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 dibutryl 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 E1 (PGE1). 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 PGE1 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-E1 cells (a cloned osteoblast line) were plated at 3.5x10⁶ cells/well for 4 well plates and 8.3x10⁵ cells/well for 6 well flats for coverslips and ³H-thymidine
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-HCl containing 1mM MgCl₂, 50mM KCl, 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 MgCl₂, 50mM KCl, 10mM Tris-HCl 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 densitometry.
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 PGA2 induction of gaad 153. It was suggested that the PGA2 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 portion of bar.](image)

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, PGE1, 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 dmPGA1 inhibited DNA synthesis, in the
Growth responses of a number of cell types (27-34) have been shown to be mediated by growth factors. In addition to being a feature of proliferating cells, growth factors can also affect differentiation, morphogenesis, and development.

Protein kinase C (PKC) has been shown to be involved in the regulation of cell growth and differentiation. PKC is activated by growth factors, and this activation is thought to be mediated by the Ras signaling pathway. The activated PKC then phosphorylates and activates downstream targets, including transcription factors and kinases.

The role of PKC in growth regulation is complex and depends on the cell type and the growth factor involved. PKC can either promote cell proliferation or inhibit it, depending on the context. For example, PKC activation can lead to the activation of the extracellular signal-regulated kinases (ERK), which promote cell growth, or the activation of the phosphatase PTP1B, which inhibits cell growth.

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The activation of PKC can also lead to the activation of the Akt/protein kinase B (Akt/PKB) pathway, which promotes cell survival and survival. The inhibition of Akt/PKB by, for example, the mTOR inhibitor rapamycin, leads to the induction of cell growth arrest and apoptosis.

In conclusion, PKC plays a crucial role in the regulation of cell growth and differentiation, and its activation by growth factors is a key event in the pathogenesis of many diseases, including cancer and inflammation.
growth in our studies are totally compatible and in full agreement with other data suggests (24). The concentration of PGE2 (6 ng/ml) which stimulates c-fos and
have shown the effects of PGE2 to increase DNA synthesis is from 2.10
Numerous reports of PGE2 stimulating DNA synthesis in a variety of cells
showed an inhibition by prostaglandins
mRNA used to study c-fos expression and its connection with glioblastoma
mechanisms involved in the osseous response to PGE2.
Receptors and signals are under way in my laboratory to define the signal transduction
(21). It is possible that PGE2 shares similar mechanisms to the other bone growth
super-induced suppression that now protein is not needed for c-fos expression (Figure
other growth factors, where cytokine-receptors are added with prostaglandin, the message is
through activation of one or more c-fos transcriptional factors. In addition, as with
by a number of extracellular stimuli within 20 minutes to 2 hours after their addition
messenger (27). Growth factors, serum and phosphatases up regulate c-fos messenger
receptors and super-induced activation of protein kinase and stimulation of c-fos
signal transduction that occur through activating the tyrosine kinase domain of their
expression of c-fos is unknown. Other growth factors like EGF and PDGF have
in a variety of cell types (21,22,23). The mechanism of PGE2 in activating the
eexpression much in the same manner as other growth factor EGF, PDGF and TGF-
of addition of PGE2 (Figure 2). This demonstrates that PGE2 regulates early gene
expression (30). c-fos transcriptional message is increased by at least 2 fold within minutes
genes and then again expression during proliferation and osteoblasts in growth in vivo (28,29). Using these cells, we found that PGE2 induces immediate-early
this purpose, we employed the MDA-MB-231 breast cancer cell line, which was established
on osteoblast-like DNA replication and early gene expression in cultured osteoblasts. For
mechanisms of growth regulation by PGE2, we have looked at the direct effects of PGE2
consistencies of ectopic progesterone (AP-1), which binds to DNA in a sequence-specific
and transiently activated by growth factors (25,26). EGF and Ins are the major

RECEPTORS
published in the literature on PGE2 stimulation of osteoblast growth both \textit{in vivo} and \textit{in vitro}. We asked the question whether the concentration of prostaglandin needed for \textit{c-fos} expression correlates with the concentration needed for increased DNA synthesis and osteoblast cell growth.

**Figure 2. Prostaglandin stimulation of \textit{c-fos}.**

Osteoblasts were serum deprived for 48 hours in 0.5% FCS before addition of PGE2. \textit{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 \textit{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 G1, preceding S-phase DNA synthesis by several hours (20). When this G1 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 G1/S boundary (20). This observation of a G1 block of the cell cycle is supported by the work of other laboratories that have demonstrated that dexamethasone arrests hepatoma and adenocarcinoma cells in G1 (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 PGE2 synthesis since addition of exogenous prostaglandin to the glucocorticoid-treated cells increased cell growth to control levels. Using synchronous osteoblasts we found that PGE2 synthesis activity is highest during the G1 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 350,000 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 PGE2. 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 E2 acts as a growth factor in both in vitro and in vivo (30-33). The growth inhibitory prostaglandins like dmPGA1 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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