient these findings to future investigations along the same or tangental directions.

It would be appropriate to initially temper any expectations about the eventual outcome of research on prostaglandins and cancer by making the obvious note that prostaglandins are biological modifiers or hormones and not "circuit breakers." Whether the study involves host immunosuppression, tumor differentiation, or cell cycle kinetics, the best outcome of manipulation by prostaglandins will be a modification of the process and is not likely to be a stop or start response.

Human carcinoma specimens produce prostaglandins, mainly PGE_2. Substantial amounts of PGI_2 are also noted for some tumors. When the cells which comprise the <u>in situ</u> tumor are separated into subpopulations based on adherence and phagocytic criteria, it is found that the

adherent cells, or macrophage-enriched fraction, is primarily responsible for the prostaglandins found in the tumor. The range of PGE_2 produced by the adherent cells is large, reflecting heterogeneity of tissue macrophages (Forster and Landy, 1981; Goldyne, Kennedy and Stobo, 1980). This heterogeneity may reflect the infiltration of different classes of blood monocytes into the tumor (Goldyne and Stobo, 1979) or this may represent different degrees of macrophage activation and differentiation (Shaw et al., 1979; Hibbs, 1977. Reports of macrophage heterogeneity are so extensive, in fact, that it is almost surprising that there is statistical difference between the adherent and the nonadherent-nonphagocytic populations with respect to prostaglandin production. This finding, coupled with the stimulating influence of reconstituted macrophages on tumor cell clonogenicity, raises some direct, fundamental questions of the role of prostaglandins in human cancer: first, what is the effect of macrophage-produced prostaglandins on tumor cell clonogenicity?; second, do the tumor cells in some way "encourage" resident macrophage production of prostaglandins, and if so, what is the chemical or molecular (possibly antigenic) nature of this "encouragement"?; third, are there differences in the prostaglandins produced (either qualitatively or quantitatively) by macrophages infiltrating primary tumors versus metastatic lesions?; and fourth, are the prostaglandins influencing other host cells which may in turn modulate tumor growth?

Initiating the discussion of these questions by briefly expounding the findings of my research, the point can be taken that the

starting point for assigning a role (or roles) for prostaglandins in human cancer begins at the infiltrating macrophage within the tumor. From the seventeen carcinoma samples studied, the adherent cell subpopulation (macrophage enriched) is the predominant source of tumor prostaglandins rather than the cancer cells themselves. It would be most rewarding to delineate the influence of tumor cells on the biochemical activity of infiltrating macrophages.

The clonogenic experiments of adherent cell depletion and subsequent reconstitution described herein provide some insight into the role of macrophage-derived prostaglandins on tumor cell clonogenicity. It is clear that the infiltrating macrophage stimulates clonogenicity of tumor cells in vitro. The absence of macrophages causes a reduction in the number of cells which grow to form colonies in soft agar. Reconstituting the autologous macrophages shows a dose response stimulation of clonogenicity in vitro.

Since the analysis of cell incubation supernatants showed that the adherent cells were the predominant producers of prostaglandins in tumors, it is tempting to suggest that the macrophage-produced prostaglandins (possibly the E series primarily) stimulate tumor colony formation. Resoundingly, the data of the effects of inhibition of prostaglandin synthesis by flurbiprofen suggests that the opposite tendency exists: inhibition of prostaglandin synthesis causes a stimulation in tumor colony formation in vitro. These findings prompt the fourth question listed earlier: is the effect of macrophage-produced prostaglandins on tumor cell clonogenicity mediated through

some other host cell? My research shows that macrophages support tumor cell colony formation, and suggests that this support is not a direct effect of prostaglandins on the tumor cells. I hypothesize that macrophage produced prostaglandins act on other host cells which, in response, act to condition the local environment to promote tumor cell colony formation in vitro or its equivalent in vivo.

Future investigations designed to test this hypothesis are conceivable. One possibility would be to further divide the nonadherent-nonphagocytic cells into a greater number of subpopulations by density gradient fractionation. The reconstitution of macrophages to combinations of these fractions of cells would act to reveal additional cell-to-cell interactions which originate as prostaglandin production by macrophages. The adherent cells also could be subfractionated using density gradients in an attempt to elucidate the degree of macrophage heterogeneity within the tumor. This particular study would be revealing in regards to the tumor induced modification of macrophage function.

In addition to the heterogeneity of macrophages, tumor cells are also considerably heterogeneous. Indeed, the tumor cells breech a gamut of variety from near-normal to aggresively malignant (Steele, 1978). The process of tumor differentiation or dedifferentiation leaves great room for transient responsiveness to potential modulation by prostaglandins. This consideration is elegantly addressed by Alexander (1982). A tumor population is diverse and possibly evolving by clonal expansion (Nowell, 1976). Along the route from

normal cell to neoplastic cell, the characteristic change experienced by a developing tumor is loss of responsiveness to normal homeostatic controls. Depending on the normal role of prostaglandins in each specific tissue homeostatic repertoire, the tumor may either respond to prostaglandin modulation or discontinue responding. The normal action of prostaglandins on tissue homeostasis is certainly a needed understanding on which to consider their role in cancer.

Parallels have been drawn between malignancy and pregnancy from the standpoint of host support and immuno non-responsiveness (Gleicher, Deppe and Cohen, 1971; Rocklin, Kitzmiller and Kay, 1979; Salmon, 1980). In both cases a primarily competent immune system allows the growth of antigenic tissue within the host. This parallel is taken further by Pearson (1980), who recommends PGE_2 as a macrophage tumoricidal activator which would abort tumors. These approaches to the cancer problem bring to the forefront the complex and tangled field of tumor-immune interactions. Clear evidence exists for the action of prostaglandins as immunodepressants (Weinstein and Melmon, 1977) which suppress B and T lymphocyte function. Goodwin and Webb (1980) review the action of the prostaglandins on immunity giving support for immunodepression and immunostimulation. The primary immunosuppressive action of prostaglandins is the inhibition of lymphocyte proliferation (Goodwin and Webb, 1980). Prostaglandins may act to suppress host immunity which would otherwise eliminate the neoplasia.

An additional question to acknowledge concerning the production of prostaglandins by tumor-associated adherent cells, is why do these cells produce elevated prostaglandin levels?

Salmon and Hamburger (1977) presented a hypothesis of the macrophage as a two edged sword. This hypothesis was subsequently expanded to relate tumor and fetal development (Salmon, 1980). During fetal development, with the presentation of fetal antigens, the macrophage would stimulate tissue development and assist in immuno-suppression during organogenesis. After a timespan of several decades, progeny macrophages when presented with fetal antigens or other tumor associated antigens would stimulate neoplastic tissue development and subvert the immune response against the foreign tissue. This model warrants testing, especially in regards to the activity of both macrophages and prostaglandins in fetal development and in cancer.

APPENDIX A

NON-SPECIFIC ESTERASE STAIN FOR MACROPHAGES

Materials:

1. Fixative:

Na_2HPO_4	20 mg
KH_2PO_4	100 mg
dH_2O	30 ml
acetone	45 ml
30% formaldehyde	25 ml

Mix; adjust pH to 6.6; KEEP REFRIGERATED; use 2-3x then discard.

2. Pararosaniline:

pararosaniline hydrochloride (Sigma P-3750)	1.0 gm
2 N HCl (warm)	25.0 ml

3. 4% sodium nitrite solution:

sodium nitrite	100 mg
d H_2O	2.5 ml

**********PREPARE FRESH FOR EACH USE**********

4. M/15 Sorenson's Phosphate buffer (pH 6.3)

Na_2HPO_4	2.128 gm	1.064 gm
KH_2PO_4	6.984 gm	3.492 gm
d H_2O	1000 ml	500 ml

5. Alpha naphthyl butyrate solution:

naphthyl butyrate (Sigma N-8000)	1.0 gm
dimethyl formamide (Sigma D-4254)	50.0 ml

Mix; KEEP IN FREEZER (will not freeze)

6. Methyl green counterstain (0.5%):

methyl green	500 mg
d H_2O	100 ml

STORE IN REFRIGERATOR; FILTER BEFORE USE.

Procedure:

1. Cytocentrifuge in serum for best morphology.
2. Fix in cold fixative in coplin jar for 30 seconds.
3. Rinse by 5 dips each in four cars of d H_2O
4. Air dry for 30 minutes.
5. Filter approximately 1 ml pararosaniline solution.
6. Mix filtered solution with an equal volume of FRESHLY PREPARED 4% sodium nitrite for hexazotization. Allow to stand for 1 minute before use.
7. Mix in sequence:

M/15 buffer	44.5 ml
hexazot. Paroros.	0.25 ml
napth.butyr soln	3.0 ml

8. Filter mixture into coplin jar. (Use only once)
9. Stain slides in coplin jar by placing jar into 37°C water bath for 45 minutes.
10. Rinse slides by dipping into jars of d H_2O.
11. Drain slides and counterstain in 0.5% methyl green for 15 sec. (or counterstain in Giemsa).
12. Rinse repeatedly in d H_2O.
13. Air dry for 30 minutes and coverslip with PERMOUNT.

Interpretation:

Non specific esterase containing cells are easily distinguished by the presence of multiple intensely red-stained granules in the cytoplasm as compared with the green counterstain of the esterase-negative cells.

Nonspecific esterase activity is very strong in monocytes, macrophages, histiocytes, and megakaryocytes. It is very weak or absent in neutrophils, basophils and eosinophils. It is usually absent from lymphocytes and plasma cells, although occasionally these cells will contain a SINGLE, intensely-stained granule.

APPENDIX B

LATEX BEAD DETERMINATION OF PHAGOCYTIC CELLS

1. Dilute DIFCO latex beads (DIFCO Bacto-Latex 0.81; #3102-65) to 2.5×10^9 particles/ml. (1:10 dilution of stock)
2. Prepare cell suspension at 1×10^5 cells/ml.
3. Mix 0.5 ml cells with 20 µl latex bead solution.
4. Incubate for 60 minutes at 37°C, 5% CO_2.
5. Wash the cells X2 to remove non-phagocytized beads.
6. Resuspend the cells in 0.5 ml media.
7. Add 0.1 ml FCS. Mix.
8. Prepare cytocentrifuge prep: 0.3 ml per chamber.
9. Fix slides immediately in abs. methanol (or ethanol).
10. Stain in Wright's stain.
11. Examine slides by oil immersion microscopy. Five (5) beads or more per cell is scored as positive for phe phagocytosis.

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