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Osteotropic Cytokines mediate human osteoblast-like cell eicosanoid production

Anurag Grover

Thesis submitted to the College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements
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ABSTRACT
Osteotropic Cytokines mediate human osteoblast-like cell eicosanoid production.

Anurag Grover

Because of its high incidence and devastating socio-economic consequences, osteoporosis is an important disorder of the aging population. To treat and to prevent this disorder the basic physiological mechanisms regulating bone cell biology must be understood. Bone remodeling is regulated by systemic hormones such as parathyroid hormone, glucocorticoids, estrogen, 1,25-dihydroxyvitamin D_3 $(1,25(OH)_2 D_3)$, and by local factors such as cytokines, growth factors, and prostaglandins, which affects cells of the osteoclast or osteoblast lineage.
The regulation of bone metabolism by hormones has been extensively studied, but it is now clear that locally produced, nonsystemic, autocrine and paracrine factors play a direct and important role in bone remodeling. Eicosanoids (e.g. prostaglandins, HETEs, leukotrienes) are key components of a target cell's early responses to various stimulators including growth factors, cytokines, and hormones.

We have examined the effects of the cytokines tumor necrosis factorα (TNF), transforming growth factorβ (TGFβ) and the effect of cyclooxygenase inhibitor ibuprofen on prostaglandin and 15-HETE biosynthesis by normal adult human osteoblast-like (hOB) cells. In the present studies, conditioned media harvested from hOB cell cultures were assayed by eicosanoid specific EIAs to determine quantitatively the effects of cytokines on PGE_2, 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$, PGD_2, thromboxane (TXB_2) and on 15-HETE biosynthesis. EIA analysis indicated that PGE_2 was the predominant PG metabolite released by unstimulated hOB cells and its synthesis was increased more than other PGs in cells treated with TGFβ and TNFα, both separately and in combination. TXB_2 was not detected in control samples, or in samples stimulated with cytokines. 15-HETE was produced by the hOB cells, and was sensitive to modulation by the cytokines and ibuprofen.

The possibility that the up-regulation of PGE_2 synthesis elicited by cytokines might have resulted from an increased level of the terminal PGE_2 synthases (cPGES and mPGES) was tested. Transcripts for cPGES and mPGES was assessed by the reverse-transcriptase polymerase chain reaction using specific primers for human cPGES and mPGES. Upon dual cytokine stimulation, a significant up-regulation of cPGES and mPGES mRNA was observed.

The steady-state level of 15-Lipoxygenase type-II mRNA in the hOB cells was determined in control samples and specimens treated with TGFβ and/or TNFα, IL-3 and IL-1β. A significant increase in 15-LOX type-II mRNA level was evident in specimens treated with TNFα alone (47.9 ± 22 fold-basal).

Thus, these studies indicate that TGFβ and/or TNFα modulate AA metabolism and upregulation of PGE_2 synthesis elicited by these cytokines might have resulted from an increased level of the terminal PGE_2 synthases in hOB cells.

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ABBREVIATIONS

AA	arachidonic acid
BSA	bovine serum albumin
COX	cyclooxygenase
cPGES	cytosolic prostaglandin E_2 synthase
DMEM	Dulbecco's Modified Eagle Medium
EIA	enzymeimmunoassay
FCS	fetal calf serum
HETE	hydroxyeicosatetraenoic acids
hOB	human osteoblast-like
IL-1	interleukin-1
LOX	lipooxygenase
mPGES	microsomal prostaglandin E_2 synthase
NSAID	non-steroidal anti-inflammatory drugs
OB	osteoblasts
OC	osteoclasts
PG	prostaglandins
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PG I_2	prostaglandin G/H synthase
PG H_2	prostaglandin I_2
PLA_2	phospholipase A_2
PTH	parathyroid hormone
RIA	radioimmunoassay
RT-PCR	reverse transcriptase-polymerase chain reaction
TGF-β	transforming growth factor-beta
TLC	thin layer chromatography
TNF-α	tumor necrosis factor-alpha
TxB_2	thromboxane
$6\text{-k-}PGF_{1\alpha}$	6-keto- prostaglandin F_α

INTRODUCTION

Osteoporosis is a skeletal disorder in which bone strength is compromised due to the loss of bone density and bone quality (Inzerillo et al., 2002). It is characterized by low bone mass as well as by the micro architectural deterioration of bone with a consequent increase in bone fragility and sensitivity to fractures following minimal trauma (Riggs et al., 1992). Efforts have been primarily concentrated on the development of drugs that block bone resorption by decreasing the formation of osteoclasts or the activity of the osteoclasts. Inhibition of bone resorption can be accomplished by reducing either osteoclast generation (with estrogen or selective estrogen receptor modulators) or osteoclast activity (with calcitonin or bisphosphonates) (Stepan et al., 2003). Parathyroid hormone has become available as the first anabolic agent that increases bone formation (Hodsman et al., 1997). To date, these drugs and hormones have been the backbone of osteoporosis treatment and the prevention of fractures (Allen, 1993, Prestwood et al., 1998).

Notwithstanding the available effective therapies, osteoporosis is the most commonly occuring bone disease, and it is expected that the prevalence of osteoporosis will increase as the population ages (Fontanges et al., 2004). It affects approximately 28 million people in the United States, mostly post menopausal women, due to an accelerated rate of bone loss at and following the menopause (Hightower, 2000). Among significant chronic ailments in the U.S., 28 million people are affected with osteoporosis compared with 52 million and 42 million afflicted by hypercholesterolemia and by hypertension, respectively (Natarajan et al.,

2003). Health care costs of fractures resulting from osteoporosis were estimated to be $10 to $18 billion per year in the U.S.A in 1997, and these costs are predicted to grow to more than $60 billion per year by the year 2020 (Ray et al., 1997).

Bone is a dynamic living tissue that protects the body's internal organs, provides sites for muscle attachment for locomotion, and serves such physiological functions as maintaining calcium and phosphate balances in the body, and providing a site for the production of haemopoietic tissues (Rho et al., 2004). In adults, bone undergoes a continous process of remodeling, that is, the replacing of old and damaged bone with newly formed bone (Eriksen et al., 1994; Jilka et al., 1994). Osteoporosis results from defective bone remodeling regulation: the main problem is an imbalance between the rates of bone resorption and bone formation such that resorption exceeds formation particularly in cancellous bone (Raisz et al., 1984). In adults, approximately 25% of cancellous bone is resorbed and replaced every year, as compared with only 3% of cortical bone (Seeman, 2003). Cancellous bone has a high surface-to-volume ratio with 70 to 85% of the surface of the trabecular bone in contact with the bone marrow (Islam et al., 1990). In addition to their anatomical concurrence, cells of the bone and the haemopoietic bone marrow share progenitors, produce and respond to some of the same cytokines and colony-stimulating factors, and are ultimately essential for each other's function (Prockop, 1997).

Bone is composed of four different cell types (Fig. 1.). Osteoblasts, osteoclasts, and bone lining cells are present on bone surfaces, whereas osteocytes permeate the mineralized interior. Osteoblasts, lining cells, and osteocytes are all cells of the osteoblast lineage and osteoclasts are derived from precursors originating

in the bone marrow (Manolagas et al., 1995; Manolagas et al., 1995). The precursors

of osteoblasts are multipotent mesenchymal stem cells, which also give rise to bone

marrow stromal cells, chondrocytes, muscle cells, and adipocytes (Owen, 1985;

Ghilzon et al., 1999; Hirschi et al., 2002) whereas the precursors of osteoclasts are

hematopoietic cells of the monocyte/macrophage lineage (Vaes, 1988; Suda et al.,

1992; Roodman, 1996). Long before osteoblasts could be cultured, the existence of

multipotent mesenchymal stem cells was suspected, based on the evidence that

fibroblastic colonies formed in cultures of adherent bone marrow cells can

differentiate, under suitable stimuli, into each of the above mentioned cells; these

progenitors were named colony forming unit fibroblasts (CFU-F) (Castro-Malaspina

et al., 1984; Pittenger et al., 1999). When CFU-F are cultured in the presence of β-

glycerophosphate and ascorbic acid, the majority of the colonies form a mineralized

bone nodule; these bone-forming colonies are known as CFU-osteoblast (CFU-OB)

(McCulloch et al., 1991; Malaval et al., 1994). Osteoblast precursors may originate

not only from stromal mesenchymal progenitors of the marrow, but also from

pericytes, mesenchymal cells that are adherent to the endothelial layer of blood

vessels penetrating bone tissue (Schor et al., 1995). Whereas osteoclast precursors

reach bone from the circulation, osteoblast precursors most probably reach bone by

the migration of progenitors from neighboring connective tissues (Manolagas, 2000).

Fully differentiated osteoblasts produce and secrete proteins that make up the

organic phase of bone matrix (Rifas et al., 1989). The proteinaceous matrix is

subsequently mineralized under the control of the same cells (Masi et al., 2001;

Mulari et al., 2004). A major protein product of the bone-forming osteoblast is type

I collagen and it is a useful bone-formation marker (Abildgaard et al., 2004). This polymeric protein is initially secreted in the form of a precursor, which contains peptide extensions at both the amino-terminal and the carboxyl end of the molecule. The propeptide extensions are proteolytically removed. Further extracellular processing results in mature three-chained type I collagen molecules, which then assemble themselves into a collagen fibril (Imamura et al., 1998). Individual collagen molecules become interconnected by the formation of pyridinoline cross-links, which are unique to bone (Hanson et al., 1992). Bone-forming osteoblasts synthesize a number of other proteins that are incorporated into the bone matrix, including osteocalcin and osteonectin, which constitute 40% to 50% of the non-collagenous proteins of bone (Rodan et al. 1991). Osteocalcin (OC) is widely used as a marker for *in vivo* osteoblastic activity and bone formation (Gerdhem et al., 2004). Mice deficient in osteocalcin develop a phenotype characterized by higher bone mass and improved bone quality, suggesting that osteocalcin functions normally to limit bone formation without compromising mineralization (Ducy et al., 1996). Osteonectin may mediate deposition of hydroxyapatite and binds growth factors (Termine et al., 1981; Delany et al., 1998). Mice deficient in osteonectin exhibit decreased osteoclast and osteoblast numbers and reduced bone remodeling suggesting that under normal conditions osteonectin may play a role in the birth or survival of these cells (Delany et al., 2000). A number of other minor proteins such as osteopontin, bone sialoprotein, fibronectin, vitronectin, and thrombospondin serve as attachment factors that interact with integrins (Grzesik et al., 1994). In addition to being the cells that produce the osteoid matrix, mature osteoblasts are essential for its

mineralization, the process whereby deposition of hydroxyapatite crystals occurs (Hessle et al., 2002). Osteoblasts are thought to regulate the local concentrations of calcium and phosphate in such a way as to promote formation of hydroxyapatite crystals (Boskey, 1996; Boskey, 1998). Mineralization of the matrix increases the density of bone by displacing water, but does not alter its volume (Robinson et al., 1957; Neuman et al., 1958). Osteoblasts express very high amounts of alkaline phosphatase, which is anchored to the outer surface of the plasma membrane (Stein et al., 1993). Alkaline phosphatase has been long thought to play a role in bone mineralization. Consistent with this, deficiency of alkaline phosphatase due to genetic defects leads to a condition hypophosphatasia characterized by defective bone mineralization (Whyte, 1994; Wennberg et al., 2000). However, the precise mechanism of mineralization and the exact role of alkaline phosphatase in this process remain ambiguous.

Mature osteoclasts are usually large (50 to 100 μm diameter), multinucleated cells with abundant mitochondria, numerous lysosomes, and free ribosomes. They are usually found in contact with a calcified bone surface and within lacunae that are the result of their own resorptive activity. Their most remarkable morphological feature is the ruffled border, a complex system of finger-shaped projections of the membrane, the function of which is to support the resorption of the calcified bone matrix (Roodman, 1996; Boskey, 1998). This structure is completely surrounded by another specialized area, called the clear zone. The clear zone delineates the area of attachment of the osteoclast to the bone surface and seals off a distinct area of the bone surface, essentially forming a secondary lysosome, that lies immediately

underneath the osteoclast, that will be acidified and excavated. The ability of the clear zone to seal off this area of bone surface allows the formation of an acidic microenvironment suitable for the operation of the resorptive apparatus.

Acidification of the secondary lysosome is created by the action of an ATP-driven proton pump located in the ruffled border membrane. The mineral component of the matrix is dissolved in the acidic environment of the resorption site. The protein components of the matrix, mainly collagen, are degraded by matrix metalloproteinases and cathepsins that are secreted by the osteoclast into the area of bone resorption (Bossard et al., 1996). The degraded bone matrix components are endocytosed along the ruffled border within the resorption lacunae and transcytosed to the membrane area opposite the bone, where they are released (Salo et al., 1997). Active osteoclasts exhibit a characteristic polarity, that is, the nuclei of the osteoclasts are typically located in the part of the cell most removed from the bone surface and are interconnected by cytoskeletal proteins (Watanabe et al., 1995). Another characteristic of osteoclasts is the presence of high amounts of the phosphohydrolase enzyme, tartrate-resistant acid phosphatase (TRAPase). This aspect is widely used as marker for osteoclasts in bone specimens (Udagawa et al., 1990; Hayman et al., 1996).

Some mature osteoblasts are ultimately buried within refilled lacunae of mineralized matrix, and these buried cells are responsible for maintainence of matrix (Buckwalter et al., 1996). These cells are the osteocytes and are characterized by a striking stellate morphology, resembling the dendritic network of the nervous system (Marotti et al., 1990; Nijweide et al., 1996). Osteocytes are regularly spaced

throughout the mineralized matrix and communicate with each other and with osteoblasts, which in turn communicate with cells of the bone marrow stroma (Fig. 1.). A syncytium extends from the entombed osteocytes all the way to the blood
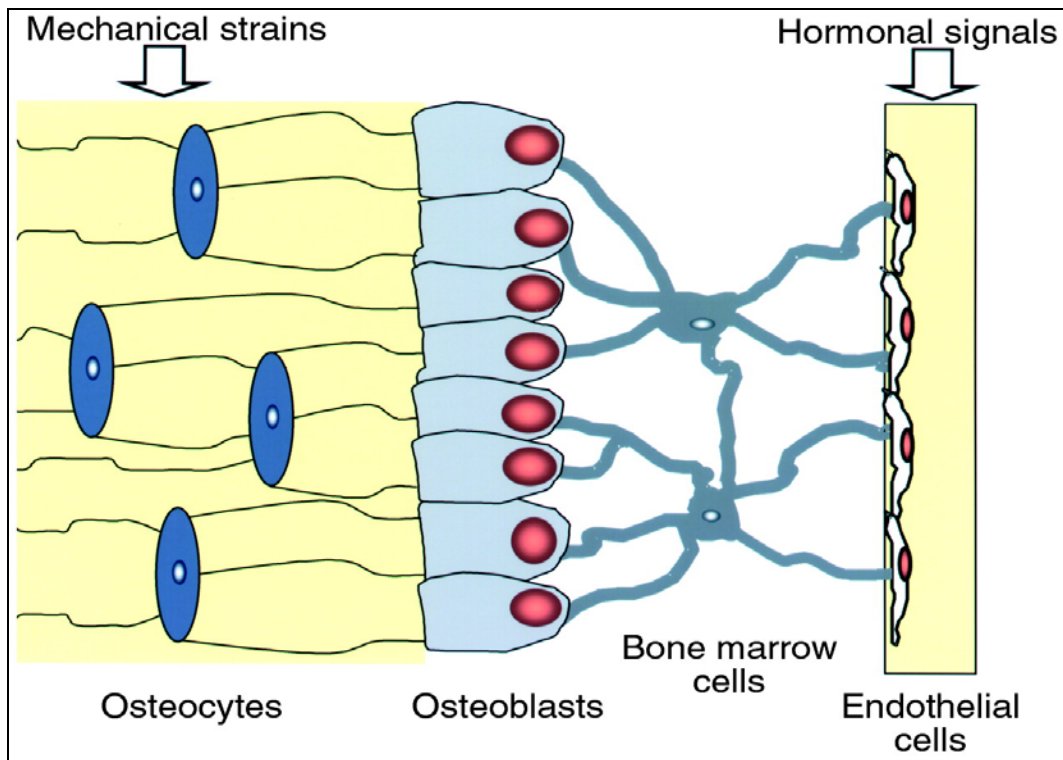
Figure 1. *Functional syncytium comprising osteocytes, osteoblasts, bone marrow stromal cells, and endothelial cells. [Adapted from Journal of Clinical Investigation 104:1363–1374, 1999.]*

vessel wall (Marotti et al., 1996). Consequenly, the strategic location of osteocytes makes them excellent candidates for mechanosensory cells able to detect the need for bone augmentation or reduction during functional adaptation of the skeleton, and the need for repair of microdamage. The disruption of the osteocyte network would be likely to increase bone fragility (Weinstein et al., 1998; Tomkinson et al., 1998). Bone lining cells are flat, elongated, dormant cells that cover bone surfaces that are undergoing neither bone formation nor resorption (Parfitt, 1994).

During development and growth, the skeleton is sculpted to achieve its shape and size by the removal of bone from one site and deposition at a different one; this process is called modeling. Once the skeleton has reached maturity, regeneration continues in the form of a periodic replacement of old bone with new at the same location (Frost, 1973). This process is called remodeling and is responsible for the complete renewal of the adult skeleton every 10 years. The purpose of remodeling in the adult skeleton is not entirely clear, although in bones that are load bearing remodeling most likely serves to repair fatigue damage and to prevent excessive aging and its consequences. Therefore, the most likely function of bone remodeling is to prevent the accumulation of old or damaged bone.

Removal of bone i.e., resorption, is the task of osteoclasts, while formation of new bone is the function of osteoblasts. Bone resorption and bone formation are not independently regulated processes (Parfitt, 1984). In the uninjured adult skeleton, all osteoclasts and osteoblasts belong to a unique transitory structure, known as a basic multicellular unit or BMU (Parfitt, 1994). Although during modeling one cannot distinguish anatomical units analogous to the BMU by itself, sculpting of the growing skeleton requires spatial and sequential orchestration of the destination of osteoblasts and osteoclasts, although with different rules and coordinates to those operating in the BMU of the remodeling skeleton. The BMU in cortical bone is approximately 1–2 mm long and 0.2–0.4 mm wide, comprises a team of osteoclasts in the front, a team of osteoblasts in the back, a central vascular capillary, a nerve supply, and associated connective tissue (Parfitt, 1994). In healthy human adults, it is estimated that 3–4 million BMUs are initiated per year and about 1 million are

operating at any moment. Each BMU begins at a particular place and time (origination) and advances toward a target, which is a region of bone in need of replacement, and for a variable distance beyond its target (progression) and eventually comes to rest (termination) (Parfitt, 1996). Osteoclasts adhere to bone and subsequently remove it by acidification and proteolytic digestion. As the BMU advances, osteoclasts leave the resorption site and osteoblasts move in to cover the excavated area and begin the process of new bone formation by secreting osteoid, which is eventually mineralized into new bone.

Table 1. *Vital statistics of adult bone remodeling (Parfitt, 1994).*

- Lifespan of BMU - 6–9 months
- Speed - 25μm/day
- Bone volume replaced by a single BMU - 0.025 mm^3
- Lifespan of osteoclasts - 2 weeks
- Lifespan of osteoblasts (active) - 3 months
- Interval between successive remodeling events at the same location - 2–5 years.
- Rate of turnover of whole skeleton - 10% per year

The lifespan of the BMU is 6–9 months, which is much longer than the lifetime of its constituent cells (Table 1). As a result, a continuous supply of new osteoclasts and osteoblasts derived from their respective progenitors in the bone marrow is essential for the origination of BMUs and their progression on the bone surface. Consequently, the balance between the supply of new cells and their lifespan are key determinants of the number of either cell type in the BMU and the work performed by each type of cells which is significant for the maintenance of bone homeostasis.

"Remodeling" of bone begins early in fetal life, and once the skeleton is fully formed in young adults, most of the skeletal metabolic activity is in this form

(Eriksen, 1986; Erlebacher et al., 1995; Reddi, 1997). The bone remodeling cycle

involves a series of highly regulated steps (Fig. 2.) that depend on the interactions of

the osteoblastic cells and the osteoclastic cells (Raisz et al., 1983; Raisz, 1999). The

initial "activation" stage involves the interaction of osteoclast and osteoblast
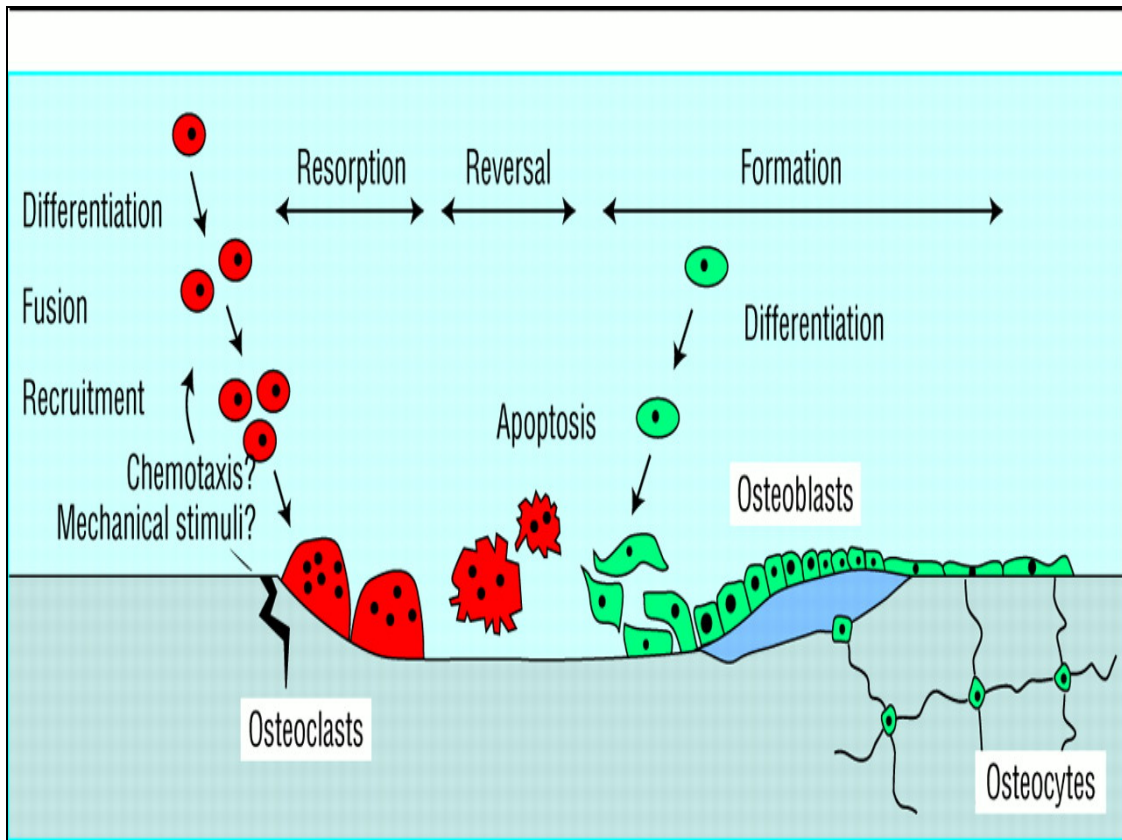
Fig. 2. *Bone remodeling**: Remodeling begins with attraction of osteoclasts to the
target site, probably triggered by mechanical stimuli or release of chemotactic
substances from areas of microdamage in bone. Mature osteoclasts remove bone
during the phase of bone resorption. After resorption is complete, osteoclasts
undergo apoptosis during the reversal phase, and bone formation begins as
osteoblasts are recruited to the site. Some osteoblasts become trapped within bone
matrix and differentiate to form osteocytes, which act as sensors of mechanical
strain in bone (Ralston, 1997).*

precursor cells leading to the differentiation, migration, and fusion of mononuclear

cells to produce the large, multinucleated osteoclasts. These osteoclast cells attach to

the mineralized bone surface and initiate resorption by the secretion of hydrogen ions and lysosomal enzymes, particularly cathepsins, which can degrade all the components of bone matrix, including collagen, at low pH. Osteoclastic resorption produces irregular, scalloped cavities called lacunae in the trabecular bone surface, and called cylindrical Haversian canals in cortical bone. Once the osteoclasts have completed their work of bone removal, there is a "reversal" phase during which mononuclear cells, possibly of the macrophage lineage, are seen on the bone surface. The events during this stage are not well understood, but they may involve further degradation of collagen, the deposition of proteoglycans to form the so-called cement line, and the release of growth factors to initiate the formation phase. During the final "formation" phase of the remodeling cycle, the cavity created by resorption can be completely filled in by successive layers of osteoblasts, deposition of mineralizable matrix.

Most simply put, the challenges of understanding the complexities of skeletal modeling and remodeling, are illustrated by the influences that osteoblasts have on osteoclasts and vice versa (Marks et al., 1988; Mundy, 1994). The development and differentiation of osteoblasts and osteoclasts, and consequently bone remodeling, is controlled by growth factors and cytokines produced in the bone and bone marrow microenvironment (Manolagas, 1998). Several systemic hormones, as well as mechanical signals, also exert potent effects on osteoclast and osteoblast development and differentiation (Raisz, 1993; Mosley, 2000).

The metabolic functions of the skeleton are served in large part by two major calcium-regulating systemic hormones, parathyroid hormone (PTH) (Dempster et al.,

1993; Hodsman et al., 1997) and 1,25-dihydroxy vitamin D_3 (Li et al., 1998). PTH regulates serum calcium concentration. It is a potent stimulator of bone resorption and has biphasic effects on bone formation. There is an acute inhibition of collagen synthesis with high concentrations of PTH, but prolonged intermittent administration of this hormone produces increased bone formation, a property for which it is being explored clinically as an anabolic agent (Dempster et al., 1993). 1,25-dihydroxy vitamin D_3 has its greatest effect on intestinal calcium and phosphate absorption, but has direct effects on bone and other tissues (Li et al., 1998). It is probably critical for the differentiation of osteoblasts and osteoclasts and can stimulate bone resorption and formation under some experimental conditions (Norman, 1992). A third hormone, calcitonin, which can inhibit bone resorption, may be important in skeletal development but appears to play little role in physiologic calcium regulation in adult humans (Whitfield et al., 2002; Doggrell, 2003; Stepan et al., 2003).

The most significant systemic hormone in regards to maintaining normal bone turnover is estrogen (Pacifici, 1996; Pacifici, 1998). Estrogen deficiency leads to an increase in bone remodeling in which resorption outstrips formation and bone mass decreases. This can be observed not only in postmenopausal women, but also in men with defects either in the estrogen receptor or in the synthesis of estrogen from testosterone (Bilezikian et al., 1998). The mechanisms by which estrogen regulates bone turnover are still not well understood, although studies in animals suggest that estrogen acts by changing either the production or activity of local factors that regulate osteoblast and osteoclast precursors (Lorenzo, 1992; Pacifici, 1998). Estrogen treatment produces a decrease in both formation and resorption of

bone associated with decreased remodeling, but overall increases bone mass. This increase may simply be a result of the filling in of the resorption space.

There is a concept that there must be local factors that also regulate bone remodeling (McLeod et al., 1998). The identification of these local factors has occurred only during the last 30 years. Among the first of the local factors to be identified were cytokines. Originally these "osteoclast-activating factors", which could be produced by inflammatory cells, particularly macrophages, were implicated in the local bone loss associated with periodontal disease and inflammatory arthritis (Lorenzo, 1992). At about the same time prostaglandins, particularly prostaglandin E_2, were shown to be potent stimulators of bone resorption that could also be important in inflammatory bone loss (Kawaguchi et al., 1995).

A large number of cytokines and growth factors that can affect bone cell functions have now been identified. The only factors capable of initiating osteoblastogenesis from uncommitted progenitors are bone morphogenetic proteins (BMPs) (Rosen et al., 1996; Sakou, 1998). BMPs have been long implicated in skeletal development during embryonic life and fracture healing. More recently, it has become apparent that BMPs, and in particular BMP-2 and BMP-4, also initiate the commitment of mesenchymal precursors of the adult bone marrow to the osteoblastic lineage (Abe et al., 2000). Other factors such as transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs), and members of the fibroblast growth factor (FGF) family can all stimulate osteoblast differentiation (Bonewald et al., Hakeda et al., 1996; Inui et al., 1998; Mills et al., 1997). However, although TGF-β, PDGF, FGF, and IGFs are able to

influence the replication and differentiation of committed osteoblast progenitors toward the osteoblastic lineage, they cannot induce osteoblast differentiation from uncommitted progenitor cells.

A large group of cytokines and colony-stimulating factors that are involved in hematopoiesis also stimulate osteoclast development (Takahashi et al., 1991; Fujikawa et al., 2001). This group includes several interleukins (IL-1, IL-3, IL-6, IL-11), tumor necrosis factor-α (TNF-α), granulocyte macrophage-colony stimulating factor (GM-CSF) and M-CSF. As opposed to the above listed cytokines that stimulate osteoclast development, IL-10, IL-12 and IL-18 inhibit osteoclast development (Horwood et al., 2001; Owens et al., 1996).

Recently, some of the proteins that are responsible for the interaction between cells of the osteoblastic and osteoclastic lineage have been identified. These proteins belong to the family of tumor necrosis factor receptors (Takahashi et al., 1999; Yasuda et al., 1999; Khosla, 2001). Osteoblast precursors express a molecule called TRANCE or RANKL, which can activate cells of the osteoclast lineage by interacting with a receptor called RANK (Receptor Activator of NF-κB) (Takahashi et al., 1999; Suda et al., 1999). A third molecule, osteoprotegerin (OPG), can be produced by cells of the osteoblast lineage or by other cells in the marrow (Gori et al., 2000). OPG acts as a decoy receptor for TRANCE, blocking its interaction with RANK and inhibiting osteoclast formation (Aubin et al., 2000; Hofbauer et al., 2000; Khosla, 2001). Recent studies have shown that knocking out the OPG gene in rodents produces severe osteoporosis characterized by excessive bone resorption (Kostenuik et al., 2001).

Prostaglandin E_2 (PGE_2) was identified as a product of unstimulated and complement-stimulated fetal rat long bones (Klein et al., 1970, Raisz et al., 1979). Subsequently PGA_2, PGI_2, PGD_2, PGE_2, and $PGF_{2\alpha}$ were found in bone cultures (Klein et al., 1970). The discovery that PGI_2 could be synthesized by the bone itself was of substantial interest, as its short half-life would be of less consequence if it was synthesized by cells on or immediately adjacent to the bone surface (Raisz et al., 1979).

There are four known PGE_2 receptors, EP_{1-4} and one prostaglandin $F_{2\alpha}$ receptor in bone (Suda et al., 1996; Nemoto et al., 1995). Little is known about prostaglandin receptor functioning in bone cells, but there are indications that PGE_2 stimulates osteoblastic differentiation in bone marrow cultures by activating the EP_4 receptor, an effect that may involve recruitment of uncommitted osteogenic precursors (Weinreb et al., 1999). In addition, a role of EP_4 in PGE_2-induced osteoclast formation has been shown, suggesting a therapeutic potential of EP_4 antagonists in some clinical conditions with accelerated bone resorption (Ono et al., 1998; Suzawa et al., 2000). The EP_1 subtype of PGE_2 receptor plays a role in the autoamplification of PGE_2 (Funk et al., 1993; Suda et al., 1998; Suda et al. 2000). The autoamplification of PGE_2 production is considered to be important in elongating the otherwise short-lived PGE_2 action in certain physiological conditions such as mechanical stress and fracture healing, as well as the pathological inflammatory bone loss (Suda et al., 1998). A role for EP_2 receptor has been suggested in rapidly growing skeletal tissue (Nemoto et al., 1997).

Prostaglandins are abundant in bone and are produced by cells of the osteoblastic lineage (Raisz et al., 1993; Okada et al., 2003). They are complex regulators of bone metabolism, being potent stimulators of bone resorption in organ culture (Klein et al., 1970; Kawaguchi et al., 1995; Raisz, 1999), but inhibiting bone resorption by isolated osteoclasts (Raisz, 1995; Kawaguchi et al., 1995). This inhibitory effect was demonstrated in isolated osteoclasts but is difficult to exhibit when osteoblasts are also present. PGE_2 can mediate resorptive responses to cytokines and growth factors in bone (Miyaura et al., 2000; Sakuma et al., 2000).

Although the effects of prostaglandins on bone resorption have been studied to a much greater extent than those on bone formation, it seems likely that their effects on bone formation will be of at least equal importance in regulation of bone metabolism (Vrotsos et al., 2003). As with resorption, prostaglandins can also stimulate and inhibit bone formation. High concentrations of prostaglandins can inhibit collagen synthesis and reduce procollagen (I) mRNA levels in bone (Raisz et al., 1993). The stimulatory effect can be observed in vitro either at low concentrations or in the presence of cortisol and is probably indirect effect (Flanagan et al., 1994; Gronowicz et al., 1994). Although the anabolic effect of prostaglandins is indirect, it is a potent effect and may predominate in vivo (Jee et al., 1985; Jee et al., 1987; Mori et al., 1990; Miller et al., 1994).

Prostaglandins are formed by most cells in our bodies and act as autocrine and paracrine lipid mediators (i.e., signal at or immediately adjacent to their site of synthesis) (Samuelsson, 1983; Six et al., 2000). They are not stored but are synthesized de novo from membrane-released arachidonic acid when cells are

activated by mechanical trauma or by specific cytokine, growth factor, and other stimuli (e.g., collagen and adenosine diphosphate in platelets, bradykinin and thrombin in endothelium). A host of enzymes regulate cellular levels of arachidonic acid, keeping it esterified until mobilized by phospholipase A_2 (PLA_2). The control of arachidonic acid release from membranes has undergone several paradigm shifts in recent years with the continuing identification of new PLA_2 members (Six et al., 2000). Despite this, type IV cytosolic PLA_2 ($cPLA_2$) remains the key player for eicosanoid production.

The levels of free arachidonic acid are normally very low since the liberated fatty acid is rapidly metabolized (Samuelsson, 1987). Free arachidonic acid is a substrate for two distinctively different enzymatic pathways, one leading to the synthesis of PGs, the other to the hydroperoxy- and hydroxy-eicosatetraenoic acids referred to as HPETEs and HETEs, respectively (Ford-Hutchinson, 1990). The two pathways have in common the insertion of molecular oxygen into the fatty acid, although PGs have an additional cyclopentane ring. Lipoxygenases (LOXs) are a family of enzymes that catalyze the incorporation of molecular oxygen into polyunsaturated fatty acids at specific positions to form active metabolites such as HETEs. Three major LOXs have been characterized in mammals and designated as 5-, 12-, and 15-LOX, that produce 5(S)-, 12(S)-, 15(S)- hydroperoxides (hydroxyperoxyiecosatetranoic acids, HPETEs) respectively, products that are reduced to 5(S)-, 12(S)-, 15(S)- hydroxyeicosatetranoic acids (HETEs). The three enzymes are known mainly from the blood cells in which they are robustly expressed—the 5*S*-lipoxygenase in leukocytes, the 12*S*-lipoxygenase in platelets,

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and the 15*S*-lipoxygenase in reticulocytes, macrophages and eosinophils (Brash et al., 1997). Nevertheless selective expression is well documented in other tissues, for example, both the 12*S*- and 15*S*-lipoxygenases are detected in skin (Nugteren et al., 1987; Takahashi et al., 1993). 12-lipoxygenase generates from arachidonic acid the bioactive lipid 12-hydroxyeicosatetranoic acid (12-HETE), which has been shown to promote growth in chinese hamster ovary fibroblasts (Wen et al., 2000; Limor et al., 2001), and the proliferation of pancreatic cancer cells (Brash et al., 1997).   Studies have suggested that the HETEs may activate osteoclasts and increase bone resorption (Meghji et al., 1988). However, very little is still known about the exact roles the HETEs play as regulators of bone remodeling.

Arachidonic acid released by the cytosolic isoform of the PLA_2 ($cPLA_2$) is alternatively presented to prostaglandin H synthase (referred to colloquially as COX for cyclooxygenase) pathway and the fatty acid is then metabolized into an intermediate prostaglandin, PGH_2. PGHS exists as two isoforms referred to as PGHS-1 (COX-1) and PGHS-2 (COX-2) (Smith et al., 2000). In simple terms, COX-1 is the enzyme responsible for basal, constitutive prostaglandin synthesis, whereas COX-2 is important in various inflammatory and "induced" conditions (Crofford, 1997; Vane et al., 1998). The constitutive COX-1 is mainly utilized in immediate PGH_2 biosynthesis, which occurs within several minutes after stimulation with Ca^{2+} mobilizers, whereas the inducible COX-2 mediates delayed PGH_2 biosynthesis, which lasts for several hours following proinflammatory stimuli. COX-1 and COX-2 have been reported to exhibit subtle differences in AA requirements, that is COX-2 is favored over COX-1 at low AA concentrations

(Murakami et al., 1999), and differences in their subcellular localizations (Vane et al., 1998). The COX enzymes are monotypically inserted into the ER and nuclear membrane with the substrate-binding pocket precisely oriented to take up released arachidonic acid. The crystal structures of COX-1 and COX-2 are remarkably similar, with one notable amino acid difference that leads to a larger "side-pocket" for substrate access in COX-2 (Smith et al., 2000).

Vane and coworkers found that the analgesic, antipyretic and anti-inflammatory properties of acetylsalicylate (aspirin) were based on the inhibition of prostaglandin synthesis (Vane, 1971). Vane showed that the acidic anti-inflammatory analgesics decreased pro-inflammatory prostaglandin concentrations by inhibiting cyclooxygenases. The cyclooxygenase activity of PGH synthase is the target site of aspirin, ibuprofen and related nonsteroidal anti-inflammatory drugs (NSAIDs) (Abramson et al., 1985; Higgs et al., 1987); most NSAIDs, including aspirin, are competitive inhibitors of COX-1 (Kulmacz et al., 1985). NSAIDs, which possess analgesic, anti-inflammatory and antipyretic properties, are a heterogeneous group of substances without any uniform chemical properties, but nevertheless share the same therapeutic and side effects. However, the selective blockade of the COX-2 isoform should lead to the inhibition of inflammation and pain without impeding the COX-1-dependent prostaglandin production (Vane, 1994). This hypothesis led to an intensive worldwide search for selective COX-2 inhibitors; since 1999 rofecoxib (Vioxx®) and celecoxib (Celebrex®) have been available in the markets.

Downstream of the cyclooxygenases, the product PGH_2 can be further metabolized into various important eicosanoids, specifically PGE_2, $PGF_{2\alpha}$, PGD_2,

PGI$_2$, and thromboxane A$_2$ (Smith, 1992) (Fig. 3.). The coupling of PGH$_2$ synthesis

and metabolism by down stream enzymes is intricately coordinated in a cell-specific

fashion. Thromboxane synthase is found in platelets and macrophages, prostacyclin
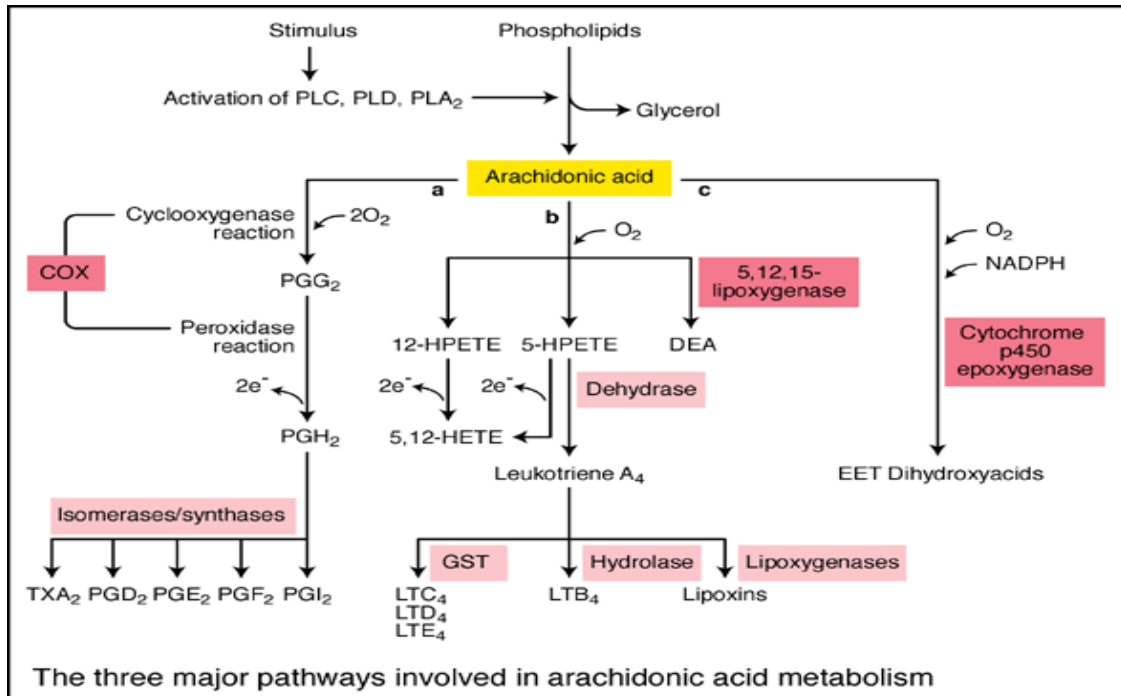
Fig. 3. *The three major pathways involved in arachidonic acid metabolism .*
(a) The cyclooxygenase (COX) pathway results in the formation of prostaglandin
G2 (PGG2) from arachidonic acid by a cyclooxygenase reaction. In a subsequent
peroxidase reaction, PGG2 undergoes a two-electron reduction to PGH2. Both of
these reactions are catalysed by COX (prostaglandin synthase H). PGG2 serves as a
substrate for cell-specific isomerases and synthases, producing other eicosanoids.
(b) The lipoxygenase pathway forms hydroperoxyeicosatetraenoic acids (HPETEs)
and dihydroxyeicosatetraenoic acid (DEA) by lipoxygenase and subsequently
converts these to (1) hydroxyeicosatetraenoic acids (HETEs) by peroxidases, (2)
leukotrienes (e.g. LTC4) by hydrase and glutathione S-transferase (GST), and (3)
lipoxins by lipoxygenases. (c) The epoxygenase pathway forms epoxyeicosatrienoic
acid (EET) and dihydroxyacids by cytochrome p450 epoxygenase.

synthase is found in endothelial cells, both of which belong to the cytochrome P-450

family and are reportedly localized in the endoplasmic reticulum (ER) and

perinuclear membranes (Walsh, 1989; Ramos et al., 1995; Walsh, 2004). PGF$_{2\alpha}$

synthase in lung and liver are cytosolic proteins with high homology to the aldo-

reductase family (Suzuki et al., 1999; Suzuki-Yamamoto et al., 1999; Suzuki-Yamamoto et al., 2000). PGD_2 synthases, which isomerize PGH_2 to PGD_2, occur in two distinct forms, the haemopoietic PGDS (H-PGDS) is the key enzyme for production of the D series of prostanoids in the immune system and mast cells (Kanaoka et al., 1997), and the lipocalin-type PGDS (L-PGDS) that is abundantly present in the central nervous system (Urade et al., 2000; Urade et al., 2002).

Two proteins that exhibit PGE_2 synthase (PGES) activity have been identified to date; they are the constitutive cytosolic PGES (cPGES), which is identical to p23 (Nakatani et al., 2002), and the inducible, perinuclear membarane-bound PGES (mPGES) (Murakami et al., 2002). Thus, PGE_2 synthase activity is present in both cytosolic and membrane fraction of cells, and the optimal catalytic activity of each enzyme require glutathione (Tanioka et al., 2000; Nakatani et al., 2002). Cytosolic PGES (cPGES), a GSH-requiring enzyme constitutively expressed in a wide variety of cells, is functionally linked to COX-1, not COX-2, to promote immediate PGE_2 production (Tanioka et al., 2000). The expression of mPGES is strongly induced by proinflammatory stimuli *in vitro* and at inflamed sites *in vivo* and is down regulated by anti-inflammatory glucocorticoids (Stichtenoth et al., 2001). $mPGE_2$ synthase activity can also be induced by proinflammatory stimuli in leukocytes (Fournier et al., 1997). Induction of mPGES-1 expression has also been observed in various systems in which COX-2-derived PGE_2 has been implicated to play a critical role, such as inflammation, fever, pain, female reproduction, tissue repair and cancer (Jakobsson et al., 1999; Murakami et al., 2000; Claveau et al., 2003; Thoren et al., 2003; Kamei et al., 2003).

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The present studies were undertaken to examine the effects of TNF-α and TGF-β, known mediators of osteoblast biology and bone remodeling, on PG and HETE biosynthesis in the human osteoblast-like (hOB) cell model system. It is possible that modulation of PGE_2 synthesis by cytokines in various cells and tissues may also have resulted, in part, from increased amounts of PGE_2 synthases (cPGES and mPGES), which are downstream of COX enzymes in the arachidonic acid metabolic pathway for PGE_2 production. To examine this possibility transcripts for cPGES and mPGES were detected in hOB cells, by reverse-transcriptase polymerase chain reaction using specific primers for human cPGES and mPGES.

Even though no cell culture model can exactly duplicate *in vivo* physiology, non-transformed cells approximate normal biology to a greater extent than do cancerous cells (Sporn et al., 1980). Therefore, in order to best mimic *in vivo* bone biology, all of the experiments made use of cultured hOB cells. hOB cells are grown from femoral bone explants and cultured in T-175 flasks, passaged twice, and then used for experimentation. These hOB-like cells represent a fairly mature osteoblast phenotype and respond appropriately to hormone and cytokine treatments (Robey and Termine 1985; Keeting et al., 1991). The use of hOB cells obtained from human bone explants avoids the problems of understanding and application of data acquired from whole animal models, or from animal derived bone cells to human bone. However, as with the other model systems there are limitations attendant with this model system. For example, primary human cell cultures are derived from a heterogeneous population, thus interstrain variations in characterstics and responses of the cells can sometimes be large, thereby making analysis challenging.

Furthermore, hOB cells have a limited life span and they grow slowly in culture limiting the variables being tested on a single strain of cells. The difficulties associated with studying such cells are counterbalanced with their suitability as a model system representing authentic human osteoblasts.

The objectives of these studies were focused to: 1) describe the spectrum of PGs and HETEs produced by hOB cells under basal and cytokine-stimulated conditions and 2) to determine steady-state mRNA levels of cPGES or mPGES which could lead to increased PGE_2 production.

MATERIALS AND METHODS

Experimental Subjects

Cancellous bone explants were obtained as surgical waste generated from the femoral head during routine bone grafting procedures. The harvest of this waste material following informed consent was approved by the West Virginia University Internal Review Board. Patient records were evaluated, and explants were not taken from patients with diagnosed osteoporosis or from patients presenting with endocrine disorders known to affect bone. Bone explants used in the EIA experiments were from 3 men aged 61 to 77 (median age of 64), and from 3 women aged 59 to 84 (median age of 80). Bone explants used in cPGES RT-PCR studies were from 3 women aged 23 to 85 (median age of 48) and 1 man aged 64. Bone explants used in mPGES RT-PCR studies was from 2 men aged 75 and 64, 2 women aged 23 and 48. Bone explants used in LOX RT-PCR studies were from 3 men aged 61 to 75 (median age of 68).

hOB Cell Cultures

Cancellous bone explants were prepared and placed into culture according to the method developed by Robey and Termine (1985), as previously described by Cissel et al. (1996). The essential elements of this method include stripping the explants in a 2 hour collagenase (1 mg/ml; Gibco, Grand Island, NY) digestion after which the explants were placed in a calcium-free, phenol red-free mixture of DMEM: Ham's F12K (1:1) (Biofluids, Rockville, MD) supplemented to 8% heat-inactivated fetal calf serum (FCS; Gibco). These culture methods yield nearly

homogeneous cell cultures that display multiple aspects of the mature osteoblast phenotype (Cissel et al. 1996, Borke et al., 1988). The hOB cell phenotype is stable through at least 4 passages (Marie, 1994). All experiments described in this work used hOB cells subcultured in 2nd or 3rd passage.

Reagents

Recombinant human tumor necrosis factor-α and recombinant human transforming growth factor-β were purchased from R&D Systems (Minneapolis, MN). IL-1β and IL-3 were purchased from BioRad. Stock solutions of each were prepared according to the supplier's instructions, and stored at -20° C for no more than 4 months. PGE$_2$, 6-keto PGF$_{1\alpha}$, PGF$_{2\alpha}$, PGD$_2$, TXA$_2$ and 15-HETE-Monoclonal Enzyme Immunoassay (EIA) Kits were obtained from Cayman Chemical Co. (Ann Arbor, MI). RT-PCR One-Step kits were obtained from Invitrogen (Carlsbad, CA). Other chemicals and reagents used were of the highest quality grade available. Oligonucleotide primers for human glyceraldehyde-3-phosphate dehydrogenase (GAPdH), cPGES, mPGES and 15-Lipoxygenase type II were purchased from Invitrogen. The oligonucleotide primers used were as follows: mPGES, forward 5′-ATGCCTGCCCACAGCCTG-3′, and reverse 5′-TCACAGGTGGCGGGCCGC-3′; for cPGES, forward 5′-ATGCAGCCTGCTTCTGCA-3′, and reverse 5′-TTACTCCAGATCTGGCAT-3′ (Stichtenoth et al., 2001); for 15-lipoxygenase type II (Tang et al., 2002): forward 5'-AACTCACCCCCACCACCATACACA-3' and reverse 5'-TTCCCGCCTCCATCTCCCAAAGT-3', primers that amplify a 351 bp fragment

and cover nucleotides 2234-2584 in the 3'-UTR (3'-untranslated region).

Amplification specificity of cPGES, mPGES, 15-lipoxygenase type II and GAPdH

sequences was assured by sequencing the subcloned gel purified PCR products from

our experiments (Univ. California-Davis Sequencing Laboratory).

hOB Cell Incubations

hOB cells were subcultured at 50,000 cells/well into 24-well plates (EIAs) or

at 150,000 cells/well into 12-well plates (RNA assays) in medium supplemented to 1

mM calcium and 10% (v/v) FCS (see flow chart below). In all experiments the

calcium concentration of the media was thereafter maintained at 1 mM.

<div style="border:1px solid black; padding:10px;">

	48 hrs		48 hrs		24hrs(conditioned media) or 6-8hrs(RNA)	

Subculture $\longrightarrow$ **Media change** $\longrightarrow$ **Media change** $\longrightarrow$ **Harvest Conditioned Media**
(10% FCS)   (1% FCS)   (10% FCS ± Cytokines)

</div>
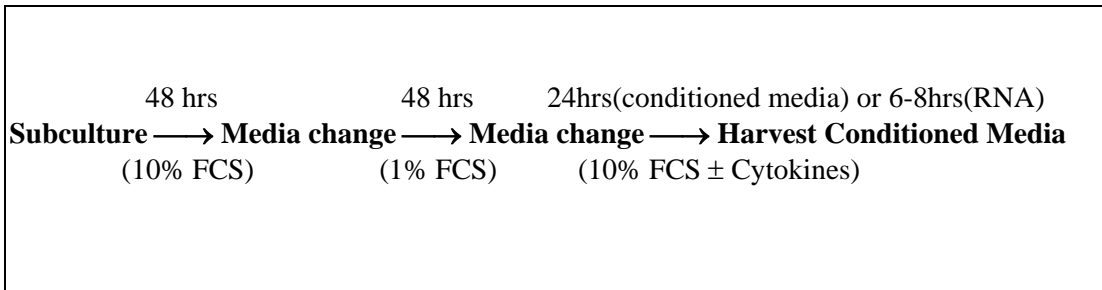
Forty-eight hours after subculturing, media were replaced and the FCS was reduced

to 1% for an additional 48 hours. Media were changed and the experimental

manipulations were initiated by the addition of 20 nM TNF-α, 40 pM TGF-β, TNF-

α plus TGF-β, TNF-α and TGF-β plus ibuprofen, or vehicle for 24-hours when

harvesting conditioned media for EIA studies, or for 6-8 hours when harvesting total

RNA.   For 15-lipoxygenase type II RT-PCR studies, hOB cells were exposed to

growth factors and cytokines at final concentrations as follows: TGF-β at 40 pM;

TNF-α at 400 pM alone, and in combination with TGF-β; IL-1β and IL-3 at 300 pM.

These concentrations of TGF-β, TNF-α, and IL-1β elicit their maximum effects on hOB cell PGE_2 production (Xu et al., 1997). The concentration of IL-3 was based on the literature (Li et al., 1992).

Immunochemical Assays for PGs

hOB cells were subcultured in 24-well plates at a density of 50,000 cells/well. After the 48 hour attachment, the cells were placed in 1% FCS for an additional 48 hours. Following the replacement of 1% with 10% FCS, cytokines, or vehicle, were added to the media as described. After treatment, the conditioned media were collected and were stored at -80^oC until assayed. PGE_2, 6-keto $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGD_2, TXA_2 and 15-HETE were measured in hOB cell conditioned media using specific EIA kits according to the supplier's instructions (Cayman Chemical). Each sample was assayed at least two dilutions and performed in duplicate. The detection limit for different prostaglandins were PGE_2 15 pg/ml, 6-keto $PGF_{1\alpha}$ 11 pg/ml, $PGF_{2\alpha}$ 9 pg/ml, PGD_2 3.1pg/ml, TXA_2 11pg/ml and 15-HETE 170 pg/ml at 80% B/B_0. Briefly, the assay is based upon the competition between PG or 15-HETE in the sample or standards and the respective PG or 15-HETE acetylcholine-esterase conjugate (tracer) for a limited amount of specific monoclonal antibody.

The limits of detection at $(80\% B/B_0)$ for different kits were as follows: Prostaglandin E_2- 15 pg/ml, 6 keto-Prostaglandin $F_{1\alpha}$ -11 pg/ml, Prostaglandin $F_{2\alpha}$ - 9 pg/ml, Prostaglandin D_2 - 3.1 pg/ml, Thromboxane B_2 - 11pg/ml and 15-HETE - 170 pg/ml. The intra- and interassay coefficient of variations (CVs) for: PGE_2 were 4.8% and 13.2% respectively, 6-keto$PGF_{1\alpha}$ were 5.2% and 12.0% respectively,

PGF$_{2\alpha}$ were 4.4% and 12.9% respectively, PGD$_2$ were 5.2% and 2.9% respectively, 15-HETE were 5.7% and 8.7% respectively.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR analysis was preferred over Northern blotting to assess specific mRNA levels due to cell culture limitations and the high costs associated with TNF treatments. hOB cells were subcultured at a density of 150,000 cells/well in 12-well plates in 10% FCS medium. After 48 hours, media were changed to 1% FCS for 48 hours. Following the replacement of 1% with 10% FCS, the cytokines were added to the media 6-8 hours before harvesting the cells for RNA preparations. Total RNA was isolated using the TRIZOL Reagent (Invitrogen) and DNAase treated. RNA yields were estimated by absorbance at 260 nm, and the reverse transcriptions and amplifications were performed starting with 50 ng of total RNA per sample, using the RT-PCR One-Step kit according to the manufacturer's recommendations. Thirty-five cycles of amplifications were performed for cPGES at 50°C, 32 cycles for mPGES at 65°C, 35 cycles for 15-lipoxygenase type II at 65°C and 25 cycles for GAPdH at 55°C, leaving each product in its linear range. Hybridization intensities in these experiments were determined using the BioRad Fluor-S^{TM} MultiImager gel documentation system with their software. All data are presented as "Fold-basal" in which the band intensity of the treated specimens was compared with that of its respective control after correcting for concurrently amplified human GAPdH levels in the control and treated specimens.

Statistical Analyses

Data were analyzed using the SAS-JMP statistical package. Data for PGs and HETE production as measured by EIA are presented as "Fold-basal" to represent the response of the cells to treatment compared with basal production. Because hOB cells are derived from the heterogeneous human population, variation between cell strains makes a normalization method (i.e.,"Fold basal") desirable to assist in permitting meaningful comparisons. Data are presented as the mean ± SEM of N experiments using different hOB cell strains. Intra-assay coefficient of variation (CV) was calculated by comparing variation between replicate samples using the formula CV=(Std. Dev./Mean)*100. Inter-assay coefficient of variation (CV) was calculated by comparing variation between samples obtained from similar experiments performed on different days using the similar kit using the formula CV=(Std. Dev./Mean)*100. In the RT-PCR studies, individual band intensities were first normalized to the samples respective GAPdH band intensity. These values were then expressed as a percentage of control sample value, and treatment effects were analyzed.

The data were analyzed by the paired t-test (e.g. cytokine effects on PGE_2 production by hOB cells) using the SAS-JMP statistical package. Statistical significance was examined by these tests using $p < 0.05$ as the significance level.

RESULTS

Effects of cytokines on biosynthesis of arachidonic acid metabolites

Previously published TLC analyses (Fig. 4; with permission from Dr. Philip

E. Keeting) and EIA studies from this laboratory guided investigations into EIAs to
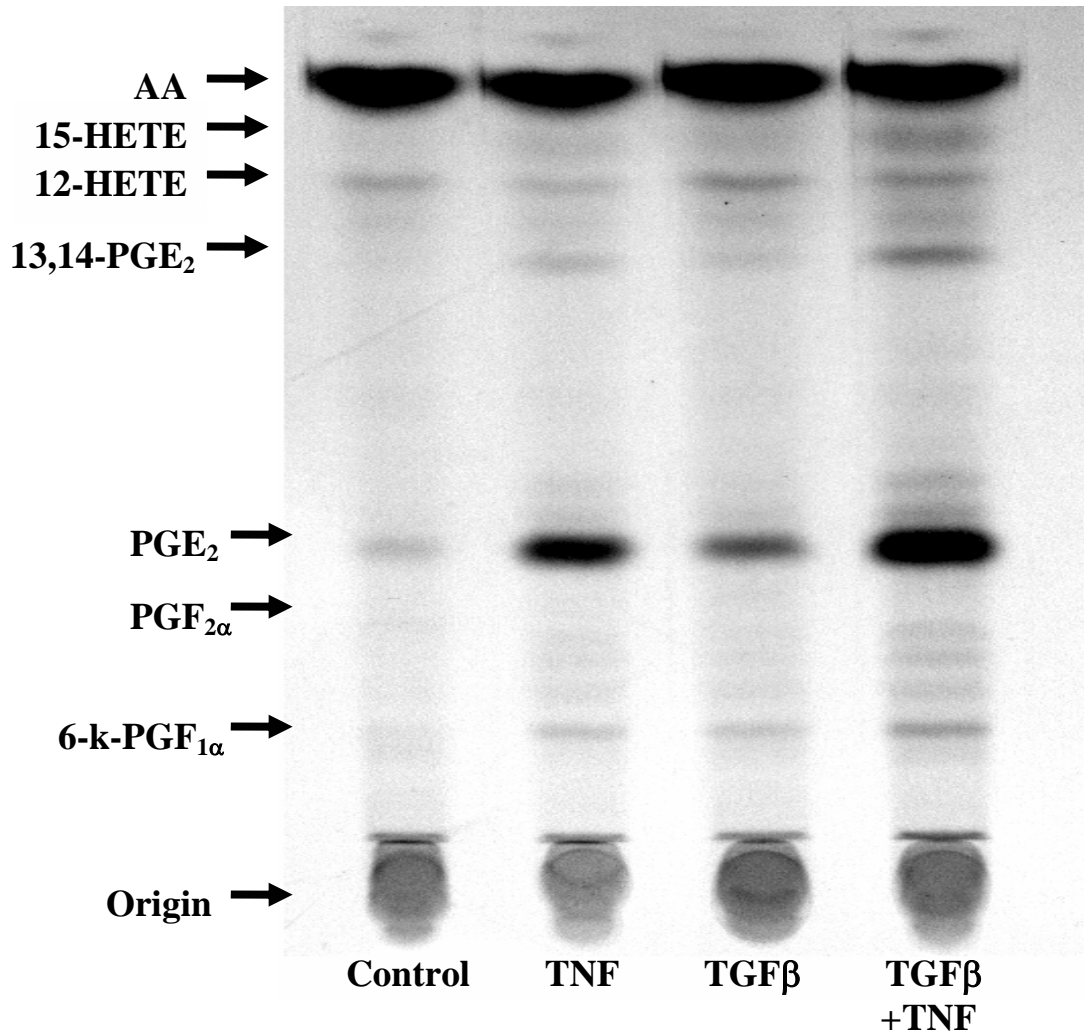

Figure 4. *hOB cells were preradiolabeled with 1-[^{14}C]arachidonic acid for 24 hours, and stimulated with or without cytokines, as indicated. Twenty-four-hour cell-conditioned media were collected, extracted, products separated by thin layer chromatography, and the TLC plates used to expose x-ray film. Metabolites were identified by the comigration of authentic standards.*

further define and characterize the role of cytokines in the modulation of PG and HETE production in hOB cell cultures. These TLC studies indicated that the application of TGFβ or TNFα alone, and in combination, elevated $1\text{-}[^{14}\text{C}]\text{-PGE}_2$ formation several fold, and produced modest increases of perhaps two-five fold in the levels of $1\text{-}[^{14}\text{C}]\text{-}6\text{-keto-PGF}_{1\alpha}$ in hOB cells. In some of these studies a band corresponding to $PGF_{2\alpha}$ was observed. Bands corresponding to the migration of authentic 12-HETE and 15-HETE were also seen in some experiments. The 15-HETE band was increased in cytokine stimulated specimens, and appeared to be decreased when aspirin or ibuprofen, both inhibitors of COX, was included. 12-HETE formation did not appear to vary by treatment. Production of other PG metabolites, such as TxB_2 or 5-HETE, by the hOB cells was not detected using the TLC procedure.

In the present studies, conditioned media harvested from hOB cell cultures were assayed by prostalandin specific EIAs to determine quantitatively the effects of cytokines on PGE_2, $6\text{-keto-PGF}_{1\alpha}$, $PGF_{2\alpha,}$ PGD_2, thromboxane (TXB_2) and on 15-HETE biosynthesis. The main product of PGH_2 in the conditioned media was found to be PGE_2, with lesser amounts of $6\text{-keto-PGF}_{1\alpha}$, $PGF_{2\alpha,}$ and PGD_2 detected (Figure 5 and Table 2). TXB_2 was not detected in control samples, or in samples stimulated with cytokines. 15-HETE was produced by the hOB cells, and was sensitive to modulation by the cytokines and ibuprofen.

EIA analysis indicated that PGE_2 was the predominant PG metabolite released by unstimulated hOB cells and its synthesis was increased more than other PGs in cells treated with TGFβ and TNFα, both separately and in combination,

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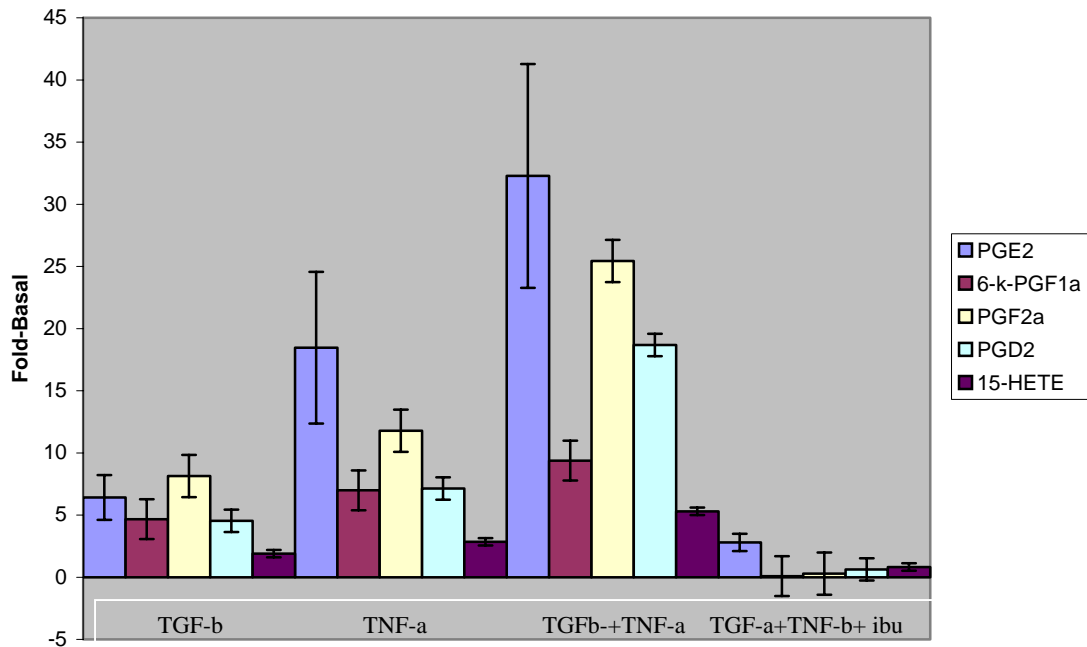**Increases in PGs and 15-HETE when hOB cells treated by cytokines**

Fig. 5. Summary of PGs and 15-HETE stimulation by TGF-β and TNF-α

hOB cells were treated with 20 nM TNF-α and 40 pM TGF-β in combination or separately. In some specimens, fifty μM of ibuprofen was used in combination with 20 nM TNF-α and 40 pM TGF-β for 24 hrs in 10% FBS supplemented medium. PGs and HETEs were assayed by EIA (N=5).

agreeing with previous observations (Xu et al., 1997) (Table. 2). Basal production of

PGE_2 in control samples was measured at 9.5 ± 5.1 ng/ml (N=5). TNFα alone and in

combination with TGFβ over a 24 hr incubation significantly increased PGE_2 levels

(N=5, $p<0.05$). In combination the cytokines increased PGE_2 formation to 32.3 ± 9

fold-basal (N=5, $p<0.05$), a response dramatically different from the increase elicited

by TGFβ alone (6.4 ± 1.8 fold; $p<0.05$ vs. control), but not significantly different

from the response to TNFα (18.5 ± 6.1 fold; $p<0.05$ vs. control). Samples treated

with TGFβ and TNFα in the presence of ibuprofen (2.8 ± 0.7 fold; $p<0.05$ vs.

control; $p<0.05$ vs. TGFβ, TNFα or Both) exhibited an attenuated response. A

significant difference in PGE_2 formation was found between TNFα treated samples

(18.5 ± 6.1 fold-basal) vs. TGFβ plus TNFα plus ibuprofen (2.8 ± 0.7 fold basal)

(Table 2).

6-keto-$PGF_{1\alpha}$ was the second most predominant PG found in hOB cells,

although the amounts found did not approach the amount of PGE_2 under either basal

(0.5 ± 0.1 ng/ml vs. 9.5 ng/ml) or maximal stimulated conditions (4.7 ± 0.2 ng/ml vs.

306.9 ± 45 ng/ml). The cytokine dependent increase in 6-keto-$PGF_{1\alpha}$ formation in

hOB cells was a significant 9.4 ± 1.6 fold-basal in specimens treated with TGFβ and

TNFα in combination (Table 3). Ibuprofen inclusion blocked the cytokine

stimulated increase (0.1 ± 0.0 fold-basal). TNFα increased 6-keto-$PGF_{1\alpha}$ formation

significantly to 7.0 ± 2.0 fold-basal.

Basal production of $PGF_{2\alpha}$ in control samples was 0.2 ± 0.0 ng/ml (N=5)

(Table 4). $PGF_{2\alpha}$ production was stimulated by cytokines individually, and in

Table 2. Effect of cytokines and Ibuprofen on PGE$_2$ synthesis by hOB cells

Treatment	Fold Basal
Control	1.0 ± 0.0
TGF-β	6.4 ± 1.8
TNF-α	18.5 ± 6.1^a
TNF-α+TGF-β	32.3 ± 9.0^a
TNF-α+TGF-β+ ibuprofen	$2.8 \pm 0.7^{b,c}$

hOB cells were treated with 20 nM TNF-α and 40 pM TGF-β in combination or separately. Fifty μM of ibuprofen was used in combination with 20 nM TNF-α and 40 pM TGF-β for 24 hrs in 10% FBS supplemented medium. PGE$_2$ was assayed by EIA (N=5). The data are presented as the mean $\pm$ SEM of five individual cell strains. The basal production of PGE$_2$ was 9.5 $\pm$ 5.1 ng/ml.

[a], p<0.05, by comparison with control (by student's t-test).
[b], p<0.05, by comparison with TNF-α (by student's t-test).
[c], p<0.05, by comparison with TNF-α+TGF-β (by student's t-test).

Table 3. Effect of cytokines and Ibuprofen on 6-keto-PGF$_{1\alpha}$ synthesis by hOB cells

Treatment	Fold Basal
Control	1.0 ± 0.0
TGF-β	4.7 ± 1.6
TNF-α	7.0 ± 2.0[a]
TNF-α+TGF-β	9.4 ± 1.6[a]
TNF-α+TGF-β+ ibuprofen	0.1 ± 0.0[b,c]

hOB cells were treated with 20 nM TNF-α and 40 pM TGF-β in combination or separately. Fifty μM of ibuprofen was used in combination with 20 nM TNF-α and 40 pM TGF-β for 24 hrs in 10% FBS supplemented medium. 6-keto-PGF$_{1\alpha}$ was assayed by EIA (N=5). The data are presented as the mean ± SEM of five individual cell strains. The basal production of 6-keto-PGF$_{1\alpha}$ was 0.5 ±0.1 ng/ml.

[a], p<0.05, by comparison with control (by Tukey-Kramer's method).
[b], p<0.05, by comparison with TNF-α (by Tukey-Kramer's method).
[c], p<0.05, by comparison with TNF-α+TGF-β (by Tukey-Kramer's method).

Table 4. Effect of cytokines and Ibuprofen on PGF$_{2\alpha}$ synthesis by hOB cells

Treatment	Fold Basal
Control	1.0 ± 0.0
TGF-β	8.1 ± 1.7^b
TNF-α	$11.8 \pm 4.5^{a,b}$
TNF-α+TGF-β	25.4 ± 4.0^a
TNF-α+TGF-β+ ibuprofen	0.3 ± 0.1^b

hOB cells were treated with 20 nM TNF-α and 40 pM TGF-β in combination or separately. Fifty μM of ibuprofen was used in combination with 20 nM TNF-α and 40 pM TGF-β for 24 hrs in 10% FBS supplemented medium. PGF$_{2\alpha}$ was assayed by EIA (N=5). The data are presented as the mean $\pm$ SEM of five individual cell strains. The basal production of PGF$_{2\alpha}$ was 0.2 $\pm$ 0.0 ng/ml.

[a], p<0.05, by comparision with control (by Dunnett's method)
[b], p<0.05, by comparision with TNF-α and TGF-β in combination (by student's t-test).

combination, reaching a maximum increase to 25.4 ± 4.0 fold-basal (N=5) with the combination treatment of TGFβ plus TNFα. TNFα individually elevated PGF$_{2\alpha}$ production significantly to 11.8 ± 4.5 fold-basal.

There was also a significant difference in the stimulated PGF$_{2\alpha}$ synthesis between the combined application of TGFβ and TNFα (25.4 ± 4.0 fold-basal) versus treatment of TGFβ alone (8.1 ± 1.7 fold-basal) or TNFα alone (11.8 ± 4.5 fold-basal). Ibuprofen treatment blocked the cytokine stimulated increase in PGF$_{2\alpha}$ production (0.3 ± 0.1 fold-basal).

PGD$_2$ production by hOB cells had not been reported previously. In the present studies, the level of PGD$_2$ in unstimulated samples was measured at 0.02 ± 0.01 ng/ml (N=5). The capacity of the cytokines in combination and individually to modulate PGD$_2$ formation was examined, and TGFβ and TNFα in combination stimulated PGD$_2$ formation significantly (18.7 ± 2.9 fold-basal; $p<0.05$). This increase in PGD$_2$ formation with TGFβ plus TNFα in combination was significantly different from samples treated with TGFβ alone (4.5 ± 0.9 fold-basal), TNFα alone (7.1 ± 1.9 fold-basal), and TGFβ and TNFα plus ibuprofen (0.6 ± 0.1 fold-basal). TNFα alone elevated PGD$_2$ formation significantly over basal PGD$_2$ formation (7.1 ± 1.9 fold-basal) (Table 5).

The basal level of 15-HETE in hOB cell samples was 0.5 ± 0.1 ng/ml (N=5) (Table 6). TGFβ and TNFα applied in combination elevated 15-HETE biosynthesis significantly (5.3 ± 0.8 fold-basal). There was a significant difference in the 15-HETE levels between application of TGFβ and TNFα in combination compared to

Table 5. Effect of cytokines and Ibuprofen on PGD$_2$ synthesis by hOB cells

Treatment	Fold Basal
Control	1.0 ± 0.0
TGF-β	4.5 ± 0.9^c
TNF-α	$7.1 \pm 1.9^{a,c}$
TNF-α+TGF-β	18.7 ± 2.9^a
TNF-α+TGF-β+ ibuprofen	$0.6 \pm 0.1^{b,c}$

hOB cells were treated with 20 nM TNF-α and 40 pM TGF-β in combination or separately. Fifty μM of ibuprofen was used in combination with 20 nM TNF-α and 40 pM TGF-β for 24 hrs in 10% FBS supplemented medium. PGD$_2$ was assayed by EIA (N=4). The data are presented as the mean $\pm$ SEM of four individual cell strains. The basal level of PGD$_2$ was 0.02 $\pm$ 0.05 ng/ml.

[a], p<0.05, by comparision with control (by student's t-test).
[b], p<0.05, by comparision with TNF-α (by student's t-test).
[c], p<0.05, by comparision with TNF-α+TGF-β (by student's t-test).

Table 6. Effect of cytokines and Ibuprofen on 15-HETE synthesis by hOB cells

Treatment	Fold Basal
Control	1.0 ± 0.0
TGF-β	1.9 ± 0.3^c
TNF-α	$2.9 \pm 0.9^{a,c}$
TNF-α+TGF-β	5.3 ± 0.8^a
TNF-α+TGF-β+ ibuprofen	$0.8 \pm 0.1^{b,c}$

hOB cells were treated with 20 nM TNF-α and 40 pM TGF-β in combination or separately. Fifty μM of ibuprofen was used in combination with 20 nM TNF-α and 40 pM TGF-β for 24 hrs in 10% FBS supplemented medium. 15-HETE was assayed by EIA (N=5). The data are presented as the mean ± SEM of five individual cell strains. The basal level of 15-HETE was 0.5 ± 0.1 ng/ml.

[a], *p<0.05, by comparision with control (by student's t-test).*
[b], *p<0.05, by comparision with TNF-α (by student's t-test).*
[c], *p<0.05, by comparision with TNF-α and TGF-β (by student's t-test).*

treatment with TGFβ alone (1.9 ± 0.3 fold-basal) or with TNFα alone (2.9 ± 0.9 fold

basal). Ibuprofen application inhibited the cytokine stimulated increase in 15-HETE

(0.8 ± 0.1 fold-basal) (Table 6).

EIAs were also performed to measure TXB_2 in unstimulated hOB cells, and

cells treated with cytokines separately and in combination. TXB_2 was undetectable.

Effect of cytokines on Prostaglandin E$_2$ synthases

The possibility that the up-regulation of PGE$_2$ synthesis elicited by cytokines might have resulted from an increased level of the terminal PGE$_2$ synthases (cPGES and mPGES) which act downstream of COX enzymes in the arachidonic acid metabolic cascade for PGE$_2$ production was tested. Transcripts for cPGES and mPGES was assessed by the reverse-transcriptase polymerase chain reaction using specific primers for human cPGES and mPGES (Stichtenoth et al., 2001). cPGES and mPGES cDNAs had 482 and 458 nucleotides respectively.

cPGES mRNA in hOB cells was detected in unstimulated control samples (Fig. 5.). Upon dual cytokine stimulation, an up-regulation of cPGES mRNA was observed. A non-significant change in the cPGES mRNA level was observed in specimens stimulated singly with TGFβ (2.5 ± 0.4 fold basal, N=4), or with TNFα (4.6 ± 1.4 fold basal, N=4). The conjoint treatment of TGFβ and TNFα significantly increased the steady state level of cPGES mRNA (5.3 ± 1.3 fold-basal, N=4, $p<0.05$).

The mRNA levels of mPGES, was assessed utilizing RT-PCR technique, and the outcome for mPGES mRNA assays was generally similar to that of cPGES in response to the proinflammatory cytokines. mPGES mRNA expression was assayed in hOB cells following treatment with TGFβ (2.7 ± 0.7 fold-basal, N=4), or with TNFα versus control samples (4.5 ± 1.6 fold-basal, N=4). Neither response reached significance in these studies. TGFβ plus TNFα in combination significantly increased the mPGES mRNA level (5.3 ± 1.7 fold basal, N=4, $p<0.05$) (Fig. 6.).

Figure 6. *RT-PCR based experiments tested whether the mRNA level of cPGES was sensitive to TGFβ and/or TNFα treatment. The top panel illustrates the results of an experiment using hOB cells from a 23 year old woman. Specimens were treated with or without TGFβ, TNFα, or TGFβ plus TNFα for 6-8 hours. The bar graphs represent the relative levels of cPGES mRNA after normalizing to GAPdH levels in the pooled samples (N=4).*

*, $p < 0.05$, by comparision with control samples (Student's t-test).

| mPGES → | |
| GAPdH → | |

Control   TGFβ   TNFα   TGFβ
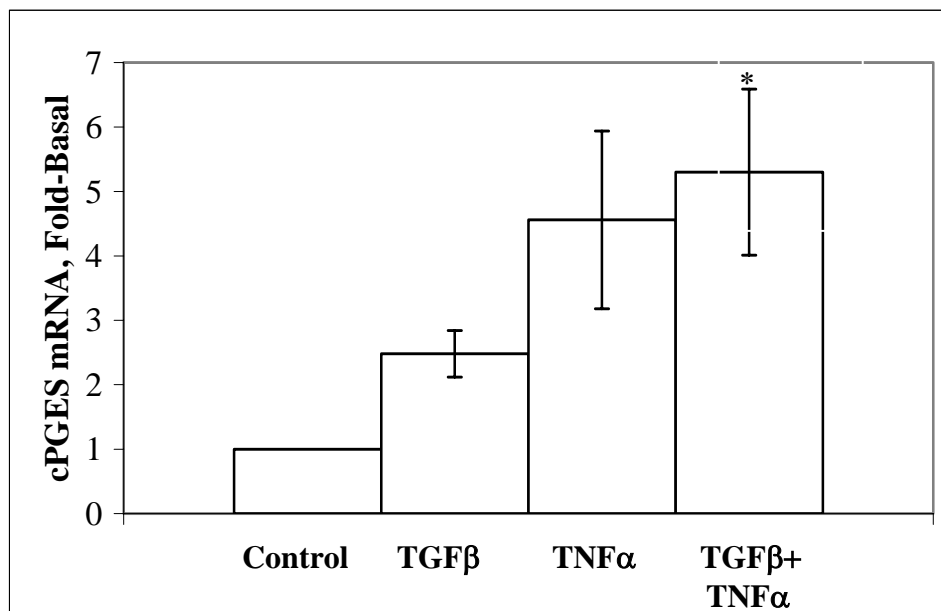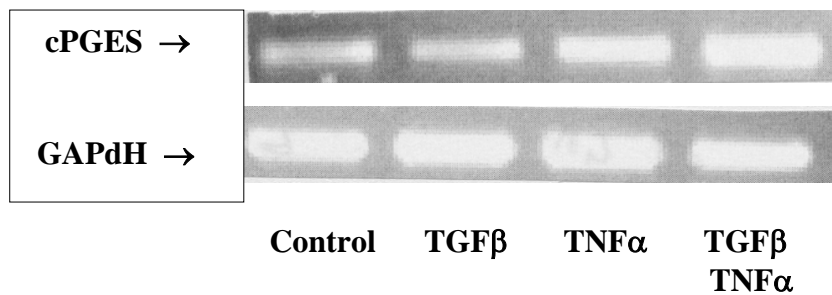   TNFα

Figure 7. *RT-PCR based experiments tested whether the mRNA level of mPGES was sensitive to TGFβ and/or TNFα treatment. The top panel illustrates the results of an experiment using hOB cells from a 75 year old man. Specimens were treated with or without TGFβ, TNFα, or TGFβ and TNFα for 6-8 hours. The bar graphs represents the relative levels of mPGES mRNA after correcting for GAPdH levels in the various samples (N=4).*

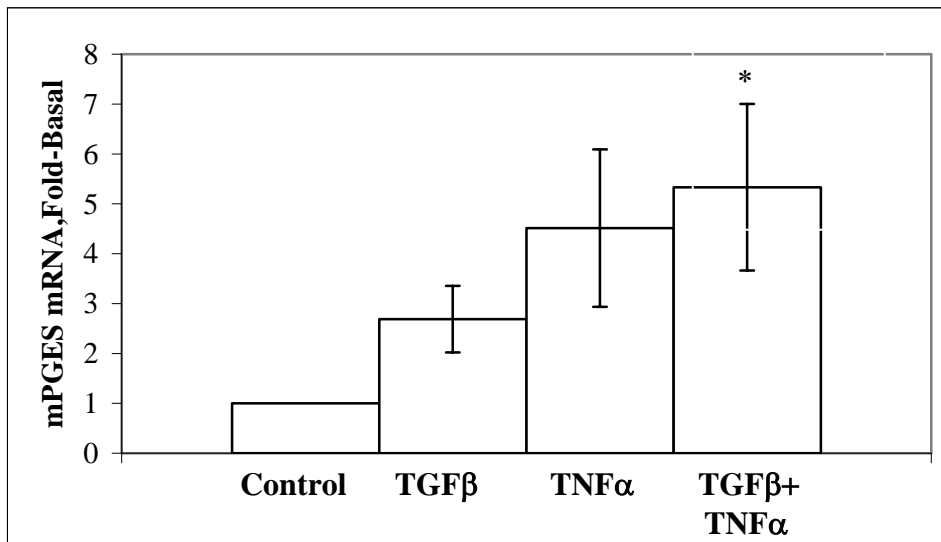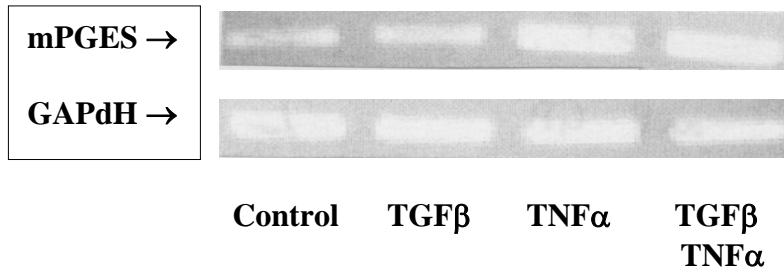**, p<0.05, by comparision with control samples (Student's t-test).*

Effect of cytokines on 15-lipoxygenase type-II

The steady-state level of 15-Lipoxygenase type-II mRNA in the hOB cells was determined in control samples and specimens treated with TGFβ and/or TNFα, IL-3 and IL-1β (Fig. 7a,b). A significant increase in 15-LOX type-II mRNA level was evident in specimens treated with TNFα alone (47.9 ± 22 fold-basal). The specimens treated with following treatments elicited a non-significant increase in 15-LOX type-II mRNA level, with TGFβ alone (2.7 ± 1.1 fold-basal); TGFβ plus TNFα (11.6 ± 4.6 fold-basal); IL-3 (5.7 ± 2.9 basal) and with IL-1β (14.2 ± 9.3 fold-basal).

1=Control; 2=TGFβ; 3=TNFα;
4=TGF /TNFα; 5=IL-3; 6=IL-1β

Figure 8a. *RT-PCR based experiments tested whether the mRNA level of 15-lipoxygenase type-II was sensitive to TGFβ and/or TNFα, IL-3 and IL-1β treatments. Specimens were treated with or without TGFβ, TNFα, or TGFβ plus TNFα, IL-3, or IL-1β for 6-8 hours. The top panel illustrates the results of an experiment using hOB cells from a 75 year old man.*
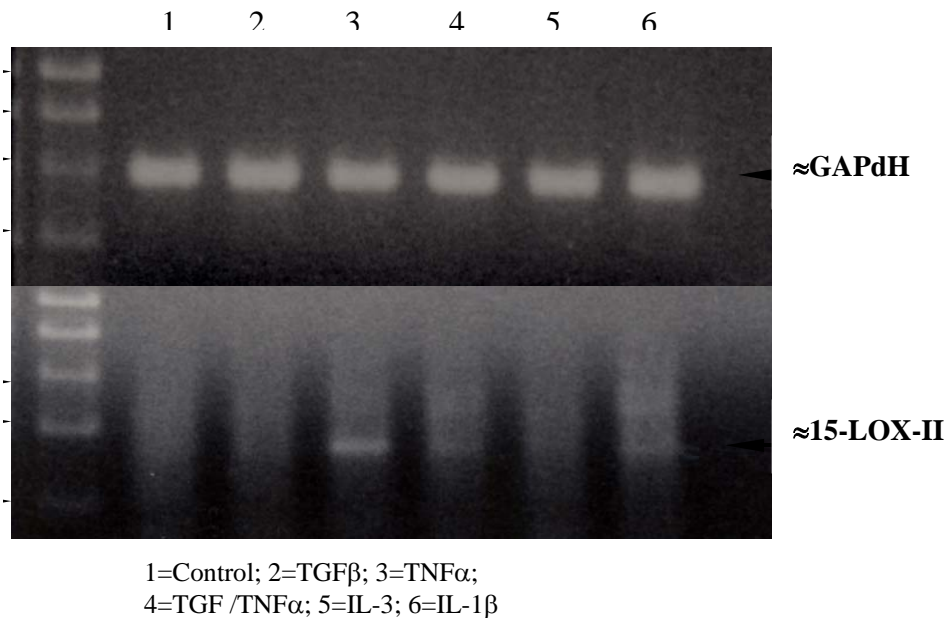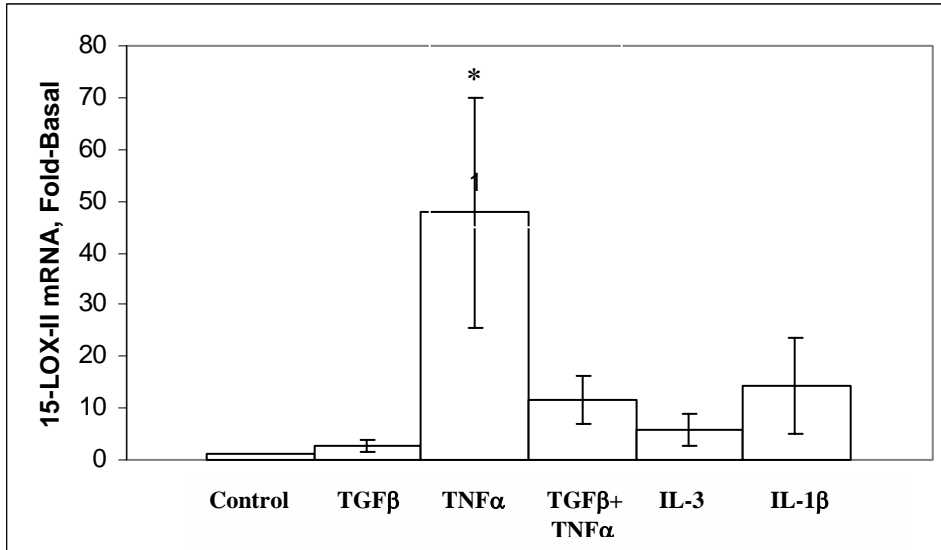
Figure 8b. *RT-PCR based experiments tested whether the mRNA level of 15-lipoxygenase type-2 was sensitive to TGFβ and/or TNFα, IL-3 and IL-1β treatments. *, significantly different from control and other treatments at p<0.05, N=3 (by student's t-test).*

DISCUSSION

Our enzyme-immunoassay data show that the predominant prostaglandin metabolite found in the conditioned media of unstimulated and cytokine-challenged hOB cells was PGE_2. Basal PGE_2 production may have been primarily the result of a constitutive COX-1 activity (Smith et al., 1996). The treatment with TGFβ or TNFα, alone and in combination, increased PGE_2 biosynthesis; the maximum response was a 32-fold increase following stimulation by the conjoint application of TGFβ plus TNFα, probably due to increased COX-2 expression and activity (Smith et al., 1996; Xu et al., 1997). Researchers have demonstrated the involvement of COX-2 induction and subsequent increased PGE_2 production by cytokines, such as IL-1β and TNFα, in the bone resorptive disorders of postmenopausal osteoporosis and rheumatoid arthritis joint destruction (Kawaguchi et al., 1995).

TGFβ and TNFα are products of various types of cells including hOB cells, and hOB cells are responsive to treatment with these cytokines when added exogenously (Raisz, 1993; Kuroki et al., 1994). Thus, TGFβ and TNFα serve as autocrine/paracrine regulators in osteoblast cells. Supporting this statement is the observation by Dodds et al. (1994) of TGFβ and TNFα mRNA expression in osteoblasts localized to remodeling sites in human bone sections. Cytokines regulate prostaglandin production in a tissue specific manner, and TGFβ has been reported to promote, and to oppose, augmented PG production with specificity to different tissues (Goppelt-Struebe, 1995). TNFα appears to act solely to increase PG biosynthesis (Crofford, 1997).

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The results of the present studies are consistent with the results and conclusions drawn from an earlier published report from this laboratory (Xu et al., 1997). An overall 32-fold potentiation of PGE_2 biosynthesis by TGFβ and TNFα treatment was recorded in the present studies; a 22-fold increase under these conditions was previously reported, and described as synergistic (Xu et al., 1997). Opposing/or synergistic interactions of TGFβ and TNFα are not restricted to hOB cells but are also seen in studies of rat mesangial cells, human airway epithelial cells, and fetal rat calvarial osteoblasts. In rat mesangial cells, TGFβ and TNFα in combination synergistically up-regulate fibronectin expression (Pawluczyk et al., 1998). Disparate interaction effects for TGFβ and TNFα mediation of GM-CSF and IL-8 expression in human airway epithelial cells have been reported (Jagels et al., 2000). In fetal rat calvarial osteoblasts a TNFα-stimulated increase in secretory PLA_2 release was opposed by TGFβ, as was a TNFα- dependent increase in COX-2 mRNA expression (Pruzanski et al., 1998).

In our studies the inhibitory effect of ibuprofen on PGE_2 production in hOB cells was obvious. The non-steroidal antinflammatory drugs (NSAIDs, e.g., ibuprofen) inhibit prostaglandin biosynthesis by inhibiting the cyclooxygenase enzymes. The present results were consistent with the earlier published report by Xu et al. (1997).

It should be noted that the present study is limited to *in vitro* experiments. In fact, PGE_2, particularly in the bone cells, has a very important role since it is known to have potent effects not only on bone resorption (Kawaguchi et al., 1995; Raisz 1999; Miyaura et al., 2000; Sakuma et al., 2000), but also on bone

formation *in vivo* (Jee et al., 1985; Jee et al., 1987; Mori et al., 1990; Miller et al., 1994). The recognized resorption effect of prostaglandins on bone has been repeatedly confirmed since it was first reported by Klein and Raisz (1970). Numerous reports indicate that PGE_2 stimulates bone resorption in mouse and rat bone cultures (Farndale et al., 1988), participates in the development of the bone resorbing osteoclasts from mononuclear cells, and can directly regulate osteoclast activity (Raisz et al., 1975; Roodman, 1996). The osteogenic effects are mainly seen when a substantial amount of exogenous PGE_2 was applied locally or systemically (Raisz et al., 1993; Kawaguchi et al., 1995), and PGE_2 can prevent ovariectomy-induced cancellous bone-loss in rats (Jee et al., 1990; Mori et al., 1992; Welch et al., 1993). Thus, the regulatory actions of PGE_2 in bone are diverse, and include the mediation of cyclic nucleotide accumulation (Partridge et al., 1981; Klein-Nulend et al., 1990; Yu et al., 1976; Atkins et al., 1977), cell proliferation (Hakeda et al., 1987; Raisz et al., 1990) are among its other recognized actions (Raisz et al., 1983; Raisz, 1988; Shen et al., 1986; Evans et al., 1990).

Prostaglandin production is a multi-step process (Smith et al., 1996). Esterified arachidonic acid is released from membrane glycerophospholipids by activated phospholipases. The activity of the downstream cyclooxygenase enzyme is rate-limited by liberated arachidonic acid availability (Smith et al., 1996), which is supplied through phospholipase A_2 hydrolysis of membrane glycerophospholipids. This available free arachidonic acid can then be used as a substrate by the COX enzymes to form the unstable transitional cyclic endoperoxide PGH_2. Further,

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downstream of the cyclooxygenases, terminal conversion of PGH_2 to PGE_2 takes place, which may be catalyzed by either of two isoforms of PGE_2 synthase (PGES).

The regulatory mechanisms of PGE_2 production may involve multiple regulation mechanisms. One mechanism regulating cytokine-stimulated PGE_2 production could be effects on release of arachidonic acid from membrane glycerophospholipids by the activity of cytosolic PLA_2 ($cPLA_2$). This enzyme is critical for PG biosynthesis (Fujishima et al., 1999) and provides the necessary substrate for the newly expressed COX-2 protein after cytokine stimulation. It has been reported that $TNF\alpha$ mediates PG biosynthesis by activating $cPLA_2$ and by increasing the rate of phospholipase A_2 synthesis in bone cultures (Yanaga et al., 1992; Kawaguchi et al., 1996), an effect that may be duplicated in hOB cells (Secreto et al., submitted to Prostaglandins & Other Lipid Mediators). Pro-inflammatory cytokines have also been reported to stimulate PLA_2 activity and/or expression in mesangial and epithelial cells (Pfeilschifter et al., 1989; Newton et al., 1997).

The second PGE_2 production regulatory site is the inducible cyclooxygenase-2 gene. Previous work reported from this laboratory (Xu et al., 1997) demonstrated that $TGF\beta$ and $TNF\alpha$ can independently elevate the levels of COX-2 mRNA resulting in increased formation of PGE_2. When both cytokines were added to specimens concurrently, the independent stimulation of hOB cell COX-2 mRNA levels, protein levels, and prostaglandin biosynthesis was further elevated (Xu et al., 1997; Secreto et al., 2003). Some researchers have reported that $TGF\beta$ can stimulate COX-2 mRNA expression in MC3T3-E1 cells (Pilbeam et al., 1997). Our result

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shows that COX-2 activity is regulated by TGFβ and TNFα in hOB cells, as is evident by the stimulation of PGE$_2$ production by these cytokine treatments. This conclusion is supported by the inhibitory effects of the NSAID ibuprofen on PGE$_2$ formation.

The identification of an inducible PGE synthase in hOB cells opens up new avenues for studying the regulation of PGE$_2$ biosynthesis by pro-inflammatory cytokines. The third regulatory site for PGE$_2$ production is the membrane-associated PGES (mPGES), which was originally designated microsomal glutathione S-transferase 1-like 1 (MGST1-L1). This is an inducible enzyme, which is coordinately induced with COX-2 and is functionally coupled with COX-2 (Jakobsson et al., 1999; Murakami et al., 2002). mPGES expression is induced by proinflammatory cytokines in various cells and tissues, now including hOB cells as indicated by the results of the present studies, and is down-regulated by dexamethasone, accompanied by changes in COX-2 expression and subsequent PGE$_2$ production (Murakami et al., 2000).

The present study indicates the presence of the mRNAs for the microsomal and the cytosolic PGES enzymes in hOB cells. mPGES mRNA message in hOB cells appeared to increase modestly with TGFβ or TNFα treatment. The concurrent treatment of hOB cells with both cytokines increased mPGES mRNA levels by 5-fold, to a level that was significantly different from that observed in unstimulated control hOB cells. These results are consistent with the accumulated evidence from various studies which show that mPGES is an inducible enzyme, the expression of which is markedly increased, in a manner similar to COX-2, in various cells and

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tissues following several usually proinflammatory stimuli (Jakobsson et al., 1999; Soler et al., 2000; Stichtenoth et al., 2001; Murakami et al., 2002; Yamagata et al., 2001; Han et al., 2002). Proinflammatory cytokines are reported to induce mPGES mRNA levels in synovial cells (Stichtenoth et al., 2001), thus, mPGES might also be involved in the pathophysiology of the rheumatoid arthritis joint destruction. The nearby osteoblasts may then contribute to the PGE_2 levels found in synovial fluids, perhaps, thereby exacerbating the localized inflammation. mPGES is reported to be expressed and up-regulated transcriptionally by proinflammatory cytokines in the macrophage cell line RAW 264.7 (Naraba et al., 2002). Lately, membrane-associated PGE_2 synthase (mPGES) has been identified in the mouse coculture system of osteoblasts and bone marrow cells (Saegusa et al., 2003). In cultured mouse primary osteoblasts, both mPGES and cyclooxygenase-2 were induced by the bone resorptive cytokines interleukin-1, tumor necrosis factor-α, and fibroblast growth factor-2 (Saegusa et al., 2003). Induction of mPGES was also seen in mouse long bone and bone marrow *in vivo* by intraperitoneal injection of lipopolysaccharide. An antisense oligonucleotide blocking mPGES expression inhibited not only PGE_2 production, but also osteoclastogenesis and bone resorption stimulated by the cytokines (Saegusa et al., 2003). Unlike NSAIDs, which inhibit COX activity and suppress not only PGE_2 but also production of other possibly essential prostanoids, an inhibitor of this PGE_2 specific enzyme (PGES) could provide a highly selective treatment with low side effects on other aspects of tissue homeostasis.

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cPGES mRNA expression also increased in a similar fashion that of mPGES in hOB cells. These findings are in contrast with earlier reports that found cytosolic PGES (cPGES) was expressed ubiquitously and constitutively, and that mRNA levels were unaltered by proinflammatory stimuli in a wide variety of cells and tissues, where cPGES promotes COX-1 mediated immediate PGE_2 production (Tanioka et al., 2000). cPGES is expressed constitutively both in the mouse coculture system of osteoblasts and bone marrow cells, and mouse long bone in *vivo* without being affected by bone resorptive stimuli (Saegusa et al., 2003). This COX-1 and cPGES coupling is assumed to contribute to the basal production of PGE_2, which plays a role in the maintenance of tissue homeostasis.

Our EIA data suggests cytokine treatments individually had little effect on 6-keto-$PGF_{1\alpha}$ biosynthesis, but that the conjoint application of TGFβ and TNFα elevated 6-keto-$PGF_{1\alpha}$ by five-fold over basal production in unstimulated cells. Ibuprofen, when administered along with TGFβ and TNFα, blocked the formation of this prostanoid. These results are in line with the earlier reported RIA and TLC data by this laboratory (Xu et al., 1997). 6-keto-$PGF_{1\alpha}$ is the second most abundant PG produced in bone (Raisz, 1995; Kawaguchi et al., 1995). PGI_2, the precursor of 6-keto-$PGF_{1\alpha}$, reportedly inhibits osteoclastic bone resorption, and completely abolishes the normally intense cytoplasmic motility of isolated osteoclasts (Chambers et al., 1983). In contrast, some investigations have shown that PGI_2 is a very potent stimulator of bone resorption in fetal rat long bone organ culture (Dewhirst, 1984). Because PGI_2 is formed by bone it may affect bone mineral metabolism. PGI_2 is a potent vasodilator and inhibitor of platelet aggregation

(Navamani et al., 1997). Since blood vessels produce PGI_2 it is possible that PGI_2 release, perhaps augmented by the resident osteoblasts, may be responsible for the frequent association between vascular invasion and resorption of bone or calcified cartilage in physiologic remodeling and pathologic osteolysis (Raisz et al., 1979).

The pattern of $PGF_{2\alpha}$ production in hOB cells on stimulation with cytokines was similar to that of PGE_2; its production was elevated a significant 25-fold by the concurrent application of TGFβ and TNFα. This increase in $PGF_{2\alpha}$ production after cytokine stimulation suggests synergistic effects of TGFβ and TNFα over individual administration of these cytokines in hOB cells, whose additive effects only reached 19.9 fold-basal. The synthesis of $PGF_{2\alpha}$ was completely blocked by ibuprofen in cells when administered along with the combination of TGFβ and TNFα. The basal level of $PGF_{2\alpha}$ in hOB cells was somewhat similar to that of 6-keto-$PGF_{1\alpha}$, although it was only about $1/20^{th}$ of the basal level of the most abundant PG, PGE_2. These results are in agreement with the previously published TLC data, which suggested that $PGF_{2\alpha}$ levels increased after stimulation with TGFβ and TNFα together (Xu et al., 1997).

$PGF_{2\alpha}$ is produced by bone cells, and exogenous $PGF_{2\alpha}$ can stimulate bone resorption (Raisz et al., 1990). Bone resorption was stimulated in mouse calvaria *in vitro* by both PGE_2 and $PGF_{2\alpha}$, each in a dose-dependent manner, with PGE_2 being more potent than $PGF_{2\alpha}$ (Katz et al., 1981). $PGF_{2\alpha}$ also effected bone formation in contrast to the effects of PGE_2 in cultured neonatal mouse parietal bones (Raisz et al., 1990). Studies suggest that some of the effects of $PGF_{2\alpha}$ on bone resorption,

formation may be mediated by its capacity to stimulate an increase in endogenous

PGE_2 production (Raisz et al., 1990).

PGD_2 was the PG metabolite found in least amount in hOB cells; its

production was elevated by stimulation with TGFβ and TNFα, either individually or

in combination, in a similar pattern to that of the other prostaglandins measured.

Administration of TGFβ and TNFα in combination elevated the PGD_2 levels to

about an 18-fold increase over basal and ibuprofen inclusion blocked the PGD_2

formation stimulated by TGFβ and TNFα. PGD_2 has a notable stimulatory activity

on alkaline phosphatase activity and osteoblast calcification, but the effective form

of PGD_2 is probably a metabolite, Δ12-PGJ_2 (Koshihara et al., 1989). Δ12-PGJ_2

enhanced the transcription of type I procollagen (alpha 1) mRNA levels in

osteoblasts (Tasaki et al., 1991). It has been reported that PGJ_2 and its derivatives

are effective activators of peroxisome proliferator-activated receptors alpha and

gamma (PPAR-∝ and PPAR-ϒ, respectively) (Kliewer et al., 1995). It has been

recently reported that nuclear hormone receptor peroxisome proliferator activated

receptor-ϒ, when activated, promotes adipogenesis and inhibits osteogenesis from

bone marrow progenitors (Nuttall et al., 2004). A bone-selective PPAR-ϒ modulator,

capable of acting as an antagonist, may be required to exploit PPAR-ϒ as a target for

osteoporosis therapy (Akune et al., 2004; Pei et al., 2004).

Prostaglandins are important mediators that play a multiple roles in biological

events, such as fever, pain, inflammation, tumorigenesis, gastrointestinal protection,

vascular circulation, and bone metabolism (Raisz, 1995; Kawaguchi et al., 1995;

Funk, 2001; Harris et al., 2002). Prostaglandin E_2, $PGF_{2\alpha}$, and PGI_2 are

multifunctional regulators with both stimulatory and inhibitory effects on bone

formation and resorption (Raisz, 1995; Kawaguchi et al., 1995). The research

community has viewed the major effect of PGs as the stimulation of bone resorption

since PGE_2 was first shown to increase cyclic AMP and stimulate resorption in

cultured fetal rat long bones more than 30 years ago (Klein and Raisz, 1970), but the

work of Jee and others has now identified PGE_2 as an anabolic agent in bone as well

(Jee et al., 1985; Jee et al., 1987; Mori et al., 1990; Miller et al., 1994).

Prostaglandins exert versatile actions in diverse tissues and cells through

specific cell surface receptors. Molecular biological studies revealed the primary

structure of eight types of prostanoid receptor from various species. The prostanoids

act on the eight types and subtypes of the receptors. They are the PGD receptor (DP),

the EP_1, EP_2, EP_3, and EP_4 subtypes of the PGE receptor, the PGF receptor (FP), the

PGI receptor (IP), and the thromboxane A_2 receptor (TP) (Samuelsson et al., 1978;

Moncada et al., 1985; Halushka et al., 1989; Coleman et al., 1990; Negishi et al.,

1995). These receptors can recognize the structural differences of prostanoid

molecules. The binding affinities of these receptors to prostanoid molecules are

determined primarily by the cyclopentane ring structures of ligands. For example,

the DP receptor shows the highest affinities to PGD_2 and PGD_1, but affinities to other

prostanoids are at least 2 orders of magnitude less. One exception is the IP receptor,

which shows an affinity to PGE_1 almost comparable to PGI analogs such as iloprost.

This receptor, however, can bind PGE_2 with much lower affinity, suggesting that the

IP receptor can discriminate a difference in the side chains. The term "Kd"

represents the affinity of a ligand for a particular receptor and it's inversely related to

receptor affinity. The activation of membrane receptors and target cell responses is proportional to the degree of receptor occupancy.

The affinities of the prostanoid ligands for their specific receptors are as follows: EP_1 receptor subtype had a Kd value of 21 nM, EP_2 receptor subtype had a Kd value of 11 nM, EP_3 receptor subtype had a Kd value of 3 nM, and the EP_4 receptor subtype had a Kd value of 8.2 nM for radioligand $[^3H]PGE_2$ (Coleman et al., 1994; Bastepe et al., 1997). Thus, a PGE_2 response would be first seen with PGE_2 binding the EP_3 receptor subtype because it has Kd value of 3 nM for radioligand $[^3H]PGE_2$. While other EP receptors would require greater amounts of PGE_2 to be come activated. The FP receptor type had Kd value of 1.3 nM for radioligand $[^3H]PGF_{2\alpha}$; IP receptor type had Kd value described as <10 nM for radioligand $[^3H]$6-keto $PGF_{1\alpha}$, and mouse DP receptor type had Kd value of 43 nM for radioligand $[^3H]PGD_2$ (Coleman et al., 1994). Of the 8 receptors, EP_4, EP_1, EP_2, EP_3, $PGF_{2\alpha}$ (FPR), and PGI_2 receptors have been found in bone and in osteoblastic cell lines (Miyaura et al., 2000; Suzawa et al., 2000; Nemoto et al., 1995; Wang et al., 1999).

The actions of prostaglandins produced by hOB cells will depend on the Kd value for the prostanoid and its specific receptor. For example, the Kd value of the EP_3 receptor for PGE_2 is 3 nM, and PGE_2 production in hOB cells is approximately 27 nM. The FP receptor type has Kd value of 1.3 nM for radioligand $PGF_{2\alpha}$, indicating that hOB cell production of $PGF_{2\alpha}$ (~ 0.56 nM) should be sufficient to activate the FP receptor population in bone. However, hOB cell culture model doesnot exactly duplicate *in vivo* bone physiology, and the concentration of the PG

products in the microenvironment of the cells wouldn't reflect the dilution by the cell culture media, and should be rather higher.

15-HETE was also measured in hOB cell cultures by EIA. Its basal level, 0.5 ng/ml, was in the general range of 6-keto-PGF$_{1\alpha}$ and PGF$_{2\alpha}$ basal levels. TGFβ and TNFα in combination elevated 15-HETE production in comparision to the levels measured in specimens treated with either TGFβ or TNFα. 15-HETE production was inhibited by the COX inhibitor ibuprofen. These results are in agreement with thin layer chromatography (TLC) studies performed earlier in this laboratory, which demonstrated a band that co-migrated with authentic 15-HETE in hOB cell conditioned media, and that 15-HETE biosynthesis was eliminated in the presence of the NSAIDs aspirin or ibuprofen (P.Keeting, personal communication). A band tentatively identified as 12-HETE was also noted in the TLC studies, which appeared unaffected by cytokine addition. These findings are not inconsistent with work that showed that the amounts of all metabolites of lipoxygenases and cyclooxygenases were decreased by aspirin or indomethacin pretreatment in the perfused hamster lung tissue (Uotila et al., 1981; Schalin et al., 1982). However, typically the 15-LOX is not viewed as an NSAID target.

The steady-state level of hOB cell 15-lipoxygenase-II mRNA was responsive to modulation by TNFα, and by TGFβ plus TNFα in combination. TGFβ alone had no demonstrable effect on 15-LOX-II mRNA levels in the hOB cells. Opposing interaction effects for TGFβ and TNFα mediation of 15-lipooxygenase-II mRNA expression in hOB cells was suggested, and when added concurrently TGFβ limited the TNFα-mediated increase in 15-LOX-II mRNA levels.

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5-HETEs play an important role in bone research as suggested by studies on the involvement of 5-HETEs in bone resorption by activating osteoclasts (Gallwitz et al. 1993) and it might act as negative regulator of bone formation (Traianedes et al., 1998). While our studies do not address the role of the 15-HETEs in hOB cells and in bone remodeling, the identification 15-HETE production in hOB cells may serve to stimulate investigations aimed at such topics. Thus, the roles of the 15-HETEs and 15-LOX activity in bone remodeling and cellular signaling in osteoblasts is not clear since their presence was not previously documented; however, since they are produced in these cells, they may serve some mediator roles in bone remodeling, and thus, in the etiology and progression of bone disorders such as osteoporosis. However, it has been documented that 15-HETE has no proinflammatory capacity, but that it can inhibit the formation and the chemotactic response of neutrophils to leukotriene B4 (LTB4), a potent mediator of inflammation (Fogh et al., 1989). 15-HETE inhibits carageenan-induced arthritis by lowering leukotriene B4 concentrations in the synovial fluid of dogs, indicating that it possesses anti-inflammatory properties (Fogh et al., 1989).

Localized bone loss in rheumatoid arthritis results from the activation of an inflammatory immune response, which increases both the number and the activity of osteoclasts (Rehman et al., 2001). Elevated TNF-α levels can promote osteoclastogenesis by stimulating the osteoblasts/stromal cells and possibly T lymphocytes production of RANKL and M-CSF. In addition, recent *in vitro* studies have shown that TNF-α, in the presence of M-CSF, directly induces the formation of multinucleated cells containing tartrate-resistant acid phosphatase that are fully

capable of resorbing bone (Azuma et al., 2000; Kobayashi et al., 2000). Several anti-inflammatory cytokines such as IL-10, IL-11, and IL-13 act by suppressing the production of inflammatory cytokines or by neutralizing them (Rehman et al., 2001). 15-HETE, because of its anti-inflammatory properties, may act by suppressing the production of inflammatory cytokines or by neutralizing them and may play a significant role in preventing bone loss.

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