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PROSTAGLANDIN REGULATION OF GENE EXPRESSION AND GROWTH IN NORMAL AND MALIGNANT TISSUES

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INTRODUCTION

Role of prostaglandins in cell growth

Prostaglandins bind with cell surface receptors and cause an activation of adenylate cyclase and generation of adenosine 3'5' cyclic monophosphate (cAMP) (1,2). C-AMP is thought to play a central role in the regulation of cell growth since the growth of many types of cultured cells is arrested by adding exogenous dibutyryl adenosine 3'5' cyclic monophosphate (dbcAMP) (3,4). In 1971, a report by Pastan and Johnson demonstrated that growth of L-929 cells was inhibited by prostaglandin E_1 (PGE_1). Like epinephrine and glucagon, many prostaglandins are able to trigger the adenylate cyclase enzyme in the cell membrane and stimulate a rise in cAMP; it was therefore postulated that the block in cell growth caused by PGE_1 was solely due to the increased intracellular concentration of cAMP (5). Since that time many investigators have shown that prostaglandins with an α,β unsaturated ketone ring structure inhibit cell growth (1,6-10) in normal and malignant cells. In this paper, we demonstrate using cell mutants that the growth inhibitory prostaglandins do not require cAMP or protein kinase mediated mechanism. In addition, we discuss the finding that inhibition of endogenous prostaglandin synthesis causes inhibition of osteoblast cell growth.

MATERIALS AND METHODS

Osteoblast and S-49 cell lines

MC3T3-EI cells (a cloned osteoblast line) were plated at 3.5×10^6 cells/well for 4 well plates and 8.3×10^5 cells/well for 6 well flats for coverslips and ^{3}H-thymidine

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incorporation. Cell cultures were cultured in 10% fetal bovine serum (Hyclone Labs Inc. Logan UT) in minimum essential media alpha (aMEM) from the University of California Cell Culture Facility (San Francisco, CA), containing antibiotic and L-glutamine (Sigma Chemical Co., St. Louis, MO). The S-49 Wt, Cyc- and Kin-cells were supplied by the University of California Cell Culture Facility. They were growth in DME supplemented with 5% Horse Sera and L,-glutamine. Cell numbers were determined using a Coulter Counter ZBI.

DNA synthesis

^{3}H-thymidine incorporation was used to determine DNA synthesis. Triplicate samples were incubated with ^{3}H-thymidine (Dupont/NEN Products, Wilmington, DE) at a concentration of 3 μCi/well for 15 minutes. Cells were washed three times with 6% TCA (5°C) and then washed three more times with equal volumes of 95% ethanol (5°C). Cells were allowed to air dry for 5 minutes before solubilizing in lysis buffer. After 30 minutes were aliquoted for liquid scintillation counting and protein assay as previously described (23).

Reverse transcriptase polymerase chain reaction (rtPCR)

These assays were completed using protocols from Cetus, Inc. (Perkin Elmer Cetus Norwalk, CT). One ug of RNA is reverse transcribed in 20ul of 10mM Tris-HC1 containing lmM MgC12, 50mM KC1, 1mM dNTP's, 2.5uM random hexamers, RNase inhibitor and 2.5U of reverse transcriptase (RT). The reaction is incubated for 10 minutes at room temperature and then at 420 for 15-45 minutes in order to generate cDNA. Then 4-10ul of the new cDNA is put into 78 ul of polymerase chain reaction master mix containing 2mM MgC12, 50mM KCI, 10mM Tris-HCI and 2.5 U of AmpliTaq (Perkin Elmer) for 26-35 cycles with appropriate primers. We have quantitative rtPCR with documentation of linearity of signal for PCR cycles as well as linearity of signal from varying amounts of RNA in the initial RT. The signal is further identified by northern blotting with known probes, base pair size of product and restriction analysis of the PCR band. Internal standards include multiplex PCR using primers for β actin. The ethidium bromide stained DNA gels are quantitated using radioisotopic incorporation and/or video densitrometry.

RESULTS AND DISCUSSION

Reports of the induction of *gaad* 153 mRNA in prostaglandin cell cycle arrest have shown that protein kinase inhibitor 2-aminopurine decreased PGA_2 induction of gaad *153*. It was suggested that the PGA_2 mediated growth arrest was working through a protein kinase since the use of the broad spectrum protein kinase inhibitor reduced the *gaad 153* signal (11).

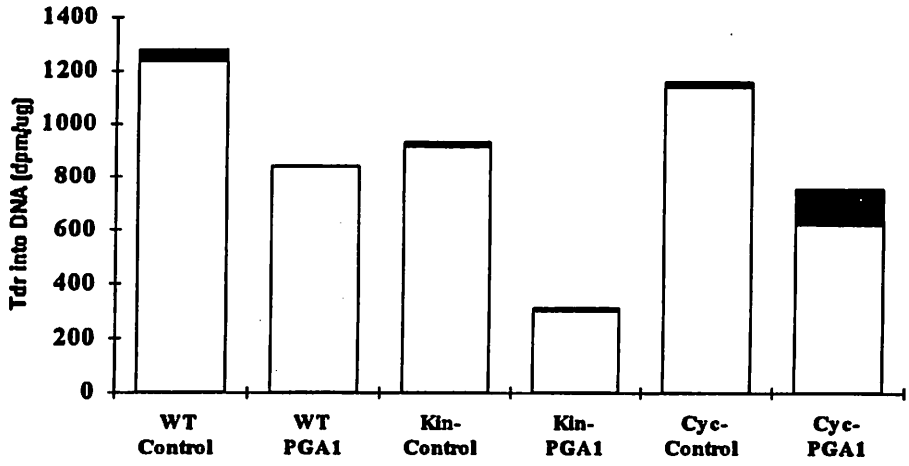

Figure 1. Effect of prostaglandin A1 on S49 Wild Type (WT), Kin- and Cyc- cells. The cells were in stationary suspension culture with the two hour 50 mM prostaglandin treatment. Standard deviation is shown by top protion of bar.

In order to fully disassociate the effect of the prostaglandins from that of induction of cAMP synthesis of protein kinase A in normal and malignant tissues, we designed experiments using a S-49 lymphoma cell kin- and cyc- variants that were first isolated by Bourne, et. al. (12). The kin- cells lack protein kinase activity and the cyc- variant fail to respond to five effectors, adrenergic amines, PGE_1, cholera toxin, guanine nucleotides and fluoride ion, that normally stimulate adenylate cyclase to increase cAMP in the wild type S-49 cells. The cyc- phenotype possesses the catalytic unit of adenylate cyclase, but lacks the N protein component needed for coupling the hormone receptor and adenylate cyclase components which are necessary for transmitting the external hormonal signal to activate adenylate cyclase (12). As seen in Figure 1, we demonstrate that $dmPGA_1$ inhibited DNA synthesis, in the

three S-49 cell types suggesting that the adenylate cyclase/protein kinase A pathway is not mediating the growth inhibitory action of $dmPGA_1$. These data show that growth inhibition does not depend upon c-AMP or protein kinase A activity. In addition, using cyc- cells we have demonstrated that $dmPGA_1$ inhibits cell growth, and blocks the cell cycle at the G_1/S boundary independent of cAMP mediated pathways (13). It is of interest that these prostaglandin arrested cells maintain their G_1 oncogene profile of increased *c-fos* and *c-myc* oncogene expression without progression into S-phase of the cell cycle (13).

Prostaglandins affect the growth of the cyc- cells in a somewhat paradoxical manner. The prostaglandins containing an α,β unsaturated ketone are inhibitors of thymidine incorporation into DNA, with dimethyl PGA_1 being more effective in growth inhibition than PGB_1. This was expected since these two prostaglandins share a common ring structure of cyclopentenone, and agree with our previous findings (14-16). There is a significant increase of thymidine incorporation into DNA caused by dimethyl PGE_2 in these adenylate cyclase mutants. Since prostaglandins can up regulate (PGE_2 and $PGF_{2\alpha}$) and specifically inhibit (PGA_1, PGB_1) cell growth, I hypothesized that the α,β unsaturated ketone prostaglandins might be interfering with normal physiological regulation of growth by naturally synthesized prostaglandins. We therefore decided to study cells from organs that were known to have a prostaglandin mediated growth.

Role of prostaglandins in natural bone growth

Prostaglandin E_2 has been postulated to be one of the key regulators of local bone formation and has been shown to stimulate bone growth *in vivo* (17,18). Studies by Webb Jee et al. have demonstrated that PGE_2 stimulates new bone formation *in vivo* by 120% over a 21 day period (12,18). Osteoblasts have been shown to synthesize relatively large amounts of prostaglandin E_2 (PGE_2) (19,20) and stimulation of cultured bone cells by growth factors increases prostaglandin synthesis (21,22). However, the intracellular mechanisms for prostaglandin-mediated bone growth remain unknown. Oncoproteins Fos and Jun are thought to be central to growth responses of a number of cell types (23,24). Transcription of *c-fos* is rapidly

and transiently activated by growth factors (25,26). Fos and Jun are the major constituents of activator protein-1 (AP-1), which binds to DNA in a sequence-specific fashion to promote transcription of specific genes (27). To gain insight on the mechanism of growth regulation by PGE_2, we have tested the direct effects of PGE_2 on osteoblast DNA replication and early gene expression in cultured osteoblasts. For this purpose, we employed the MC3T3 osteoblast cell line, which was established from newborn mouse calvaria and retains the capacity to differentiate and mineralize in vitro (28,29). Using these cells, we found that PGE_2 induces immediate-early genes and actin gene expression during prostaglandin-stimulated osteoblast growth in culture (30). *c-fos* transcript message is increased by at least 20 fold within minutes of addition of PGE_2 (Figure 2). This demonstrates that PGE_2 regulates early gene expression much in the same manner as other growth factors EGF, PDGF and TGFb, in a variety of cell types (21,22,31). The mechanism of PGE_2 in activating the expression of *c-fos is* unknown. Other growth factors like EGF and PDGF have signal transduction that occurs through activating the tyrosine kinase domain of their receptors and subsequent activation of protein kinase and stimulation of *c-fos* message (25). Growth factors, serum, and phorbol esters up regulate *c-fos* message by a number of extracellular stimuli within 30 minutes to 2 hours after their addition through activation of one or more *c-fos* transcriptional factors. In addition, as with other growth factors, when cyclohexamide is added with prostaglandin, the message is super-induced suggesting that new protein is not needed for *c-fos* expression (Figure 2). It is possible that PGE_2 shares similar mechanisms to the other bone growth factors and studies are underway in my laboratory to define the signal transduction mechanisms involved in the osteoblast response to PGE_2.

rtPCR used to study c-fos *expression and its connection with glucocorticoid growth inhibition by prostaglandins*

Numerous reports of PGE_2 stimulating DNA synthesis in a variety of cells have shown the etfective range for PGE_2 to increase DNA synthesis is from 2-10 μg/ml (20,33). The concentration of PGE_2 (4 μg/ml) which stimulate *c-fos* and growth in our studies are totally compatible and in full agreement with other data

published in the literature on PGE_2 stimulation of osteoblast growth both *in vivo* and *in vitro*. We asked the question whether the concentration of prostaglandin needed for *c-fos* expression correlates with the concentration needed for increased DNA synthesis and osteoblast cell growth.

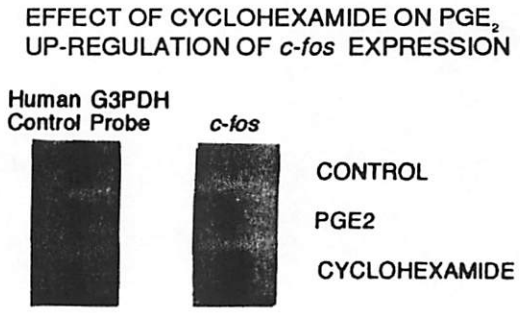

Figure 2. Prostaglandin stimulation of *c-fos*. Osteoblasts were serum deprived for 48 hours in 0.5% FCS before addition of PGE_2. *c-fos* expression was increased within 30 minutes of addition of the PG. When cyclohexamide was added with the prostaglandin, there was a super induction of the *c-fos* message. This is representative of two experiments.

If prostaglandin is a growth stimulator in the osteoblast, then inhibition of its endogenous synthesis would by necessity decrease osteoblast cell growth. Glucocorticoids are known to decrease prostaglandin synthesis and theoretically should decrease osteoblast growth. This is evidenced clinically, since elevated levels of glucocorticoids have been associated with osteoporosis since 1932 when Harvey Cushing first noted that an excess of endogenous glucocorticoid was accompanied by bone loss (34). Since that time, numerous investigations have shown that bone mass is subnormal in both Cushing's syndrome patients as well as patients treated with steroids for asthma and rheumatoid arthritis (35,36). Using synchronous osteoblast cultures, we have found that endogenous prostaglandin synthesis increases in late G_1, preceding S-phase DNA synthesis by several hours (20). When this G_1 prostaglandin synthesis was reduced by dexamethasone, over 40% of cells in synchronized culture failed to incorporate tritiated thymidine, showing a block in progression of the cell cycle at the G_1/S boundary (20). This observation of a G_1 block of the cell cycle is supported by the work of other laboratories that have demonstrated that dexamethasone arrests hepatoma and adenocarcinoma cells in G_1 (37,38).

We have found that glucocorticoids decreased osteoblast growth (Figure 3) and decreased *c-fos* mRNA expression (Figure 3). This reduction of bone growth and *c-fos* expression by glucocorticoids is probably due to its inhibition of PGE_2 synthesis since addition of exogenous prostaglandin to the glucocorticoid-treated cells increased cell growth to control levels. Using synchronous osteoblasts we found that PGE_2 synthesis activity is highest during the G_1 stage of the cell cycle preceding S-phase DNA synthesis by several hours. In data not shown, we have found that COX-2 is increased upon addition of serum suggesting that the early increase in prostaglandin synthesis is mediated through increased COX-2 activity.

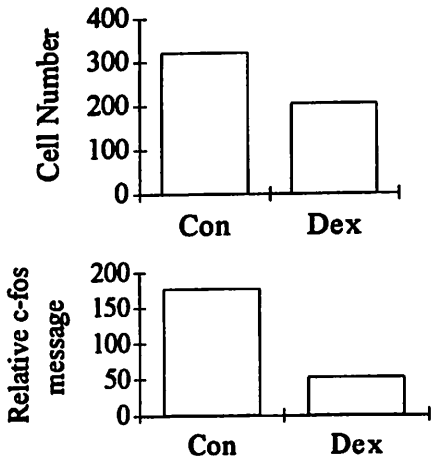

Figure 3. MC3T3E1 cells were grown in 2% FCS aMEM media for 48 hours before seeding for experiment. Osteoblast cells were seeded at 350,00 cells per well and treated for 24 hours with 200nM dexamethasone. In the first graph, the increase in cell number is shown following 24 hours of treatment. The second set of bars shown the relative abundance of *c-fos* message after treatment with dexamethasone. Messenger RNA was extracted, reverse transcribed and reacted with Taq polymerase for 31 cycles using GeneAmp (Cetus) protocols. Values represent typical experimental results. Results were corrected by using internal standard concentrations.

Assuming that growth regulation by the prostaglandins is mediated by early events like its elevation of *c-fos* message, then the decrease of prostaglandin synthesis by dexamethasone should cause a decrease in the message. As expected there is a decrease in *c-fos* message in glucocorticoid treated cells. What was not expected was the rapid loss of the *c-fos* message within one hour of treatment with dexamethasone. We believe this to be due to the decrease in prostaglandin synthesis since the *c-fos* activity was recovered with addition of exogenous PGE_2. These results suggest that the glucocorticoids inhibit bone formation by interfering with the normal eicosanoid growth regulation in the osteoblast.

Possible clinical applications to cancer growth regulation.

Our data have demonstrated that prostaglandins can up and down regulate growth of lymphoma cells and of osteoblast cells. In recent years it has become apparent that regulation of cell cycle is a multi-phasic action of combined actions which can be receptor mediated, ion flux facilitated and regulated at the level of gene expression. These studies have demonstrated that prostaglandins can increase or decrease DNA synthesis, depending on specific prostaglandin structure. Studies by this laboratory and others have demonstrated that prostaglandin E_2 acts as a growth factor in both *in vitro* and *in vivo* (30-33). The growth inhibitory prostaglandins like dmPGA$_1$ could interfering with endogenous prostaglandins interacting with normal growth regulatory pathways. Growth inhibition by the α,β unsaturated prostaglandins is not mediated through cAMP or cAMP dependent protein kinases. Do prostaglandins affect growth in other cell systems in the same way? We are currently exploring the role of prostaglandins as growth regulators of cancer. Of particular interest are the colorectal cancers whose growth are down regulated by NSAIDS. It is possible that the prostaglandins are regulating the growth of these cancers in the same manner they regulate osteoblast growth. Which part of the eicosanoid growth regulation is affected in the colorectal cancers remains unknown, but is now an important focus our research program.

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