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Regulation of cytokine production in the rat osteoblast by transforming growth factor-BETA

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Yale University

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by John L. Phillips
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PHOTO, XEROGRAPH, AND ILLUSTRATION CREDITS

pg 5 T. Brailsford Robertson, from Australian Journal of Experimental Biology and Medical Science, volume IX, preface, 1932.

pg 5 Rita Levi-Montalcini and Stanley Cohen, from "Nobel Prize Winners", New York, 1987, HW Wilson Co., ISBN O 8242 0756-4.

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GLOSSARY & ABBREVIATIONS

AASF Amyloid-A Stimulating Factor (IL-1)
AP Anaphylatoxin
APC Antigen Presenting Cell
BAC Macrophage line, BAC 1.2F5, used in M-CSF assay[139b].
BCDF B-Cell Differentiation Factor (Interleukin-6)
BCGF-1 B-Cell Growth Factor-1 (Interleukin-4)
BCGF-2 B-Cell Growth Factor-2 (Interleukin-5)
BFU-E Burst Forming Unit-Erythroid
BDP Bone-Derived Protein
BMP-2B Bone Morphogenetic Protein-2B (BMP-4)
BSF-1 B-Cell Stimulatory Factor-1 (Interleukin-4)
BSF-2 B-Cell Stimulatory Factor-2 (Interleukin-6)
C3bBP Product derived after C3 factor proteolysis with serum B factor and properdin.
CFU-Eo Colony Forming Unit-Eosinophil
CFU-GEMMs Colony Forming Unit-Granulocyte/Eosinophil/Monocyte/Megakaryocyte
CFU-GM Colony Forming Unit-Granulocyte/Monocyte
CFU-MEG Colony Forming Unit-Megakaryocyte
CM Conditioned Media
CSF Colony Stimulating Factor
CSF-1 Colony Stimulating Factor-1 (Macrophage-Colony Stimulating Factor)
CRF Corticotropin Releasing Factor
CTLL IL-2 responsive T-cell line, CTLL-2/CT-6, used in second part of IL-1 assay[213b].
D-10 IL-1/lectin responsive T-cell line
DAG Diacylglycerol
DEP Diethylpyrocarbonate
EGF Epidermal Growth Factor
EHAA Eagle's Hank's Amino Acid (Bruff)
EP Endogenous Pyrogen (Interleukin-1)
FBS Fetal Bovine Serum
FGF Fibroblast Growth Factor
G-CSF Granulocyte-Colony Stimulating Factor
GBE Glucocorticoid Binding Element
GM-CSF Granulocyte-Macrophage Colony Stimulating Factor
GRE Glucocorticoid Response Element
GP Granulocyte Pyrogen (Interleukin-1)
H-1 Hemopoetin-1 (Interleukin-1)
HAF Hepatocyte Activating Factor (Interleukin-1)
HGF Hybridoma Growth Factor (Interleukin 6)[1]
HSF Hepatocyte Stimulatory Factor (Interleukin-6)

IGF-1 Insulin-like Growth Factor-1
IFN-α Interferon-alpha
IFN-γ Interferon-gamma
IL-1 Interleukin-1 (LAF,LEM,HAF,H-1,GP,EP)
IL-1R Interleukin-1 receptor
IL-2 Interleukin-2 (T-cell Growth Factor)
IL-3 Interleukin-3 (Multi-Colony Stimulating Factor)
IL-4 Interleukin-4 (BCGF-1, BSF-1)
IL-5 Interleukin-5 (BCGF-2, Eosinophil Differentiaion Factor)
IL-6 Interleukin-6 (HSF, BCF-2)
IL-7 Interleukin-7, a stromal cell derived growth factor for pre-B and T cells
IL-8 Interleukin-8 (Neutrophil Activation/Attractive Protein-1)
IP_3 Inositol triphosphate
LAF Lymphocyte Activating Factor (IL-1)
LAK Lymphokine Activated Killer cells
LBRM T-cell Lymphoma line, LBRM 33-1A5.47, used in first part of IL-1 assay[213b].
LEM Leukocyte Endogenous Mediator (IL-1)
LPS Lipopolysaccharide
M-CSF Macrophage-Colony Stimulating Factor
NGF Nerve Growth Factor
OAFs Osteoclast Activating Factors (Includes IL-1ß and TNF-ß)
PBS Phosphate Bufered Saline
PC Phosphotidylcholine
PDGF Platelet-derived Growth Factor
PI Phosphotidylinositol
PIP_2 Phosphotidylinositol diphosphate
PKA Protein kinase A
PKC Protein kinase C
PMA Phorbol 12-myristate 13-acetate
PTH-R Parathyroid hormone receptor
PTHrP Parathyroid Hormone Related Protein
rhIL-2 Recombinant human Interleukin-2
Synergen Molecule which synergizes with TGF-ß for cytokine release
TGF-α Transforming Growth Factor-Alpha
TGF-ß Transforming Growth Factor-Beta
TBP Transforming Growth Factor Binding Protein
TGFR-ß Receptor for Transforming Growth Factor-Beta
TIE Transforming Growth Factor Inhibitory Element
TNF-α Tumor Necrosis Factor alpha

καὶ γάρ ὄστεον οὔ ξύλου
ποιεῖται οὔτε λίθου, ἀλλά μην
τῶν θαυμαστῶν

For bone is not made of wood, indeed not stone, but wondrous things.

I. ABSTRACT

Osteoblast activities include bone formation, osteoclast regulation, and the secretion of a class of hormones called cytokines. Osteoblast-derived cytokines include Interleukin-6 (IL-6) and two colony stimulating factors (CSFs): Granulocyte-Macrophage CSF (GM-CSF), and Macrophage-CSF (M-CSF). Osteoblasts secrete cytokines after exposure to several well-characterized mitogens such as lipopolysaccharide (LPS), phytohemagglutinin (PHA), and Interleukin-1. What roles osteoblast-derived cytokines play in normal bone remodeling is unknown.

Transforming Growth Factor-Beta (TGF-ß) is a ubiquitous 23-25 kDa homodimeric protein stored in platelets and bone. TGF-ß was originally discovered as an inducer of neoplastic transformation in normal fibroblasts but has since been implicated in matrix homeostasis, immunomodulation, development and hematopoesis. Bone is the body's largest source of TGF-ß and the protein becomes activated after osteoclast bone resorption. No definitive role for TGF-ß in bone remodeling is known.

This thesis presents experiments which tested the effect of TGF-ß on osteoblast cytokine production and release. Data from *in vitro* cytokine assays of conditoned media suggest that TGF-ß upregulates IL-6 and GM-CSF secretion through a novel synergistic interaction with other low concentration molecules including LPS, IL-1, and parathyroid hormone (PTH), as a group referred to here as *synergens*. M-CSF secretion was unaffected by TGF-ß alone or in combination with any synergen. 90% of the GM-CSF and IL-6 release observed is achieved between 2.5 and 6 hours after exposure to TGF-ß and a given synergen. Furthermore, the synergy between TGF-ß and a given molecule is TGF-ß-dependent. The

osteoblast response was not altered by exposure to indomethacin. RNA analysis suggested that the synergy between TGF-ß and IL-1 may involve TGF-ß upregulation of the IL-1 cell surface receptor. These observations are the first to implicate TGF-ß as a stimulatory molecule for cytokine release from osteoblasts and suggest new aspects of bone remodeling as well as a possible role for the osteoblast in hematopoesis.

II. INTRODUCTION Growth factors and essential principles; The Discovery of Transforming Growth Factor-ß; Osteoblast-derived cytokines; Thesis investigation.

Throughout the life of the normal cell, there exists a tightly controlled series of programs designed to insure the orderly and regulatable growth and proliferation of the cell. These controls have enabled cells to survive the environmental fluctuations throughout evolution and everyday life: Cells survive starvation by slowing growth to minimal levels and cells maximize growth in environments consistent *with* growth. At any given moment, a cell must be aware of both its internal state as well as the external world. Without normal growth and proliferation controls, there can be dire consequences: An inability to respond appropriately to favourable environmental stimuli could result in decreased cell growth and premature senescence. Alternatively, an inability to check growth and to maintain quiescence, could result in inappropriate, sustained, and non-suppressable growth, an extreme condition known as cancer.

Understanding how normal cells control the transitions between the states of quiescence and growth is paramount towards understanding why certain cells, i.e. neoplastic cells, *cannot* control these transitions (Figure II.i). The evolution of multicellular life presented a particular problem for cellular growth. Multicellular organisms required each cell to regulate growth in a manner appropriate to the growth of surrounding cells. How cells communicate to ensure such regulation is exceedingly complex and has intrigued investigators for at least 70 years. In 1916, Australian Brailsford Robertson (Figure II.ii) discovered that a murine

pituitary extract stimulated the growth of juvenile mice. Robertson purified a substance capable of achieving the same effect, a substance

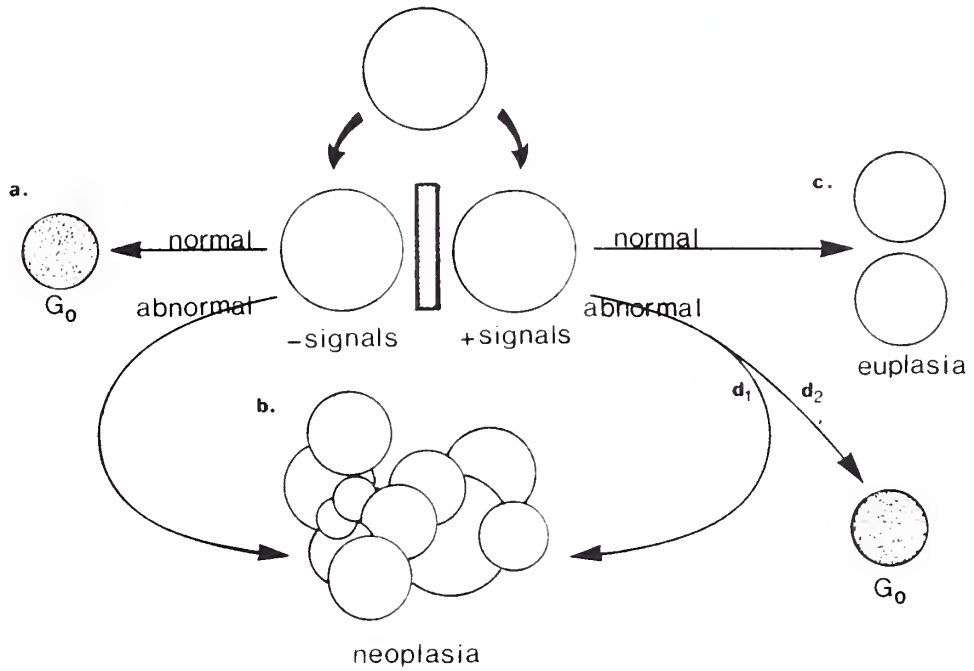


Figure II.i. Normal and abnormal cellular growth pathways. When presented with an environment devoid of growth signals normal cells respond by entering the quiescent state of G_0 (a). Neoplastic cells, however, may continue to proliferate irregularly (b). An environment replete with growth signals may induce normal cells to divide regularly (c), whereas abnormal cells may either grow unchecked as a neoplasia (d_1) or cease growth and enter G_0 (d_2).

he called *tethelin*.[22] At the same time, J. M. Byrne and Eric Horning of Melbourne, Australia were attempting to grow mammalian cells *in vitro* using tissue extracts from normal and neoplastic tissue[82]. In 1929, after Byrne and Horning published their findings in the Australian Journal of Science, Robertson eagerly sent Byrne and Horning samples of tethelin to test on their *in vitro* cultures. Working with chick enterocytes, cells cultured in the presence of tethelin grew faster than enterocytes cultured in chick plasma alone[22]. The significance of tethelin and tissue extracts towards the growth of cells was immediately appreciated and tethelin was accepted as the first 'growth factor' determined through *in vitro*

analysis. The daunting task had only begun, however, in determining what specifically a 'growth factor' was, how growth factors worked, and what role(s) the factors played in normal *in vivo* physiology.

Upon Robertson's death in 1929 and Horning's departure from Australia shortly thereafter these early efforts unfortunately came to a halt. In 1951, growth factor work eventually resumed when Rita Levi-Montalcini and Stanley Cohen (figure II.ii) isolated nerve growth factor (NGF) and epidermal growth factor (EGF)[45,126]. The characterization of these two molecules spawned a renewed and intense effort to understand growth factor biology and growth factor

Figure II.ii. Pioneers in growth factor research. Australian T. Brailsford Robertson (1884-1930), left, isolated the first 'growth factor' from mouse pituitaries which he called *tethelin* (Greek:'growing') a molecule whose identity is still undetermined. Rita Levi-Montalcini (born 1909), top right, and Stanley Cohen (born 1922), bottom right, both received Nobel prizes for medicine in 1986 for their work on Nerve Growth Factor and Epidermal Growth Factor, respectively, the first two Nobel laureates in the growth factor field.

roles in physiology, guided by the ultimate goal of utilizing *in vivo* growth factor capabilities for clinical needs. By 1963, several new growth factors were isolated including Fibroblast Growth Factor (FGF), Platelet-Derived Growth Factor (PDGF) and Insulin-like Growth Factors 1 and 2 (IGFs-1 and -2). In 1972, Gery et al. isolated Lymphocyte Activating Factor (LAF) derived from macrophages[67,68]. Because LAF was derived from a type of white cell, and functioned as a growth mediator between white cells, LAF was eventually known as *inter*leukin-1, or IL-1. IL-1 stimulated great interest not only because its potent T-cell stimulatory ability seemed immunologically relevant, but because IL-1 had other functions not directly leading to the 'usual' processes of growth, such as mitosis or proliferation. IL-1 was, for example, observed to stimulate cartilage resorption[98], induce the intrinsic pathway of the clotting cascade[128], chemoattract acute phase cells[100], and cause profound fever[13,24]. Furthermore, IL-1 induced T-cell growth by inducing the synthesis of interleukin-2, or IL-2, originally known as T-cell Growth Factor (TCGF)[124]. It appeared that certain growth factors had diverse roles in homeostasis and disease states. With attention shifted to the immune system, many growth factors were characterized which appeared to be specifically active in the growth and activity of immune cells. Molecules like Interleukin-2 were joined with six other interleukins (interleukins-3 through 8) and the Colony Stimulating Factors or CSFs: Granulocyte-Monocyte CSF (GM-CSF), Granulocyte-CSF (G-CSF), and Macrophage-CSF (M-CSF). Thus, a new class of growth factors were found which promoted changes in cellular activity in addition to stimulating

growth. These factors became broadly known as *cytokines*. Investigators soon demonstrated that cytokines could not be limited by activity or in source to cells of the immune system but could be isolated from cells as diverse as astrocytes[132b], kidney epithelial cells[137c], endometrium[154,167b], and cells of bone[87]. The roles cytokines play in these and other systems is not definitively known.

Growth factor research was made even more intriguing with the discovery of cytokines important to *excessive* growth and frank neoplastic change. Such molecules include the Transforming Growth Factors Alpha and Beta (TGF-α & TGF-ß)[5,62,166]. These molecules were orginally observed in 1983 to induce neoplastic transformation in normal fibroblasts growing in soft agar. Transforming Growth Factors have generated great interest because of their possible role in wound repair, tissue differentiation and matrix formation.

Transforming Growth Factor-ß (TGF-ß) is decidedly unique among cytokines, however, in that it has been generally regarded as an inhibitory molecule of normal cells, often implicated in the suppression of normal growth.

For example, Transforming Growth Factor-ß is 10,000-100,000 times as immunosuppressive as cyclosporin-A[9]. TGF-ß is ubiquitous, is stored primarily in the matrix of bone and is active only after release from binding proteins[28,35,167,183]. Bone is the richest source of TGF-ß in the body[34,35] but no clear role is yet defined for TGF-ß in bone tissue biology. TGF-ß is now being tested in clinical trials as an immunosuppressive adjuvant in chemotherapy, and as a topical agent in wound healing[9,167,183a]. The obvious importance of this molecule for clinical pursuits

necessitates a clear understanding of TGF-ß's actions in normal and abnormal human physiology.

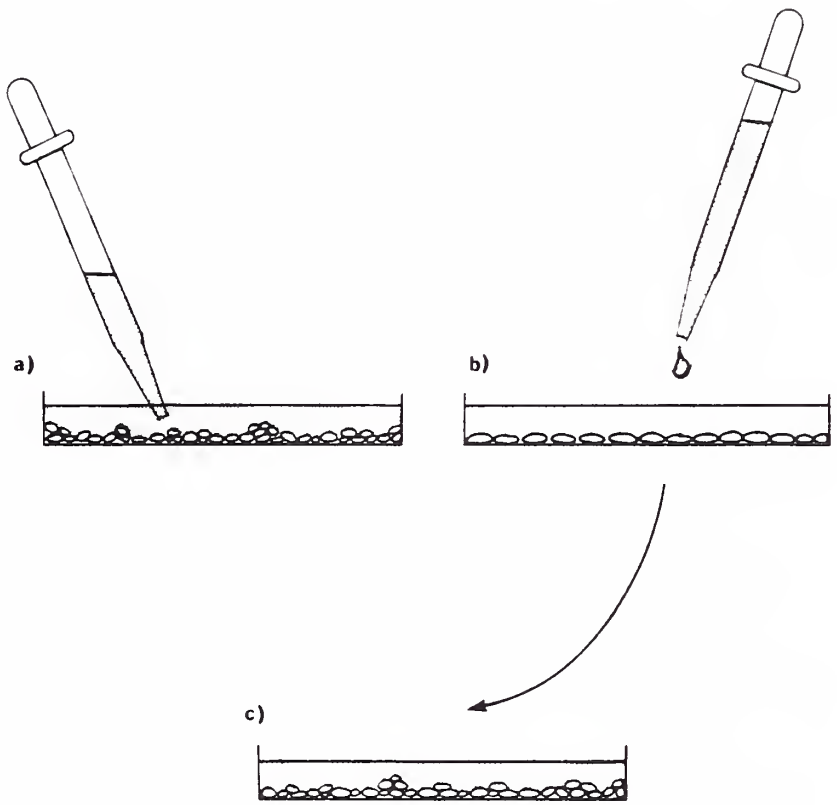


Figure II.iii. Original observations for the existence of transforming growth factors. (a) Supernatants from neoplastic fibroblasts were aspirated and (b) added to cultures of normal fibroblasts in vitro. The normal fibroblasts, in turn, were observed to undergo transformation (c) and assume the neoplastic phenotype of the originally transformed cultures[4].

As mentioned above, cytokines are made by diverse populations of cells. Perhaps few cells in the body are as active in this regard as the osteoblast, the cell that forms bone. Evidence of cytokine production by bone cells is well-established, but the roles cytokines play in bone metabolism and bone resorption is unknown[37,87]. Recalling that TGF-ß is stored in bone, is the body's largest source of the cytokine, and bone resorptive processes can activate TGF-ß, one may question if there is a relationship between TGF-ß and the

release of cytokines from osteoblasts. This thesis investigates the ability of TGF-ß and other cytokines to modulate the release of the known osteoblast-derived cytokines. To focus the implications of the data presented in this work, short discussions of the cells, cytokines and genes relevent to the interpretation of the data will be presented as follows.

A. TRANSFORMING GROWTH FACTOR- BETA TGF-ß can transform 'primed' cells; TGF-ß is produced ubiquitously and stored in bone; There are multiple types of TGF-ßs but TGF-$ß_1$ is studied in the present work; TGF-ß is normally latent but is activated by low/ high pH and specific enzymes.

It has long been conjectured that normal cells influence their own growth by releasing particular autocrine hormones which, in turn, stimulate the cell itself, or in a paracrine fashion, stimulate neighboring cells. Cancer cells may support their growth, in part, by over producing or responding inappropriately to normal autocrine hormones. Such a hypothesis gained credence when it was observed that supernatants from certain retrovirally transformed cells could transform previously normal fibroblasts growing in soft agar (Figure II.iii above)[4,62,166]. The supernatants, it was thought, contained molecules produced by the transformed cells, which stimulated the transformed cells' growth and induced transformation in healthy cells.

The molecules were referred to as Transforming Growth Factors (TGFs), initiating much effort towards the isolation and characterization of the still-theoretical material. In 1983, several workers eventually isolated two disparate proteins which had conferred upon supernatants an ability to transform healthy

cells[133b]. One of the proteins, TGF-Alpha (TGF-α) was found to be related to Epidermal Growth Factor (EGF) and had equal affinity for the EGF-receptor as did EGF itself. The second protein, TGF-Beta (TGF-ß) was unrelated to EGF and was a ligand for its own

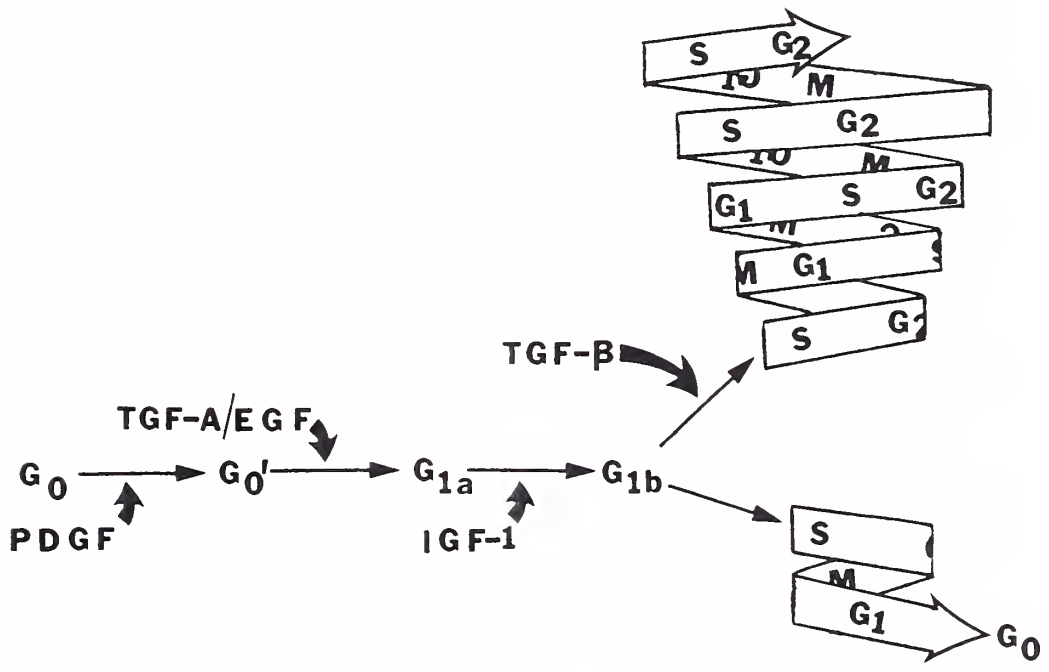


Figure II.iv. Growth factor induction of active growth cycles. Quiescent cells can be activated into active growth after certain 'priming' events, initiated by Platelet-derived Growth Factor (PDGF). Either Epidermal Growth Factor (EGF) or Transforming Growth Factor-alpha (TGF-α) can then induce a cell to enter the first part of G_1. Insulin-like Growth Factor-1 (IGF-1) continues the induction of a normal cycle of growth. Cells exposed to TGF-ß, however, may enter cell cycles which are not soon terminated, but continue in the unchecked manner typical of neoplastic growth.

receptor complex[91,167,183]. As illustrated in figure II.iv, it was found that cells could be transformed by the TGFs only after promotion by factors normally present in serum, primarily Platelet-Derived Growth Factor (PDGF), and Insulin-like Growth Factor-1 (IGF-1). TGF-α could substitute for EGF because the molecules

were ligands for the same receptor. Once a cell had been thus promoted, TGF-ß alone could induce transformation[91]. Workers have since found that TGF-α is produced only by certain neoplastic cells *in vitro*. TGF-ß, however, is ubiquitous, produced by many normal cells, especially developing tissues and tissues undergoing repair. TGF-ß is present in high concentrations in platelets (1500 molecules per cell)[5,195] and in bone (>30 mg/kg)[35,167], suggesting that the molecule is important to soft tissue wound healing and mineralized tissue remodeling. It is now appreciated that TGF-ß is an important molecule in normal growth processes and that an understanding of TGF-ß biology *in vivo* will have many implications for basic science and clinical medicine. A great deal of information concerning TGF-ß has been accumulated, a description of which is beyond the scope of this work. A summary of the major elements of TGF-ß biology relevent to the data presented in this thesis follows.

TGF-ß is a homodimeric protein, linked by sulfide bonds, with a molecular weight of 23-25,000 Daltons. By 1985, it was realized that several TGFs-ß existed, differing in messenger RNA and amino acid length and source of isolation. The original TGF-ß, and the one studied in this thesis, was referred to as TGF-ß1, isolated from human platelets[195], where it is stored in alpha-type granules[167]. TGF-ß2 was isolated from porcine platelets and bovine bone. TGFs-ß3-5 have not yet been isolated, but tentative cDNAs for these putative proteins have been identified in öocyte gene libraries[73,183]. Depending on the TGF-ß subspecies, TGF-ß mRNAs range from 1.5-6.5 kilobases, with a processed message of approximately 1200

kilobases in length. The mRNAs are translated into 380-420 amino acid TGF-ß precursors which is cleaved on both the amino end and the carboxyl end to yield a 180 a.a. product. All TGF-ßs are generally equivalent in activity, despite their structural differences and, therefore, this discussion will refer to them all as TGF-ß, unless otherwise stated. TGF-ß is active in many tissues, as will be described shortly, but only after it has been released from a protein complex known as TGF binding protein (TBP). As shown below in figure II.v, cleavage of the last 112 amino acids from the carboxyl end of the original protein produces a TGF-ß homodimer. Cleavage of the first 29 amino acids from the remnant precursor produces a sulfide-linked dimer which in turn binds to the TGF-ß. The two dimers are themselves covalently linked to a larger, 125-160 kDa 'modulator' protein[167].

The roles of these proteins are not known, but Kanzaki et al. found that the binding protein was not necessary for the latency of TGF-$ß_1$. Kanzaki suggests that the protein functions as a mediator of targeting, supported by the observation that both the binding protein, and the precursor protein both have the so-called "RGD" sequence- a fibronectin/vitreonectin cellular recognition site established by an Arginine-Glycine-Aspartic Acid triplet[101].

TGF-ß is released in its active form by low or high pH (<4.0 or >8.5) or by *in vitro* chaiotropic agents[124,128]. TGF-ß release *in vivo* is most likely to be achieved, therefore, in microenvironments capable of generating such pH gradients. One site may be that of healing wounds where platelet-derived TGF-$ß_1$ could be activated by

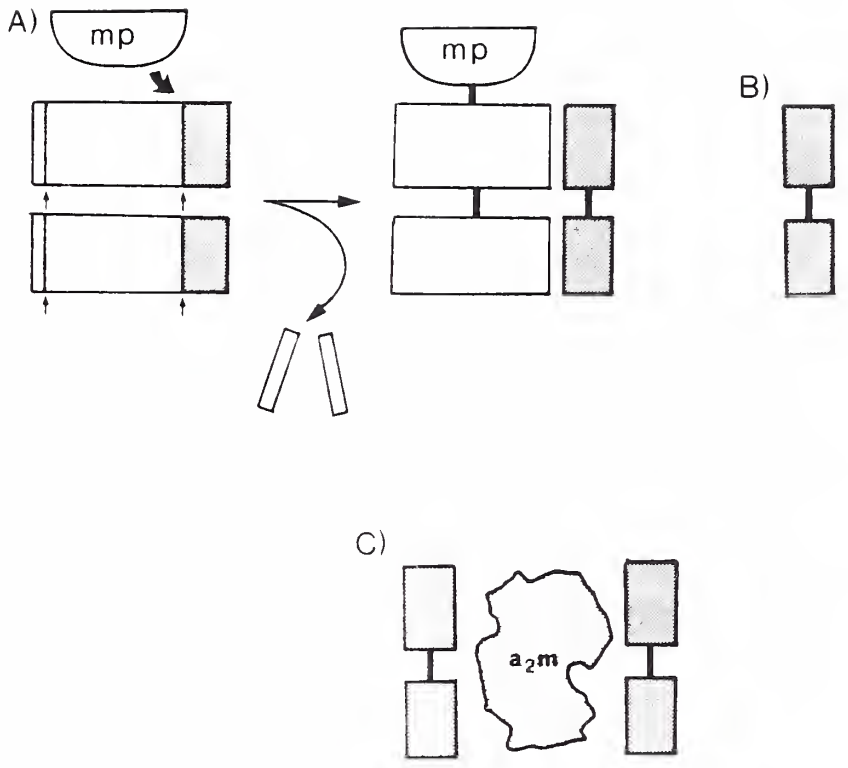


Figure II.v. Cleavage forms of TGF-ß. (A.) Two precursor molecules are cleaved at sites indicated by arrows producing mature TGF-ß and a large dimer to which the dimer TGF-ß is bound. A 125-160 kDa 'modulator protein' (mp) is, in turn, bound to the two dimers, but a true function for the modulator protein is still unknown. (B.) Free, mature TGF-ß as a 75 kDa homodimer. (C.) Two TGF-ß molecules bound covalently by alpha$_2$-microglobulin (a_2m), a proposed 'scavenger' protein for TGF-ß and other potent growth factors.

macrophage-derived sialidases, endoglycosidases, and other protonogenic proteases which can establish extremely low, local pH gradients (<3.7). A particularly unique site is that of resorption lacunae in bone, where resident osteoclasts resorb bone by utilizing basolateral proton pumps which can lower lacunar pH to approximately 3.0. Since latent TGF-ß$_1$ is stored in bone, resorption can release many molecules of TGF-ß$_1$ directly into a resorption lacuna while the low pH of the lacuna functions to activate TGF-ß itself[152]. These mechanisms of TGF-ß activation could insure that a responsive cell will not be affected by TGF-ß unless the molecule is activated, as in conditions of inflammation, wound

healing, or bone resorption. This strategem insures that the potency of TGF-ß will be made manifest only where and when such a molecule is neccessary.

Confirming the importance of *activated* TGF-ß to its autocrine and paracrine function are observations concerning a human lung carcinoma cell line, A549. A549 cells are some of the most active producers of latent forms of TGF-ß and express many TGF-ß receptors. Active TGF-ß, however, when added to A549 cells *in vitro*, greatly *inhibits* cell growth, suggesting that A549 cells have lost some ability to activate the TGF-ß that the cells themselves release[199]. A cell, apparently, like the A549 cell, which releases TGF-ß and expresses receptors for the molecule, must also have activating mechanisms to utilize any of the autocrine capablities which TGF-ß may confer.

TGF-ß may be important in controlling cell growth by regulating the activity of the retinoblastoma gene product, RB. The retinoblastoma gene product may function as a growth suppressor because loss of function of the RB gene can lead to oncogenesis. Reintroduction of the RB product into proliferating cells which do not produce such product slows growth. The activity of the RB product is thought to be regulated post-translationally via protein phosphorylation states: Underphosphorylated RB is associated with growth-arrest and G_1 whereas phosphorylated RB is found in S phase and G_2/M. TGF-ß was found to inhibit RB phosphorylation. SV40 virus large T antigen binds the RB product and this large T antigen specifically inhibits the growth suppressive activity of TGF-ß[120].

Activated, processed TGF-ß has been found to bind avidly to alpha$_2$-microglobulin, the 240 kDa serum protein. Alpha$_2$-microglobulin, or other TGF-ß binding serum proteins, may be one of the physiologic 'scavenging' mechanisms whereby the availability of activated TGF-ß is regulated (Figure II.v)[198]. TGF-ß acts as a ligand for two glycoprotein membrane-bound receptors and one betaglycan glycosaminoglycan receptor. The glycoprotein receptors, TGFR-ß I and II, are thought to mediate the effects of TGF-ß whereas the betaglycan may function in pericellular TGF-ß presentation[133b].

TGF-ß is remarkedy pluripotent and its actions on cells are greatly dependent on cell type and differentiation state, TGF-ß concentration, and the presence of other growth factors. There has been much work on how TGF-ß affects the physiology of hepatocytes[185], myocytes and myocardial infarction[159,189a/b], steroid synthesis[89], bone cells[3,33], alveolar cells[134], angiogenesis[41], and development[163]. Findings from this work have great implications for both TGF-ß research as well as clinical applications of TGF-ß, findings which can not be summarized in this forum. What follows is, therefore, a description of TGF-ß effects in systems which are especially pertinent to this thesis.

TGF-ß and the Immune System. Suppression of B- and T-cells; Inhibition of IgG/IgM production; Antagonism of IL-1 and other growth factors; Chemotactin and stimulant for macrophages.

TGF-ß decreases B- and T-cell growth and clonal expansion, decreases the generation of lymphokine-activated killer cells (LAKs), and decreases cytotoxic T-cell (CTL) growth[58,140]. Recently, TGF-ß has been found to inhibit thymocyte growth, especially

thymocyte growth induced and supported by Interleukin-7. In this regard, TGF-ß is immunosuppressive to the femto-molar range, making TGF-ß 10,000 to 100,000 times as suppressive as cyclosporin- A[9,114]. TGF-ß is, however, remarkably pleiotropic. While some reports describe TGF-ß as a potent inhibitor of acute arthritic events[19], others describe TGF-ß as a potent *inducer* of synovial inflammation[4,197b].

TGF-ß works to decrease antibody production of the IgG and IgM classes yet increases production of the IgA classes[44,106]. The hypothesis that TGF-ß might function to increase the populations of IgA-bearing cells along respiratory and gastrointestinal epithelium is now being investigated[197a]. TGF-ß anatagonizes many of the affects of Interleukin 1 (IL-1), IL-2, and IL-3, Tumor Necrosis Factor-alpha (TNF-α), and Interferon alpha (IFN-α) on cytotoxic T-cell function and the development of Lymphocyte-Activated Killer Cells (LAKs)[58]. TGF-ß downregulates IL-1-induced IL-6 production by human monocytes[144].

Whereas TGF-ß is generally inhibitory for lymphocyte function, it tends to be stimulatory for macrophage and monocyte function. TGF-ß is a chemoattractant for monocytes and macrophages, and increases macrophage membrane receptors for TNF-α, PDGF-AA, Fibroblast Growth Factor (FGF), IL-1, TGF-α, and TGF-ß itself[130,197a,197b]. TGF-ß is also extremely potent in suppressing the superoxide (O^-) respiratory burst of macrophages and polymorphonuclear lymphocytes (PMNs), leaving the general impression that TGF-ß suppresses the destructive effects of

macrophages and acute cell activities while facilitating macrophage-derived factor function and the processes of tissue repair[191].

TGF-ß and the Cell Matrix. Upregulation and support of matrix proteins via three mechanisms.

Perhaps few other fields in TGF-ß research have proven as intriguing as that of cell matrix biology and how the molecule regulates matrix formation and the activity of mesenchymal cells. TGF-ß, the "gadabout growth factor"[9], was eventually assigned a role that subserved many of its ostensibly disparate activities: the upregulation of matrix proteins[133c]. Three matrixogenic mechanisms have been thus far studied:

(1) TGF-ß increases matrix molecule production: TGF-ß induces the production of certain matrix proteins like collagen types I, III, IV and V, fibronectin, thrombospondin, osteopontin, osteonectin, SPARC, and chondroitin and dermatan sulfates[2,41,131,133a,148,196]. TGF-ß directly stimulates the promotors for α_2(I) collagen as well as fibronectin, an influence mediated by an NF-1 binding site[49]. Furthermore, TGF-ß directly increases fibronectin gene transcription. Most recently, Heine et al. have shown that TGF-ß co-localizes with collagen I & III, fibronectin and glycosaminoglycans during lung branching morphogenesis[80]. Such observations suggest that TGF-ß may be important in the regulation of matrix formation in development as well as in mature stages of life.

(2) TGF-ß increases receptors for matrix proteins: TGF-ß induces the expression of the matrix protein receptors classed as *Integrins*, the transmembrane links between the extracellular and intracellular worlds[79,93,133a].

(3) TGF-ß inhibits matrix proteases: TGF-ß can both inhibit the expression of enzymes which can degrade the matrix proteins, as well as induce the production of enzymes which inhibit the matrix-degrading enzymes[57,164]. Inhibition of the matrix protease *transin*, a member of the metalloproteinase family, has recently been found to involve a specific sequence in the transin gene promotor, the TGF-ß inhibitory element (TIE)[108,134,135]. After TGF-ß stimulation of the cell, the TIE sequence is bound by a trans-activating factor containing the c-fos protein, *fos*, thus implicating the c-fos oncogene as a mediator of TGF-ß function in both cis- and trans-inhibition of transin gene expression and thus of matrix regulation[108].

TGF-ß and Bone. TGF-ß is stored in bone; TGF-ß is activated by osteoclasts; TGF-ß may promote bone formation via several mechanisms; TGF-ß may upregulate osteoblast cytoskeletal proteins.

TGF-ß is stored primarily in two locations in the body: in the alpha granules of platelets and in the matrix of bone[33,35]. There are approximately 1500 TGF-ß molecules per platelet making platelets the richest source of the hormone. TGF-ß in bone, however, is dispersed equally throughout the body skeleton making bone the *largest* source of TGF-ß in the body (0.3 mg/kg)[179]. While platelet-derived TGF-ß has been postulated to have roles in tissue repair, bone-derived TGF-ß is thought to have roles in the regulation of bone remodeling, the continuous life-long process of resorbing and replacing bone with new mineralized tissue. Embryologically, TGF-ß is actively expressed in the axial skeleton and, in older embryos, in the cytoplasm of osteoblasts involved in ossification processes[30].

Osteoblasts and certain osteosarcoma cells, have very high messenger RNA levels for TGF-ß, and secrete high concentrations of

the hormone[28,35]. Recalling that TGF-ß is generally inhibitory for the growth of normal cells, it is surprising to observe that TGF-ß is mitogenic for osteoblasts and inhibitory for osteoclasts, suggesting a pro-bone forming effect of the hormone[33,34,47,157]. These effects appear independent of prostaglandin production[130]. Mackie et al. have described a TGF-ß-induced increase in bone formation while Chenu et al. have suggested that TGF-ß acts as a chemotactic agent for osteoblast migration into a remodeling locus[42,130]. Although TGF-ß upregulates extracellular matrix formation[158], Lomri et al. have shown that TGF-ß can increase intracellular cytoskeletal proteins as well[127]. TGF-ß upregulates collagens I & III production[3] but inhibits production of collagen type II[88]. Particularly relevent to *in vivo* physiology of the hormone is the observation that osteoclasts activate TGF-ß from its latent form when the hormone is released from the bone matrix. The activated TGF-ß may then, in high local concentrations, upregulate the restoration of bone removed by the osteoclasts which activated the hormone.

The Bone Morphogenetic Proteins (BMPs) are important hormones in osteogenesis and development and belong to the TGF-ß superfamily. BMP-2B is a known inducer of endochondral bone and limb bud cartilage formation. TGF-ß inhibits some of the effects of BMP-2B in chondrogenesis and may modulate the effects of the BMP family *in vivo*[41b].

There is little data in the literature on the regulation of osteoblast cytokine secretion by TGF-ß. Such a relationship between TGF-ß and cytokine secretion could have profound effects on not only bone remodeling itself, but on the development of the neighboring colonies

of bone marrow cells which could be stimulated by bone cell-derived cytokines. As this thesis presents the first data supporting a role for TGF-ß activation of osteoblast-cytokine secretion, it would be advantageous to discuss briefly the major properties known about the bone cell-derived cytokines studied in this work, as follows concerning Interleukin-6 (IL-6), Interleukin-l (IL-1), GM-CSF and M-CSF.

B. INTERLEUKIN-1 Coley's toxins and LPS; Discovery of IL-1; IL-1A and IL-1ß are processed differently; IL-1ß is studied in this thesis.

In the late 19th century, William B. Coley, a New York City surgeon, observed that several patients of his with terminal cancer of comparable stage and who contracted low-grade Gram negative bacterial infection had less morbidity and metastatic disease than similar patients free of infection. Reviewing experiments by Germany's Pfeiffer on 'Gram negative' cell walls, Coley conjectured that there was some component of the causative bacteria which enabled the patient's body to fight neoplastic disease. Eventually, Coley isolated fractions of Gram negative bacterial cell walls, eventually called 'Coley's toxins', which he used with considerable success to treat several patients with bone and soft tissue sarcomas[46,202]. By World War I, however, use of Coley's toxins fell into disfavor partly because the use of such toxins was accompanied by undesirable side effects including extreme nausea, confusion and a profound fever. It would not be known for fourty years that the effects of Coley's endotoxins were due to a molecule known as lipopolysaccharide (LPS). It would be sixty years

before it was known that Coley's toxins, or the LPS moiety of endotoxin, had been inducing the body's production of a hormone called Interleukin-1, the isolation of which has revolutionized immunological science. In 1942, Menkin et al. characterized the physiologic responses to the administration of endotoxins[136]. After an intravenous dose of 25 mg/kg of endotoxin, animals

Figure II.vi. Dr. William B. Coley, Surgeon (1926), 1862-1936. In the late 19th and early 20th centuries, Coley became the first investigator to use biologic molecules in attempts to potentiate the immune reponse to cancer. His preperations of endotoxins were later determined to contain lipopolysaccharide (LPS), one of the most potent inducers known of IL-1 synthesis and release, the probable mediator of endotoxin immunopotentiation. Photograph courtesy of the New York Academy of Medicine Library.

expressed a profound, acute fever often as high as 39.5 degrees Celsius. Menkin termed the causative molecule *pyrexin* and believed that fever was caused by endotoxin-induced pyrexin and not by endotoxin itself[136]. In 1949, Beeson eventually isolated a protein candidate in granulocytes for pyrexin, a protein he termed 'granulocyte pyrogen' or GP[12]. There was much debate on whether pyrexin and GP were actually pyrogenic or whether endotoxin itself caused fever. In 1970, however, Bodel was the first to show that a 14-15,000 Dalton protein, isolated from endotoxin-stimulated granulocytes, could cause fever, a molecule termed the Leukocyte Endogenous Mediator, or LEM[18]. By 1971, several laboratories, including Gery et al.[67], Oppenheim et al.[151], and Calderon et al.[27] began describing a soluble factor produced by stimulated macrophages which increased thymocyte DNA synthesis. The activating entity was termed Lymphocyte Activating Factor, or LAF, produced by macrophages exposed to bacterial endotoxins (LPS) or lectins (PHA)[17]. Using similarly-activated macrophages, laboratories began describing a wide variety of activities induced by a soluble factor(s). Besides LEM and LAF above, other workers described an inducer of acute phase protein synthesis called Hepatocyte Activating Factor, or HAF, an inducer of muscle wasting termed *catabolin*, an inducer of bone resorption termed Osteoclast Activating Factor, a stimulator of primitive hemopoetic cells called Hemopoeitin-1, and an inducer of serum proteins known as Amyloid-A Stimulating Factor[50,51,55,139,156]. However, with the initial purification of LAF by Blyden and Handschumaker in 1974[18], it was found that

all of the above activites could be attributed to one, single molecule[51]. With the observation that the molecule was macrophage-derived and could stimulate other white cells, including T-cells, the molecule was eventually known as Interleukin-1 (IL-1). Additional characterizations of IL-1 determined that at least two related proteins exist with IL-1 activity, differing in isoelectric point (pI) and various tertiary configurations- IL-1 α and IL-1 ß with pIs of 7.0 and 5.0, respectively[111a,207]. Despite differences in homology and trans-species conservation, IL-1 α and IL-1- ß are ligands for the same receptor and have, in general, identical modes of action. The IL-1 α gene is located on chromosome 2 in the mouse and is remarkable for the absence of any upstream enhancer sequences like TATA, or CAAT[63]. The 10206 base pair gene is transcribed into a 2.3 kilobase (kB) message, and translated into a 268-271 amino acid precursor of 38 kDa[111b,141]. IL-1 ß is also located on murine chromosome 2, but has several TATA and CAAT enhancer sequences just upstream of the Start point. The 7008 base pair gene is transcribed into a shorter 1.4-1.8 kB mRNA, and translated into a similar 33-38 kDa precursor of 266-269 amino acids[63]. Both proteins must be cleaved in an as-of-yet undetermined process to yield the mature 17-18 kDa polypetides[16,50]. Neither protein has an N-terminal hydrophobic secretory signal suggesting that IL-1s are released by a unique mechanism, possibly via cleavage by species-specific membrane proteins. Kobayashi et al. have presented the model whereby precursor IL-1 α is phosphorylated and then associated with a calcium-dependent acidic phospholipid

on the membrane outer surface which releases mature IL-1 α[111b]. In contrast, IL-1 ß, which is released earlier and faster than IL-1 α, is thought to be bound without phosphorylation to a trans-membrane processing protein, which releases mature IL-1-ß. Recently, Bakouche et al. have presented data suggesting that mature IL-1 is released via lysosomal vesicles[8a,8b].

In 1982, Auron et al. eventually cloned a cDNA from activated human macrophages, and both species of human IL-1 cDNAs were cloned, sequenced and expressed by 1985[7].

Cell Sources of IL-1 In Vivo. Produced mostly by activated macrophages, but also by T-cell classes, keratinocytes and osteoclasts.

By far, the largest source of Interleukin-1 is derived from the phagocytic leukocytes: peritoneal and tissue macrophages and blood monocytes, from which IL-1 was first purified in low picomolar concentrations[18]. Kupper et al. have shown that non-stimulated keratinocytes are active secretors of IL-1 α, a finding that continues to have great implications in immunodermatology[116a,116b]. Muegge described IL-1 production by killer T-cells in the late 1980's and then found IL-1 in γ/δ T-cells as well. Although mice make more IL-1 α than IL-1 ß, IL-1 ß is believed to be the more active isoform in macrophage biology and is the IL-1 species studied in this thesis.

Activities of IL-1 Perhaps few other molecules in biological research have been implicated in as many diverse systems of physiology as Interleukin-1. Summarized here will be only those activities of the hormone which are most relevent to the studies in this thesis, although the interested reader may be referred to several excellent reviews [50,55].

i. Stimulation of T- and B-cells. Induces IL-2 production; IL-1 may activate B-cells.

Larrson et al. first presented data in 1980 demonstrating that T-cell production of T-cell derived growth factor (TCGF), now known as Interleukin-2 (IL-2), was supported by a 20 kDa molecule derived from macrophages, later identified as Interleukin-1[123]. The observations were some of the first to characterize the crucial T-cell/macrophage axis, perhaps the most important arm of the early activation of the immune system. IL-1 secretion and consequent IL-2 production can greatly increase T-cell clonal expansion and proliferation. Furthermore, IL-1 can synergize with IL-2 to enhance IL-2 responsiveness[132].

B-cells can be stimulated at precursor stages and after exposure to antigen by T-cell derived cytokines like B-cell Growth Factor (BCGF) and Interferon-gamma (IFN-γ) and possibly IL-2[125]. Several laboratories have shown that B-cells in quiescence can be brought to G_1, driven into mitosis[181], and eventual immunoglobulin secretion by Interleukin-1 and the T-cell-derived cytokines[125].

ii. Stimulation of acute phase cells, acute phase proteins and features of inflammation.

IL-1 is a potent chemotactin and degranulator for neutrophils, eosinophils and other macrophages, implicating the hormone as an amplifier and director of early stages of inflammatory events. IL-1, formerly studied as Hepatocyte Stimulatory Factor (HSF), can induce a large spectrum of acute phase proteins from liver cells *in vitro* (see below) [156,161]. IL-1 also induces arachidonic acid metabolism, with the consequent

production of leukotrienes and prostaglandins[50]. Glucocorticoid therapy for inflammation may down regulate prostaglandin synthesis in at least two ways- inhibition of cylooxygenases, and by inhibiting transcription of the IL-1 gene via a Glucocorticoid Binding Element (GBE) [50].

iii. Induction of M-CSF, GM-CSF, and IL-6 synthesis.

Kaushanksy et al.[104] and Zucali et al.[214] have presented data on dermal fibroblasts showing that IL-1 can induce the production of Granulocyte-Monocyte CSF (GM-CSF) and Granulocyte-CSF (G-CSF). IL-1 did not, however, induce the production of Macrophage-CSF (M-CSF) in the Kaushansky group's studies. Stanley et al.[184], and Bartelmez et al.[10], studied Hemopoetin-1 (H-1), a growth factor isolated in 1985 from human bladder carcinoma cells and later determined to be IL-1 itself. Data indicated that although H-1 alone had no effect on colony stimulation *in vitro*, H-1 synergized with other colony stimulating factors, like M-CSF (CSF-1) and Interleukin-3 (IL-3), to induce hematopoesis at earlier stages than either M-CSF or IL-3 alone.

Several workers have also described the induction of Interleukin-6 (IL-6) production in fibroblasts by IL-1[177]. Released IL-6, as reviewed above, may stimulate both hematopoesis as well as the synthesis of C-reactive and acute phase proteins[65,161]. IL-6 may mediate some IL-1 effects. Such evidence implicates IL-1 not only in early stages (0-12 hours) of immunoreactivity against foreign invasion, but the later stages by inducing potentially immunoreactive cell precursors towards maturity.

iv. Interactions with complement. C5a induces macrophage IL-1 synthesis; IL-1 can activate C3 and Factor B.

Macrophages do not constitutively secrete IL-1, but will produce large amounts when exposed to complement products such as C5a[142b]. In turn, IL-1 stimulates hepatocyte production of Complement factors 3 and B[156,161]. As more species of animal systems were described for IL-1 activities, it became clear that the molecule had an extraordinary degree of conserved homology, even over presumably large evolutionary divisions. Human IL-1, for example, shares 95 % homology with primate IL-1 (chimpanzee) and 90% homology with the horseshoe crab (Figure II.xii). Starfish Factor B, derived from coelemic phagocytes, is now known as Interleukin-1[11]. It was the presence of active IL-1 in echinoderms that suggested IL-1 was extremely old, since horseshoe crabs are believed to have changed little since the late Jurassic period (160-140 million years ago). There is evidence that IL-1 may induce the alternate pathway of the complement cascade and is induced by complement products like C5a (see figure II.xii)[142]. Such observations suggest that IL-1 may have operated in simple immune systems, like those of the echinoderms- devoid of antibody production but dependent upon coelemic phagocytes and phagocyte-derived IL-1 to induce inflammatory changes.

v. Pyrogenesis and prostaglandin E_2 production . IL-1 ß induces fever by increasing hypothalamic PGE_2 mediated perhaps by CRF.

IL-1 was originally studied as the LEM, or Leukocyte Endogenous Mediator, or simply, the Endogenous Pyrogen (EP), as the inducer of acute rises of body core

temperature to levels of fever. Although there exist other cytokine pyrogens, like IL-6, Tumor Necrosis Factor and Interferon α, IL-1 is at least 50 times as potent, acting as a pyrogen in doses of 2 ng/kg[55]. IL-1, and the other pyrogenic cytokines, most likely induce fever by inducing the synthesis of prostaglandin

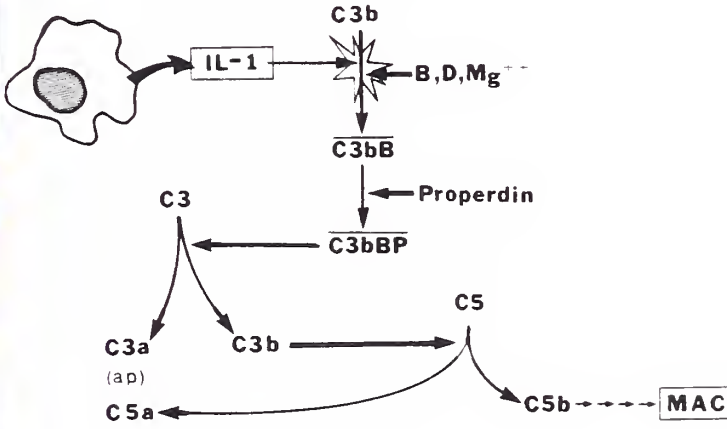


Figure II.vii. Left, the horseshoe crab, *Limulus polyphemus*, one of the oldest animal species on earth. The crab's cœlom is patrolled by host phagocytes which are active producers of an Interleukin-1 species that is 95% homologous to human IL-1, and which may be involved in a complement-dependent type of primitive immune system. Right, IL-1 activation of the alternate complement cascade, effective in generating Membrane Attack Complexes (MACs) in the absence of antibody. IL-1 induces C3 and B synthesis in hepatocytes thus fueling the cascade. Production of C3a and C5a may contribute to IL-1 mediated inflammation and hypersentitivity reactions. (ap) = anaphylatoxins.

E_2 (PGE_2). Endotoxin, or LPS exposure, leads to increased levels of PGE_2 in the brain, spinal fluid and in the hypothalamus[24,174]. As shown below in figure II.xiii., released PGE_2 in the brain may trigger heat controlling centers in the pre-optic nucleus of the anterior hypothalamus which, in turn, transmits thermogenic information to the periphery causing the

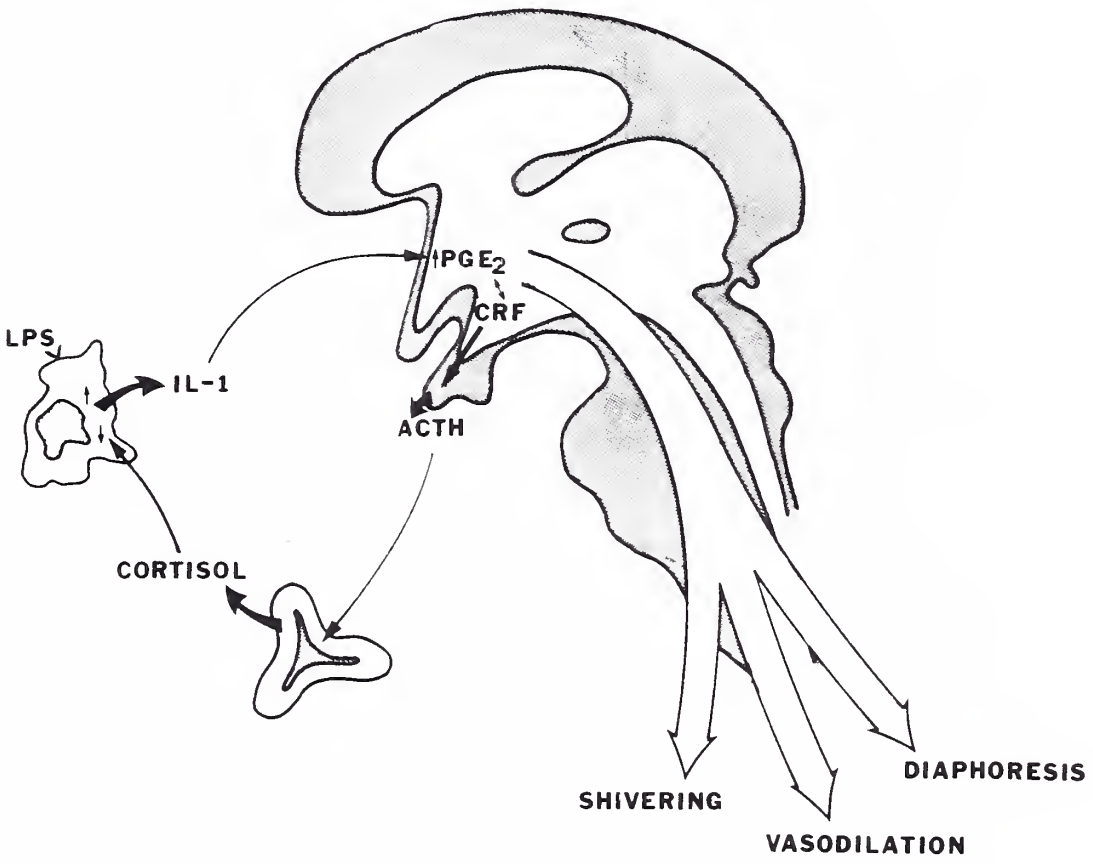


Figure II. viii. IL-1 pyrogenesis and the IL-1 pituitary-adrenal axis. Endotoxin-induced release of IL-1 leads to increased hypothalamic PGE_2, resetting hypothalamic autonomic outflow to raise body temperature. The PGE_2 also functions to release CRF from the hypothalamus which induces the release of ACTH from the anterior pituitary. Cortisol, produced in the adrenal cortex after ACTH stimulation, functions to directly suppress macrophage production of IL-1, thus acting as the negative feedback arm of the axis.

body temperature to rise. IL-1 may have direct effects on the hypothalamus as well. Although it is believe IL-1 resets hypothalamic temperature regulation via prostaglandin synthesis, Busbridge et al. have presented evidence suggesting at least some of IL-1's pyrogenicity may be mediated by Corticotropin Releasing Factor (CRF)[24]. It was first noted in 1986 by Besedovsky et al.[14] and Uehara et al.[193] that IL-1 participated in a hypothalamic pituitary-adrenal axis by inducing hypothalamic release of CRF and consequent adrenocorticotropic hormone (ACTH) from the anterior pituitary. Glucocorticoids released from stimulated adrenal glands then inhibited in a feedback mechanism ACTH release, CRF release and IL-1 release from leukocytes. Clinicians had used endotoxin itself as a common clinical test for anterior pituitary-adrenal axis function[24]. However, Busbridge et al. found that IL-1 ß-induced fever could be blocked by both anti-CRF antibody and CRF receptor antagonists. IL-1 α-induced fever could not be similarly blocked. IL-1 ß mRNA is expressed in the hypothalamus but IL-1 α mRNA is not[24]. Further studies, especially *in vivo* studies of brain tissue, will be needed to clarify these observations. IL-1-induced fever is probably an important mechanism of heightening the immune system in the bodys general response towards foreign invasion, although the exact function of hyperthermia in pathologic states is not yot clear.

vi. Interleukin-1 and Bone. Osteoblasts express receptors for IL-1 while osteoclasts do not. IL-1-induced effects on osteoclasts may be regulated via osteoblast-derived factors.

As early as the 1880s, clinical descriptions of multiple myeloma have included reports of abnormal bone

resorption and associated sequellae: hypercalcemia, profound osteoporosis, bone fractures and bone pain. Bone pathology is a hallmark of multiple myeloma and is one of the most debilitating aspects of the disease. Myeloma cells can elicit hormones as Osteoclast Activating Factor (OAFs) which activate osteoclasts to resorb bone unchecked by normal controls[94]. OAFs include Tumor Necrosis Factor ß and IL-1ß. Some workers suggest IL-1 is less important *in vivo* as an OAF[83] but IL-1 *in vitro* is 10 times more potent than TNF for decreasing proteoglycan synthesis in cartilage[171].

Osteoblasts express receptors for IL-1 while osteoclasts do not[167c]. IL-1, however, has been found to increase osteoclast development, osteoclast precursor number[69,187], and osteoclast collagenase production[78]. IL-1 stimulates osteoblast production of Macrophage-Colony Stimulating Factor which may then act, in a paracrine fashion, to induce the development of osteoclasts[77b,128b,206]. Paracrine activity by osteoblasts may be important in mediating the other effects of IL-1 on osteoclast development outlined above.

IL-1 may induce bone resorption by increasing local prostaglandin E (PGE) production[110,172] but other workers suggest a direct resorptive effect[147]. PGE as a single agent is a potent inducer of matrix resorption in vivo[110]. IL-1 induced bone resorption is decreased by TGF-ß[157]. Such findings suggest that IL-1 may be an important mediator in the regulation of bone remodeling.

The IL-1 receptor and IL-1 signal transduction

The determination and analysis of the IL-1 receptor was greatly facilitated by the cloning and expression of purified IL-1 from isolated cDNAs in 1986[50,56,207]. Early experiments reported receptor protein candidates with several molecular weights, but after the isolation of receptor cDNAs, it was determined that the IL-1 receptor was a single glycoprotein of 80 kDa[15]. The complete amino acid sequence has been determined including a membrane spanning domain of 21 amino acids[2,21]. Putative protein kinase C acceptor (Gly-x-Gly-Ser-x-Ser)[90,92] and cAMP binding sites have also been identified[29,77]. Despite this initial data, however, it is still unclear how the IL-1 receptor transduces ligand binding into intracellular signalling[2,66,138,150].

Many hormone receptors transduce extracellular stimulation by utilizing second messenger systems to activate specific intracellular kinases: cyclic nucleotide hydrolysis for activation of cGMP/cAMP-dependent kinases or phospholipid hydrolysis for calcium/diacyl glycerol-dependent kinases[14,92,153]. There is much debate concerning which second messenger system predominates in the IL-1-stimulated cell. Many transcription factors and co-factors are activated by IL-1 so the second messenger systems which mediate the IL-1 response may be complex.

Mizel et al. provide evidence of protein kinase A activation by cAMP hydrolysis in IL-1 stimulated T-cells[150]. In contrast, other workers report that IL-1 does *not* raise T-cell cAMP levels and has no effect on intracellular levels of diacylglycerol, calcium, or phosphoglycerides[66,138,150]. O'Neill et al. have found that a

stimulatory G protein activates a novel serine kinase in IL-1-exposed T-cells. Rosoff et al.[168] have presented data concerning Jurkatt T-cells that suggest IL-1 may transduce its signal not via classical PI hydrolysis, but rather through a novel hydrolysis of membrane-bound phosphotidylcholines (PCs). The Rosoff group showed that

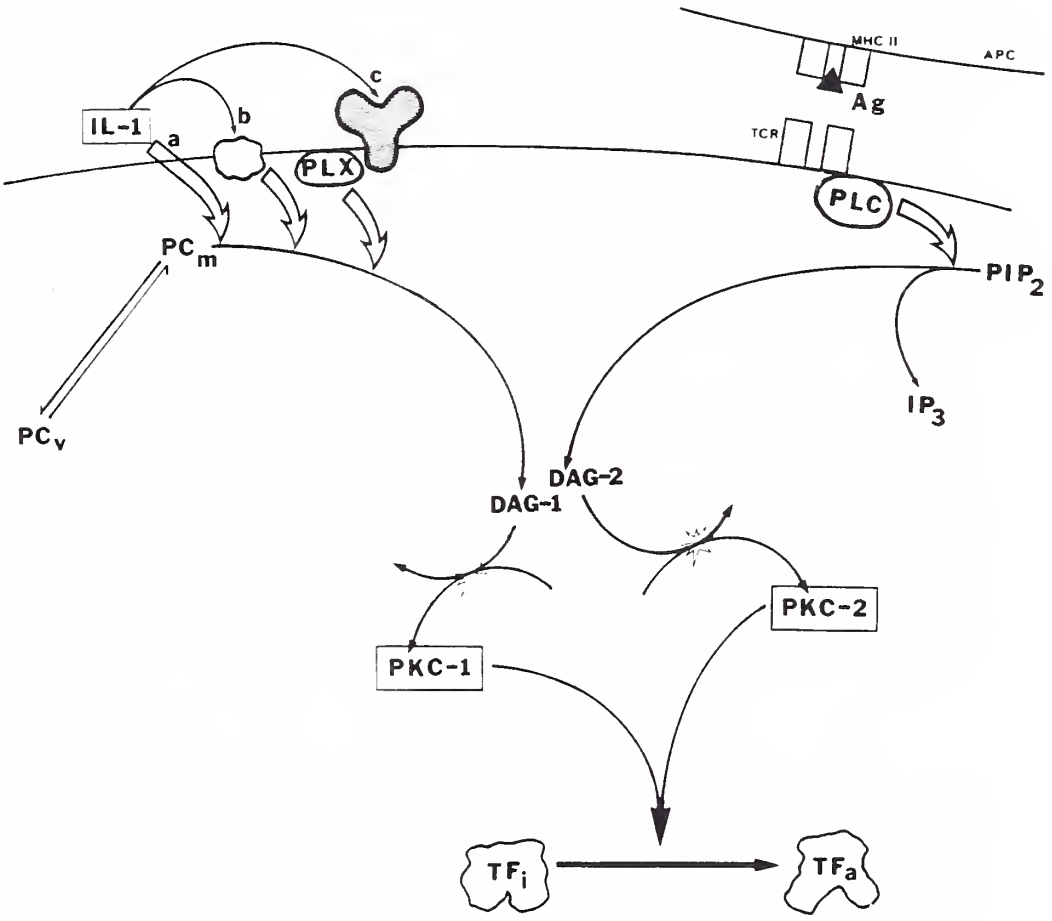


Figure II.ix. Theories of signal transduction by the IL-1 receptor in T-cells. As suggested by Rosoff et al.[168], T-cell co-stimulation by IL-1 and anitgen may be achieved through the production of differing species of diacylglycerols (DAGs) and the synergistic activation of trasncription factors (TFs). As illustrated above, antigen stimulation of the T-cell receptor induces classic PI turnover with the production of free intracellular calcium (not shown) and diacylglycerol species 2 (DAG-2). IL-1 induces hydrolysis of rapidly-shuttled phosphatidylcholine (PC) for the production of calcium (not shown) and diacylglycerol species 1 (DAG-1).

DAG-1 and -2 activate species-specific protein kinases C (PKC-1 and -2), respectively. PKC-1 and -2 synergize to activate transcription factors (TFs) or enhancers. IL-1 may have hydrolyzing capabilities and may hydrolyze PC directly (a). Alternatively, IL-1 may hydrolyze PC via a membrane-bound hydrolase (b) or through a classic IL-1R and an associated phospholipase (c). IP_3=inositol triphosphate, MHC=multiple histocompatability, APC=antigen presenting cell, PLC=phospholipas C, PLX=putative phospholipase, PIP_2= phosphotidylinositol diphosphate, PC_v=vacuolar PC, PC_m=membrane-bound PC, TF_i/TF_a=inactive/active TF.

PC hydrolysis after IL-1 stimulation produces diacylglycerol (DAGs) species with different fatty acid side chains than DAGs produced by PI hydrolysis after antigen stimulation. Furthermore, IL-1 induces PC hydrolysis on outer cell membranes and on many internal membranes as well, liberating DAG molecules throughout the Jurkatt cell cytoplasm. Jurkatt T-cells, however, as assayed by Rosoff et al.[168], have no IL-1 receptors, or at least less than 200 receptors per cell. Rosoff suggested that IL-1 may have dual effects on a cell. First, IL-1 may be a ligand for a low number, high-affinity IL-1 receptor. Secondly, IL-1 may have intrinsic PC-hydrolyzing ability and may directly initiate outer membrane-bound PC hydrolysis without a receptor intermediate.

Signal transduction by IL-1 is still, therefore, unclear, but an important model arises with particular relevance to this thesis as presented in figure II. ix. : IL-1 induces PC hydrolysis, via unknown pathways, to increase DAG production throughout the cell. Concomitantly, a secondary signal, antigen, lectin or OKT3 antibody against the T-cell receptor (TCR), initiates TCR signal transduction via classical PI hydrolysis with the production of other DAG species and free intracellular calcium. The two differing species of DAGs may act on different protein kinases C or A and

synergize for transcription factor or co-factor cativity. The utilization of two seperate signalling pathways which converge upon and synergistically activate common trans- or cis- gene activators may be an important mechanism of cellular activation with importance to the discussion of data presented in this work.

C. INTERLEUKIN 6 Discovery of the roles of IL-6 in B-cell maturation; IL-6 production; *In vivo* sources of the hormone.

The immune system relies heavily upon B-cells to produce antibody against specific antigens foreign to the host. The only antibody producing B-cells, however, are those B-cells which are fully differentiated and appropriately stimulated with antigen. These two conditions may be achieved by the presence of T-cells and antigen-presenting macrophages. By 1982, it was clear that certain soluble growth factors could substitute for the apparent function of T-cells and macrophages in the process of B-cell stimulation[176,213]. In 1986, three of the putative growth factors were isolated and cloned[109]. As illustrated in figure II. vi below, the first factor was Interleukin-4 (IL-4), originally called B-Cell Growth Factor-1 (BCGF-1) or B-Cell Stimulatory Factor 1 (BSF-1) because the molecule functioned to activate early B-cell progenitors exposed to antigen. The second factor was Interleukin-5 (IL-5), then known as BCGF-2, which functioned to induce growth in activated B-cells. The third factor functioned to induce the differentiation of growing, activated B-cells into antibody-producing B-cells. This third factor, originally called B-cell Differentiation Factor (BCDF) or B-cell

Stimulatory Factor 2 (BSF-2), is now known as Interleukin-6 (IL-6). Like Tumor Necrosis Factor (TNF) and Interleukin-1 (IL-1), IL-6

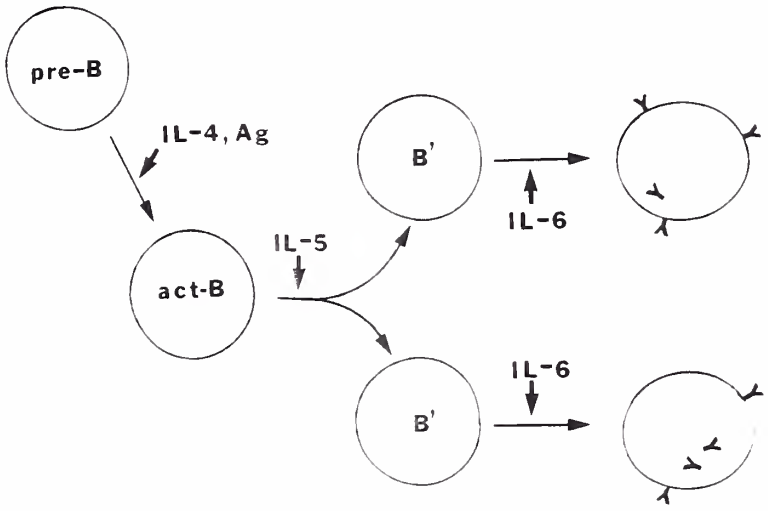


Figure II.x. The B-cell growth factors. Pre-B cells are activated (act-B) by IL-4 and express receptors for IL-5, which in turn, stimulates activated cells to assume a 'primed' state (B'). Only then can IL-6 induce such cells to synthesize and secrete antibody. IL-7 may funtion to upregulate pre-B cell growth and differentiation.

was also studied as the major inducer of acute phase protein synthesis, and in this regard IL-6 was also known as Hepatocyte Stimulatory Factor (HSF)[31,165]. Like many growth factors, IL-6 is a small protein of 184 amino acids. Mouse and human IL-6 DNA is 60% homolgous for the gene coding region, but 90% homologous over 300 base pairs at the extreme 5' end of the gene suggesting an important regulatory site. Additionally, the 5'-flanking sequences of each exon in the gene is similar to previously identified transcription enhancer elements like the serum reponse elements (SRE) of c-fos, the Early Growth Response genes, or the glucocorticoid receptor binding element (GRE)[109,188]. Although mouse and human IL-6 protein are only 42% homologous, the two species are 100% homologous in the position of the four cysteine residues in the molecule. Such cysteine homology is also shared by

a hematopoietic growth factor called G-CSF suggesting that cysteine residues may be important to IL-6 and G-CSF activity.

IL-6 is produced by T- and B-cells, monocytes and macrophages, fibroblasts, keratinocytes, endothelial cells, some tumor cells, and osteoblasts[60,76,84]. IL-1 is a potent inducer of IL-6 production in these cells, an effect inhibited by TGF-ß[144].

Roles of IL-6 in the immune system. T-cell activation; Mediation of IL-1ß; Lennert's disease.

Unlike resting B-cells, resting T-cells express surface receptors for IL-6. Once stimulated with IL-6, T-cells express receptors for Interleukin-2 (IL-2), the T-cell growth factor[109]. IL-6 also stimulates the production of serine esterases in T-cells, an enzyme crucial to T-cell cytotoxicity[109]. IL-6 was identified as the soluble factor in addition to IL-2 that was necessary for the induction and growth of cytotoxic T-cells (CTLs)[39]. *In vivo*, T-cell stimulation by IL-6 may most likely be achieved by constitutive IL-6 production by macrophages. IL-6 may mediate the effects of IL-1-ß, an effect ablated by TGF-ß[31,144]. The importance of this cellular relationship is illustrated clinically in Lennert's T-cell lymphoma, a non-Hodkin's lymphoma. In Lennert's disease, massive infiltration of lymphoid tissue by macrophage-derived histiocytes is accompanied by the growth of T-cell lines which are dependent upon the presence of the infiltrating macrophages[109]. IL-6 *in vitro* may substitute for macrophages in supporting the growth of Lennertine T-cells because antibody to IL-6 completely abrogates the growth effects of the macrophages upon the T-cells[175].

Roles of IL-6 in hematopoiesis. Blast cell activation with IL-3 for granulocyte-monocyte lines; Role in megakaryocytopoiesis; Activity in multiple myeloma; Uses of IL-6 in transplantation medicine.

IL-6 has a potent ability to induce hematopoietic stem cells from the quiescence of G_0 into the active growth of G_1. A second growth factor, Interleukin-3, can stimulate such activated blast cells to grow and differentiate along various lineages into blast cell colonies. Although granulocyte-monocyte lines typically develop from stimulated blast cells, Koike et al. have shown that IL-6 also upregulates megakaryocytopoiesis[112]. Murray et al. demonstrated that IL-6 mRNA is clearly expressed in 4-day old murine blastocysts suggesting that IL-6 may be important in embryonic stem cell development and the first stages of hematopoiesis[143].

The synergistic interaction of IL-6 with IL-3 for the establishment and growth of blast cell colonies *in vivo* may also be important in clinical science; IL-6 and IL-3 have already been used to super-induce the formation of bone marrow cultures in preparation for transplantation. In fact, Okana et al. has observed that transplanted marrow cells which were pre-cultured with IL-6 before exposure to IL-3 had a survival rate of 90% while non-cultured marrows had only a 20% survival rate[149b].

IL-6 is a strong stimulator of growth in myeloma cells and plasmacytoma cells, and anti-IL-6 abrogates such growth. Myeloma cells also produce IL-6 and are therefore likely to maintain cell growth via this autocrine mechanism[109].

Roles of IL-6 and the endocrine system. The pituitary-adrenal axis; Suppressionof IL-6 with dexamethasone.

As illustrated in figure II. vii below, IL-6 induces the release of corticotropin releasing hormone (CRH), which functions to induce the release of adrenocorticotropic hormone (ACTH)[146]. ACTH stimulates adrenal cortex cells to produce glucocorticoids, which, in turn, function to inhibit IL-6 production. Both IL-6 and glucocorticoids function to induce acute phase proteins[31,32]. Glucocorticoid suppression of IL-6 production suggests an important feedback mechanism in the regulation of the molecular response to stress.

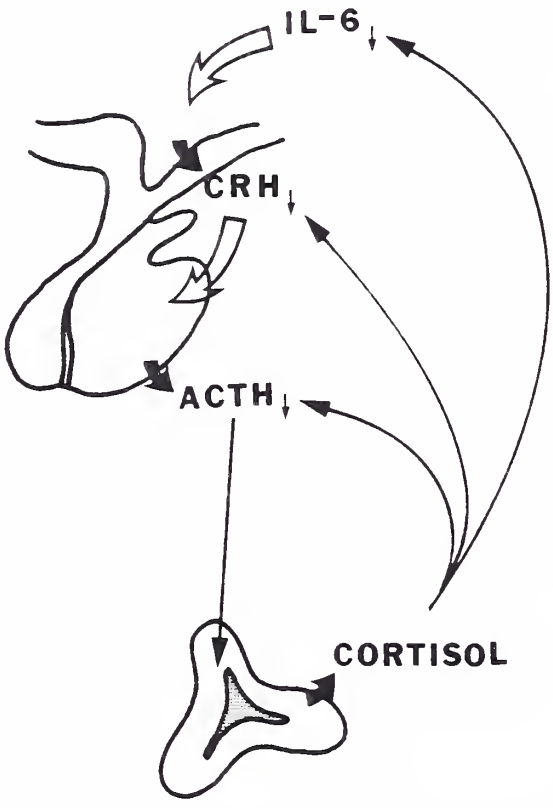


Figure II.xi. The pituitary-adrenal IL-6 axis. IL-6 induces the release of corticotropin releasing hormone (CRH) from the hypothalamus. CRH, in turn, induces adrenocorticotropic hormone (ACTH) release from the anterior pituitary. Finally, adrenal cortisol, produced secondary to ACTH stimulation, functions to both down-regulate hypothalamic IL-6 receptors as well as decrease systemic IL-6 production itself.

IL-6 in bone. Produced by osteoblasts in response to various stimuli; Implicated in osteoclast formation; Role in osteobiology unclear.

Normal primary osteoblasts do not constitutively express IL-6 mRNA but will transcribe IL-6 genes and produce and secrete IL-6 in response to numerous stimuli including exposure to lipopolysaccharide (LPS)[85,87], Interleukin-1 (IL-1)[171,172], and parathyroid hormone (PTH)[84]. The role of IL-6 in normal or pathologic bone and calcium metabolism, however, is not clear.

There have been reports that IL-6 can stimulate proliferation and differentiation of osteoclast precursors[117], potentiate IL-1-mediated bone resorption[117] and inhibit osteoclast activity[64]. Glucocorticoids may inhibit IL-1-induced bone resorption by decreasing IL-6 mRNA production and hormone secretion[94].

Although these studies implicate IL-6 in a feedback mechanism between osteoblasts and osteoclasts to regulate local ratios of bone formation to bone resorption, data has not been widely confirmed. IL-6 may be produced by osteoblasts in fracture sites. This IL-6 may chemoattract and activate local acute and chronic phase cells of inflammation and repair. Evidence is presented is this thesis that suggests LPS-, IL-1- or PTH-induced IL-6 production by osteoblasts can be greatly accelerated and increased by cell priming with Transforming Growth Factor-Beta.

THE COLONY STIMULATING FACTORS (CSFs)

Every second of human life consumes 500,000 granulocytes, a number of cells which must consistently be replaced to avoid a systemic decrease in granulocyte number and the life-threatening condition of granulocytopenia. Granulocytes, and all other cell types normally present in the blood, arise from progenitors in the bone marrow, as the final cellular stage of long lineages in hematopoetic development. The reverse pyramid of blood cell formation is thought to be initiated by the growth of one single stem cell, a pluripotent progenitor of all cell types. During times of blood cell need such as infection or anemia, the marrow can produce up to ten times the baseline number of cells in as little as 48 hours suggesting that the development of stem cells into fully differentiated cell types can be regulated. In the 1960's, the development of semi-solid media enabled researchers to establish cell culture systems for cells normally present only in bone marrow; cell progenitors which eventually differentiate into mature colonies of granulocytes, monocytes/macrophages, megakaryoctyes, etc. Using semi-solid media systems, it was found that cell types could not sustain cell division for more than 48 hours in media alone. Rather, cell colonies could be initiated and sustained when certain fractions of marrow serum were included in culture. Specfic regulatory molecules were isolated from serum, and, since the molecules were identified by their ability to induce the growth of cell colonies, such molecules were referred to as Colony Stimulating Factors, or CSFs[137b]. Because the CSFs were present in extremely small amounts *in vivo* , it

required many cycles of high-performance liquid chromatography to concentrate the molecules to analytical levels, in some cases, one-million times the concentration *in vivo*. Eventually, four distinct CSFs were isolated and cloned from murine and human bone marrow. As the properties of the CSFs began to be elucidated it was found that the molecules affected not only disparate branches of the hematopoetic family tree, but affected cells at certain stages of differentiation (figure II.xii). Therefore, one CSF affected the early, common progenitor for both granulocytes and monocytes, and is therefore called Granulocyte-Macrophage CSF, or GM-CSF. A second induced the growth of only those cells which developed into macrophages (Macrophage-CSF, or M-CSF) and a third, Granulocyte-CSF (G-CSF), was an inducer of relatively well-differentiated pre-granulocyte colonies. The fourth, Interleukin-3, was by the far the earliest effector of cellular growth, inducing the proliferation of almost all cell types, including stem cells themselves. Since the CSFs are important in normal cellular growth, the hormones have also been implicated in disorders of *excessive* white blood cell growth (leukemia, thrombocythæmia), and of *reduced* white blood cell growth (granuloctyopenia, thrombocytopenia). Successful clinical trials in the treatment of neutropenia (acquired and intrinsic) have only heightened the interest in studying the CSFs as important hormones in disease and medical treatments.

Despite the importance of the CSFs in blood cell formation, they possess other activities *in vivo* , and are made by non-marrow resident cell types like fibroblasts[43], keratinocytes[116a], and

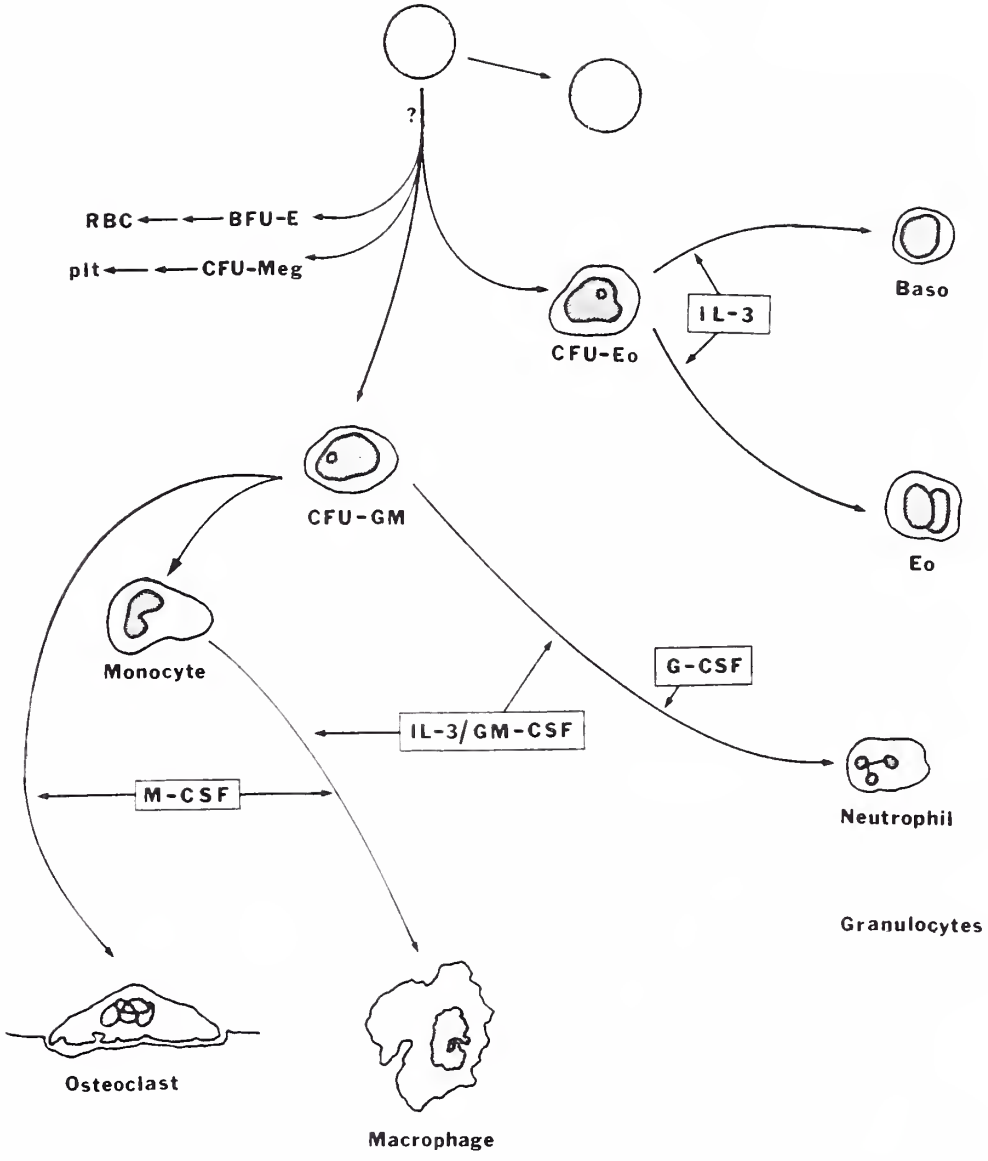


Figure II.xii. Hematopoetic growth factors and cell lineages. An unknown factor induces stem cell proliferation and early maturation. It is not known how early ultimate cell fates are determined. Blood Forming Units-Erythroid (BFU-E), Colony Forming Units-Megakaryocyte (CFU-Meg) and -Eosinophil (CFU-Eo) are formed early. CFUs-Granulocyte/Monocyte (CFU-GM) form an immense pool of precursors, from which are derived all neutrophils, macrophages, osteoclasts, and other tissue phagocytes.

osteoblasts[85]. Osteoblasts make large amounts of GM-CSF and M-CSF in response to various stimuli like lipopolysaccharide (LPS), Interleukin-1, and parathyroid hormone[60,86,87,204].

D. GRANULOCYTE-MACROPHAGE CSF (GM-CSF). GM-CSF is a monomeric protein whose cDNA was eventually isolated

and cloned in 1985 by Wong and coworkers[209]. Four cysteine residues are required for the two disulfide intramolecular bonding necessary for activity. Precursor proteins are processed only at the amino end to yield a 23-24,000 Dalton (Da) mature molecule, capped by an alanine-proline dimer at the amino terminus. The size of the GM-CSF species is determined by the number of the two asparagine sites in the protein which are glycosylated[52]. Double-site glycosylation will yield molecules which are 50% carbohydrate by weight[53b]. The sugar moieties have no apparent function *in vitro* and, in fact, decrease the growth promoting activities of the molecule.

GM-CSF & Hematopoesis. Stimulation of multiple lineages at different points of maturity.

GM-CSF affects granulocyte and monocyte formation by stimulating certain progenitor cells at various time points in hematopoesis, from very early to very late. As illustrated above in figure II.xii, GM-CSF has been shown to stimulate some of the first daughters of stem cell division. The stimulated cells form colonies, or colony forming units (CFUs), from which are derived all non-lymphoid blood cells; granulocytes, erthyrocytes, monocytes, and megakaryocytes and thus are called CFU-GEMMs. GM-CSF stimulation of CFU-GEMMs induces the production of more differentiated colonies of cells, committed to four other lineages. One, an erythroid burst forming unit (BFU-E) is further stimulated by GM-CSF, IL-3 or erythropoetin to form erythroid CFUs and then reticulocytes. A second lineage, a megakaryocytic CFU (CFU-MEG) is first stimulated by IL-3 and GM-CSF and then by erythropoetin to form mature megakaryocytes. A third lineage,

CFU-Eo, is stimulated by IL-3 to form basophils and by both IL-3 and GM-CSF to form eosinophils. The fourth lineage develops into a CFU for granulocytes and monocytes, CFU-GM. Stimulation of CFU-GMs by IL-3 and GM-CSF induces the production of monocytes. GM-CSF stimulation of monocytes, in turn, induces maturation into tissue and blood macrophages. Stimulation of CFU-GMs by IL-3, GM-CSF, and Granulocyte-CSF (G-CSF) produces mature neutrophils.

Sources of GM-CSF *in vivo*. Lack of evidence for constitutive release in marrow; Production og GM-CSF by activated T-cells, macrophages, and osteoblasts.

The requirement that the marrow produce blood cells at different rates throughout an individual's life points to the regulation required for the production of GM-CSF and the other hematopoietic hormones. Furthermore, normal cells thus far examined constitutively express minute quantities of GM-CSF messenger RNA, suggesting that the hormone is produced only when needed. Isolatable quantities of hormone are obtained from cells, such as murine alveolar cells, only after stimulation, i.e. with endotoxins[23]. T-cells[209], stromal cells[211], and osteoblasts[86] will all produce quantities of GM-CSF after appropriate stimulation with endotoxin or tumor necrosis factor (TNF). Such observations support the hypothesis that the rapid induction of acute phase cell growth during times of infection is achieved after the release of GM-, G-, and M-CSFs from activated cell types. Although one may implicate GM-CSF in the maintainence of normal, steady-state hematopoesis, no definitive data yet exist to show that the hormone is constitutively produced in the bone

marrow. Because of GM-CSF's low concentration *in vivo*, it may be difficult to detect constitutive secretion of the hormone by marrow stromal cells into microenvironments to induce CFU activity.

GM-CSF and osteoblasts. Produced by stimulated osteoblasts; Hypersecretion in the transgenic TAX mouse; Growth factor in osteosarcoma.

In 1988, Horowitz et al.[86] showed that osteoblasts secreted large quantites of GM-CSF when appropriately stimulated with 1-10 nM parathyroid hormone or 1-10 μg/ml of lipopolysaccharide (LPS), the mitogenic component of Gram negative bacterial cell wall endotoxin. *In situ* hybridization data suggested that no messenger RNA for GM-CSF is consitutively expressed in osteoblasts but message levels are greatly increased after a 24 hour hour exposure to LPS[86]. GM-CSF is not mitogenic for osteoblasts but may positively affect the development of osteoclasts[128b,167c]. GM-CSF does not affect levels of other bone-regulating hormones like PTH, calcitonin, or 1,25 $(OH)_2$-vitamin D_3[87]. A new approach to the study of the hormone in bone has been established using a mouse strain transgenic for the transactivating (TAX) gene from the Human T-cell Leukemia Virus -1 (HTLV-1)[169]. The transgenic animals were originally designed to study the effects of the TAX gene on immune function but were observed to have striking bone abnormalities, such as evidence of greatly increased rates of bone turnover and 10-fold increases in osteoclast number[169]. The TAX gene, by definition, activates other genes in a *trans* fashion, three of which are Lymphotoxin, Interleukin-2 (T-cell Growth Factor) and GM-CSF.

Lymphotoxin, or Tumor Necrosis Factor-ß, was previously identified to be an activator of osteoclasts[70] and was thought to be at least one of the Osteoclast Activating Factors (OAFs)[171]. The production of such OAFs by the TAX gene was especially intriguing since many patients who succumbed to T-cell leukemia were not victims of the leukemic T-cells *per se*, but were victims of flagrant hypercalcemia consequent to increased bone resorption. Such observations have fueled interest in the roles of GM-CSF not only in HTLV-1-induced bone resorption but in normal, osteoporotic, and Pagetic bone resorption as well. Evans et al. reported that recombinant human GM-CSF stimulated osteoblast proliferation, but inhibited osteoblast alkaline phosphatase activity and 1, 25 (OH)2 vitamin D3-induced osteocalcin synthesis[59]. Additionally, observations that GM-CSF is a growth factor for osteosarcoma and acute and chronic myeloid leukemic cells has implicated the hormone in neoplastic processes of liquid and mineralized tissues as well[43,194]. Some reports have implicated GM-CSF and other colony stimulating factors as inducers of cartilage degradation by chondrocytes[98].

GM-CSF and clinical applications. Treatment of intrinsic and acquired leukopenias; Limited, yet promising results;

GM-CSF functions to maintain the biological activity of those cells induced by the hormone. That is, GM-CSF not only induces granulocytopoesis, it prolongs and enhances neutrophil and eosinophil phagocytosis, chemotaxis and antibody-dependent cytolysis[137a], and enhances macrophage syntheses of prostaglandin E and plasminogen-activators[43]. Donahue et al. showed that continuous infusion of human GM-CSF into *Macaca*

fasicularis and *mulatta* monkeys induced a five-fold increase in white cell counts within 36 hours without toxic effects[53a]. Later experiments showed that GM-CSF infusion could also hasten the recovery from marrow transplantation-induced pancytopenia[53b,170]. Laboratory successes prompted the use of GM-CSF in clinical trials for patients with myelodysplastic syndromes[195], aplastc anemia[26,40], and the leukopenia of the acquired immunodeficiency syndrome (AIDS)[74,75]. In one study, continuous intravenous infusion GM-CSF daily for two weeks in patients with the myelodysplastic syndrome increased granulocyte counts 5- to 373-fold[195]. Monocytes, eosinophils, and lymphocytes also increased. Dramatic increases in circulating neutrophils, eosinophils, and monocytes in AIDS patients after GM-CSF treatment fell to background 1-2 days after treatment was stopped[75]. Such clinical successes point to both the enourmous potential of GM-CSF in medical treatments of hematologic disease, as well as the hormone's short course of action.

E. MACROPHAGE-CSF (M-CSF). Exists as two different forms *in vivo*; M-CSF induces the development of monocyte lines into macrophages. The M-CSF receptor is encoded by c-fms.

M-CSF was the first hematopoetic growth factor identified by analysis of spleen cell colony proliferation and was originially called, therefore, Colony Stimulating Factor 1 (CSF-1)[137b,192]. cDNAs for M-CSF were obtained only after the native amino acid sequence of the protein was used to predict the nucleotide sequences of disparate regions of M-CSF mRNA[105]. Synthetically produced oligonucleotides were then used to identify and eventually isolate a

complete cDNA for the M-CSF gene. Various cell lines transcribe the M-CSF gene into two species of mRNAs, a 1.8 kilobase message and a 4.0 kilobase message. The 4.0 kB mRNA is produced after a 894 base insertion into the 1.8 kB mRNA[105,210]. As shown

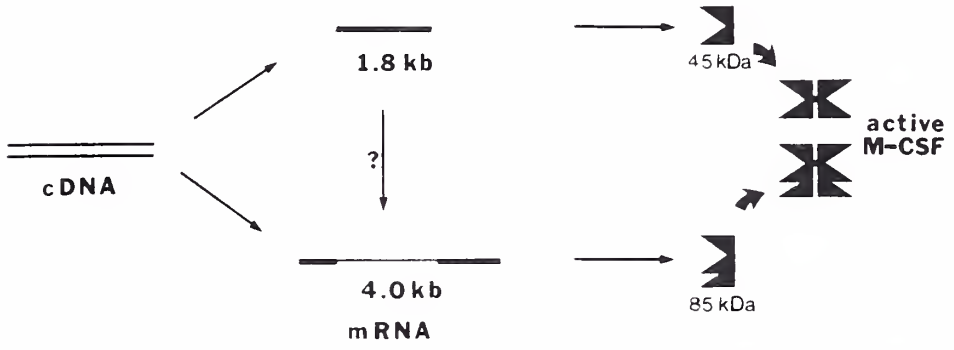


Figure II.xiii. M-CSF processing. Two forms of active dimeric M-CSF exist, a 45 and a 85 kDa proteins, both derived from a 1.8 and a 4.0 kb mRNA splice product, from the same DNA transcript, respectively. The smaller mRNA differs from the larger in that codons for 298 amino acids are inserted between residues 181 and 182 of the smaller species. There is some evidence that this may occur, in part, post-transcriptionally.

above in figure II.xiii, the M-CSF mRNAs are post-translationally processed into two different species of M-CSF hormone; the 1.8 kb mRNA yields a 18-26 kDa protein and the 4.0 kb mRNA codes for a 35-45 kDa molecule[210]. The two proteins are then N-glycosylated to attain molecular weights of 45 kDa, and 85 kDa, respectively. The two proteins then form dimers with one other molecule of the same weight species to yield an active M-CSF hematopoetin. Thus, two different forms of the hormone exist *in vivo* ; a dimer consisting of two 45 kDa glycoproteins and another consisting of two 85 kDa glycoproteins[137a].

As shown in figure II.xii, M-CSF induces promonocytes and monocytes to differentiate into macrophages. In this respect, M-CSF is unlike GM-CSF in that M-CSF affects cell types at relatively late

stages of maturation. M-CSF also increases macrophage cytotoxicity and appears to increase monocyte/macrophage longevity[43]. M-CSF modulates hematopoiesis further by inducing production of interferon-gamma and Tumor Necrosis Factor-alpha from monocytes[200].

M-CSF is unlike the three other CSFs in that there are 10-50 times the number of receptors for M-CSF on a given cell than there are for GM-CSF, IL-3, or G-CSF[25,137b,205,209]. The observation that the M-CSF receptor, a 165 kDa glycoprotein, is identical to the product coded by the proto-oncogene *c-fms* may implicate abnormal transduction of signal by M-CSF receptors in neoplastic growth[173].

M-CSF and bone. M-CSF enhances osteoclast proliferation; Op/op mice have osteopetrosis and are homozygous for a defective M-CSF gene; Osteopetrosis resolves with M-CSF therapy.

M-CSF enhances the development of osteoclast precursors[77b,128b,206,212]. The finding that osteoblasts produce M-CSF in response to PTH and PTHrP suggests further that osteoblasts may regulate osteoclast activity via paracrine hormone production[203].

Wiktor-Jedrzejczak and coworkers have shown that M-CSF may have particular importance in bone cell biology, as illustrated by the researcher's study of the bone disease, osteopetrosis. Osteopetrosis is a disorder of bone remodeling consequent to a profound deficiency of osteoclasts, the bone resorbing cell. Children born with the disease have extremely brittle bones which are resistant to normal bone growth. Osteopetrotic children can be effectively cured, however, by a successful transplant of bone marrow. Normal osteoclasts eventually grow out from monocytic

lines in the marrow and repopulate bone matrices throughout the body (figure II.xii). To study the disease process further, a mouse strain was developed which was homozygous recessive for osteopetrosis, the so-called op/op mouse[206]. Op/op mice are smaller than normal littermates, have a lower birth weight, have no teeth, and have bones typical of osteopetrosis which are devoid of osteoclasts. Because the mice also have low numbers of circulating moncytes and macrophages, the M-CSF gene was implicated in the disease etiology. Wieslaw-Jedrzejczak et al. eventually demonstrated that tissues from op/op mouse were devoid of M-CSF whereas op/+ and +/+ littermates had normal levels of the hormone[206]. Furthermore, only the larger 4.6 kB mRNA species was detected in op/op mice, whereas normal littermates expressed both 2.3 and 4.6 kB species. Eventually, a stop codon was identified in the middle of an important reading frame in the op/op mouse

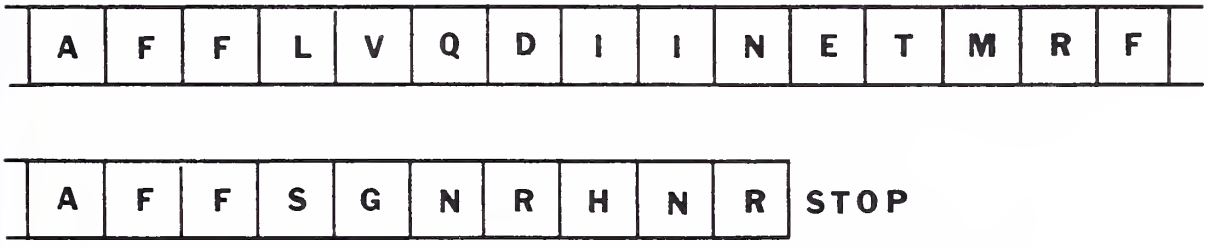


Figure II.xiv. The wild vs. op/op M-CSF amino acid sequence from residues #54-68 of exon 4. An insertional mutation of thymidine in a codon for leucine produces a codon translates for serine and produces frameshift mutations distally until a TGA stop codon is read producing a truncateded molecule unable to act as a ligand for the M-CSF receptor.

mRNA for M-CSF, an oligonucleotide error which precluded production of the hormone (Figure II.xiv)[212]. Administration of recombinant murine M-CSF replenished osteoclasts in the op/op mice

and cleared the animals of disease. These experiments demonstrated both the importance of a specific hematopoetin in the etiology of a particular disease as well as the role such hematopoetins may play in normal bone physiology.

F. Thesis Investigation.

The discussion of TGF-ß and the cytokines above points to the diverse activities of these hormones in cell growth and regulation. Many of TGF-ß's roles include modulating the effects of other growth factors. Mineralized tissue may be unique in this regard because bone is not only a large source of active TGF-ß but of several osteoblast-derived growth factors. Any relationship between active TGF-ß and the regulation of osteoblast-derived cytokine production, however, as not yet been defined. Understanding the regulation of osteoblast-derived cytokine production may help towards describing the function of these growth factors *in vivo*.

This thesis presents experiments which tested the affects of TGF-ß on fetal rat osteoblast production of IL-1, IL-6, GM-CSF, and M-CSF, as assayed with known growth factor-dependent cell lines. This work presents a novel description of growth factor interactions which may have relevance to the paracrine regulation of bone remodeling and hematopoesis *in vivo*.

III. MATERIALS AND METHODS

A. Cell Preparation.

1. Primary cultures of fetal rat osteoblasts. Fetal rat calvaria were obtained at 22 days gestation and isolated under sterile conditions. Primary cells were obtained through a sequential collagenase digestion procedure as detailed by Wong and Cohn[208]. After a 20 minute digestion in 3 ml isotonic salt with 0.1 % collagenase, 0.05 % trypsin, and 4mM EDTA at room temperature the first eluted cell suspension was saved as population #1. The eluant obtained through a second 20 minute digestion was discarded, but the cells recovered from the 3rd, 4th, and 5th digestions were saved, and pooled, as populations # 3-5. Analysis of known cellular markers for osteoblasts (alkaline phosphatase activity, response to parathyroid hormone, synthesis of collagen I & III) has previously determined that cells from populations #3-5 are predominantly (> 95%) osteoblasts and osteoblast precursors[99b]. As described in III.C.5 and V.ii, osteoblast preparations were assayed for interleukin-1 activity (IL-1) to determine the presence of contaminating macrophages which constitutively release IL-1. No significant IL-1 activity was found in any preparation of osteoblast CM (data not shown). The LBRM assay for IL-1 activity can detect IL-1 concentrations down to 10 pg/ml[213b]. The absence of detectable IL-1 activity is therefore suggestive that the osteoblast preparations were reasonably devoid of macrophage activity (data not presented).

All preparations of primary osteoblast cultures were kindly provided by Dr. M. Centrella, St. Francis Hospital, Hartford, Connecticut.

2. C3H/HeJ mouse bone marrow cells. Total bone marrow cell cultures were obtained from a C3H/HeJ mouse (Jackson Laboratories, Maine). The animal was killed with cervical dislocation. The two hind legs were cleaned with

50% ethanol and shaven. The femurs were isolated and removed to a sterile field. The metaphyses of each bone were removed and the indwelling marrow cells expelled with PBS introduced by a 10 cc syringe. The cells were collected, washed three times in PBS and counted. The cells were placed into Costar culture plates for experimentation as described in IV. D and maintained in Dulbecco's modified Eagles medium (DMEM) with nonessential amino acids, penicillin, streptomycin, 20 mM HEPES, 100 ug/ml L-ascorbic acid, and 10 % fetal calf serum (FCS).

All preparations of C3H/HeJ mouse bone marrow cells were made by the author.

B. Preparation of Conditioned Medium (CM). Cells suspensions were plated out in 6 well tissue culture plates (Costar) at a concentration of 5 x 10^4 cells/well, and grown in Dulbecco's modified Eagles medium (DMEM) with nonessential amino acids, penicillin, streptomycin, 20 mM HEPES, 100 ug/ml L-ascorbic acid, and 10 % fetal calf serum (FCS). Cells were cultured in the serum containing media until the cells reached sub-confluence, at which time the media was withdrawn, the cells washed twice in phosphate buffered saline (PBS), and serum-free media added to the culture wells. The cells were maintained in the serum-free media 60-72 hours until confluent. The media was removed, the cells washed twice with PBS, and fresh serum-free media was returned to the wells along with the appropriate inducing agents for each experimental protocol as noted in the text. A typical experiment maintained the cell monolayers for 48 hours at 37°C in 5% CO_2. Following the culture period, the CM was collected, filtered through Millipore syrnige-held membranes, and frozen at -20.C.

A series of 'priming' experiments are described and presented in sections IV. v and xv and were conducted as follows: Confluent, serum-deprived cells were exposed to a single agent in serum-free media for a 2 hour time period.

The cells were then washed twice with PBS and a second agent was then provided to the cells in fresh serum-free media for 46 hours. The supernatant was then removed and saved as a CM and analyzed for cytokine activity as described below in sections IV. v and xvi. Appropriate control CMs were obtained for each experiment. For example, if a cell group was exposed to TGF-ß for 0-2 hours and then LPS for 2-48 hours, the three experimental controls would include (1) CM from cells exposed to serum-free media for 0-2 hours and again for hours 2-48, (2) CM from cells exposed to TGF-ß for 0-2 hours and then to serum-free media alone for 2-48 hours, and (3) CM from cells exposed to serum-free media alone for 0-2 hours and then to LPS for 2-48 hours.

A series of 'time course' experiments were conducted in which confluent osteoblasts were prepared as described above and then treated with indicated agents in fresh serum-free media. Conditioned media was then removed and saved for later analysis after 6, 22 and 48 hours of treatment. A second experiment used the same protocol but analyzed CMs removed after 1, 2.5 and 6 hours of treatment.

All CMs were prepared by Drs. M. Centrella and T. McCarthy, St. Francis Hospital, Hartford, Connecticut unless otherwise specified in section IV.

C. Cytokine Assays.

1. Granulocyte-Monocyte Colony Stimulating Factor (GM-CSF). GM-CSF activity was determined using the HT-2 T-cell assay as was initially reported by Kupper et al.[116b]. Briefly, as outlined in table III.i below, HT-2 cells were maintained in suspension in continuous culture in Eagles Hanks Amino Acid (EHAA) medium with 10% fetal bovine serum (FBS), penicillin, and streptomycin. Cells were washed three times in phosphate buffered saline (PBS) (Gibco, Grand Island, N.Y.) with 2% FBS, resuspended in EHAA with 10% FBS, and plated into wells of 96 well tissue culture plates (Costar) at a concentration of

2×10^4 cells/well. Varying concentrations of test CM were added to the cells. A final volume of 200 µl was achieved with complete media. Triplicate cultures were incubated for 48 hours at 37 °C in 5% CO_2. HT-2 cells proliferate in response to GM-CSF as well as Interleukin-2 (IL-2). Recombinant human interleukin-2 (r-hu IL-2) is, therefore, used as a positive control in the HT-2 assay.

HT-2 cells respond to GM-CSF, IL-2, and IL-4[116c]. IL-2 and IL-4 activity has never been identified in the preparations of osteoblasts used in the these present studies (M. Horowitz, unpublished). Osteoblasts do not produce IL-2 suggesting that HT-2 cell-inducing activity in a given CM is due to the presence of GM-CSF. To test for the presence of GM-CSF in a given CM, a goat antibody against rat GM-CSF was used at a concentration of 1:1000 in assays as specified in section IV. iv. The antibody was made and provided by Dr. J.

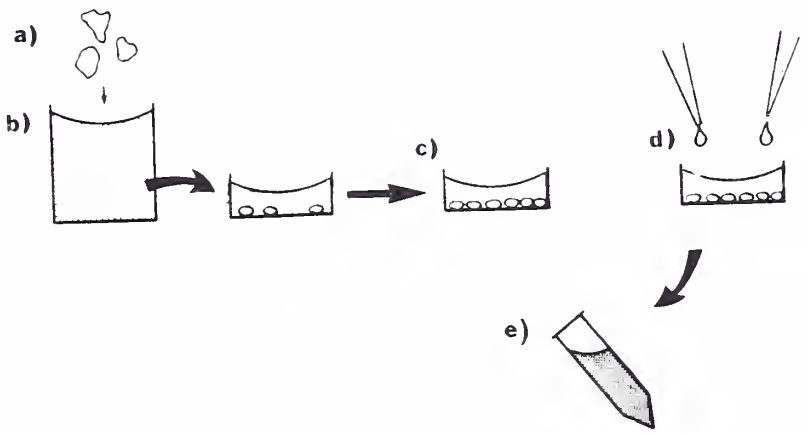


Figure III.i. Preparation of conditioned media. a) Parietal bones dissected from fetal rat pups at 22 days gestation. b) Bones digested with .1% collagenase to obtain populations of osteoblasts. c) Osteoblasts grown to confluence and serum-deprived for 24 hours. d) Reagents added to cultures for given time interval. e) Supernatants collected as conditioned media (CM).

Schreurs, DNAX (Palo Alto, California). The antibody preparation against GM-CSF does not cross-react with IL-2, IL-3, G-CSF, M-CSF, or any other known cytokine (J. Schreurs, unpublished).

HT-2 cells were grown for 44 hours, pulsed with 1 μCi/well of ^{3}H-Thymidine (Specific Activity: 70-85 Ci/mmol) (New England Nuclear, Cambridge, Mass.) and harvested at 48 hours onto glass fiber filters using an automated sample harvester (Cambridge Technologies, Cambridge, Mass.). Incorporated radioactivity was measured with a Beckman scintillation spectrometer (Fullerton, Ca.). Data are presented as the mean of the triplicate cultures ± the standard error (SE). All data presented are representative of multiple experiments. All assays for GM-CSF were made by the author.

2. Interleukin 6 (IL-6). IL-6 activity was determined using the IL-6 dependent hybridoma developed by Aarden et al. and Landsorp et al. known as B 13.29, or the subclone, B-9[1,121]. B-9 cells are 15-20% more sensitive than the B 13.29 parent line to Hybridoma Growth Factor (HGF) as assayed by Aarden et al[1,121]. B-9 cells will proliferate in response to a minimum of .06-.125 units/ml HGF (IL-6) and maximally respond to ~2 units/ml HGF (IL-6)[1]. The cell line does not proliferate in response to other cytokines and is presumed IL-6 dependent[1,121]. B-9 cells were maintained in EHAA medium, penicillin, streptomycin, glutamine, and 2% FBS. The cells were washed three times in PBS with 2% FBS, resuspended in EHAA with 2% FBS, and plated in 96-well tissue culture plates at a concentration of 10^{4} cells/well. Varying concentrations of test CM were added to the cells, and a positive control was established using the supernatant from the human bladder carcinoma cell line, T24. Cells were grown for 56 hours and then pulsed with 1 μCi/well of ^{3}H-Thymidine (Specific Activity: 3-4 Ci/mmol) (New England Nuclear, Cambridge, Mass.) and harvested at 72 hours with an automated sample harvester (Cambridge Technologies, Cambridge,

Mass.), and measured for radioactivity with a Beckman scintillation spectrometer (Fullerton, Ca.). Data was presented as the mean of each triplicate ± the SE.

A monoclonal mouse antibody against IL-6 was prepared by Lansdorp et al.[121]. The antibody was used in experiments to block the activity of CMs which had B-9 cell inducing activity. The antibody failed to block the activity of CMs over a broad range of concentrations and there was some evidence that the antibody preparation itself was stimulatory for B-9 cells (data not presented). The presence of IL-6 in given CMs could not be thus confirmed. However, the specificity of B-9 cells for IL-6-dependent growth is highly suggestive that the activity of a given CM is due to IL-6. In subsequent experiments the stimulatory activity of the CM was confirmed using a second neutralizing anti-IL-6-antibody (M. Horowitz, pers. comm.).

All assays for IL-6 were made by the author.

3. Macrophage Colony Stimulating Factor (M-CSF). M-CSF activity was determined using the growth factor dependent macrophage cell line, BAC 1.2F5[139b,184]. Morgan reported that BAC cells proliferate in response to a minimum of 50-100 units/ml CSF-1 (M-CSF) with >98% viability and maximally proliferate in response to ~3000 units/ml CSF-1. BAC cells have not been shown to proliferate in response to other cytokines except M-CSF[139b]. BAC 1.2F5 cells were maintained in Iscoves medium supplemented with 10% FBS and 10% L929 cell CM as a source of M-CSF. Cells were washed in PBS and plated into 96 well tissue culture plates at a final concentration of 1 X 10^4 cells/well. Varying amounts of CM were added to each well. The cells were cultured for a total of 72 hours, pulsed with 1 µCi/well of ^{3}H-Thymidine (3-4 Ci/mmol specific activity) for the last 16 hours of culture. BAC cells are adherent and were therefore prepared for harvesting by discarding the cell supernatants, and washing each well twice with 50 µl/well of PBS. Cells were swollen open by the addition of 50 µl/well of H_2O, incubated for five minutes, and then by the

addition of 50 µl of 1 N NaOH for five minutes. Lysate from each well was then harvested onto glass fiber filters using an automated sample harvester (Cambridge Technologies, Cambridge, Mass.). Radioactivity was measured using a Beckman scintillation spectrometer (Fullerton, Ca.), and the data was presented as the mean ± the SE of each triplicate.

All assays for M-CSF were made by the author.

4. Interleukin-1 (IL-1). IL-1 activity was determined using the LBRM 33-1A5.47 (LBRM) T-cell lymphoma and CTLL-2/CT-6 (CTLL) T-cell lines[213b]. Zlotkin et al. originally reported that LBRM cells proliferate maximally to co-stimulation by IL-1 (25% supernatant from P388 D1 SN macrophages, a DBA/2-derived tumor line, Amer. Type Cult. Collec.) and phytohemagglutiinn (PHA) (50 µg/ml)[2113b]. The LBRM cells produce a maximum of 640 units/ml Interleukin-2 (IL-2) in response[213b]. One unit of IL-2 is defined as the minimum dilution of CM capable of achieving 90% viability of 4000 HT-2 cells for 24 hours[213b].

The LBRM assay for IL-1 activity is performed in two parts: First, the IL-1 responsive T-cell lymphoma cell line LBRM 33-1A5.47 is cultured in the presence of osteoblast-derived CMs. After 12 hours the supernatants are removed and filtered. Second, the CTLL-2/CT-6 T-cell line is then cultured with the filtered CMs. The LBRM cells produce IL-2 in response to any IL-1 in the test CMs. The CTLL cells proliferate in response to the IL-2 in the supernatants derived from the LBRM cell culture. CTLL cells also respond to high concentartions of IL-4 which LBRM cells have not be shown to produce[213b].

LBRM cells are maintained in EHAA with 10 % FBS. The cells were washed in PBS three times and resuspended in 96 well tissue culture plates with the CMs at varying dilutions. The cells were cultured for 12 hours in the presence of phytohaemagglutin (PHA) at 1:200 final concentration. Recombinant IL-1 was included as a positive control. At 48 hours, the wells

were mixed, and the plates were spun at 1500 r.p.m. for 3 minutes to pellet cells. 50 µl from each well was removed and placed into wells of a 96 well plate, with CTLL cells plated at a concentration of 2 x 10^4 cells/well. The CTLL cells were cultured for 44 hours and then pulsed with ^{3}H-Thymidine (spec. activ. 70-85 Ci/mmol) for a final 4 hours, harvested with an automated sample harvester onto glass fiber filters (both Cambridge Technologies, Cambridge, Mass.), and radioactivity was measured using a Beckman scintillation spectrometer (Fullerton, Ca.).

All assays for IL-1 were made by the author.

TABLE III.i

CYTOKINE	GM-CSF	IL-6	M-CSF	IL-1
ASSAY CELL	HT-2	B-9	BAC	LBRM/ CTLL
CELL TYPE	T-CELL	Hybridoma	Macr.	Macr./T-cell
MEDIA	EHAA	EHAA	ISCOVE	RPMI/EHAA
ASSAY TIME (hours)	48	72	72	12/48
PULSE TIME (hours)	4	16	16	-/4
POSITIVE CONTROL	IL-2	T24	L929	IL-1/2

D. RNA Analaysis

1. RNA isolation. Total cellular ribonucleic acid was isolated according to the procedures of Chirgwin et al.[42b]. Briefly, confluent monolayers of osteoblasts were lysed with a guanidinium lyze buffer (GTC) [4 M Guanidine Thiocyanate (American Bioanalytical), 0.5 % NaN-lauryl sarcosine (Sigma), 1 M Na Citrate, pH 7.0, 0.7 % v/v 2-mercaptoethanol, and 0.33% v/v 30% anti-foam

A (Sigma), all Millipore-filtered three times and stored at 4° C]. Lysate was drawn into a 10 ml syringe through a 23-gauge needle to shear DNA:DNA hybrids. Sheared lysate was then layered on top of a 3 ml 5.7 M Cesium Chloride solution (Gallard-Schlesinger Ind.), with 25 mM Na Citrate, pH 5, in a 15 ml SW 41 polyallomer tube (Beckman). Tubes were then topped off with guanidinium lyse buffer. Tubes were centrifuged in a SW 41 rotor for 20 hours at 35,000 r.p.m. at 20° C. Supernatant was removed and discarded leaving a clear pellet of crude RNA. Pellet was resuspended in 400 µl of Diethylpyrocarbonate-treated (500 pg/ml) (Sigma) H_20, and 1/10 volume 4 M NaCl was added. The solution was transferred to micro-centrifuge tubes and RNA extracted with an equal volume of water-saturated phenol. The acqueous (upper) layer was removed and placed into a new micro-centrifuge tube. 2.5 volumes of cold 100% ethanol was added to tubes and solution was frozen on dry ice (-70° C). Tubes were spun for 20 minutes in a microcentrifuge, and the supernatant was withdrawn and discarded. Procedure was repeated to resuspend and rextract RNA a second time to rid RNA of any residual cesium chloride. After a second extraction, RNA was resuspended in 50 µl of DEP'd H20, 1:1000 dilutions of 1 µl aliquots from each sample were made to test for optical density at wave lengths of 260 and 280 nm using a Spectronic 601 spectrophotometer (Milton Ray Company). For RNA, 1.0 units of OD at 260 nm corresponded to 40 µg/ml of RNA. A ratio of 0D 260/280 approaching 2.0 indicated reasonable purity of RNA preperation. RNA samples were prepared by the author and Andrea Fields, Yale University.

2. RNA electrophoresis. RNA preparations were brought to concentrations of 2 mg/ml. 10 µl (20 µg) of RNA was then mixed with 20 µl of glyoxal buffer [1 M glyoxal (Eastman Kodak), 0.1 % SDS (Boehringer-Mannheim), 10 mM Phosphate buffer (pH 6.8 mono/dibasic), 50 % vol/vol Spectrograde DMSO, brought to volume with DEP'd H20] and incubated at 50° C for one hour. 15 µl

(10 μg) RNA was loaded into each lane of a 1.5 % agaorse gel made up in 10 mM Phosphate buffer, and was electrophoresed into the gel matrix body at 0.5 volts/cm. Once the RNA front was in the body of the gel, the voltage was increased to 5 volts/cm until the gel front had traversed at least 60 % of the gel length. The elctrophoresis was then stopped. Electrophoresis was performed by the author and Andrea Fields, Yale University.

3. Northern blotting. After electrophoresis, gel was removed and placed upside down on an ethanol/0.3% H_2O_2 cleansed industrial sponge soaked in a pan of 20 X SSC (3M NaCl, 0.3M Na citrate, pH 7.0). A square of Zeta bind (Amersham) blotting paper was cut to fit the gel, was soaked in H_2O one minute to wet, and was placed onto the gel. A pipette was used to force out bubbles between blot and gel interface. A piece of filter paper (Whatman) of the same proportions as the gel was wetted in H_2O and placed on the blotting paper followed by five similar but dry pieces of filter paper. Two Diaper-liners (Johnson & Johnson) were then placed on top of the filter paper, and a plastic plate placed on top of liners. A 500 gm bottle was placed on plate as a weight. The apparatus was left in place for 15-20 hours and then the Zeta-bind was removed, and briefly rinsed for 15 seconds in 2X SSC and then let dry for 10 minutes. Blotted RNA was cross-linked with a hand-held short-wave ultraviolet light source for 3 minutes. Hybrization was performed by the author and Andrea Fields, Yale University.

4. Radiolabeling of cDNA IL-1R probes. Probes of complimentary DNA (cDNA) for murine IL-1R were labeled with ^{32}P-ATP/CTP by random priming of a IL-1R insert. The IL-1R cDNA was carried in a pGEM-2 vector and was kindly provided by Simone Carding, Yale University. The IL-1R 300kb insert was prepared and radiolabeled by Andrea Fields, Yale University after double digestion with ECOR1 and Pst1 for 30 minutes at 37° C. The fragment was isolated using a Gene Clean kit (Boeringer-Mannheim) protocol and radiolabeled

using the Random Priming kit (Boehringer-Mannheim) protocol. Briefly, the linear piece of cDNA was denatured by boiling for 5 minutes, and 50 ng of cDNA was placed in a 1.5 ml tube. To this was added 1 µl each of dGTP and dTTP (50 mM stcok), 2 µl reaction buffer ("hexanucleotide mixture in 10 x concentrated reaction buffer"), 50 µCi each of ^{32}P dCTP and ^{32}P dATP (New England Nuclear), and 1 µl of Klenow fragment (2 u/µl). The mixture was placed in a 37.0 °C water bath for 30 minutes. A second 1 µl of Klenow (2 u/µl) was added to the reaction and the reaction was left overnight at room temperature (22.5° C). The reaction was stopped with enough kit stop buffer to increase reaction volume to 100 µl. Radio-labeled cDNA was isolated after passing mixture over a Sephadex G-50 spin-column centrifuged at 1500 r.p.m. for 5 minutes.

5. Hybridization. Filters were pre-hybridized for 30 minutes at 65° C in 10 mls of 1% Bovine Serum Albumin (Fraction V, Sigma), 7% Sodium Dodecyl Sulfate (Boehringer-Mannheim), 500 mM Sodium Phopshate buffer, and 25 mM EDTA, all sealed in a Seal-a-meal® bag. Radionucleotide-labeled probe was added at 1-10 x 10^6 cpm/ml and hybridized for 15-20 hours at 65° C. Following hybridization, fluid was drained and disposed and blot was washed twice in 65° C Wash buffer A (5% SDS, 0.5 % BSA, 50 mM Sodium Phosphate Buffer, 2.5 mM EDTA) and then twice in buffer A for 15 minutes at 65° C each.

A second buffer of 1% SDS, 50 mM Sodium Phosphate buffer, and 2.5 mM EDTA (Wash B) was used to wash the blot three more times at 65° C. Blot was then briefly dried by blotting with Kim-wipes and then placed in an envelope of saran wrap. Blot was placed in an X-ray exposure holder (Kodak) and covered with one piece of X-OMAT AR type film (Kodak). An intensifying screen (Parker X-ray) was placed on film and holder was sealed, and placed in a 70° C freezer until exposure time as noted in the text.

Hybridization was performed by the author and Andrea Fields, Yale University.

IV. RESULTS

STUDIES OF GM-CSF: TGF-ß and LPS

IV.i. TGF-ß does not induce the release of GM-CSF. To determine whether TGF-ß could induce GM-CSF release from osteoblasts, various concentrations of TGF-ß were added to serum-deprived osteoblasts. As described in III.B, the conditioned media (CM) was collected after 48 hours and tested for GM-CSF activity with the T-cell line HT-2. As presented in figure IV.i, 0.1-10 ng/ml of TGF-ß did not induce GM-CSF activity in various dilutions of tested CMs, compared to both experimental controls (no TGF-ß) and to assay controls (no tested CM).

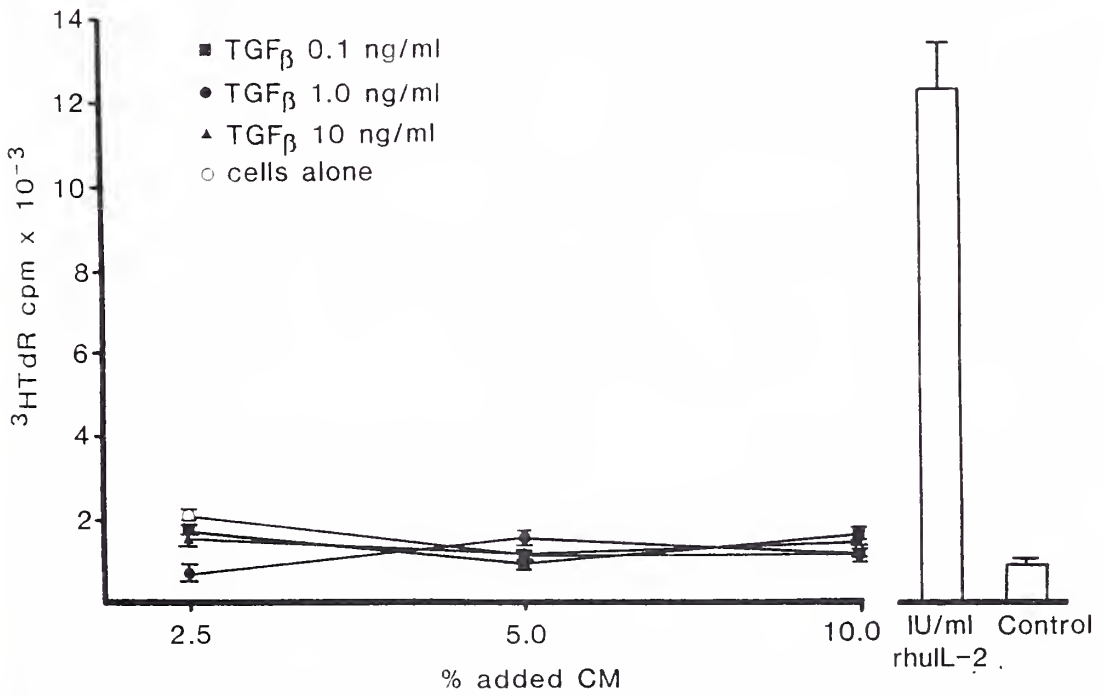


Figure IV.i. HT-2 cell assay for TGF-ß-induced GM-CSF activity in test CMs. Included are negative test controls (no TGF-ß), positive assay controls (recombinant human Interleukin-2), and negative assay controls (no CM).

IV.ii. Lipopolysaccharide (LPS) induction of GM-CSF release is dose-dependent. Although 10 µg/ml of LPS had been known to induce GM-CSF release from osteoblasts, the effect of lower doses of LPS on osteoblasts was unclear. As described in III.B, serum-deprived osteoblasts were exposed to LPS at concentrations of 10 ng/ml to 10 µg/ml for 48 hours. CMs were tested for GM-CSF activity using the HT-2 T-cell assay. As shown in figure IV.ii below, LPS, at concentrations greater than 1.0 µg/ml, induced considerable GM-CSF activity whereas LPS at concentrations less than 1.0 µg/ml induced no detectable GM-CSF activity.

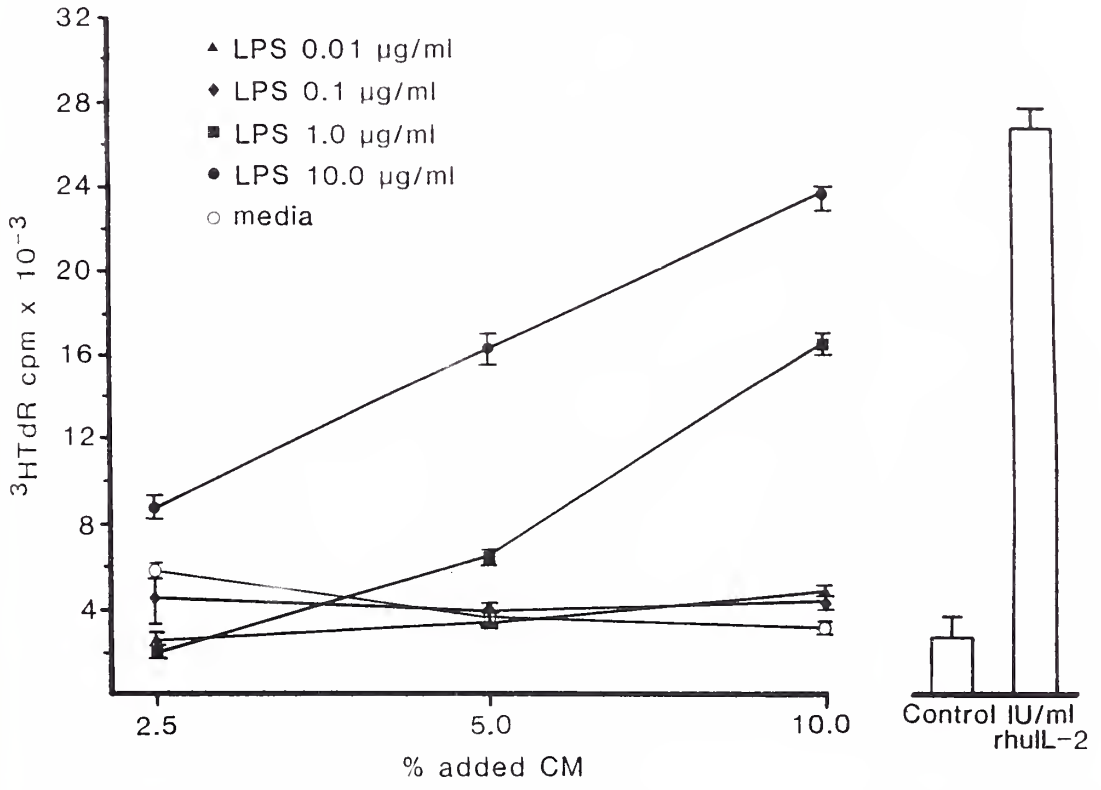


Figure IV.ii. HT-2 assay for GM-CSF activity of CMs from osteoblasts cultured with different concentrations of lipopolysaccharide (LPS). Included are a test control (no LPS), a negative assay control (no CM), and a positive assay control (recombinant human IL-2).

IV.iii. TGF-ß and low dose LPS synergize for release of GM-CSF from osteoblasts. From the previous experiment, it had been determined that

neither 10 ng/ml of TGF-ß nor LPS at concentrations < 100 ng/ml induced GM-CSF release from osteoblasts. To test whether TGF-ß would change the response of osteoblasts to sub-stimulatory levels of LPS (<100 ng/ml), osteoblasts were exposed to either TGF-ß alone, to substimulatory LPS alone (10 or 100 ng/ml), or to TGF-ß (10 ng/ml) together with LPS (10 or 100 ng/ml). As presented in figure IV.iii below, neither TGF-ß alone nor LPS alone induced GM-CSF activity in CMs. However, TGF-ß combined with LPS synergized to induce a substantial increase in GM-CSF activity.

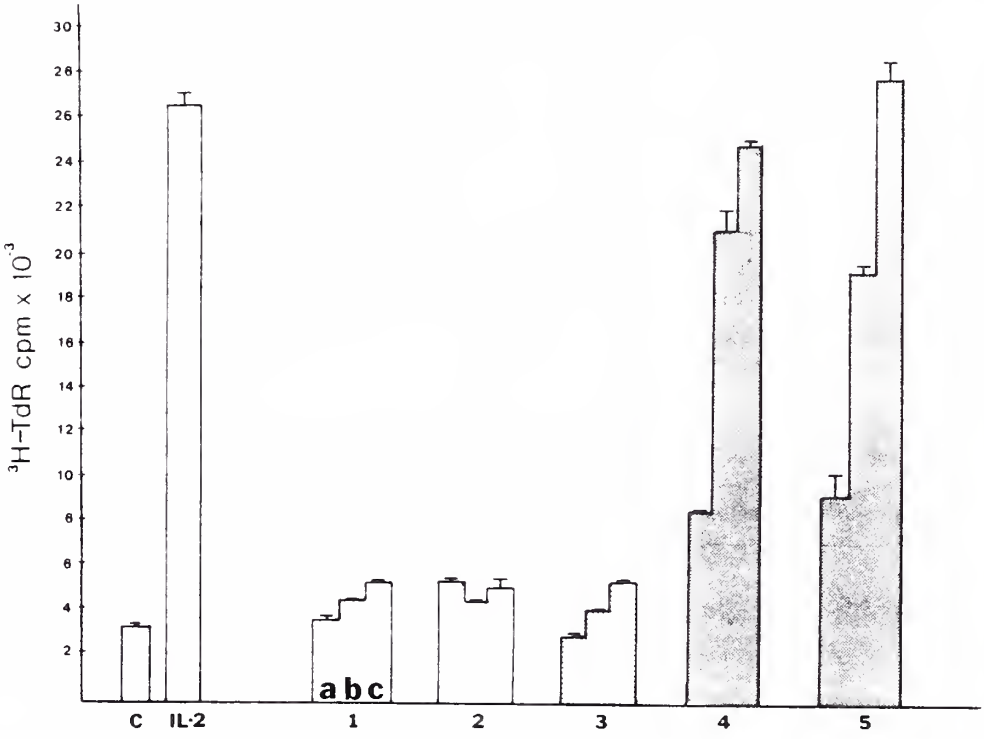


Figure IV.iii. HT-2 assay for GM-CSF activity of CMs from osteoblasts cultured with either TGF-ß alone or with LPS alone, or with TGF-ß and LPS together. Included are a negative assay control (no CM) and a positive assay control (10% rhIL-2). 1=LPS at 10 ng/ml, 2=LPS at 100 ng/ml, 3=TGF-ß at 10 ng/ml, 4= LPS at 10 ng/ml + TGF-ß at 10 ng/ml, 5=LPS at 100 ng/ml + TGF-ß at 10 ng/ml. a=2.5% CM, b=5% CM, and c=10% CM. 10% negative experimental control CM (no agents) induced 1.8 x 10^3 ^{3}H-TdR cpm.

IV.iv. TGF-ß/LPS-induced GM-CSF activity in tested CMs can be neutralized with goat antibody against mouse GM-CSF. To determine whether the observed increases in GM-CSF activity of CMs, as presented in figure IV.iii, was specifically due to GM-CSF, the same CMs were re-tested in the HT-2 assay with either supplemental goat antibody to mouse

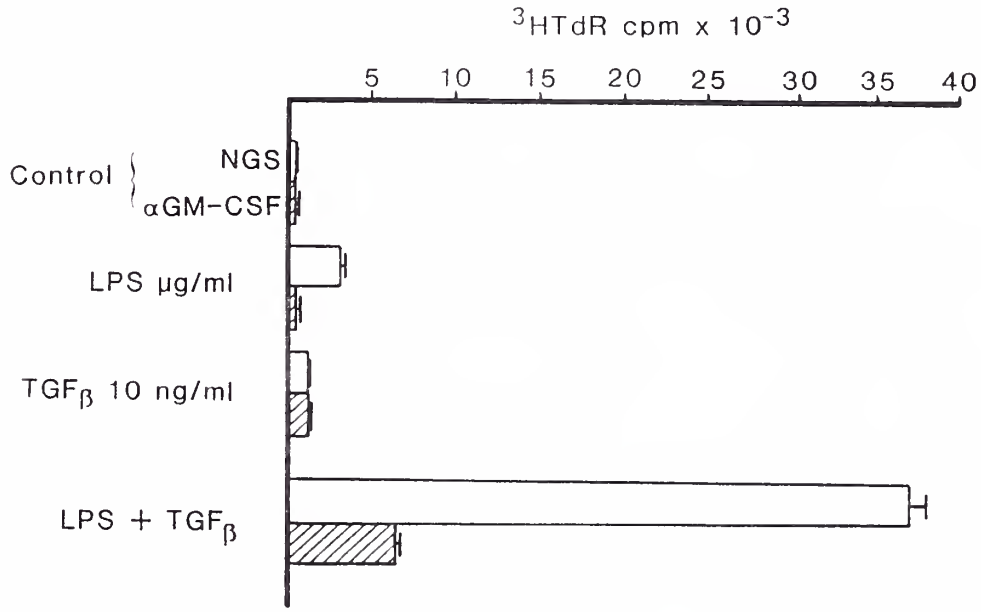


Figure IV.iv. HT-2 assay for GM-CSF activity of 10% CMs co-cultured with either heat-inactivated goat serum or goat antibody to mouse GM-CSF. Slashed histograms represent samples assayed in the presence of a 1:1000 dilution of the goat antibofy and the clear histograms represent samples assayed in the presence of a 1:1000 dilution of the inactivated serum. Background controls include normal goat serum alone (NGS) and goat anti-mouse GM-CSF alone (GM-CSF). Other controls not presented include 10% recombinant human IL-2 (42450 ±320 cpm) and assay background activity (450 ± 35 cpm). Standard deviations are also represented.

GM-CSF or with heat-inactivated normal goat serum. As shown in figure IV.iv above, the TGF-ß /LPS-induced increase in GM-CSF activity was blocked to within background levels by goat anti-mouse GM-CSF but not by normal heat-inactivated goat serum.

IV.v. TGF-ß/LPS synergy for GM-CSF release is TGF-ß dependent. As described in III. B, a series of 'priming' experiments were conducted in which osteoblasts were exposed to either TGF-ß alone for two hours, or to low-dose LPS alone for two hours. The cells were then washed, and then incubated with the complementary synergen for the next 46 hours (Cells incubated with TGF-ß for hours 0-2 were exposed to LPS for hours 2-48). As shown below in figure IV.v., there was no GM-CSF activity in CMs from cells cultured with LPS alone for hours 0-2 and then with TGF-ß for

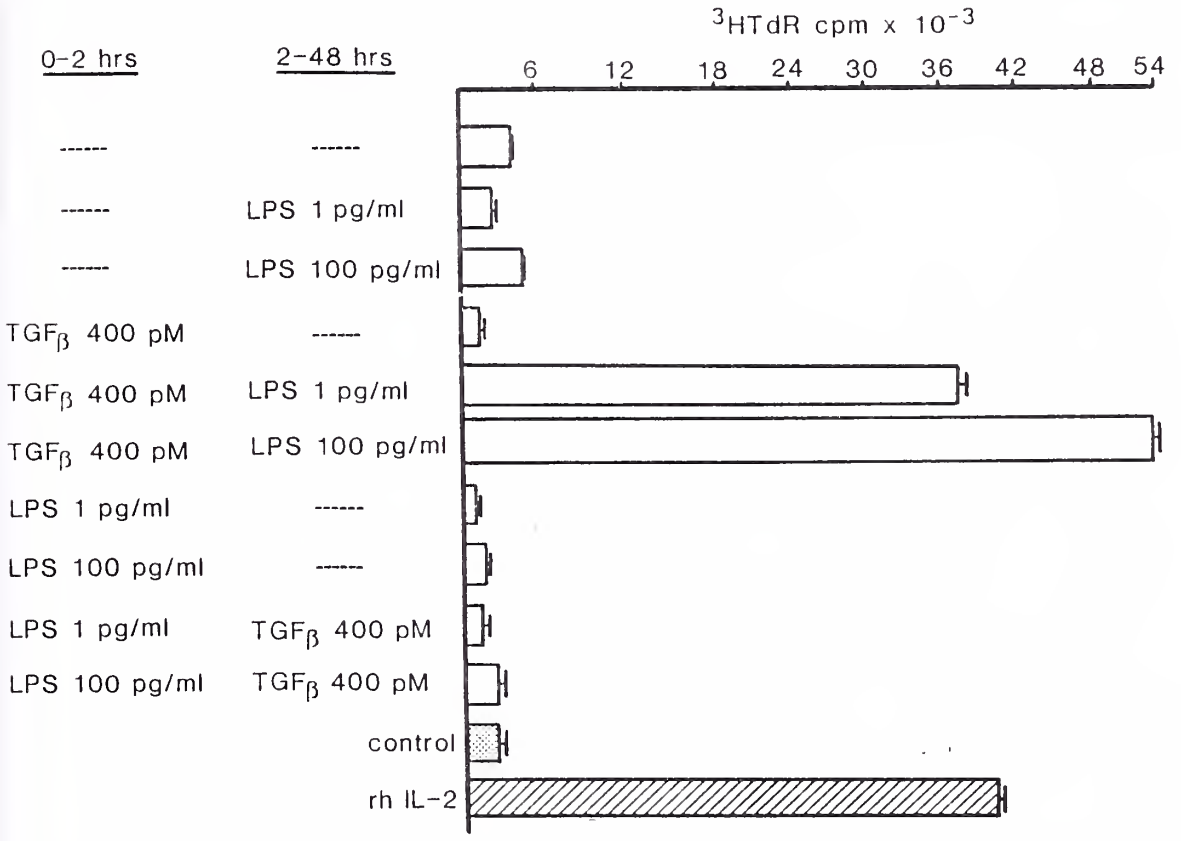


Figure IV.v. HT-2 assay for GM-CSF activity in 10% CMs from osteoblasts 'primed' with either TGF-ß or with LPS and then exposed to the corresponding *synergen*. Included are negative (assay cells) and positive assay controls (10% rhIL-2) as described above.

hours 2-48. However, cells incubated with TGF-ß for hours 0-2, and then LPS for hours 2-48 produced CMs with the high GM-CSF activity seen in previous experiments.

IV.vi.a/b. Most of the TGF-ß/LPS-induced release of GM-CSF occurs between hours 2.5 and 6. TGF-ß and LPS induced large increases in GM-CSF activity of CMs after 48 hours of cell treatment, as described above. To decribe a more complete spectrum of activity, two experiments were conducted. In the first (IV.vi.a.), CMs were collected at hours 0, 6, 22, and 24 and analyzed for GM-CSF activity while the second (IV.vi.b) analyzed CMs collected at hours 0, 1, 2.5, and 6.0. As shown below in figure IV.vi.a. below, CMs at time zero (CMs collected before addition of agents to media) contained no detectable GM-CSF activity compared to

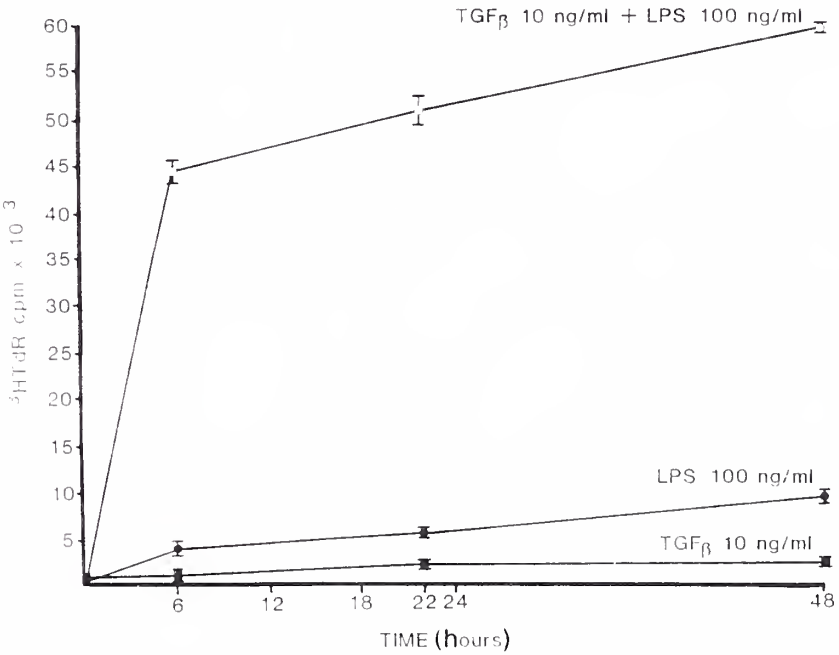


Figure IV.vi.a. HT-2 assay for GM-CSF activity of indicated 10% CMs. Values not included are the assay control (850 ± 20), experimental negative control [no agents] (920 ± 15), and experimental positive control [10% recombinant human IL-2] (52250 ± 650).

background samples. CMs at six hours of incubation contained ten to twenty times the GM-CSF activity of relevant background samples. CMs at 22 hours incubation contained 15% more activity than 6 hour CMs, and CMs at 48 hours contained 20% more activity than CMs at 22 hours. LPS as a single agent induced a small increase in GM-CSF activity in six hour CMs, but the level of activity plateaued over the next 42 hours. The second experiment, presented as figure IV.vi.b below, tested CMs at hours 0, 1, 2.5, and 6 to determine GM-CSF release at earlier time points than discussed in IV.vi.a. As shown below, no GM-CSF activity was detected in the zero, 1, or 2.5 hour CMs. The 6 hour CMs contained the high GM-CSF

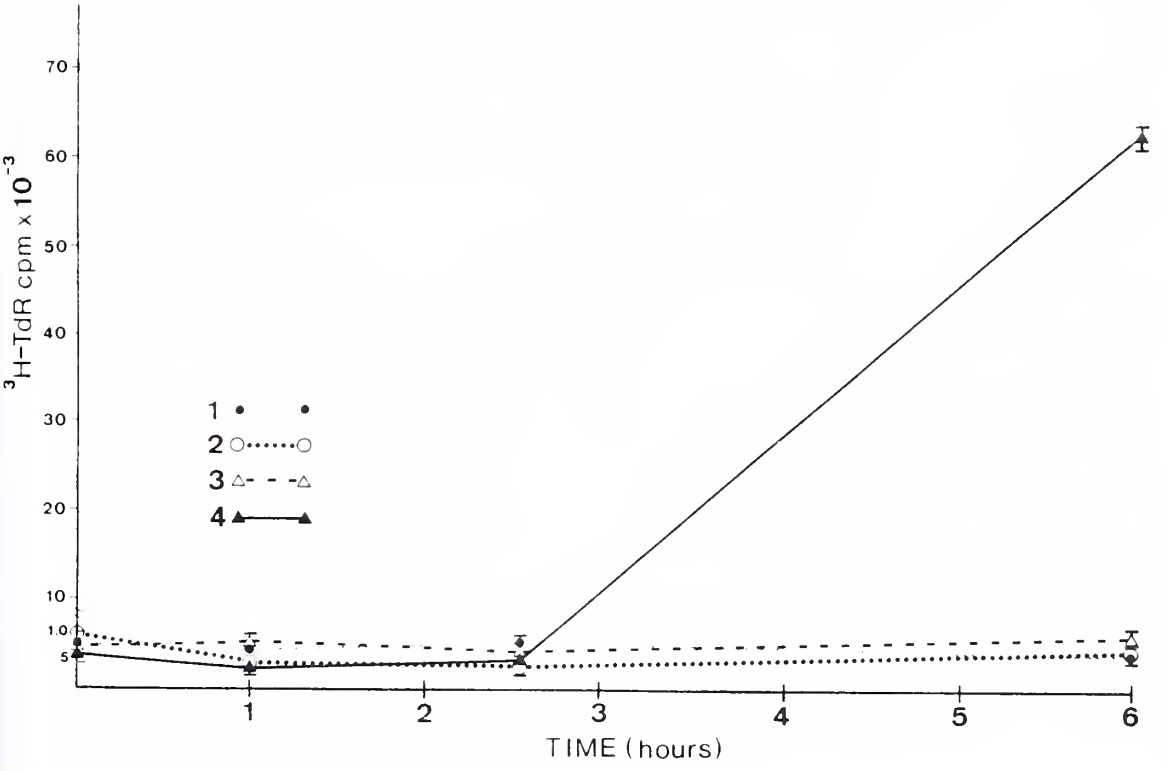


Figure IV.vi.b. HT-2 assay for GM-CSF release over hours 0-1, 1-2.5, and 2.5-6. Histograms represent the GM-CSF activity for each 10% CM grouped according to time points. 1=Control, 2=LPS at 10 ng/ml, 3=TGF-ß at 10 ng/ml, 4=2+3. Positive assay control of 10% rec. human IL-2 induced 69,000 ± 550 cpm in HT-2 cells, while a negative assay control of assay cells alone yielded 650 ± 40 cpm.

activity levels observed previously. The data from IV.vi. a and b suggest that the effect of TGF-ß and LPS on osteoblasts for GM-CSF release is obtained after a minimum of 2.5 hours of exposure to the synergens, maximizes quickly, and is short-lived.

IV.vii. The rate of GM-CSF release is at a maximum between hours 0-6, a rate 95% higher than the rate of release between hours 6 & 22 and between 22 & 48.

As presented in IV.vi.a, the largest increase in GM-CSF release occurs between hours 0 and 6, while smaller increases occur over the subsequent 42 hours. This difference is presented in figure IV. vii as the rates of change in GM-CSF release. As illustrated below, TGF-ß and LPS induce an increase in the rate of GM-CSF release to a maximum between hours zero and six. The rate of release over the next 16 hours drops 95% to

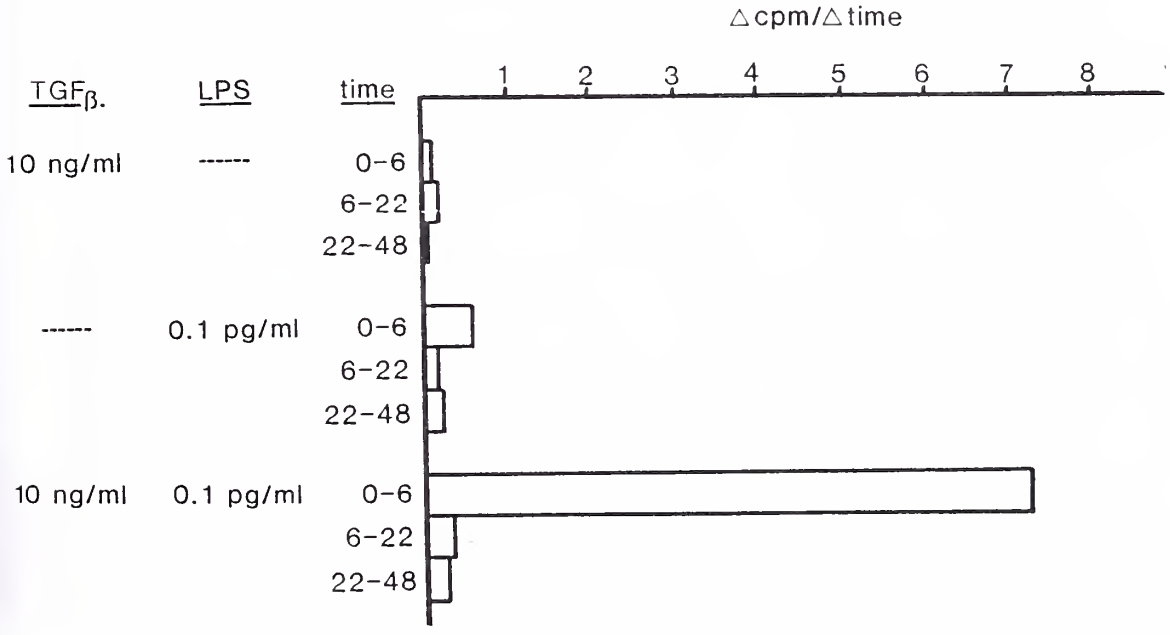


Figure IV.vii. Rates of change in GM-CSF release in cells exposed to TGF-ß and LPS for different intervals of time. The incorporated radioactvity in HT-2 cells induced by a given sample of CM is quantitated as counts per minute (cpm). The increase in cpm value (Δcpm) is divided by the time interval in which a given CM was produced (Δtime), either 6 hours, 22 hours, or 48 hours. The ratio of the cpm value of a given CM to the time value of a given CM is presented as the rate of change (Δcpm/Δtime) in GM-CSF release.

levels statistically equivalent to background. Finally, the rate of GM-CSF release over hours 22-48 remains unchanged from the 6-22 hour release rate and is essentially background.

STUDIES OF GM-CSF: TGF-ß and PTH

IV.viii. PTH alone does not induce GM-CSF release; PTH > 20nM is a poor inducer of GM-CSF release in combination with TGF-ß. Cells were cultured with one of four concentrations of parathyroid hormone (.02, .2, 2.0 or 20.0 nM). Additionally, some cells were also incubated with TGF-ß at 10 ng/ml (400 picomolar [pM]). CMs were collected after 48 hours incubation and assayed for GM-CSF. As presented in figure IV.viii below, PTH alone (at any concentration) did not induce significant GM-CSF release. TGF-ß also failed to increase cytokine release. Only PTH at a concentration of 20.0 nM, when combined with 400 pM TGF-ß, induced a significant increase in GM-CSF release.

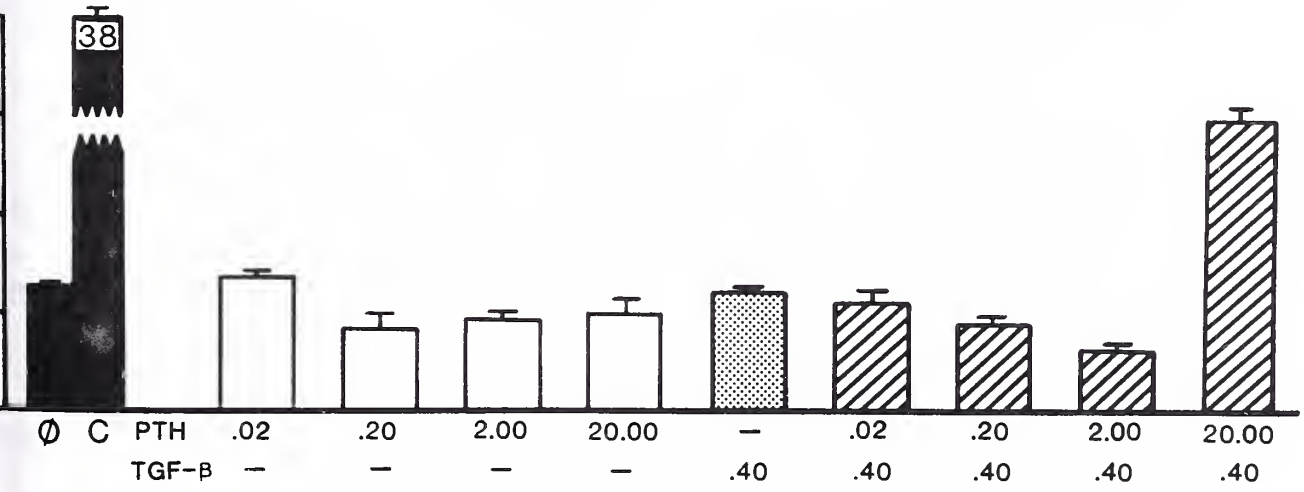


Figure IV viii. HT-2 assay for GM-CSF activity of 10% CMs from cells cultured with .02 - 20 nM parathyroid hormone (PTH) alone or with PTH and TGF-ß, as indicated. The assay control (no CM) value was 6275 ± 340, the positive control (10% recombinant human IL-2) value was 32,575 ± 965.

STUDIES OF GM-CSF: TGF-ß and Interleukin-1

IV. ix. Low-dose Interleukin-1 alone does not induce GM-CSF release, but low-dose IL-1 synergizes with TGF-ß to induce a 15-fold increase in GM-CSF release.

It was previously known that concentrations of Interleukin-1 (IL-1) greater than 10 µg/ml induced GM-CSF release from osteoblasts (data not shown), but the effects of lower doses was unclear. To test low-dose IL-1, and whether TGF-ß could effect IL-1 action, cells were incubated in either 1, 10 or 100 nanogram (ng) IL-1. Other cells were also exposed to 10 ng/ml TGF-ß (400 pM). After 48 hours of incubation, CMs were collected and tested for GM-CSF activity. As presented in figure IV.ix, neither 1 or 10 ng/ml IL-1 induced any significant GM-CSF activity in the tested CMs.

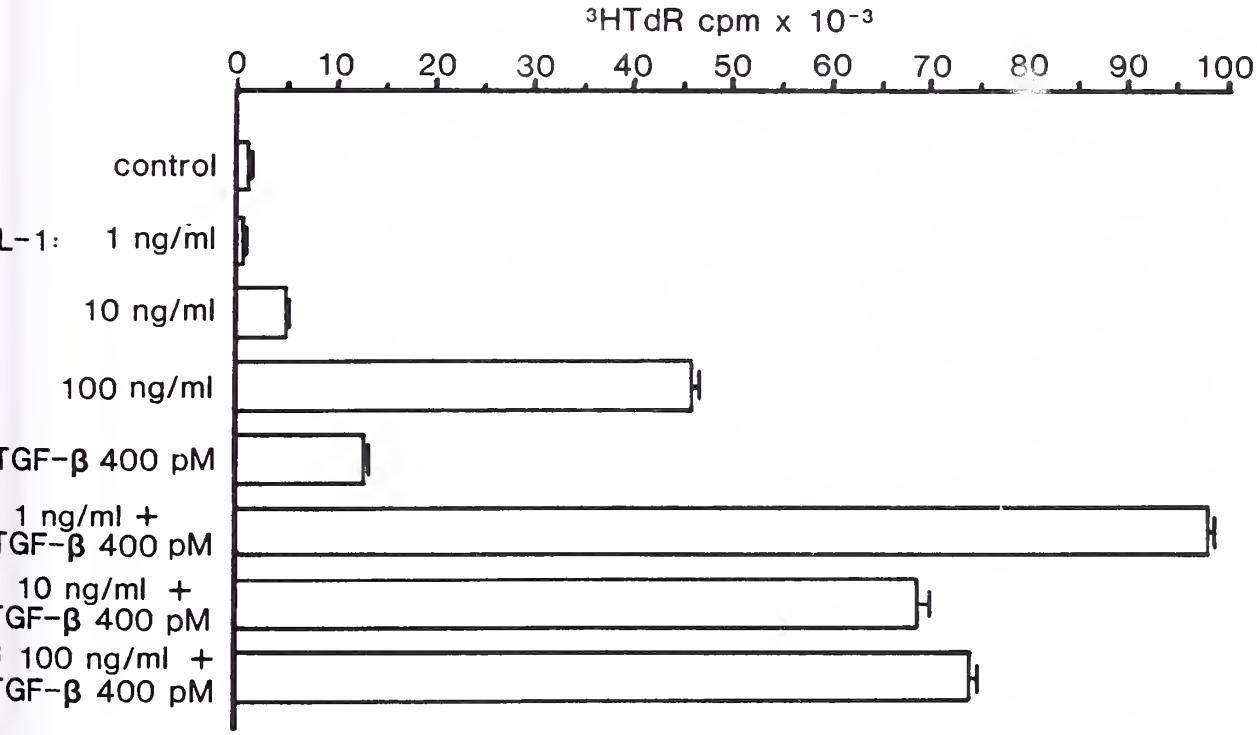


Figure IV.ix. HT-2 assay for GM-CSF activity of CMs from cells cultured with IL-1 alone (1, 10 or 100 ng/ml) or IL-1 with TGF-ß (10 ng/ml [400 pM]). Experimental control no agents) included. Assay controls: HT-2 cells alone=3; 10% rhIL-2=78.

Both concentrations of IL-1, however, synergized with TGF-ß to produce a large increase in GM-CSF activity in tested CMs.

IV.x. IL-1/TGF-ß synergy is unchanged by indomethacin. As described in III. B, osteoblasts *in vitro* were exposed to either TGF-ß alone or to low-dose IL-1 alone or to both agents together. Additional cells were similarly cultured but with the addition of the non-steroidal anti-inflammatory agent

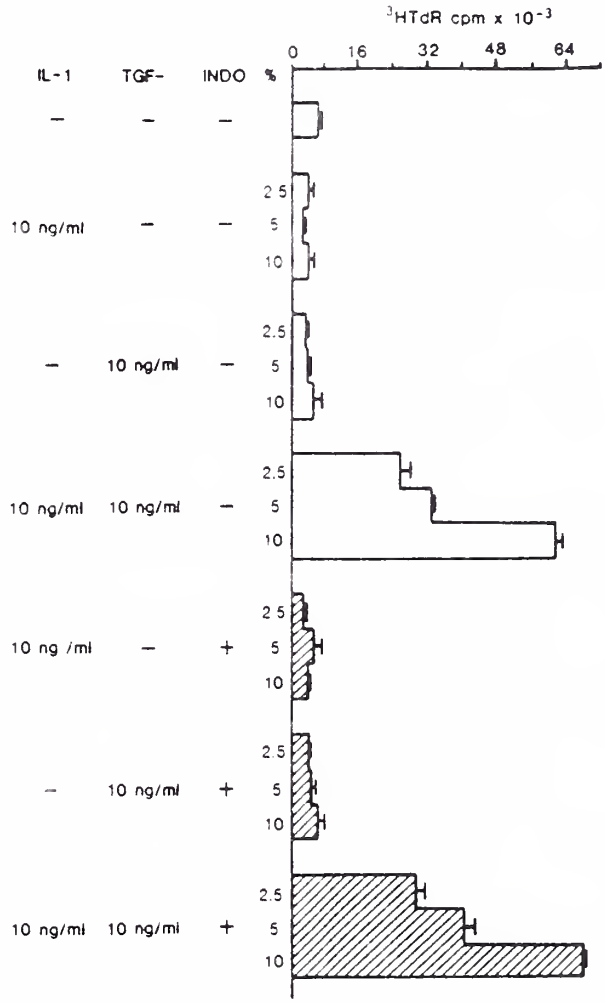


Figure IV.x. HT-2 assay for GM-CSF activity in a series of cyclooygenase inhibition experiments with 10^{-6} M indomethacin. Agents were added to cell cultures for the period of time as indicated. Relevant controls are represented as above. Negative assay control (assay cells alone) induced 7 x 10^3 ^{3}H TdR cpm activity in HT-2 cells while the positive assay control (10% rhIL-2) induced 68 x 10^3 ^{3}H TdR cpm activity. CMs at 10% dilution.

indomethacin at a concentration of 10^{-6} M. As shown above in figure IV.x, there was no GM-CSF activity in CMs from cells cultured with the IL-1 alone or with TGF-ß alone. As expected, TGF-ß and IL-1 synergized to increase GM-CSF release greater than 10 fold. The addition of 10^{-6} M indomethacin to the cultures did not change the GM-CSF release response of the osteoblasts cultured with either IL-1 or TGF-ß alone. There was a slight (<5%) increase in the GM-CSF released by osteoblasts cultured with all three agents (IL-1, TGF-ß, and indomethacin).

IV.xi. Maximum GM-CSF release by TGF-ß/IL-1 synergy is achieved between hours 2.5 and 6 of stimulation, is rapid, and short-lived. As discussed above in IV.vi a and b regarding the time-course of TGF-ß/LPS synergy, the time-course for TGF-ß/IL-1 synergy was determined by analyzing CMs for GM-CSF activity obtained at 0, 1, 2.5, and 6 hours of exposure to TGF-ß alone, IL-1 alone, or to both agents together. As presented in figure IV.xi below, no or little GM-CSF activity was observed in CMs at hours 0, 1, or 2.5. CMs collected at 6 hours of exposure to both TGF-ß and IL-1, however, contained 30 times the activity of control samples. Although not included below, CMs obtained at 6, 22, and 48 hours were also tested for GM-CSF activity. Similar to the results of experiment IV.vi.a above concerning TGF-ß with LPS, CMs from hours 22 and 48 showed less than 5% more GM-CSF activity than CMs at hour 6 (not shown). The data of IV.vi a/b (TGF-ß + LPS) and IV.xi (TGF-ß + IL-1) suggest that TGF-ß synergizes with LPS or with IL-1 to induce GM-CSF release from osteoblasts. The effect occurs after 2.5 hours of

treatment, peaks after about 6 hours of treatment, and declines dramatically soon thereafter.

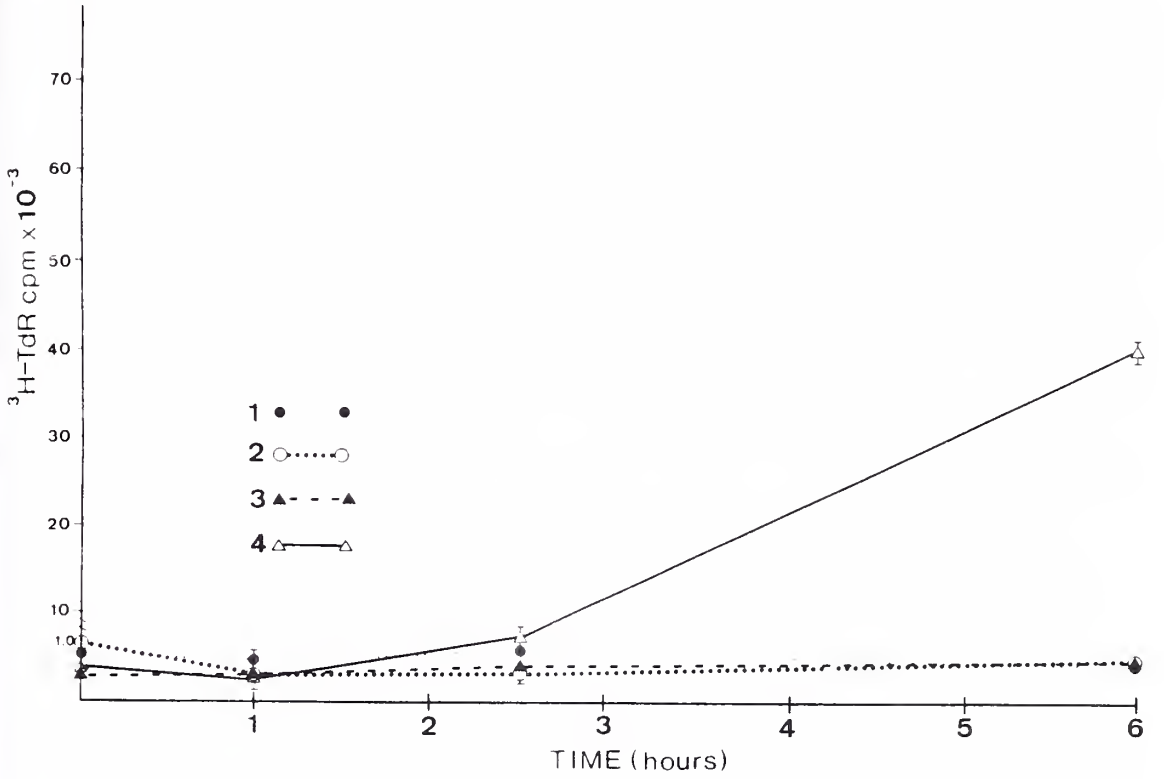


Figure IV.xi. HT-2 assay for GM-CSF activity in 10% CMs collected at specified time points, grouped as histograms. 1=Control, 2=IL-1 10 ng/ml, 3=TGF-ß at 10 ng/ml, and 4=2+3. Positive control of 10 % recombinant human IL-2 induced 69,000 ± 550 cpm and an assay background control was 620 ± 40 cpm.

IV.xii. <u>TGF-ß increases mRNA levels for the interleukin-1 receptor.</u> To determine the effect of TGF-ß, LPS, and IL-1 on osteoblast mRNA production, osteoblasts were cultured with 10 ng/ml TGF-ß alone, 10 ng/ml IL-1, 10 ng/ml LPS alone, TGF-ß + IL-1 (each at 10 ng/ml), or TGF-ß + LPS (each at 10 ng/ml). According to the procedures outlined in III.D.1-5, samples of mRNA from each treatment group were obtained. 10 µg RNA from each sample were electrophoresed , transferred to nitrocellulose membranes, and hybridized with ^{32}P-labeled linear cDNA for the interleukin-1 cell surface receptor (IL-1R). The autoradiograph

obtained, as presented below in figure IV.xii, indicate that TGF-ß may increase the amount of intracellular mRNA for the IL-1R. IL-1 had no effect, nor did the synergen increase the amount of IL-1R mRNA when combined with TGF-ß. LPS also had no effect. The data does not indicate whether TGF-ß increases mRNA production or message stability. The data suggests, however, that the synergy between TGF-ß and IL-1 for GM-CSF release may be achieved, in part, by a TGF-ß-mediated upregulation of cell surface receptors for IL-1.

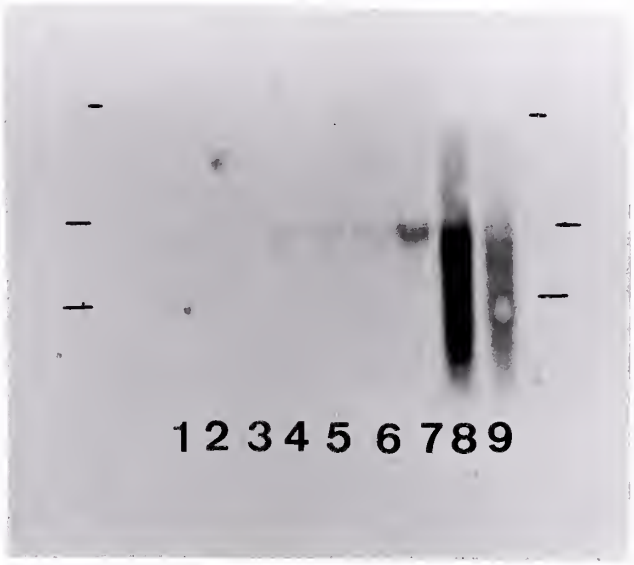


Figure IV.xii. Effect of TGF-ß, IL-1, LPS, TGF-ß + IL-1, and TGF-ß + LPS on IL-1 cell surface receptor mRNA levels. 48 hour autoradiograph of mRNA hybridization with a ^{32}P-labeled (random primed) linear strand of cDNA for the IL-1R. Precisely 10 µg of RNA was electrophoresed in each lane and hybridized with 5-10 x 10^6 counts/ml of cDNA probe. 1=control, 2=IL-1 10 ng/ml, 3=LPS 10 ng/ml, 4=TGF-ß 10 ng/ml, 5=2+4, 6=3+4, 7=transformed osteoblast line MC3T3 positive control, 8=resting D-10 T-cell mRNA, 9=IL-1 (10^{-7})/ConA (2.5 µg/ml)-stimulated D-10 positive control. A hybridization control with an unrelated probe was not prepared from this blot. The specificity of the IL-1R cDNA in previous experiments (data not shown), however, suggests that the bands represented above are those of the IL-1R. Blot prepared by A. Fields, Yale University.

A. STUDIES OF INTERLEUKIN-6: TGF-ß and LPS

IV.xiii.TGF-ß induces IL-6 release. Osteoblasts were cultured with 10.0 ng/ml of TGF-ß for 48 hours. CMs were collected and assayed for IL-6 activity as described in III.C ii. As shown below in figure IV. xiii, 10 ng/ml TGF-ß induced IL-6 activity in dilutions of tested CMs. Similar increases in IL-6 activity were observed in repeat experiments, although the absolute level of IL-6 activity induced by 10 ng/ml TGF-ß varied (data not presented). These findings are in marked contrast to the results of figures IV.i and iii which show that TGF-ß has *no* effect on GM-CSF activity in tested CMs.

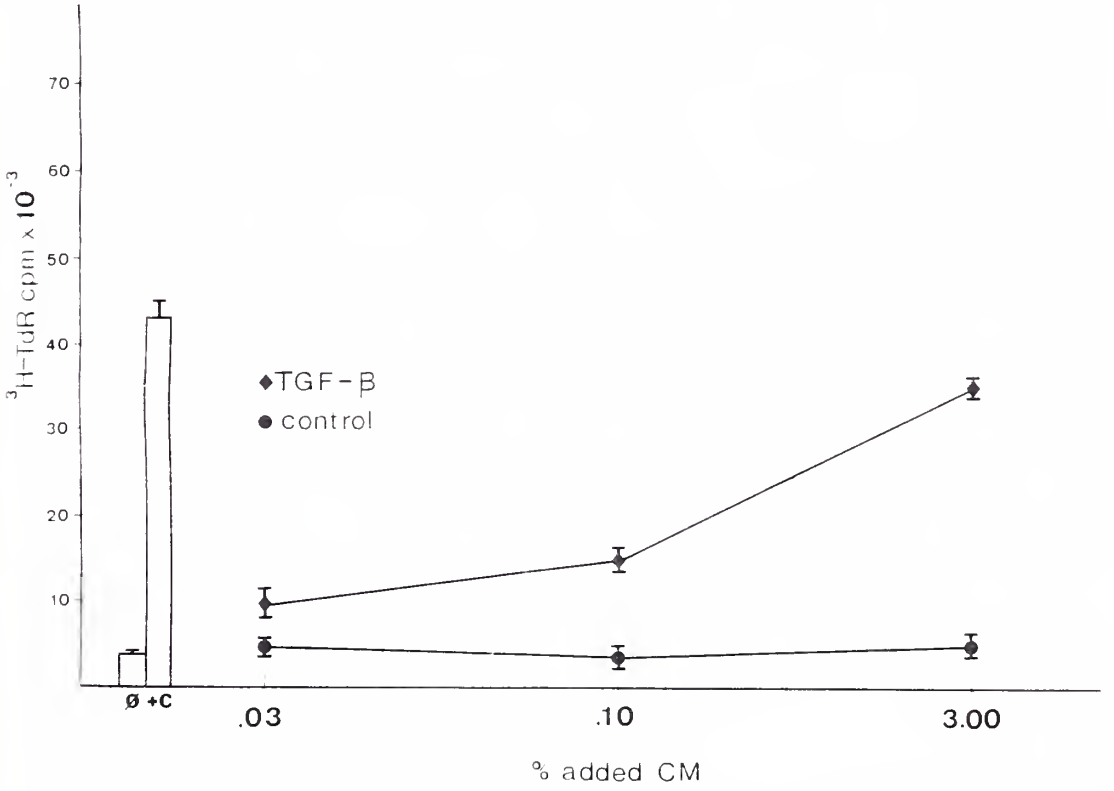


Figure IV.xiii. B-9 assay for IL-6 activity of CMs collected from cells incubated with different concentrations of TGF-ß. Negative (ø) and positive (+C) controls (media alone and 1.0% T24 CM, respectively) are included as indicated. Ordinate values are presented as ^{3}H-Thymidine uptake by stimulated B-9 assay cells. Control=CM without agents.

IV.xiv. IL-6 release is induced by LPS at concentrations greater than 10^3 ng/ml. Cells were incubated with LPS at concentrations of 0.1 ng/ml 1.0 ng/ml, 100 ng/ml and in a second experiment, of 10^3 ng/ml or 10^4 ng/ml for 48 hours. CMs were collected and assayed for IL-6 activity using the B-9 B-cell hybridoma line as descibed in III.C ii. The experiments are presented together below in figure IV. xiv for comparison, separated by a cross-hatch along the x-axis. As the figure suggests background CM activity CMs collected after exposure to 10^4 ng/ml of LPS had twice the amount of IL-6 activity as assay and test background samples. LPS concentrations equal to or less than 10^3 ng/ml failed to induce any IL-6 activity greater than relevant background samples.

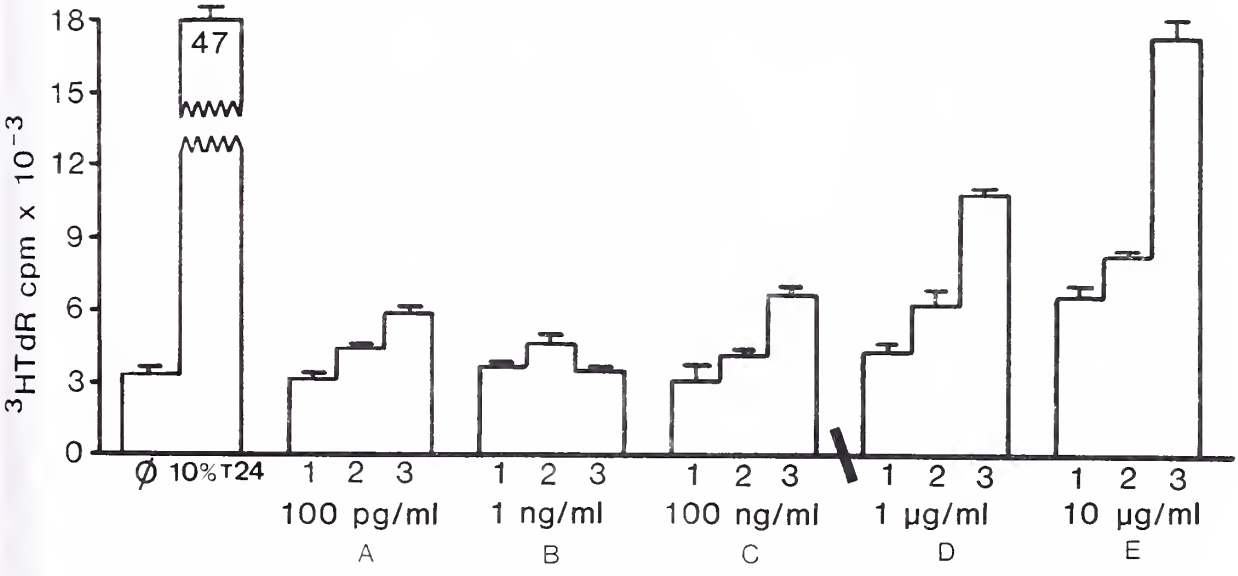


Figure IV.xiv. B-9 assay for IL-6 activity in CMs after exposure to different concentrations of LPS. The negative and positive controls for groups A, B, and C are included as indicated above. For groups D and E the negative control was 4 x 10^3 ^{3}H TdR cpm and the positive control (1.0 % T24 CM) was 60 x 10^3 ^{3}H TdR cpm. For each histogram, 1=.03% CM dilution, 2=.1% and 3=.3%. The x-axis is cross-hatched as described above to represent the inclusion of data from the second, comparable experiment.

IV. xv. TGF-ß and LPS synergize for IL-6 release from osteoblasts. Cells were cultured for 48 hours with LPS alone (at four concentrations), with TGF-ß alone (at 10 ng/ml) or with both agents together. Data is presented in figure IV.xv and illustrate that LPS and TGF-ß as single agents stimulate small or no release of IL-6 activity into CMs. Cells cultured with both TGF-ß and LPS, however, produced CMs with large increases in IL-6 activity over background. The most striking increase in IL-6 production occured in the groups combining TGF-ß and the lower LPS concentrations (10 and 100 ng/ml). These two groups of CMs had IL-6 activity similar to the groups combining TGF-ß and higher LPS concentrations (10^3 and 10^4 ng/ml).

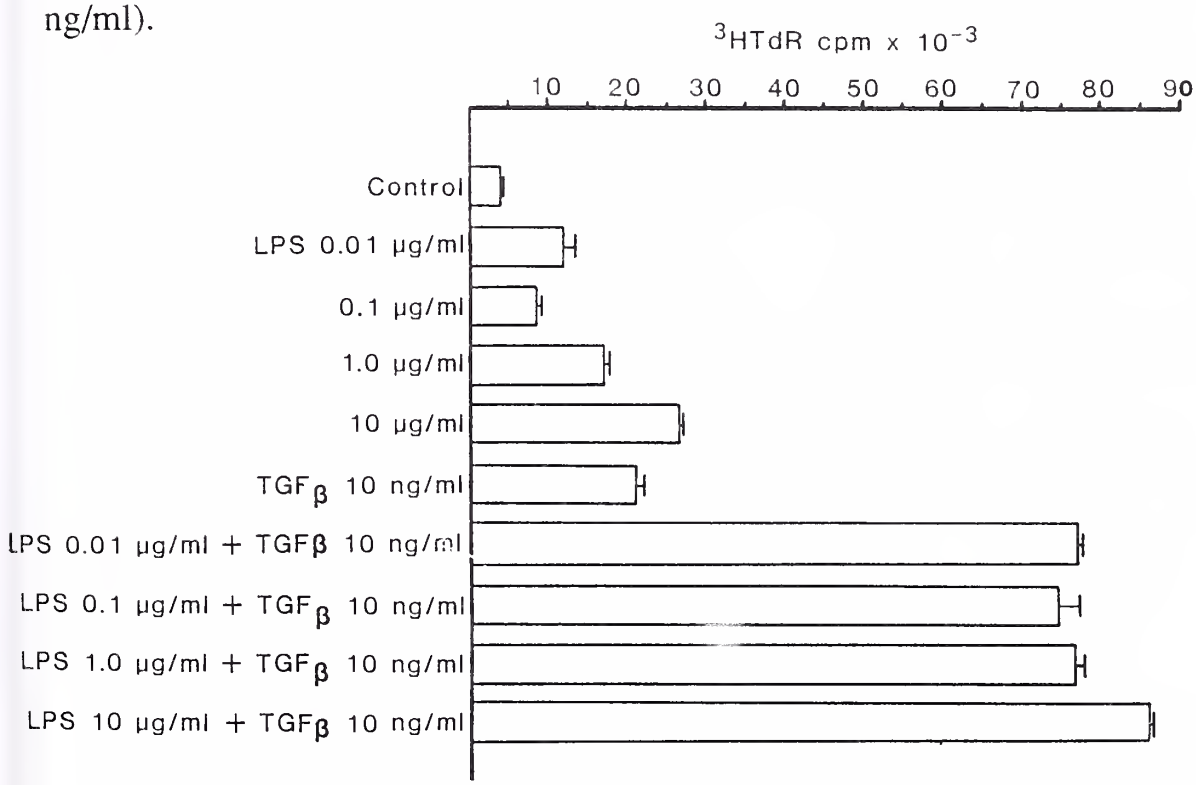


Figure IV.xv. B-9 assay for IL-6 activity in CMs after exposure to different concentrations of LPS alone, TGF-ß alone, and to both agents together for 48 hours. A negative experimental control is included. Negative assay control (B-9 cells alone) = 5 x 10^3 ^{3}H-TdR. Positive assay control (1% T24 CM) = 48 x 10^3 ^{3}H-TdR cpm. CMs tested at .3%

IV.xvi. Release of IL-6 from osteoblasts is more dependent upon TGF-ß than upon LPS. A series of 'priming' experiments were carried out as described in III. B, and similar to the experiments with GM-CSF in IV.v. Data from IV. xv suggested that for the induction of IL-6 activity, low dose LPS may interact with TGF-ß as productively as higher dose LPS. The concentration of LPS used in IV.xvi was therefore lower than that used in experiment IV. xv in order to test the low concentration limit of LPS to synergize with TGF-ß.

TGF-ß as the 'priming' agent: Data is presented below in figure IV.xvi and illustrates that cells exposed to TGF-ß for hours 0-2 manifested little enhancement of IL-6 activity in CMs. Similarly, LPS at 1 pg/ml and 100 pg/ml for hours 2-48 also did not induce IL-6 release. However, cells first exposed to TGF-ß for hours 0-2, washed and then exposed to 1 or 100 pg/ml of LPS for hours 2-48 produced CMs with high IL-6 activity.

LPS as the 'priming' agent: Cells exposed to 1 pg/ml or 100 pg/ml of LPS for hours 0-2 did not produce CMs with increased IL-6 activity. Similarly, cells exposed to TGF-ß for hours 2-48 also failed to show increases in IL-6 release. Cells exposed to 1 pg/ml of LPS, washed and then exposed to 10 ng/ml of TGF-ß produced CMs with two- to three-fold the IL-6 activity of background samples. Cells exposed to 100 pg/ml of LPS, washed and exposed to 10 ng/ml of TGF-ß produced CMs with higher IL-6 activity.

The data in figure IX. xvi suggest (1) that cells can be 'primed' with TGF-ß and activated by LPS for IL-6 release and (2) cells can also be 'primed' by LPS, in a dose-dependent manner, for IL-6 release. These

results are in contrast with the results of IV.v in which GM-CSF release was only obtained after cell 'priming' with TGF-ß. LPS in IV.v failed to primed cells for GM-CSF release.

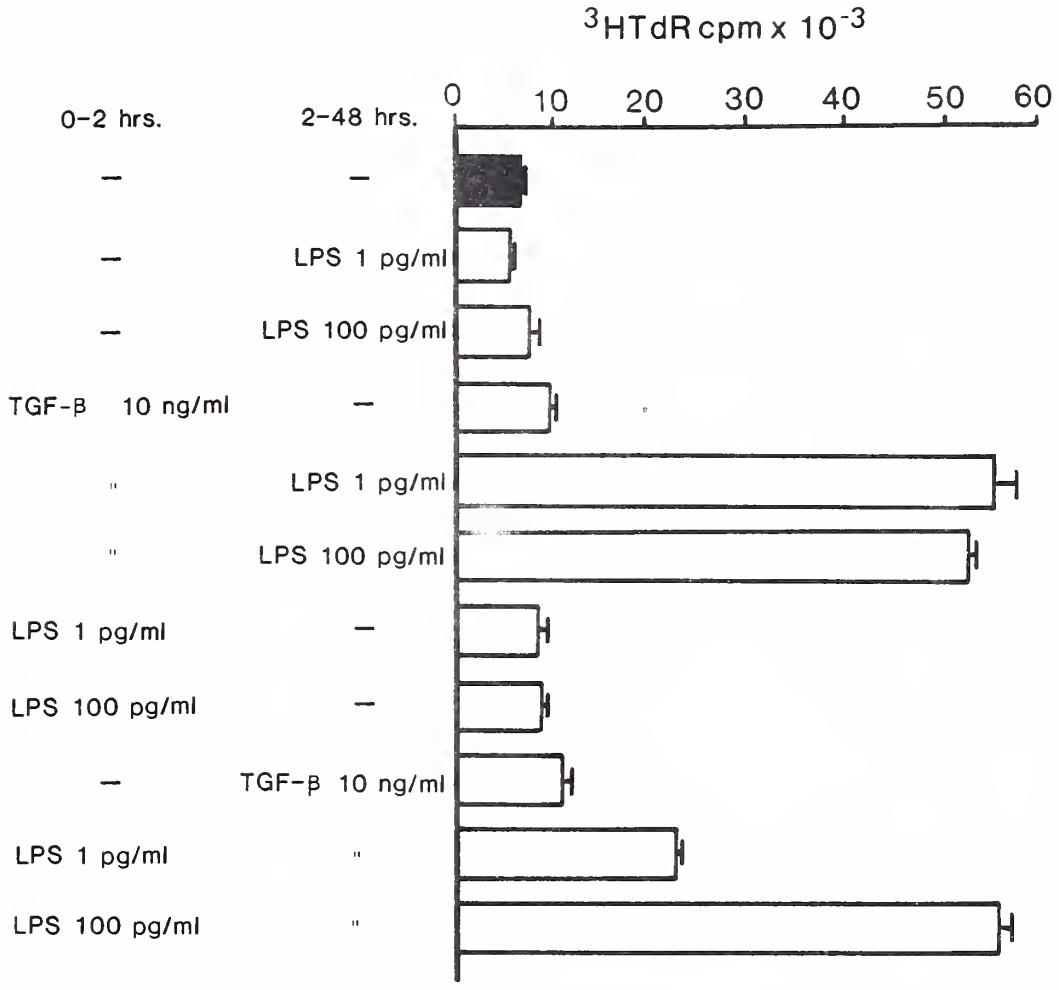


Figure IV.xvi. B-9 assay for IL-6 activity of CMs from cells 'primed' with either TGF-ß or LPS, and then exposed to the corresponding *synergen*. Experimental controls are included. Negative and positive (1.0 % T24 CM) assay controls for this experiment were 7 x 10^3 ^{3}H-TdR and 60 x 10^3 ^{3}H-TdR, respectively. CMs tested at .3 % dilution.

B. STUDIES OF INTERLEUKIN-6: TGF-ß and IL-1

IV.xvii. Low-dose IL-1 alone does not induce IL-6 release but low-dose IL-1 synergizes with TGF-ß to induce a 12-fold increase in IL-6 release.

To test the effect of lower doses of IL-1 on IL-6 release and the effect of

TGF-ß on IL-1 activity, cells were cultured with either IL-1 alone (1, 10 or 10 ng/ml) or with IL-1 and TGF-ß (10 ng/ml). CMs were collected after 48 hours, and tested for IL-6 activity. As presented below in figure IV.xvii, low-dose (1 ng/ml) IL-1 did not induce IL-6 activity in tested CMs. However, 1-100 ng/ml IL-1 synergized with TGF-ß to induce a large increase in the IL-6 activity of tested CMs.

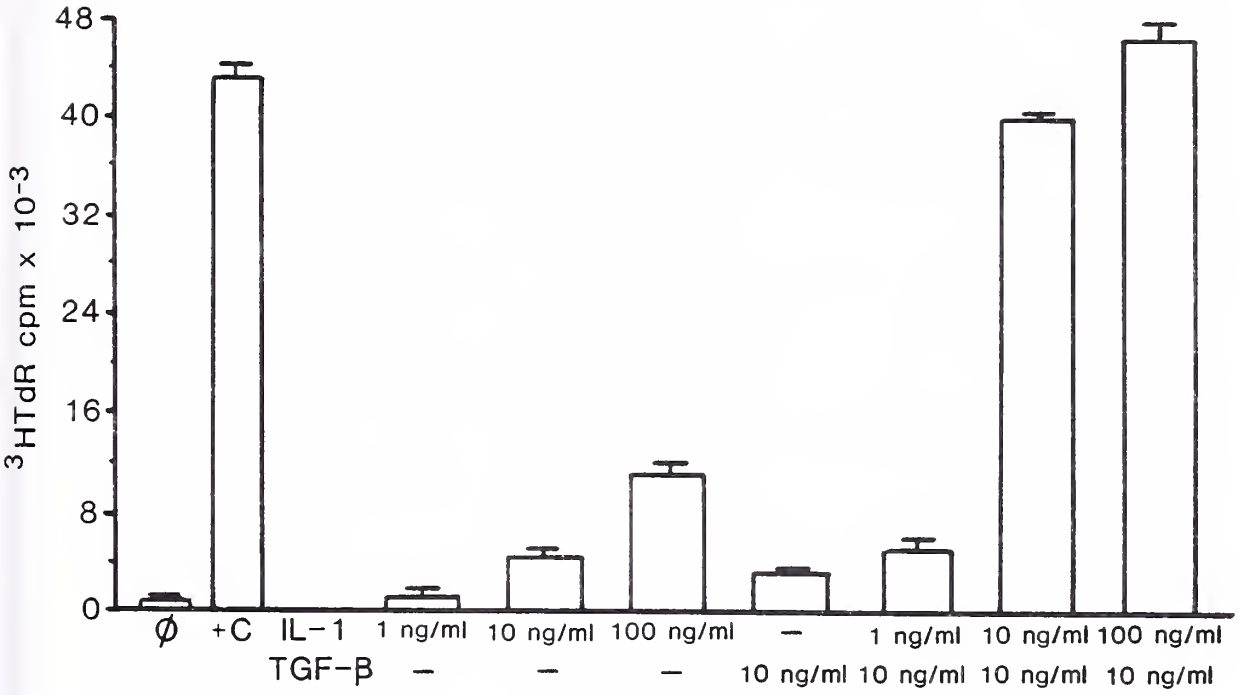


Figure IV.xvii. B-9 assay for IL-6 activity of CMs from cells cultured with either IL-1 alone (1, 10 or 100 ng/ml), TGF-ß alone (10 ng/ml) or with IL-1 and TGF-ß together . Negative (ø) and positive (+C: 1.0 % T24 CM) assay controls are included as described in III.B and table III.i. Experimental background control (no agents) was 1 x 10^3 ^{3}H T-dR cpm. All CMs tested at 0.3 % dilution.

IV.xviii. IL-1/TGF-ß synergy for IL-6 release is not affected by indomethacin exposure.. Prostaglandins are potent inducers of IL-6 release

and may mediate at least part of IL-1 induced bone resorption. To determine whether prostaglandin production by cells derived from rat parietal bone played any role in the observations presented above the following experiment was conducted. Osteoblasts *in vitro* were exposed to

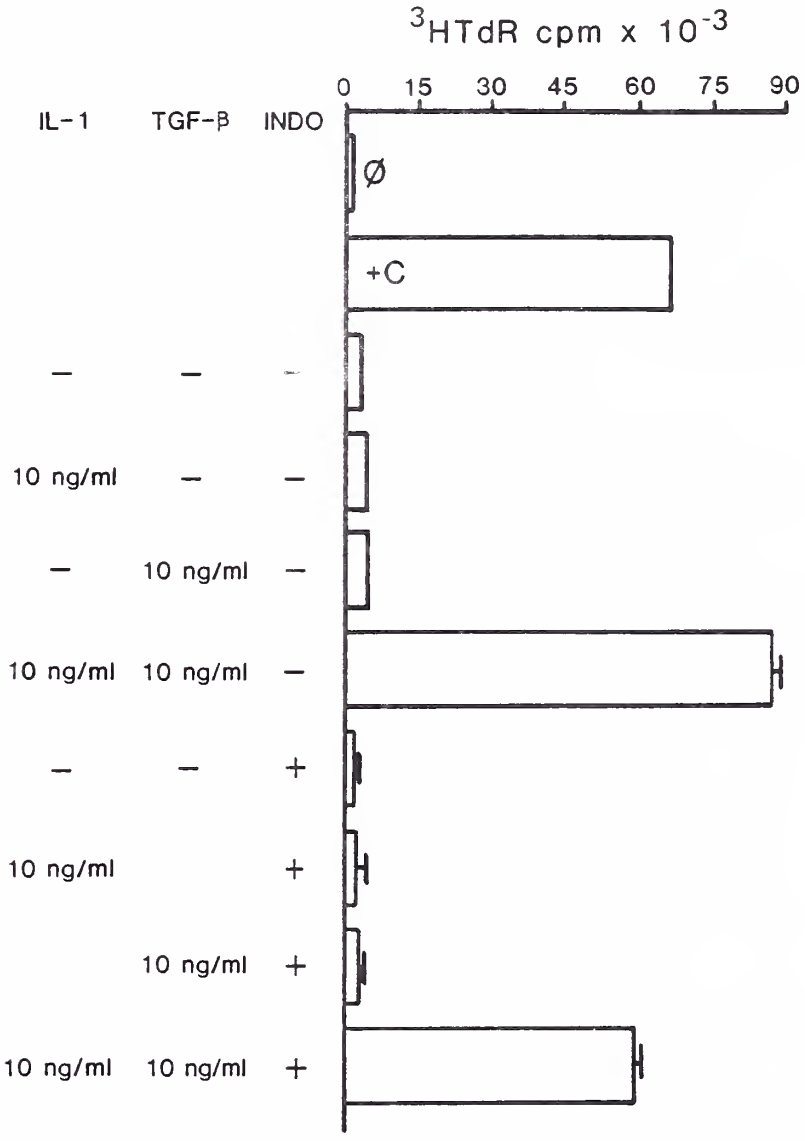


Figure IV.xviii. B-9 assay for IL-6 activity in a series of cyclooxygenase inhibition experiments with indomethacin. Agents were added to cell cultures for the period of time as indicated. Relevant controls are included as indicated: Negative assay control= ø, positive asay control (1.0% T24 CM) =+C. CMs tested at 0.3 % dilution.

either TGF-ß alone or to low-dose IL-1 alone or to both agents together. Additional cells were similarly cultured but with the addition of the non-steroidal antiinflammatory agent indomethacin, a potent inhibitor of prostaglandin production. As shown above in figure IV.xviii, there was no IL-6 activity in CMs from cells cultured with the IL-1 alone or with TGF-ß alone. As expected, TGF-ß and IL-1 synergized to increase IL-6 release >10 fold. The addition of indomethacin to the cultures did not change the IL-6 release response of the osteoblasts cultured with either IL-1 or TGF-ß alone. There was a slight (<5%) increase in the IL-6 released by osteoblasts cultured with all three agents (IL-1, TGF-ß, and indomethacin).

IV.xix.a./b. Most of the TGF-ß/synergen-induced release of IL-6 occurs between hours 0-6, specifically, between hours 2.5 and 6. TGF-ß/LPS and TGF-ß/IL-1 induced large increases in IL-6 activity of CMs after 48 hours of cell treatment, as described above in IV. xv, xvi and xvii. To decribe a more complete spectrum of activity, CMs were collected at hours 0, 6, 22, and 48 of TGF-ß/synergen treatment and analyzed for IL-6 activity as described in III. C 2. A second experiment collected CMs at hours 0, 1, 2.5, and 6 hours after TGF-ß/synergen exposure. As shown below in figure IV.xix.a below, CMs at time zero (CMs collected before addition of agents to media) contained no detectable IL-6 activity compared to background samples. IL-1 alone failed to induce IL-6 activity at any time point. TGF-ß alone induced no activity in IL-6 activity after 6 and 22 hours of treatment but induced activity after 48 hours of treatment. TGF-ß and IL-1 together, however, induced a large increase in IL-6 activity at 6 hours with a 15% increase between hours 6 and 22. All 48 hour CMs are essentially 22 hour CMs which remain in culture dishes for

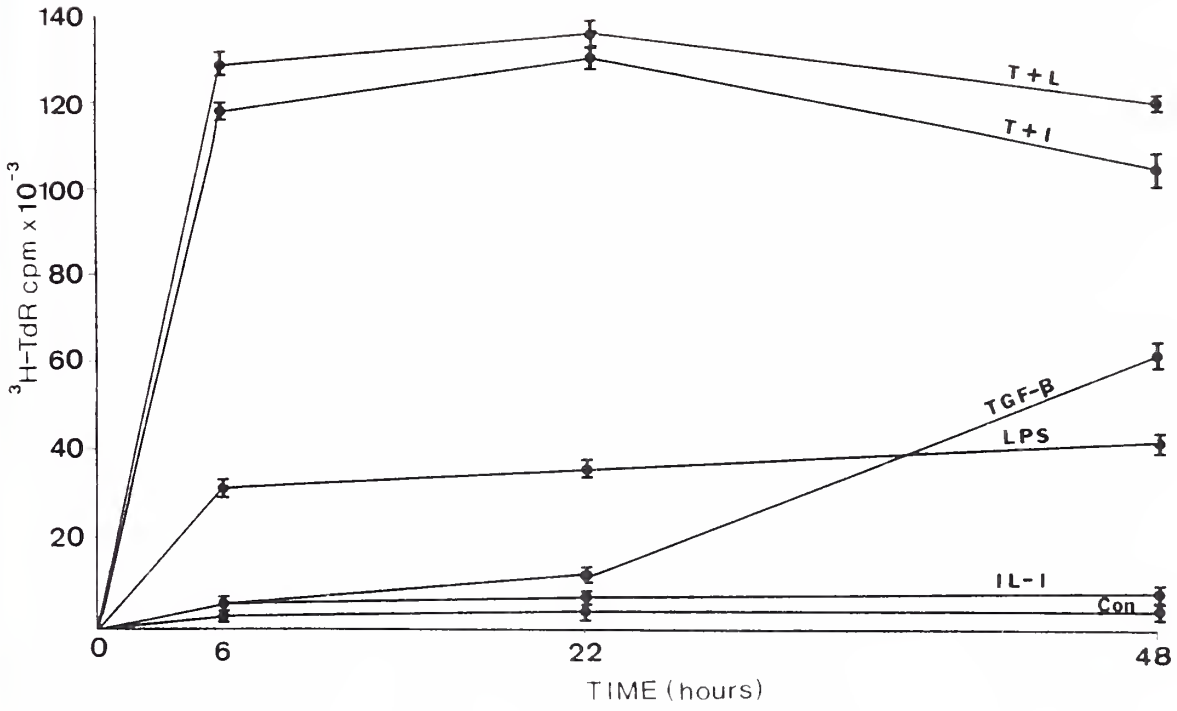
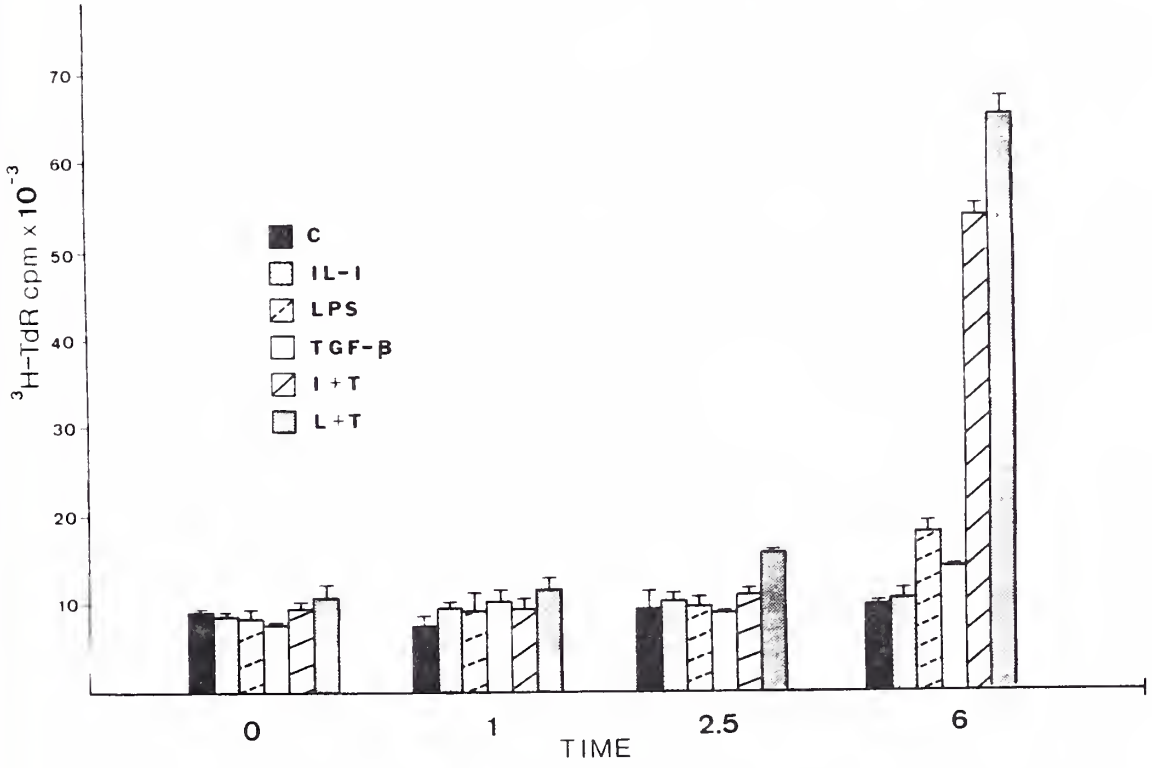


Figure IV.xix.a. B-9 assay for IL-6 release over hours 0-6, 6-22, and 22-48 of exposure to TGF-ß alone, LPS alone, IL-1 alone, TGF-ß with LPS, or TGF-ß with IL-1. Ordinate values are presented as the counts per minute of ^{3}H-Thymidine incorporation by B-9 cells after culture with a given .3% CM sample. Abscissal values are the length of time of CM exposure to a given agent or agents. Negative and positive (1.0 % T24 CM) assay controls were 5 x 10^3 ^{3}H-TdR cpm and 85 x 10^3 ^{3}H-TdR cpm, respectively. Negative experimental control at each time point was always less than 5 x 10^3 ^{3}H-TdR cpm.

an additional 26 hours. It was therefore noteworthy that there was an apparent *decrease* in IL-6 activity in 48 hour CMs from 22 hour CMs, as presented in figure IV. xix a. This difference may not accurately reflect true changes in IL-6 quantity in 48 hour CMs. Unless there is an active degredation or binding of IL-6 molecules between 22 and 48 hours, it is assumed that at least an equal quantity of IL-6 remains at 48 hours and that the decrease in IL-6 activity at 48 hours is an assay artefact.

LPS as a single agent induced a small increase in IL-6 activity in 6 hour CMs, but the level of activity remained the same over the following 42 hours. TGF-ß and LPS together induced a large increase in IL-6 at 6 hours with additional, smaller increases occurring up to 22 hours. As discussed above, 48 hour CMs from cells exposed to both TGF-ß and LPS apparently had less IL-6 activity than CMs from 22 hours.

The second experiment concerning the time interval 0 to 6 hours showed that the largest increases in IL-6 activity was achieved between hours 2.5 and 6, as presented in figure IV.xix b below. There was no



IV.xix.b. B-9 assay for IL-6 activtiy in .3% CMs collected at 0, 1, 2.5 and 6 hours after exposure to indicated agents. are devoid of IL-6 activity. IL-1, LPS and TGF-ß as single agents fail to induce significant increases in IL-6 activity at the time points indicated. TGF-ß/IL-1 and TGF-ß/LPS induce comparably large increases in IL-6 activity only after 6 hours of treatment. These data suggest that the greatest rate of IL-6 release by TGF-ß and a given synergen occurs between 2.5 and 6 hours of treatment. Control=media alone, IL-1=10 ng/ml, LPS=10 ng/ml, TGF-ß=10 ng/ml. Negative assay control (assay cells alone) was 8000 ^{3}HTdR cpm of activity and the positive control (T24) was 65000 ^{3}HTdR cpm of activity.

detectable IL-6 activity of the CMs between hours 0 and 2.5. As the data from IV.xix a/b suggest, the kinetics of TGF-ß synergy with LPS for IL-6 release is similar to the kinetics of TGF-ß synergy with IL-1: Maximum release is obtained between hours 2.5 and 6 of treatment, with a large decrease in the rate cytokine release over hours 6 through 48. These kinetics, summarized below in IV.xx, are also similar to the kinetics of the above *synergens* for GM-CSF release (IV.vii and IV.xi) suggesting that the effects of TGF-ß for cytokine release may be achieved through common pathways.

IV.xx. The rate of IL-6 release is at a maximum between hours 0-6, a rate 18-20 times higher than the rate of release between hours 6 & 22 and between 22 & 48.

As presented in IV.xix.a. above, the largest increase in IL-6 release occurs between hours 0 and 6, while smaller increases occur over the subsequent 42 hours. These differences are presented in figure IV.xx below as the rates of change in IL-6 release, or Δcpm/Δtime. As illlustrated in figure IV. xx, TGF-ß and LPS induce an increase in the rate of IL-6 release to a maximum between 0 and 6 hours of treatment . The rate of release over the next 16 hours drops 90-95% to levels comparable to background. Finally, the rate of IL-6 release over hours 22-48 remains unchanged from the 6-22 hour release rate and is essentially background.

Data from figure IV.xix.b suggests that cytokine released between 0 and 6 hours is released mostly between hours 2.5 and 6 suggesting that there is at least a 2.5 hour exposure period required before any IL-6 can be released.

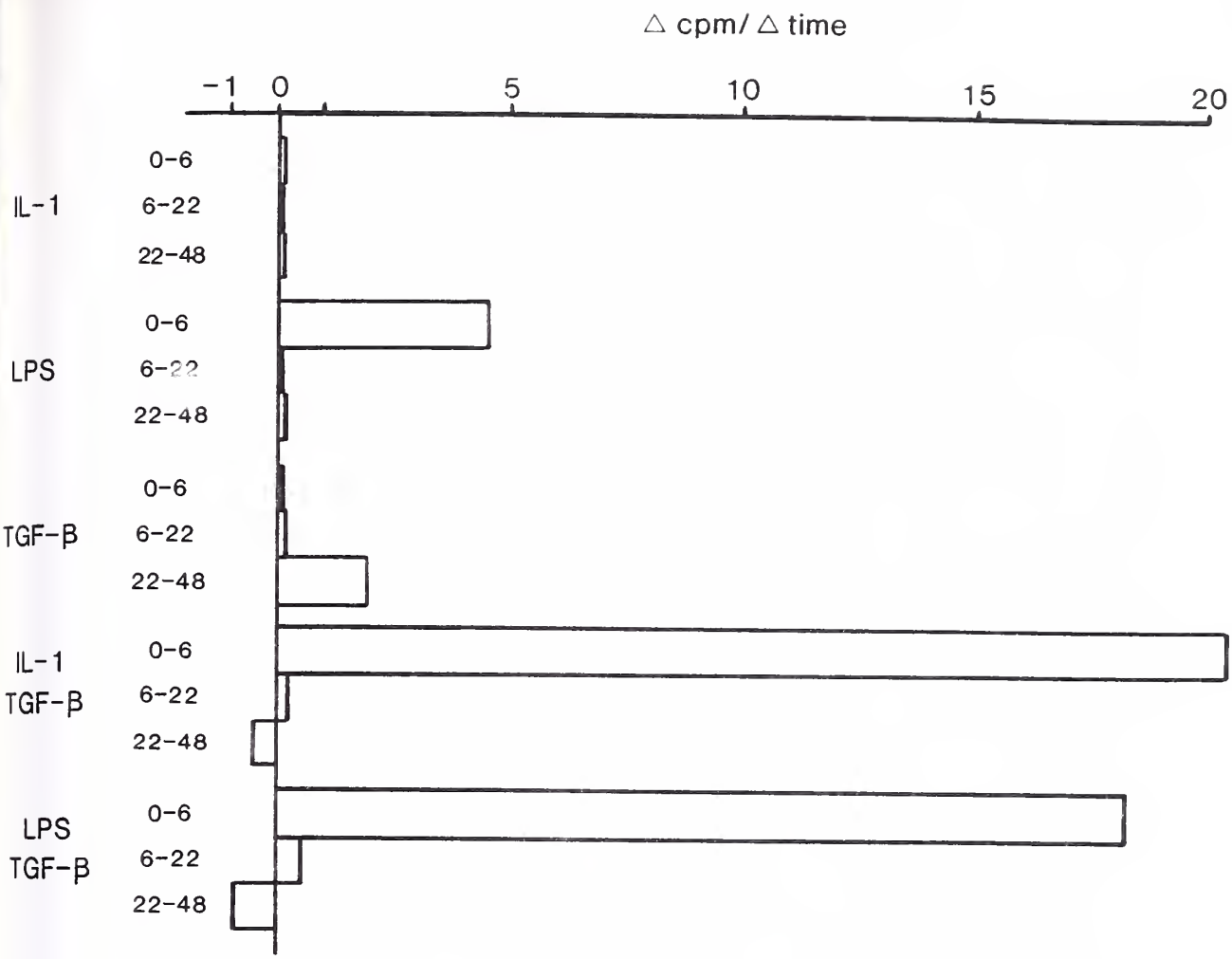


Figure IV.xx. Rates of change in IL-6 release in cells exposed to TGF-ß + LPS or TGF-ß + IL-1, or single agents, for different intervals of time. The rate of change was determined as described in III. E using the data presented in IV.xviii a. The incorporated radioactivity in B-9 cells induced by a given sample of CM is quantitated as counts per minute (cpm). The cpm value (Δcpm) is divided by the time interval in which a given CM was produced (Δtime), either 6 hours, 22 hours, or 48 hours. The ratio of the change in cpm value of a given CM to the time difference between given CMs is presented as the rate of change (Δcpm/Δtime) in IL-6 release.

C. STUDIES OF INTERLEUKIN-6: TGF-ß and PTH

IV.xxi. Parathyroid hormone (PTH) alone does not induce IL-6 release, but higher doses of PTH synergize with TGF-ß to induce a large increase in IL-6 release. Experiment IV.viii suggested that PTH had no clear effects on osteoblast release of GM-CSF. To determine the effect of PTH on IL-6 release and whether TGF-ß could change the profile of PTH

activity, cells were cultured with 0.02 to 20.0 nM PTH alone, TGF-ß alone (0.4 nM or with both PTH and TGF-ß (0.4 nM). As described in III.C 2, CMs were collected after 48 hours incubation and assayed for IL-6 activity in the B-9 hybridoma assay. Data presented below in figure IV.xxi suggest that PTH alone does not induce IL-6 release. 2 or 20 nM PTH synergizes with TGF-ß to induce an increase in IL-6 activity in tested CMs. Note that the effect of 2.0 nM PTH with TGF-ß is largely additive for the induction of IL-6 release.

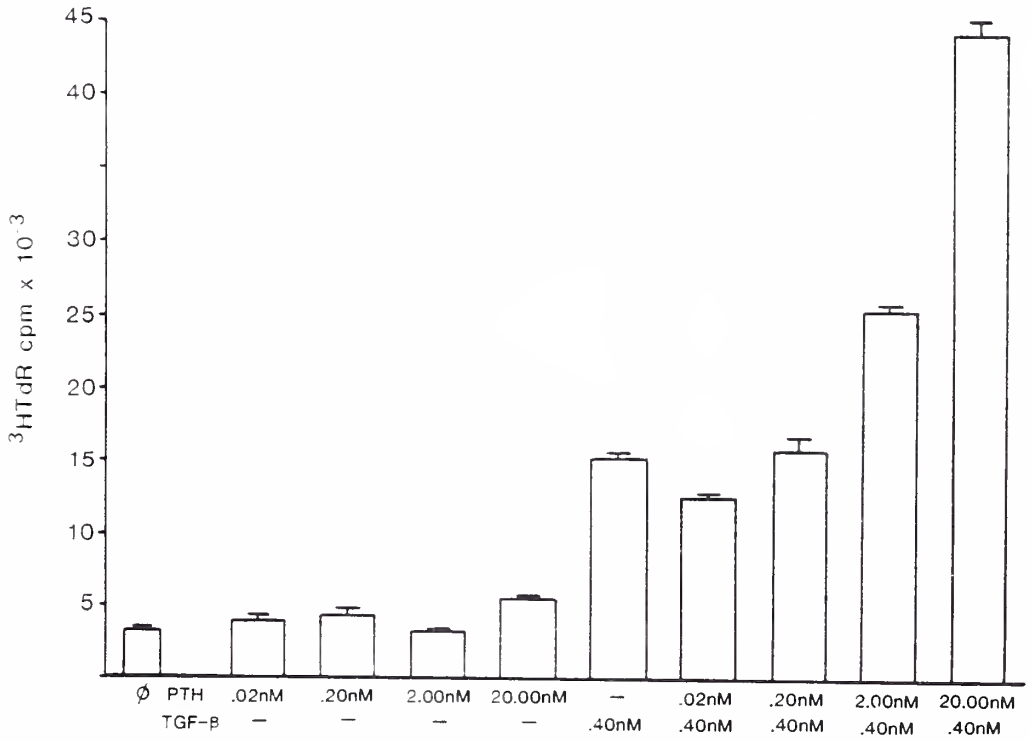


Figure IV. xxi. B-9 assay for IL-6 activity of .3% CMs from cells cultured with either 2.0 nM or 20 nM PTH alone, or with both PTH (.02, 0.2, 2.0 or 20 nM) and TGF-ß (0.4 nM). Negative experimental control (ø) is included. Negative and positive (1.0 % T24 CM) assay controls were 4 x 10^3 ^{3}H-TdR cpm and 45 x 10^3 ^{3}H-TdR cpm, respectively.

IV. xxii. PTH/TGF-ß synergy for IL-6 release may be TGF-ß dependent. As described in III. B, a series of 'priming' experiments were done in which osteoblasts were exposed to TGF-ß alone for two hours at a concentration of 400 pM. The cells were then washed, and then incubated with PTH (.20, 2.0 or 20 nM) for the next 46 hours. The converse experiment, namely PTH 'priming' followed by TGF-ß exposure, was not performed. As shown below in figure IV.xxii, there was no IL-6 activity in CMs from cells cultured with TGF-ß alone for hours 0-2, and then with fresh media alone for hours 2-48. However, cells incubated with TGF-ß for hours 0-2 and then PTH for hours 2-48 produced CMs with the high IL-6 activity seen in previous experiments.

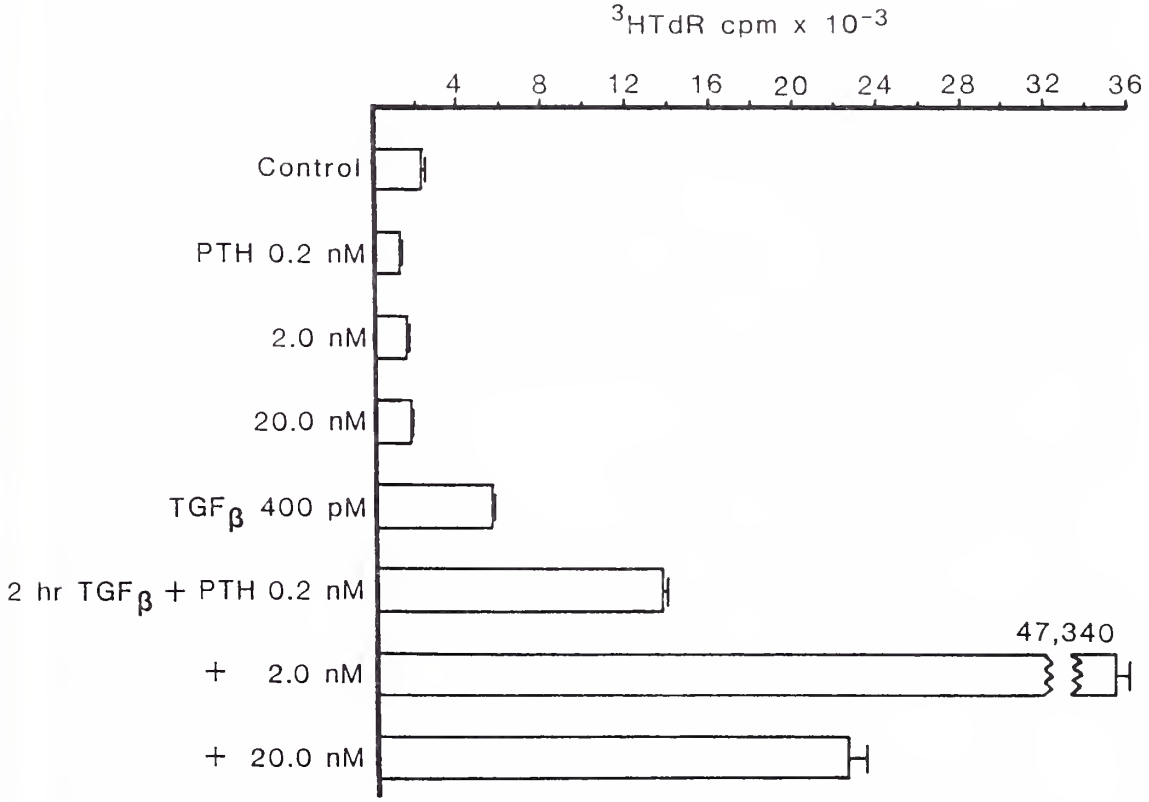


Figure IV.xxii. B-9 assay for IL-6 activity in a series of 'priming' experiments. Agents were added to cell cultures for the period of time as indicated. Experimental control is included. Negative and positive (1.0 % T24 CM) assay controls were 4 x 10^3 ^{3}H-TdR and 45 x 10^3 ^{3}H-TdR cpm, respectively. CMs asayed at a .3% dilution.

C. STUDIES OF M-CSF

IV. xxiii. TGF-ß does not stimulate the secretion of M-CSF. As described in III.B, osteoblasts were cultured in media alone or in media with TGF-ß at three different concentrations, 0.1 ng/ml, 1.0 ng/ml or 10 ng/ml. CMs were collected and assayed for Macrophage-CSF (M-CSF) using the BAC M-CSF-dependent macrophage line.

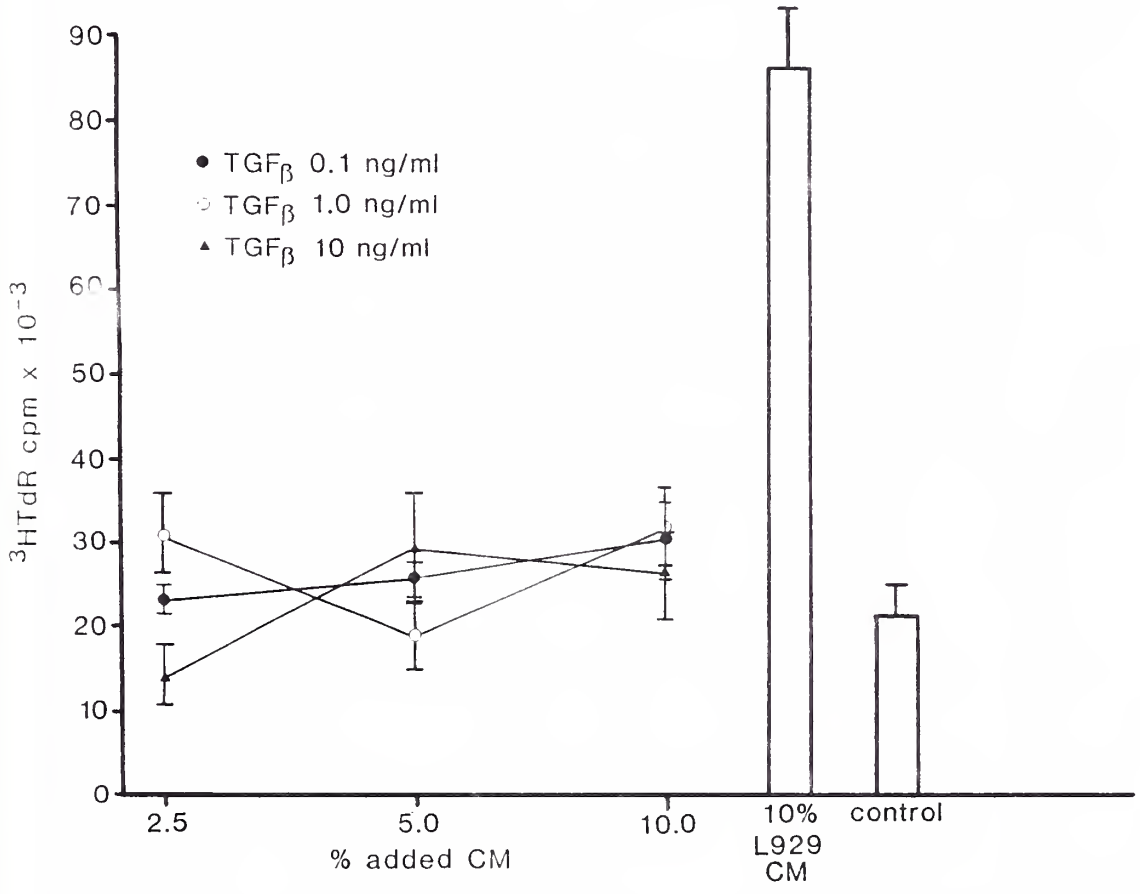


Figure xxiii. BAC cell assay for M-CSF activity of CMs from osteoblasts cultured with one of three concentrations of TGF-ß: 100 pg/ml, 1.0 ng/ml, or 10 ng/ml. The abcissal values correspond to the counts per minute of incorporated tritiated thymidine by the BAC cells in the M-CSF assay. The ordinate values are indicated per diagram. Assay positive (10 % L929 CM) and negative controls are included while the experimental control (10 % media alone) value was 26 x 10^3 ^{3}H-TdR cpm.

As presented in figure IV.xxiii above, TGF-ß at the concentrations tested did not induce any M-CSF release from osteoblasts detectable in the CMs

IV.xxiv. TGF-ß and LPS fail to synergize for M-CSF release from osteoblasts. Osteoblasts were cultured with either TGF-ß, LPS, or TGF-ß and LPS together as described in section III. B. CMs were tested for M-CSF activity as outlined above and in table III.i. As presented in figure IV. xxiv below, 100 ng/ml LPS induced M-CSF activity in tested CMs as reported previously[87]. However, TGF-ß had little or no effect on the osteoblast response to either dose of LPS for M-CSF release.

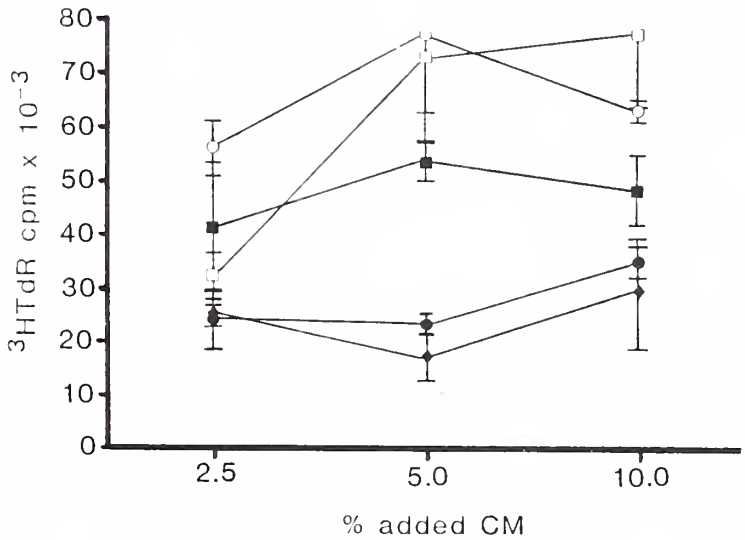


Figure IV.xxiv. BAC cell assay for M-CSF showing relative amounts of activity of tested CMs. ● =TGF-ß at 10 ng/ml, ◆ = LPS 10 ng/ml, ○ = LPS 100 ng/ml, ■ = TGF-ß at 10 ng/ml + LPS at 10 ng/ml, □ = TGF-ß at 10 ng/ml + LPS at 100 ng/ml. The abcissal values correspond to the counts per minute of incorporated tritiated thymidine by the BAC cells in the M-CSF assay. The ordinate values are indicated as % CM tested. The negative and positive (10 % L929 CM) assay control values were 14 and 135 x 10^3 ^{3}H-TdR cpm, respectively. The negative experimental control (media alone) value was 22 x 10^3 ^{3}H-TdR cpm.

D. BONE MARROW INDUCTION STUDIES

IV. xxv. TGF-Beta and IL-1 do not induce the proliferation of total bone marrow cells from C3H/HeJ mice. As described in III. A, bone marrow cells were obtained from the long bones of C3H/HeJ mice. C3H-HeJ mice carry a recessive gene which renders them unable to respond to most forms of lipopolysaccharide (M. Horowitz, personal communication). C3H/HeJ cells were therefore selected to test any stimulatory effect of LPS on the assay cells described in IV.A-C and to test the effect of LPS and TGF-ß on total marrow populations. Marrow cells were

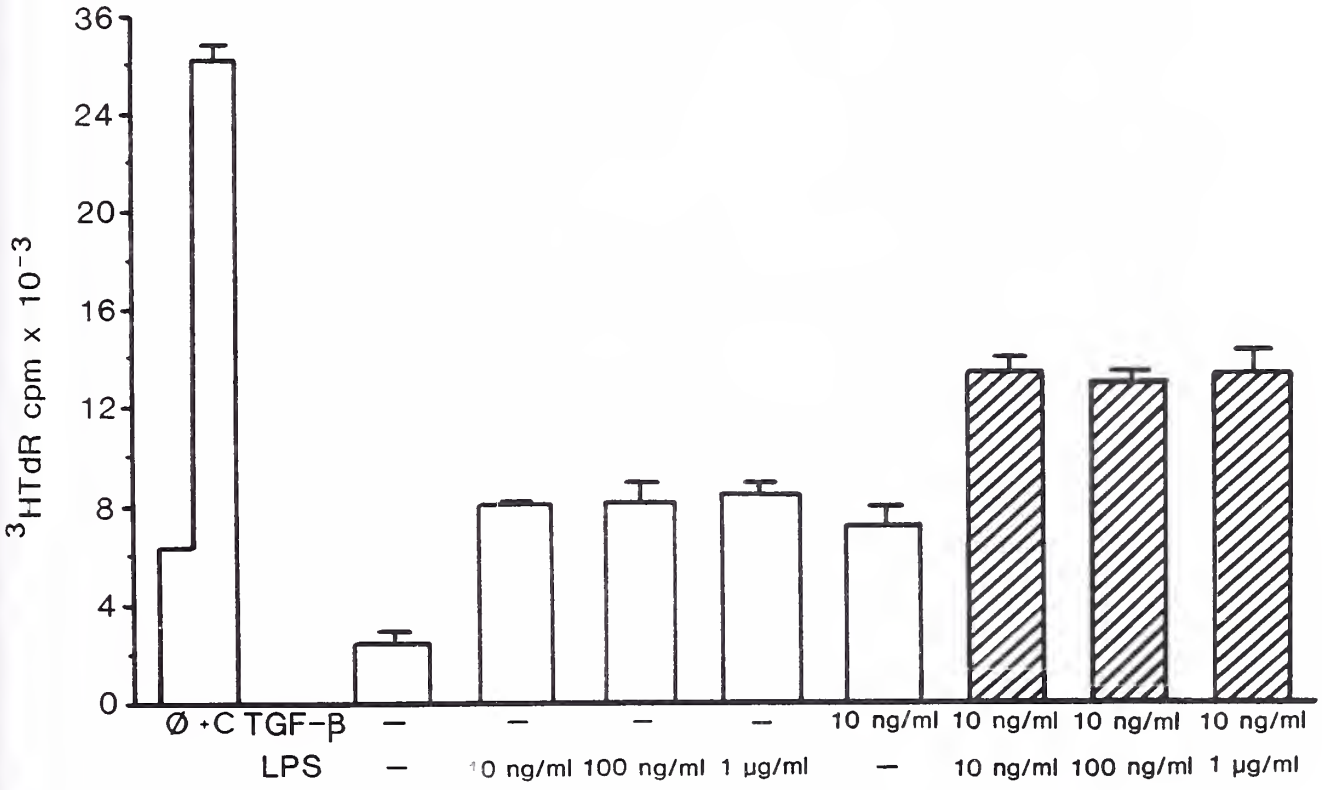


Figure IV.xxv. 12 hour incorporation of tritiated thymidine by C3H/HeJ marrow cells during exposure to CMs from experiment IV.xxiii. Ordinate values are indicated. Negative (ø) and positive (+C:L929) assay control groups are indicated.

cultured with TGF-Beta (10 ng/ml), with LPS (10-1000 ng/ml) or with both agents together. As shown in figure IV.xxv above, the marrow cells exposed to both TGF-ß and any concentration of LPS showed a small increase in proliferation over control groups as measured by uptake of tritiated thymidine. The additive effect of LPS and TGF-ß for marrow cell proliferation is less the synergistic effects of LPS and TGF-ß presented in previous sections. One may note, however, that the present experiment utilized total marrow populations and not specific assay cells (i.e. HT-2) responsive to a limited number of cytokines. The marrow population contains *all* cell types- the number of cells represented which are responsive to TGF-ß/LPS-induced cytokines may be too small to mount an observably high increase in proliferation

E. STUDIES WITH PLATELET-DERIVED GROWTH FACTOR

IV. xxvi. PDGF and IL-1 synergize to increase GM-CSF production.

TGF-ß may control the bimodal growth response of osteoblasts to the hormone via the autocrine elaboration of PDGF-AA, -AB, and/or -BB and the control of the PDGF receptor[102,103]. IL-1 has been found to influence mitogenesis of fibroblasts and smooth muscle cells (SMCs) by an analogous induction of PDGF release. Osteoblasts are known secretors of PDGF-BB but express receptors for both PDGF-AA and PDGF-BB. It was hypothesized that the synergy between TGF-ß and IL-1 for cytokine release may be due to an autocrine PDGF loop. To test the hypothesis, osteoblasts were cultured in media alone, with PDGF-AA, with PDGF-BB, with IL-1 or with IL-1 plus one of the two PDGF isoforms. CMs were assayed for GM-CSF activity. Data presented below in figure IV. xxvi

show that IL-1 and PDGF-BB synergized to enhance GM-CSF release from osteoblasts but the effects of IL-1 and PDGF-AA was not as pronounced.

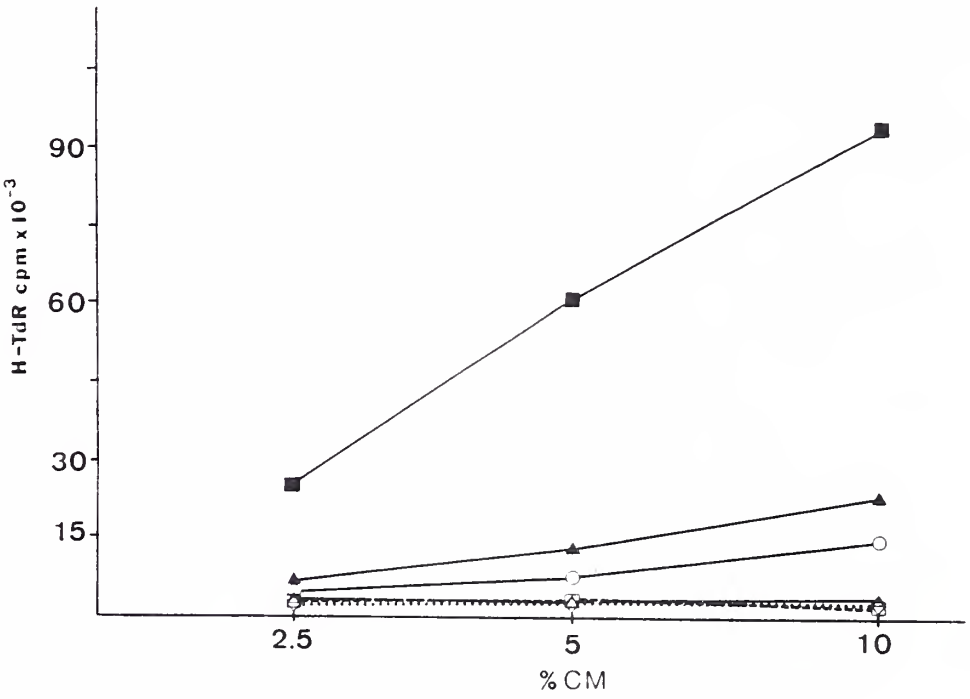


Figure IV. xxvi. HT-2 assay for GM-CSF activity: Effects of IL-1 and PDGF isoforms on the release of three cytokines from osteoblasts. ◇= assay control, ○=IL-1 10 ng/ml, △=PDGF-AA 10 ng/ml, □=PDGF-BB 10 ng/ml, ●=IL-1 + PDGF-AA each 10 ng/ml, ■=IL-1 + PDGF-BB each 10 ng/ml. Negative and positive (10% rh-IL-2) controls had values of 0.7 x 10^{-3} ^{3}H-TdR cpm and 95.0 x 10^{-3} ^{3}H-TdR cpm , respectively. CMs tested at .3% dilution.

V. DISCUSSION Summary of findings; Possible mechanisms of TGF-ß synergy and roles in bone remodeling and hematopoesis; Unifying concepts of TGF-ß in cell biology; Future studies

Normal bone remodeling is a dynamic process in which the concerted action of osteoblasts and osteoclasts can replace the entire human skeleton with new bone in less than three years. Normal bone formation requires the osteoblast to regulate its growth, to respond appropriately to extracellular signals and to function cooperatively among a population of other cells. Abnormal bone formation and bone disease is the result of defects in these processes. The complexity of normal bone formation and the difficulty of understanding the process of bone disease may be appreciated by the gross examination of pathologic specimens of bone. The following case of osteomyelitis from the American Civil War is particularly striking, as the severity of disease is seldom seen in today's world of antibiotics.

On 15 June, 1865, Corporal Walter Ford of the 1st United States Colored Troops was wounded in the left leg by a 1/4 inch ball fired in an antebellum incident in Petersburg, Virginia. Taken to a Baltimore hospital, the 20 year-old Ford struggled with erysipelas, a recurrent wound infection and "continual pain"*. The corporal eventually died of "general fatty degeneration and leukocythaemia"* in February of 1866. Army surgeons had been collecting traumatized bone specimens throughout the war to medically categorize the spectrum of war injuries and so preserved Ford's left femur, now in permanent storage at the Army Medical Museum in Washington, D.C. As shown in figure V.i a below, the femur was

*U.S. Army Surgeon's Report, 1882

was fractured completely midshaft. An irregular bone callous joined the proximal ends with disorganised, abnormal bone occurring in areas far from the immediate trauma locus. In contrast, in figure

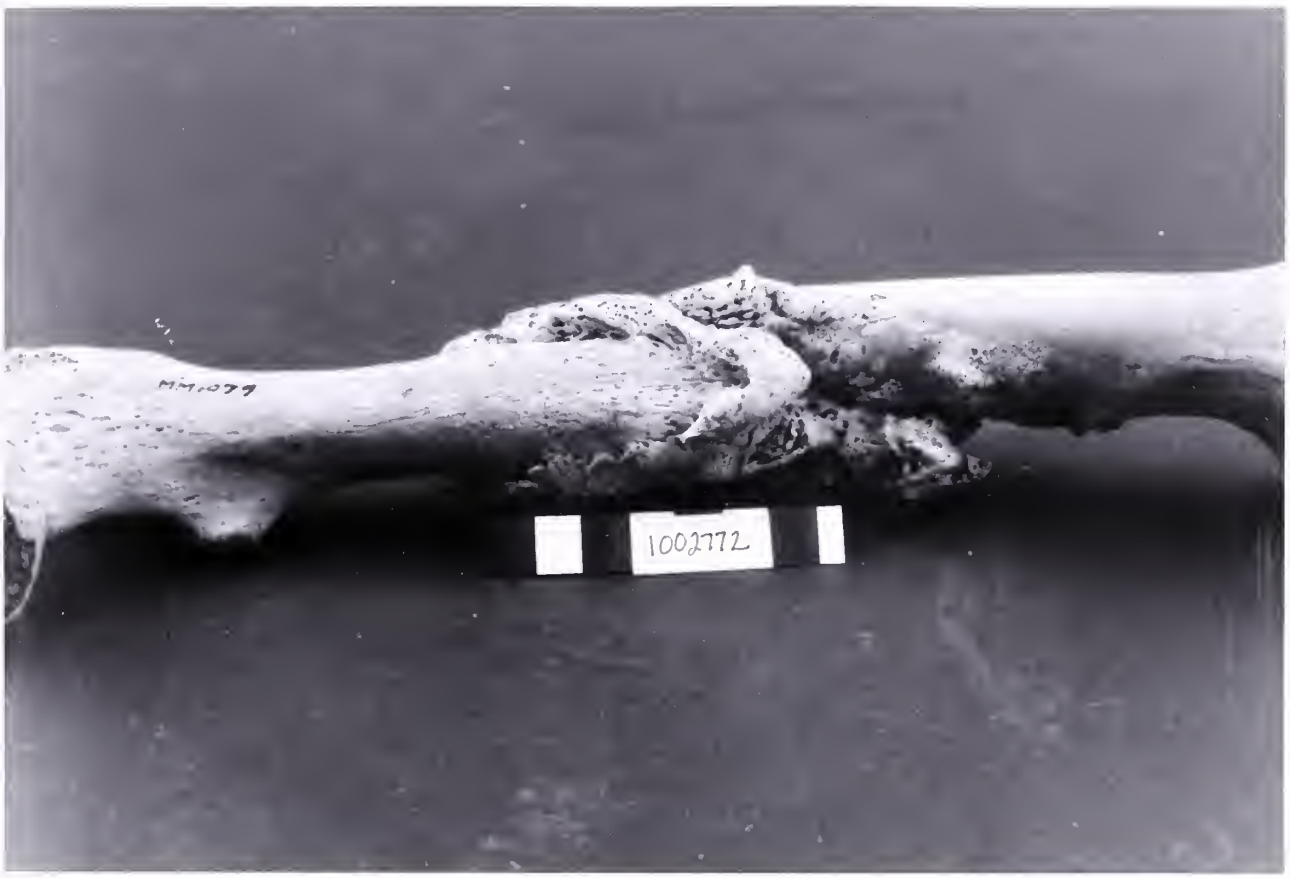


Figure V.i.a. Left femur of Corporal Walter Ford, 1st United States Colored Troops, who sustained a "shot fracture" of the leg in June, 1865, suffered months with recurrent infections and eventually succumbed in February, 1866. The haphazard and irregular bone formation typical of osteomyelitic remodeling which produces a brittle, porous bone. Photograph courtesy of the Department of Defense, Armed Forces Institute of Pathology.

V.i.b, is the normal bone formation of a similar fracture of the right femur from a Peruvian male dated circa 2000 B.C. Corporal Ford's case is important in that it addresses aspects of bone biology essential to this thesis: First, what signals do osteoblasts use to form bone in a regulated, polarized fashion in normal or traumatic bone remodeling? Second, how can such signals be changed in an infected

locus to produce distorted, haphazard bone? And third, are defects in these processes important in the development of bone diseases and cancer? A better understanding of these challenging issues may

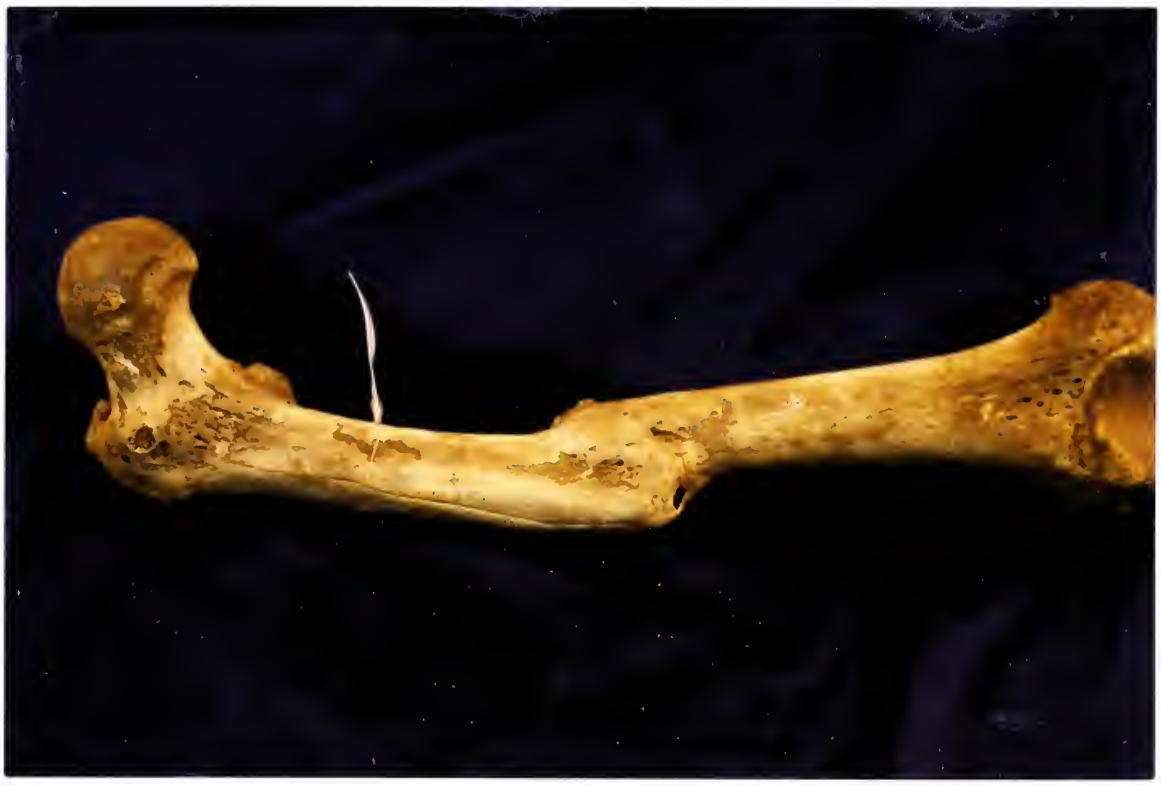


Figure V.i.b. Right femur of a Peruvian male, dated 2000 B.C. Fracture most likely the result of blunt injury or fall, suggesting the wound was closed and sterile. The presumably aseptic fracture has been healed by a regular, organized process of bone remodeling in marked contrast with that of figure V.i.a. Photograph courtesy of the Department of Defense, Armed Forces Institute of Pathology.

come since the discovery of several unique endocrine properties of the normal osteoblast and its environ:

It has been recently demonstrated that osteoblasts are among the body's most active producers of cytokines, a hormone class implicated in the control of hematopoesis and other growth processes. Osteoblast cytokine production has been studied for roles in bone metabolism and in fracture healing, but a definitive role for cytokines in bone remodeling is not yet clear[29,87].

Transforming Growth Factor-Beta (TGF-ß) is a platelet-derived hormone which is stored in the matrix of bone. TGF-ß is a pleotropic protein whose actions range from immunosuppression to matrix upregulation[167]. This thesis presents experiments which studied the effects of TGF-ß on osteoblast cytokine release in vitro. We present evidence that TGF-ß has multiple effects upon osteoblast cytokine release which can be summarized below and in figure V.ii:

(1) TGF-ß induces IL-6 but not GM-CSF release from osteoblast *in vitro*.

(2) Parathyroid hormone (PTH), and low dose lipopolysaccharide (LPS) and Interleukin-1 (IL-1) are *not* inducers of cytokine release from osteoblasts *in vitro*.

(3) TGF-ß synergizes with low dose LPS* or IL-1* to induce the secretion of both GM-CSF and IL-6 from osteoblasts *in vitro*.

(4) TGF-ß synergizes with PTH* to induce the release of IL-6 from osteoblasts *in vitro*.

(5) The enhanced cytokine production by TGF-ß and a *synergen* is not ablated by indomethacin.

(6) TGF-ß functions to "prime" osteoblasts to respond to LPS and PTH.

(7) The *rate* of cytokine release is at a maximum between 0 and 6 hours after exposure to TGF-ß and a

*Any molecule discussed in this thesis which synergizes with TGF-ß for cytokine release will, for clarity, be referred to as a *synergen*.

synergen. The rate of cytokine release falls to baseline thereafter.

(8) TGF-ß fails to upregulate the release of M-CSF from osteoblasts *in vitro* and does *not* enhance LPS-induced release of M-CSF.

These experiments are the first to characterize a synergistic relationship between TGF-ß and other molecules to enhance cytokine release from bone cells.

Furthermore, this is one of the first reports of an ability of TGF-ß to upregulate cytokine release from any cell type. The observations are unusual in that TGF-ß has previously been regarded as a negative growth regulator and as an immunosuppressive agent.

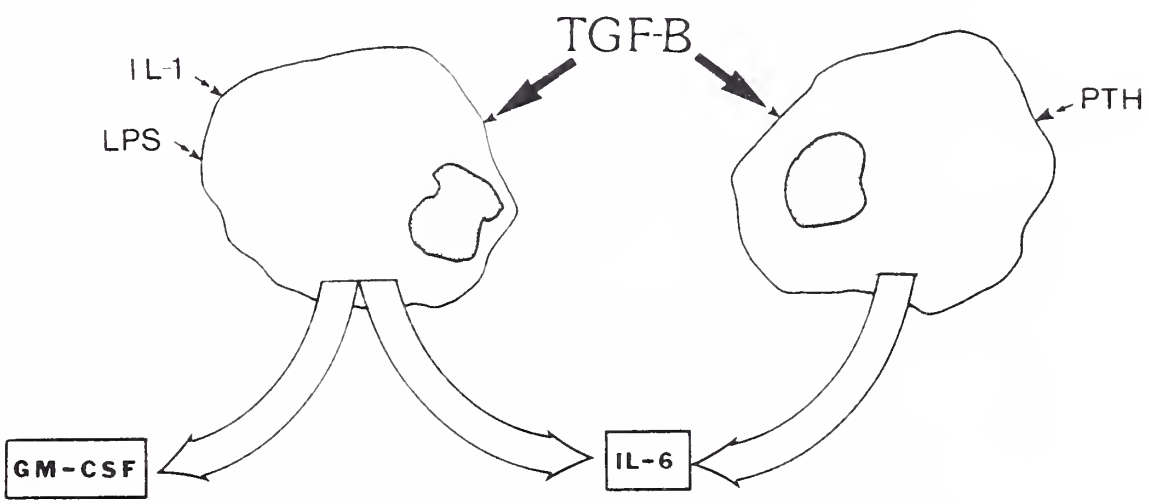


Figure V.ii. Effects of TGF-ß and various synergens on osteoblast cytokine release. TGF-ß and PTH will synergize to induce the release of IL-6 from osteoblasts. TGF-ß can also synergize with either IL-1 or LPS to induce the release of IL-6 as well as GM-CSF.

Its ability, however, to upregulate fibroblast matrix formation and to induce neoplastic growth in normal fibroblasts underlies the pluripotent affects of TGF-ß on cell biology.

According to the data presented in this thesis, TGF-ß confers upon osteoblasts the ability to respond to several unrelated molecules in a similar way with greatly upregulated cytokine release. The characteristics of each response, however, suggest that osteoblasts respond to TGF-ß in a regulated, hormone-specific manner, and that the response is important to the cell as it is preserved even after 24 hours of serum deprivation. These hypotheses are supported by data which are discussed separately in the following sections: Cell Purity, Cell Dormancy and Reactivation, Response Rapidity, Cytokine Specificity, TGF-ß dependence, Mechanisms of Synergy, and New Elements of Bone Remodeling and Hematopoesis.

Cell Purity. No method in the contemporary literature achieves cell cultures from bone which are 100% osteoblasts. The extraction procedure by Wong et al.[208], however, produces osteoblast cultures with 95% purity as determined by analyses of specific protein marker profiles[33b]. Wong et al. employed a collagenase digestion technique to isolate fetal osteoblasts and is the protocol used in this thesis. Known contaminating cells include fibroblasts and tissue macrophages. Fibroblasts are known producers of some cytokines. The fibroblast contribution to the magnitude of cytokine release observed in these experiments may be small, based on the actual number of such cells identified in osteoblast preparations[33b,208]. Macrophages, however, despite low

numbers, can secrete Interleukin-1, which may potently alter the osteoblast response to TGF-ß. To test this possibility, all CMs assayed were analyzed for inherent Interleukin-1 activity as described in III. A and C using the LBRM assay. No significant IL-1 activity was detected in any conditioned medium from cultured osteoblasts, regardless of the type of added stimulatory reagent (data not presented). The sensitivity of the LBRM assay allows a determination of IL-1 activity from concentrations as small as 10 pg/ml (Horowitz, M., pers. comm. and Zltonik et al.[213b]). The cultures of osteoblasts used in these studies were, therefore, relatively pure. The cytokine activity of each tested CM was most likely derived from osteoblast activity and not from contaminating co-inhabitants of fetal rat parietal bone.

Cell Dormancy and Reactivation. As discussed in section II. B, all osteoblasts cultured for these studies were serum-deprived *in vitro* for 24 hours in media alone prior to experimentation. Cells cannot grow *in vitro* in serum-deprived media, but will survive for periods of time. Normal cells respond to the serum-deprived state by shutting off cellular activities not essential towards the maintainence of basic cellular integrity. Serum-deprived cells leave the active cell cycle and enter the G_0 phase of cell life- a 'housekeeping' period of dormancy in which there exists minimal energy expenditure, gene activity or protein production. Our observation that TGF-ß induces cytokine release in such dormant cells suggests that the ability to produce and release cytokines is preferentially maintained in the G_0 osteoblast and that TGF-ß may be

an important hormone for activation of this response *in vivo*. What remains to be determined is the importance of cytokines, and the rapid upregulation of cytokine production in the quiescent osteoblast.

Response Rapidity. Preliminary experiments tested CMs from osteoblasts cultured with TGF-ß and LPS after 48 hours of exposure. To obtain a more complete response curve, additional experiments analyzed CMs from osteoblasts after 1, 2, 6, 12 and 48 hours of exposure to TGF-ß and LPS. As presented in figures IV.vi.a/b and xix a/b, data revealed that 95% of the GM-CSF and IL-6 activity found in the CMs after 48 hours of exposure was found in 6-hour CMs but not in 2.5-hour CMs. The relative rates of cytokine release over the five timepoints were determined as the ratio of the change in cytokine cell-proliferating activity (Δ CPM) to the change in time (Δt) and presented in figures IV. vii and xx. The rate of release for both GM-CSF and IL-6 is at a maximum from hours zero to six. From 6-22 hours, the release rate drops 75-90% and from 22-48 hours, the rate returns to near baseline values. These results suggest that the effect achieved by TGF-ß and a given synergen on GM-CSF or IL-6 release is rapid and short-lasting. How and why the osteoblast maintains secretory pathways to respond quickly after appropriate stimulus, even after 48 hours of serum-deprivation, is not clear.

Cytokine Specificity Osteoblasts are known to secrete three cytokine hormones: two colony stimulating factors (M-CSF and GM-CSF) and Interleukin-6 (IL-6). All three molecules were tested

in CMs derived from stimulated osteoblasts. Only two of the three, GM-CSF and IL-6, were affected by TGF-ß stimulation with a given synergen, either IL-1 of LPS. PTH was a unique synergen in that TGF-ß/PTH elevated the release of IL-6 but did not change the release of GM-CSF (Figures V.ii). The release of the third osteoblast-derived cytokine, M-CSF, was not changed by the TGF-ß/synergen combinations studied. The interchangeability of LPS and IL-1 as synergens for TGF-ß stimulation suggest that LPS and IL-1 may utilize similar intracellular pathways of activation in concert with those utilized by TGF-ß. PTH, however, may utilize still other pathways to specify release of IL-6. Several questions arise. First, how do the roles of the cytokines differ in the local environment when IL-6 is released singly (TGF-ß + PTH) or in combination with GM-CSF (TGF-ß + IL-1 or TGF-ß + LPS)? Second, what is the significance of the exclusion of M-CSF from these reactions, besides the assumption that its release utilizes pathways unaffected by TGF-ß. Would the release of M-CSF in addition to the other hematopoetic cytokines be a biologic redundancy or counteract the effects of these other molecules? The described roles of M-CSF in bone biology are limited but include a role as growth promoter for osteoclasts[128b,206,212]. GM-CSF and IL-6 may function to upregulate osteo*blast* activities which could be lessened by M-CSF-induced osteo*clast* activity.

Dependence of Synergy on TGF-ß A set of 'priming' experiments were conducted in which cells were exposed to TGF-ß for two hours, washed, and exposed to LPS. Conversely, other cells

were exposed to LPS as the 'priming' agent, washed, and then exposed to TGF-ß. Results from figures IV.v and xvi demonstrate that the increases in cytokine release previously observed were duplicated when cells were initially exposed to TGF-ß and then to LPS. Little or no increase in cytokine release was seen when cells were first exposed to LPS and then to TGF-ß. Cells could also be primed for IL-6 release by TGF-ß with subsequent exposure to PTH (Figure IV. xxii).

These observations suggest that the synergy between TGF-ß and a given *synergen* for cytokine release may be dependent upon TGF-ß itself. TGF-ß may possibly function to prepare the osteoblast to respond to a secondary stimulus, such as LPS or PTH, with large releases of active cytokine. Studies with TGF-ß as a cell primer for IL-1 are to be performed. Clearly, any effect that TGF-ß imparts upon the cell lasts long enough to augment the stimulatory effect of a subsequent agent. What enables TGF-ß to 'prime' the osteoblast for cytokine release is not clear.

Mechanisms of Synergy. It is difficult to determine the roles of TGF-ß in osteoblast stimulation and those of a given synergen but some assumptions may be made. First, activation of the cell by TGF-ß may utilize pathways different than those of LPS or PTH since cells can be 'primed' by TGF-ß and not by these two synergens. Second, activation of the TGF-ß-mediated pathway(s) is the rate-limiting step in cytokine release since the process is TGF-ß dependent. Third, the pathways utilized by TGF-ß in enhancing

cytokine release must be readily activated, within hours of exposure, to high levels, even in the serum-deprived state.

Three hypotheses arise which may unite the observations presented above concerning the role of TGF-ß and its synergens in cytokine secretion: (1) TGF-ß functions to upregulate the number or activity of surface receptors for a given synergen; (2) TGF-ß functions to upregulate second messenger systems common to TGF-ß and its synergens; and (3) TGF-ß induces the release of Platelet-Derived Growth Factor (PDGF) which, in turn, activates the osteoblast in concert with another synergen. Each hypothesis is supported by data in the present work and data in the literature and will be briefly discussed as follows.

a. Upregulation of synergen surface receptors by TGF-ß.

A qualitative analysis of osteoblast IL-1 receptor mRNA was performed in which cells were exposed to TGF-ß, a synergen, and to both agents together. The results presented in figure IV. xii suggest that osteoblasts exposed to TGF-ß have more mRNA for the surface receptor for Interleukin-1 (IL-1R) than control cells. The messsage level is unchanged by IL-1 itself. This is in contrast with the fibroblast studies by Dubois et al.[54] who found that TGF-ß suppressed fibroblast expression of IL-1R. Transcriptional studies will determine whether this affect is secondary to enhanced mRNA stabilization or to increased message synthesis. If TGF-ß enhances expression of functional IL-1R, it remains unclear what pathways the IL-1R activates for cytokine release. The synergy between TGF-ß

and IL-1 for GM-CSF release may, therefore, be a two-step process in which TGF-ß first increases IL-1R levels allowing the cell to respond to a previously sub-stimulatory level of IL-1 (Figure V.iii.a). Increased osteoblast sensitivity to IL-1 may be particularly important for the cell's response to early infection when local IL-1 levels are low.

b. Upregulation of synergen second messenger systems by TGF-ß.

TGF-ß may function to 'prime' cells by activating second messenger systems from quiescence or baseline state. The TGF-ß receptor may act to upregulate proteins maintained in the quiescent state for the trans-activation of a broad range of regulated genes. Alternatively, TGF-ß may simply enhance a rate-limiting step for second messenger production by a synergen[102].

Most growth factors induce the production of second messenger signals, like diacylglycerol (DAG) and intracellular calcium ($[Ca^{++}]_i$), by enhancing phosphotidylinsoitol (PI) turnover[168]. Cells can be stimulated by agents which substitute for DAG and $[Ca^{++}]_i$, such as phorbol esters and calcium ionophores, resepectively[138,150]. PI turnover may not be the sole mechanism to achieve this end. Many primary cells require *two* growth factor signals- one that enhances PI turnover and one that does not[168]. T-cells, for example, proliferate maximally only after co-stimulation by antigen and IL-1. Antigen increases PI turnover but IL-1 does not. As presented in II. B, Rosoff et al. showed that IL-1 stimulates the production of another phospholipid, phosphotidylcholine (PC)[168]. PC hydrolysis produces a species of diacylglycerol (DAG)

distinct from DAGs produced after PI hydrolysis. As shown in figure II. ix, the two DAG species activate species-specific kinases which synergize to activate transcription factors.

TGF-ß may prime osteoblasts to respond to IL-1 by enhancing IL-1- specific DAG production or DAG-dependent kinase activity from baseline state. This would allow the rapid up-regulation of second messenger systems while maintaining the specificity of IL-1-induced pathways. Alternatively, TGF-ß may itself produce DAG-species which synergize with those derived after IL-1 stimulation for protein kinase C activation.

The second messenger pathways important in LPS activation of the cell are not as well-characterized as IL-1, but most likely involve hydrolysis of the cyclic nucleotides, cAMP or cGMP[20,61]. TGF-ß may function to upregulate the activity of the second messenger system by enhancing or activating distal enzymes such as protein kinase C or A or other LPS-specific serine kinases.

The 'priming' of the cell by TGF-ß, as discussed above, would correspond to the rapid activation by TGF-ß of rate-limiting steps in second messenger systems. Subsequent ligand binding by a synergen would then utilize the activated second messenger system for full cell stimulation and cytokine production. Second messenger upregulation by TGF-ß would provide for a rapid, efficient process of specific kinase activation without the need for transcriptive or translative processes.

c. PDGF as a mediator of TGF-ß/synergen stimulation of osteoblasts.

Workers have shown that a TGF-ß-controlled autocrine loop exists involving Platelet-derived Growth Factor (PDGF)[10,95]: Low

levels of TGF-ß upregulate PDGF-AA release from smooth muscle cells and high TGF-ß levels downregulate PDGF-α receptors. The combined effect may explain in part the bimodal effect on cellular growth seen over a range of TGF-ß concentrations in

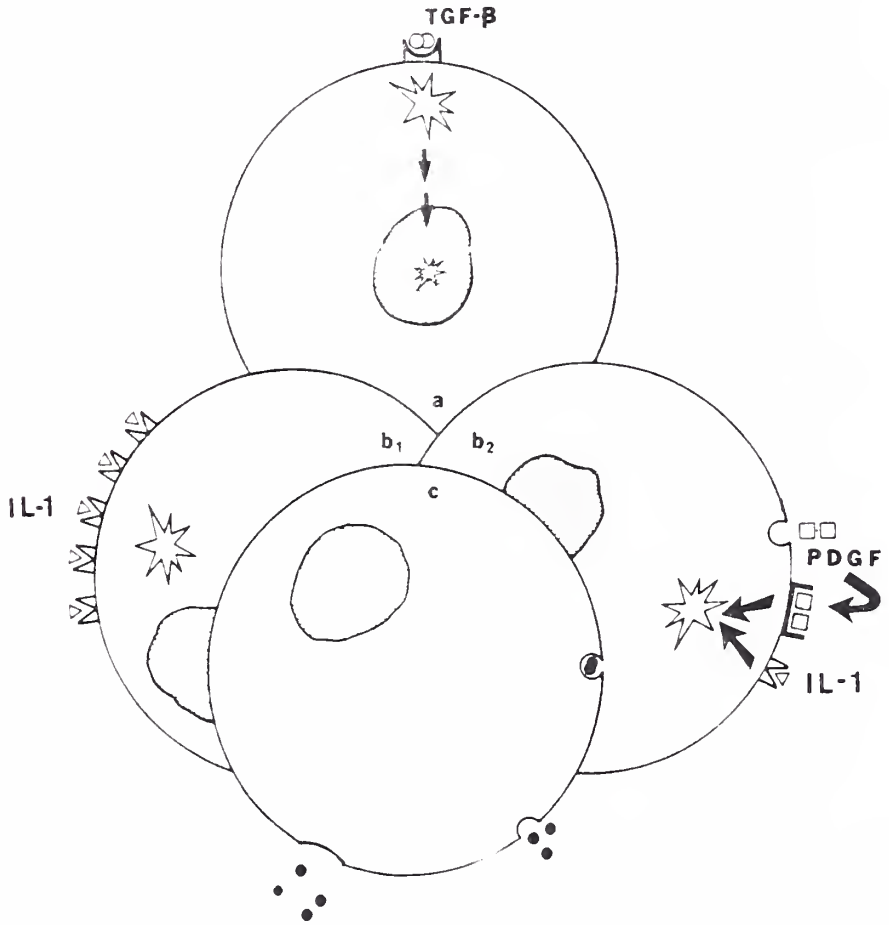


Figure V.iii. Hypothetical activation sequences of TGF-ß synergy with IL-1 for cytokine release. a) TGF-ß ligand induces post-receptor events which upregulates cytoplasmic and/or nuclear events for cell priming. TGF-ß-induced events lead to b_1) upregulation of receptors for IL-1 and/or b_2) production of PDGF. In the latter hypothesis depicted in figure b_2, IL-1 synergizes with autocrine PDGF dimer. The result of either b_1 or b_2 leads to c) cytokine release. Adapted from concept by Palumbo et al. (with permission)[154].

fibroblasts, smooth muscle cells, chondrocytes, and osteoblasts[34,167]. Like TGF-ß, IL-1-induced mitogenesis has also been found to involve autocrine production of PDGF-AA[160] and modulated by TGF-ß[155].

PDGF exists in isoform mixtures derived from the three possible combinations of hormone subunits, A and B: AA, AB, or BB. Isoform AA is a ligand for α-type PDGF receptors and isoforms AB and BB are ligands for ß-type receptors. The PDGF A chain is efficiently secreted and is a potent inducer of cytokine production in fibroblasts[103,119]. The B chain remains cell associated, has a 25-fold higher transforming ability for fibroblasts, and is the homolog of the v-*sis* oncogene product. Osteosarcoma cells translate both PDGF-A and -B mRNA (c-sis) into processed polypeptide whereas normal human and rat osteoblasts produce only PDGF-AA[71,72,81], implicating PDGF-BB and v-*sis* in neoplastic bone cell growth. In bone cell studies, PDGF-BB enhances osteoblast chemotaxis[190], DNA synthesis and replication[36], collagen synthesis and bone formation[34,159]. PDGF-AA has lower activity in these studies than AB or BB isoforms.

Centrella et al. have shown that osteoblast PDGF receptors preferentially bind PDGF-BB over -AA although osteoblasts produce mostly the PDGF-AA isoform[37,38a]. This phenomenon is most likely a consequence of PDGF receptor type expression by the osteoblast, type ß > α. IL-1 α, however, can increase PDGF-AA binding to induce levels of mitogenesis equivalent to levels induced by PDGF-BB[38b]. PDGF-AA action on osteoblasts may be thus controlled by other local factors.

Released PDGF-AA induces the oligomerization of cell surface receptors for other PDGF ligands and the oligomerized receptors synergize for tyosine kinase activity[178]. Multiple post-receptor events are complex and involve a physical association with four other enzymes including phospholipase C gamma (PLC-γ)[115,122], phosphatidyl inositol 3-phosphate kinase (PI-3pK)[5], the serine-threonine kinase raf-1[162], and GTPase Activating Protein (GAP)[103]. TGF-ß and IL-1 may thus synergize by converging on common PDGF-signalling pathways upregulated by TGF-ß priming and enhanced further by IL-1 activation.

To identify a role of PDGF expression in the synergistic elaboration of cytokines by TGF-ß and IL-1, osteoblasts were exposed to IL-1 and PDGF-AA or PDGF-BB with the appropriate controls. As presented in figure IV. xxiv, PDGF-BB synergized with IL-1 for GM-CSF release from osteoblasts. Production of IL-6 was not assayed. The effect of PDGF-AA with IL-1 was less than the effect of PDGF-BB. Experiments in which osteoblasts are exposed to both TGF-ß and IL-1 but in the presence of antibody to the PDGF-BB ligand and/or receptor are to be performed. Such an experiment would be likely to determine the role of PDGF-BB in TGF-ß mediated cytokine enhancement.

Cytokine release induced by TGF-ß and a synergen from previous experiments occurs within 6 hours. It is unclear how TGF-ß induces PDGF release and how IL-1 and PDGF then induce cytokine release within a 6 hour time frame.

Two distinct growth factor signals are often required for cell stimulation. Fibroblasts can be stimulated by PDGF, which enhances

phosphotidylinositol turnover, if the cells are also exposed to a growth factor which does not enhance PI turnover[168]. As discussed in V.vi.b, such a 'second molecule' may be IL-1. PDGF and IL-1 may, therefore, synergize for cytokine release by activating two separate pathways of cell stimulation which converge upon a common point for cytokine production, i.e. a transcription factor.

It remains to be determined if PDGF may be significant in mediating the synergy between TGF-ß and LPS or between TGF-ß and PTH.

Common Themes of TGF-ß Activity. The hypotheses discussed above and presented in figure V. iii suggest that TGF-ß acts as an early signal for generalized activation of the dormant cell. As presented in figure V. iv, TGF-ß may initially activate a cell from quiescence while the cell's ultimate response is determined by the presence of other serum growth factors. TGF-ß was originally described as a transforming growth factor with such a role, as discussed in II. A. TGF-ß, isolated from neoplastic cells *in vitro*, was observed to facilitate the transition from G_0 to G_1 in normal cells and eventually induced neoplastic growth in cells exposed to PDGF-BB, EGF, and IGF-1[91]. In normal conditions, as in the osteoblast studied in this work, TGF-ß may be one of a few extracellular molecules, present in serum, which maintains the cell in the activated state. The absence of TGF-ß from the extracellular mileau could imply the absence of other nutrients as well. The absence of TGF-ß allows the cell to become quiescent and conserve metabolism in periods of nutrient scarcity. The presence of TGF-ß,

however, would enable the cell to respond to other signals for structural protein synthesis, mitosis, or cytokine release. Considering the rapidity of cytokine release from the dormant cell, it is unclear if cytokines are important only as paracrine growth factors, or are important as autocrines in the processes of cell reactivation.

These hypotheses do not consider the source of released cytokine. Translational inhibition studies would quickly determine whether new cytokine is produced or stored cytokine is released following cell stimulation. The latter possibility seems unlikely unless cytokines are so significant to the cell that they are stored for long periods in the dormant cell, ready for release when the cell is appropriately stimulated.

New Elements in Bone Remodeling and Hematopoesis. The role of TGF-ß and its synergens in cytokine release by osteoblasts remains unclear. Perhaps, as implicated above, TGF-ß functions as a cell 'activator', enhancing second messenger or receptor systems utilized by synergens (Figure V.iv). The roles for TGF-ß and its synergens may elude scientific determination for some time, but the *effect* of these molecules is clear: greatly increased cytokine release. As suggested previously, such a response may be important to the newly resuscitated cell since cytokine release is maximally observed less than 6 hours after stimulation from quiescence. What remains is to determine how cytokine release is important to the osteoblast, and what roles released cytokines may play in normal growth processes. Possible clues are suggested with analysis of the anatomy

and biology of bone remodeling. Osteoblasts, the bone-forming cells, are situated in close proximity to the osteoclasts, the bone-resorptive cells, and to osteoclast resorptive pits or lacunae. Situated close to these cells, and adjacent to and supported by the bone matrix

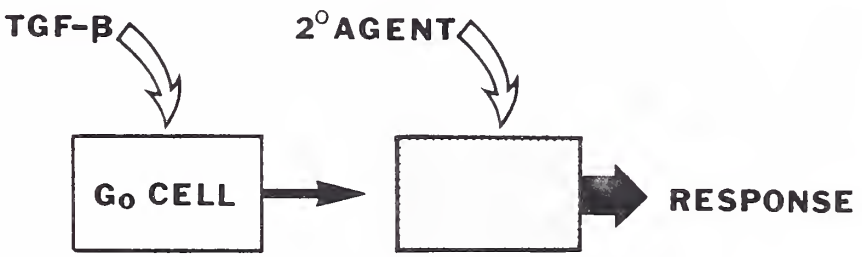


Figure V.iv. Common role of TGF-ß in cell activation. A common concept for TGF-ß's roles in growth transformation, matrix formation, and cytokine release may be that TGF-ß functions to induce changes in the G_0 cell. These intracellular changes alone are insufficient to induce protein release but act to 'prime' the cell to respond to a secondary growth signal.

itself, lies the bone marrow. Given the observations presented in this thesis, a possible model arises concerning the role of TGF-ß and its synergens in bone remodeling and hematopoesis itself: As depicted in figure V.v, osteoclasts resorb bone and release from the matrix inactive TGF-ß, as described on paes 12-13. The protons used by the osteoclast to resorb bone accumulate in the pit, and this low pH, combined with osteoclast enzymes, activates latent TGF-ß. The active TGF-ß diffuses the short distance to the osteoblast and binds to the TGF-ß receptor. Meanwhile, parathyroid hormone which has diffused into the remodeling locus, stimulates PTH receptors on the same osteoblast. The TGF-ß and the PTH synergize to induce osteoblast production of Interleukin-6. The IL-6 diffuses back and may enhance osteoclast resorptive activity, or diffuse across the

resorptive locus and enter the bone marrow to induce IL-6-dependent marrow lines (B-cells)[64].

Other models arise when osteomyelitis or a fracture locus is

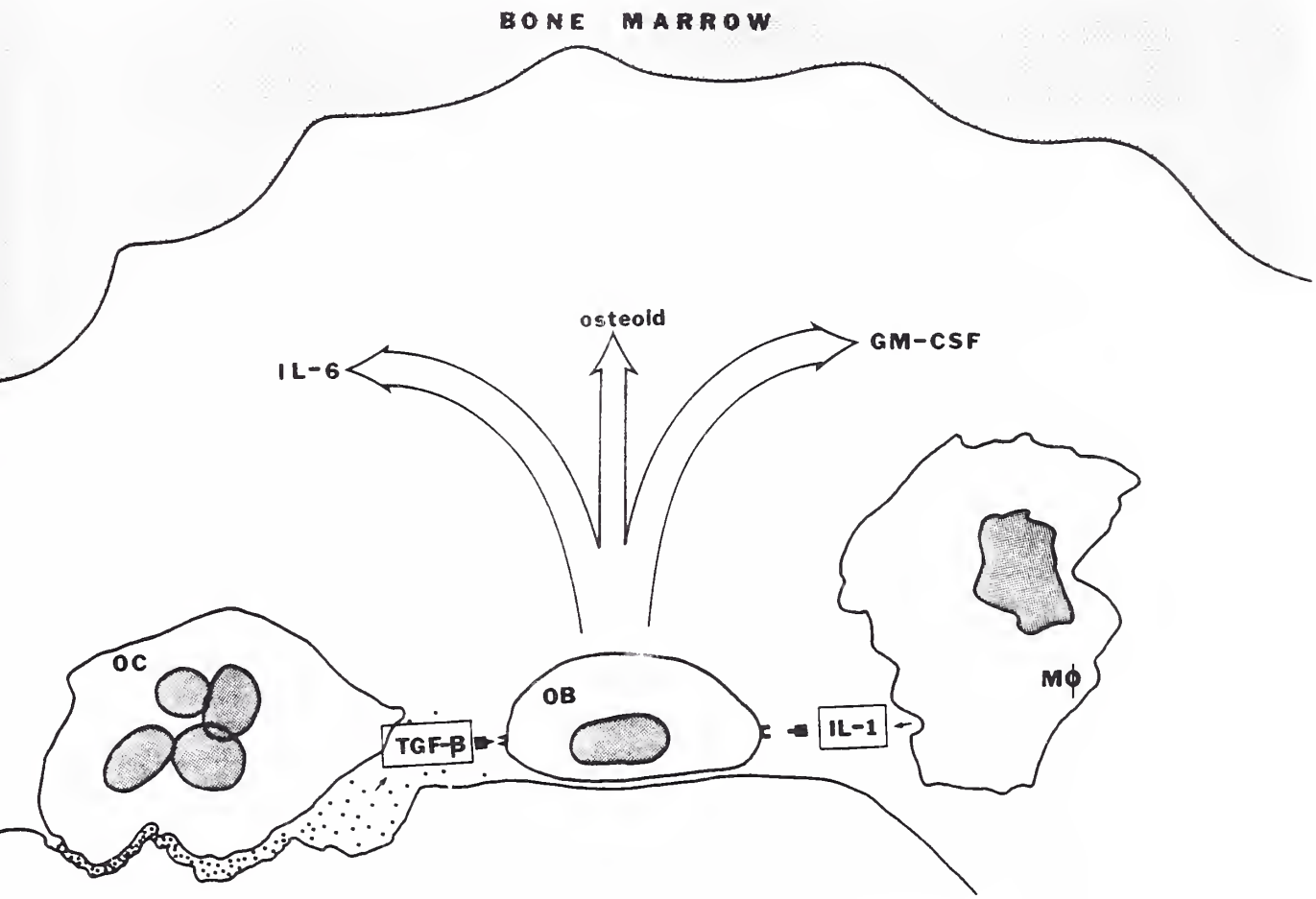


Figure V.v. Model for osteoblast cytokine release at the resorption locus, as described below, for TGF-ß and IL-1 synergy.

superimposed upon normal remodeling. Active infections, for example, would provide IL-1 from recruited bone and blood

macrophages. IL-1 would synergize with TGF-ß to release both GM-CSF as well as IL-6, an effect perhaps mediated by PDGF. The GM-CSF could diffuse into the marrow space and induce the growth of and act as a chemoattractant for neutrophilic and monocytic lines. These cells may migrate into the remodeling locus to focus and augment the processes of bone healing from infection or trauma. Gram-negative bacterial osteomyelitis would also provide LPS, the active component of endotoxin, providing another synergen for TGF-ß dependent cytokine release. Continuous stimulation of cytokine production by exogenous LPS and LPS-induced IL-1 release may ablate local control and feedback mechanisms in cytokine-mediated bone remodeling. Haphazard osteoclast and osteoblast activity may give rise to bone with the classic characteristics of unchecked and untreated osteomyelitis as seen in in figure V.i.

Bone fracture, however, with accompanying hemorrhage and clot, would provide many molecules of PDGF-AB and BB, which may mediate many of the effects of TGF-ß for GM-CSF and IL-6 production. TGF-ß-induced osteoblast activity may also lead to increased osteoid formation and bone repair[157].

With several ways to provide GM-CSF and IL-6, TGF-ß and its synergens could enhance the healing changes required in osteomyelitic and fractured mineralized tissue. What arises from these models is an impression that the local environment of the bone remodeling locus is intimately regulated on the local level by processes initiated by TGF-ß. Whether in the pathologic conditions of trauma or osteomyeltis, or in normal bone remodeling, TGF-ß

may be an important mediator between osteoclasts, osteoblasts, and cell lines of the bone marrow.

There is some evidence in the literature that supports the hypothesis that osteoblast-derived cytokines may be important in the support of normal hematopoetic processes in the bone marrow[43,87]. The evidence presented in this thesis suggests that TGF-ß may be an important molecule in the normal control of such a relationship between the stromal cell of the bone matrix (osteoblasts) and stromal cells of the bone marrow (stem cells).

Cytokines doubtless evolved in cells which were dependent upon other cells for survival. *Para*crine hormone activity most likely existed long before the multi-organ relationships in *endo*crine activity since the first complex 'organisms' were most likely two- or three-celled. The multi-cellular construction of the body increases the importance of paracrine relationships since the diverse biological activites of each cell type may be intimately controlled on the local level. Osteoblasts are no different than other cells in that osteoblasts regulate normal growth and function by responding appropriately to extracellular signals. Cytokines may be important molecules in the paracrine orchestration of cellular activity and growth in a localized area. The evidence presented in this thesis further implicates cytokines and TGF-ß in local control mechanisms. What remains to be determined is the significance of such activities in normal bone biology and how these activities are involved in the pathogenesis of bone disorders.

VI. REFERENCES

1. Aarden, L., Degroot, E., Schaap, O., Lansdorp, P., Production of Hybridoma Growth Factor by Human Monocytes, Eur. J. Immunol. 17, 1411-16, 1987.

1b. Abraham, R.T., Ho, S.N., Barna, T.J., McKean, D.J., Transmembrane Signaling during Interleukin-1-dependent T Cell Activation, J.B.C., 262, 2719-2728, 1987.

2. Appling, W.F., O'Brien, W.R., Johnston, D.A., Duvic, M., Synergistic Enhancement of Type I and III Collagen Production in Cultured Fibroblasts by Transforming Growth Factor-B and Ascorbate, FEBS Letters, 1989, 250 (II), 541-544.

3. Allen, J.B., Manthey, C.L., Hand., A.R., Ohura, K., Ellingsowrth L., Wahl, S.M., Rapid Onset Synovial Inflammation and Hyperplasia Induced by Transforming Growth Factor-B, J. Exp. Med. 171, 231-247, 1990.

4. Assoian, R., Komoriya, A., Meyers, C., Miller, D., Sporn, M., Transforming Growth Factor-beta in Human Platelets, J. Biol. Chem. 258, 7155-7160, 1983.

5. Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P., Cantley, L.C., PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells, Cell 57, 167-165, 1989.

6. Auron, P.E., Webb, A.C., Rosenwasser, L.J., Mucci, S.F., Nucleotide Sequence of Human Monocyte Interleukin-1 Precursor cDNA, P.N.A.S. 81, 7907-7911, 1984.

7. Bakouche, O., Brown, D.C., Lachman, L.B., Subcellular Localization of Human Monocyte Interleukin-1: Evidence for an Inactive Precursor Molecule and a Possible Mechanism for IL-1 Release, J. Immunology 138, 4249-4255, 1987.

8. Bakouche, O., Brown, D.C., Lachman, L.B., Liposome Expressing IL-1 Biological Activity, J. Immunol. 138, 4256-4262, 1987.

9. Banks, P., Grounding the Gadabout Growth Factor, TGF-B, J. NIH Res. Nov-Dec (I), 93-96, 1989.

10. Battegay, E.J., Raines, E.W., Seifert, R.A., owen-Pope, D.F., Ross, P., TGF-ß Induces Bimodal Proliferation of Connective Tissue Cells via Complex Control of an Autocrine PDGF Loop, Cell 63, 515-525, 1990.

11. Beck, G., Habicht, G.S., Isolation and Characterization of a Primitive Interleukin-1-like Protein from an Invertebrate, *Asterias forbesi*, P.N.A.S. 83, 7429-7433, 1986.

12. Beeson, P.B., Temperature-Elevating Effect of a Substance Obtained from Polymorphonucelar Leukocytes, J.C.I. 27, 524-526, 1948.

13. Berridge, M.J., Inositol Triphosphate and Diacylglycerol: Two Interacting Second Messengers, Annu. Rev. Biochem. 56, 159-163, 1987.

14. Besedovsky, H., del Rey, A., Sorkin, E., Dinarello, C.A., Immunoregulatory Feedback between Interleukin-1 and Glucocorticoid Hormones, Science 233, 652-654, 1986.

15. Bird, T.A., Saklatvala, J., Studies on the Fate of Receptor-Bound-^{125}I-Interleukin-1-ß in Porcine Synovial Fibroblasts, J. Immunol. 139, 92-97, 1987.

16. Black, R. A., Kronheim, S.R., Cantrell, M., Deeley, M.C., March, C.J., Prickett, K.S., Wignall, J., Conlon, P.J., Cosman, D., Hopp, T.P., Mochizuki, D.Y., Generation of Biologically Active Interleukin-1 ß by Proteolytic Cleavage of the Inactive Precursor, J. Biol. Chem. 263, 9437-9442, 1988.

17. Blyden, G., Handschumaker, R.E., Purification and Properties of Human lymphocyte activating Factor (LAF), J. Immunology 118 (V), 1631-1638, 1977.

18. Bodel, P., Studies on the Mechanism of Endogenous Pyrogen Production. III. Human Blood Monocytes, J. E. M. 140, 954-965, 1974.

19. Brandes, M.E., Allen, J.B., Ogawa, Y., Wahl, S.M., Transforming Growth Factor Beta 1 Suppresses acute and Chronic Arthritis in Experimental Animals J.C.I. 87 (3), 1108-1113, 1991.

20. Brandwein, S.R., Skamene, E., Aubut, J-A., Gervais, F., Nesbitt, M.N., Genetic Regulation of Lipoplysaccharide-Induced Interleukin-1 Production by Murine Peritoneal Macrophages, J.Immunol. 128, 4263-4269, 1987.

21. Bron, C., MacDonald, H., Identification of the Plasma Membrane Receptor for Interleukin-1 on Mouse Thymoma Cells, FEBS Lett. 219, 365-368, 1987.

22. Burgess, A.W., The Origins of Growth Factors: Tethelin Lost, T.I.B.S. 14, *116-119*, 1989.

23. Burgess, A.W., Camakaris, D., Metcalf, J., Purification and Properties of Colony Stimulating Factor from Mouse Lung-Conditioned Medium, J.Biol. Chem. 252, 1998, 1977.

24. Busbridge, N.J., Dascombe, M.J., Tilders, F.J.H., van Oers, J.W.A.M., Linton, E.A., Rothwell, N.J., Central Activation of Thermogenesis and Fever by Interleukin-1ß and Interleukin-1α Involves Different Mechansims, Biochem. Biophys. Res. Comm. 162 (II), 591-596, 1989.

25. Byrne, P.V., Guilbert, L.J., Stanley, E.R., Distribution of Cells Bearing Receptors for a Colony Stimulating Factor (CSF-1) in Murine TIssues, J.C.B. 91, 848, 1981.

26. Cairo, M., Hematopoietic Growth Factors: A New Frontier in Immunotherapy, J. Ped. S1-S3, March, 1991.

27. Calderon, J., Unaue, E.R., Two Biologic Activities Regulating Cell Proliferation Found in Cultures of Peritoneal Exudate Cells, Nature 253, 359, 1975.

28. Canalis, E., Interleukin-1 has Independent Effects on Deoxyribonucleic Acid and Collagen Synthesis in Cultures of Rat Calvariae, Endocrinology 118, 74-81, 1986.

29. Canalis, E., McCarthy, T., Centrella, M., Growth Factors and the Regulation of Bone Remodeling, J. Clin. Inves. 81, 277-281, 1988.

30. Carrington, J.L., Roberts, A.B., Flanders, K.C., Roche, N.S., Reddi, H.A., Accumulation, Localization, and Compartmentation of TGF-ß During Endochondral Bone Development, J. Cell. Biol. 107, 1969-1975, 1988.

31. Castell, J.V., Andus, T., Kunz, D., Heinrich, P.C., IL-6: The Major Regulator of Acute-Phase Protein Synthesis in Man and Rat, in "Interleukin-6", Annals of the N.Y. Acad. Sci. 557, 87-101, Ed.Sehgal, P.B., et al.

32. Castell, J.V., Gomez-Lechan, M.J., David, M., Hirano, T., Kishimoto, T., Heinrich, P.C., Recombinant Human IL-6 (IL-6/BSF-2/HSF) Regulates the Synthesis of Acute Phase Proteins in Human Hepatocytes, FEBS Letters 232, 347-351, 1988.

33. Centrella, M., Canalis, E., Transforming and Nontransforming Growth Factors are Present in Medium Conditioned by Fetal Rat Calavariae, P.N.A.S. 82, 7335-7339, 1985.

34. Centrella, M., McCarthy, T.L., Canalis, E., Transforming Growth

Factor-ß is a Bifunctional Regulator of Replication and Collagen Synthesis in Osteoblast-Enriched Cell Cultures from Fetal Rate Bone, J. Biol. Chem. 262 (VI), 2869-2874, 1987.

35. Centrella, M., McCarthy, T.L., Canalis, E., Skeletal tissue and Transforming Growth Factor-ß, FASEB J. Dec., (II), 3066-3073, 1988.

36. Centrella, M., McCarthy, T.L., Canalis, E., Platelet-Derived Growth Factor Enhances Deoxyribonucleic Acid and Collagen Synthesis in Osteoblast-Enriched Cultures from Fetal Rat Parietal Bone, Endocrinology 125, 13-19, 1989.

37. Centrella, M., McCarthy, T.L., Ladd, C., Canalis, E., Expression of Platelet-derived Growth Factor (PDGF) and Regulation of PDGF Binding are Both Isoform Specific in Osteoblast-Enriched Cultues from Fetal Rat Bone, J. Bone Min. Res. 5, S86, 1990.

38a. Centrella, M., McCarthy, T., Kusmik, W. , Canalis, E., Relative Binding and Biochemical Effects of Heterodimeric and Homodimeric Isoforms of Platelet-Derived Growth Factor in Osteoblast-Enriched Cultures from Fetal Rat Bone, J. Cell. Phys. 147, 420-426, 1991.

38b. Centrella, M., J. Immunol. (In Press), 1992.

39. Ceuppens, J.L., Baroja, M.L., Lorre, K., Damme, J.V., Billiau, A., Human T-cell Activation with Phytohemagglutinin: The Function of IL-6 as an Accesory Signal, J. Immunol. 141, 3868-3872, 1988.

40. Champlin, R.E., Nimer, S.D., Ireland, P., Oette, D., Golde, D.W., Treatment of Refractory Aplastic Anemia with Recombinant human GM-CSF, Blood 73, 694-699, 1989.

41. Chen, J-K., Hochi, H., McKeehan, W.L., TGF-ß Specifically Stimulates Synthesis of Proteoglycan in Human Arterial Smooth Muscle Cells, P.N.A.S. 84, 5287-5291, 1987.

41b. Chen, P., Carrington, J., Hammonds, R., Reddi, A., Stimulation of Chondrogenesis in Limb Bud Mesoderm Cells by Recombinant Human Bone Morphogenetic Protein 2B (BMP-2B) and Modulation by Transforming Growth Factor ß1 and ß2, Exp. Cell Res. 195, 509-515, 1991.

42. Chenu, C., Kurifara, N., Mundy, G.R., Roodman, G.D., Prostaglandin E_2 Inhibits Formation of Osteoclastic Cells in Long-Term Human Marrow Cultures but Is Not a Mediator of the Inhibitory Effects of Transforming Growth Factor ß, J. Bone and Mineral Res. 5, 677-829, 1990.

42b. Chirgwin, J., Przybyla, A., MacDonald, R., Rutter, W., Isolation of Biologically Active Rinbonucleic Acid from Sources Enriched in Ribonuclease, Biochemistry 18, 5294-5299, 1979.

43. Clark, S.C., Kamen, R., The Human Hematopoiesis Colony-Stimulating Factors, Science 236, 1228-1237, 1987.

44. Coffman, R.L., Lebman, D.A., Shrader, B., Transforming Growth Factor-B Specifically Enhances Ig-A Production by Lipopolysaccharide-Stimulated Murine B-Lymphocytes, J. Exp. Med. 170, 1039-1044, 1989.

45. Cohen, S., Isolation of a Mouse Submaxillary Gland Protein Accelerating Incisor Eruption and Eyelid Opening in the Newborn Animal, J.Biol. Chem. 237, 1555-1562, 1962.

46. Coley, W. B., Further Observations upon the Treatment of Malignant Tumors with the Toxins of Erysipelas and Bacteria Prodigiosus, with a Report of 160 Cases, Bull. J. Hopk. Hosp. 65, 157-162, 1896.

47. Crabb, I.D., O'Keefe, R.J., Puzas, J.E., Rosier, R.N., Synergistic Effect of Transforming Growth Factor ß and Fibroblast Growth Factor on DNA Synthesis in Chick Growth Plate Chondrocytes, J. Bone and Mineral Res. 5, 1105-1112, 1990.

48. Crawford, J., Ozer, H., Stoller, R., Johnson, D., Lyman, G., Tabbara, I., Kris, M., Grous, J., Picozzi, V., Rausch, G., Smith, R., Gradishar, W., Yahanda, A., Vincent, M., Stewart, M., Glaspy, J., Reduction by Granulocyte Colony-Stimulating Factor of Fever and Neutropenia Induced by Chemotherapy in Patients with Small-Cell Lung Cancer, N.E.J.M. 325 (3), 164-170, 1991.

49. Dean, D.C., Newby, R.F., Bourgeois, S., Identification of TGF-ß1 Responsive Elements in the Fibronectin and SV40 Early Gene Promoters (in preparation).

50. Dinarello, C.A., Interleukin-1, Rev. Inf. Dis. 6, 51-70, 1984

51. Dinarello, C.A., Goldin, N.P., Wolff, S.M., Demonstration and Characterization of Two Distinct Human Leukocyte Pyrogens, J.E.M. 139, 1369-1381, 1974.

52. Donahue, R.E., Wang, E.A., Kaufman, R.J., Foutch, L., Leary, A.C., Witek-Gianetti, J.S., Metzger, M., Herrick, R.H., Steinbrick, D.R., Shaw, G., Kamen, R., Clark, S.C., Effects of N-Linked Carbohydrates on the In-Vivo Properties of Human GM-CSF, Cold Spring Harbor Symp. Quant. Biol. 51, 685-691, 1981.

53a. Donahue, R.E., Wang, E.A., Stone, D.K., Kamen, R., Wong, G.G., Sehgal, P.K., Nathan, D.G., Clark, S.C., Stimulation of Hematopoiesis in Primates by Continuous Infusion of Recombinant Human GM-CSF, Nature 321, 872-875, 1986.

53b. Donahue, R.E., Cold Spring Harbor Symp. Quart. Biol. 51, 885, 1981.

54. Dubois, C.M., Ruscetti, F.W., Palaszynski, E.W., Falk, L.A., Oppenheim, J.J., Keller, J.R., Transforming Growth Factor ß is a Potent Inhibitor of Interleukin 1 (IL-1) Receptor Expression: Proposed Mechanism of Inhibition of IL-1 Action, J. Exp. Medicine 172, 737-744, 1990.

55. Durum, S.K., Schmidt., J.A., Oppenheim, J.J., Interleukin-1: an immunological perspective, Ann. Rev. Immunol. 3, 263-287, 1985.

56. Dwyer, S.K., Gillis, S., Sims, J.E., The Interleukin-1 Receptor and Post-Receptor Events, in "Interleukin-1, Inflammation and Disease", Eds:Bomford, R., Henderson, B., Res. Mono. Cell Tis. Phys. 16, 63-78, Elsevier, 1989.

57. Edwards, D.R., Murphy, G., Reynolds, J.J., Wheton, S.E., Doherty, A.J.P., Angel, P., Heath, J.K., TGF-ß Modulates the Expression of Collagen and Metalloproteinase Inhibition, E.M.B.O. J. 6, 1899-1904, 1987.

58. Espevik, T., Figari, I., Ranges, G., Palladino, M., Transforming Growth Factor-ß1 (TGF-ß1) and Recombinant Human Tumor Necrosis Factor-alpha Reciprocally Regulate the Generation of Lymphokine-activated Killer Cell Activity, J. Immunol. 140, 2312-2316, 1988.

59. Evans, D.B., Bunning, R.A.O., Russell, A.H., The Effect of Recombinant Human GM-CSF (rhGM-CSF) in Human Osteoblast-like Cells, Biochem. Biophys. Res. Comm. 160, 588-591, 1989.

59b. Felix, R., Cecchini, M., Hofstetter, W., Guenther, H., Fleisch, H., Production of Granulocyte-Macrophage (GM-CSF) and Granulocyte Colony-Stimulating Factor (G-CSF) by Rat Clonal Osteoblastic Cell Population CRP 10/30 and the Immortalized Cell Line IRC10/30-myc1 Stimulated by Tumor Necrosis Factor Alpha, Endocrinology 128, 661-667, 1991.

60. Feyen, J.H.M., Elford, E., DiPadova, F., Trechsel, U., Interleukin-6 is produced by bone and modulated by parathyroid hormone, J. Bone Min. Res. 4 (IV), 633-638, 1989.

61. Forehand, J. R., Pabst, M.J., Phillips, W.A., Johnston, R.B., Jr.,

Lipopolysaccharide Priming of Human neutrophils for an enhanced respiratory burst, J. Clin. Invest. 83, 74-83, 1989.

62. Frolik, C.A., Dart, I.L., Meyers, C.A., Smith, D.M., Sporn, M.B., Purification and Initial Characterization of a Type Beta Transforming Growth Factor from Human Placenta, P.N.A.S. 80, 3676-3680, 1983.

63. Furutani, Y., Notake, M., Fukui, T., Ohue, M., Nakamura, S., Complete Nuclear Sequence of the Gene for Human Interleukin-1-alpha, Nuc. Acids Res. 14, 3167-3179, 1986.

64. Garrett, I.R., Black, K.S., Mundy, G.R., Interactions Between Interleukin-6 and Interleukin-1 in Osteoclastic Bone Resorption in Neonatal Mouse Calvariae, Abstract #140, 3rd International Workshop on Cells and Cytokines in Bone and Cartilage, Calcified Tissue International, Sup. 2 to 46, 1990.

65. Gauldie, J., Richards, C., Harnish, D., Lansdorp, P, Baumann, H., Interferon $ß_2$/B-cell Stimulatory Factor type 2 Shares Identity with Monocyte-derived Hepatocyte Stimulatory Factor and Regulates the Major Acute Phase Protein Response in Liver Cells, P.N.A.S. 84, 7251-7255, 1987

66. Georgilis, K., Schefer, C., Dinarello, C.A., Klempner, M.S., Human Recombinant Interleukin 1B has no effect on intracellular calcium or on functional responses of human neutrophils, J. Immunol. 138 (X), 3403-3407, 1987.

67. Gery, I., Gershon, R.K., Waksman, B.M., Potentiation of Cultured Mouse Thymocyte Responsiveness by Factors Released by Peripheral Leukocytes, J. Imm. 107, 1778-1781, 1971.

68. Gery, I., Handshumaker, R.E., Potentiation of the T-Lymphocyte Response to Mitogens. III. Properties of the Mediators from Adherent Cells, Cell. Immunol. 11, 162-165, 1972.

69. Gowen, M., Wood, D.D., Ihrie, E.J., McGuire, M.K.B., Russell, R.G.G., An Interleukin 1 like Factor Stimulates Bone Resorption *In Vitro*, Nature 306, 378-380, 1983.

70. Gowen, M., Chapman, K., Littlewood, A., Hughes, D., Evans, D., Russell, G., Production of Tumor Necrosis Factor by Human Osteoblasts is Modulated by other Cytokines, but not by Osteotropic Hormones. Endocrinoloy 126 (II), 1250-1255, 1990.

71. Graves, D.T., Owen, A.J., Barth, R.K., Tempst, P., Winoto, A., Fors,

L., Hood, L.E., Detection of c-sis Transcripts and Synthesis of PDGF-like Proteins by Human Osteosarcoma Cultures, Science 226, 972-974, 1984.

72. Graves, D.T., Valentin-Opran, A., Degado, R., Valente, A.J., Mundy, G., Piche, J., The Potentil of Platelet-derived Growth Factor as an Autocrine or Paracrine Factor for Human Bone Cells, Connect. Tissue Res. 23, 209-218, 1989.

73. Graycar, J.L., Miller, D.A., Arrick, B.A., Lyons, R.M., Moses, H.L., Dewryck, R., Human Transforming Growth Factor-B3: Recombinent Expression, Purification, and Biological Activities in Comparison with Transforming Growth Factors-B1 and -B2, Mol. Endocri. 3 (XXII), 1977-1986, 1989.

74. Groopman, J.E., Mitsuyashu, R.T., DeLeo, M.J., Oette, D.H., Golde, D.W., Effect of Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor on Myelopoesis in the Acquired Immunodefiency Syndrome, N. Engl. J. Med. 317 (X), 593-598, 1989.

75. Groopman, J.E., Molina J-M, Scadden, D.T., Hematopoietic Growth Factors: Biology and Clinical Applications, N. Engl. J. Med. 321, 1449-1459, 1989.

76. Haegman, G., Content, J., Volckaert, G., Derynck, R., Tavernier, J., Fiers, W., Structural Analysis of the Sequence Enoding for Inducible 26-kDa Protein in Human Fibroblasts., Eur. J. Biochem. 159, 625, 1986.

77. Hanks, S.K., Quinn, A.M., Hunter, T., The Protein Kinase Family: Conserved Features and Deduced Phylogeny of the Catalytic Domains, Science 241, 42-52, 1988.

77b. Hattersley, G., Owens, J., Flanagan, A., Chambers, T., Macrophage Colony Stimulating Factor (M-CSF) is Essential for Osteoclast Formation *In Vitro*, Biochem. Biophys. Res. Comm. 177, 526-531, 1991.

78. Heath, J.K., Atkinson, S.J., Meikle, M.C., Reynolds, J.J., Mouse Osteoblast Synthesizes Collagenase in Response to Bone Resorbing Agents, Biochim. Biophys. Acta. 802, 151-154, 1984.

79. Heino, J., Ignotz, R.A., Hemler, M.E., Crouse, C., Massague, J., Regulation of Cell Adhesion Receptors by Transforming Growth Factor-B, J. Biol. Chem. 264 (I), 380-388, 1989.

80. Heine, E., Colocalization of TGF-ß1 and Collagen I and III, Fibronectin

and Glycosaminoglycan during Lung Branching Morphogenesis, Development 109, 29-36, 1990

81. Heldin, C.-H., Johnson, A., Wennergenm S., Wernstedt, C., Betscholtz, C., Westermark, B., A Human Osteosarcoma Cell Line Secretes a Growth Factor Structurally Related to a Homodimer of PDGF A-chains, Nature 319, 511-514, 1986.

82. Horning, E.S., Byrne, J.M., On Tethelin as a Tissue Culture Medium, Aust. J. Exp. Biol. Med. Sci. 6, 137-142, 1929.

83. Horowitz, M.C., Baron, R., Mart, J., Andreoli, M., Vignery, A., Osteoclast activating Factor is Distinct from the Monokine IL-1. Br. J. Rheum. 24, 162-64, 1985.

84. Horowitz, M.C., Brown, M., Insogna, K., Coleman, D., Centrella, M., Phillips, J., Weir, E., PTHrP and PTH Induce the Secretion of IL-6 by a Clonal Osteosarcoma Cell Line, pgs 471-476 in "Molecular and Cellular Biology of Cytokines", Eds: Powanda, M.C., Oppenheim, J.J., Kluger, M.J., Dinarello, C.J., A.R. Liss, Inc., 1990.

85. Horowitz, M.C., Coleman, D.L., Flood, P.M., Kupper, T.S., Jilka, R.L., Parathyroid Hormone and Lipopolysaccharide Induce Murine Osetoblast-like Cells to Secrete a Cytokine Indistinguishable from Granulocyte-Macrophage Colony Stimulating Factor, J. Clin. Inves. 83, 148-152, 1989.

86. Horowitz, M.C., Coleman, D.L., Ryaby, J.T., Einhorn, T.A., Osteotropic Agents Induce the Differential Secretion of Granulocyte-Macrophage Colony-Stimulating Factor by the Osteoblast Cell Line MC3T3-E1, J. Bone Min. Res. 4, 911-921, 1989.

87. Horowitz, M.C., Jilka, R.L., Colony Stimulating Factors in Bone Remodeling in "Cytokines and Bone Metabolism", Eds. Gowen, M., Russel, R.G.G., CRC Press, Inc., London, 1991.

88. Horton, W.E., Jr., Higginbotham, J.D., Chandrasekhar, S., Transforming Growth Factor-Beta and Fibroblast Growth Factor Act Synergistically to Inhibit Colagen Synthesis Through a Mechanism Involving Regulatory DNA Sequences, J. Cell. Phys. 141, 8-15, 1989.

89. Hotta, M., Baird, A., Differential Effects of TGF-ß in the Growth and Function of Adrenocortical Cells *in vitro*, P.N.A.S. 83, 7795-7799, 1986.

90. House, C., Wettenhall, R., Kemp, B., The Influence of Basic Residues on

the Substrate Specificity of Protein Kinase C, J. Biol. Chem. 262, 772-777, 1987.

91. Hunter, T., Oncogenes and Growth Control, TIBS, 277-180, July, 1985.

92. Hunter, T., A Thousand and One Protein Kinases, Cell 50, 823-826, 1987.

93. Hynes, R.O., Integrins: a Family of Cell Surface Receptors, Cell 48, 549-553, 1987.

94. Ishikawa, H., Tanaka, H., Tanabe, O., Asaoku, H., Nobuyoshi, M., Yamamoto, I., Kawao, M., Kuramoto, A., Effect of Glucocorticoids on the Biologic Activities of Myeloma Cells: Inhibition of Interleukin-1ß Osteoclast Activating Factor-Induced Bone Resorption, Blood 75, 715-720, 1990.

95. Ishikawa, O, LeRoy, E.C., Trojanowska, M., Mitogenic Effect of Transforming Growth Factor Beta-1 on Human Fibroblasts involves the Induction of Platelet-Derived Growth Factor Alpha Receptors, J. Cell. Phys. 145 (1), 181-186, 1990.

96. Ito, et al. Bladder Cancer Producing Granulocyte Colony-Stimulating Factor, N.E.J.M. 323 (24), 1709-c, 1990.

97. Jakubowski, A.A., Souza, L., Kelly, F., Fain, K., Budman, D., Clarkson, B., Bonilla, M.A., Moore, M.A.S., Gabrilove, J., Effects of Human Granulocyte Colony-Stimulating Factor in a Patient with Idiopathic Neutropenia New Eng. J. Med. 320 (I), 38-42, 1989.

98. Jasin, H.E., Dingle, S.T., Human Macrophage Cell Factors Mediate Cartilage Matrix Degradation Through Chondrocyte Activation, J. Clin. Inves. 68, 571-574, 1982.

99. Jennings, A.M., Shirley, J.E., Stoner, G., Regulation of Proliferation and Differentiation of Respiratory Tract Epithelial Cells by TGF-ß, Exp. Cell. Res. 167, 539-549, 1986.

99b. Jilka, R, Cohn, D.Role of Phosphodiesterase in the Parathormone-Stimulated Adenosine 3'-5'-Monophosphate Response in Bone Cell Populations Enriched in Osteoclasts and Osteoblasts, Endocrinology 109, 743-747, 1981.

100. Kampschmidt, R.F., Upchurch, H.F., Eddington, C.L., Pulliam, L. L., Multiple Biological Actvities of a Partially Purified Leukocyte Endogenous Mediator, Am. J. Phys. 224, 536-541, 1973.

101. Kanzaki, T., Olofsson, A., Moren, A., Werenstadt, C., Hellman, U., Miyazono, K., Claesson-Welsh, L., Heldin, C-H., TGF-B1 Binding Protein: A Component of the Large Latent Complex of TGF-B1 with Multiple Repeat Sequences, Cell 61, 1051-1061, 1990.

102. Kaplan, D., Whotman, M., Schaffhausen, B., Pallas, D.C., White, M., Cantley, L., Roberts, T.M., Common Elements in Growth Factor Stimulation and Oncogenic Transformation: 85 kD Phosphoprotein and Phosphatidylinositol Kinase Activity, Cell 50, 1021-1029, 1987.

103. Kaplan, D., Morrison, D.K., Wong, G., McCormick, F., Williams, L.T., PDGF ß-Receptor Stimulates Tyrosine Phosphorylation of GAP and Association of GAP with a Signalling Complex, Cell 61, 125-133, 1990.

104. Kaushansky, K., Lin, N., Adamson, J.W., IL-1 Stimulates Fibroblasts to Synthesize GM- and G-CSF, J. Clin. Inves. 81, 92-97, 1988.

105. Kawasaki, E.S., Ladner, M.B., Wang, A.M., Arsdell, J.V., Warren, M.K., Laoyne, M.Y., Schmeicken, U.L., Lee, M., Wilson, K.J., Bossman, A., Stanley, E.R., Ralph, P., Mark, D.F., Molecular Cloning of a Complementary DNA Encoding Human Monocyte-Specific Colony Stimulating Factor (CSF-1), Science 230, 291, 1985.

106. Kehrl, J.H., Taylor, A.S., Delsing, G.A., Roberts, A.B., Sporn, M.B., Fauci, A.S., Further Studies of the Role of Transforming Growth Factor-ß in Human B- Cell Function, J. Immunol. 143 (VI), 1868-1874, 1989.

107. Keller, J.R., Mcneice, I.K., Sill, K.T., Ellingsworth, L.R.W., Quesenberry, P.J., Sing, G.K., Ruscetti, F.W., Transforming Growth Factor-ß Directly Regulates Primitive Murine Hematopoetic Cell Proliferation, Blood 75 (III), 596-602, 1990.

108. Kerr, L.D., Miller, D.B., Matrisian, L.M., TGF-B1 Inhibitor of Transin/Stromelysin Gene Expression is Mediated through a Fos Binding Sequence, Cell 61, 267-278, 1990.

109. Kishimoto, T., The Biology of Interleukin-6, Blood 74 , 1-10, 1989.

110. Klein, D.C., Raisz, L.C., Prostaglandins: Stimulation of Bone Resorption in Culture, Endocrinology 86, 436-1440, 1970.

111a. Kobayashi, Y., Matsushima, K., Oppenheim, J.J., Differential Gene

Expression, Synthesis, Processing, and Release of Interleukin-1α and Interleukin-1ß, in "Interleukin-1, Inflammation and Disease", Eds:Bomford, R., Henderson, B., Res. Mono. Cell Tis. Phys. 16, 48-62, Elsevier, 1989.

111b. Kobayashi, Y., Apella, E., Yamada, M., Copeland, T.D., Matsushima, Phosphorylation of Intracellular Precursors of Human Interleukin-1-alpha, J. Immunol. 140, 2279-2287, 1988.

112. Koike, K., Nakahata, T., Kubo, T., Kikuchi, T., Takagi, M., Ishiguro, A., Tsuji, K., Naganuma, K., Okano, A., Akiyama, Y., Akabane, T., Interleukin-6 Enhances Murine Megakaryocytopoiesis in Serum-Free Culture, Blood 75, 2286-2291, 1990.

113. Kojima, et al. Cyclosporine and Recombinant Granulocyt Colony-Stimulating Factor in Severe Aplastic Anemia, N.E.J.M. 323(13), 920-c, 1990.

114. Kronke, M., Leonard, W.J., Depper, J.M., Arya, S.K., Wong-Staal, F., Gallo, R.C., Waldmann, T.A., Greene, W.C., Cyclosporin A Inhibits T-cell Growth Factor Gene Expression at the Level of mRNA Transcription, P.N.A.S. 81, 5214-5218, 1984.

115. Kumijan, D., Wahl, M.I., Rhee, S.G., Daniel, T.O., Platelet-derived growth factor (PDGF) binding promotes physical association of PDGF receptor with phospholipase C, P.N.A.S. 86, 8232-8239, 1989.

116a. Kupper, T.S, Horowitz, M., Lee, F., Coleman, D., Flood, P., Molecular Characterization of the Keratinocyte Cytokines, J.Inv. Derm 88, 501, 1987.

116b. Kupper, T., Flood, P., Coleman, D., Horowitz, M., Growth of an IL-2/IL-4 Dependent T Cell Line Induced by Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), J. Immunol. 138, 4288-4292, 1987.

117. Kurihara, N., Bertolini, D., Suda, T., Akiyama, Y., Roodman, G.D., IL-6 Stimulates Osteoclast-like Multinucleated Cell Formation in Long Term Human Marrow Cultures by Inducing IL-1 Release, J. Immunology 144, 4226-4330, 1990.

118. Kuruvilla, A.P., Shah, R., Hochwald, G.M., Liggitt, H.D., Palladino, M.A., Protective Effect of Transforming Growth Factor Beta 1 on Experimental Autoimmune Diseases in Mice P.N.A.S. 88 (7), 2918-2921, 1991.

119. LaRochelle, W.J., Giese, N., May-Siroff, M., Robbins, K.C., Aaronson, S.A., Molecular Localization of the Transforming and Secretory Properties of PDGF A and PDGF B, Science 248, 1541-1544, 1990.

120. Laiho, M., DeCaprio, J., Ludlow, J.. Livingston, D., Massagué, J., Growth Inhibition by TGF-ß Linked to Suppression of Retinoblastoma Protein Phosphorylation, Cell 62, 171-185, 1990.

121. Lansdorp, P.M., Aarden, L.A., Calafat, J., Zeiljemaker, W.P., A Growth Factor Dependent B-cell Hybridoma, Cur. Top. Micr. Immun.132, 105-113, 1986.

122. Larrodera, P., Cornet, M.E., Diaz-Meco, M.T., Lopez-Barahona, M., Diaz-Laviada, I., Guddal, P.H., Johansen, T., Moscat, J., Phopsholipase C-Mediated Hydrolysis of Phosphatidylcholine is an important step in PDGF-stimulated DNA Synthesis, Cell 61, 1113-1120, 1990.

123. Larrson, E-L., Iscove, N.N., Coutinho, A., Two distinct factors are required for induction of T-cell growth, Nature 283 (XIV), 664-666,1980.

124. Lawrence, D.A., Pircher, R., Julien, P., Conversion of a High Molecular Weight Latent ß-TGF from Chicken Embryo Fibroblasts Into a Low Molecular Weight Active ß-TGF Under Acidic Conditions, Biochem. Biophys. Res. Comm. 133, 1026-1024, 1985.

125. Leibson, J.H., Marrack, P., Kappler, J., B-cell Help Factors, J.Immunol. 129, 1398-1406, 1982.

126. Levi-Montalcini, R., Hamburger, V., Selective Growth Stimulating Effects of Mouse Sarcoma on the Senosry and Sympathetic Nervous System of the Ckick Embryo, J. Exp. Zool. 116, 321-263, 1951.

127. Lomri, A., Marie, P.J., Effects of Transforming Growth Factor ß on Expression of Cytoskeletal Proteins in Endosteal Mouse Osteoblastic Cells, Bone 11, 445-451, 1990.

128. Lyons, R.M., Keski, J., Moses, H.L., Proteolytic Activation of Latent TGF-ß from Fibroblast Conditioned Medium, J. Cell. Biol. 106, 1659-1665, 1988.

128b. MacDonald, B., Mundy, G., Clark, S., Wang, E., Kuehl, T., Stanley, E., Roodman, G., Effects of Human Recombinant CSF-GM and Highly Purified CSF-1 on the Formation of Multinucleated Cells with Osteoclast Characteristics in Long Term Marrow Cultures, J. Bone Min. Res. 1, 227-233, 1986.

129. McCartney-Francis, N., Mizel, D., Wong, H., Wahl, L. Wahl, S., TGF-ß as an Immunoregulatory Molecule, FASEB J. 2, A875, 1988.

130. Mackie, E.J., Trechsel, U., Stimulation of Bone Formation in vivo by Transforming Growth Factor-ß: Remodeling of Woven Bone and Lack of Inhibition by Indomethacin, Bone 11, 295-300, 1990.

131. Madri, J., Pratt, B., Tucker, A., Phenotypic Modulation of Endothelial Cells by Transforming Growth Factor-ß Depends upon the Composition and Organization of the Extracellular Matrix, J. Cell. Biol. 106, 1375-1384, 1988.

132. Maennel, D.N., Mizel, S.B., Diamantsetin, T., Falk, W., Induction of Intereukin-2 responsivesness in thymocytes by synergistic action of interleukin 1 and interleukin 2, J. Immunol. 134 (V), 3108-3110, 1985.

132b. Malipiero, U., Frei, K., Fontana, A., Production of Hemopoietic Colony-Stimulating Factors by Astrocytes, J. Immunol. 144 ,3816-21, 1990.

133a. Massagué, J., Like, B., Cellular Receptors for Type ß-Transforming Growth Factor, J.B.C. 260, 2636-2645, 1985.

133b. Massagué, J., Control of Cell Growth and Phenotype by Transforming Growth Factors, 1991 HHMI Bull. Res. In Progress, 301-302, 1991.

133c. Massagué, J., The Transforming Growth Factors, T.I.B.S., 237-240, 1985.

134. Masui, T., Wakefield, L.M., Leicher, J.F., LaVeck, M.A., Sporn, M.B., Harris, C.C., Type ß Transforming Growth Factor in the Primary Differentiation Inducing Serum Factor for Normal Human Bronchial Epithelial Cells, P.N.A.S. 83, 2438-2442, 1986.

135. Matrisian, L.M., Leroy, P., Rohlman, C., Gesnel, M-C, Breathmach, R., Isolation of the Oncogene and EGF Induced Transin Gene: Complex Control in Rat Fibroblasts, Molec. Cell. Biol. 6, 1679-1686, 1986.

136. Menkin, V., Chemical Basis of Fever, Science 100, 337-338, 1944.

137a. Metcalf, D., Begley, C.G., Johnson, G.R., Nicola, N.A., Vadas, M.A., Lopez, A.F., Williamson, D.J., Wong, G.G., Clark, S.C., Wang, E.A., Biologic Properties *in Vitro* of a Recombinant Human Granulocyte-Macropahge Colony-Stimulating Factor, Blood 67 (I), 37-45, 1986.

137b. Metcalf, D., The Granulocyte-Macrophage Colony-Stimulating Factors, Science 229, 16-22, 1985.

136c. Metcalf, D., Mechanisms Contributing to the Sex Difference in Levels of Granulocyte-Macrophage Colony-Stimulating Factor in the Urine of GM-CSF Transgenic Mice, Exp. Hem. 16, 794-800, 1988.

138. Mizel, S.B., Cyclic AMP and IL-1 Signal Transduction, Imm. Today 11, 390-393, 1990.

139. Moore, D.M., Murphy, P.A., Chesney, P.J., Wood, W.B., Synthesis of Endogenous PYrogen by Rabbit Leukocytes, J.E.M. 137, 1263-1274, 1973.

139b. Morgan, C., Pollard, J., Stanley, E., Isolation and Characterization of a Cloned Growth Factor Dependent Macrophage Cell Line, BAC 1.2F5, J. Cell. Phys. 130, 420-427, 1987.

140. Morris, D.R., Kuepfer, C.A., Ellingsworth, L.R., Ogawa, Y., Rabinovitch, P.S., Transforming Growth Factor-ß Blocks Proliferation but not Early Mitogenic Signalling Events in T-Lymphocytes, Exp. Cell Res. 185, 529-534, 1989.

141. Mosley, B., Dower, S.K., Cosman, D., Determination of the Minimum Polypeptide Lengths of the Functionally Active Sites of Human Interleukin-1-alpha and beta, P.N.A.S. 84, 4572-4576, 1987.

142a. Muegge, K., Tartovsky, B., Finnegan, A., Berzofsky, J.A., Young, H.A., Durum, S.K., Interleukin-1 Expression in Different T Cell Types, FASEB J. 2, 5518-A, 1988.

142b. Muegge, K., Durum, S.K., The Role of Interleukin-1 in the Immune Response, in "Interleukin-1, Inflammation and Disease", Eds:Bomford, R., Henderson, B., Res. Mono. Cell Tis. Phys. 16, 107-122, Elsevier, 1989.

143. Murray, R., Lee, F., Chiu, C., The Genes for Leukemia Inhibitory Factor and Interleukin-6 Are Expressed in Mouse Blastocysts prior to the Onset of Hemopoiesis, Mol. Cel. Biol. 10, 4953-3956, 1990.

144. Musso, T., Espinoza-Delgado, I., Pulkki, K., Gusella, G.L., Longo., D.L., Varesio, L., Transforming Growth Factor ß Downregulates Interleukin-1 (IL-1)-Induced IL-6 Production by Human Monocytes, Blood 76, 2455-2469, 1990.

145. Nagata, S., Tsuchiga, M., Asano, S., Kaziro, Y., Yamazaki, T.,, Molecular Cloning and Experimentation of cDNA for Human G-CSF, Nature 319, 415, 1986.

146. Naitoh, Y., Fukata, J., Tominaga, T., Nakai, Y., Tamai, S., Mori, K., Imura, H., IL-6 Stimulates the Secretion of Adrenocorticotropic Hormone in Conscious, Fully Moving Rats, Biochem. Biophys. Res. Comm 155, 1459, 1988.

147. Nishihara, T., Ishihara, Y., Noguchi, T., Koga, T., Membrane IL-1 induces bone resorption in organ culture, J. Immunol. 143 (VI), 1881-1886, 1989.

148 Noda, M., Yoon, K., Prince, C., Butler, W., Rodan, G., Transcriptional Regulation of Osteopontin Production in Rat Osteosarcoma Cells by Type ß Transforming Growth Factor, J. Biol. Chem. 263, 13916-13921, 1988.

149. Ohno, et al., Effect of Granulocyte Colony-Stimulating Factor after Intesnive Induction Therapy in Relapsed or Refractory Acute Leukemia, N.E.J.M. 323 (13), 871-880, 1990.

150. O'Neill, L.A.J., Bird, T.A., Saklatavala, J., IL-1 Signal Transduction, Immunology Today, 11, 392-393, 1990.

151. Oppenheim, J.J., Scheyoun, A., Kook, A.I., Enhancement of DNA Synthesis and cAMP Content of Mouse Thymocytes by Mediators Derived from Adherent Cells, J.Immunol. 116, 1466, 1976.

152. Oreffo, R.O.C., Mundy, G.R., Twardzik, D., Bonewald,L.F. Selective Release of Active human TGF-ß1 from its Recombinant Inactive Precursor by Isolated Osteoclasts, A-1647, 3rd Int'l Symp. Cells. Cytokines. Bone Cartl., 1990, Springer Int'l.

153. Ostrowksi, J., Meier, K.E., Stanton, T.H., Smith, L.L., Bomsztyk, K., Interferon-gamma and Interleukin-1-alpha Induce Transient Translocation of Phospholipase C Activity to Membranes in a ß Lymphoid Cell Line, J. Biol. Chem. 263, 13786-13790, 1988.

154. Palumbo, A., Jones, C.L., Lightman, A., Immunohistochemical Localization of Renin and Angiotensin II in Human Ovaries, Amer. J. Obstet. Gynecol. (In press), 1988.

155. Peracchia, F., Ferrari, G., Poggi, A., Rotilio, D., IL-1 Beta-induced Expression of PDGF-AA Isoform in Rabbit Articular Chondrocytes is Modulated by TGF-Beta 1, Exp. Cell. Res. 193 (1), 208-212, 1991.

156. Perlmutter, D.H., Dinarello, C.A., Punsal, P.I., Colten, H.R., Cachectin/Tumor Necrosis Factor Regulates Hepatic Acute Phase Gene Expression, J. Clin. Inves. 78, 1349-1354, 1986.

157. Pfeilschifter, J., Wolf, O., Naumann, A., Minne, H.W., Mundy, G.R., Ziegler, R., Chemotactic Response of Osteoblastlike Cells to Transforming Growth Factor ß, J. Bone and Mineral Res. 5, 825-829, 1990.

158. Pfeilschifter, J., Oechsner, M., Naumann, A., Gronwald, R., Minne, H., Ziegler, R., Stimulation of Bone Matrix Apposition *in Vitro* by Local Growth Factors: A Comparison between Insulin-like Growth Factor I, Platelet-Derived Growth Factor, and Transforming Growth Factor ß, Endocrinology 127, 69-75, 1990.

159. Pierce, G.F., Vande Berg, J., Rudolph, R., Tarpley, J., Mustoe, T.A., Platelet-derived Growth Factor-BB and Transforming Growth Factor Beta 1 Selectively Modulate Glycosaminoglycans, Collagen, and Myofibroblasts in Excisional Wounds Am. J. Pathol. 138 (3), 629-646, 1991.

160. Raines, E.W., Dower, S.K., Ross, R., Interleukin-1 mitogenic activity for Fibroblasts and Smooth Muscle Cells is due to PDGF-AA, Science 243, 393-396, 1989.

161. Ramadori, G., Sipe, J.D., Dinarello, C.A., Mizel, S.B., Colten, H.R., Post-translational Modification of Acute Phase Hepatic Protein Synthesis by Murine Recombinant Interleukin-1 (IL-1) and Purified Human IL-1, J.E.M. 162, 930-942, 1985.

162. Rapp, U.R., Cleveland, J.L., Bonner, T.I., Storm, S.M., The *raf* oncogene family. In The Oncogene Handbook, P.Reddy, T. Curren, and A. Skalka, eds. (Amsterdam:Elsevier), pp. 213-253, 1987.

163. Rappolei, D.A., Brenner, C.A., Schultz, R., Mar, D., Weib, Z., Develpmental Expression of PDGF, TGF-alpha, and TGF-ß in Preimplantated Mouse Embryos, Science 242, 1823-1825, 1988.

164. Redini, F., Lafumac, C., Purjol, J-P, Robert, L., Hornebeck, W., Effect of Cytokines and Growth Factors in the Expression of Elastase Activity by Huamn Synoviocytes, Dermal Fibroblasts and Rabbit Articular Chondrocytes, Biochem. Biophys. Res. Comm. 155, 786-793, 1988.

165. Ritchie, D.G., Levy, B.A., Adams, M.A., Fuller, G.M., Regulation of Fibrinogen Synthesis by Plasmin-Derived Fragments of Fibrinogen and Fibrin: An Indirect Feedback Pathway, P.N.A.S. 79, 175-179, 1982.

166. Roberts, A.B., Anzano, M.A., Meyers, C.A., Wideman, J., Blacher, R.,

Pan, Y-C, Stein, S., Lehrman, S.R., Smith, J.M., Lamb, L.C., Sporn, M.B., Purification and Properties of a Type Beta-Transforming Growth Factor from Bovine Kidney, Biochem. 22, 5692-5698, 1983.

167. Roberts, A.B., Sporn, M.B., The Transforming Growth Factors in Peptide Growth Factors and Their Receptors, Handbook of Experimental Pharmacology, Springer-Verlag, Heidelberg, 1990, (in press).

167b. Robertson, S., Seamark, R., Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) in the Murine Reproductive Tract: Stimulation by Seminal Factors, Rep. Fert. & Dev. 2, 359-368, 1990, .

167c. Rodan, S., Wesolowski, G., Chin, J., Limjuco, G., Schmidt, J., Rodan, G., IL-1 Binds to High Affinity Receptors on Human Osteosarcoma Cells and Potentiates Prostaglandin E_2 Stimulation of cAMP Production, J. Immunol 145, 1231-1237, 1990.

168. Rosoff, P.M., Savage, N., Dinarello, C.A., Interleukin-1 Stimulates Diacylglycerol Production in T lymphocytes by a novel mechanism, Cell 54, 73-81, 1988.

169. Ruddle, N.H., Paul, N., Horowitz, M., Troiano, N., Jay, G., Baron, R., HTLV-1 Activation of Lymphotoxin (TNF-ß) Production and High Turnover Bone Disease. #224, 3rd Int'l. Symp. Cells Cyt. Bone Cart., Springer Int'l., 1990.

170. Sakamoto, K. M., Golde, D.W., Gasson, J.C., The Biology and Clinical Applications of Granulocyte-Macrophage Colony Stimulating Factor, J. Ped. 118, S17-S19, 1991.

171. Saklatvala, J., Interleukin-1 and Connective Tissue, in "Interleukin-1, Inflammation and Disease", Eds:Bomford, R., Henderson, B., Res. Mono. Cell Tis. Phys. 16, 143-162, Elsevier, 1989.

172. Saklatvala, J., Tumor Necrosis Factor-alpha Stimulates Resorption and Inhibits Synthesis of Proteoglycans in Cartilage, Nature 322, 547-549, 1986.

173. Scherr, C.J., Retternmeier, C.W., Sacca, R., Roussel, M.F., Look, A.T., Stanley, E.P., The c-fms Proto-oncogene Product is Related to the Receptor for the Monocyte Phagocyte Growth Factor CSF-1, Cell 41, 665-676, 1985.

174. Schettini, G., Florio, T., Meucci, O., Scala, G., Landolfi, E., Grimaldi, M., Effect of Interleukin 1 Beta on Transducing Mechanisms in 235-1 Clonal Pituitary Cells. Part I: Modulation of Adenylate Cyclase Activity, Biochem. Biophys. Res. Comm. 155 (III), 1089-1096, 1988.

175. Schimizu, S., Hirano, T., Yoshioka, K., Sugai, S., Matsuda, T., Taga, T., Kishimito, T., Konda, S., IL-6 (BCSF-2)-Dependent Growth of a Lennerts Lymphoma-Derived T-cell Line, KT-3, Blood 72, 1826-1830, 1988.

176. Schimple, A., Wecker, E., Replacement of T-cell Functioning by a T-Cell Product, Nature 237, 15-17, 1972.

177. Sehgal, P.B., Walther, Z., Tamm, I., Rapid Enhancement of ß2-Interferon/B-cell Differentiation Factor BSF-2 Gene Expression in Human Fibroblasts by Diacylglycerol and Calcium Ionophore A23187, P.N.A.S. 84, 3662-3667, 1987.

178. Seifert, R.A., Hart, C.E., Phillips, P.E., Forsrtom, J.W., Ross, R., Murray, M.J., Bowen-Pope, D.F., Two Different Subunits Associate to Create Isoform Specific Platelet-Derived Growth Factor Receptors, J. Biol. Chem. 264, 8771-8778, 1989.

179. Seyedin, S.M., Thomas, T.C., Thompson A.Y., Rosen, D.M, Piez, K.A., Purification and Characterization of two Cartilage-Inducing Factors from Mouse Demineralized Bone, P.N.A.S. 82, 2267-2271, 1985.

180. Sims, J.E., March, C.J., Cosmna,. D., Widmer, M.B., MacDonald, H.R., McMahan, C.J., WignallJ.M., Grubin, C.E., Call, S.M., Friend, D., Alpert, A.R., Gillis, S.R., Urdal, D.L., Dower, S.K., cDNA Expression Cloning of the IL-1 Receptor, a Member of the Immunoglobulin Superfamily, Science 241, 585-589, 1988

181. Singer, A., Morrisey, P.J., Hathcook, K.S., Ahmed, A., Scher, T., Hodes, R.J., Role of the Major Histocompatability Complex in T-Cell Activation of B-Cell Subpopulations, J.E.M. 154, 501-514, 1981.

182. Souza, L.M., Boone, T.C., Gabrilove, J., Lai, P., Zsebo, K.M., Recombinant Human G-CSF:Effects on Normal and Leukemic Myeloid Cells, Science 232, 61-65, 1986.

183. Sporn, M.B., Roberts, A.B., Transforming Growth Factor-Beta Multiple Actions and Potential Clinical Applications, J.A.M.A. 262, 938-943, 1989.

184. Stanley, E., Cifone, M., Heard, P., Defendi, V., Factors Regulating

Macrophage Production and Growth: Identification of a Colony-Stimulating Factor and Macrophage Growth Factor, J. E. M. 143, 631-647, 1976.

185. Strain, A.J., Frazer, A., Hill, D.J., Miker, R.D.G., TGF-ß Inhibits DNA Synthesis in Hepatocytes Isolated from Normal and Regenerating Rat Liver, Biochem. Biophys. Res. Comm. 145, 436-442, 1987.

186. Sudo, H., Kodama, H-A., Amagai, Y., Yamamoto, S., Kasai, S., In Vitro Differentiation and Calcification in a New Clonal Osteogenic Cell Line Derived from Newborn Mouse Calvaria, J. Cell. Biol. 96, 191-198, 1983.

187. Takahashi, N., Mundy, G.R., Roodman, G.D., Recombinant Human Interferon-gamma Inhibits Formation of Human Osteoclast-like Cells, J. Immunol. 137, 3544-3549, 1986.

188. Tanabe, O., Akira, S., Kamiza, T., Wong, G.G., Hirano, T., Kishimata, T., Genomic Sructure of the Murine IL-6 Gene. High Degree Conservation of Potential Regulatory Sequences Between Mouse and Human, J. Immunol. 141, 3875-3880, 1988.

189a. Thompson, N.L., Bazoberry, F., Speir, E., Cassells, W., Ferrans, V., Flanders, K., Kordaiah, P., Geiser, A., Sporn, M., Acute Myocardial Infarction in Rats, Growth Factors (In Press).

189b. Thompson, N.L., Flanders, K.C., Smith, M., Ellingsworth, L.R., Roberts, A.B., Sporn, M.B., Cell Type Specific Expression of TGF-ß in Adult and Neonatal Mouse Tissue, J. Cell. Biochem. (In Press), 1989.

190. Tsukamoto, T., Matsui, T., Fukas, M., Fujita, T., Platelet-Derived Growth Factor ß Chain Homodimer Enhances Chemotaxis and DNA Synthesis in Normal Osteoblast-like Cells (MC3T3-E1), Biochem. Biophys. Res. Comm. 175, 745-751, 1991.

191. Tsunawaki, S., Sporn, M.B., Ding, A., Nathan, C., Deactivation of Macrophages by TGF-ß, Nature 334, 260-262, 1988.

192. Tuschinski, R.J., Oliver, I.T., Guilbert, L.J., Tynen, F.W., Warner, J.R., Stanley, E.R., Survival of Mononuclear Phagocytes Depends on a Lineage-Specific Growth Factor that the Differentiated Cells Selectively Destroy, Cell 28, 71, 1982.

193. Uehara, A., Gottschall, P.E., Dahl, R.R., Arimura, A., Stimulation of ACTH Release by Human Interleukin-1 ß but not by Interleukin-1 α in Conscious, Freely-Moving Rats, Biochem. Biophys. Res. Comm. 146, 1286-1290, 1987.

194. Vadhan-Raj, S., Keating, M., LeMaistre, A., Hittleman, W.N., McCreddie, K., Trujillo, J.M., Broxmeyer, H.E., Henney, C., Gutterman, J.U., Effects of Recombinant Human Granulocyte-Macrophage Colony Stimulating Factors in Patents with Myelodyspalstic Syndromes, N. Engl. J. Med. 317 (25), 1544-1552, 1987.

194b. Valtieri, M., Tweardy, D., Caracciolo, D., Jophnson, K., Mavilio, F., Altmann, S., Santoli, D., Rovera, G., Cytokine-Dependent Granulocyte Differentiation. Regulation of Proliferation and Differentiative Response in a Murine Progenitor Cell Line, J. Immunol. 138, 3829-3835, 1987.

195. Van den Eijnden-van Raaij, A., Koornneed, I., van Zoelen, E., A New Method for High Yield Purification of type beta Transforming Growth Factor from Human Platelets, Biochem. Biophys. Res. Comm. 157, 16-23, 1988.

196. Varga, J., Rosenbloom, J., Jimenez, S., Transforming Growth Factor-ß Causes a Persistant Increase in Steady-State Amounts of Type I and Type III Collagen and Fibronectin mRNAs in Normal Human Dermal Fibroblasts, Biochem. J. 247, 597-604, 1987.

197a. Wahl, S.M., McCartney-Francis, N., Mergenhagen, S.E., Inflammatory and Immunomodulatory Roles of TGF-B, Immunol. Today 10 , 258-261,1989.

197b. Wahl, S.M., Allen, J.B., Wong, H.L., Dougherty, S.F., Ellingsworth, L.R., Antagonistic and Agonistic Effects of Transforming Growth Factor-B and IL-1 in Rheumatoid Synovium, J. Immunology 145, 2514-2519, 1990.

198. Wakefield, L.M., Latent TGF-ß in Serum, J. Biol. Chem. 262, 14090-14099, 1987.

199. Wakefield, L.M., Smith, D.M., Masui, T., Sporn, M.B., Distribution and Modification of Receptor for Transforming Growth Factor-ß, J. Cell. Biol. 105, 965-975, 1987.

200. Warren, M.K., Ralph, P., J. Imm., Monocyte Growth Factor Colony Stimulating Factor 1 Stimulates Human Monocyte Production of Interferon, Tumor Necrosis factor, and Colony Stimulating Activity, 137, 2281, 1986

201. Waterfield, M.D., Scrace, T., Whittle, N., Stroobart, D., Johnson, A., Wasteson, A., Westermark, B., Heldin, C.H., Huang, J-S, Duel, T.F., Platelet-Derived Growth Factor is Structurally Related to the Putative Transfer Protein p28 sis of Simian Sarcoma Virus, Nature 304, 35-39, 1983.

202. Weeks, J. E., "Coley's Toxins" in The Status of Vaccine and Serum

Therapy in Ophthalmology, J.A.M.A. 40 (4), 267-268, 1910.

203. Weir, E., Horowitz, M.C., Insogna, K., Macrophage Colony Stimulating Factor is Principally Responsible for the Colony Stimulating Activity Secreted by Osteoblasts in Response to Parathyroid Hormone and Parathyroid Hormone Related Peptide *in vitro*, J. Clin. Inves. (submitted)

204. Weir, E., Insogna, K., Horowitz, M, A Clonal Rat Osteosarcoma Cell Line Secretes Colony Stimulating Factors In Response to Parathyroid Hormone and Lipopolysaccharide, Endocrinology 124, 899-904, 1989.

205. Whetton, A.D., Dexter, T.M., Haemopoietic Growth Factors, T.I.B.S. 11 (V), 207-211, 1986.

206. Wiktor-Jedrezczak, W., Bartocci, A., Ferran te, A.W., Ahmed-Ansari, A., Sell, K.W., Pollard, J.W., Stanley, E.R., Total Absence of Colony Stimulating Factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse, P.N.A.S. 87, 4828-4832, 1990.

207. Wolff, S.F., Auron, P.E., Dinrello, C.A., Rich, A., "Clone Controversy at Immunex", Nature 319, 270, 1986.

208. Wong, G.L., Cohn, D.V., Target Cells in Bone for Parathormone and Calcitonin are Different: Enrichment for Each Cell Type by Sequential Digestion of Mouse Calvaria and Selective Adheison to Polymeric Surfaces, P.N.A.S. 72 (VIII), 3167-3171, 1975.

209. Wong, G.C., Witek, J.S., Temple, P.A., Human GM-CSF: Molecular Cloning of the Complementary DNA and Purification of the Natural and Recombinant Proteins, Science 228, 810-813, 1985.

210. Wong, G.C., Temple, P.A., Leary, D.C., et al., Human CSF-1:Cloning and Experimentation of 4kb cDNA Encoding Human Urinary Protein, Science 235, 1504-1506, 1987.

211. Yang, Y.C., Tsai, S., Wong, G.G., Clark, S.C.(in preparation).

212. Yoshida, H., Hayashi, S., Kunisada, T., Ogawa, M., Nishikawa, S., Okamura, H., Sudo, T., Shultz, L.D., Nishikawa, S., The murine Mutation Osteopetrosis is in the coding Region of the Macropahge Colony Stiulating Factor gene, Nature 345, 442-443, 1990.

213. Yoshizaki, K., Nakagawa, T., Kaieda, T., Muraguchi, A., Yamamura, Y., Kishimoto, T., Induction of Proliferation and Ig Production in Human B Leukemic Cells by Anti-Ig and T Cell Factor, J. Immunol. 128, 1296-1299, 1982.

213b. Zlotnik, A., Daine, B., Smith, C., Activation of an Interleukin 1 Responsive T-Cell Lymphoma by Fixed P388D1 Macrophages and an Antibody against the Ag:MHC T-Cell Receptor, Cell. Immunol. 94, 447-453, 1985.

214. Zucali, J.R., Dinarello, C.A., Gross, M.A., Anderson, L., Weine, R.S., Interleukin-1 Stimulates Fibroblasts to Produce Granulocyte-Macrophage Colony Stimulating Activity and Prostaglandin E_2, J. Clin. Invest. 77, 1857-1863, 1986.

215. Zsebo, K.M., Cohen, A.M., Murdock, D.C., Recombinant G-CSF: Molecular and Biological Characterization, Immunobiology 171, 175-184, 1986.

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