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# PROSTAGLANDIN $E_2$ REGULATION OF CHONDROCYTE PROLIFERATION AND DIFFERENTIATION

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THESIS

Presented to the Faculty of
The University of Texas Graduate School of Biomedical Sciences
at San Antonio
in Partial Fulfillment
of the Requirements
for the Degree of
MASTER OF SCIENCE

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By
Ridge Morgan Gilley, A.A., B.S., D.D.S.

San Antonio, Texas

May, 1994

# PROSTAGLANDIN $E_2$ REGULATION OF CHONDROCYTE PROLIFERATION AND DIFFERENTIATION

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Dean

# DEDICATION

To Debbie, my one and only love.

and

To my parents and family, who have given me their continuous love and support throughout my life.

## ACKNOWLEDGMENTS

With heartfelt gratitude and affection, I would like to thank Dr. Barbara Boyan for her constant support and encouragement during this project. I am grateful for the faith she has put into this "fledgling researcher" and for the opportunities she has made possible for me. To Dr. Zvi Schwartz, I express my sincere admiration and loyalty. His tireless efforts and direction were instrumental to the success of this research and to him I owe my deepest thanks. I would also like to thank the laboratory and administrative staff. I particularly wish to thank Monica Luna and Ruben Gomez for their assistance in laboratory techniques, data collection, and tissue culture. In addition, my great appreciation goes to Sandy Messier and Rachel Quinn for their tremendous administrative support.

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May the God of our Fathers bless all of you.

# PROSTAGLANDIN $E_2$ REGULATION OF CHONDROCYTE PROLIFERATION AND DIFFERENTIATION

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at San Antonio

Supervising Professor: Barbara D. Boyan

Endochondral ossification involves the differentiation of chondrocytes, along with the process of extracellular matrix formation and mineralization by these cells. Vitamin $D_3$ is essential to the regulation of endochondral ossification. The chondrocyte response to vitamin $D_3$ is dependent on the level of cell maturation and the particular vitamin $D_3$ metabolite used. Growth zone chondrocytes respond primarily to 1,25-dihydroxy vitamin $D_3$ and resting zone chondrocytes respond primarily to 24,25-dihydroxy vitamin $D_3$. Prostaglandin $E_2$ production by growth zone and resting zone chondrocytes is regulated by vitamin D metabolites and is believed to be a mediator between these hormones and their final effect on the cells. Production of prostaglandin $E_2$ is increased in growth zone chondrocytes responding to 1,25-dihydroxy vitamin $D_3$, while 24,25-dihydroxy vitamin $D_3$ inhibits prostaglandin $E_2$ production in resting zone chondrocytes. Alkaline phosphatase specific activity is stimulated in both growth zone and resting zone chondrocytes responding to their primary vitamin $D_3$ stimulators; however, indomethacin ($10^{-7}$M), which blocks endogenous prostaglandin $E_2$ production, inhibits alkaline phosphatase specific activity in growth zone chondrocytes, but stimulates the enzyme activity in resting zone chondrocytes. This suggests an autocrine or paracrine role for prostaglandin $E_2$ produced in chondrocytes that are stimulated by vitamin $D_3$ metabolites.

This study examined the direct regulatory effects of prostaglandin $E_2$ on chondrocytes and whether the effects are dose- and maturation-dependent. Chondrocytes were isolated from the growth zone and resting zone of the costochondral junction of 125 g Sprague-Dawley rats and cultured in complete medium containing Dulbecco's Modified Eagle's Medium, 10% fetal bovine serum, 1% antibiotics, and 50 µg/ml ascorbic acid in 100% humidity at 37°C. Prostaglandin $E_2$ was added to confluent, fourth passage cultures at concentrations from 0.007 to 15.00 µg/ml, using vehicle alone as a control.

Exogenous prostaglandin $E_2$ significantly increased [$^3H$]-thymidine and [$^3H$]-uridine incorporation, collagen production, and cyclic AMP production. Significant increases in alkaline phosphatase specific activity were present in the cell layer and in the matrix vesicle and plasma membrane fractions. Prostaglandin $E_2$ affected growth zone and resting zone chondrocytes in a comparable manner.

The results of this study suggest that prostaglandin $E_2$ from exogenous sources has the ability to modulate cell proliferation and the level of differentiation of growth zone and resting zone chondrocytes. The effect of prostaglandin $E_2$ on cyclic AMP production supports the view that cyclic AMP acts as a second messenger for prostaglandin $E_2$-initiated events. Unlike the response of these cells to different vitamin $D_3$ metabolites, there were no great differences between the cells based upon the maturation level.

Prostaglandin $E_2$ may be an important mediator of events taking place during endochondral ossification, having an autocrine effect in chondrocytes. It modifies vitamin $D_3$ regulation and exerts its effects in the areas of cell proliferation, protein production, and calcification, which are crucial aspects in the process of bone formation.

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## INTRODUCTION AND LITERATURE REVIEW

The influence and importance of vitamin $D_3$ in the regulation of endochondral ossification has been well established(1,2). As the primary hormonal regulator of osseous metabolism, vitamin $D_3$ maintains serum calcium and phosphate at concentrations appropriate for mineralization(3,4), and has been shown to regulate the differentiation and maturation of chondrocytes in growth plate and costochondral cartilage(5,6). Specifically, vitamin $D_3$ metabolites are known to affect or modulate alkaline phosphatase(7) and phospholipase $A_2$(8) specific activity, matrix vesicle phospholipid composition(9), extracellular matrix protein synthesis and cell proliferation(10).

### A. Prostaglandins

#### 1. Prostaglandin production.

An increase in phospholipase $A_2$ activity may result in the release of free arachidonic acid from membrane phospholipids. This, in turn, may lead to the formation of prostaglandins, thromboxanes and prostacyclins through the metabolism of arachidonic acid *via* the cyclo-oxygenase pathway, and to the formation of leukotrienes and hydroxyeicosatetraenoic acids (HETE's) *via* the lipoxygenase pathway(11). Prostaglandins are 20-carbon unsaturated fatty acids that contain a cyclopentane ring(12). They are identified by a letter to designate the particular structure of the cyclopentane ring (*e.g.* prostaglandin E, F, A, B, C or D), and subdivided by the number of double bonds in the side chains (*e.g.* prostaglandin $E_1$, prostaglandin $E_2$)(12). Nearly all mammalian tissues contain cyclo-oxygenase and have the ability to synthesize prostaglandins, making the prostaglandins seemingly ubiquitous throughout the body(11,13). In this process, the release of arachidonic acid, due to phospholipase $A_2$ activity, is presumed to be the rate limiting step in the production of prostaglandins(14).

Cells of the monocyte/macrophage lineage are the major cellular source of prostaglandins(15). Other sources of prostaglandin production include platelets and neutrophils(16); however, neutrophils do not possess cyclo-oxygenase and cannot produce prostaglandins in the absence of platelets(17). Prostaglandins are not stored to any extent, therefore, their presence is dependent on *de novo* synthesis, and they are rapidly metabolized in the liver, lungs, and other tissues(12). When released into the circulation, prostaglandins have numerous effects in nearly every biologic system(11).

### 2. Role in inflammation and bone resorption.

Prostaglandins are potent mediators of acute and chronic inflammation(18). The inflammatory effects of prostaglandins vary depending upon the acting prostaglandin and the tissue concentration, and include vasodilation, vasoconstriction, platelet aggregation and increased capillary permeability(16,19). Secondary effects include the enhancement of the vascular permeability and pain-producing effects of other inflammatory mediators such as bradykinin, histamine and complement components (particularly C5a)(20). In addition, prostaglandin $E_1$ and prostaglandin $E_2$ inhibit neutrophil activation and superoxide anion formation, lymphocyte proliferation, cell-mediated cytotoxicity, and the generation of cytokines(21-25).

Numerous studies have shown that prostaglandins are potent mediators of bone resorption(26-28), and may inhibit bone collagen formation(29). Prostaglandins are proposed mediators of increased bone resorption in osteolytic lesions(30,31), periodontal disease(32-34), osteomyelitis(35), and rheumatoid arthritis(36). Stimulation of osteoclastic bone resorption by prostaglandin $E_2$ ($PGE_2$) is associated with increased osteoclast mobility and activity(37). Degradation of connective tissue extracellular matrix components in health and disease may be mediated by $PGE_2$ through its influence on cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1ß (IL-1ß), and the subsequent role of these cytokines in the induction of metalloproteinase formation(38,39). Bradykinin, TNF-α, and IL-1ß have been shown to increase production of E-series prostaglandins in osteoblasts(40-42), periodontal ligament fibroblasts(43,44), and osteoblast-like MC3T3 cells(40,44,45). Low tissue concentrations of PGE$_2$ may act synergistically with IL-1ß, resulting in a level of bone resorption comparable to high PGE$_2$ levels(46,47).

3. <u>Inhibition of prostaglandin production</u>

Considerable emphasis has been placed on the inhibition of prostaglandin production in the treatment of chronic inflammatory diseases(12). The major approach to prostaglandin inhibition is the use of non-steroidal anti-inflammatory drugs (NSAID's). The widespread use of NSAID's in the treatment of chronic inflammation arose from the discovery that aspirin and other NSAID's selectively inhibit cyclo-oxygenase(48-50). It is through this mechanism that NSAID's exert their anti-inflammatory, antipyretic, and analgesic effects(12). Steroids interfere with prostaglandin production through their inhibition of phospholipase and exhibit additional anti-inflammatory effects by stabilizing lysosomal membranes and by blocking the vascular effects of histamine and bradykinin(51).

4. <u>Role in bone formation</u>. Other *in vitro* and *in vivo* evidence suggests that prostaglandins also act as stimulators of bone matrix synthesis(52-54). While PGE$_2$

concentrations of $10^{-6}$M or greater inhibited bone collagen synthesis in 21-day fetal rat calvaria(27), a low $PGE_2$ concentration of $10^{-7}$M stimulated DNA synthesis at 24 hours and collagen synthesis at 96 hours(53). In MC3T3-E1 osteoblast-like cells, incubation with 0.1 to 2.0 µg/ml $PGE_2$ resulted in a dose-dependent elevation of collagenase-digestible and non-collagenase-digestible protein synthesis and a slight increase in DNA synthesis(55). In embryonic chick calvaria, $10^{-8}$ to $10^{-5}$M $PGE_2$ increased collagenase-digestible protein and percent collagen synthesis with little effect on non-collagenase-digestible protein and increased calcification as measured by [$^3$H]-tetracycline uptake into bone(56). The stimulation of both bone resorption and formation by prostaglandins suggests that they may be important in coupling events during ossification(53).

5. Prostaglandin $E_2$ and cyclic AMP activity. The effects of $PGE_2$ on target cells are thought to occur through receptor binding and through second messenger activity by cyclic adenosine monophosphate (cyclic AMP)(57). The $PGE_2$ receptor is believed to be a small, membrane associated protein that is structurally similar to the ß-adrenergic receptor and is coupled to guanine nucleotide proteins (G proteins)(58). G proteins act as transmembrane signaling intermediates and activate a number of pathways including the formation of cyclic AMP through the breakdown of

adenosine triphosphate by adenylate cyclase(59). Cyclic AMP activates various protein kinases which may then mediate such factors as intracellular pH(60), cellular $Ca^{2+}$ levels(61,62), and regulation of gene expression(63,64). Cyclic AMP responses to prostaglandin may be modulated primarily by protein kinase C(62,65,66). $PGE_2$ may not always act as a primary initiator of cellular responses, but by increasing cyclic AMP, it may augment other cellular signals or act as a permissive activator of gene expression(38).

Increased levels of cyclic AMP have been identified in cells undergoing chondrogenesis(67-70) and this increase appears to be important in cell-to-cell interactions during chondrogenesis, resulting in stimulation of cartilage cell differentiation(71-73). $PGE_2$ stimulates cyclic AMP production in limb mesenchyme(74-79), epiphyseal, costochondral, and condylar cartilage(80-82), human osteoblasts(42,83), osteoblast-like cells(44,84,85), and osteoclasts(86). Studies in limb mesenchyme have shown that there are dramatic rises in endogenous $PGE_2$ and cyclic AMP production in the first 2 to 3 days of culture and that this rise leads to a homogenous population of differentiated chondrocytes by day 6(76,78,79). $PGE_2$ concentrations were maximal at day 3 and fell dramatically by day 6(76). The responsiveness of the cells to $PGE_2$ appeared to be specific, *i.e.*, similar concentrations of other prostaglandins failed to produce a similar response(79). The cells were more responsive to the $PGE_2$ and cyclic AMP increases at earlier time points, rather than after differentiation had taken place, suggesting a regulatory role for $PGE_2$ and cyclic AMP in the early events of chondrogenesis(76,78,79). In a separate study, exogenous $PGE_2$ ($10^{-8}$ to $10^{-5}$M) resulted in a dose-dependent increase in cyclic AMP production. $PGE_1$ was found to be as effective as $PGE_2$ in producing this response(77). In rat condylar cartilage, $PGE_2$ stimulated the differentiation and transition of prechondroblasts to functioning chondroblasts, and of functioning chondroblasts to hypertrophic chondroblasts(82). In human osteoblasts and MC3T3-E1 cells, $PGE_2$-induced increases in cyclic AMP were correlated with alkaline phosphatase activity and the differentiation of these cells(83,84).

## B. Vitamin $D_3$ and Endogenous Prostaglandin $E_2$

$PGE_2$ is produced by growth zone and resting zone chondrocytes(87), as well as several osteoblast-like cells(88), in response to vitamin $D_3$ metabolites. The amount of $PGE_2$ produced depends on the type of cell(88) and the vitamin $D_3$ metabolite used(87). Using a well established chondrocyte model(9), the effect of vitamin $D_3$ on $PGE_2$ production by growth zone and resting zone chondrocytes has been studied. Earlier studies have shown that growth zone chondrocytes respond primarily to 1,25-dihydroxy vitamin $D_3$ [1,25-$(OH)_2D_3$], while resting zone chondrocytes respond primarily to 24,25-dihydroxy vitamin $D_3$ [24,25-$(OH)_2D_3$](7,9). The production of $PGE_2$ in chondrocyte cultures incubated with varying molar concentrations of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ for 24 hours and was measured(87). Half of the cultures were also incubated with $10^{-7}$M indomethacin (a known inhibitor of the cyclo-oxygenase pathway). Growth zone chondrocytes incubated with 1,25-$(OH)_2D_3$ showed significant increases in $PGE_2$, while indomethacin completely blocked the effect. Resting zone chondrocytes incubated with 24,25-$(OH)_2D_3$ showed a significant <u>decrease</u> in $PGE_2$ production compared to untreated controls. As before, indomethacin inhibited $PGE_2$ production. Growth zone chondrocytes incubated with 24,25-$(OH)_2D_3$ and resting zone chondrocytes incubated with 1,25-$(OH)_2D_3$ showed no significant change in $PGE_2$ production compared to untreated controls. These results demonstrate a clear maturation-dependent difference between growth zone and resting zone chondrocytes in the production of $PGE_2$ in response to vitamin $D_3$.

The response of chondrocytes to endogenous $PGE_2$ production was also examined by measuring alkaline phosphatase specific activity in the cell layers of growth zone and resting zone chondrocytes after incubation with vitamin $D_3$ metabolites (Schwartz, unpublished data). For this study, growth zone chondrocytes were incubated with varying concentrations of 1,25-$(OH)_2D_3$, while resting zone chondrocytes were incubated with 24,25-$(OH)_2D_3$. To determine the effects of $PGE_2$ inhibition, half of the cultures were incubated with $10^{-7}$M indomethacin. Growth zone chondrocytes incubated with 1,25-$(OH)_2D_3$ showed significant increases in alkaline phosphatase specific activity. In the presence of $10^{-7}$M indomethacin there was a significant decrease in alkaline phosphatase specific activity which seemed to correlate with the inhibition of $PGE_2$. Resting zone chondrocytes incubated with 24,25-$(OH)_2D_3$ demonstrated significant increases in alkaline phosphatase specific activity, while treatment with $10^{-7}$M indomethacin appeared to enhance alkaline phosphatase specific activity at the lower 24,25-$(OH)_2D_3$ concentrations ($10^{-9}$ and $10^{-8}$M) and this effect was still present at the highest 24,25-$(OH)_2D_3$ concentration($10^{-7}$M). Similar responses were seen when alkaline phosphatase specific activity was measured in the matrix vesicle and plasma membrane fractions of growth zone and resting zone chondrocytes (Schwartz, unpublished data). These results suggest that $PGE_2$ functions in an autocrine or paracrine manner(87). As a result, $PGE_2$ may be an important regulator of cellular events subsequent to vitamin $D_3$ stimulation. Considering the ubiquitous production of prostaglandins in mammalian tissues, there could be other potential sources of $PGE_2$ in local tissues that may act on calcifying cells. For example, 1,25-$(OH)_2D_3$ has been shown to markedly increase $PGE_2$ production in peripheral human blood monocytes(89).

## C. Purpose of Investigation

The primary objective of this study is to determine the regulatory effects of exogenous prostaglandin $E_2$ on growth zone and resting zone chondrocyte differentiation and proliferation, and to compare these effects to the autocrine action of $PGE_2$ found in earlier experiments. To accomplish this, the effect of $PGE_2$ on growth zone and resting zone chondrocyte alkaline phosphatase specific activity, incorporation of $[^3H]$-thymidine and $[^3H]$-uridine, collagen synthesis, and cyclic AMP production were examined.

## Materials and Methods

### A. Chondrocyte Cultures

Using a previously described method(9), chondrocytes were obtained from the resting zone and growth zone cartilage cells from the costochondral junction of 125 g male Sprague Dawley rats. The rib cages were removed by sharp dissection and placed in Dulbecco's Modified Eagle's Medium [DMEM (Gibco, Grand Island, NY)]. The resting zone and adjacent growth zone cartilage were separated, sliced and incubated overnight in DMEM at 37°C. The DMEM was replaced with two washes of Hank's Balanced Salt Solution (HBSS). The extracellular matrix was digested by sequential incubations in 1% trypsin (Gibco) for 1 hour and 0.02% collagenase for 3 hours(90). The cells were separated from tissue debris by filtration through 40 mesh nylon and collected from the filtrate by centrifugation at 500 x g for 5 minutes. The cells were plated in 25 mm culture dishes at initial densities of 25,000 cells/cm$^2$ for growth zone chondrocytes and 10,000 cells/cm$^2$ for resting zone chondrocytes(91). The cells were cultured in complete medium {DMEM containing 10% fetal bovine serum [FBS (Gibco)], 50 µg/ml vitamin C and 1% penicillin-streptomycin-fungizone} and incubated in an atmosphere of 5% $CO_2$ in air at 37°C and 100% humidity until confluent. The medium was changed every 72 hours. At the third passage, the cells were subcultured as described in the specific assays. Fourth passage cells were used for all experiments. Previous studies have determined that phenotypic expression is retained in both growth zone and resting zone chondrocyte cultures through the fourth passage(9).

Experimental media were prepared by combining $PGE_2$ (MW 352.5, Sigma, St. Louis, MO.) resuspended in ethanol with complete medium (DMEM + 1% FBS + vitamin C + antibiotic). The $PGE_2$ concentrations in experimental media ranged from 0.007 to 15.0 µg/ml ($1.88 \times 10^{-8}$ to $4.0 \times 10^{-5}$M). Ethanol combined with complete medium was used as control. The final amount of ethanol in either experimental or control media was 0.3 µl per ml of medium.

## B. Alkaline Phosphatase Specific Activity

1. Preparation of cell layers. Confluent, third passage growth zone and resting zone chondrocytes were subcultured into 24-well plates. Cell plating densities were 85,000 cells per well for growth zone cells and 35,000 cells per well for resting zone cells. At confluence, experimental media (500 µl) were added for 24 hours. At harvest, the cells were washed with phosphate buffered saline (PBS) and removed using a cell scraper. The harvested cells were centrifuged, washed again with PBS and resuspended in 500 µl 0.05% Triton X-100(92). Alkaline

phosphatase specific activity was measured as a function of the release of para-nitrophenol from para-nitrophenyl phosphate(93). The reaction was performed on 96-well plates and read on a BioRad EIA reader (Model #2550, BioRad Inc., Richmond CA,) at 405 nm.

2. Determination of protein content. Protein content was measured utilizing a Micro BCA protein assay reagent (Pierce Chemical Co., Rockford IL). The test uses a biuret reaction where $Cu^{+2}$ is reduced to $Cu^{+1}$ that reacts with bicinchoninic acid to form a product with strong absorbance at 562 nm. Bovine serum albumin was used as a standard (1.0 to 20.0 µg/ml). Results of alkaline phosphatase and protein assays are reported as specific activity (µmol Pi/mg protein/minute).

3. Preparation of membrane fractions. The alkaline phosphatase specific activity was also measured in the matrix vesicle and plasma membrane cell fractions of growth zone and resting zone chondrocytes(9). Confluent, third passage growth zone and resting zone chondrocytes were subcultured in T-75 flasks. Cell plating densities were 25,000 cells/$cm^2$ for growth zone chondrocytes and 10,000 cells/$cm^2$ for resting zone chondrocytes. Experimental media (15 ml) were added for 24 hours. At harvest, the culture medium was replaced with 1% trypsin in HBSS. The reaction was stopped with DMEM containing 10% FBS. The cells were

collected by centrifugation at 500 x g for 5 minutes, resuspended in saline, washed twice, and counted. The trypsin digest supernatant was centrifuged at 21,000 x g for 10 minutes to pellet a mitochondria/membrane fraction and at 100,000 x g for 1 hour to pellet matrix vesicles. To isolate plasma membranes, cells were homogenized in a Tenbroek homogenizer, followed by differential and sucrose density centrifugations(94). The matrix vesicle and plasma membrane fractions were resuspended in 0.9% NaCl and assayed for alkaline phosphatase specific activity and protein as before.

### C. $[^3H]$-Thymidine Incorporation

As a measure of cell proliferation, $[^3H]$-thymidine incorporation was determined. Confluent growth zone and resting zone chondrocytes were subcultured into 96-well plates. Cells were plated at densities of 15,000 cells per well for growth zone chondrocytes and 8,000 cells per well for resting zone chondrocytes. The cells were incubated for 48 hours with complete media (150 µl per well) to allow for cell attachment. At confluence, the cells were synchronized and made quiescent by incubation in DMEM with 1% FBS for 48 hours. This medium was then removed and replaced with 150 µl of experimental medium. After 20 hours of incubation with experimental medium, 50 µl $[^3H]$-thymidine (1 µCi/ml) was added to each well and the cell layer harvested 4 hours later. At the end of incubation, the cells were washed twice with 150 µl phosphate buffered saline (PBS) and fixed by washing three times with 150 µl 5% trichloroacetic acid (TCA). The TCA was removed and the cell layer air-dried. The fixed cells were dissolved in 200 µl of 1% sodium dodecyl sulfate (SDS) for 30 min at room temperature, added to scintillation vials containing 10 ml of scintillation fluid (Protein Plus, Beckman) and then counted in a Beckman scintillation counter (Model LS 6000 IC).

In a similar manner, time course experiments were conducted at 5, 12, 24 and 48 hours after addition of experimental media. Confluent cells were incubated with $PGE_2$ using the same concentration range as before, but the cells were not made quiescent prior to the addition of experimental media. [$^3$H]-thymidine was added for the last four hours of incubation. The cell harvest and DPM determination was conducted as in the earlier experiments.

### D. [$^3$H]-Uridine incorporation

The synthesis of RNA was measured by determining [$^3$H]-uridine incorporation. Confluent, third passage growth zone and resting zone chondrocytes were subcultured into 96-well plates. Cells were plated at the same densities as in the [$^3$H]-thymidine incorporation experiments, incubated for 48 hours in the presence of complete medium and the confluent cultures were made quiescent. The samples were incubated with experimental media for 5 hours and 50 µl [$^3$H]-uridine (1 µCi/ml) was added for 2 hours. At the end of incubation, the cells were harvested and the amount of [$^3$H]-uridine determined as described for [$^3$H]-thymidine experiments.

Time course experiments were conducted at 1, 3, 5, 12, and 24 hours after addition of experimental media. Confluent cells were incubated with $PGE_2$ in the same concentration range as before, but the cells were not made quiescent prior to addition of experimental media. [$^3$H]-uridine was added for the last two hours of incubation except for the 1 hour time point where [$^3$H]-uridine was added along with the experimental media. The cell harvest and DPM determination was conducted as before.

## E. Collagen Production

Collagen and non-collagen protein synthesis was determined by measuring the incorporation of [$^3$H]-proline into newly-systhesized protein. Collagenase digestion was used to separate collagenase-digestible protein from non-collagenase-digestible protein(95). Confluent, third passage growth zone and resting zone chondrocytes were subcultured into 6-well plates. Cell plating densities were 400,000 cells per well for growth zone cells and 200,000 cells per well for resting zone cells. At confluence, the cells were incubated for 24 hours with experimental medium (2 ml per well) using the same $PGE_2$ concentrations as before. The cells were labeled for 24 hours with 2 ml of complete medium containing [$^3$H]-proline (5 µCi/ml) and 50 µg/ml of ß-aminoproprionitrile. After labelling, the medium was removed and set-aside for protein extraction. The cells were harvested in two 0.5 ml portions of 0.2 N NaOH and centrifuged at 400 x g at 4°C for 10 min.

The cell and medium proteins were precipitated separately by 0.1 ml additions of 100% TCA containing 10% tannic acid and centrifuged as before. The cell and protein precipitates were combined after centrifugation, and washed three times with 10% TCA containing 1% tannic acid. The pellets were washed two more times with 1.0 ml ice-cold acetone and centrifuged after each of the washes. The final pellet was dissolved in 500 µl of 0.05M NaOH. Total protein content was measured as described above. Total incorporation of [$^3$H]-proline was determined by placing 50 µl of each sample into scintillation vials containing 10 ml of scintillation fluid, and counting in a Beckman spectrometer.

Collagenase-digestible and non-collagenase-digestible proteins were separated by a collagenase digestion reaction(96). Each sample was combined with 500 µl of a collagenase reaction mixture containing 25 units of collagenase (Type II, Clostridiopeptidase EC 3.4.24.3, Sigma), 60 µM Hepes buffer, 1.25 µM N-ethylmaleimide (NEM), 0.25 µM $CaCl_2$, and 0.08 N HCl and was then incubated at 37°C for 4 hours. The reaction was stopped by precipitation of the non-collagenase-digestible protein with 0.5 ml of 10% TCA containing 0.5% tannic acid for 5 min at 0°C. The samples were centrifuged at 400 x g for 5 min at 4°C and the supernatant (digested collagen) was transferred to a vial with 10 ml scintillation fluid. The precipitates were resuspended in 0.5 ml of 5% TCA containing 0.25% tannic acid for 24 hours at -20°C to increase residual collagenase-digestible protein solubility. The suspensions were recentrifuged and the supernatants were added to the vials containing the collagenase-digestible protein. The precipitates containing the non-collagenase-digestible proteins were resuspended in 1 ml of 5% TCA + 0.25% tannic acid and transferred to vials with 10 ml of scintillation fliud. Vials containing collagenase-digestible and non-collagenase-digestible proteins were read in a Beckman spectrometer. The non-collagenase-digestible protein values were multiplied by 5.4 to correct for the relative abundance of proline in collagen(97), and the percent collagen production was calculated by dividing the collagenase-digestible protein by the total protein in the TCA precipitate.

## F. Determination of Cyclic AMP Production

The production of cyclic AMP was measured after extraction of cellular cyclic AMP. Confluent, third passage growth zone and resting zone chondrocytes were subcultured into 24 well plates. Cell plating densities were 85,000 cells per well for growth zone cells and 35,000 cells per well for resting zone cells. At confluence and prior to addition of experimental media, the cells are washed twice with 500 µl DMEM and preincubated for 30 min in 500 µl DMEM with 0.2 mM/l isobutylmethylxanthine (IBMX, a cyclic AMP phosphodiesterase inhibitor). The preincubation medium was removed and the cells incubated for 10 min in 500 µl experimental medium, using the same $PGE_2$ concentrations as before. After 10 minutes, experimental media were removed and stored at -20°C. Cyclic AMP was extracted with 500 µl of 90% *n*-propan-1-ol at 4°C for 24 hours. The extract was evaporated, and reconstituted in 100 µl acetate buffer.

The cyclic AMP content of the extract was measured by radioimmunoassay. The assay employs a preconjugated double antibody separation in an acetate buffer (#BT-300, Biomedical Technologies Inc., Stoughton MA)(98). The $^{125}I$ tracer contains [$^{125}I$] succinyl cAMP-tyrosine methyl ester in normal rabbit IgG containing phosphodiesterase inhibitors and sodium azide. 100 µl of sample is combined with 100 µl cAMP tracer and 100 µl cAMP antibody. Reagents are mixed for 30 sec, covered, and incubated for 20 hours at 4°C. At the end of the incubation, one ml cold buffer is added, and the samples centrifuged at 2000 x g for 20 min at 4°C. The pellets are air-dried and counted in a gamma counter (Model #28150, Micromedic Systems, Horsham, PA). The values are compared to a standard curve and results are reported as pmol cyclic AMP/µg DNA.

Time course experiments were conducted at 10, 60, 180, 360, and 720 minutes. The assay was identical to the one just described, except for the time of incubation with experimental medium.

## G. DNA Extraction

For extraction of DNA contained in the cells used for the cyclic AMP experiment, the cells were fixed with 500 µl 10% TCA, collected by scraping, and then the samples were diluted with 500 µl of 0.5N $HClO_4$. DNA content was then measured utilizing a diphenylamine reaction(99,100). Samples were mixed with 2 ml of diphenylamine reagent (diphenylamine in acetic acid, $H_2SO_4$, and acetaldehyde), and incubated for 20 hours at 30°C. The optical density was read at 600 nm on a Beckman spectrophotometer (Model #DU7400) and compared to a calf thymus DNA standard.

## H. Statistical Analysis

Each experiment was conducted at least three times and the results represent typical experiments. Each data point represents the mean ± SEM of six samples. For matrix vesicle and plasma membrane samples, each experimental group represents the combined fractions of five T-75 flasks. Statistical significance was determined by comparing each data point to its untreated control using the Student's *t*-test with the Bonferroni modification. Significant differences were evaluated using a two-tailed analysis of variance ($p < 0.05$). Treatment/control ratios were determined from the results of five separate experiments and statistical significance was determined using the Wilcoxon signed rank test ($p < 0.05$). Significant values represent stimulated values compared to the untreated control within each chondrocyte cell type, but do not reflect significant differences between the two cell types.

# RESULTS

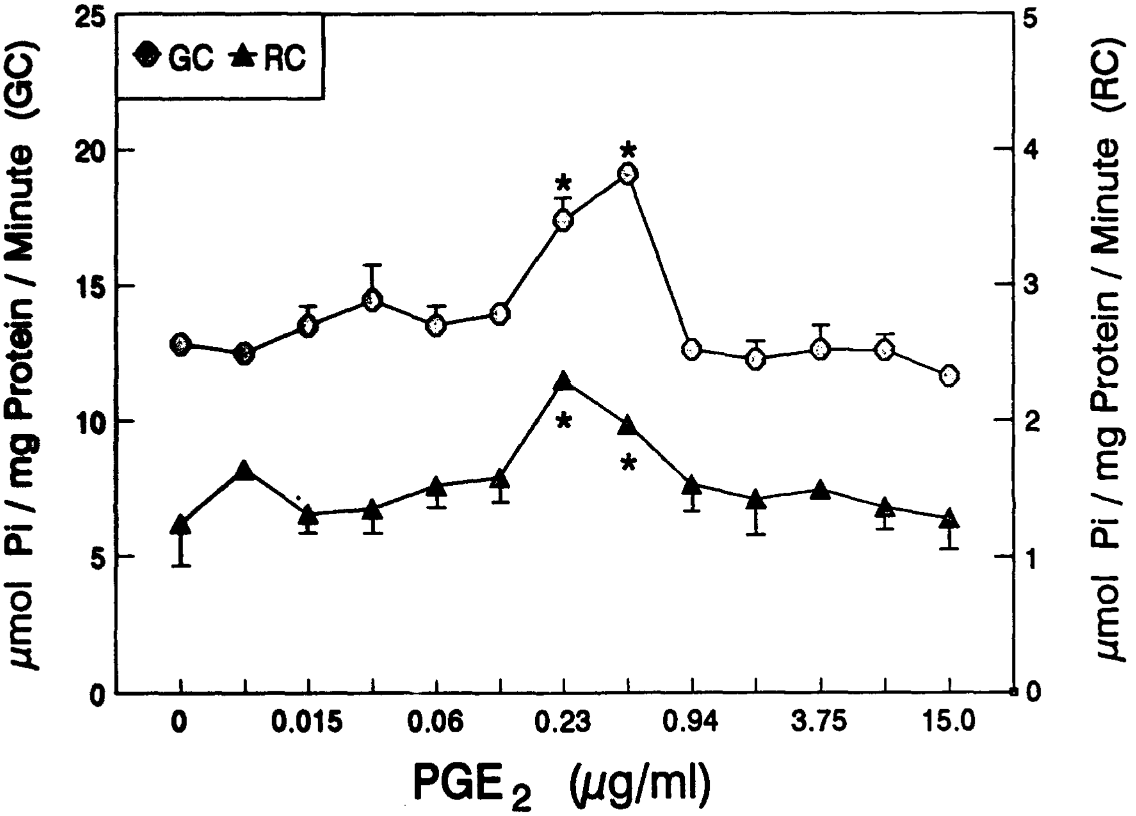
## A. Alkaline Phosphatase Specific Activity

Significant increases in alkaline phosphatase specific activity were seen in the cell layer of both growth zone and resting zone chondrocyte cultures at $PGE_2$ concentrations of 0.23 and 0.47 µg/ml (Figure 1). Enzyme specific activity in the resting zone cultures was significantly less than that observed in the growth zone irrespective of treatment. In order to account for differences in values between the two cell types, a treatment/control ratio was calculated (Figure 2). Significant increases in alkaline phosphatase specific activity of both growth zone and resting zone chondrocytes were seen at the same concentrations of $PGE_2$ as before, but the treatment/control ratio revealed that fold increases in alkaline phosphatase specific activity were similar for both cell growth zone and resting zone chondrocytes.

Matrix vesicles and plasma membranes produced by these cells also contained alkaline phosphatase specific activity that was affected by addition of $PGE_2$ to the cultures. Matrix vesicles produced by growth zone chondrocytes contained increased alkaline phosphatase specific activity over the range of 0.24 to 0.94 µg/ml $PGE_2$, while the plasma membrane fraction showed an increase at 0.24 µg/ml $PGE_2$ (Figure 3). Significant increases in alkaline phosphatase specific activity were also found in resting zone chondrocyte plasma membrane fractions after addition of 0.24 to 0.94 µg/ml $PGE_2$ to the cultures, while increases in matrix vesicles enzyme activity was only observed at 0.24 µg/ml $PGE_2$ (Figure 4). As before, alkaline phosphatase specific activity was higher in the membranes derived from growth zone chondrocyte cultures compared to those derived from the resting zone. The growth zone chondrocyte matrix vesicle and plasma membrane fractions displayed roughly a three-fold increase in alkaline phosphatase specific activity. In the resting zone chondrocytes, the matrix vesicles had a two-fold increase in alkaline phosphatase specific activity, while the plasma membrane fraction showed approximately a 50% increase in activity.

**Figure 1: Effect of $PGE_2$ on the alkaline phosphatase specific activity of resting zone (RC) and growth zone (GC) chondrocytes.**

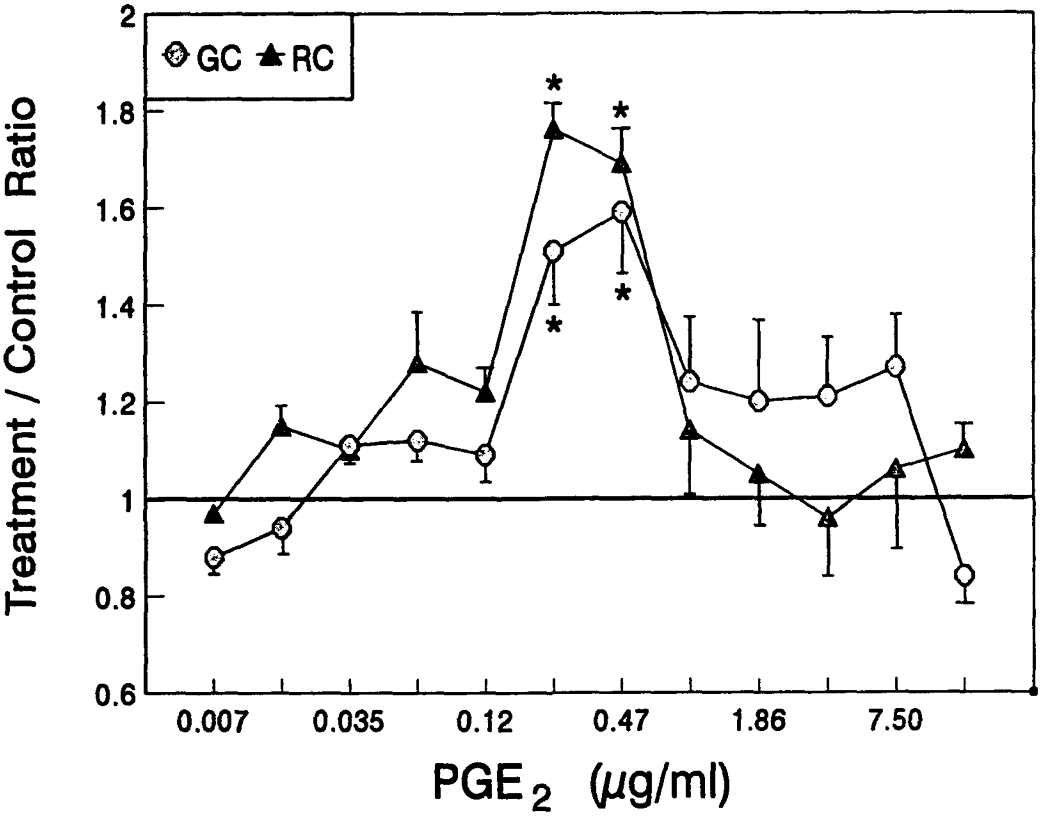
Confluent, fourth passage cultures of rat costochondral resting zone and growth zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for 24 hours and the cell layer assayed for alkaline phosphatase specific activity. Values are mean ± SEM for six cultures. Data are from one of five replicate experiments. * $p < 0.05$, treatment v. control.



GC
RC
µmol Pi / mg Protein / Minute (GC)
µmol Pi / mg Protein / Minute (RC)
0
5
10
15
20
25
0
1
2
3
4
5
*
*
*
*
0
0.015
0.06
0.23
0.94
3.75
15.0
PGE$_2$ (µg/ml)

**Figure 2: Treatment/Control Ratios showing the effect of $PGE_2$ on the alkaline phosphatase specific activity of resting zone (RC) and growth zone (GC) chondrocytes.**

Confluent, fourth passage cultures of rat costochondral resting zone and growth zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for 24 hours and the cell layer assayed for alkaline phosphatase specific activity. Values are mean ± SEM for six cultures. Data are from one of five replicate experiments. ⋆ $p < 0.05$, treatment/control.



GC
RC
Treatment / Control Ratio
2
1.8
1.6
1.4
1.2
1
0.8
0.6
*
*
*
*
0.007
0.035
0.12
0.47
1.86
7.50
PGE$_2$ (µg/ml)

**Figure 3: Effect of $PGE_2$ on the matrix vesicle (MV) and plasma membrane (PM) alkaline phosphatase specific activity of growth zone (GC) chondrocytes.**

Confluent, fourth passage cultures of rat costochondral growth zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for 24 hours and the membrane fractions were assayed for alkaline phosphatase specific activity. Values are mean ± SEM for five cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.

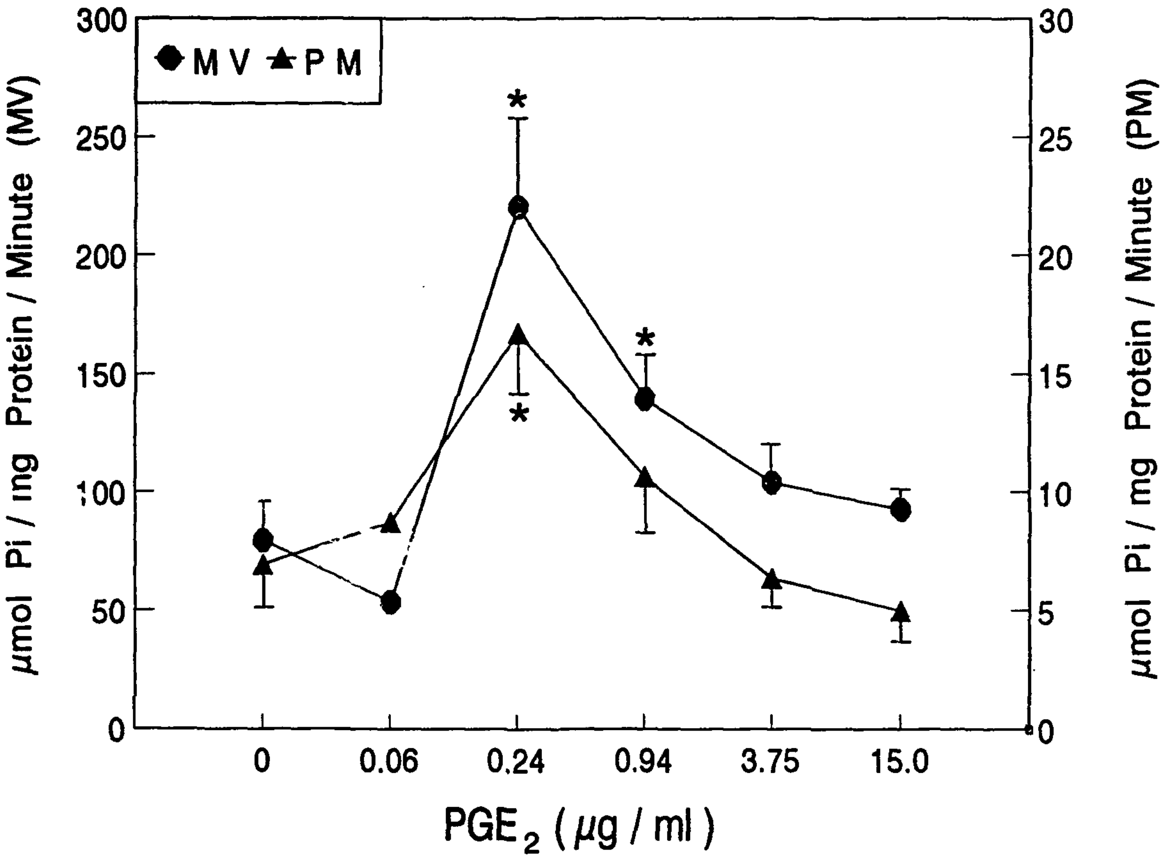
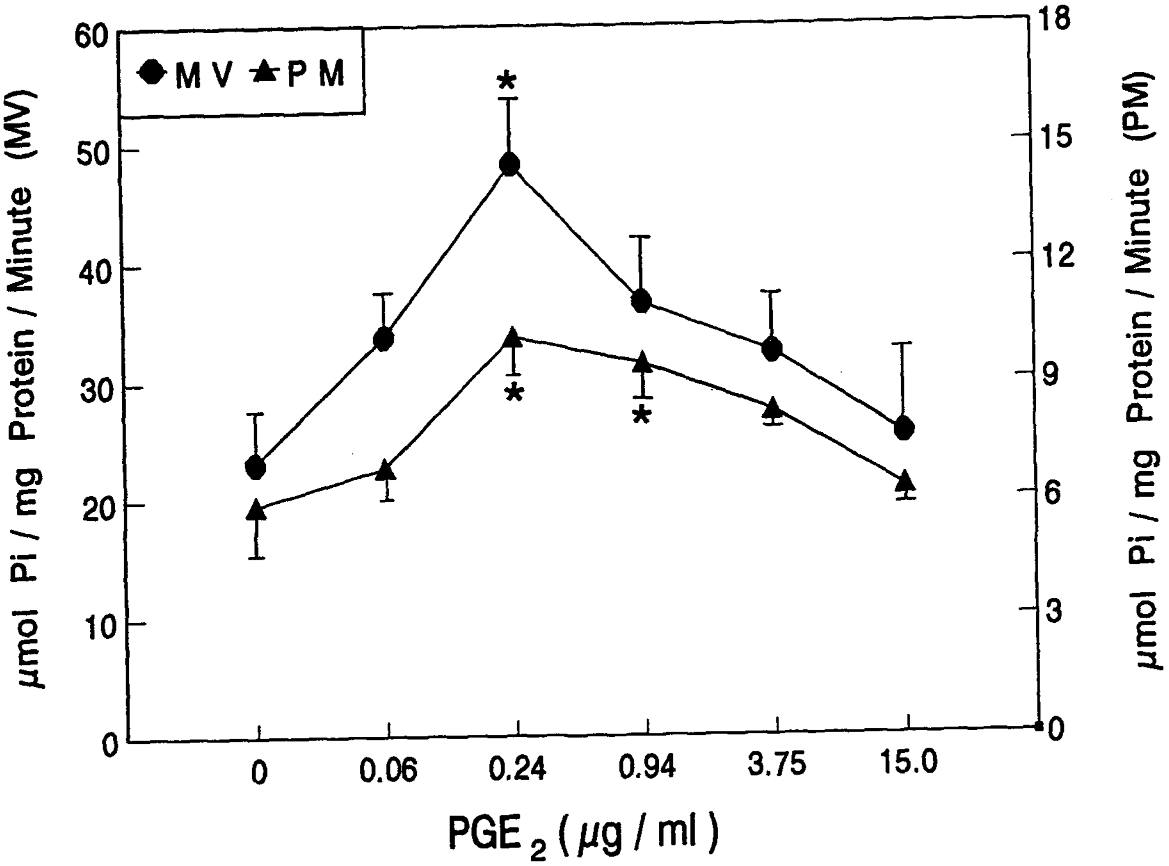

● M V ▲ P M
μmol Pi / mg Protein / Minute (MV)
μmol Pi / mg Protein / Minute (PM)
300
250
200
150
100
50
0
30
25
20
15
10
5
0
*
*
*
0
0.06
0.24
0.94
3.75
15.0
PGE$_2$ (μg / ml)

**Figure 4: Effect of $PGE_2$ on the matrix vesicle (MV) and plasma membrane (PM) alkaline phosphatase specific activity of resting zone (RC) chondrocytes.**

Confluent, fourth passage cultures of rat costochondral resting zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for 24 hours and the membrane fractions were assayed for alkaline phosphatase specific activity. Values are mean ± SEM for five cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.



● M V ▲ P M
µmol Pi / mg Protein / Minute (MV)
µmol Pi / mg Protein / Minute (PM)
60
50
40
30
20
10
0
18
15
12
9
6
3
0
*
*
*
0
0.06
0.24
0.94
3.75
15.0
PGE$_2$ (µg / ml)

### B. [$^3$H]-Thymidine Incorporation

The effect of $PGE_2$ on the incorporation of [$^3$H]-thymidine by growth zone and resting zone chondrocytes was examined (Figure 5). Significant increases in [$^3$H]-thymidine incorporation by growth zone chondrocytes were found at concentrations of $PGE_2$ ranging from 0.12 to 3.75 µg/ml. In resting zone chondrocytes, $PGE_2$ concentrations ranging from 0.12 to 1.86 µg/ml produced significant increases in [$^3$H]-thymidine incorporation (Figure 5). Resting zone chondrocytes showed peak levels of [$^3$H]-thymidine incorporation at 0.23 µg/ml $PGE_2$, while in growth zone chondrocytes, the stimulatory effect of $PGE_2$ was equivalent over all concentrations that produced significant increases in [$^3$H]-thymidine incorporation over control.

The significant increases observed in [$^3$H]-thymidine incorporation in response to $PGE_2$ were confirmed through cell number measurements of growth zone and resting zone chondrocytes following a 24 hour incubation with $PGE_2$. Both growth zone and resting zone chondrocytes exhibited significant increases in cell number over $PGE_2$ concentrations ranging from 0.23 to 3.25 µg/ml. The greatest increase in cell number was seen at 0.94 µg/ml $PGE_2$ for both types of chondrocytes.

Time course experiments revealed significant increases in [$^3$H]-thymidine incorporation for both growth zone (Figure

6) and resting zone (Figure 7) chondrocytes after 24 or 48 hours of incubation with $PGE_2$ at concentrations of 0.23 to 3.75 µg/ml. Maximum incorporation of $[^3H]$-thymidine occurred at the 24 hour time point in growth zone chondrocytes, while resting zone chondrocytes showed maximum $[^3H]$-thymidine incorporation after 48 hours. There was little change in $[^3H]$-thymidine incorporation from untreated controls after 5 or 12 hours for either cell type. The data show that increased DNA synthesis and cell proliferation occurred at nearly equivalent concentrations of $PGE_2$, and at similar times for both types of chondrocytes.

**Figure 5: Effect of $PGE_2$ on the $[^3H]$-thymidine incorporation of resting zone (RC) and growth zone (GC) chondrocytes.**

Confluent, fourth passage cultures of rat costochondral resting zone and growth zone chondrocytes were made quiescent, then incubated in complete medium containing various concentrations of $PGE_2$ for 24 hours. $[^3H]$-thymidine was added four hours prior to harvest. Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.

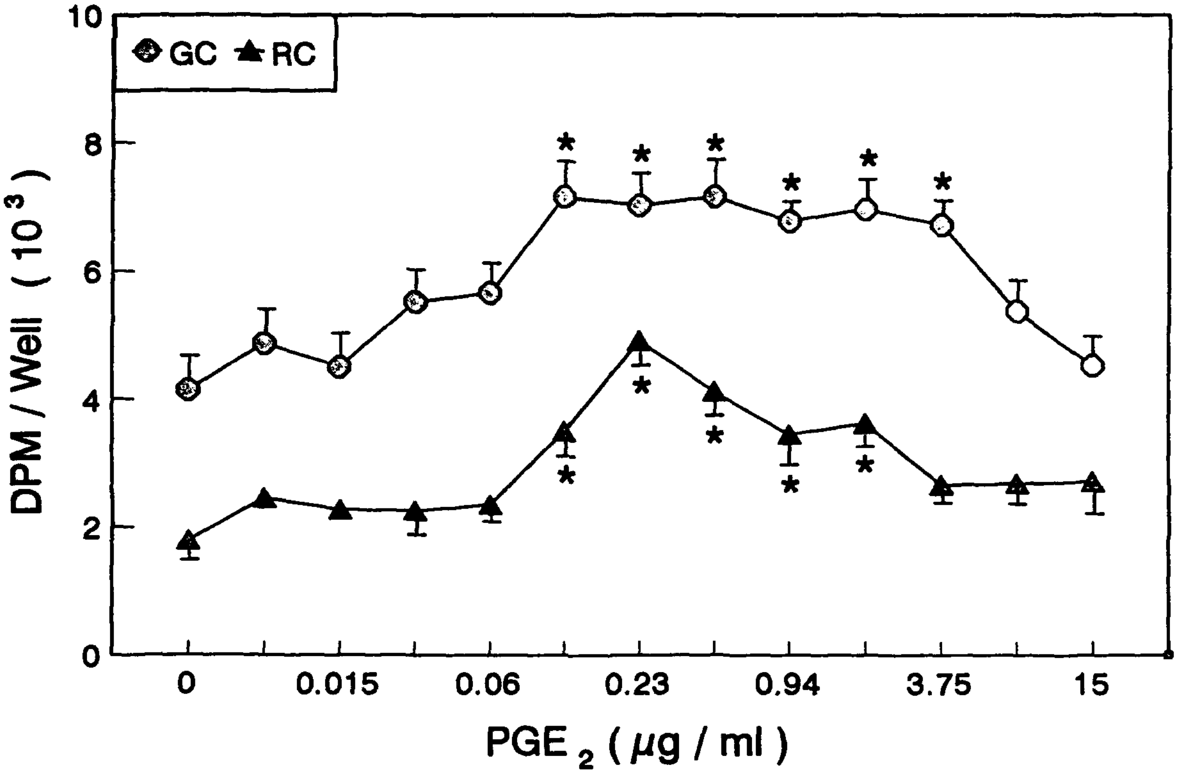

10
8
6
4
2
0
DPM / Well ( $10^3$ )
GC
RC
*
0
0.015
0.06
0.23
0.94
3.75
15
PGE $_2$ ( $\mu$g / ml )

**Figure 6: Effect of $PGE_2$ on the $[^3H]$-thymidine incorporation of growth zone (GC) chondrocytes after 5, 12, 24, or 48 hours of treatment.**

Confluent, fourth passage cultures of rat costochondral growth zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for 5, 12, 24, and 48 hours. $[^3H]$-thymidine was added four hours prior to harvest. Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.

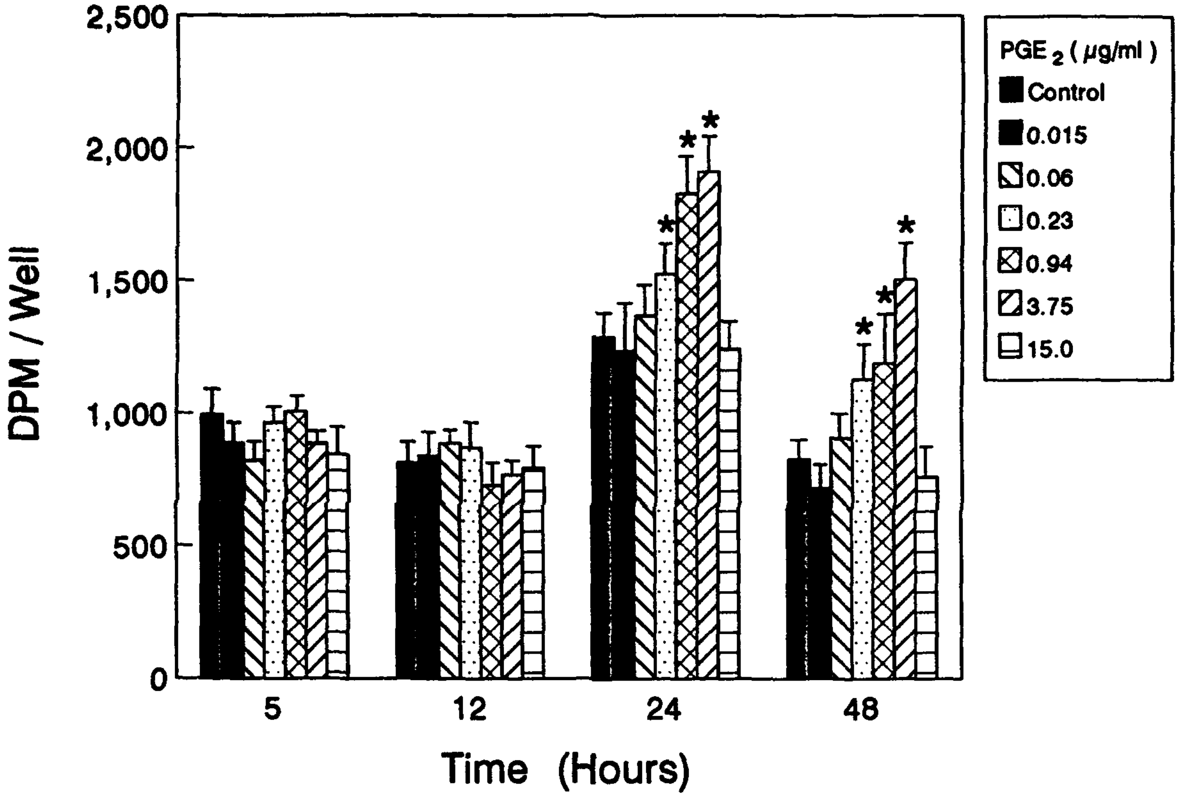
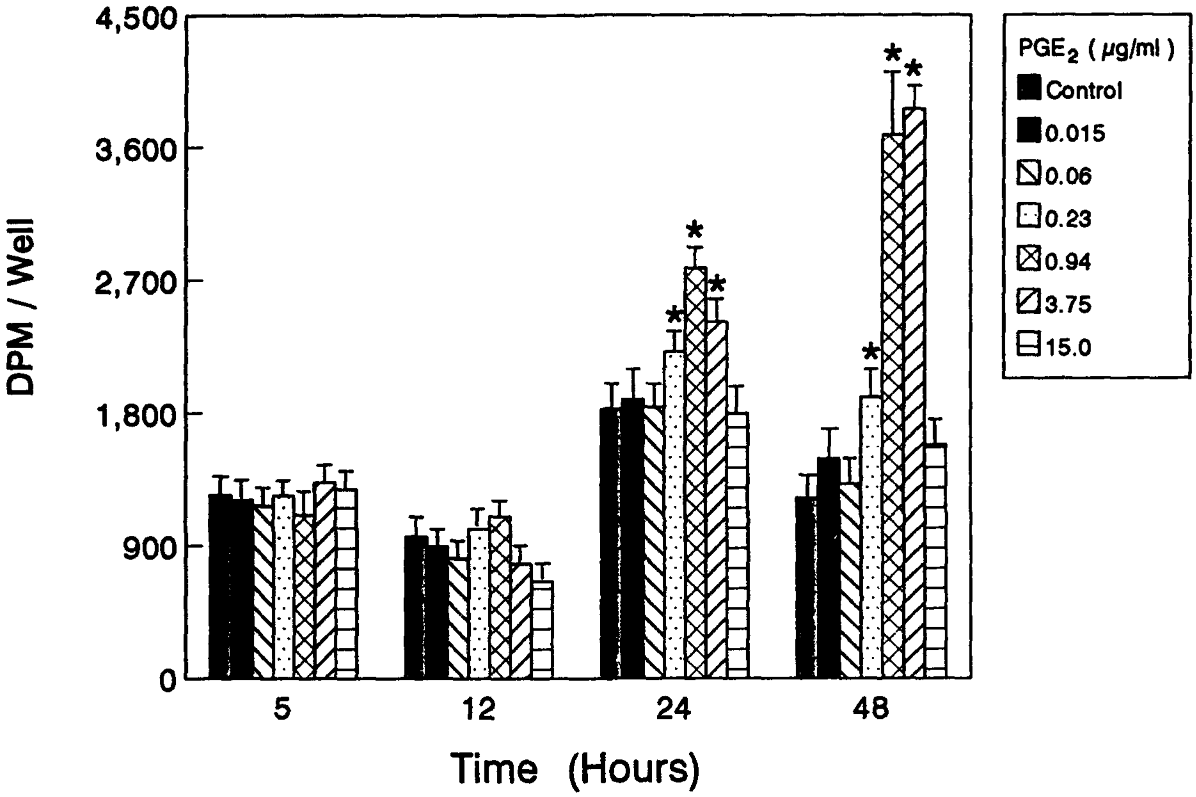

2,500
2,000
1,500
1,000
500
0
DPM / Well
5
12
24
48
Time (Hours)
PGE $_2$ ( µg/ml )
Control
0.015
0.06
0.23
0.94
3.75
15.0
*

**Figure 7: Effect of $PGE_2$ on the [$^3H$]-thymidine incorporation of resting zone (RC) chondrocytes after 5, 12, 24, or 48 hurs of treatment.**

Confluent, fourth passage cultures of rat costochondral resting zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for 5, 12, 24, and 48 hours. [$^3H$]-thymidine was added four hours prior to harvest. Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.


4,500
3,600
2,700
1,800
900
0
DPM / Well
5
12
24
48
Time (Hours)
PGE$_2$ ( μg/ml )
Control
0.015
0.06
0.23
0.94
3.75
15.0
*
*
*
*
*
*

### C. [$^3$H]-Uridine Incorporation

[$^3$H]-Uridine incorporation was used as an indicator of total RNA synthesis. PGE$_2$ elicited a dose-dependent increase in [$^3$H]-uridine incorporation by growth zone and resting zone chondrocytes. Significant increases in [$^3$H]-uridine incorporation occurred at concentrations ranging from 0.94 to 15.0 µg/ml PGE$_2$ in growth zone chondrocytes and from 1.86 to 15.0 µg/ml PGE$_2$ in resting zone chondrocytes (Figure 8).

Increases in RNA synthesis were found at earlier time points after addition of PGE$_2$ than those found for DNA synthesis. Time course experiments showed significant increases in [$^3$H]-uridine incorporation at 5, 12, and 24 hours after addition of PGE$_2$ for growth zone (Figure 9) and resting zone (Figure 10) chondrocytes at PGE$_2$ concentrations of 3.75 and 15.0 µg/ml. In addition, growth zone chondrocytes had significant increases in [$^3$H]-uridine incorporation with 15.0 µg/ml PGE$_2$ at 3 hours and 0.94 µg/ml PGE$_2$ at 24 hours.

**Figure 8: Effect of $PGE_2$ on the $[^3H]$-uridine incorporation of resting zone (RC) and growth zone (GC) chondrocytes.**

Confluent, fourth passage cultures of rat costochondral resting zone and growth zone chondrocytes were made quiescent, then incubated in complete medium containing various concentrations of $PGE_2$ for five hours. $[^3H]$-uridine was added and cultures harvested two hours later. Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.

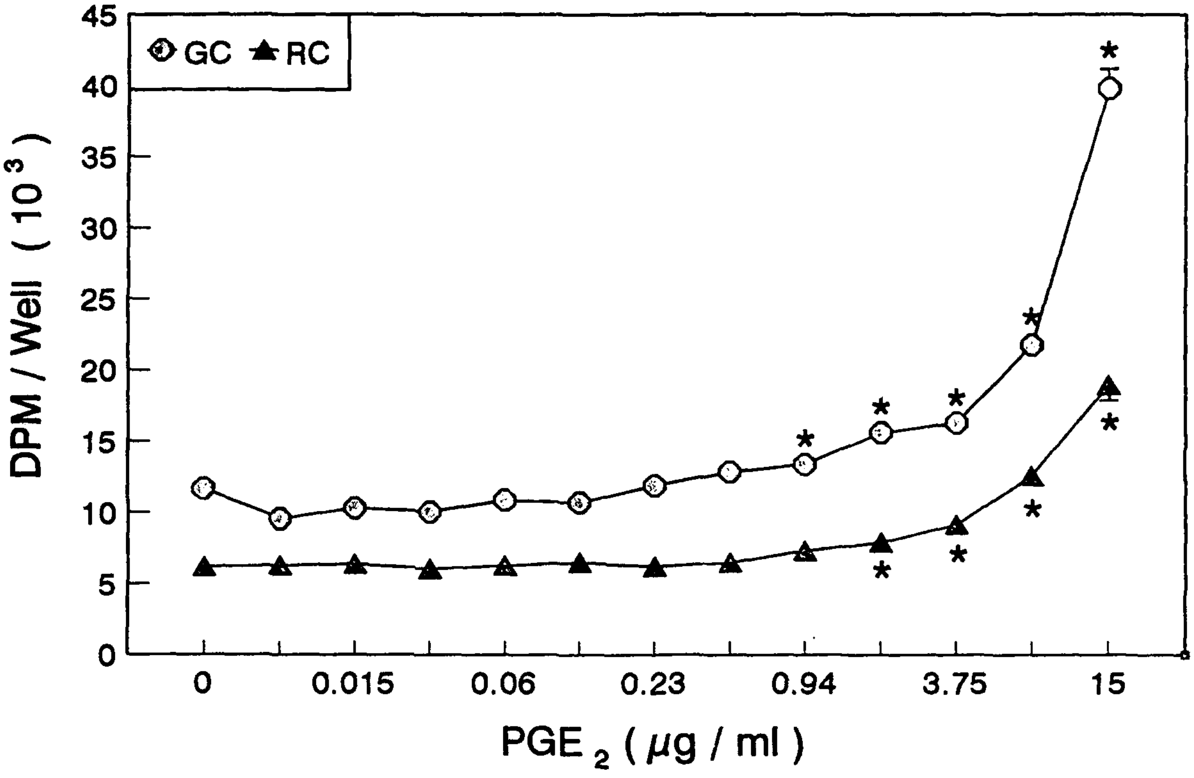
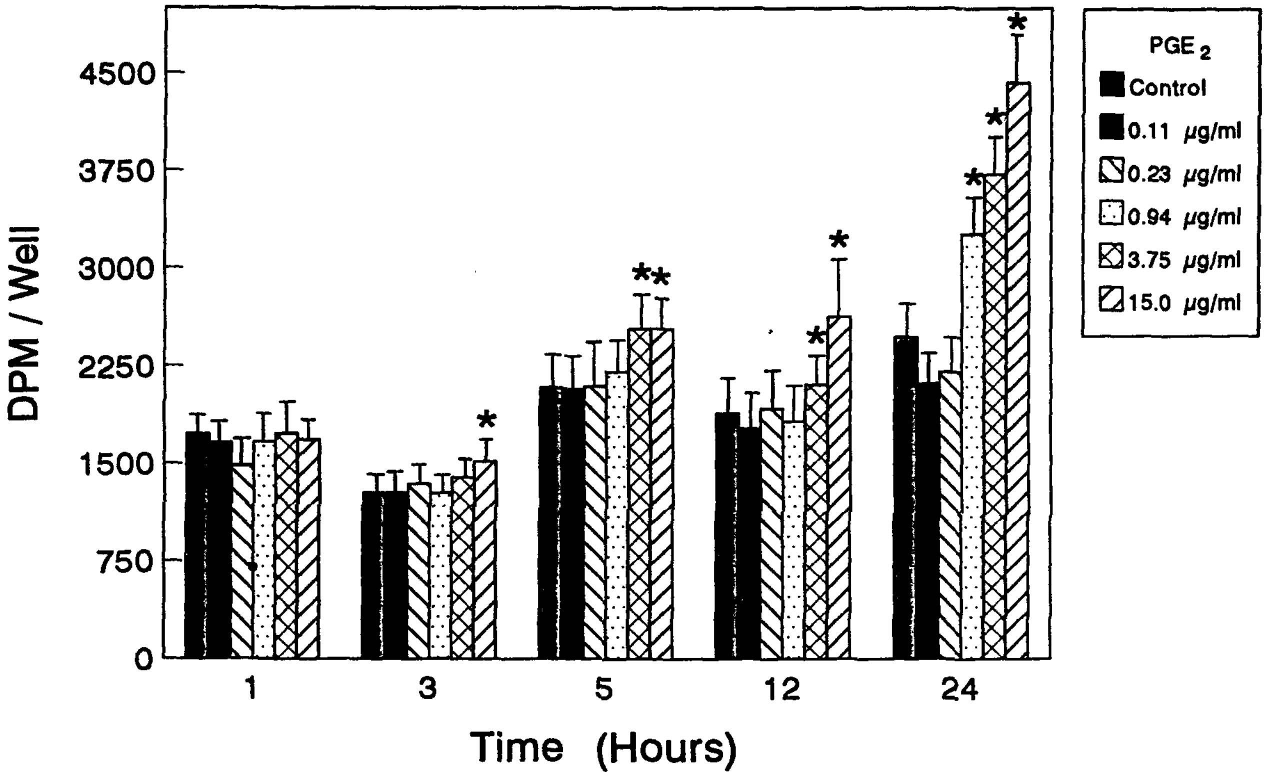

GC
RC
45
40
35
30
25
20
15
10
5
0
DPM / Well ( $10^3$ )
0
0.015
0.06
0.23
0.94
3.75
15
PGE $_2$ ( μg / ml )
*

**Figure 9: Effect of $PGE_2$ on the $[^3H]$-uridine incorporation of growth zone (GC) chondrocytes after 1, 3, 5, 12, or 24 hours of treatment.**

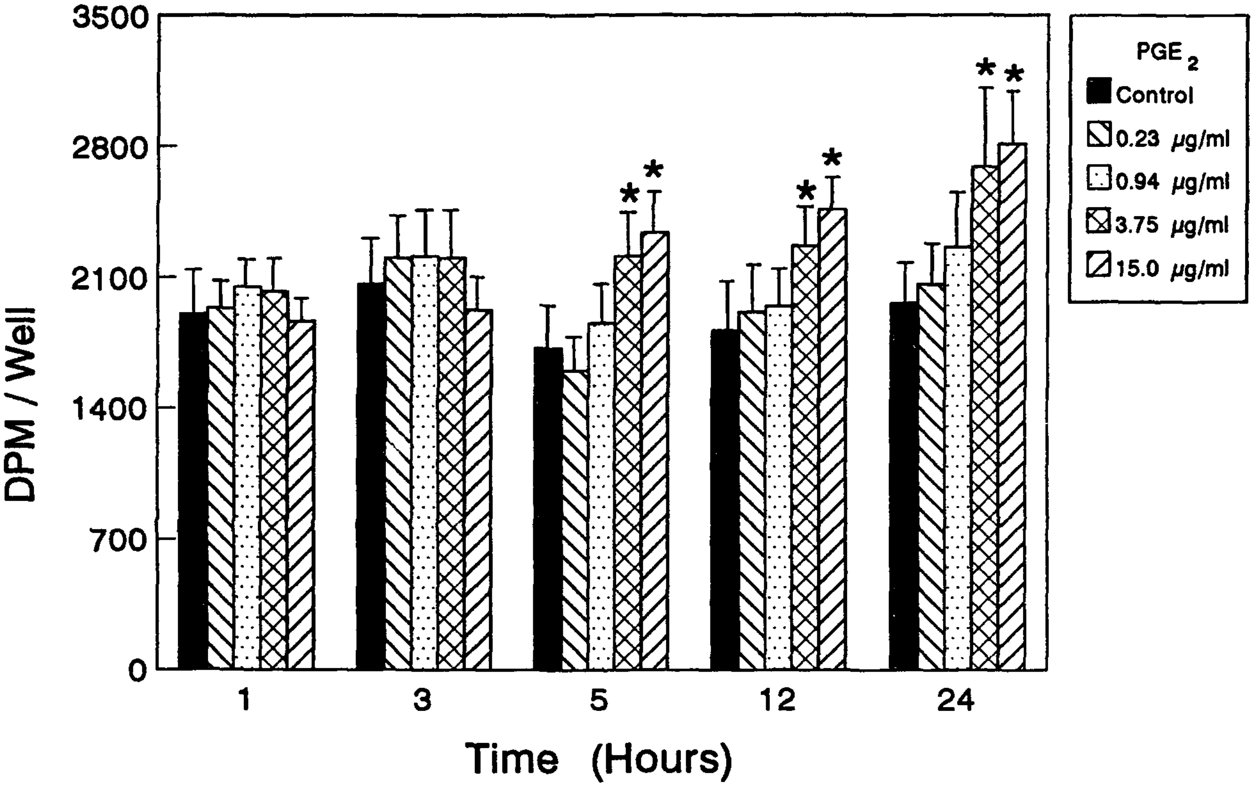
Confluent, fourth passage cultures of rat costochondral growth zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for 1, 3, 5, 12, and 24 hours. $[^3H]$-uridine was added two hours prior to harvest (except for the 1 hour time point, $[^3H]$-uridine and $PGE_2$ added simultaneously and harvested at 1 hour). Values are mean $\pm$ SEM for six cultures. Data are from one of three replicate experiments. $\star$ $p < 0.05$, treatment v. control.



PGE 2
Control
0.11 μg/ml
0.23 μg/ml
0.94 μg/ml
3.75 μg/ml
15.0 μg/ml
DPM / Well
4500
3750
3000
2250
1500
750
0
1
3
5
12
24
Time (Hours)
*

**Figure 10: Effect of $PGE_2$ on the [$^3$H]-uridine incorporation of resting zone (RC) chondrocytes after 1, 3, 5, 12, or 24 hours of treatment.**

Confluent, fourth passage cultures of rat costochondral resting zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for 1, 3, 5, 12, and 24 hours. [$^3$H]-uridine was added two hours prior to harvest (except for the 1 hour time point, [$^3$H]-uridine and $PGE_2$ added simultaneously and harvested at 1 hour). Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.


3500
2800
2100
1400
700
0
DPM / Well
1
3
5
12
24
Time (Hours)
PGE 2
Control
0.23 µg/ml
0.94 µg/ml
3.75 µg/ml
15.0 µg/ml
*

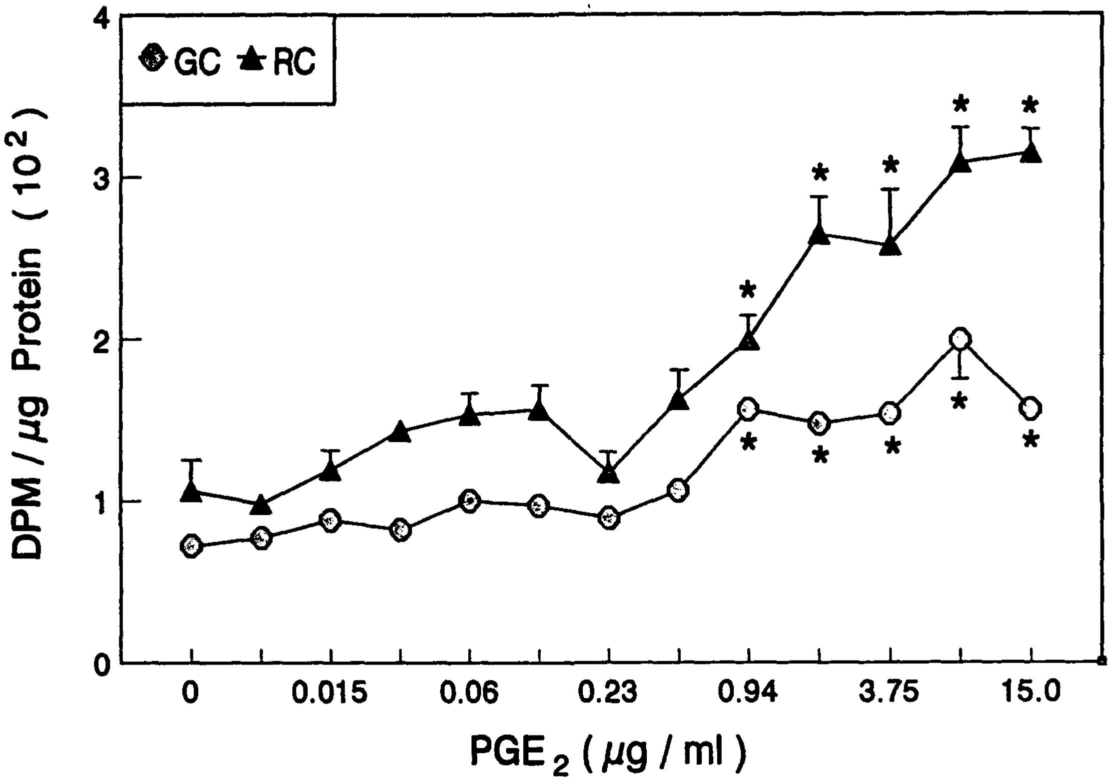
## D. Collagen Production

Collagenase-digestible protein production by growth zone and resting zone chondrocytes was significantly increased at $PGE_2$ concentrations ranging from 0.94 to 15.0 µg/ml (Figure 11). Both growth zone and resting zone chondrocytes displayed an approximate three-fold increase in collagenase-digestible protein synthesis. Significant increases in the synthesis of non-collagenase-digestible protein were only seen in growth zone chondrocytes at $PGE_2$ concentrations ranging from 0.94 to 15.0 µg/ml (Figure 12). In resting zone chondrocytes there was no significant change from baseline in non-collagenase-digestible protein synthesis.

To determine the percentage of collagen production, the amount of collagenase-digestible protein was divided by the total protein in the trichloroacetic acid precipitate. Percent collagen production by growth zone and resting zone chondrocytes was virtually identical. The percentage of collagen produced in growth zone chondrocytes increased from 4.1% in the untreated control to 7.1% to 9.4% after addition of $PGE_2$ to the cultures. Similarly, collagen production in the resting zone chondrocytes increased from 4.3% to 6.7% to 9.4% (Figure 13).

**Figures 11: Effect of $PGE_2$ on the collagenase-digestible protein production of resting zone (RC) and growth zone (GC) chondrocytes.**

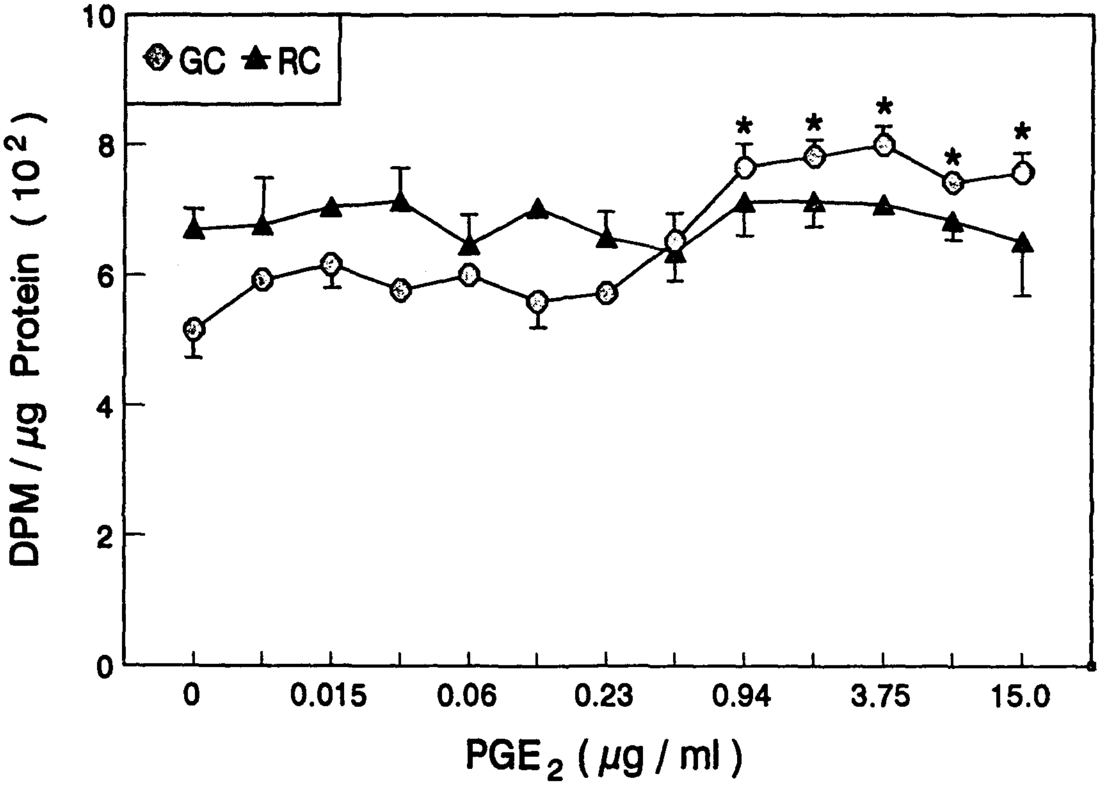
Confluent, fourth passage cultures of rat costochondral resting zone and growth zone chondrocytes were incubated in complete medium containing $[^3H]$-proline and various concentrations of $PGE_2$ for 24 hours. The label incorporated into collagenase-digestible (CDP) trichloroacteic acid precipitable protein was measured. Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. $\star$ $p < 0.05$, treatment v. control.



GC
RC
DPM / μg Protein ( 10²)
0
1
2
3
4
0
0.015
0.06
0.23
0.94
3.75
15.0
PGE₂ ( μg / ml )
*

**Figures 12: Effect of $PGE_2$ on the non-collagenase-digestible protein production of resting zone (RC) and growth zone (GC) chondrocytes.**

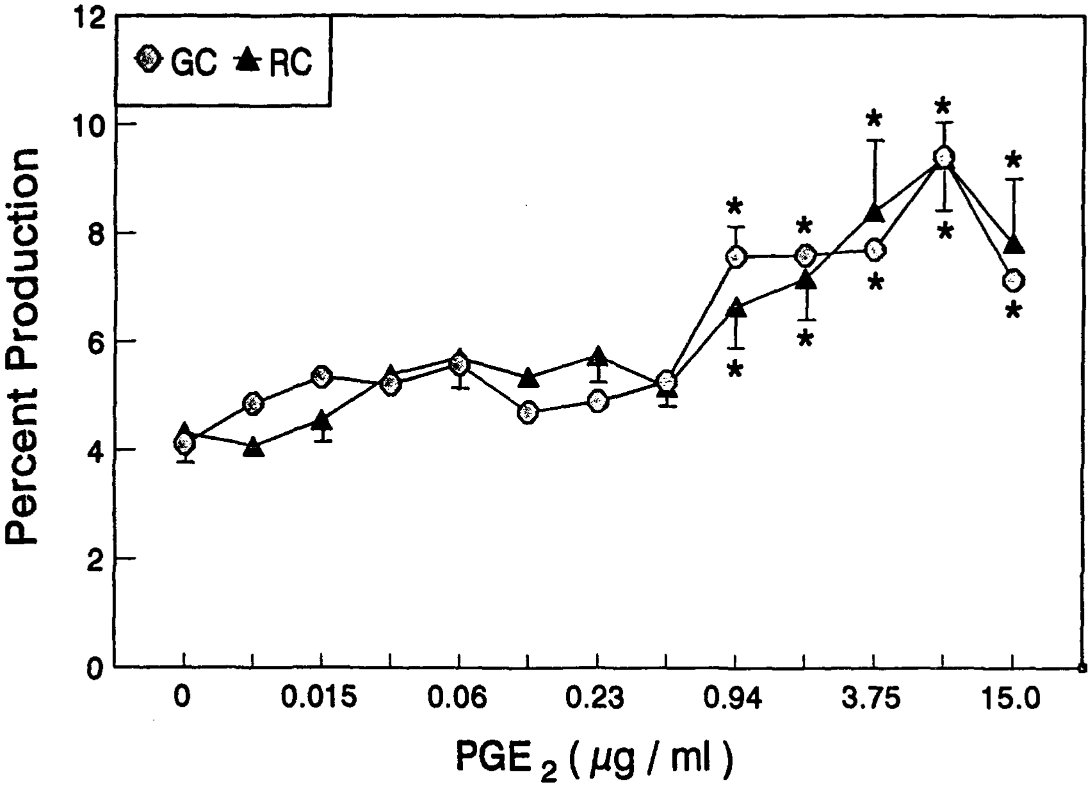
Confluent, fourth passage cultures of rat costochondral resting zone and growth zone chondrocytes were incubated in complete medium containing [$^3$H]-proline and various concentrations of $PGE_2$ for 24 hours. The label incorporated into non-collagenase-digestible (NCP) trichloroacteic acid precipitable protein was measured. Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.



GC
RC
DPM / µg Protein ( 10²)
0
2
4
6
8
10
0
0.015
0.06
0.23
0.94
3.75
15.0
PGE₂ ( µg / ml )
*

**Figures 13: Effect of $PGE_2$ on the percent collagen production of resting zone (RC) and growth zone (GC) chondrocytes.** .

Confluent, fourth passage cultures of rat costochondral resting zone and growth zone chondrocytes were incubated in complete medium containing [$^3H$]-proline and various concentrations of $PGE_2$ for 24 hours. The label incorporated into collagenase-digestible (CDP) and non-collagenase-digestible (NCP) trichloroacteic acid precipitable protein was measured and the percent collagen produced was calculated as the amount of CDP/(NCP + CDP) x 100%. Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.


GC
RC
Percent Production
PGE$_2$ ( μg / ml )
0
2
4
6
8
10
12
0
0.015
0.06
0.23
0.94
3.75
15.0
*

## E. Cyclic AMP Production

$PGE_2$ concentrations ranging from 0.12 to 15.0 µg/ml produced significant dose-dependent increases in cyclic AMP production by growth zone and resting zone chondrocytes (Figure 14). For both cell types, the level of cyclic AMP in untreated controls was similar. In addition, the magnitude of increased production was similar.

In time course experiments, growth zone (Figure 15) and resting zone (Figure 16) chondrocytes displayed significant increases in cyclic AMP production at 10 minutes after addition of $PGE_2$, but at none of the later time points. Significant increases in cyclic AMP production occurred at the same $PGE_2$ concentrations found to elicit increases in cyclic AMP production in the dose-response experiments.

**Figure 14: Effect of $PGE_2$ on the cyclic AMP production of resting zone (RC) and growth zone (GC) chondrocytes.**

Confluent, fourth passage cultures of rat costochondral resting zone and growth zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for ten minutes. Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.

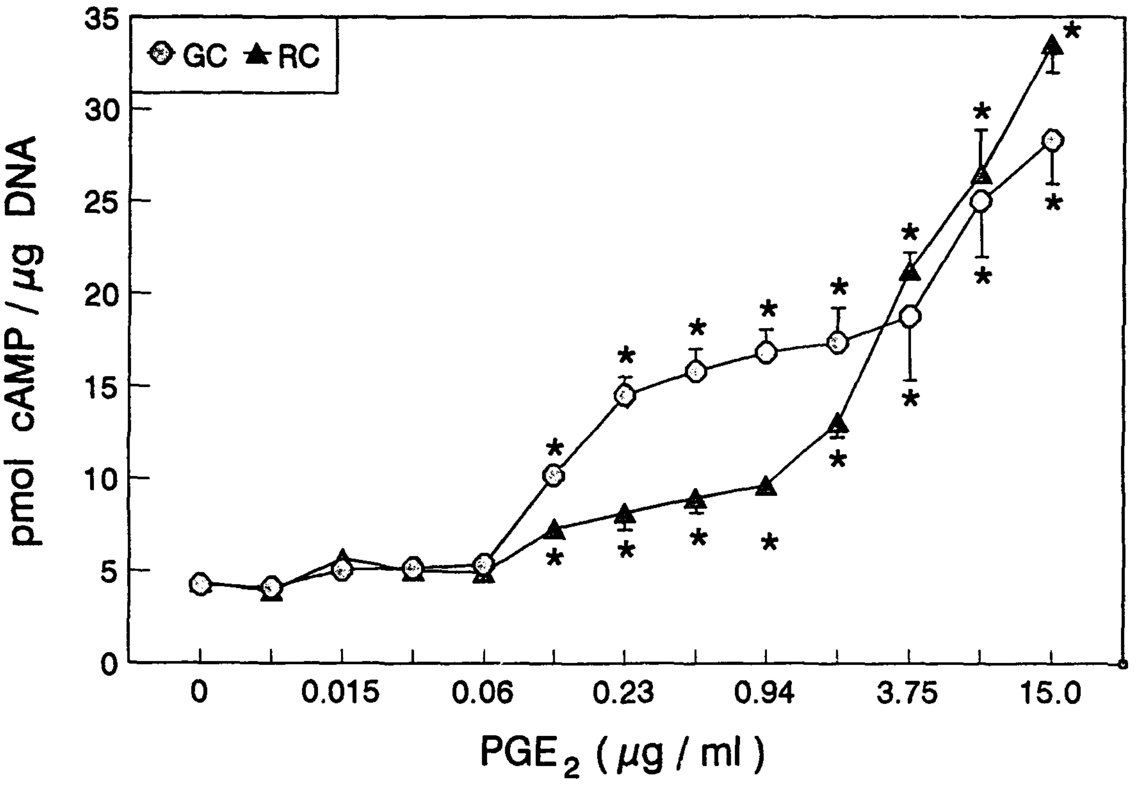

GC
RC
pmol cAMP / μg DNA
35
30
25
20
15
10
5
0
0
0.015
0.06
0.23
0.94
3.75
15.0
PGE₂ (μg / ml)

**Figure 15: Effect of $PGE_2$ on the cyclic AMP production of growth zone (GC) chondrocytes after 10, 60, 180, 360, or 720 minutes.**

Confluent, fourth passage cultures of rat costochondral growth zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for 10, 60, 180, 360, and 720 minutes. Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. $* \ p < 0.05$, treatment v. control.

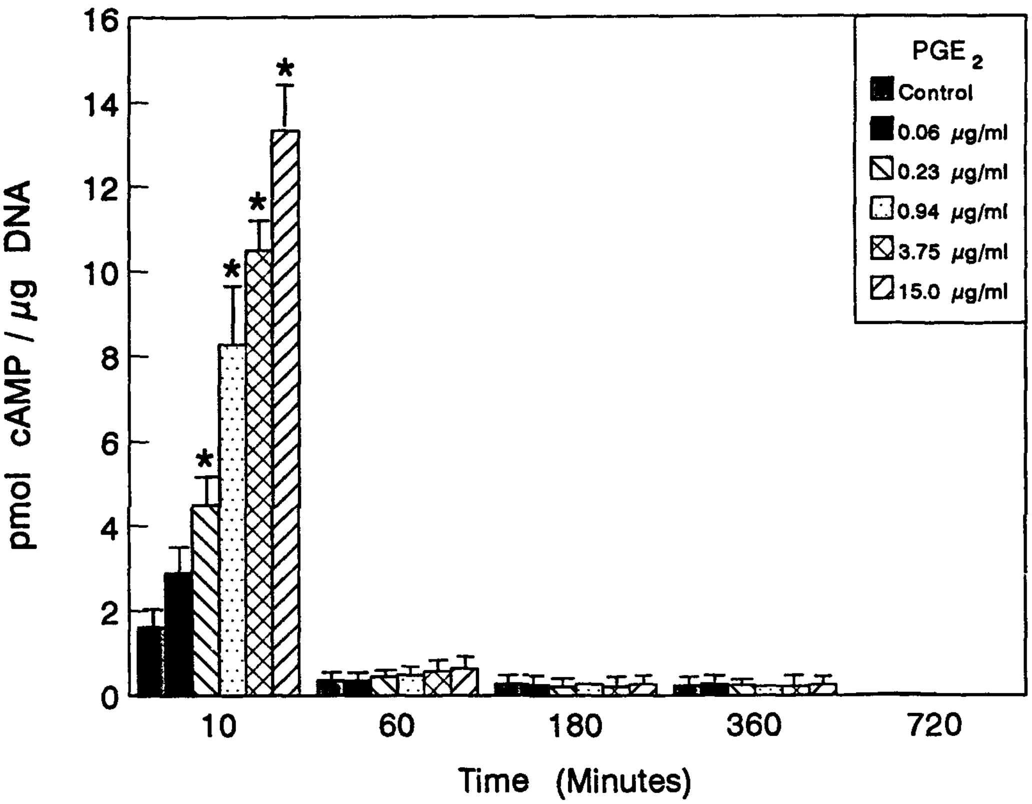
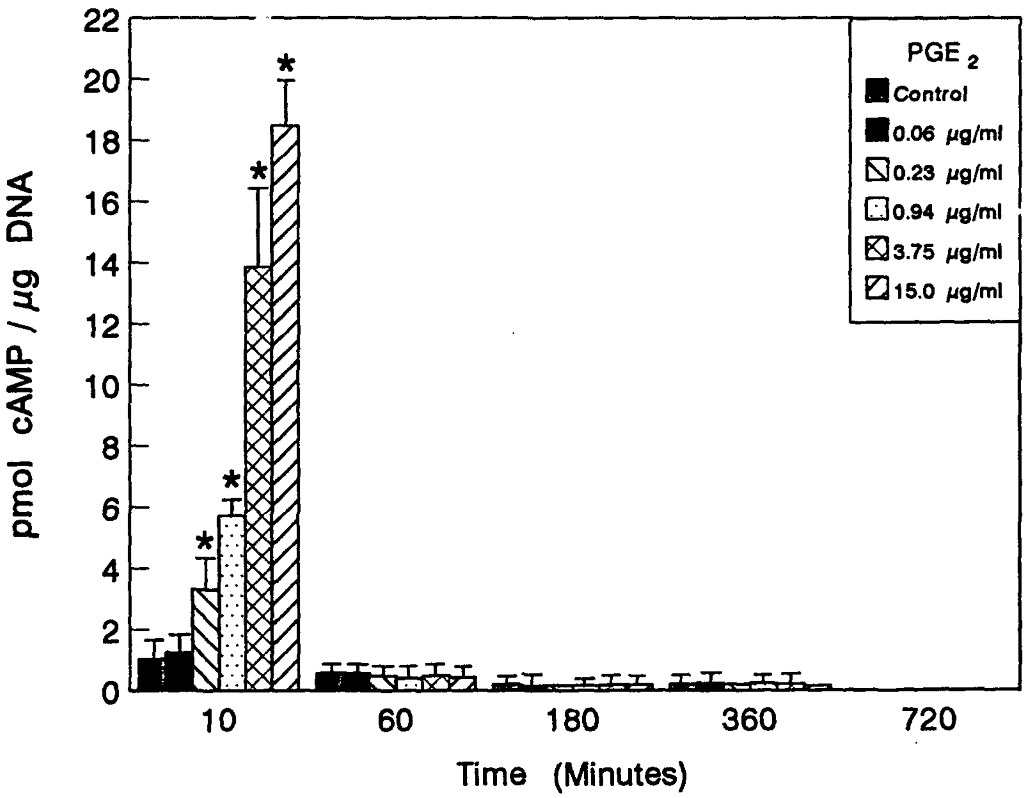

pmol cAMP / μg DNA
16
14
12
10
8
6
4
2
0
*
*
*
*
*
10
60
180
360
720
Time (Minutes)
PGE 2
Control
0.06 μg/ml
0.23 μg/ml
0.94 μg/ml
3.75 μg/ml
15.0 μg/ml

**Figure 16: Effect of $PGE_2$ on the cyclic AMP production of resting zone (RC) chondrocytes after 10, 60, 180, 360, or 720 minutes.**

Confluent, fourth passage cultures of rat costochondral resting zone and growth zone chondrocytes were incubated in complete medium containing various concentrations of $PGE_2$ for 10, 60, 180, 360, and 720 minutes. Values are mean ± SEM for six cultures. Data are from one of three replicate experiments. * $p < 0.05$, treatment v. control.


PGE 2
Control
0.06 μg/ml
0.23 μg/ml
0.94 μg/ml
3.75 μg/ml
15.0 μg/ml
pmol cAMP / μg DNA
0
2
4
6
8
10
12
14
16
18
20
22
*
10
60
180
360
720
Time (Minutes)

# DISCUSSION AND SUMMARY

The results of this study indicate that $PGE_2$ may play an important role in the regulation of chondrocyte proliferation and differentiation by its significant effects on cell proliferation, extracellular matrix synthesis, and activity of enzymes involved in calcification. It was interesting to find that the effects of exogenous $PGE_2$ on growth zone and resting zone chondrocytes did not appear to be dependent on cell maturation. Both cell types responded in a like manner and at nearly similar concentration ranges in these experiments. This is in contrast to the response of these cells to vitamin $D_3$ metabolites and to endogenous $PGE_2$ where distinct maturation-dependent differences are seen(87).

In the present study, $[^3H]$-thymidine incorporation was used as a measure of DNA synthesis and cell proliferation. Alkaline phosphatase specific activity was used as an indicator of calcification activity due to its correlation with mineralization in calcifying cells(101,102). Incorporation of $[^3H]$-uridine was used as a measure of RNA synthesis and to indicate changes in the cellular mechanism for protein formation. Collagen synthesis was determined as well as cyclic AMP production. The results show that exogenous $PGE_2$ stimulates significant increases in all of these areas in growth zone and resting zone chondrocytes.

It is important to note that for [$^3$H]-uridine incorporation, and collagen synthesis, significant increases were seen in growth zone and resting zone chondrocytes at a range of PGE$_2$ concentrations between 0.94 and 15.0 µg/ml. This supports the regulatory role of PGE$_2$ in the relationship between the cellular mechanism of protein production and formation of extracellular matrix protein in these calcifying cells.

Vitamin D$_3$ metabolites affect calcifying cells differently, depending on the stage of maturation and differentiation of the cells(9,10). 1,25-(OH)$_2$D$_3$ appears to influence more mature cells toward a higher degree of differentiation, while 24,25-(OH)$_2$D$_3$ guides less mature cells toward proliferation(10). 1,25-(OH)$_2$D$_3$ has been shown to cause inhibition of proliferation in osteoblasts(103), rat costochondral chondrocytes(10), murine condylar cartilage(104), and both human and rabbit articular cartilage(105). It has also been shown to inhibit collagen synthesis in osteoblast-like cells(106), calvaria(107,108), and avian medullary bone(109), but increases collagen synthesis in osteoblasts(103). Differential effects on alkaline phosphatase specific activity by 1,25-(OH)$_2$D$_3$ are dependent on the stage of cell growth or phenotypic maturation in costochondral chondrocytes(7), osteoblasts(103), rat calvaria(108), and osteoblast-like cells(110,111).

24,25-$(OH)_2D_3$ has been shown to increase cell proliferation in mesenchyme-derived chondrocytes(112), periosteal cells(108), and resting zone costochondral chondrocytes(10), while in human and rabbit articular cartilage 24,25-$(OH)_2D_3$ inhibits cell proliferation(105). 24,25-$(OH)_2D_3$ increases alkaline phosphatase specific activity in resting zone costochondral chondrocytes(7), but in avian growth plate chondrocytes, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ exert opposite effects(92). Recent evidence suggests that 24,25-$(OH)_2D_3$ directs the differentiation and maturation of resting zone chondrocytes into growth zone chondrocytes by inducing resting zone chondrocytes to become responsive to 1,25-$(OH)_2D_3$(112).

The results suggest that $PGE_2$, through its influence on increased cell proliferation in growth zone and resting zone chondrocytes, promotes a less differentiated type of cell, but through its influence on increased calcification activity and extracellular matrix formation promotes continued cell growth and differentiation. A similar study conducted with a chick limb chondrocyte model gave somewhat different results in that exogenous $PGE_2$ in a concentration range of $10^{-10}$ to $10^{-6}$M stimulated cyclic AMP production and $[^3H]$-thymidine incorporation, but inhibited alkaline phosphatase specific activity and extracellular matrix formation(113). This supports the view that exogenous $PGE_2$

increases proliferation, but maintains these cells in a lower stage of differentiation. Further support for this view comes from studies that have examined the effects of steroids on calcifying cells, or that have used steroids as a means to inhibit $PGE_2$ production. Dexamethasone was shown to depress cyclic AMP production and incorporation of $[^3H]$-thymidine in mouse condylar cartilage(114) and, in addition, also reduced condylar growth and collagen synthesis(115). In rat calvaria, cortisol inhibited $PGE_2$-induced $[^3H]$-thymidine incorporation and collagen synthesis, but exogenous $PGE_2$ was able to reverse the inhibitory effects(53). Osteoblasts exposed to physiologic concentrations of dexamethasone for 4 weeks were observed to grow more slowly than untreated controls; however, the cells appeared larger and more polygonal, had a significantly increased alkaline phosphatase specific activity, and exhibited a more differentiated osteoblastic phenotype(116). These results reinforce the position that $PGE_2$ promotes cell proliferation in less differentiated cells and that the inhibition of $PGE_2$ synthesis may allow for further cell differentiation.

Cyclic AMP is implicated as a second messenger in $PGE_2$ regulation(57,59,76,78). The results of the present study support the view that second messenger activity is mediated by cyclic AMP. The range of $PGE_2$ concentrations that resulted in increased cyclic AMP production is of great significance in these experiments. This range covers all of the $PGE_2$ concentrations that produced significant results in the other experiments and lends support to the role of cyclic AMP as a second messenger for $PGE_2$-initiated events. Additional studies that could explore this area more fully include the use of dibutyryl cyclic AMP to reproduce the effects of $PGE_2$ and investigation into protein kinase activation, particularly protein kinase C.

The range of $PGE_2$ concentrations used in the present study was determined based upon studies of vitamin $D_3$-stimulated $PGE_2$ production by the same chondrocyte model(87) and approximates exogenous $PGE_2$ concentrations used in other experiments(55,77,78,81,84,113). Baseline and vitamin $D_3$-stimulated values were between 40 and 135 ng $PGE_2/10^6$ cells and is represented by the $PGE_2$ concentrations used in this study of 0.06 to 0.23 µg/ml. The lower concentrations in this study are, therefore, well below baseline production levels, but the highest concentrations may reflect more pharmaceutical doses. Levels of $PGE_2$ production reported in other studies differ depending on the experimental model. $PGE_2$ production of $10^{-9}$M has been reported in chick embryonic calvaria(56) and chick growth plate chondrocytes(117). In chick limb mesenchyme, concentrations of 1.1 to 4.5 ng/ml were measured during 6-day high density micromass cultures(76), while human monocytes were shown to produce baseline levels of 60 ng/ml $PGE_2$ and levels of 140 ng/ml when stimulated by 1,25-$(OH)_2D_3$(89). In the present study, the exogenous $PGE_2$ concentrations providing significant increases in $[^3H]$-uridine incorporation and collagen synthesis are considerably higher than the $PGE_2$ concentrations produced by the cells; however, $PGE_2$ concentrations responsible for significant increases in alkaline phosphatase specific activity, $[^3H]$-thymidine incorporation, and cyclic AMP production are well within the concentrations of $PGE_2$ that can be achieved by growth zone and resting zone chondrocytes. It is quite possible that the results of RNA and collagen synthesis reflect the effects of pharmaceutical doses of $PGE_2$, particularly at the highest concentrations. If so, this would lend more support to the role of $PGE_2$ in cell proliferation and retention of lower stages of differentiation.

Although some of the exogenous $PGE_2$ concentrations used in this study are above baseline production values, the optimal concentrations of $PGE_2$ required for regulation may be quite different *in vivo*. Other factors such as cytokines and bradykinin may greatly increase $PGE_2$ production(40-45). Bradykinin has been shown to increase $PGE_2$ production 4.5 times above baseline in MC3T3 osteoblast-like cells(45). In addition, it is not known what the local concentration of $PGE_2$ is in the territorial matrix of the cells. It is possible that higher concentrations are needed to produce responses *in vitro*(88).

Baseline production of $PGE_2$ was not inhibited prior to addition of exogenous $PGE_2$ in the present study. Therefore, exogenous $PGE_2$ was added to endogenously-produced $PGE_2$. Additional information may be gained by repeated experiments evaluating the effects of exogenous $PGE_2$ in cells where baseline $PGE_2$ production is inhibited. However, in a recent study where the effects of $PGE_2$ on collagen synthesis and calcification activity were measured in chick calvaria, no significant differences were seen in cultures with and without inhibition of baseline $PGE_2$ production by $10^{-6}$M indomethacin(56).

The presence of $PGE_2$ during periods of bone induction may be very important clinically. Subcutaneous doses of 3 or 6 mg/kg/day $PGE_2$ for six weeks were shown to inhibit disuse-induced cortical bone loss in a rat limb immobilization model(118,119). $PGE_2$ was found to stimulate more bone formation than resorption and shorten the period of bone remodeling(118). A different limb immobilization study in dogs utilized aspirin, 75 mg/kg/day for four weeks and found that aspirin treatment was associated with a 65% decrease in bone $PGE_2$ and a 13% bone mass sparing effect(120). The difference in results may be due to a compensation for $PGE_2$-induced bone loss with the administration of $PGE_2$ to produce an anabolic state(119). $PGE_2$ has also been shown to inhibit lipopolysaccharide- and TNF-induced cartilage breakdown in bovine nasal cartilage(121).

The use of medications that modulate $PGE_2$ production may have an adverse effect upon bone induction. Administration of indomethacin 2 mg/kg/day significantly inhibited bone induction in heterotopic grafts of demineralized, freeze-dried bone in rats(122). Recent studies have evaluated the use of locally delivered $PGE_1$ *via* minipumps and controlled-release polymer pellets for alveolar bone augmentation in dogs(123,124). In these studies, significant increases in new bone formation was seen in a dose-dependent manner with the greatest increases seen at 8.3 mg $PGE_1$/week during a 3 week treatment. Collectively, these studies suggest that $PGE_2$ and $PGE_1$ exert significant clinical effects on bone formation and that the presence of prostaglandins may become more important in treatment schemes involving regeneration. The use of NSAID's for post-operative analgesia may inhibit prostaglandin levels that are optimal for bone induction and cartilage formation.

The results of this study confirm the view that $PGE_2$ may be an important mediator of bone induction and vitamin $D_3$ regulation. In addition to autocrine regulatory effects, $PGE_2$ from sources other than calcifying cells may produce significant effects on cell proliferation and differentiation. These regulatory effects are important in endochondral bone formation as well as in callus formation in a healing fracture and in bone augmentation procedures. The identification of optimal *in vivo* tissue concentrations of $PGE_2$ could lead to precise dosing of drugs that modulate $PGE_2$ production and result in enhanced bone formation during regenerative and augmentation procedures.

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# VITA

Ridge Morgan Gilley was born on May 2, 1959 in St. Petersburg, Florida and is the seventh child of John M. Gilley Jr. and R. Jeanne Gilley. Upon graduation from high school in 1977, he attended St. Petersburg Jr. College, was a member of the Phi Theta Kappa fraternity, and received an Associate of Arts degree with high honors in 1979. He then studied at the University of Florida in Gainesville, Florida and received a Bachelor of Science degree in Zoology in 1982. He entered the Indiana University School of Dentistry in Indianapolis, Indiana and graduated in 1986 as a Doctor of Dental Surgery.

In June, 1986, Dr. Gilley was commissioned in the United States Air Force and completed a one-year General Practice Residency at Davis-Monthan Air Force Base in Tucson, Arizona. In December, 1986, he married Deborah Kay Bunning. Upon completion of the General Practice Residency in June, 1987, he was assigned as a General Dental Officer at Royal Air Force Base, Alconbury, in the United Kingdom.

Dr. and Mrs. Gilley returned from the United Kingdom in May, 1991, and in June, 1991, Dr. Gilley entered the post-doctoral program in Periodontics at the University of Texas Health Science Center at San Antonio in conjunction with Wilford Hall Medical Center. He was admitted to candidacy for the Masters of Science degree at the University of Texas Graduate School of Biomedical Sciences at San Antonio in 1992.

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