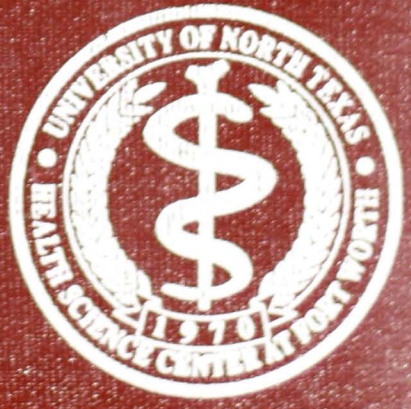
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REGULATION OF HUMAN MACROPHAGE

COLONY-STIMULATING FACTOR TRANSCRIPTION

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# REGULATION OF HUMAN MACROPHAGE COLONY-STIMULATING FACTOR TRANSCRIPTION

DISSERTATION

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For the Degree of

DOCTOR OF PHOLOSOPHY

By

Pisate John Kamthong, M.D.

Fort Worth, Texas

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AP-1 | Activator protein-1 |
| ATP | Adenosine 5'triphosphate |
| cAMP | cyclic Adenosine 5'monophosphate |
| DB-cAMP | dibutyryl cyclic Adenosine 5'monophosphate |
| DMEM | Dulbecco's modified Eagle's medium |
| EMSA | Electrophoretic mobility shift assay |
| ERK | extracellular receptor-associated kinase |
| IkB | Inhibitory kappa B |
| IKK | IkB kinase |
| IL | Interleukin |
| IL-1R | Interleukin-1 receptor |
| IRAK | IL-1R-associated kinase |
| JNK | Jun-N-terminal kinase |
| LPS | Lipopolysaccharide |
| MAPK | Mitogen activated protein kinase |
| MAPKK | Mitogen activated protein kinase kinase |
| MEK1 | Mitogen activated protein kinase kinase 1 |
| M-CSF | Macrophage colony-stimulating factor |
| NIK | NF-κB inducing kinase |

| | |
|---|---|
| NF-κB | Nuclear factor kappa B |
| PI-3K | Phosphatidylinositol 3'-kinase |
| PKA | Protein kinase A |
| PKC | Protein kinase C |
| PMA | Phorbol 12-myristate 13-acetate |
| PPARα | Peroxisome proliferator-activated receptor alpha |
| RNI | Reactive nitrogen intermediates |
| TAK1 | Transforming growth factor beta-activated kinase 1 |
| TNF | Tumor necrosis factor |
| TRAF6 | TNF receptor-activated factor 6 |

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# CHAPTER I

# INTRODUCTION

## *Background*

The role of macrophage colony-stimulating factor (M-CSF) in hematopoiesis has been firmly established mainly by using bone marrow cell cultures. Semi-solid culture of bone marrow cells that were independently developed by Bradley and Metcalf in 1966 and Pluznik and Sachs in1965, has been the standard method to study proliferation and differentiation of hematopoietic cells since the mid-1960s. It supports the clonal expansion of the hematopoietic colonies in vitro. Thus provides the means to functionally assay the hematopoietic progenitor cells and aides the discovery of growth factors regulating the progenitor cell differentiation.

Macrophage colony-stimulating factor (M-CSF) was initially identified as a hematopoietic growth factor that stimulates the proliferation, differentiation and survival of monocytes, macrophages, and their progenitors (Robinson *et al.* 1969; Stanley *et al.* 1971). M-CSF is produced by a large variety of cells throughout the body. It can be purified from various body fluids as well as the conditioned media of several cell lines and tissues, such as leukocytes, placenta, lung, pancreatic cancer cells and spleen (Metcalf 1984; Stanley and Guilbert 1981; Yunis 1983). The sources of M-CSF recently have been extended to include liver parenchymal cells (Ezure *et al.* 1997) and thyrocytes (Kasai *et al.* 1997). It was also previously called colony- stimulating factor from human

urine (CSF-HU) attributing to the source of human urine growth factor that stimulated the formation of small aggregates consisting of granulocyte clusters in a soft agar culture system of human bone marrow cells (Metcalf 1974). At first without the knowledge about biochemical structures of CSFs, several colony-stimulating factors, later proven to be M-CSF, were recognized by their sources, i.e. mouse L-cell CSF, mouse uterus CSF, human lung-conditioned-medium CSF. Later, researchers in the field adopted the reclassification of CSF subtypes using the predominant colony types stimulated by the factor in semi-solid bone marrow cell cultures. M-CSF is for CSF that predominantly stimulates macrophage colony formation. Granulocyte colony-stimulating factor (G-CSF) refers to a granulocyte-active material, such as peritoneal cell-conditioned medium CSF (Horiuchi and Ichikawa 1977). CSF stimulating both types of colonies is called granulocyte-macrophage colony-stimulating factor, GM-CSF. M-CSF was also termed colony stimulating factor 1 (CSF-1), described as the first CSF to be purified (Stanley 1977). G-CSF was later called CSF-2. The existence of these two biochemically distinct CSFs was first identified in this lab (Wu *et al.* 1981). By the same virtue, GM-CSF sometimes was referred to as CSF-3. All of the three CSFs were later identified as distinct peptides encoded by different genes. Sachs and Pluznick group at Rehovot introduced alternative nomenclature for CSFs. The term macrophage granulocyte inducer-1M, MGI-1M, refers to M-CSF. MGI-1G is equivalent to G-CSF, and MGI-GM is used for GM-CSF.

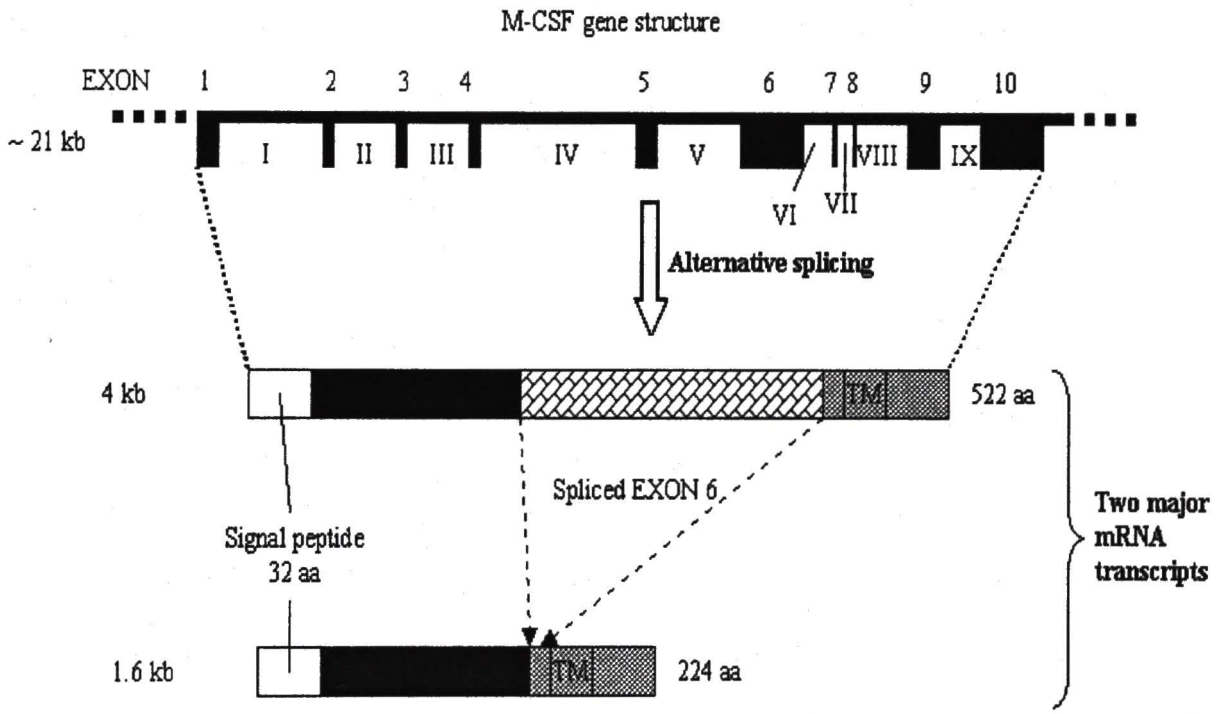
M-CSF stimulates differentiation of progenitor cells (colony-forming unit macrophage, CFU-M) to mature monocytes, and prolongs the survival of monocytes (Motoyoshi *et al.* 1997). It enhances expression of differentiation antigens (Hashimoto *et*

*al.* 1997) and stimulates chemotactic, phagocytic and the killing activities of monocytes (Wang *et al.* 1988). It also stimulates production of several cytokines such as granulocyte- macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and interleukin (IL)-6 by priming monocytes, and directly stimulates production and secretion of IL-8 and reactive nitrogen intermediates (Motoyoshi and Takaku 1991).

***Molecular structure of M-CSF***

Complimentary DNA (cDNA) encoding human M-CSF was first cloned from pancreatic cancer cell line MIA PaCa-2 (Kawasaki *et al.* 1985). The gene for human M-CSF was assigned to the short arm of chromosome 1 at region p13-p21 (Morris et al., 1991), where a single copy of M-CSF gene is located. The gene comprises 10 exons spanning 21 kilobases (kb) of the genome (Ladner *et al.* 198[illegible]). Five cDNAs have been cloned from alternative mRNA transcripts ranging in size from 1.5 kb to 4.4 kb (Stanley *et al.* 1994; Kawasaki *et al.* 1985). This heterogeneity is due to various combinations of a differential splicing of the coding region in exon 6 and of the 3'-untranslated region of exons 9 and 10 (Fixe and Praloran, 1998). The alternative splicing in the exon 6 affects two intracellular proteolytic cleavage sites (Fig. 1), a site of glycosaminoglycan addition, a site of O-glycosylation and the other two sites of N-glycosylation (Stanley *et al.* 1994). Alternative splicing of non-coding exons 9 or 10 modifies the stability of the mature mRNAs. Alternative splicing in combination with heavy post-translational glycosylations generates different mature M-CSF isoforms. The secreted form is encoded by two

**Figure 1. M-CSF gene stucture and major mRNA transcripts** M-CSF gene spans ~ 21 kb and comprises 10 exons and 9 introns (in Roman numerals). The signal peptide (open boxes), partial coding sequences (filled boxes), and transmembrane regions (TM) are identical in both major functional forms of M-CSF. The shorter form which becomes membrane-bound lacks the sequence from spliced exon 6.


M-CSF gene structure
EXON 1 2 3 4 5 6 7 8 9 10
~ 21 kb
I II III IV V VI VII VIII IX
Alternative splicing
4 kb
TM
522 aa
Spliced EXON 6
Signal peptide
32 aa
1.6 kb
TM
224 aa
Two major mRNA transcripts

transcripts of 2.5 and 4.0 kb, from which 85-kDa M-CSF and PG-M-CSF are made, contain full-length coding sequence and differ only in the 3'-noncoding regions (Hamilton 1993). The sequence of the cDNA predicted a translated protein of 522 amino acids including a signal peptide of 32 amino acid. These contain the complete exon 6 encoding an amino acid sequence, which is cleaved by proteases, leading to the rapid secretion of the protein from the cell (Rettenmier *et al.* 1988). The other two transcripts of 1.6 and 3.1 kb encode the M-CSF protein associated with the cell membrane (Ladner et al., 1987 and Pogue-Geile *et al.* 1995). Since in these transcripts the splicing out of the large segments of exon 6 deletes the region of 298 amino acid residues containing the proteolytic cleavage site, the product is stably expressed at the cell surface and is also biologically active (Stein *et al.* 1990). Although 4.0, 3.8, 2.0 and 1.4 kb mRNA have been detected in Northern blot analysis of murine M-CSF, only 4.0 and 2.0 kb transcripts were cloned. Both murine 4.0 and 2.3 kb cDNA possess full coding sequence and differ only in 3'-untranslated region similarly to human isoforms (Ladner *et al.* 1988).

Other variations in naturally occurring M-CSF include differences in glycosylation and glycosaminoglycan additions, the latter of which may modulate bioactivity (Partenheimer *et al.* 1995). Each M-CSF subunit contains a four-helical bundle that is linked end to end in the M-CSF dimer via a single interchain disulfide bond, several other disulfide bonds appear to stabilize the helices by crosslinking loops at the end of the molecule (Pandit et al., 1992). It is theoretically possible to have differences in the two subunits of the molecule, and heterodimers with regard to glycosaminoglycan addition. Three major M-CSF species have been reported in mouse

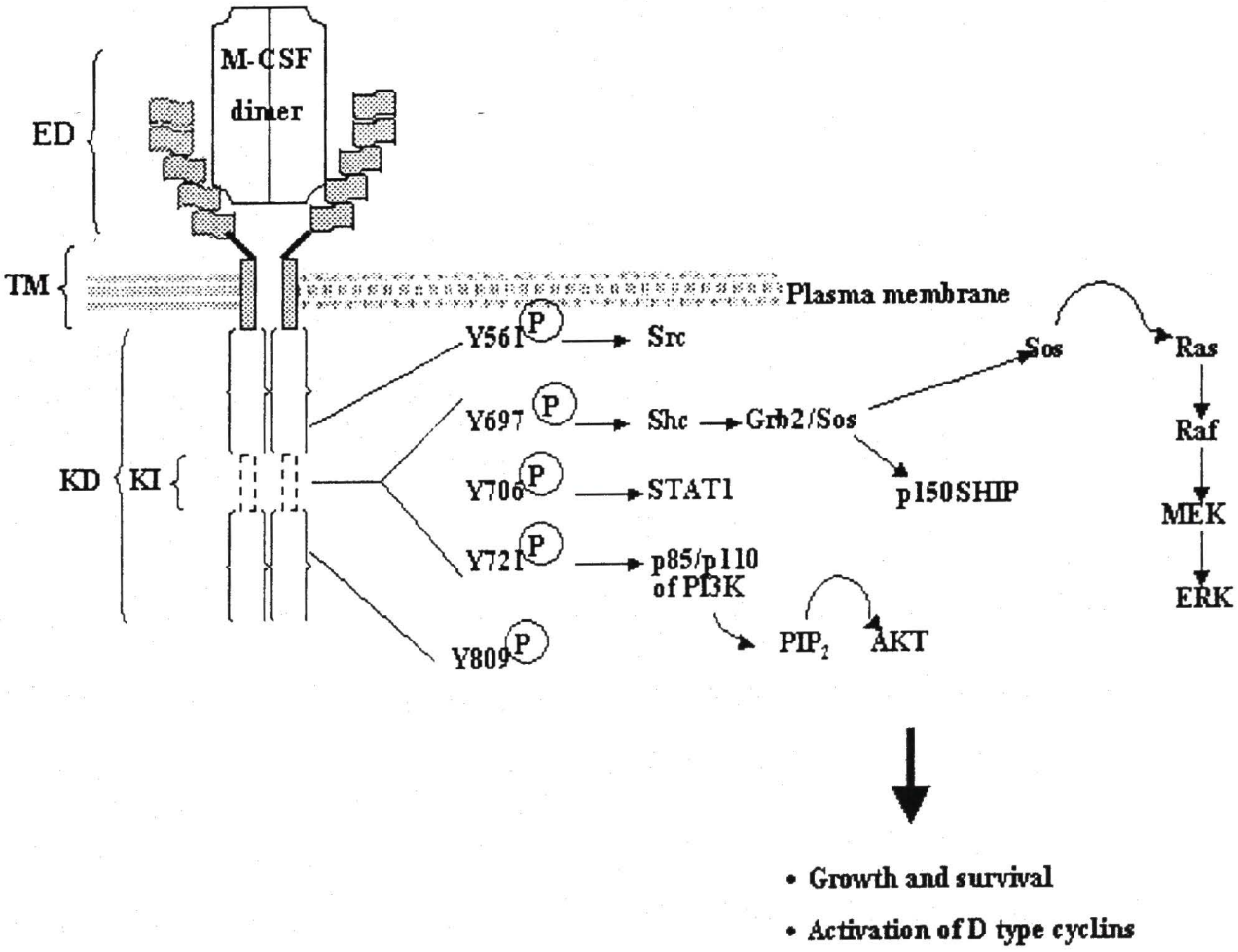
L-cells; an 80/80 kDa homodimer, a 50/50 kDa homodimer, and an 80/50 kDa heterodimer (Price *et al.* 1992). Similar heterogeneity of the dimers have been observed in both naturally occurring and recombinant human M-CSF (Koths *et al.* 1997). Human M-CSF expressed in CHO-3ACSF-69 cells appeared as an 85-kDa (homodimer of 43 kDa subunit) and a high molecular weight species of greater than 200 kDa, heterodimer of 43 kDa and a 150-200 kDa subunit (Suzu *et al.* 1998). The high molecular weight M-CSF subunit is an extensively glycosylated form containing an additional c-terminal peptide and conjugated to chondroitin sulfate glycoaminoglycan called proteoglycan-type M-CSF (PG-M-CSF). This form of M-CSF binds to collagen type V of the extracellular matrix, and is not present in human body fluids (Suzu *et al.* 1992). It is also found, in vitro, to bind to low-density lipoprotein (LDL) via a chodroitin chain, and to basic fibroblast growth factor via an additional c-terminal peptide (Suzu *et al.* 1994). PG-M-CSF has also been isolated from the mouse spinal cord neuron conditioned medium. Human M-CSF may be classified into three forms: the soluble (secreted) form, the proteoglycan form, and the glycoprotein membrane-bound form. The biologic activity of the soluble and membrane forms has been clearly demonstrated. However, contrary to earlier data, proteoglycan macrophage colony-stimulating factor (PG-M-CSF) that has been found to bind low-density lipoprotein has now also been found to be biologically active. It induces proliferation of cells of the mononuclear phagocytes and rapidly induces receptor phosphorylation with a similar potency to the soluble form (Suzu *et al.* 1997). The physiologic relevance of PG-M-CSF is still unclear, currently it is believed

that its effect is exerted and contained locally. Therefore, PG-M-CSF may modulate growth of specific cell types in a localized microenvironment.

***The M-CSF receptor (M-CSF-R or CSF-1R) and signal transduction***

All known forms of M-CSF bind to a cell surface receptor encoded by the c-fms proto-oncogene (Hampe *et al.* 1989). The M-CSF receptor (CSF-1R) is a member of ligand-activated tyrosine kinase receptor family, which includes c-kit, platelet-derived growth factor (PDGF) receptor, and fibroblast growth factor (FGF). It consists of a single transmembrane domain, extracellular ligand-binding domain with five immunoglobulin repeats, and two intracellular tyrosine kinase domains flanking a non-catalytic sequence called the kinase insert (KI) (Sherr *et al.* 1990). The binding of M-CSF to the extracellular domain results in receptor dimerization and stimulates the intrinsic tyrosine kinase activity of the intracellular receptor domain (Fig. 2). The observed 2:1 stoichiometry of soluble M-CSF receptor extracellular domain:ligand in vitro binding experiment supports the belief that the dimeric nature of M-CSF facilitates the dimerization of the receptor that initiates signal transduction within the cell (Koths 1997). At least four tyrosine residues in the intracellular region of the human M-CSF receptor found to be phosphorylated upon binding of the ligand, namely; Y697, Y706, Y721 in the KI domain, and Y809 in the kinase domain (Motoyoshi 1998). M-CSF stimulation causes three Src family kinases, Src, Fyn and Yes to associate with M-CSF-R and results in activation of these kinases. Mutation of Y809 autophosphorylation site of the receptor reduces both binding and enzymatic activation of these kinases (Roussel *et al.* 1990).

**Figure 2. Structure of M-CSF receptor and receptor-mediated signaling** Diagramatic cartoon depicting the M-CSF receptor. A tyrosine kinase receptor with an extracellular ligand binding domain (ED) composed of five Ig domains, a short transmembrane domain (TM) consistent with a single membrane spanning helix, and an intracellular kinase domain (KD) split by the kinase insert (KI) domain. The numbers stating the positions of tyrosine (Y) residue that become phosphorylated upon the binding of the ligand and their associated signaling pathways.


M-CSF
dimer
ED
TM
KD
KI
Plasma membrane
Y561
P
Src
Y697
P
Shc
Grb2/Sos
Sos
Ras
Raf
MEK
ERK
p150SHIP
Y706
P
STAT1
Y721
P
p85/p110
of PI3K
PIP2
AKT
Y809
P
• Growth and survival
• Activation of D type cyclins

As a result, fibroblasts expressing this mutant receptor are unable to form colonies in semi-solid culture or proliferate in presence of M-CSF. Signaling through Src family kinases is likely to convey mitogenic response to M-CSF. Non-catalytic M-CSF-R KI domain is required for the binding and activation of PI3'-kinase through its p85 subunit, which can be disrupted by mutation of Y721 (Reedijk *et al.* 1992). Grb2 associates with Y697 within the KI domain of the receptor (Van der Geer and Hunter 1993).

***Other biological roles of M-CSF***

Other than its activity in hematopoietic system, emerging evidence has implicated the involvement of M-CSF in several biological processes, which are important in both human physiological and pathological states.

*Placenta and breast development* --- The first clue in non-hematopoietic function of M-CSF was the finding that M-CSF elevates 1000-fold in mouse uterus during pregnancy (Pollard *et al.* 1987). Later on, activation of trophoblastic M-CSF-R by the locally increased expression of M-CSF by endometrial epithelium was found essential for placental development and embryo implantation (Pollard *et al.* 1997). M-CSF not only induces the differentiation of immature to mature trophoblasts, but also enhances the production of chorionic gonadotropin and placental lactogen (Pollard *et al.* 1987). Furthermore, increased expression of M-CSF and its receptor has also been demonstrated

in lactating mammary glands as well as breast carcinoma tissues (Sapi and Kacinski 1999). Investigation of the mammary gland development in *op/op* mice, variant of recessive mutation of M-CSF gene, revealed defective ductal growth, lobuloalveolar system, and inability to secrete milk proteins. M-CSF is clearly crucial in multiple stages of mammary gland development (Pollard *et al.* 1994). In placenta, the intense expression of M-CSF and CSF-1R observed in syncytio- and invasive trophoblast are regulated by estradiol 17β and progesterone (Daiter *et al.* 1992). Through its proximal glucocorticoid response elements (GREs) in CSF-1R promoter sequence, glucocorticoids are the only known physiologic regulator that significantly increases CSF-1R expression without any effect on M-CSF (Sapi *et al.* 1995). M-CSF expression in mammary gland is mainly stimulated by insulin and prolactin (Sapi *et al.* 1998).

*Skeleton and tooth development* --- M-CSF deficient *op/op* mice are osteopetrotic consequent to lack of osteoclast function and abnormal bone resorption, have severely reduced numbers of macrophages, and have reduced fertility. It is evident that M-CSF is critical for neonatal murine osteoclast formation. Injection of recombinant human M-CSF resulted in partly correction of bone deformity in these mice neonates (Felix *et al.* 1994). M-CSF has also been found crucial for human osteoclast survival by in vitro isolated fetal bone resorption studies (Sarma and Flanagan 1996). Specifically, PG-M-CSF that binds to the collagen type V in extracellular matrix of human bone could differentiate osteoclast progenitors to mature osteoclasts (Ohtsuki *et al.* 1993). Moreover, M-CSF in combination with IL-4 can induce human peripheral blood monocyte culture to

differentiate into tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like multinucleated giant cells (Agakawa *et al.* 1996). Differentiation of mononuclear cells to osteoclast in the presence of M-CSF absolutely requires a membrane-bound factor expressed by cells of the osteoblastic lineage, which is not produced by other cell types (Chambers *et al.* 1993). Similar finding has been reported that an uncharacterized endothelial membrane-bound factor, not expressed by smooth muscle cells, enhances M-CSF-dependent monocyte development (Antonov *et al.* 1997). These findings initiate controversial issue whether M-CSF plays a role in macrophage development or its role is restricted to proliferation and survival.

Little is known about how tissue-specific differentiation of the various M-CSF-dependent cells occurs. It is not clear whether M-CSF plays a role in macrophage development or whether its role is restricted to proliferation and survival. Crossing transgenic mice expressing human apoptosis-blocking gene *Bcl2* with *op/op* M-CSF-deficient mice partially cured the osteopetrotic defect in these animals (Lagasse and Weissman, 1997). Furthermore, spontaneous macrophage differentiation was observed in monocytes overexpressing *Bcl2* obtained from these animals, which did not undergo apoptosis in serum-free and cytokine-free medium. It's suggestive that monocytes possess an intrinsic program to differentiate in the absence of M-CSF.

17β-estradiol has been found to reduce M-CSF expression as well as inhibit osteoclast formation and bone resorption in human bone marrow cultures from both men and women (Sarma *et al.* 1998). On the other hand, bone marrow stromal cell cultures from ovarectomized mice, which lack of estrogen, showed up-regulated expression of M-

CSF and increased its ability to support osteoclastogenesis (Kimble *et al.* 1996). These findings also implicate the role of M-CSF in postmenopausal osteoporosis.

Tooth eruption requires alveolar bone resorption and the presence of the dental follicle, a loose connective tissue sac that surrounds each tooth. This bone resorption involves the follicle in that mononuclear cells enter the follicle to form osteoclasts that resorb bone to form the eruption pathway. Because it has been demonstrated that in vivo injection of M-CSF accelerates tooth eruption and M-CSF mRNA is present in vivo in the dental follicle of the rat (Wise and Lin, 1994), M-CSF is believed to involve with tooth eruption. Interleukin 1 alpha (IL-1 alpha) stimulates increased transcription of the CSF-1 gene in a concentration- and time-dependent manner. Moreover, CSF-1 itself has an autocrine effect on transcription of the CSF-1 gene. MCP-1, NF kappaB and c-fos expression also coincide with M-CSF in dental follicle (Wise *et al.* 1999). Thus regulation of gene expression for CSF-1 by IL-1 alpha and CSF-1 may play a part in signaling the onset of tooth eruption. Nevertheless, the role of M-CSF and mechanism of its function in tooth development is still unclear. M-CSF expression in dental follicles may support the osteoclasts, which are crucial in remodeling of bone-derived tissue.

*Immunological defences* --- M-CSF activates the anti-microbial activities of macrophages both in vitro and in vivo. It enhances their phagocytic capacity, their production of reactive oxygen radicals, and their killing abilities against both bacteria and fungi. In humans, several clinical studies demonstrated the usefulness of M-CSF injections in patients with invasive fungal infection (Roilides *et al.* 1996). The direct

effect on monocyte proliferation as well as indirect effect on enhancing recovery of neutrophils, especially administered in post-chemotherapy patients, significantly reduced the incidence of febrile neutropenia and sepsis (Motoyoshi, 1998). Injections of M-CSF into patients resulted in the enhancement of killing activities of monocytes against bacteria, such as *Staphyloccus aureus* and *Listeria monocytogenes* (Kayashima *et al.* 1991).

*Cholesterol metabolism and atherosclerosis* --- In a clinical study of M -CSF on chronic neutropenia in children, it was found that injections of M-CSF reduced the plasma cholesterol level up to 30% (Motoyoshi and Takaku 1989). M-CSF injection was later reported to reduce plasma cholesterol levels in normal as well as Watanabe heritable hyperlipidemic (WHHL) rabbits, by enhancement of the clearance of lipoproteins containing apolipoprotein B-100 via both low density lipoprotein (LDL) receptor-dependent and -independent pathways (Shimano *et al.* 1990). Furthermore, intravenously injected WHHL rabbits with M-CSF remarkably decreased accumulation of cholesterol ester in the aorta of M-CSF-treated animals, compared to those of controls receiving saline injections, and the percentage of the surface area of the aorta with macroscopic plaque in animals treated with M- CSF was much less than that in the controls. Thus, M-CSF has been shown to prevent the progression of atherosclerosis in WHHL rabbits by influencing macrophage function.

M-CSF increases the uptake of acetylated LDL into macrophages by the up-regulation of scavenger receptor expression, indicating that M-CSF may induce the progression of atherosclerosis; however, it also stimulates the efflux of free cholesterol

from macrophages by the upregulation of HDL (high- density lipoprotein) receptor expression and by the activation of cholesterol ester hydrolase, indicating that M-CSF may prevent the progression of atherosclerosis (Ishibashi *et al.* 1990). It has also been reported that the amount of PG-M-CSF, which could bind to LDL through the chondroitin sulfate chain, increased in atherosclerotic lesions (Suzu *et al.* 1994). This finding indicates the pathophysiological significance of PG-M-CSF in atherosclerotic change.

Macrophage colony-stimulating factor is intricately involved in the process of atherosclerosis. The absence of M-CSF largely protects against this disease, even in the presence of hyperlipidemia. It could, therefore, be considered that this cytokine exerts a deleterious effect on the blood vessel wall. Indeed, the beneficial effect of M-CSF deficiency has been convincingly demonstrated by the protective effect produced in apolipoprotein E knockout mice (which spontaneously develop atherosclerosis) when they were crossed with M-CSF deficient *op/op* mice (Qiao *et al.* 1997). It also appears that M-CSF production provides protection against atheroma formation because immunoreactive M-CSF is increased in atherosclerotic lesions of hyperlipidemic rabbits after treatment with M-CSF, and this correlates with reduced cholesterol content of foam cells (Hayes *et al.* 1997). Strangely enough, others have provided evidence that enhanced expression of M-CSF induces smooth muscle proliferation and foam cell formation, both of which are part of the pathologic process of atherosclerosis (Herembert *at al.* 1997). This is consistent with the finding that medial smooth muscle cells in atheromatous lesions aberrantly expressed *c-fms*. Additionally,

this coincides with smooth muscle cell proliferation and foam cell formation. It appears that this abnormal expression of *c-fms* is induced by platelet-derived growth factor in the presence of tranforming growth factor beta (Kubo *et al.* 1997).

Initially, it was not clear whether M-CSF protects against or enhances atherosclerosis. However, it is likely that atherosclerosis generally occurs because of a minor change in some or all of the normal pathways involved in maintaining a lipid-free intima rather than a dramatic overproduction or underproduction of particular factor such as M-CSF. It is speculated that under normal physiologic states (one in which M-CSF is detectable in the circulation) the sequence of events is as follows: vessel walls continually suffer minor insults, the consequence of which is infiltration of the intima by lipids. These lipids do not accumulate, but they are cleared from the vessel wall by macrophages which permeate the endothelium as monocytes (an M-CSF-dependent process, as demonstrated in *op/op* mice), It is only when lipid accumulates that abnormal persistent elevation of M-CSF expression occurs, presumably in an attempt to eliminate the damaging agent. The high level of M-CSF itself then becomes part of the disease process of atherosclerosis, enhancing the life-span of macrophages so that their continued presence exerts deleterious lipid accumulative, cytotoxic, and growth-promoting effects on the vessel wall, the recent report that the anti-apoptotic effect of M-CSF is antagonized in the presence of low- density lipoprotein suggests that the damaging effect of M-CSF can be counteracted (Flanagan *et al.* 1998).

*The kidney pathologies* --- Evidences have indicated that elevated levels of M-CSF, which may contribute to the development of atherosclerosis, are associated with chronic renal failure and uremia (Le Meur *et al.* 1996). Actually atherosclerosis is a common complication of chronic renal insufficiency. M-CSF is found to involved in pathogenesis of lipid-induced glomerular sclerosis (Miyazaki *et al.* 1997) and LPS-induced nephritis (Utsunomiya *et al.* 1996). There are mesangial cell proliferation, macrophage recruitment, and an increase in the number of mesangial cells expressing M-CSF in both conditions. Moreover, there is aberrant expression of c-fms mRNA in LPS-induced nephritis. Analogous to the finding in atheromatous aorta, local production of M-CSF is required to recruit macrophages and initiate the diseases. It's also possible that LPS enhances M-CSF expression in the kidney.

*The central nervous system* --- The fact that the *op/op* M-CSF-deficient mice have reduced response to visual and auditory stimuli provides a clue of intracortical role for the cytokine (Pollard *et al.* 1997). However, the brains of M- CSF-deficient mice are normal in size and histology compared with wild- typed animals, suggesting that M-CSF has subtle effects on cell-to-cell interactions. Research now implicates M-CSF in the pathogenesis of Alzheimer's disease. M- CSF has been found to be produced, in an oxidant-sensitive nuclear factor κB-dependent pathway, by neurons in response to amyloid-beta peptide via its receptor (Yan *et al.* 1997). Brains with Alzheimer's disease have been found to increase expression of M-CSF in neurons that are in close proximity to amyloid plaques. Finally, M-CSF levels were significantly elevated in the

cerebrospinal fluid of patients with the disease compared to age-matched control subjects. Because the expression of *c-fms* by microglia is well escablished (Liu *et al.* 1994), it's speculated that secreted M-CSF induces microglial proliferation, activation, and survival, which is considered co be involved in neuronal degeneration.

*Neoplastic diseases* --- Macrophage colony-stimulating factor and *c-fms,* are commonly aberrantly expressed by malignant epithelium. From existing experimental data, It is unclear whether this serves as an advantage to the neoplasm or the individual affected. There are two sides of the coin to consider here. M-CSF may feed forward the expression of its gene and c-fms in tumor expressing c-fms, thus aide the tumor growth or metastasis. On the other hand, M-CSF enhances antibody-dependent cell-mediated cytotoxicity of monocytes by the upregulation of Fc receptor expression of monocytes (Munn *et al.* 1989). It also enhances cytotoxic activity of monocytes by the activation of phagocytic activity (Munn *et al.* 1990), by the priming of production of tumor necrosis factor (Sakurai *et al.* 1994), and by the direct stimulation of production and secretion of reactive nitrogen intermediates (Douzono *et al.* 1995). M-CSF has been found to have a protective effect against the development of melanoma metastasis, and an inverse correlation has been found between M- CSF levels produced by M-CSF gene-transduced human ovarian carcinomas and the ability to form tumors in nude mice Motoyoshi et al, 1998). Tumor associated macrophages, present in the stromal environment of most tumors, interact with the tumor to produce cytokines and various molecules that can favor either the growth of the primary tumor and metastases or the tumoricidal activity of

macrophages. M-CSF locally produced by tumoral cells, is a chemotactic factor that favors tumor infiltration by monocytes (Mantovani *et al.* 1992). Human monocytes after incubation with M-CSF more effectively killed human leukemia cell lines, such as K562, U937, Daudi, and HL60 (Suzu *et al.* 1989). Mice serum pre-injected with M-CSF contained a higher TNF level after triggering by lipopolysaccharide (LPS), and had a higher cytotoxic activity against mouse TNF-sensitive cells, L929, in vitro, which was neutralized by the addition of anti- TNF antibody (Sakurai *et al.* 1994). Mice serum injected with M-CSF contained a higher RNI and a higher cytotoxic activity against mouse TNF-resistant leukemic cells, L1210, in vitro. This cytotoxic activity was neutralized by the addition of myoglobin which is known to be a scavenger of reactive nitrogen intermediates. Injections of M-CSF alone increased the survival rates of mice previously inoculated with a small number of Ll210 cells. Combination therapy with M-CSF and anti-cancer drugs, such as cyclophosphamide or adriamycin, effectively reduced leukemic cells in mice previously inoculated with a large number of L1210, and increased their survival rates (Douzono *et al.* 1995).

***What is known about M-CSF expression***

Expression of M-CSF is enhanced by a variety of cytokines and chemicals reported earlier from this lab and others, such as IL-1, TNF, phorbol ester, endotoxin, and calcium/calcium ionophore. The most recently discovered include IL-10 (Owens et al., 1996), and transforming growth factor-β (Hong et al., 1997). The former enhances the growth-promoting and differentiation effect on monocytes (Hashimoto et al., 1997). The

latter induces its effect via a signaling pathway involving hydrogen peroxide (Flanagan and Lader 1998). Unlike GM-CSF expression, which appears to be regulated both transcriptionally and posttranscriptionally, or strictly posttranscriptionally regulated G-CSF expression, M-CSF induced by FBS or IL-1 in the fibroblasts is primarily regulated at the transcriptional level (Falkenburg *et al.* 1991).

Using MIA PaCa-2 cells as an M-CSF producing cell line, IL-1 stimulated M-CSF expression detected by biological activity assay and northern blot analysis (Ku *et al.* 1992). In addition to IL-1, other effectors including endotoxin, phorbol esters, and calcium, also increased colony-forming activity in biological assays. In contrast, cAMP strongly inhibited M-CSF expression. Furthermore, previous results from this lab have also shown that cAMP elevating agents such as pertussis toxin, cholera toxin, forskolin and theophylline can inhibit M-CSF expression (Ku 1990). Similar findings were demonstrated in human lung fibroblast cell line CCL202 (Ku 1990).

Study of early gene expression related to the expression of M-CSF was performed earlier in this lab. Immediately after stimulation of promyelocytic leukemia cell line U937 with 12-O-tetradecanoylphorbol 13-acetate (PMA), initial strong expression of c-fos was observed by Northern blot analysis, followed by c-jun expression, within 30 min of stimulation. Later M-CSF mRNA was detected at 3 h of stimulation (Liu and Wu 1992). Fos and Jun are part of the AP-1 transcription factor, which consists of either homodimer Jun/Jun or heterodimer Jun/Fos. In the promoter region of murine M-CSF gene, AP-1 cis-binding site has been demonstrated to be crucial for the basal M-CSF expression (Harrington *et al.* 1991).

Linker scanning of various truncations of 570 bp sequence upstream to human M-CSF transcription start site ligated to CAT reporter gene assay revealed that the nucleotide region –416 to –339 conveys the responsiveness to pro-inflammatory TNF treatment in myeloid leukemia HL-60 cells. It was consequently demonstrated by electromobility shift assay and DNA-footprinting that the binding site of NF-κB present in that nucleotide sequence (Yamada *et al.* 1991). Using the same M-CSF promoter/CAT reporter gene technique, NF-κB has also been shown to be involved with M-CSF expression in mouse L-cells induced by minimally modified LDL (Rajavashisth *et al.* 1995).

From the studies described, it becomes clear that the transcription factor NF-κB is also conceivably involved in human M-CSF gene expression. The importance of AP-1 has been demonstrated mainly in basal expression of murine M-CSF gene. Roles of other putative cis-acting elements on the 5'-noncoding region of human M-CSF gene, such as IRE, NF-IL-6 and SP-1, have never been investigated. Other transcription factor binding sites like CTF/NF1, EGR-1, SP-1/SP-3 have been shown to be protected by nuclear extracts of proliferating fibroblasts in DNase I protection assays of murine M-CSF promoter (Harrington *et al.* 1997), but fail to demonstrate conclusive role in transcriptional induction using in reporter gene assay or mutagenesis studies.

Most published studies deal with identifying functions of the M-CSF protein in various biological processes. Less is known about the mechanism leading to the expression of the M-CSF gene. Understanding of signaling pathways regulating the M-CSF gene expression will help to elucidate the roles of M-CSF gene in pathogenesis of

human diseases like atherosclerosis and osteoporosis, which are among common public health problems in the United States. The elucidation of these mechanisms could lead to innovative interventions of the conditions. Although previous studies in this lab demonstrated that cAMP and cAMP elevating agents such as pertussis toxin, cholera toxin, forskolin, and theophylline could inhibit M-CSF production (Ku 1990), it remained unclear what was the underlying signaling mechanism. First, the step at which cAMP exerts its inhibitory effect on M-CSF gene expression needs to be assessed. Characterization of signaling pathways induced by PMA, IL-1 or LPS plausibly aides the understanding of regulation of M-CSF gene expression. These may lead to clarification how cAMP elevation antagonizes M-CSF expression. Intracellular cAMP elevation is currently the only factor shown to negatively affect M-CSF gene expression. Therefore, insight of its signaling mechanism could broaden the knowledge about the aforementioned diseases caused by abnormal M-CSF expression and potentially lead to their novel therapeutic management.

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CHAPTER II

# CYCLIC AMP ATTENUATES INTERLEUKIN-1-STIMULATED M-CSF EXPRESSION

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Running title: cAMP attenuates M-CSF expression

# SYNOPSIS

Macrophage colony-stimulating factor (M-CSF) is a multifunctional cytokine attributed with key biological functions beyond the first discovered role in promoting proliferation of myeloid cell lineage. The human pancreatic cancer cell line MIA PaCa-2, from which M-CSF gene is originally cloned, was used to study regulation of M-CSF expression. Expression of M-CSF was inducible by interleukin-1alpha (IL-1α), lipopolysaccharide (LPS) and PMA as demonstrated by a biological activity assay, northern blot analysis and reverse transcriptase polymerase chain reaction (RT-PCR). Treatment of the cells with forskolin or dibutyryl-cAMP attenuated the expression of M-CSF induced by IL-1α or LPS, but not by PMA. Electromobility shift assays showed that IL-1α predominately activated NF-κB, while PMA preferentially activated AP-1. The activation of NF-κB, but not AP-1, could be attenuated by cAMP elevation. Relative RT-PCR demonstrated that the expression of a 1.6 kb M-CSF mRNA transcript was more effectively induced by IL-1α than that of 4.0 kb transcript. By and large the induced expression of both mRNA transcripts can be attenuated by cAMP. M-CSF promoter – driven luciferase reporter gene assays revealed that cAMP elevation attenuated the IL-1 induced transcription activation of the M-CSF promoter, but it had no effect on PMA-induced transcription. Our findings suggest that cAMP regulates M-CSF gene expression at transcriptional level and its inhibitory effect involves NF-κB signaling pathway.

# INTRODUCTION

Emerging experimental evidence implicates the essential roles of macrophage colony-stimulating factor (M-CSF) in several important biological processes other than proliferation & survival of monocytes/macrophages, such as placental development, bone formation, tooth eruption, and enhancing tumoricidal activity (Flanagan et al., 1998). Furthermore, recent reports also suggest that M-CSF is required in pathogenesis of atherosclerosis (Rajavashisth et al., 1998) and development of osteoporosis (Kimble et al., 1996 and Srivastava et al., 1998). While most studies have focused on the effects of M-CSF and the signal transduction after engagement of the M-CSF receptor, a limited amount of information regarding regulation of the M-CSF gene expression itself exists.

M-CSF is encoded by a single gene on the long arm of chromosome 1 (Pettenati et al., 1987), which comprises 10 exons spanning 21 kb (Ladner et al., 1987). Alternative splicing occurring within exon 6 primarily generates mRNA transcripts that derive soluble or membrane-bound forms of the M-CSF. The major species of mRNA transcripts present is a 4.0 kb product, which contains the full coding sequence and gives rise to a translated protein of 522 amino acids Wong et al., 1987). This form of M-CSF is synthesized as an integral membrane glycoprotein and it is intracellularly processed and subsequently becoming soluble growth factor. The 1.6 kb mRNA transcript, which lacks the sequence of exon 6, is minimally expressed. Its protein product is bound to the cell membrane more stably and can be cleaved and released to the extracellular fluid slowly (Rettenmier et al., 1988). Both forms of M-CSFs are biologically active. A distinctive

function of the membrane-bound form is believed to be providing a cell surface biological activity for local cell-cell signaling (Rubin et al., 1997).

Previous studies from this lab and others have shown that IL-1, TNF, phorbol ester, endotoxin, cGMP and calcium/calcium ionophores stimulated M-CSF production in human pancreatic cancer cell line MIA PaCa-2 cells, as well as the human lung fibroblast cell line CCL202 (Ku et al., 1992). Contrarily, cAMP and cAMP elevating agents such as forskolin, pertussis & cholera toxins and theophylline reduced the IL-1-induced M-CSF production as detected by a biological activity assay (Liu et al., 1992).

The 5'-flanking region (570 base pairs upstream) of the gene was reported to contain putative cis-acting sites for transcription factors AP-1, NF-κB, EGR-1 and NF-IL6 (Harrington et al., 1991). The interaction between these regulatory transcription elements would expectedly contribute to M-CSF gene expression. A region containing the AP-1 binding site is required for the basal expression of murine M-CSF gene (Harrington et al., 1997). The increased expression of c-Fos and c-Jun was observed by northern blot assay before the expression of M-CSF in U937 cells stimulated with PMA (Liu et al., 1992). Therefore, it appears that AP-1 is possibly one of the important regulatory elements for M-CSF expression.

The NF-κB element is a ubiquitous transcription factor, which can activate a number of proinflammatory cytokines and other genes (Christman et al., 1998). NF-κB activation is a major signaling pathway stimulated by IL-1, one of the potent acute phase response cytokines. A segment of the 5'-flanking region of M-CSF gene (position -419 to –304) that includes the binding site for NF-κB is required for TNF-induced M-CSF gene

expression in HL-60 cells as well as for minimally modified LDL-mediated M-CSF induction in mouse L-cells (Yamada et al., 1991 and Rajavashisth et al., 1995).

Activation of the protein kinase A (PKA)-dependent signaling pathway by cAMP is an important pathway to regulate cytokine expression. Activation of PKA downregulates the expression of IL-2 and IL-2 receptor (Borger et al., 1996 and Vairo et al., 1990), as well as IL-3 and GM-CSF (Christman et al., 1998) in human T-cells. Several agents which raise intracellular cAMP, such as 8-bromo-cAMP, 3-isobutyl-1-methylxanthine, cholera toxin, and prostaglandin E2, inhibit DNA synthesis in bone marrow-derived macrophages (Ohtsuki et al., 1994). And we have also shown that cAMP negatively regulates M-CSF expression.

The present studies have examined the functional significance of transcription factors NF-κB and AP-1, as well as their interactions with cAMP-dependent signaling during IL-1, LPS, and PMA-induced activation of the M-CSF gene expression in MIA PaCa-2 cells. Our results indicate that the M-CSF gene can be induced via at least two distinct signaling pathways. IL-1 and LPS stimulation preferentially depend on the activation of transcription factor NF-κB, which could be attenuated by intracellular cAMP elevation. On the other hand PMA, which predominately utilizes transcription factor AP-1, was not affected by cAMP-dependent signaling. The promoter/reporter assays confirmed that cAMP elevation negatively affected the IL-1 signaling upstream to the M-CSF gene transcription.

## MATERIALS AND METHODS

***Materials*** --- All of standard tissue culture reagents were obtained from Hyclone (Logan, UT, U.S.A.). Recombinant IL-1α (rHu. IL-1α(117-271) Ro24-5008 was a gift from Hoffmann La Roche, Inc. (Nutley, NJ, U.S.A.). Bacto® Lipopolysaccharide (S. typhosa LPS) was obtained from Sigma (St. Louise, MO, U.S.A.). [γ-$^{32}$P]-ATP and [α-$^{32}$P]-dCTP were purchased from Dupont NEN (Boston, MA, U.S.A.). The plasmids pGL2basic and pSV-β-galactosidase were from Promega Corp. (Madison, WI, U.S.A.). FuGene-6 was supplied by Roche (Indianapolis, IN, U.S.A.). Unless otherwise stated all other biochemical reagents were purchased from Sigma (St. Louise, MO, U.S.A.).

***Cell culture and treatment*** --- The human pancreatic carcinoma cell line MIA PaCa-2 obtained from the American type Culture Collection (ATCC) was cultured in Dulbecco Modified Eagle's Medium (DME) supplemented with 7.5% FCS and 2.5% horse serum. Subculture of cells is done by treatment with 0.05% trypsin/0.02%EDTA and resuspended in fresh DME with 10% serum. Cells were cultured at 37 °C in a humidified incubator under 6% $CO_2$. When 90% confluence was reached, cells were washed twice with PBS before switching to serum-free DME. In specified cultures, recombinant IL-1α, PMA, or LPS were added to the cell cultures at final concentration of 17 ng/ml, 20 ng/ml, 1 μM and 100 ng/ml, respectively. Cells were incubated further and harvested at specified times.

***Assay for M-CSF activity*** --- The soft-agar colony assay of bone marrow cells was carried out by a procedure described elsewhere [14]. Mouse bone marrow cells were

obtained from C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME, U.S.A.). Colony counts were performed using a dissection microscope at day 5, and aggregates of over 50 cells were scored as colonies. One unit is defined as the ability to form one colony. For morphological analysis of the colonies the entire soft-agar plates were mixed, dried, and stained with hematoxylin (Sigma) according to (the company's) instructions.

***Nuclear protein extraction*** --- Nuclear protein extracts were prepared according to Dignam et al. [22]. After incubation under a specific treatment, 1.5-2 x $10^6$ cells were washed twice with PBS before being scraped into buffer A [10mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 5.5 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] and briefly centrifuged. Cell pellets were consequently resuspended in buffer A, kept in ice for 15 min after addition of 10% Nonidet P-40, cells were homogenized in a Dounce homogenizer and centrifuged at 12,000 rpm for 10 seconds at 4 °C. The nuclear pellets were then resuspended in extraction buffer [20 mM HEPES (pH 7.9), 0.55 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 mg of leupeptin per 1 ml], homogenized, and centrifuged at 12,000 rpm for 2 minutes at 4 °C. Samples in the supernatants were collected and stored at –70°C until used. Concentrations of the nuclear protein samples were determined by the BioRad® protein assay according to manufacturer's protocol.

EMSA Synthetic oligonucleotides with the sequences corresponding to cis-acting elements of the M-CSF promoter were $^{32}$P-5'-end-labeled by T4 polynucleotide kinase. Equal amounts (2-10 μg) of nuclear extract from each of various cell treatments were

incubated in 20 μl of binding reaction [final concentrations; 40 mM KCl, 15 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5% glycerol, and 1 μg poly(dIdC)] for 10 min. Equal amounts (approximately 1 ng of DNA) of the radiolabeled oligonucleotide probe (60,000 cpm) were added to the reaction mixture, and further incubated for additional 25 min at room temperature. DNA protein complexes were separated from unbound DNA probe by electrophoresis on native 5% polyacrylamide gels at 140 V in 45 mM Tris-borate (pH 8.0) containing 1mM EDTA and autoradiographed. Oligonucleotide probes (Biosynthesis, Lewisville, TX) with the following sequences; NF-κB 5'-agttgaGGGACTTTCCaggc-3' (position -368 to –377 nucleotides of the M-CSF promoter) and AP-1 5'-cgcttgATGTGTCAGTccggaa-3' (position -538 to –531 and –344 to –337 nucleotides of the M-CSF promoter) were used. The gel supershift antibodies against p50 and c-Jun/AP-1 were purchased from Santa Cruz Biotech (Santa Cruz, CA). The mutant NF-κB and mutant AP-1 gel shift oligonucleotides were also purchased from Santa Cruz Biotech.

***RNA extraction and Northern blot analysis*** --- Total cellular RNA was isolated using TRIpure™ reagent (Boehringer Manheim) per manufacturer's protocol. Samples containing equal RNA (15 to 20 μg) were fractionated in 1.2% agarose gels and transferred to nylon membranes. The membranes were then hybridized according to a standard protocol [23] with either cloned M-CSF cDNA or with probes generated by PCR for M-CSF or human 18S rRNA specific cDNA. The probes were radiolabeled with [$\alpha$-$^{32}$P]-dCTP by random priming using Klenow fragment from Promega (Madison, WI, U.S.A.) per manufacturer's directions. After hybridization, InstantImager™ Electronic

Autoradiopraphy (Packard, Meriden, CT) was used to detect and quantify hybridization signals.

***Relative RT-PCR*** --- Total RNA (1 μg) from cells was reverse-transcribed using Moloney murine leukemia reverse-transcriptase from Life Technologies (Gaithersburg, MD, U.S.A.) at 42 °C for 1 hr. A 2 μl aliquot of the diluted cDNA is amplified by PCR using recombinant Taq polymerase according to the manufacturer's instructions. The M-CSF PCR primers were designed as previously described (Ohtsuki et al., 1994). M-CSF-A (sense): 5'-catgacaaggcctgcgtccga-3' (nt 489-409), M-CSF-B (antisense): 5'-aagctctggcaggtgctcctg-3' (nt 762-782), M-CSF-C (antisense): 5'-gccgcctccacctgtagaaca-3' (nt 1547-1567). Human 18S rRNA primers: 5'-acggctaccacatccaag-3' (sense) and 5'-cgcccgcccgctcccaaga-3' (antisense) (Torczynski et al., 1985) were used in the PCR reactions for loading control. Amplicons corresponding to the full length 4.0 kb (using M-CSF-A and M-CSF-B primers) and the exon 6 spliced 1.6 kb (using M-CSF-A and M-CSF-C primers) M-CSF mRNAs with molecular size of 395 bp and 286 bp, respectively, were electrophoresed on 2% agarose gel to determine the size. Signals were subsequently confirmed by southern hybridization with radiolabeled M-CSF cDNA. The human 18S PCR product is 200 bp in length.

***Plasmid Constructs, Cell Transfection and Reporter Gene Assay*** --- Plasmid containing the 5'-region of human M-CSF gene promoter was kindly provided by Dr. Hisashi Yamada, Jeikei University Medical School, Japan. After digestion by SstI and HindIII, 570 bp fragment of 5'-M-CSF promoter was directionally subcloned upstream to the firefly luciferase gene in the plasmid pGL2 from Promega Corp. (Madison, WI, U.S.A.)

at SacI/HindIII site deriving p570Luc and confirmed by sequencing. In all transfections, 1 μg of pSV-β-galactosidase were used to monitor the transfection efficiency. MIA PaCa-2 cells were seeded 2 x $10^5$ cell/35-mm plate 24 hours before transfection. Cells were washed twice with PBS and switched to serum-free DME media 6 hours before transfection. Transfection of mixture plasmid DNA into cells was performed by using Fugene-6 according to manufacturer's protocol. Transfected cells were stimulated with IL-1 or PMA with or without forskolin 12 hours after transfection , then incubated further for 12 hours until harvested for luciferase assay (Promega) per manufacturer's protocol. The assay for β-galactosidase was performed as previously described [25]. Induction of luciferase activity is reported as the average from two separate experiments in triplicate, normalized to the β-galactosidase activity.

***Data Analysis*** --- Densitometry of the autoradiographed bands from Northern blot and EMSA were analyzed quantitatively by Alpha Imager™ program version 3.23 (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). All values are expressed as mean ± S.E.M. compared to controls and among separate experiments. Two-tail paired Student's t test was used to determine the significance of changes in densitometric measurements and colony-forming activities. A significant difference was considered for p value of less than 0.05.

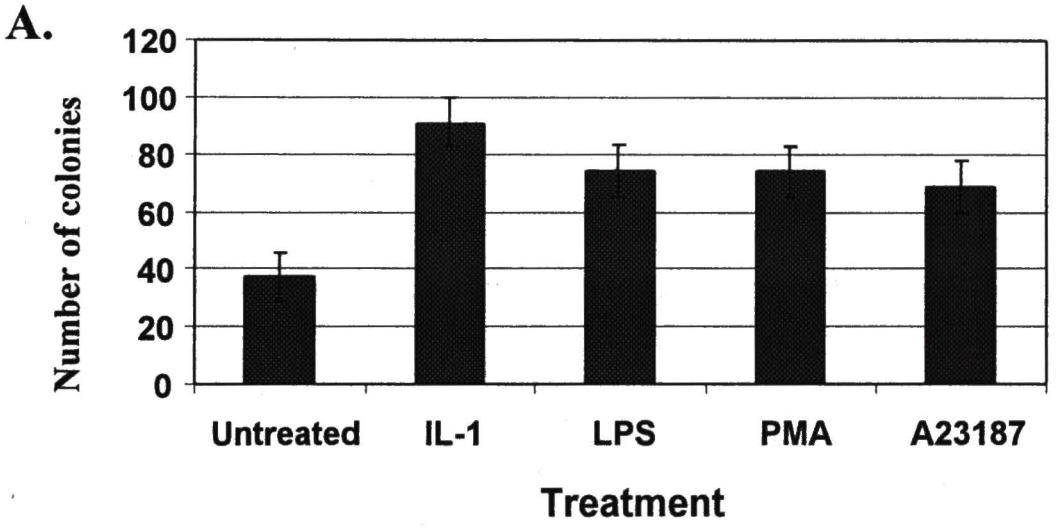
# RESULTS

***Stimulation of colony forming activity by MIA PaCa-2 cells*** --- Cultured MIA PaCa-2 cells produced a low basal level of M-CSF without any stimulation as apparent by 40±2 colonies formed in CSF-1 biological activity assay. By contrast, 17 ng/ml IL-1 enhanced M-CSF secretion into the media by nearly three fold. LPS 100 ng/ml, PMA 10 ng/ml and calcium ionophore A23187 1 μM also stimulated M-CSF production in MIA PaCA-2 cells about 2.5 fold over controls (Fig. 1A).

***Effects of cAMP on M-CSF-dependent colony forming activity*** --- The role of intracellular cAMP elevation on M-CSF expression was examined by addition of either 1 μM DB-cAMP (data not shown) or 5 μM forskolin to the cultured MIA PaCa-2 cells stimulated by IL-1, LPS, or PMA. Either DB-cAMP or forskolin treatment alone had no effect on basal bone marrow cell colony forming activity. Intracellular cAMP elevation by either forskolin or DB-cAMP markedly attenuated both IL-1-induced and LPS-induced M-CSF production (~ 50% reduction, $p=0.006$ and $p=0.010$ respectively), while there was no effect on PMA-induced M-CSF production (Fig. 1B). This result suggests that the effect of PMA is mediated through different signaling pathway to induce M-CSF expression from IL-1 and LPS.

Northern analyses revealed that IL-1 induced the mRNA expression of M-CSF in a time-dependent manner. The peak of induction occurred between 4 and 6 hours after IL-1 stimulation (data not shown). Intracellular cAMP elevation by forskolin significantly lowered both IL-1- and LPS-induced expression of M-CSF at 6 hours after

**Figure 1. Stimulation of mouse bone marrow colony forming activity and the effect of intracellular cAMP elevation** (A) MIA PaCa-2 cells were cultured with effectors for 48 hours at the final concentrations: IL-1 = 17 ng/ml, LPS = 100 ng/ml, PMA = 10 ng/ml and calcium ionophore A23187 = 1 μM. The conditioned media were harvested and assayed for biological activity as detailed earlier. (B) Cultured MIA PaCa-2 cells were treated the same way in presence (hatched and crosshatched bars) or absence (unfilled and filled bars) of 5 μM Forskolin. Data are means of colony numbers ± S.E.M. for triplicate determinants from one representative of three similar experiments, * and ** depict significant difference ($p < 0.05$) between samples treated with effector only versus ones with forskolin added.


A.
120
100
80
60
40
20
0
Number of colonies
Untreated
IL-1
LPS
PMA
A23187
Treatment

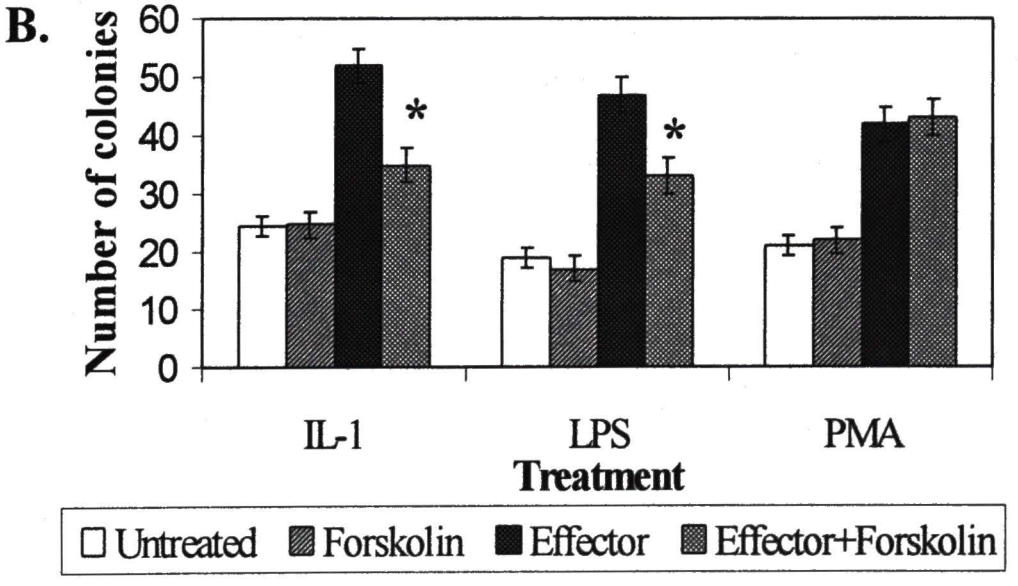
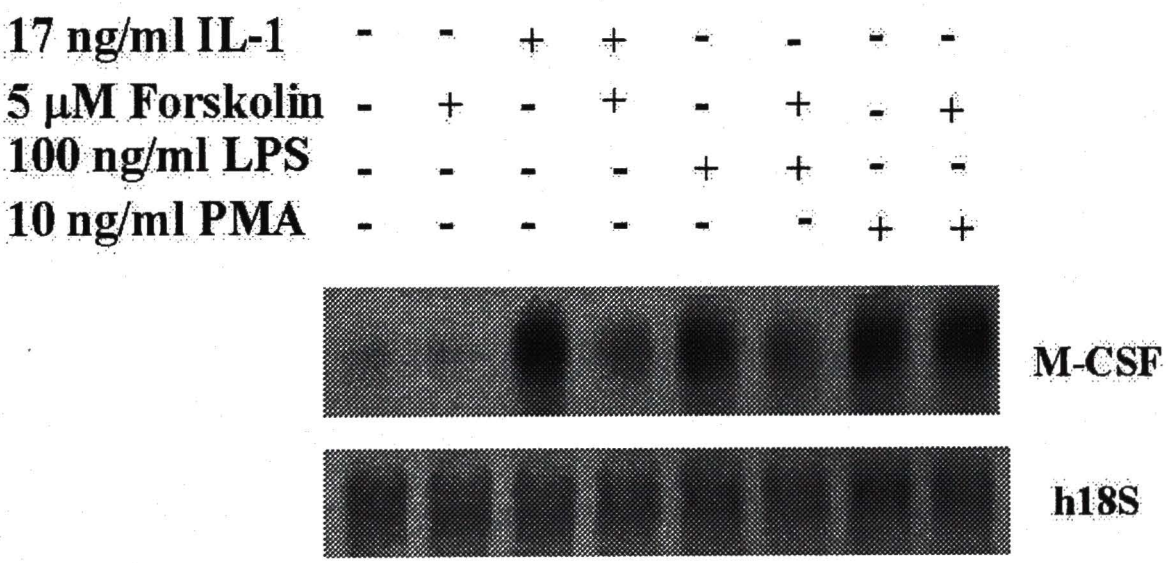

B.
60
50
40
30
20
10
0
Number of colonies
*
*
IL-1
LPS
PMA
Treatment
Untreated
Forskolin
Effector
Effector+Forskolin

**Figure 2. Effect of forskolin on stimulated M-CSF mRNA expression** Total RNA (20 μg /lane) from MIA PaCa-2 cells treated with IL-1, LPS, or PMA in presence or absence of 5 μM forskolin were hybridized with labeled M-CSF cDNA fragment in northern analyses. RNA loading was determined by quantification of human 18S rRNA. The blot is representative of three independent similar experiments.

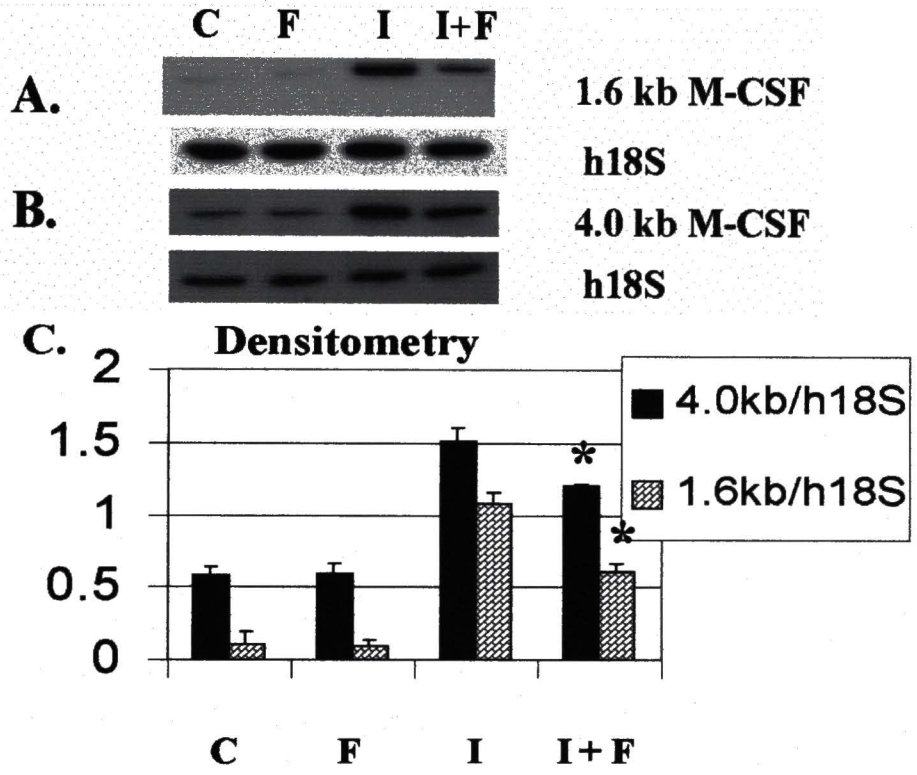

17 ng/ml IL-1 - - + + - - - -
5 μM Forskolin - + - + - + - +
100 ng/ml LPS - - - - + + - -
10 ng/ml PMA - - - - - - + +
M-CSF
h18S

treatment. On the other hand, no attenuation of PMA-induced mRNA induction was observed (Fig. 2). The attenuation of M-CSF biological activity, in conditioned media, by forskolin treatment is consistent with a decrease of intracellular mRNA accumulation, suggesting that cAMP elevation affect the expression of M-CSF at the transcriptional level.

Relative RT-PCR was also performed to detect any changes in the minor mRNA transcript of M-CSF (1.6 kb mRNA) concomitant with those of the major mRNA transcript (the 4.0 kb mRNA), because 1.6 kb transcripts are rarely detected by northern blot. Total RNA from MIA PaCa-2 cells were cultured with or without IL-1 and forskolin for 4 hours. IL-1 induced expression of both 4.0 kb and 1.6 kb M-CSF mRNA transcripts increased significantly over the untreated control (Fig. 3 A&B). Forskolin treatment alone did not cause any apparent change of the mRNA over the control. Inducibility of the 1.6 kb mRNA transcripts by IL-1 was approximately 11 fold over the control, while that of 4.0 kb transcripts were induced at about 3 fold higher level. The expression of both 1.6 kb and 4.0 kb transcripts were significantly attenuated by forskolin $p=0.003$ and 0.001 respectively. Similar to the degree of inducibility, forskolin attenuated the IL-1-induced 1.6 kb transcripts to a greater extent (~ 50%) than the 4.0 kb transcripts (~30%) (Fig. 3C).

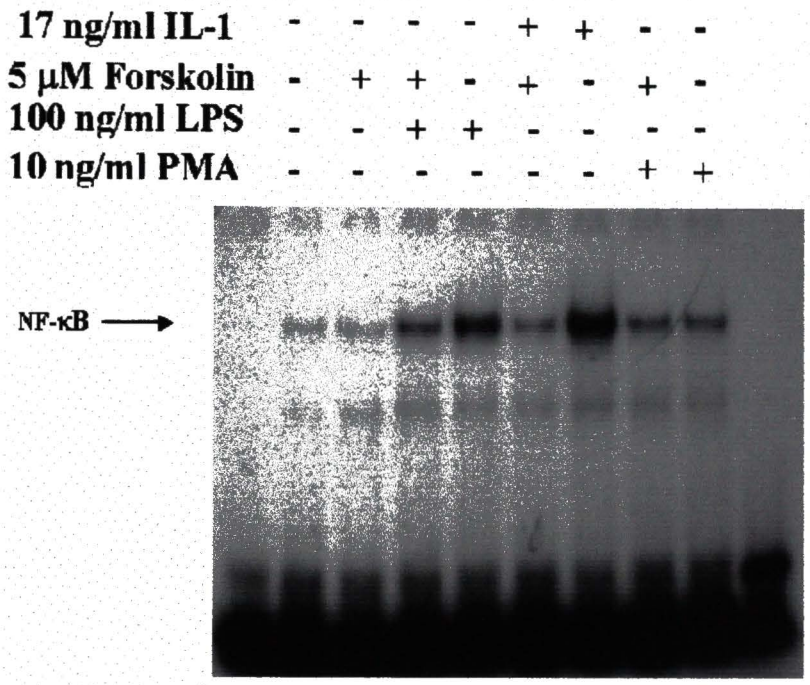
***Activation of NF-κB*** --- Either 17 ng/ml IL-1α, 10 ng/ml PMA, or 100 ng/ml LPS were added to the cultured MIA PaCa-2 cells for 60 min and a nuclear protein extract from each treatment was prepared. The doses of stimuli used throughout the study were the lowest concentration that yielded maximum M-CSF biological activity as determined

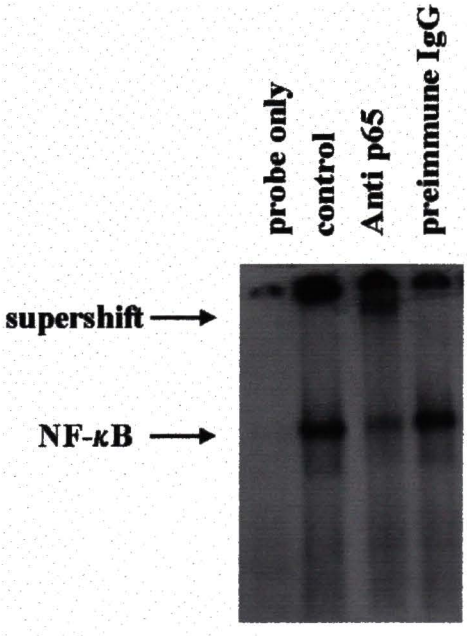
**Figure 3. Expression of M-CSF mRNA transcripts and effect of forskolin** Relative RT-PCR detecting induction of the mRNA transcripts of (A.) membrane-bound (1.6 kb) and (B.) soluble (4.0 kb) M-CSF in MIA PaCa-2 cells stimulated by 17 ng/ml IL-1 for 6 hours, either with or without 5 μM forskolin. Densitometry measurement is shown in (C), * represents significant difference ($p < 0.05$) between cells treated with IL-1 only (**I**) versus ones with 5 μM forskolin (**I+F**) for both 4.0 kb and 1.6 kb M-CSF mRNA transcripts.




| | | |
|---|---|---|
| I | = | IL-1 only |
| F | = | forskolin only |
| I + F | = | IL-1 + forskolin |

.**Figure 4. NF-κB activation complex in stimulated MIA PaCa-2 cells** (A) MIA PaCa-2 cells were harvested and extracted for nuclear proteins 1 hour after treatment by IL-1, LPS, or PMA. Cells were pre-incubated with 5 μM forskolin or 1 μM DB-cAMP for 3.5 hours before the effectors were added where specified. NF-κB oligonucleotide probes were incubated with 10 μg of nuclear extract/lane and resolved on 5% polyacrylamide gel. Mutant NF-κB probe was used with IL-1 treated extract in lane 10, lane 1 was wild type probes without nuclear extract. (B) Anti-p65 subunit antibody 1 μg (5 μl) or 1 μg preimmune IgG was added to the binding reaction containing 10 μg of IL-1 treated nuclear extract and wild type NF-κB oligonucleotides, further incubated for 15 min, and resolved on 5% polyacrylamide gel.


17 ng/ml IL-1 - - - - + + - -
5 μM Forskolin - + + - + - + -
100 ng/ml LPS - - + + - - - -
10 ng/ml PMA - - - - - - + +
NF-κB


probe only
control
Anti p65
preimmune IgG
supershift
NF-κB

previously by a mouse bone marrow cell colony-forming assay. Next, equal amounts of protein (10 μg) from each extract were assayed for NF-κB activity by EMSA. NF-κB binding complex appeared as a sharp single band on the autoradiogram (Fig. 4A Lanes 5&7). In these experiments, NF-κB was activated strongly by IL-1α and to the lesser degree, by LPS. PMA treatment either with or without forskolin weakly activated NF-κB binding. No significant activation of NF-κB was observed when the cells were treated by forskolin alone (Fig. 4A Lanes 6&7). Elevation of intracellular cAMP level, by pretreatment of the MIA PaCa-2 cells with 5 μM forskolin three hours prior to the stimulation by IL-1 or LPS treatment, markedly attenuates NF-κB activation (Fig. 4A Lane 4&6). When a mutant NF-κB probe that differed from wild-type sequence by 1 nt was used with the IL-1 treated extract to confirm the band identity, no binding was observed (Fig. 4A Lane 10). This indicated the binding complex was sequence-specific. A polyclonal antibody to the NF-κB p50 subunit, which recognizes epitope corresponding to amino acids 3-19 within amino terminal domain, was used in supershift experiment. The antibody further retarded the protein-DNA complex and the band appeared to be supershifted from the original position (Fig. 4B). By contrast, control generic IgG had no effect on the protein-DNA complex. This result further confirmed the identity of the NF-κB binding complex formed.

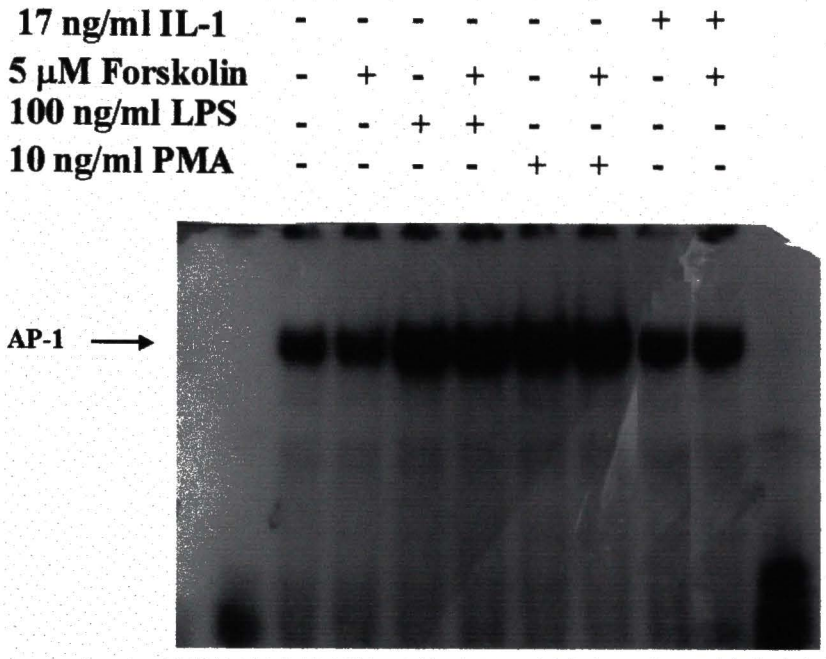
***Activation of AP-1*** --- similar nuclear protein extracts were used in EMSA with the oligonucleotides containing the consensus-binding sequence for AP-1. AP-1 activation was also detected as a single band on the corresponding autoradiogram (Fig. 5A). PMA

and LPS strongly increased the binding of AP-1 (from integrated optical density area of 1614 in control to 4584 and 4288 respectively) (Fig. 5A Lane 4&6). While IL-1 elicit apparently much weaker AP-1 activation (at 2599 in optical density) (Fig. 5A Lane 8). On the other hand, no AP-1 binding appeared when the mutant radiolabeled AP-1 probe was used with the PMA treated nuclear extracts (Fig. 5A Lane10). Forskolin did not significantly change AP-1 binding by itself (1586 optical density) comparing to control. Furthermore it did not have apparent effect on PMA, LPS or IL-1-induced AP-1 binding (4474, 4239, and 2546 respectively) (Lane 5, 7, &9 Fig. 5A). Here, a supershift experiment was performed with a polyclonal antibody to c-Jun/AP-1, which was designed to tether to residues 247-263 within the DNA binding domain of AP-1. The supershift band was clearly apparent, but the intensity of retardation band at the original position was reduced only minimally (Fig.5B).

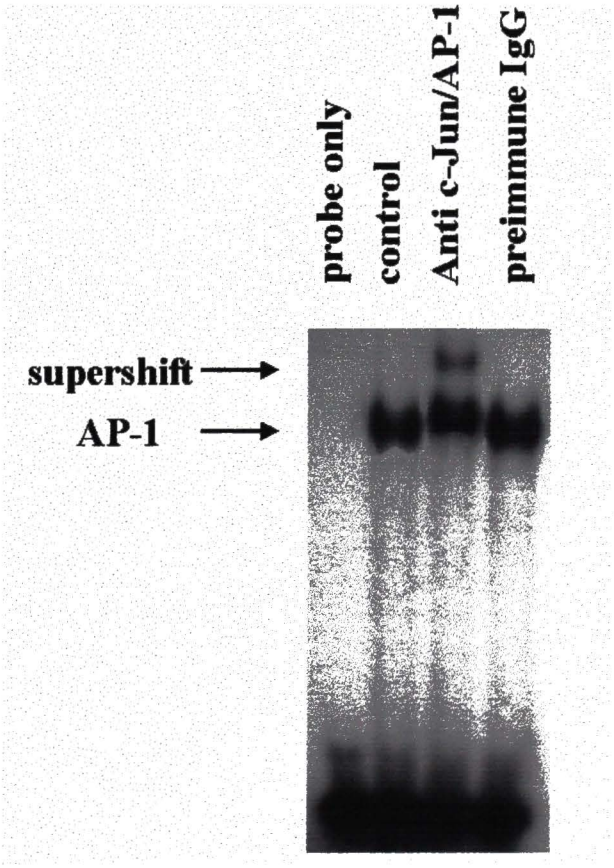
The binding between transcription factors and the oligonucleotides in the protein-DNA complexes is sequence-specific for both NF-κB and AP-1. Mutant probes, which differ from the consensus oligonucleotides by one amino acid, could not elicit binding. Thus, the identity of the protein-DNA complexes was proven. The supershift caused by the specific antibodies to NF-κB and c-Jun/AP-1 further confirmed the involvement of these transcription factor proteins in the formation of these protein-DNA complexes. This protein-DNA complex formation reflects the first step in the gene transcription that transcription factors engage in the preinitiation complex at the corresponding cis-acting binding sites. Anti-c-Jun/AP-1 antibody formed the distinct supershift band without significant weakening of the original AP-1 binding (Fig. 4B). This was likely due to

**Figure 5. AP-1 activation complex in stimulated MIA PaCa-2 cells** (A) Equal amount of $^{32}$P-labelled AP-1 oligonucleotide probe was incubated with 6 μg /lane of nuclear protein from MIA PaCa-2 stimulated with indicated treatment. Mutant $^{32}$P-labelled AP-1 probes incubated with PMA treated extract was in lane 10, lane 1 was probe only. (B) Identity of AP-1 binding was also confirmed by supershift experiment using Anti c-Jun/AP-1 antibody 1 μg (5 μl) or preimmune IgG 1 μg to incubate with PMA-treated nuclear extract and wild-type AP-1 probes at 4 °C for 12 hours before resolving on 5% polyacrylamide gel.

A.


17 ng/ml IL-1 - - - - - - + +
5 μM Forskolin - + - + - + - +
100 ng/ml LPS - - + + - - - -
10 ng/ml PMA - - - - + + - -
AP-1

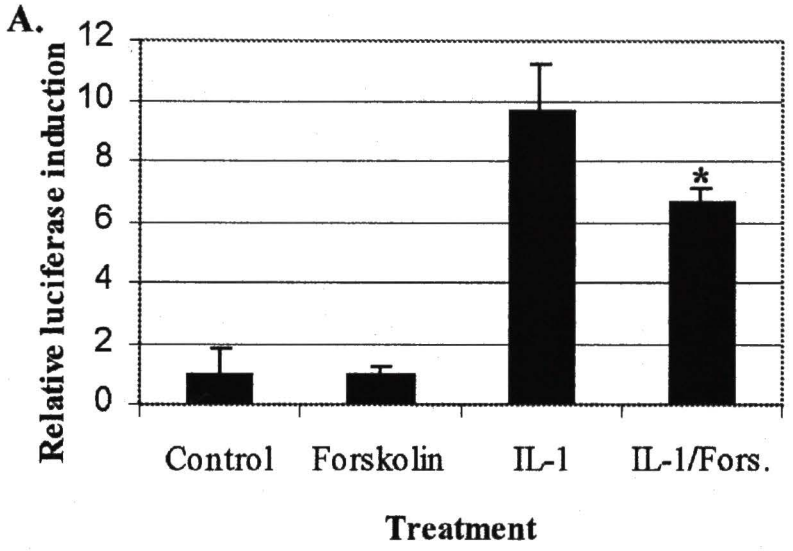
B.

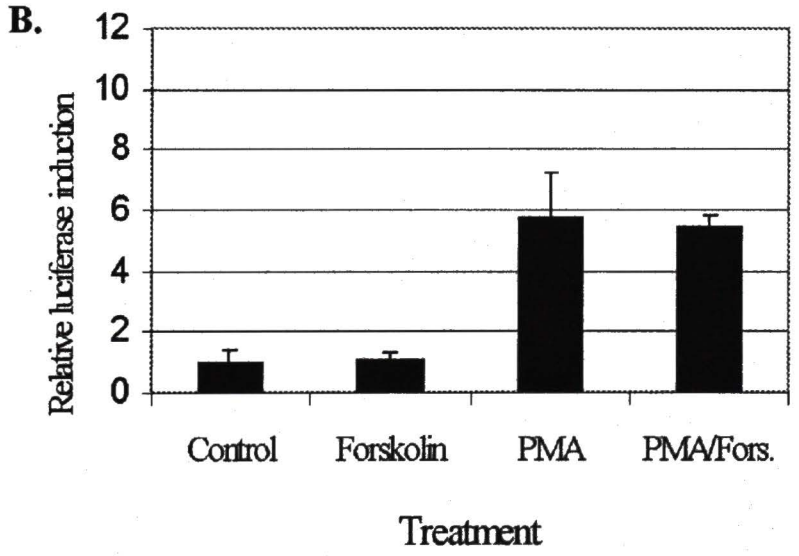

probe only
control
Anti c-Jun/AP-1
preimmune IgG
supershift
AP-1

insufficient quantity of the antibody used comparing to the amount of nuclear protein extract.

*Transcription activity of M-CSF gene promoter* --- To determine the IL-1 and PMA inducibility of M-CSF gene promoter at the level of gene transcription, we examined its functional potential on a reporter gene. MIA PaCa-2 cells were transfected with the plasmid containing the firefly luciferase cDNA under control of full-length M-CSF promoter (p570Luc). We detected approximately 10-fold increase in relative luciferase activity in the transiently transfected MIA PaCa-2 treated with 17 ng/ml IL-1 comparing to untreated cells after 12 h (Fig. 6 A.). Similarly, transfected cells treated with 20 ng/ml PMA yielded about 6-fold increase in luciferase activity (fig. 6 B.). A control reporter plasmid containing luciferase gene driven by CMV promoter yielded no significantly different luciferase activity between untreated and either IL-1 or PMA treated transfections, suggesting that induction of luciferase activity requires specific elements in the human M-CSF gene promoter (data not shown). Forskolin alone did not cause significant change in luciferase activity comparing with the untreated. Co-treatment with forskolin distinctively attenuated IL-1- induced relative luciferase activity from 9.65±0.804 to 6.67±0.415 ($p=0.011$). By contrast, forskolin had no significant effect on luciferase activity induced by PMA in transfected MIA PaCa-2 (from 5.73±0.972 to 5.40±0.289, $p=0.374$).

**Figure 6. Effect of forskolin on transactivation of (A) IL-1-induced, and (B) PMA-induced M-CSF promoter-driven luciferase activity** MIA PaCa-2 cells were transiently transfected with M-CSF promoter/luciferase gene construct 12 hours prior to switching to serum-free media containing specified treatments and incubated further for 12 hours, then cells were harvested for reporter gene assays. (A) Transfected cells were treated with 5 μM forskolin, 17 ng/ml IL-1, or combination of both. (B) Treatments were 5 μM forskolin, PMA 10 ng/ml, or combination. The data are expressed as the activity of the luciferase relative to β-galctosidase expressed as the means ± S.E.M. of two experiments, each of which was performed in triplicate. The asterisk states significant difference ($p < 0.05$) between transfected cells treated with IL-1 only versus ones co-treated with 5 μM forskolin.


A.
Relative luciferase induction
12
10
8
6
4
2
0
*
Control
Forskolin
IL-1
IL-1/Fors.
Treatment


B.
Relative luciferase induction
12
10
8
6
4
2
0
Control
Forskolin
PMA
PMA/Fors.
Treatment

# DISCUSSION

In addition to DB-cAMP, forskolin and theophylline were reported earlier to increase intracellular cAMP level and suppress IL-1-induced M-CSF production (Ku et al., 1992). In this study, our results show that intracellular cAMP elevation attenuated the IL-1-induced and LPS-induced M-CSF production by approximately 50%, while the PMA-induced M-CSF production in MIA PaCa-2 was unaffected by forskolin. The changes in mRNA message detected by northern hybridization correlated well with the changes in biological activities for both stimulatory and inhibitory stimuli. Along with the evidences from previous reports in various cells indicated that expression of M-CSF is regulated primarily at the level of gene transcription (Falkenburg et al., 1991 and Satriano et al., 1993). Two conjectures may be drawn, firstly M-CSF gene expression is regulated at the transcriptional level and secondly, cAMP elevation adversely affects the M-CSF gene expression at or upstream to the transcriptional level. Of which the result from promoter/reporter gene assays strongly supported. Recent reports demonstrated that LPS receptor or toll-like receptor (TLR 2 and 4) conferred LPS responsiveness through activation of NF-κB via the signaling molecules shared by IL-1 signaling (Zhang et al., 1999 and Chow et al., 1999). Thus, it is reasonable to expect that both IL-1- and LPS-induced M-CSF expression was affected by cAMP elevation in similar manner.

Differential effects of intracellular cAMP elevation on these particular different signaling pathways, which regulate the same target genes, are not unprecedented. Activation of the cAMP-dependent signaling pathway by DB-cAMP or PGE2 resultes in the downregulation of IL-4 in concanavalin A- or anti-CD3 plus anti-CD28-activated

human T lymphocytes. By contrast, neither DB-cAMP nor prostaglandin E2 affect IL-4 expression in PMA plus A23187-activated T cells (Borger et al., 1996). Other inducible genes for which expression can be attenuated by cAMP elevation include IL-2 and nitric oxide synthase (Tsuruta et al., 1995 and Mustafa & Olson 1998). These genes are commonly regulated by NF-κB, so it is probable that cAMP negatively affects one of the signaling components in NF-κB activation pathway. A critical step in the signal-induced activation of NF-κB is the site-specific phosphorylation of its inhibitor, IκB. This commits the latter for degradation by the ubiquitin-proteasome pathway (Dignam et al., 1983). In El-4 cells, the suppressive effect of cAMP on IL-2 expression involved the inhibition the binding of p50/p65 heterodimers to the NF-κB site as well as the alteration of the complexes bound to the NF-AT site (Tsuruta et al., 1995). The mechanism that forskolin employs to downregulate LPS-stimulated inducible nitric-oxide synthase induction was attributed to its ability to prevent the degradation of IκBα and also to induce IκBα expression (Mustafa et al., 1998). Recently both the NIK as well as its downstream enzyme complex IKKs were identified as upstream signaling molecules of IκB (Roebuck et al., 1999). This provides the possibility that any of these signaling components can be the target, upon which cAMP exerts its inhibitory effect.

NF-κB and AP-1 are inducible transcription factors critical for the expression of many genes involved in the inflammatory response, including adhesion molecules and cytokines. The enhancer region of the M-CSF gene contains two NF-κB binding sites as well as two AP-1 binding sites. IL-1 generally is regarded as activator of both NF-κB and

AP-1. In MIA PaCa-2 cells, IL-1α strongly activated NF-κB, but not AP-1. A comparable circumstance was found in TNFα , a cytokine with similar signaling mechanism to IL-1. TNF and IL-1induce both AP-1 and NF-κB in A549 epithelial cells, but only NF-κB in HMEC-1 (Kim and Maniatis, 1997). PMA distinctively activated AP-1, while it evoked a modest activation of NF-κB in MIA PaCa-2 cells. PMA acts as diacylglycerol analog and activates protein kinase C as well as phospholipase C. This suggests that M-CSF gene induction can be achieved through at least two different signal transduction pathways. The preferential activation of one transcription factor over the other by one specific stimulus trigger the question whether activation of both NF-κB and AP-1 simultaneously are required to induce M-CSF gene expression. A current evolving model of transcriptional activation comes from the studies of the IFNγ gene expression. This illustrates that an assortment of transcription factors in the enhancer region cooperatively induces the transcription activation to the varying degrees and all of the cis-acting elements are required in a specific architectural context in order to exert maximum transcription activation (Merika et al., 1998). Albeit IL-1 did not increase AP-1 binding over the unstimulated cells in EMSA, a noticeably high basal AP-1 activation could sufficiently cooperate with NF-κB or another transcription factors to enhance the M-CSF gene transcription. On the other hand, the observation that IL-1α and PMA preferentially activate one transcription factor but not the other may reflect that each of them does not evoke a maximum M-CSF gene induction. Nevertheless, all three stimuli induced the expression of M-CSF in MIA PaCa-2 cells to the comparable degree as observed in northern analysis. However, NF-κB and AP-1 are not the only known

inducible transcription factors for which binding sites are present on M-CSF enhancer region. SP-1 binding has also been shown to be activated by acute phase cytokines and in turn increases M-CSF expression [4].

The expression of membrane-integrated form of M-CSF (1.6 kb mRNA) seems to be more readily induced by IL-1α than that of major soluble 4.0 kb form. Interferon-γ stimulation also results in much higher inducibility in the expression of 1.6 kb mRNA than that of the 4.0 kb mRNA (Ohtsuki et al., 1994). These findings fit the hypothesis that stromal cells may efficiently produce membrane-bound M-CSF when they are stimulated by acute phase cytokines in the inflammatory state. This in turn may contribute to increase the local concentration of M-CSF at the inflammatory sites. Although the implication of the expression of minimally expressed 1.6 kb mRNA is still not fully understood, our results suggest that both forms of mRNAs are attenuated by cAMP elevation. This further supports that cAMP elevation affects M-CSF regulation at or upstream to transcriptional level.

The M-CSF promoter-luciferase reporter gene assays demonstrated that both IL-1 and PMA were able to induce transcription activation of the full-length M-CSF promoter to a similar extent. The finding that cAMP elevation negatively affected only IL-1 induced- but not AP-1 induced- transcription activation of the same promoter sequence, in conjunction with the finding that IL-1 preferentially activated NF-κB, narrows the target of cAMP attenuation of M-CSF expression to NF-κB dependent signaling pathway upstream of the gene transcription.

Both NF-κB and AP-1 have their distinctive roles in M-CSF gene induction. Our findings delineate that attenuation of induced M-CSF gene expression by cAMP elevation involved NF-κB-dependent signaling and suggest that it affects transcription regulation. IL-1α not only upregulats M-CSF gene expression, but also affects the alternative splicing of the gene.

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# CHAPTER III

## CHARACTERIZATION OF SIGNAL TRANSDUCTION PATHWAYS OF HUMAN MACROPHAGE COLONY-STIMULATING FACTOR TRANSCRIPTION

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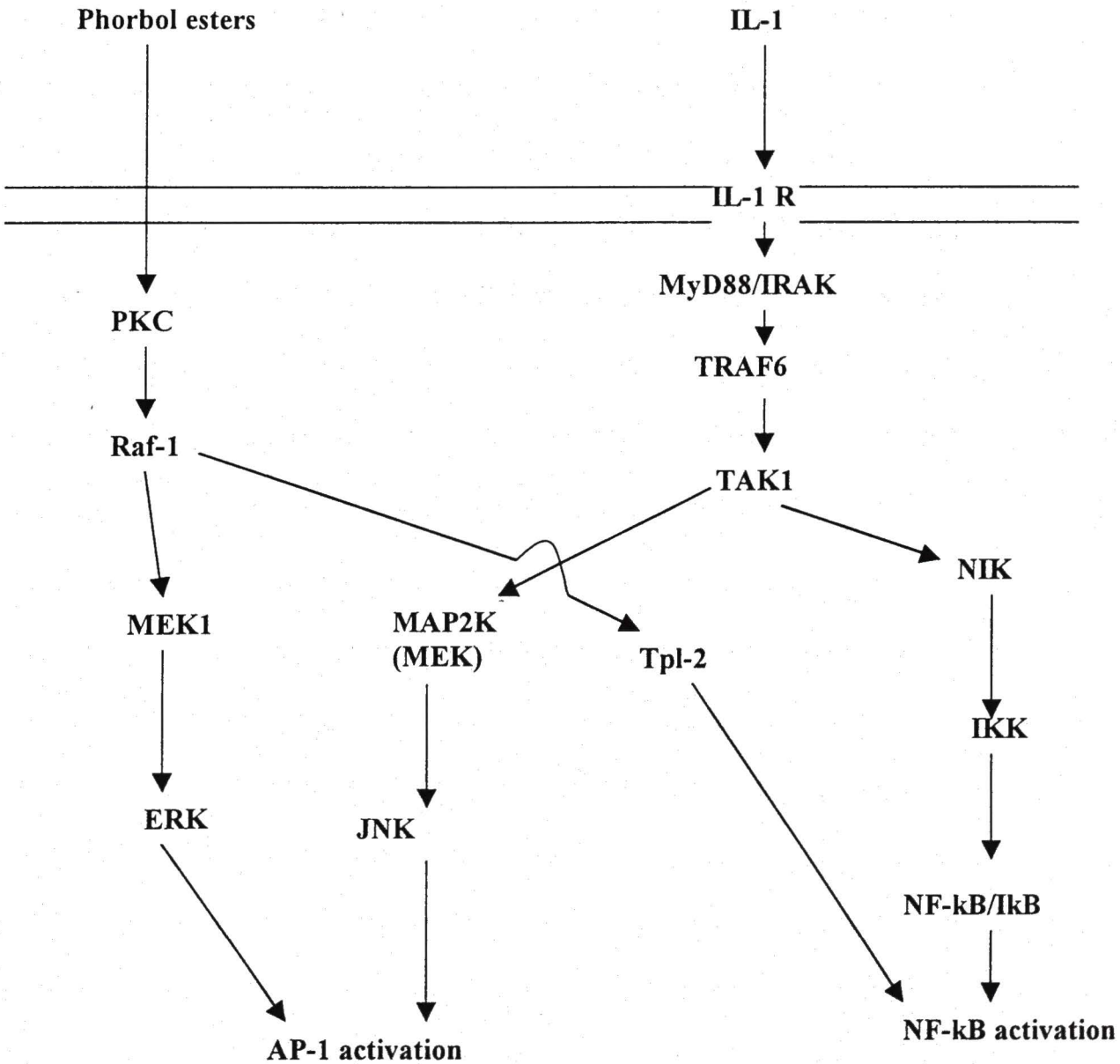
Running title: characterize M-CSF expression signaling

# PREFACE

The results of the previous investigation demonstrated that there seems to be two different signaling pathways regulating M-CSF gene transcription. Both IL-1 and PMA can induce M-CSF expression in MIA PaCa-2 cells through activation of NF-κB and AP-1 respectively. Whether these two signaling pathways are overlapping is still not certain. Although the finding that intracellular cAMP elevation antagonizes only IL-1-induced M-CSF transcription and NF-κB activation suggests that these two signaling pathways are independent of each other. However, further characterization of these signaling pathways is likely to yield more insight to understand regulation of human M-CSF gene expression.

The model depicted in Fig. 1 shows signaling pathways leading to activation of transcription factor NF-κB and AP-1 by IL-1 and PMA. The molecular events occurring from the IL-1 receptor (IL-1R) signaling have been characterized recently. The adapter protein MyD88 recruits two distinct putative Ser/Thr kinases, IL-1R-associated kinase (IRAK) and IRAK-2 to the receptor complex (Muzio *et al.* 1997). IRAK and IRAK-2 subsequently interact with the adapter molecule TNF receptor-activated factor (TRAF) 6, which bridges them to the protein kinase NF-kB-inducing kinase (NIK) (Cao *et al.* 1996; Malinin *et al.* 1997). Lastly, NIK activates the IκB kinase (IKK) complex that directly phosphorylates IκBα and releases NF-κB for nuclear translocation (DiDonato *et al.* 1997). TAK1 is a MAP kinase kinase kinase (MAP3K or MEKK) identified as a kinase that links TRAF6 to the NIK/IKK cascade as well as MAP2K (Ninomiya-Tsuji *et al.* 1999). This model suggests that IL-1 signaling not only initiates kinase cascade leading

Figure 1. Model for activation of NF-κB and AP-1 by IL-1 and phorbol esters. Interleukine-1 (IL-1), IL-1 receptor (IL-1R), IL-1R-associated kinase (IRAK), tumor necrosis factor receptor –associated factor 6 (TRAF6), NF-κB-inducing kinase (NIK), IκB kinase (IKK), protein kinase C (PKC), mitogen-associated protein kinase (MAPK), mitogen-associated protein kinase kinase (MEK), extracellular receptor-associated protein kinase (ERK), and jun-N-terminal protein kinase (JNK).

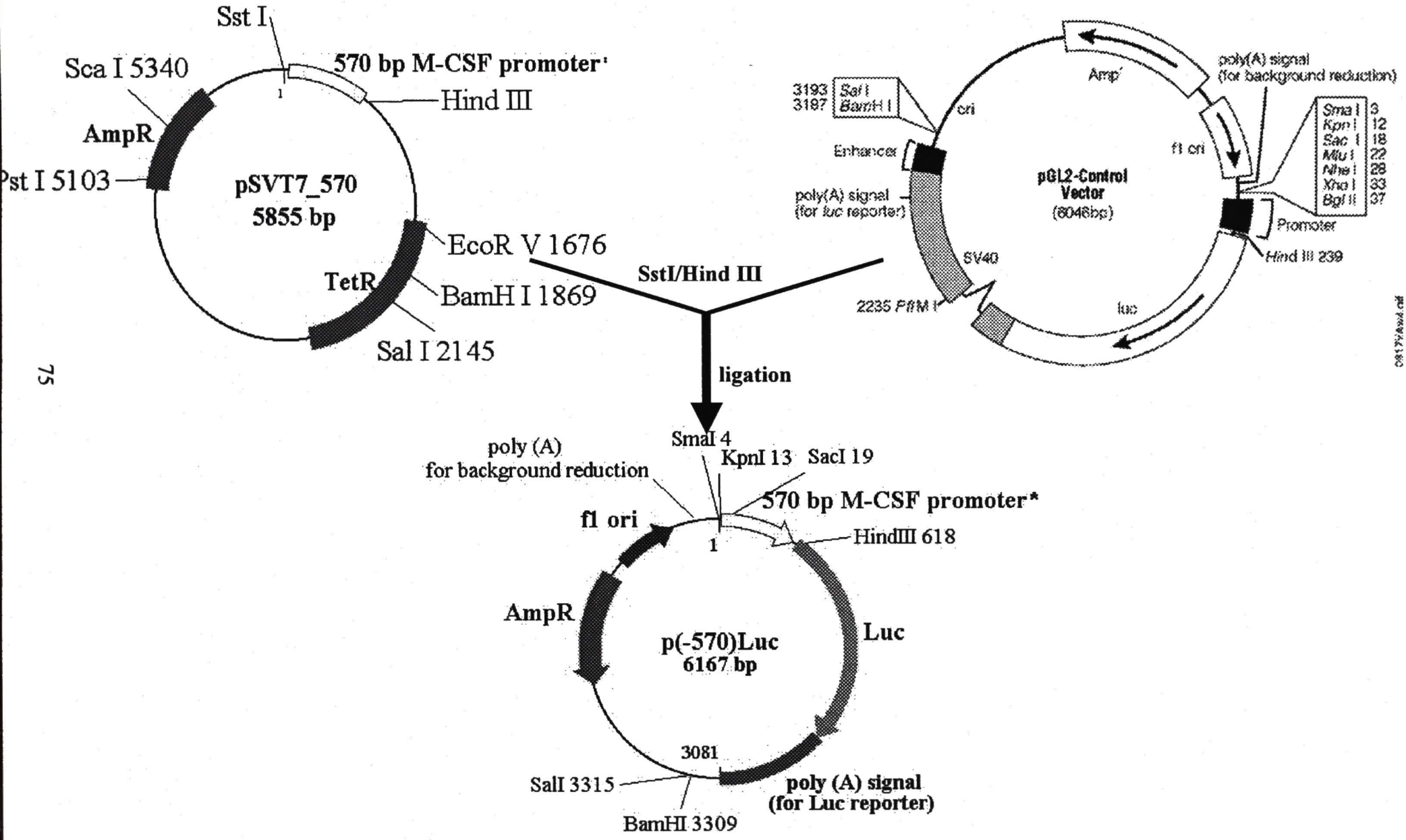

Phorbol esters
IL-1
IL-1 R
MyD88/IRAK
PKC
TRAF6
Raf-1
TAK1
NIK
MEK1
MAP2K
(MEK)
Tpl-2
IKK
ERK
JNK
NF-kB/IkB
AP-1 activation
NF-kB activation

to phosphorylation of IκB and activation of NF-kB via NIK/IKK, but also possesses a branching point via TAK1 leading to AP-1 activation via MEK and JNK.

Pathway that leads PMA to AP-1 activation is also well characterized. PMA can substitute for diacylglycerol in activating protein kinase C (Nishizuka 1995). PKC in turn phosphorylates and activates Raf-1, which initiates MAPK cascade involving activation of several kinases include MEK1, ERK and JNK (Van Biesen *et al.* 1996). A component of AP-1, c-jun, is among the target protein with docking site for both ERK and JNK (Kolch 2000). Moreover, ternary complex factors (TCF; such as Elk-1 and Sap1α) are also downstream target of ERK. These TCFs can enhance the c-fos promoter, thus increase expression of another component of AP-1 (Treisman 1994). Additionally, Tpl-2, a Raf-associated protein has been suggested to increase the level of active NF-κB by enhancing the proteolysis of an NF-κB precursor molecule p105 (Belich *et al.* 1999). This provides a possible mechanism for PMA to activate NF-κB as well.

Utilization of promoter-reporter gene contructs in transient transfection of the cells is a powerful tool in studying regulation of gene transcription. A series of plasmids containing serially deleted 5'-M-CSF promoter sequence was kindly provided by Dr. Hisashi Yamada. The strategy of subcloning the promoter sequences into pGL2basic luciferase reporter vector is depicted in Fig. 2. Since Sac 1 and Sst 1 are isoschizomers, Sst I and Hind III can be used to digest 5'-M-CSF promoter sequences from pSVT7_M-CSFs, as well as to digest pGL2basic to prepare the subcloning site. After isolation and purification, the fragments containing 5'-M-CSF promoter sequences can be directionally ligated to Sst I/Hind III digested pGL2basic. In order to characterize signal transduction

**Figure 2. Strategy of subcloning the serially deleted M-CSF promoter sequences into pGL2basic vector.** The serial deleted M-CSF promoter sequences were packaged in pSVT7 plasmid vector at the position depicted by 570 bp M-CSF promoter. The DNA fragment containing these sequences can be digested from the vector by restriction enzymes Sst I and Hind III. The fragments can then be ligated directly to Sac I/Hind III digested pGL2basic vector. Sst I and Sac I are isoschizomers. Asterisks designate the position substituted by fragments containing 490, 406, 343, 248 or 95 bp M-CSF promoter sequences in the deleted constructs.


Sst I
Sca I 5340
570 bp M-CSF promoter*
Hind III
AmpR
pSVT7_570
5855 bp
Pst I 5103
EcoR V 1676
TetR
BamH I 1869
Sal I 2145
SstI/Hind III
ligation
poly(A) signal
(for background reduction)
Amp'
3193 SalI
3197 BamH I
ori
Enhancer
f1 ori
pGL2-Control
Vector
(6048bp)
poly(A) signal
(for luc reporter)
Promoter
Hind III 239
SV40
2235 PflM I
luc
Sma I 3
Kpn I 12
Sac I 18
Mlu I 22
Nhe I 28
Xho I 33
Bgl II 37
poly (A)
for background reduction
SmaI 4
KpnI 13
SacI 19
570 bp M-CSF promoter*
f1 ori
HindIII 618
AmpR
p(-570)Luc
6167 bp
Luc
3081
SalI 3315
BamHI 3309
poly (A) signal
(for Luc reporter)

pathways contributing to M-CSF gene transcription, attempt is made to block either NF-κB or AP-1 activation using available pharmacological agents that inhibit the signal transduction at various steps of these interconnected pathways.

# INTRODUCTION

A computer-assisted motif search of the sequence of 5'-flanking region of the M-CSF gene up to 570 nucleotides upstream to the transcription starting site (Ladner *et al.* 1988; Murakami *et al.* 1989) revealed putitive binding sites for currently known enhancer elements; AP-1 (5' terminus of AP-1 at –534 relative to the transcription start site), C/EBPα or NF-IL6 (-504), NF-κB (-387 and –377), AP-1 (-340) and SP-1 (-177 and –159) as depicted in figure 1. Proximal to the transcription starting site the sequence contained a possible TATA box (TTAAAA) at –26 and CAT box (CATAAA) at –54, which could function as binding sites for basic transcription machinery (Yamada *et al.* 1991). Lastly, at position –567 there is a nuclear factor of activated T cell 1 (NFAT-1) - like sequence (GAGGAAGA: underlined denotes the base different from consensus sequence GAGGAAAA) that scores p=0.97 on searching for similarity (Chen et al., 1995) to consensus NFAT-1 using nucleotide weight matrices (Randak *et al.* 1990).

NF-κB activation is a major signaling pathway stimulated by IL-1, one of the potent acute phase response cytokines. Evidence from our lab and others (Brach *et al.* 1991; Yamada *et al.* 1991) correlates NF-κB activation with M-CSF gene expression in several different cell types. We also demonstrated in the previous chapter that PMA stimulates AP-1 activation and human M-CSF expression. In most circumstances stimuli like PMA, can induce activation of both NF-κB and AP-1. However, experimental results in MIA PaCa-2 cells showed that IL-1 strictly stimulates NF-κB with no effect on AP-1 activation, whereas PMA induces only AP-1 without NF-κB activation. Only IL-1-

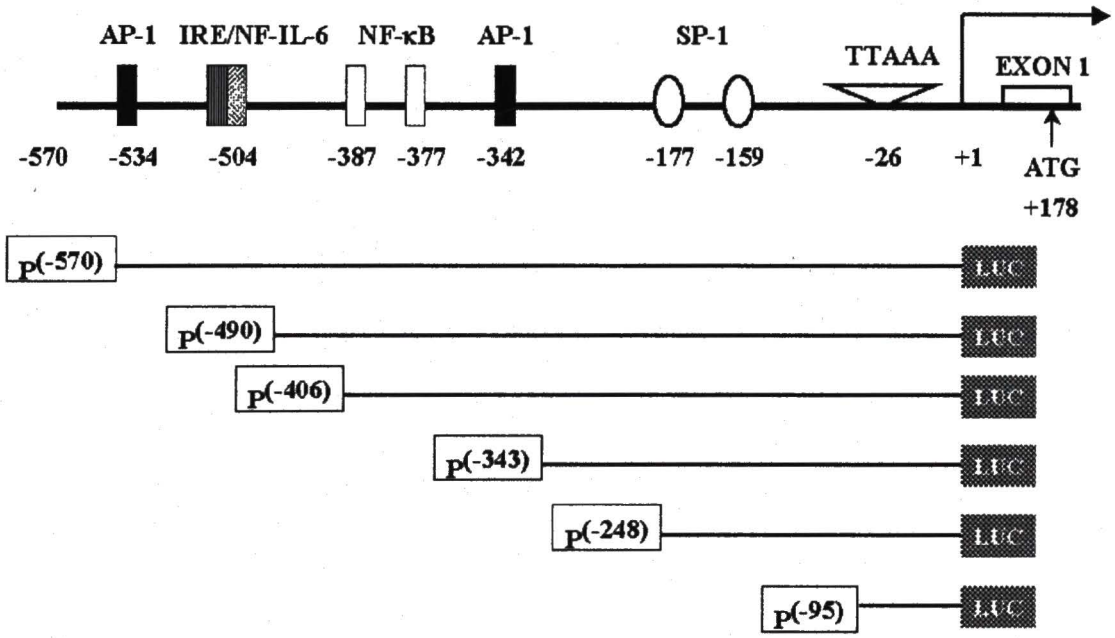
induced M-CSF transcription can be antagonized by cAMP, but not the PMA- induced M-CSF transcription. Moreover, the differential sensitivity to cAMP elevation observed between NF-κB and AP-1 activation led us to believe that at least two distinct signaling pathways can be employed to induce human M-CSF expression. Here, the roles of NF-κB and AP-1 activation pathways in human M-CSF transcription were further characterized using BAY11-7082 and PD98059 -- specific inhibitors that block key steps in NF-κB and AP-1 signaling pathways respectively.

Protein kinase C, a serine/threonine kinase, plays an important role in the regulation of cell growth and differentiation. PKC belongs to a family of up to 11 isoforms, which show different sensitivities to activators, including DAG, phospholipid and calcium (Mellor *et al.* 1998). PKC activation induces the transcription of immediate early response genes such as c-jun and c-fos that comprise the AP-1 transcription factor through either Jun homodimerization or Jun/Fos heterodimerization (Karin *et al.* 1997). The expression of c-Fos and c-Jun preceded the expression of M-CSF in monocytic leukemia U937 cells stimulated with PMA (Liu *et al.* 1992). In MIA PaCa-2 cells, PMA stimulates AP-1 and in turn induces M-CSF expression. It appears that AP-1 is an important regulatory element for M-CSF expression. Furthermore, preincubation of mesangial cells with calphostin C, a PKC inhibitor, reduced both PMA- and TNF-alpha-induced M-CSF mRNA transcripts (Kamanna *et al.* 1996). Therefore, the role of PKC in human M-CSF promoter-directed transcription needs to be evaluated.

**Figure 3. Luciferase reporter genes driven by 5'-M-CSF promoter sequences**

Schematic diagram showing cis-acting sites for enhancers on 5'-flanking region of M-CSF gene corresponding to the serially-deleted constructs of M-CSF promoter/luciferase reporter gene.

## 5'-M-CSF enhancer/reporter constructs



# MATERIALS AND METHODS

***Materials*** --- All of standard tissue culture reagents were obtained from Hyclone (Logan, UT, U.S.A.). Recombinant IL-1α (rHu. IL-1α(117-271) Ro24-5008 was a gift from Hoffmann La Roche, Inc. (Nutley, NJ, U.S.A.). Bacto® Lipopolysaccharide (S. typhosa LPS) was obtained from Sigma (St. Louise, MO, U.S.A.). [γ-$^{32}$P]-ATP and [α-$^{32}$P]-dCTP were purchased from Dupont NEN (Boston, MA, U.S.A.). The plasmids pGL2basic and pSV-β-galactosidase were from Promega Corp. (Madison, WI, U.S.A.). FuGene-6 was supplied by Roche (Indianapolis, IN, U.S.A.). Unless otherwise stated all other biochemical reagents were purchased from Sigma (St. Louise, MO, U.S.A.).

Cell culture and treatment --- The human pancreatic carcinoma cell line MIA PaCa-2 obtained from the American type Culture Collection (ATCC) was cultured in Dulbecco Modified Eagle's Medium (DME) supplemented with 7.5% FCS and 2.5% horse serum. Subculture of cells is done by treatment with 0.05% trypsin/0.02%EDTA and resuspended in fresh DME with 10% serum. Cells are culture at 37 °C in a humidified incubator under 6% $CO_2$. When 90% confluence was reached, cells were washed twice with PBS before switching to serum-free DME. In specified cultures, recombinant IL-1α, PMA, or LPS was added to the cell cultures at final concentration of 17 ng/ml, 20 ng/ml, 1 μM and 100 ng/ml, respectively. Cells were incubated further and harvested at specified times.

***Plasmid Constructs, Cell Transfection and Reporter Gene Assay*** --- Plasmid containing the 5'-region of human M-CSF gene promoter was kindly provided by Dr. Hisashi Yamada, Jeikei University Medical School, Japan. After digestion by SstI and HindIII, 570 bp fragment of 5'-M-CSF promoter was directionally subcloned upstream to the firefly luciferase gene in the plasmid pGL2 from Promega Corp. (Madison, WI, U.S.A.) at SacI/HindIII site deriving p570Luc and confirmed by sequencing. In all transfections, 1 μg of pSV-β-galactosidase were used to monitor the transfection efficiency. MIA PaCa-2 cells were seeded 2 x $10^5$ cell/35-mm plate 24 hours before transfection. Cells were washed twice with PBS and switched to serum-free DME media 6 hours before transfection. Transfection of mixture plasmid DNA into cells was performed by using Fugene-6 according to manufacturer's protocol. Transfected cells were stimulated with IL-1 or PMA with or without forskolin 12 hours after transfection , then incubated further for 12 hours until harvested for luciferase assay (Promega) per manufacturer's protocol. And assay for β-galactosidase was performed as previously described. Induction of luciferase activity is reported as the average from two separate experiments in triplicate normalized by β-galactosidase activity.

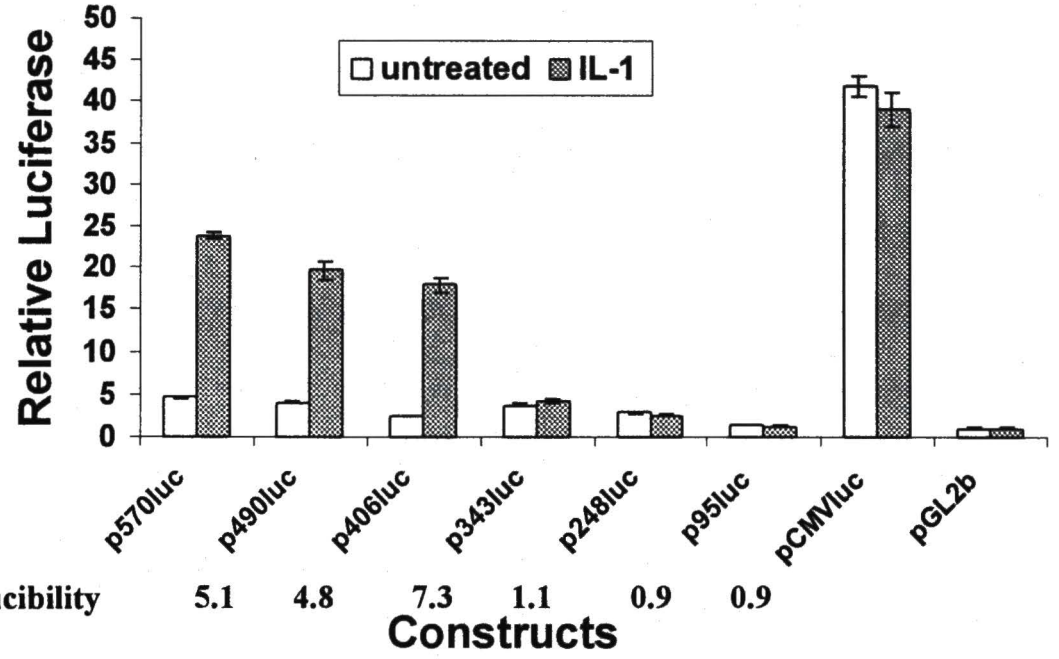
***Data Analysis*** --- Densitometry of the autoradiographed bands from Northern blot Kinase assays and EMSA were analyzed quantitatively by Alpha Imager™ program version 3.23 (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). All values are expressed as mean ± S.E.M. from independent experiments. Two-tail paired Student's t test was used to determine the significance of changes in densitometric measurements and relative luciferase activity. A significant difference was considered for p value of less than 0.05.

# RESULTS

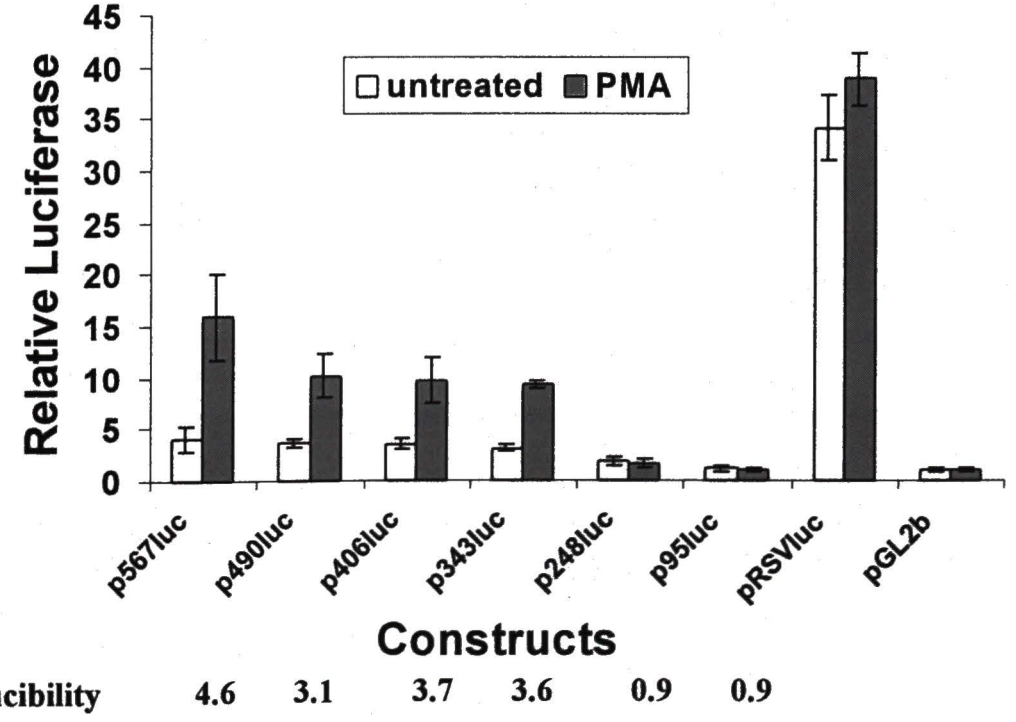
***Functional Analysis of the Human M-CSF Promoter*** --- To examine the role of 5' regulatory sequences play in controlling inducible M-CSF gene transcription, the activity of a series of human M-CSF promoter-luciferase reporter gene constructs was assessed in transient transfection assays in MIA PaCa-2 cells. Varying lengths of 5'-flanking region between positions –570 and +15 were inserted upstream of the luciferase gene in the pGL2basic plasmid vector. The diagram (Fig. 1) shows 5'- position of each of the deleted constructs and the included cis-acting sites for the transcription factors. The deleted constructs (truncated at the positions –570, -490, -406, -343, -248, and -95) were designed to assess the relative contribution of the transcription factor binding site for AP-1, NF-IL6, tandem repeated NF-κB, the other AP-1, and SP-1 respectively. All of the reporter gene constructs were transfected into MIA PaCa-2 cells, and the luciferase activity that resulted from each construct was determined with or without stimulation of the transfected cells with IL-1 or PMA. The luciferase activity from each of the luciferase reporter constructs was normalized for the transfection efficiency variation by beta-galactosidase activity from the co-transfected pSV-β-galactosidase. The relative promoter activity of these constructs normalized to the promoterless-vector pGL2basic, of which activity was designated = 1, was charted in upper panel of figure 2 showing activity in un-stimulated (open bars) and IL-1-stimulated (shaded bars) transfected cells. The lower panel represents the promoter activity in un-stimulated (open bars) and PMA-stimulated (shaded bars) cells.

**Figure 4. Functional analysis of truncated human M-CSF promoter/luciferase reporter constructs.** MIA PaCa-2 cells were transiently transfected with deleted M-CSF promoter/luciferase gene constructs 12 hours prior to switching to serum-free media containing specified treatments and incubated further for 12 hours, then cells were harvested for reporter gene assays. (A) Transfected cells were incubated for 12 hours in the absence (unfilled bars) and presence (shaded bars) of 17 ng/ml IL-1. (B) Transfected cells were incubated in the absence (unfilled bars) and presence (filled bars) of PMA 10 ng/ml. The data are expressed as the activity of the luciferase normalized to β-galctosidase expressed as the means ± S.E.M. of two experiments, each of which was performed in triplicate. Inducibility is defined as ratio between relative luciferase of stimulated (filled or shaded bars) versus un-stimulated (unfilled bars) transfected cells.

A.


Relative Luciferase
50
45
40
35
30
25
20
15
10
5
0
untreated
IL-1
p570luc
p490luc
p406luc
p343luc
p248luc
p95luc
pCMVluc
pGL2b
Inducibility 5.1 4.8 7.3 1.1 0.9 0.9
Constructs

B.

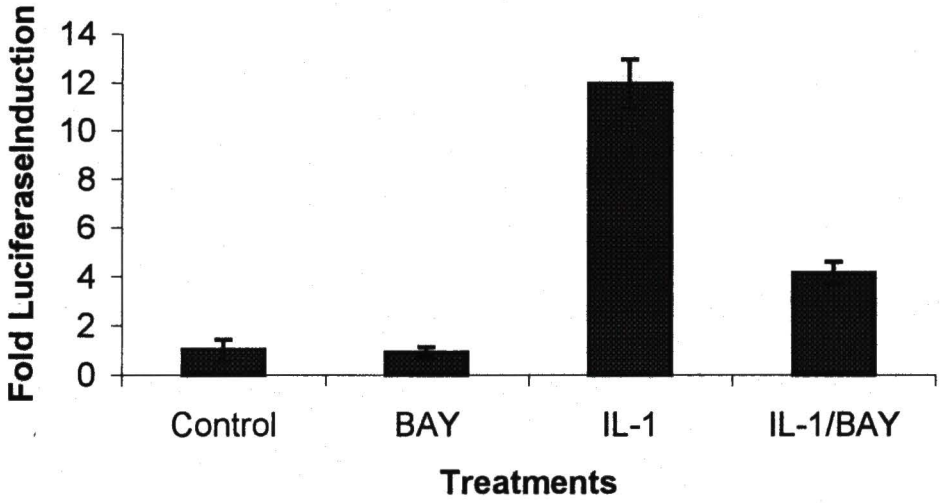

Relative Luciferase
45
40
35
30
25
20
15
10
5
0
untreated
PMA
p567luc
p490luc
p406luc
p343luc
p248luc
p95luc
pRSVluc
pGL2b
Constructs
Inducibility 4.6 3.1 3.7 3.6 0.9 0.9

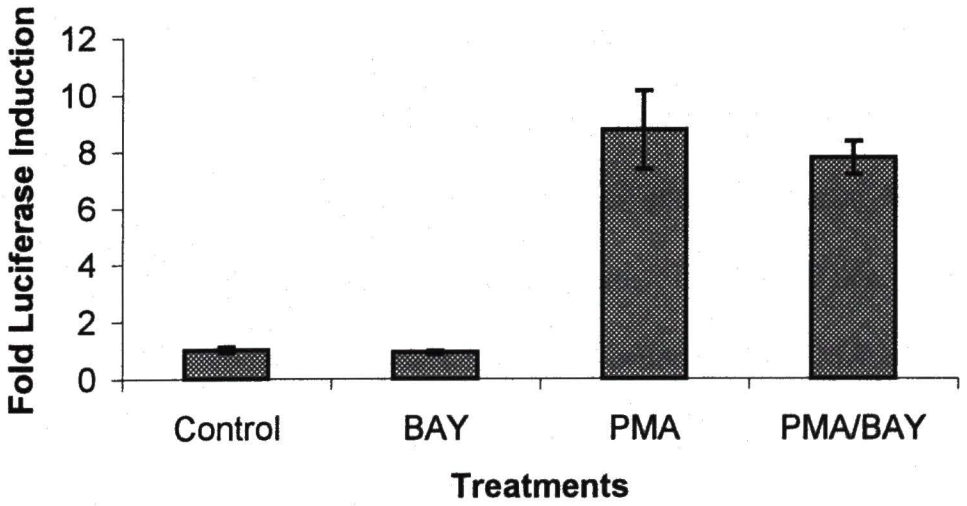
Significant luciferase activity was detected in cells transfected with the full-length human M-CSF constructs, p570luc, and stimulated with IL-1. The level of induction of this construct in IL-1-stimulated cells compared with the basal level of expression in un-stimulated transfected cells was 5.1-fold for these experiments. A control reporter plasmid containing luciferase gene driven by cytomegalovirus (CMV) promoter yielded no significantly different luciferase activity between untreated and IL-1-treated transfected cells, suggesting that induction of luciferase activity requires specific elements in the human M-CSF gene promoter. The removal of 5'-flanking sequence upstream to nucleotide – 490 decreased the extent of induced-luciferase activity approximately 20% of that observed in the full-length construct. However, the ratio of induction of IL-1-stimulated cells compared with un-stimulated cells with the shorter construct remained relatively unchanged. Further removal of the sequences between positions –490 and –406 did not significantly change the relative level of activity of the construct or its inducibility by IL-1. Nevertheless, truncation of the sequences between positions –406 and –343 resulted in significant drop in the induced-luciferase activity (about 80%) yielded by the construct and apparently total loss of inducibility by IL-1. It should be also noted that this construct yielded the highest inducibility by IL-1 at 7.3-fold. These suggest that the regulatory elements, which contribute to IL-1 inducibility of M-CSF gene transcription, lie within that nucleotide segment. This segment between positions –406 and –343 includes the two tandem repeats of NF-κB binding sites. Further deletion of 5'-flanking region beyond nucleotide –343 yielded relatively no additional loss of IL-1 inducibility.

When PMA was used instead of IL-1 to stimulate the transfected cells, the level of induction of the full-length construct in PMA-stimulated cells was 4.5-fold compared with the basal level of expression in un-stimulated transfected cells. Similarly, a control reporter plasmid containing luciferase gene driven by rous sarcoma virus (RSV) large T promoter yielded no significantly higher luciferase activity in PMA-treated transfected cells compared to the untreated cells, signifying that induction of luciferase activity by PMA also requires specific elements in the human M-CSF gene promoter. Deletion of 5'-flanking sequence upstream between nucleotide –570 and – 490 decreased the luciferase activity approximately 30% and dropped the inducibility to approximately 3-fold of that observed in the full-length construct. Further removal of the sequences between positions –490 and –343 did not significantly change the relative activity level of each of the constructs or their inducibility. But further truncation of the sequences between positions –343 and –248 resulted in tremendous drop of roughly 90% in luciferase activity and significant loss of PMA inducibility. The loss of function profile indicates that the regulatory element, which contributes inducibility of M-CSF promoter by PMA, resides within that –343 to –248 segment. AP-1 came up as the only known putative transcription factor-binding site in that particular sequence. Further truncation of 5'-flanking region to nucleotide –95 yielded no additional loss of promoter activity.

***Effects of BAY11-7082 on Human M-CSF Promoter*** --- BAY11-7082, an anti-inflammatory agent that specifically inhibits NF-κB activation, was used to assess the involvement of NF-κB in transcription activity of the M-CSF gene promoter. Full-length

**Figure 5. Effect of BAY11-7082.** MIA PaCa-2 cells were transiently transfected with full-length M-CSF promoter/luciferase gene construct (p-570Luc) 12 hours prior to switching to serum-free media containing the specified treatments for another 12 hours, then cells were harvested for reporter gene assays. The effect of BAY11-7082 on IL-1-induced M-CSF transcription is shown in filled bars (upper panel). The lower panel depicts the effect of BAY11-7082 on PMA-induced M-CSF transcription (shaded bars). The data are expressed as the activity of the luciferase normalized to β-galctosidase expressed as the means ± S.E.M. of two experiments in triplicate.


Fold LuciferaseInduction
14
12
10
8
6
4
2
0
Control
BAY
IL-1
IL-1/BAY
Treatments


Fold Luciferase Induction
12
10
8
6
4
2
0
Control
BAY
PMA
PMA/BAY
Treatments

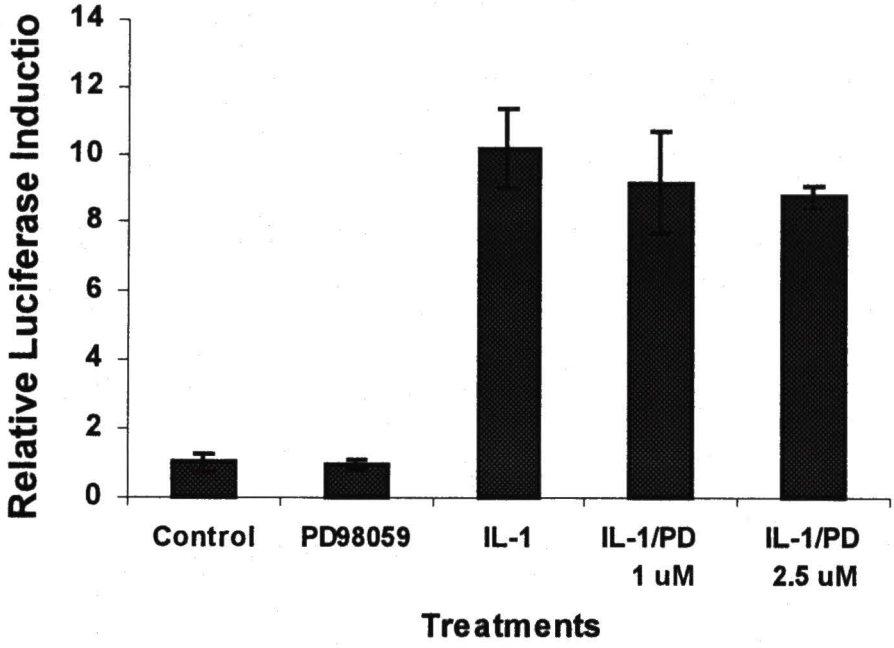
human M-CSF promoter-luciferase reporter construct was transfected into MIA PaCa-2 cells 12 h prior as described in methods. Then transfected cells either were left un-stimulated, or stimulated with 17 ng/ml IL-1, 10 μM BAY11-7082, or combination of both for another 12 h. The level of induction in IL-1-stimulated cells compared with the level of expression in un-stimulated transfected cells was 11-fold for these experiments. BAY11-7082 did not affect the promoter activity by itself (Fig.3 upper panel), but it significantly inhibited the luciferase activity in cells stimulated with IL-1 by approximately 60% ($p=0.0013$).

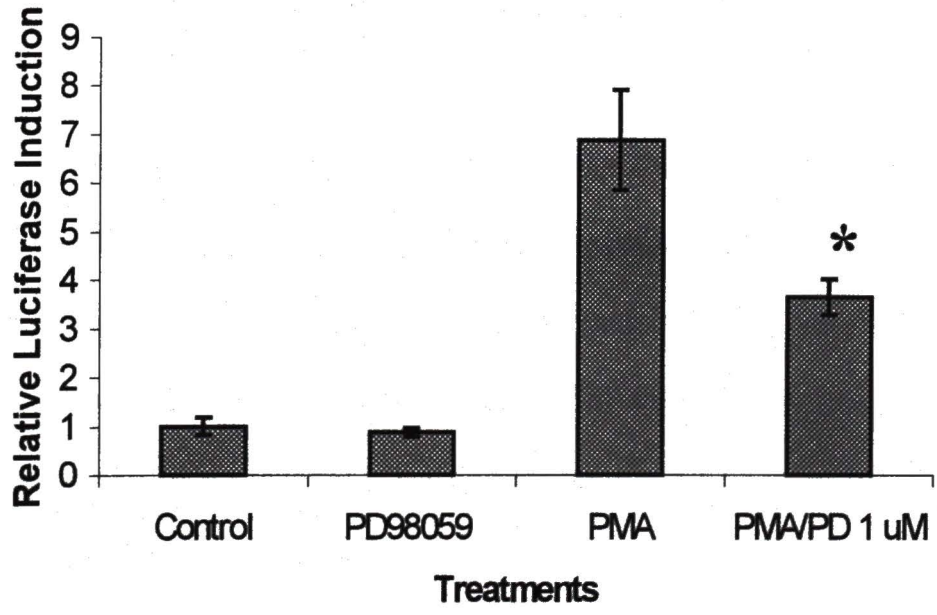
In similar experiments, which IL-1 was substituted with PMA, the level of induction in PMA-stimulated cells compared with the level of expression in un-stimulated cells was 9-fold. However, addition of BAY11-7082 yielded no significant difference in relative luciferase activity compared with the level of expression in transfected cells stimulated with PMA only ($p=0.3805$ & $0.4668$).

These experiments suggest that inducibility of human M-CSF promoter by IL-1 is dependent on NF-κB binding element, but PMA-induced human M-CSF promoter activity depends exclusively on AP-1 binding site. The p(-343)luc construct, which includes AP-1 without NF-κB binding site, was responsive to PMA but not IL-1. Not until AP-1 binding site was absent, p(-248)luc, that PMA inducibility was not demonstrable.

***Effects of MAP Kinase Kinase (MEK) Inhibitor on Human M-CSF Promoter*** --- PD98059, a selective and cell-permeable inhibitor of MEK that acts by inhibiting the

**Figure 6. Effect of PD98059.** MIA PaCa-2 cells were transiently transfected with full-length M-CSF promoter/luciferase gene construct (p-570Luc) 12 hours prior to switching to serum-free media containing the specified treatments for another 12 hours, then cells were harvested for reporter gene assays. The effect of PD98059 on IL-1-induced M-CSF transcription is shown in filled bars (upper panel). The lower panel depicts the effect of PD98059 on PMA-induced M-CSF transcription (shaded bars). The data are expressed as the activity of the luciferase normalized to β-galctosidase expressed as the means ± S.E.M. of two experiments in triplicate. The asterisk (*) states significant difference ($p < 0.05$) between transfected cells treated with PMA only versus ones with PD98059 added.


Relative Luciferase Inductio
14
12
10
8
6
4
2
0
Control
PD98059
IL-1
IL-1/PD 1 uM
IL-1/PD 2.5 uM
Treatments

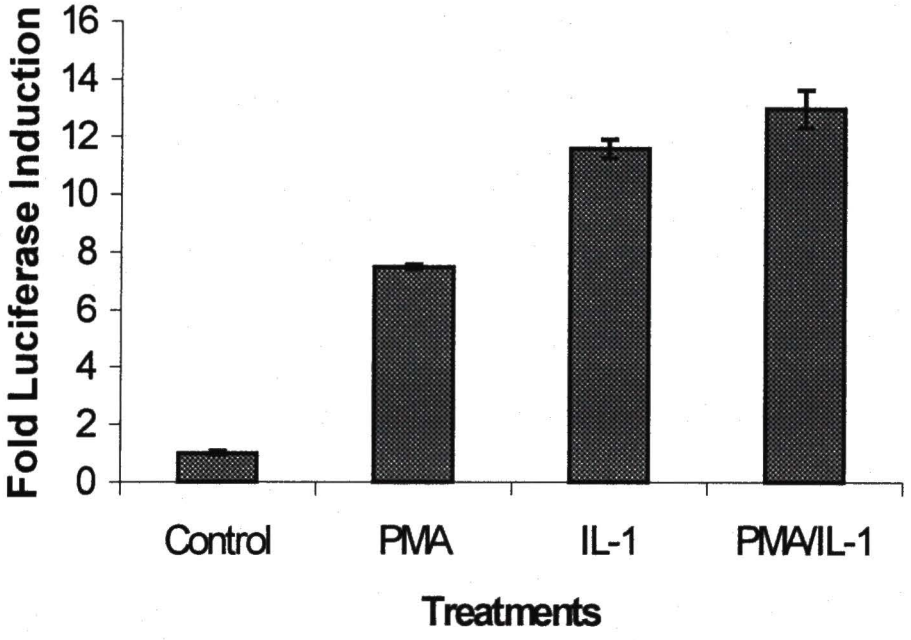

Relative Luciferase Induction
9
8
7
6
5
4
3
2
1
0
*
Control
PD98059
PMA
PMA/PD 1 uM
Treatments

activation of MEK-1 and subsequent phosphorylation of MAP kinase substrates (Bleul *et al.* 1996). The contribution of MAP kinase cascade on human M-CSF transcription was evaluated by using PD98059 to treat transfected MIA-PaCa-2 cells. We detected approximately 7-fold increase in relative luciferase activity in the transiently transfected MIA PaCa-2 treated with 20 ng/ml PMA comparing to untreated cells after 12 h (Fig. 4, top panel). PD98059 (1 μM) alone did not cause significant change in luciferase activity compared with the untreated. Co-treatment with PD98059 distinctly attenuated PMA-induced relative luciferase activity from 6.22±0.79 to 3.23±0.32 ($p=0.0017$). By contrast, PD98059 even at the higher concentration of 2.5 μM had no statistically significant effect on luciferase activity induced by IL-1 (Fig. 4, bottom panel) in transfected MIA PaCa-2 (from 9.86±2.64 to 7.48±0.81, $p=0.2267$).

Taken together, PMA is dependent on AP-1 activation through the mitogen-activated protein kinase cascade, specifically ERK-1 or ERK-2 which are the downstream kinases to MEK1, to induce human M-CSF transcription. By contrast, IL-1-induced M-CSF transcription is a relatively MEK-independent pathway.

***No Synergism between IL-1 and PMA on Human M-CSF Transcription*** --- Because IL-1- and PMA-induced human M-CSF transcription pathways seem to be separate and independent of each other, we set the experiments to examine if IL-1 and PMA could enhance each other effect on induction of human M-CSF transcription. Full-length human M-CSF promoter-luciferase reporter plasmids were transfected into MIA PaCa-2 cells as described earlier. Then the transfected cells were treated with IL-1, PMA,

**Figure 7. Effect of IL-1 and PMA on human M-CSF promoter.** MIA PaCa-2 cells were transiently transfected with full-length M-CSF promoter/luciferase gene construct (p-570Luc) 12 hours prior to switching to serum-free media containing the specified treatments for another 12 hours, then cells were harvested for reporter gene assays. The transfected cells were incubated in PMA only, IL-1 only, and combination of PMA & IL-1. The data are expressed as the activity of the luciferase normalized to β-galctosidase expressed as the means ± S.E.M. of two experiments in triplicate.

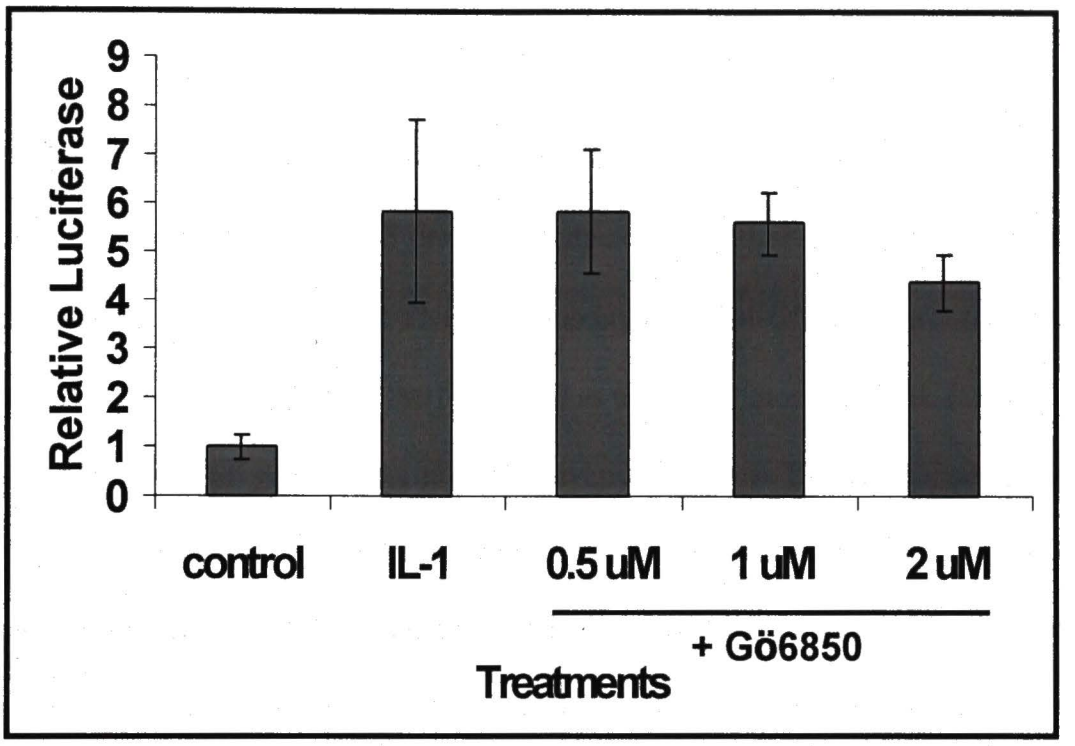

Fold Luciferase Induction
16
14
12
10
8
6
4
2
0
Control
PMA
IL-1
PMA/IL-1
Treatments

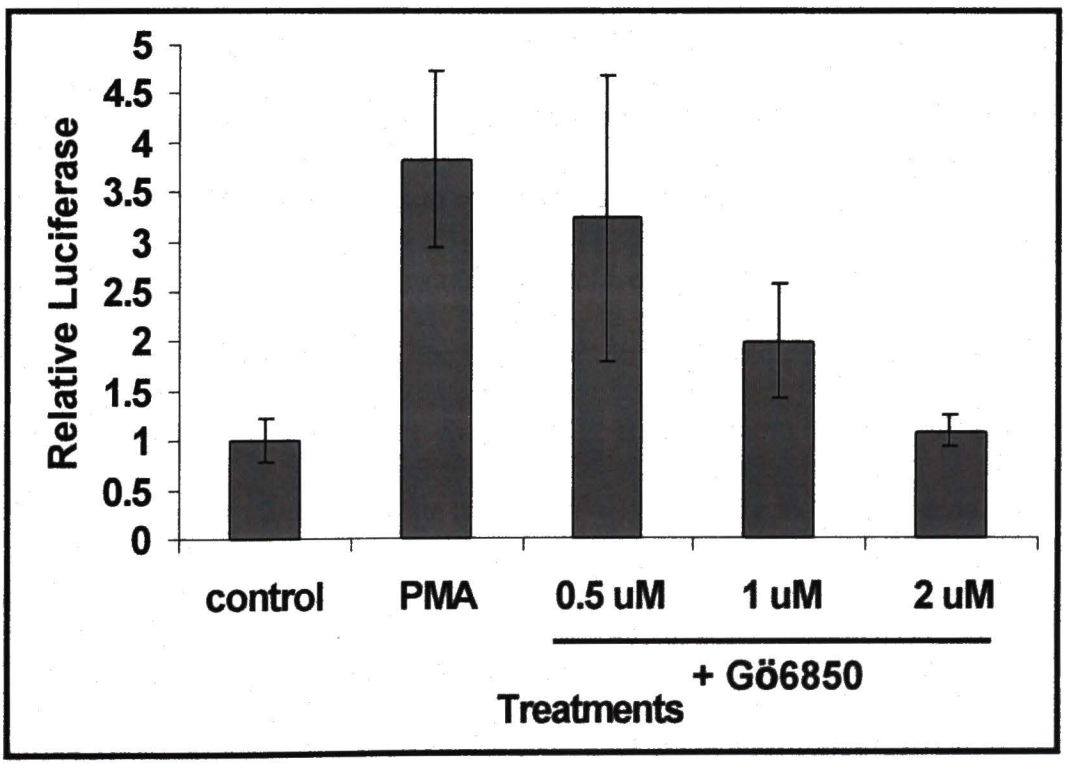
or both IL-1 and PMA. The induction of luciferase activity by only IL-1 or PMA was 9.9 –fold and 6.4–fold compared to control consecutively. The combination of both stimuli achieved slightly higher luciferase activity than IL-1 only 11-fold, but the increase was not statistically significant (p=0.035). Thus, there is no synergism between IL-1 and PMA on induction of human M-CSF promoter activity.

***Effects of Protein Kinase C (PKC) Inhibitor on Human M-CSF Promoter*** --- Gö 6850 is a highly selective cell-permeable inhibitor of conventional PKC, which acts as a competitive inhibitor for the ATP-binding site of PKC (Ku *et al.* 1997). We detected approximately 7-fold increase in relative luciferase activity in the transiently transfected MIA PaCa-2 treated with 20 ng/ml PMA comparing to untreated cells after 12 h (Fig. 4, lower panel). Gö 6850 at concentration of 1 μM alone did not cause significant change in luciferase activity compared with the untreated. Co-treatment with Gö 6850 distinctly attenuated PMA-induced relative luciferase activity from 6.88±1.03 to 3.65±0.363 (p=0.00007). By contrast, Gö 6850 had no statistically significant effect on luciferase activity induced by IL-1 in transfected MIA PaCa-2 (from 10.25±1.19 to 8.82±0.34, p=0.0675; Fig. 4, upper panel).

Altogether, PMA is dependent on PKC activation to induce human M-CSF transcription. By contrast, IL-1-induced M-CSF transcription is much less sensitive to PKC inhibitor Gö 6850.

**Figure 8. Effect of PKC inhibitor – Gö6850.** MIA PaCa-2 cells were transiently transfected with full-length M-CSF promoter/luciferase gene construct (p-570Luc) 12 hours prior to switching to serum-free media containing the specified treatments for another 12 hours, then cells were harvested for reporter gene assays. The effect of Gö6850 on IL-1-induced M-CSF transcription is shown in filled bars (upper panel). The lower panel depicts the effect of Gö6850 on PMA-induced M-CSF transcription (shaded bars). The data are expressed as the activity of the luciferase normalized to β-galctosidase expressed as the means ± S.E.M. of two experiments in triplicate. The asterisk (*) states significant difference ($p < 0.05$) between transfected cells treated with PMA only versus ones with Gö6850 added.


Relative Luciferase
9
8
7
6
5
4
3
2
1
0
control
IL-1
0.5 uM
1 uM
2 uM
+ Gö6850
Treatments


Relative Luciferase
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
control
PMA
0.5 uM
1 uM
2 uM
+ Gö6850
Treatments

# DISCUSSION

The region –406 to –344 of the M-CSF promoter, which includes functional NF-κB elements, contributes to M-CSF promoter inducibility by IL-1. The same promoter region had been reported to confer TNFα inducibility to the M-CSF gene transcription in HL-60 monocytes (Yamada *et al.* 1991), as well as minimally modified LDL (MM-LDL)-mediated M-CSF gene transcriptional activation in mouse L-cells (Rajvashisth *et al.* 1995). Moreover, this particular sequence of the M-CSF promoter when put in front of minimal TK promoter also elicited maximal activation in TNF-stimulated cells (Yamada et al., 1991). Gel shift experiments further established that a presence of functional NF-κB elements in this region was essential for both TNFα and MM-LDL stimulation (Yamada *et al.* 1991; Rajvashisth *et al.* 1995). Involvement of NF-κB in IL-1-induced M-CSF gene expression was also supported by electromobility shift assays demonstrated in chapter II. In addition, co-transfection of a dominant negative allele of NF-κB-inducing kinase (NIK) with murine M-CSF promoter luciferase reporter gene into hemangioendothelioma-derived cell line Py-4-1 led to a 3.5-fold decrease in M-CSF gene promoter activity (Green *et al.* 2000). Since NIK is an upstream kinase controlling NF-κB activity, this further emphasizes the role of NF-κB signaling in M-CSF gene transcription.

The sequence between –343 and –248 that contains AP-1 binding site is the minimum required for the M-CSF promoter to convey PMA inducibility. Along with the

AP-1 activation demonstrated in chapter II, it strongly suggests that PMA induce M-CSF transcription through AP-1 activation.

MEK inhibitor (PD98059) did not affect IKK activation of IκBα phosphorylation in vivo, whereas it completely inhibits $p90^{rsk}$ activation in T cells (Trushin *et al.* 1999). This finding is similar to our observation here that PD98059 had no significant effect on IL-1-induced M-CSF transcription. The reflection that the MEK inhibitor spares the signal transduction pathway leading to NF-κB activation from the mitogen-activated protein kinase pathway. The differential effects of BAY11-7082 on IL-1- and PMA-induced M-CSF transcriptions further support the notion that there are at least two distinct and mutually independent signaling pathways leading to human M-CSF transcription activation. This may be of future value in selectively inhibiting and differentiating specific target functions in signal transduction, such as NF-κB versus AP-1 activation.

When two seemingly distinct signaling pathways that lead to the same cellular event are simultaneously activated, higher activation may be expected. Surprisingly, PMA addition concurrent to stimulating the transfected cells with IL-1 yielded no significant change in M-CSF promoter activity compared to IL-1 only. Limitation of shared cellular resources could be the reason. The p65 component of NF-κB binds to the coactivator CBP (cyclic AMP responses element binding protein [CREB]-binding protein), as well as its analog p300 in order to enhance the activity of basal transcription machinery (Gerritsen *et al.* 1997). AP-1 also depends on CBP to exert its function in transcription (Banister *et al.* 1995). The levels of CBP/p300, transcriptional coactivator

that integrates multiple signal transduction pathways in the nuclei, are limiting (Hottingen *et al.* 1998). Furthermore, recruitment of CBP by ligand-binding domain of multiple nuclear receptors had been reported to inhibit AP-1 activation (Kamei *et al.* 1996). Thus, limiting nuclear signaling components could prevent the synergism between signals from IL-1 and PMA stimulation simultaneously.

We have seen in the previous chapter that PMA upregulated M-CSF expression by activation of AP-1, the downstream target of the mitogen-activated protein kinase pathway, presumably via PKC activation. Previous studies had shown that Inhibition of PKC by the broad-spectrum isoquinoline derivative H-7 reduced approximately 40% of M-CSF activity in phorbol ester-induced MIA PaCa-2 cells (Ku 1990), and abolished induction of M-CSF RNA in M-CSF-induced blood monocytes (Brach *et al.* 1991). H-7 inhibits both PKC and PKA at a pretty close concentration, which makes it hard to distinguish. The effect of Gö 6850 observed here emphasizes that PMA-induced M-CSF transcription indeed is conventional PKC-dependent.

Conventional PKC (cPKC) isoforms, which are calcium- and PMA-responsive, had been identified to activate of IKK complex activity in $CD^{3+}$ T cells (Lallena *et al.* 1999). This might suggest cPKC involvement in the downstream NF-κB activation and its subsequent M-CSF expression. By contrast, we found that PKC inhibitor Gö 6850 did not affect IL-1-induced M-CSF transcription in MIA PaCa-2 cells. We have shown in previous chapter that PKC activation by PMA did not elicit NF-κB activation in MIA PaCa-2 cells. FRTL-5 thyroid cell is another cell type that was documented to render no NF-κB activation upon PKC induction by PMA (Mori *et al.* 1999). Furthermore,

overexpression of PKCζ isoenzyme did not result in induction of luciferase reporter gene driven by two NF-κB binding sites of the HIV enhancer region in COS-7 fibroblast-like cells, whereas in the same experiments NF-κB-dependent luciferase induction was observed concomitantly with Rho GTPases overexpression (Montaner *et al.* 1998). Altogether, IL-1 activates NF-κB and its subsequent M-CSF transcription by a protein kinase C-independent mechanism in MIA PaCa-2 cells.

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CHAPTER IV

# INHIBITOR OF NUCLEAR FACTOR-κB INDUCTION BY cAMP ANTAGONIZES INTERLEUKIN-1-INDUCED HUMAN MACROPHAGE COLONY-STIMULATING FACTOR EXPRESSION

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Running title: IκB antagonizes M-CSF expression

# PREFACE

The two previous investigations demonstrated that intracellular cAMP elevation antagonizes NF-κB activation and M-CSF transcription induced by IL-1. Among inhibitors of signaling molecule investigated, only NF-κB inhibitor -- BAY11-7082 adversely affects M-CSF promoter-driven luciferase activity induced by IL-1. It's reasonable to speculate that intracellular cAMP elevation interferes with NF-κB activation pathway. In lieu of recent elucidation of the components of NF-κB signaling pathway (depicted in Fig. 1) there are two feasibly tested possibilities that intracellular cAMP elevation affects NF-κB activation pathway. Firstly, whether intracellular cAMP elevation modulates IKK activity and resulting in decreased IκB phosphorylation & degradation (1). Secondly, cAMP elevation increases cytosolic IκB availability through the IκB gene induction (2). Both possibilities certainly can bring about lower activation of NF-κB signaling and may be the basis of how intracellular cAMP elevation antagonizes IL-1-induced human M-CSF expression.

**Figure 1. Cartoon depicting signaling via IL-1 receptor.** Upon binding of the ligand IKK complex is recruited to the receptor by other signaling adaptor proteins and phosphorylated by NIK. Phosphorylated IKK complex become activated and then phosphorylates the IκB. Phosphorylated IκB is targeted to degraded by ubiquitin/proteasome system. P65/p50 subunit of NF- κB is then released to translocate into the nucleus. There are two proposed mechanisms that intracellular cAMP elevation antagonizes IL-1 signaling. (1) cAMP interferes with NIK/IKK kinase axis and the resulting IKK activation. (2) cAMP induces IκB induction.

IL-1
IL-1R
RIP
TRAF6
NIK
IKKα
IKKβ
(-)
cAMP↑
Forskolin,
DB-cAMP
(1)
P
P
P
P
IκB
IκB
IκB
P
P
Ubiquitination
& Degradation
Cell
membrane
(2)
Nucleus
(+)
IκB gene
M-CSF gene
NF-κB
AP-1

## SYNOPSIS

We have recently reported that interleukin-1 (IL-1α) can induce human macrophage colony-stimulating factor (M-CSF) expression through nuclear factor-κB (NF-κB) activation, and treatment of human pancreatic cancer cells MIA PaCa-2 with forskolin or cAMP attenuated the NF-κB activation as well as M-CSF expression. In this study, we have further investigated the mechanism of cAMP attenuation. MIA PaCa-2 cells were incubated with forskolin or dibutyryl-cAMP and then stimulated with IL-1 for 1 h. Cell lysates were immunoprecipitated by anti-IκB kinase-β (IKKβ) antibody and assayed the immune complex for kinase activity using recombinant inhibitor of NF-κB (IκBα) as substrate. The total cellular protein levels of IKKβ were measured by respective western blot. The results show that the level of IKK protein remains constant in the presence of cAMP, forskolin and/or IL-1, while IKK activity was robustly stimulated by IL-1. Nonetheless, DB-cAMP or forskolin did not affect the IKK activation induced by IL-1. This experiment suggests that elevated cAMP has no effect on IKK activity. IκBα protein level decreased markedly in IL-1 treated cells comparing to the untreated. By contrast, cells treated with cAMP or forskolin possessed discernibly higher IκBα level. In addition, we observed that forskolin potentiated and prolonged the IL-1-induced IκBα mRNA levels, while it did not stabilize the IκBα mRNA message. Wholly, these studies indicate that elevated cAMP antagonizes IL-1-induced M-CSF transcription by up-regulating IκBα gene induction and its consequent attenuation of NF-κB activation.

# INTRODUCTION

An expanding number of reports suggest novel roles of macrophage colony-stimulating factor other than its first discovered function in myeloid lineage hematopoiesis (Flanagan et al., 1998). For example, recent studies implicated that M-CSF played crucial roles in the pathogenesis of two major public health concerns: atherosclerosis (Rajavashisth et al., 1998) and osteoporosis (Kimble et al., 1996 and Srivastava et al., 1998). Overexpression of M-CSF as well as its receptor in several carcinomas, especially those of reproductive tracts, such as breast, ovary, uterus, and prostate gland also implicated the importance of M-CSF as auto/paracrine growth factor in tumor growth. The receptor-mediated mechanism of M-CSF has been extensively investigated. However, only limited information is available on the signal transduction leading to M-CSF expression.

We recently reported that forskolin and dibutyryl cAMP both attenuated IL-1-induced NF-κB activation in human pancreatic cancer cell line MIA PaCa-2, from which the M-CSF gene originally cloned, and correlatively reduced M-CSF expression at RNA message level as well as M-CSF promoter induction (Kamthong et al., 2000). Other inducible genes of which expressions were antagonized by cAMP elevation include IL-2 (Mary et al., 1987), IL-2 receptor (Rincon et al., 1988) and nitric oxide synthase (Mustafa and Olson, 1998). The common feature in all of these is that NF-κB dependent signaling is crucial in the regulation of these genes, so it is probable that cAMP negatively affects one of the signaling components of the NF-κB activation pathway.

The NF-κB dimers in most mammalian cells are sequestered in the cytosol by inhibitory IκB isoforms, such as IκBα, IκBβ, or IκBε (Baldwin et al., 1996). A critical step in the signal-induced activation of NF-κB is the site-specific phosphorylation of the conserved N-terminal serine residues of IκB. Specifically, they are Ser-32 and Ser-36 in IκBα. This commits IκB to degradation by the ubiquitin-proteasome pathway, and the freed NF-κB subunits are then translocated to function in the nucleus. Receptor-mediated signals, such as IL-1, tumor necrosis factor (TNF), and bacterial lipopolysacharide, lead to membrane recruitment of signaling proteins, such as TNF receptor-associated factors-2 or 6 and receptor interacting protein. These signaling pathways converge at activation of upstream NF-κB–inducing kinase (NIK) (Malinin et al., 1997). NIK then phosphorylates and activates a multiprotein IκB kinase (IKK) complex which is directly responsible for phosphorylating IκBs.

Diverse mechanisms of NF-κB activation blockade by drugs, especially antiinflammatory agents, or chemical and physical agents have been reported. Aspirin and sodium salicylate bind to IKKβ and thus inhibit its kinase activity supposedly by preventing the ATP binding to IKKβ (Yin et al., 2000), while sulindac also inhibits IKKβ even more potently (Kwak et al., 2000). Antiinflammatory cyclopentenone prostaglandins, which are synthesized in late stages of inflammatory episodes, modify the IKK subunits and inhibit their kinase activity (Rossi et al., 2000). Alternatively, the mechanism that forskolin employs to down-regulate induction of LPS-stimulated

inducible nitric-oxide synthase was attributed to its ability to prevent the degradation of IκBα and also to induce IκBα mRNA formation (Mustafa and Olson, 1998).

Here, we hypothesized that intracellular cAMP elevation could either interfere with NF-κB activation through NIK/IKK activation axis, or increase cytosolic IκB availability for complex formation with NF–κB through IκB gene induction. Either one would likely cause the decrease in NF-κB dependent M-CSF transcription. The present studies have examined how cAMP antagonizes IL-1-induced NF-κB activation and the subsequent NF-κB-dependent M-CSF gene expression.

## MATERIALS AND METHODS

***Materials*** --- All of standard tissue culture reagents were obtained from Hyclone (Logan, UT, U.S.A.). Recombinant human IL-1α (aa 117-271) Ro24-5008 was a gift from Hoffmann La Roche, Inc. (Nutley, NJ, U.S.A.). [$\gamma$-$^{32}$P]-ATP and [$\alpha$-$^{32}$P]-dCTP were purchased from Dupont NEN (Boston, MA, U.S.A.). The mutant NF-κB gel shift oligonucleotides, anti-IKKβ antibody, purified IκBα protein (aa 1-317) and protein A-agarose beads were obtained from Santa Cruz Biotech (Santa Cruz, CA, U.S.A.) BAY11-7082 and proteasome inhibitor II were acquired from Calbiochem (La Jolla, CA, U.S.A.).FuGene-6 was supplied by Roche (Indianapolis, IN, U.S.A.). Unless otherwise stated, all other biochemical reagents were purchased from Sigma (St. Louise, MO, U.S.A.).

***Cell culture and treatment*** --- The human pancreatic carcinoma cell line MIA PaCa-2 obtained from the American type Culture Collection (ATCC) was cultured in Dulbecco Modified Eagle's Medium (DME) supplemented with 7.5% fetal calf serum and 2.5% horse serum. Subculture of cells was done by treatment with 0.05% trypsin/0.02%EDTA and resuspended in fresh DME with 10% serum. Cells were cultured at 37 °C in a humidified incubator under 6% $CO_2$. When 80% confluence was reached, cells were washed twice with PBS before switching to serum-free DME. To stimulate the cells, recombinant IL-1α was added to the cell cultures at final concentration of 17 ng/ml. Cells were incubated further and harvested at specified times.

***Nuclear protein extraction*** --- Nuclear protein extracts were prepared according to Dignam et al. [12]. After incubation under a specific treatment, 1.5-2 x $10^6$ cells were washed twice with PBS before being scraped into buffer A [10mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, pH 7.9),1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5 mM phynylmethylsulfonyl fluoride (PMSF)] and briefly centrifuged. Cell pellets were consequently resuspended in buffer A, kept on ice for 15 min, 10% Nonidet P-40 added, homogenized in a Dounce homogenizer, and centrifuged at 12,000 rpm for 10 seconds at 4 °C. Then Nuclear pellets were resuspended in extraction buffer [20 mM HEPES (pH 7.9), 0.55 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 10 mg of leupeptin per 1 ml], homogenized, and centrifuged at 12,000 rpm for 2 minutes at 4 °C. Samples in the supernatants were collected and stored at –70°C until used. Concentrations of the nuclear protein samples were determined by BioRad protein assay according to manufacturer's protocol.

***EMSA*** --- Synthetic oligonucleotides with the sequences corresponding to cis-acting elements of the M-CSF promoter are $^{32}$P-5'-end-labeled by T4 polynucleotide kinase. Equal amounts (10 μg) of nuclear extract from each of various cell treatments were incubated in 20 μl of binding reaction [final concentrations; 40 mM KCl, 15 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5% glycerol, and 1 μg poly(dIdC)] for 10 min. Equal amounts (approximately 1 ng of DNA) of the radiolabeled oligonucleotide probe (60,000 c.p.m.) were added to the reaction mixture, and further incubated for additional 25 min at room temperature. DNA protein complexes were separated from unbound DNA probe by electrophoresis on a native 5% polyacrylamide

gels at 140 V in 45 mM Tris-borate (pH 8.0) containing 1 mM EDTA and then autoradiographed. Oligonucleotide probes (Biosynthesis, Lewisville, TX) with the following sequences; NF-κB 5'-agttgaGGGACTTTCCaggc-3' (position -368 to –377 nucleotides of the M-CSF promoter) was used. The upper-case letters indicate the concensus sequences for the NF-κB binding site.

***Plasmids, Cell Transfection and Reporter Gene Assay*** --- Luciferase reporter plasmids driven by the 5'-region of human M-CSF gene promoter was generated as stated elsewhere [5, 11]. In all transfections, 1 μg of pSV-β-galactosidase were used to monitor the transfection efficiency. MIA PaCa-2 cells were seeded 2 x $10^5$ cell/35-mm plate 24 hours before transfection. Cells were washed twice with PBS and switched to serum-free DME media 6 hours before transfection. Transfection of mixture plasmid DNA into cells was performed by using Fugene-6 according to manufacturer's protocol. Transfected cells were stimulated with IL-1 with or without addition of DB-cAMP or BAY11-7082 12 hours after transfection, then incubated further for 12 hours until harvested for luciferase assay (Promega) per manufacturer's protocol. And assay for β-galactosidase was performed as previously described [13]. Induction of luciferase activity is reported as the average from two separate experiments in triplicate normalized by β-galactosidase activity.

***Assay of IKK Kinase Activity*** --- After IL-1 and forskolin or DB-cAMP treatment, cells were washed with ice-cold 5 mM EDTA in phosphate-buffered saline PBS and were lysed on ice in TN buffer (containing 50 mM Tris (pH 7.5), 250 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, 50 mM NaF, 20 mM β-glycerophosphate, 1 mM $NA_3VO_4$, 1 mM

dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM EDTA, and 1 mM EGTA) by aspiration through a 21 gauge needle. Cells were spun at 10,000xg for 10 min at 4°C, and cell lysates were immunoprecipitated using 2 μg of anti-IKKβ antibody and 50 μl protein A-agarose. Aliquots of immunoprecipitate were then incubated with in 25 μl of kinase buffer (containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 20 mM ATP, and 10 μCi of [$\gamma$-$^{32}$P] ATP) at 30°C for 30 min. Protein bands were resolved by SDS-polyacrylamide gel electrophoresis and autoradiographed.

***RNA extraction and Northern blot analysis*** --- Total cellular RNA was isolated using RNAstat-60 reagent from Teltest (Friendswood, TX, U.S.A.) per manufacturer protocol. Samples containing equal RNA (15 to 25 μg) were fractionated in 1.2% agarose gels and transferred to nylon membranes. The membranes were then hybridized according to standard protocol [13] with either cloned M-CSF or GAPDH cDNA, or with probes generated by PCR for M-CSF or human 18S rRNA specific cDNA. The probes were radiolabeled with [$\alpha$-$^{32}$P]-dCTP by random priming using Klenow fragment from Promega (Madison, WI, U.S.A.) per manufacturer's direction. After hybridization or rehybridization, membranes were autoradiographed.

***Data Analysis*** --- Densitometry of the autoradiographed bands from Northern blot Kinase assays and EMSA were analyzed quantitatively by Alpha Imager™ program version 3.23 (Alpha Innotech Corporation, San Leandro, CA, U.S.A.). All values are expressed as mean ± S.E.M. from independent experiments. Two-tail paired Student's t test was used

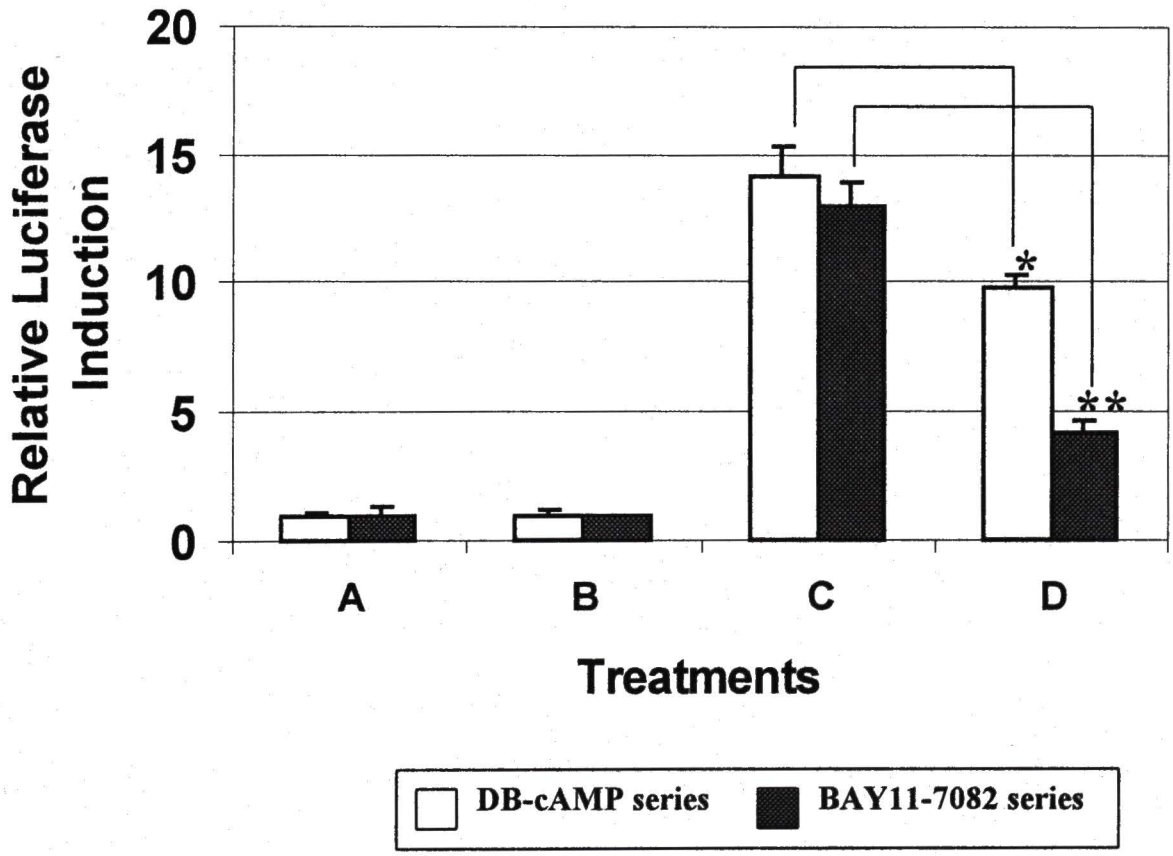
to determine the significance of changes in densitometric measurements and relative luciferase activity. A significant difference was considered for p value of less than 0.05.

## RESULTS

***NF-κB blockade and forskolin negatively affected M-CSF transcription in a similar manner*** --- BAY11-7082, a specific inhibitor of inducible IκBα phosphorylation, was used to compare with cAMP which we reported earlier to attenuate transcription activity of the M-CSF gene promoter. MIA PaCa-2 cells were transfected with the plasmid containing the firefly luciferase cDNA under control of full-length M-CSF promoter (p570Luc). We detected approximately 14 -fold increase in relative luciferase activity in the transiently transfected MIA PaCa-2 treated with 17 ng/ml IL-1 comparing to control untreated cells after 12 h (Fig. 2). DB-cAMP or BAY11-7082 alone did not cause significant change in luciferase activity comparing with control cells (p=0.4174 and 0.9389, respectively). The transfected cells appeared normal and the BAY11-7082 concentration used was well within the range 5 to 20 μM used in other reports without any signs of cytotoxicity. DB-cAMP significantly attenuated IL-1- induced relative luciferase activity from 13.5 ± 1.15 to 8.8 ± 0.41 (p=0.012) in Fig. 2 unfilled bars. In similar fashion, BAY11-7082 distinctly antagonized relative luciferase activity induced by IL-1 (from 12.9 ± 0.94 to 5.7 ± 0.46, p=0.014) in Fig. 2 filled bars.

***Effect of cAMP, BAY11-7082 and proteasome inhibitor II on NF-κB activation*** --- As previously indicated, cells were pre-incubated with 2 μM DB-cAMP, 10 μM BAY11-7082, or 1 μM proteasome inhibitor II for 1 h before they were treated with 17 ng/ml IL-1 for 60 min, then a nuclear protein extract from each treatment was prepared. NF-κB

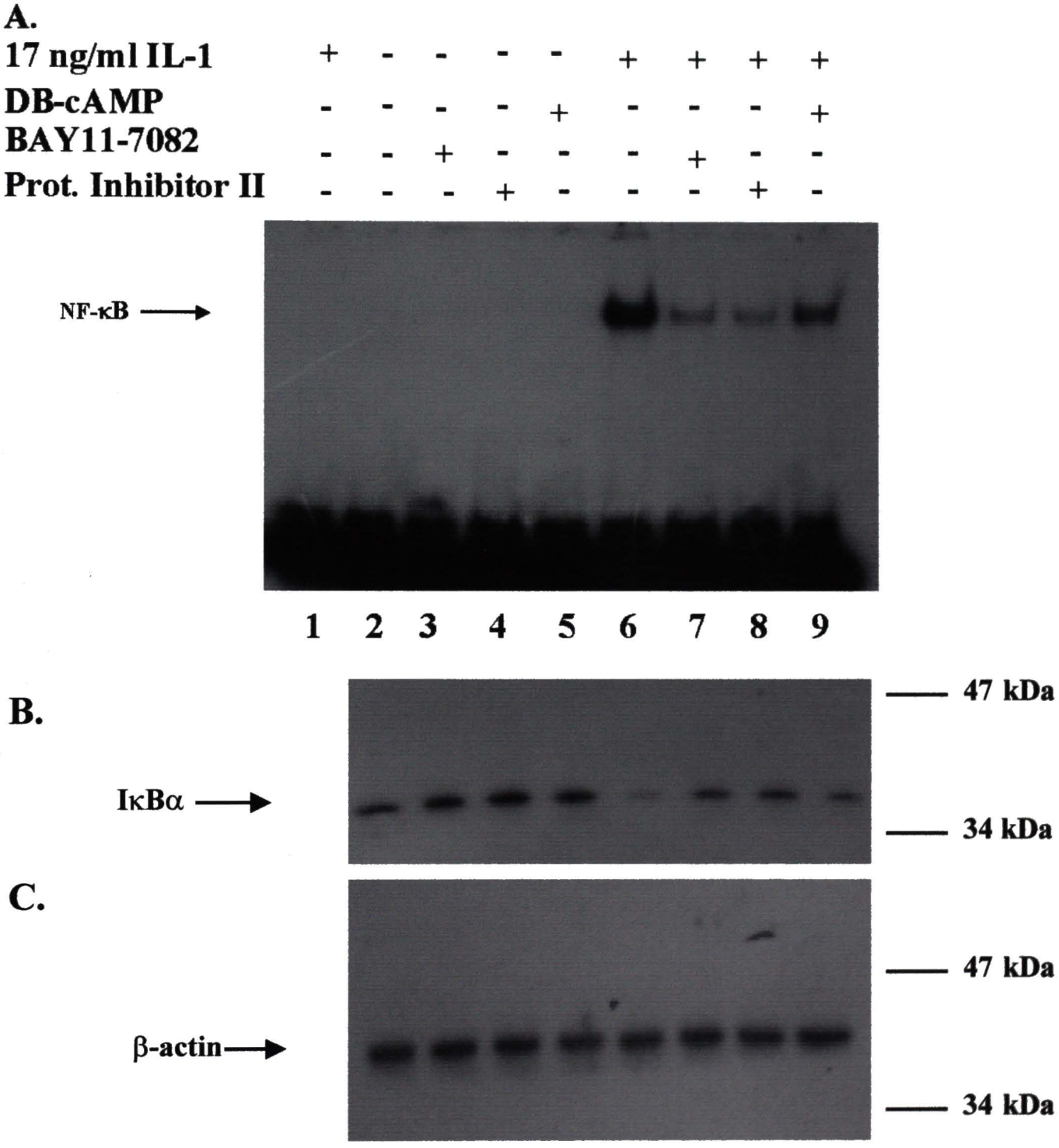
**Figure 2. Effects of DB-cAMP and BAY11-7082 on transactivation of IL-1-induced M-CSF promoter-driven luciferase activity.** MIA PaCa-2 cells were transiently transfected for 12 hours prior to switching to serum-free media containing specified treatments and incubated further for 12 hours, then cells were harvested for reporter gene assays. A. Transfected cells were left un-stimulated. B. Cells were treated with 2 μM DB-cAMP or 10 μM BAY11-7082 only. C. 17 ng/ml IL-1 was added to transfected cells. And D. cells were treated with IL-1 plus DB-cAMP or BAY11-7082. The data are the activity of the luciferase relative to β-galactosidase expressed as the means ± S.E.M. of two experiments, each of which was performed in triplicate. The asterisks mark significant difference ($p < 0.05$) between transfected cells treated with IL-1 only versus ones co-treated with 2 μM DB-cAMP or 10 μM BAY11-7082 within each series.


Relative Luciferase Induction
20
15
10
5
0
A
B
C
D
*
**
Treatments
DB-cAMP series
BAY11-7082 series

binding complex from nuclear extract appeared on the autoradiogram as a sharp single retarded band that was demonstrated earlier to be supershifted with anti-p65 antibody (data not shown). NF-κB was activated strongly by IL-1α (Fig. 3A, lane 6). A mutant NF-κB probe that differed from wild-type NF-κB binding site by 1 nt was used with the IL-1 treated extract as a negative control, and no binding was observed (Fig. 3A Lane 1). Elevation of intracellular cAMP level, by pretreatment of the MIA PaCa-2 cells with 2 μM DB-cAMP 1 h prior to the stimulation by IL-1, significantly attenuated NF-κB activation (Fig. 3A Lane 9). Attenuation of NF-κB activation by BAY11-7082 or proteasome inhibitor II were both even more pronounced than DB-cAMP (Fig. 3A lane 7 and 8).

Corresponding cytosolic proteins (100 μg) from the same treatments as above were resolved on SDS-PAGE, transferred to nitrocellulose membrane, and probed in western blot with an antibody against IκBα (Fig. 3B, apparent molecular weight 37 kDa), which showed level of IκBα present in cytosol of the cells in the specified treatment. This also reflected varying amounts of IκBα that was not degraded by ubiquitin-proteasome pathway after phosphorylated by upstream IKK. As expected, IL-1 markedly caused degradation of IκBα (Fig. 3, panel B lane 6) comparing to control in lane 2, and it distinctly activated NF-κB. By contrast, BAY11-7082, proteasome inhibitor II, or DB-cAMP alone each produced discernibly slight increase in IκBα protein without causing any change in NF-κB activation comparing to control (Fig. 3, lane 3, 4, and 5). Furthermore, each of these three compounds effectively preserved the levels of

**Figure 3. NF-κB activation in IL-1-stimulated MIA PaCa-2 cells is affected by DB-cAMP and BAY11-7082.** (A) MIA PaCa-2 cells were harvested and extracted for nuclear proteins 1 h after they were treated with the agents indicated (+). As indicated, cells were pre-incubated with 2 μM DB-cAMP, 10 μM BAY11-7082, or 1 μM Proteasome Inhibitor II for 1 h before the IL-1 was added. NF-κB oligonucleotide probes were incubated with 10 μg of nuclear extract per lane and resolved on 5% polyacrylamide gel in lanes 2 to 9. Mutant NF-κB probe was used with IL-1 treated extract in lane 1 as a negative internal control. Corresponding cytosolic proteins (100 μg) were resolved on SDS-PAGE, transferred to nitrocellulose membrane, and probed with an antibody against IκBα (B) and then beta-actin (C).


A.
17 ng/ml IL-1 + - - - - + + + +
DB-cAMP - - - - + - - - +
BAY11-7082 - - + - - - + - -
Prot. Inhibitor II - - - + - - - + -
NF-κB
1 2 3 4 5 6 7 8 9
B.
IκBα
47 kDa
34 kDa
C.
β-actin
47 kDa
34 kDa

IκBα protein in the cytosol in the presence of IL-1 (Fig. 3, panel B lane 7, 8, and 9) and significantly antagonized the IL-1-induced NF-κB activation (Fig. 3, panel A lane 7. 8, and 9). Availability of IκBα in the western blot was roughly in inverse relation with the level of NF-κB activation observed in that particular treatment. The western blots were stripped and reprobed for beta-actin protein with apparent molecular weight of 42 kDa (Fig. 3C), which was present equally across every treatment. This result indicated that IL-1-induced protein degradation is specific for IκB.

***Forskolin and DB-cAMP did not affect IKKβ activity*** --- IKKβ rather than IKKα has been reported to play central role in cytokine-inducible NF-κB activation [17]. In order to investigate if intracellular cAMP elevation could modulate IKKβ activity. MIA PaCa-2 cells were pre-incubated in 5 μM forskolin or 1 μM dibutyryl cAMP for 2 h before cells were treated with 17 ng/ml IL-1 for 1 h, and subsequently assayed for IKK activity in the anti- IKKβ immunoprecipitated complex using recombinant IkBα (aa 1-317) as a substrate. IkBα is the most preferable substrate of IKKβ [18]. Immune complex from IL-1 treated cells significantly induced IkBα phosphorylation comparing to that from untreated cells (Fig. 4 panel C, lane 5 & lane 2). There was no autoradiographed band observed in the kinase reaction of immune complex from IL-1 treated cells without recombinant IkBα, which was used as internal negative control (Fig. 4 panel C, lane 8). Similarly, the kinase reaction using pre-immune rabbit serum immunoprecipitates did not result in phosphorylation of IkBα (Fig. 4 panel C, lane 1). IL-1 also induced IKKβ

**Figure 4. Intracellular cAMP elevation did not affect IKKβ activity.** MIA PaCa-2 cells were pre-incubated in 5 μM forskolin or 1 μM dibutyryl cAMP for 1 h prior to addition of 17 ng/ml IL-1 for another 1 h. Cell lysates containing 200 μg of protein were immunoprecipitated with an antibody directed against IKKβ and 50 μl of protein A-agarose. Kinase assays containing recombinant IkBα (aa 1-317) as a substrate were resolved on 10% SDS-polyacrylamide gel electrophoresis and autoradiograghed. Fractions of the kinase assay reactions were analyzed by western blots using polyclonal antibodies against IKKβ and IκBα. The plus (+) and minus (-) signify the presence (+) or absence (-) of the agents in the cell treatments before the immunoprecipitated complexes were acquired. Pre-immune rabbit serum was used instead of anti- IKKβ in immunoprecipitation in lane 1. Immune complex from IL-1-treated cells was used in the kinase reaction in lane 8 without the substrate, recombinant IκBα.

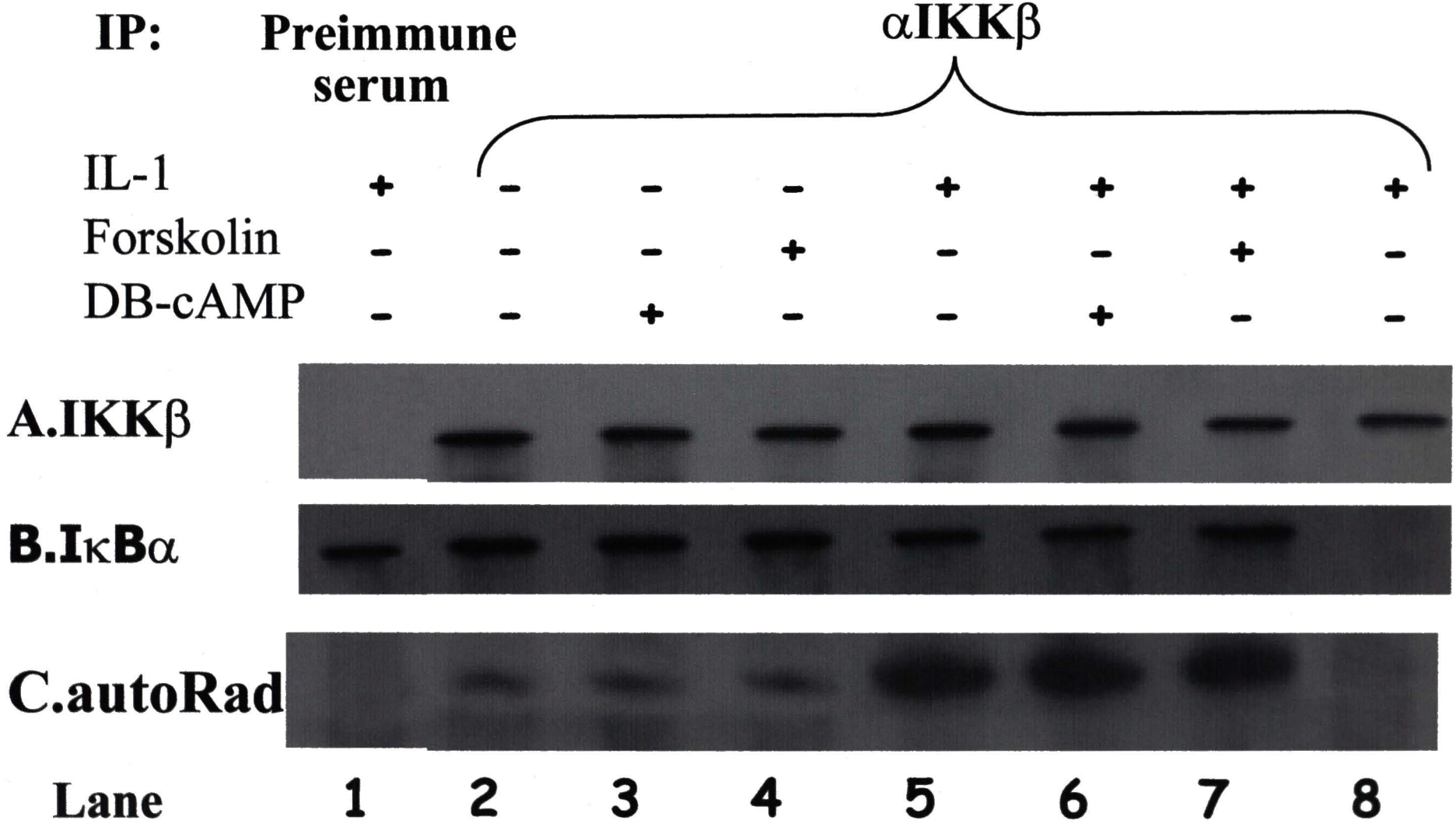
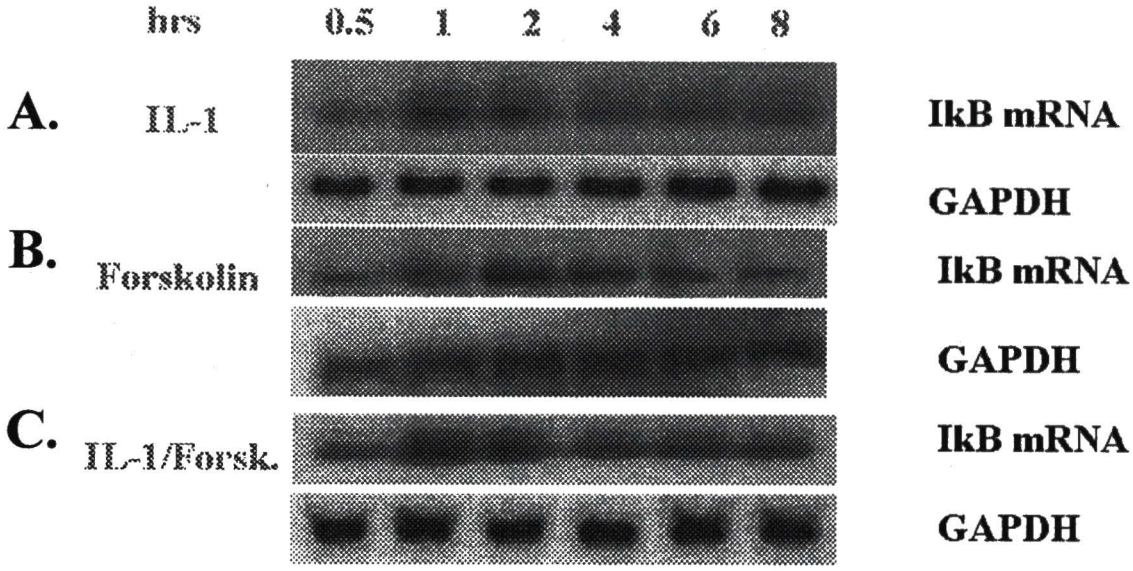
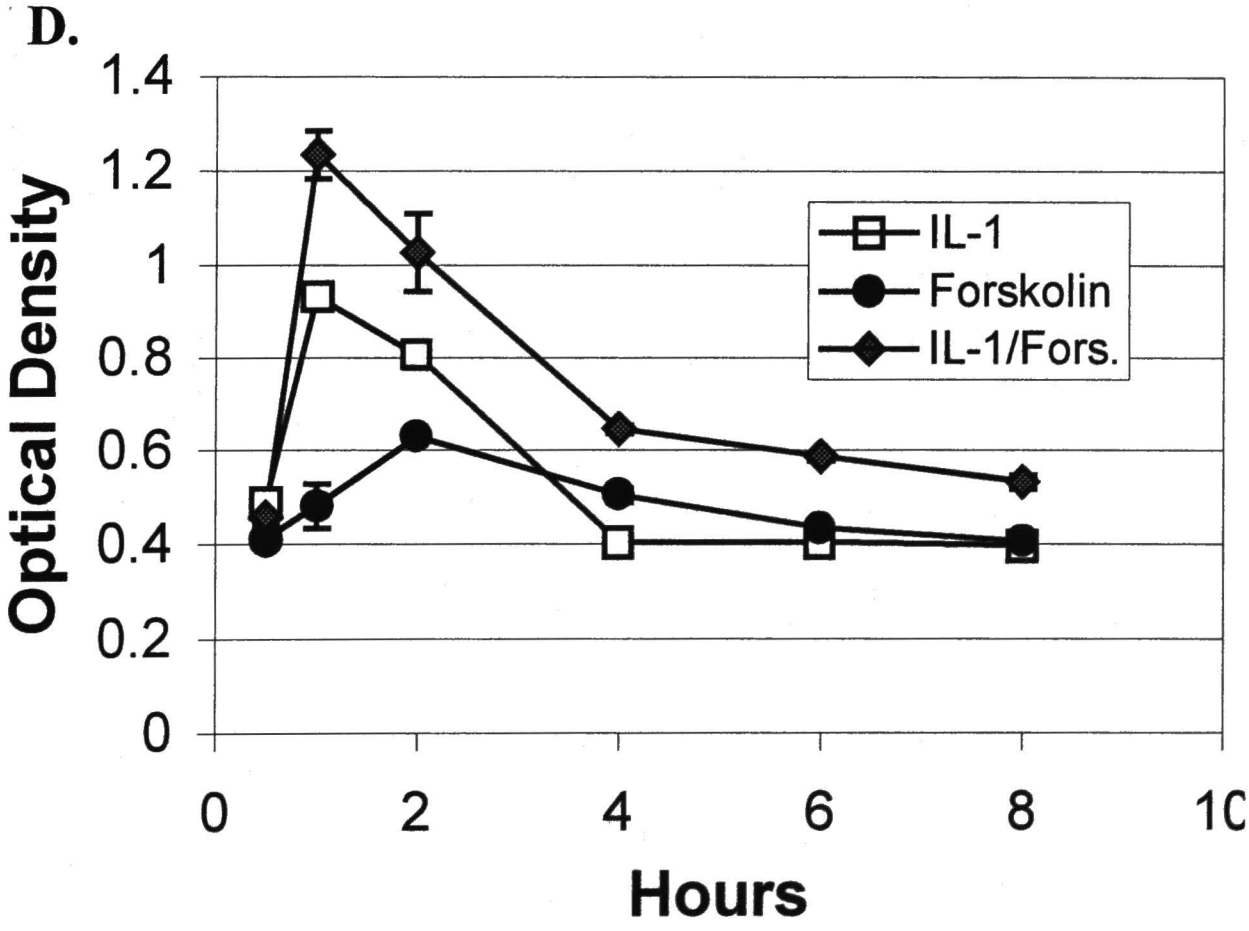

IP:
Preimmune serum
αIKKβ
IL-1
Forskolin
DB-cAMP
+ - - - + + + +
- - - + - - + -
- - + - - + - -
A.IKKβ
B.IκBα
C.autoRad
Lane
1 2 3 4 5 6 7 8

**Figure 5. Kinetics of forskolin and IL-1-induced IκBα mRNA levels.** MIA PaCa-2 cells were incubated with forskolin (5 μM) or IL-1 (17ng/ml) or were pretreated with forskolin for 2 h and then added IL-1 for various periods of time. After cell lysis and RNA purification, northern blots were hybridized with a cDNA probe for IκBα, stripped, and then probed for GAPDH. The chart shows ratio between the signal density of each IκBα mRNA and corresponding GAPDH mRNA at the specified time. The data expressed as means ± S.E.M. of two independent experiments. Closed circles (•) represent values from cells treated with forskolin, open squares (□) stand for IL-1 treatment, and closed diamonds (♦) signify cells pretreated with forskolin before IL-1 stimulation.


hrs
0.5
1
2
4
6
8
A.
IL-1
IkB mRNA
GAPDH
B.
Forskolin
IkB mRNA
GAPDH
C.
IL-1/Forsk.
IkB mRNA
GAPDH

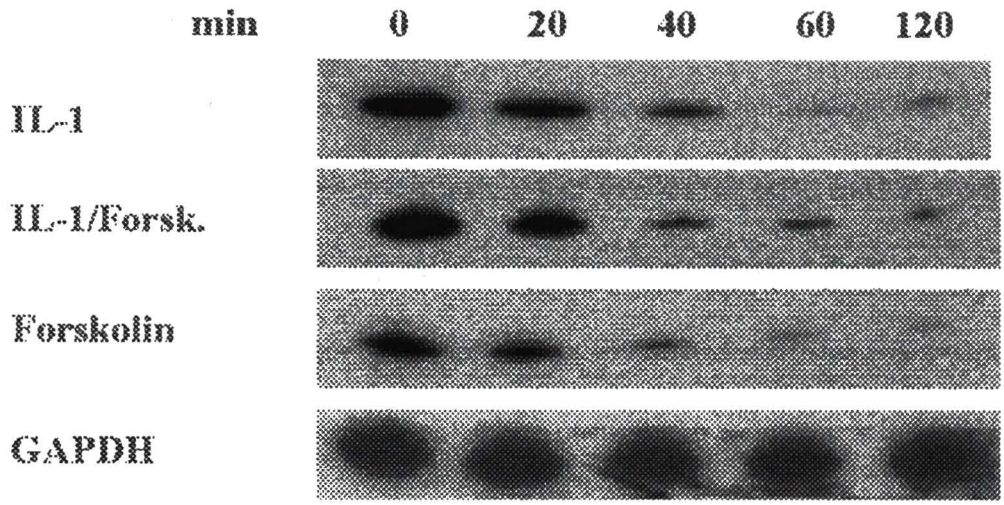

D.
Optical Density
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
Hours
IL-1
Forskolin
IL-1/Fors.

northern blots were hybridized with a cDNA probe for IκBα. The level of IκBα mRNA level in un-stimulated MIA PaCa-2 cells was undetectable (data not shown). The plot (Fig. 5) provides graphical presentation of the IκBα mRNA signal density changes in each treatment, corrected for loading variations by corresponding GAPDH mRNA density at the specified time points after stimulation. In cells treated with forskolin (Fig. 5 panels B&D, closed circles), IκBα mRNA peaked at 2 h after stimulation and the mRNA level fell off gradually to the base line by 6 h. By contrast, in the presence of IL-1 (Fig. 5 panels A&D, open squares), IκBα mRNA peaked more rapidly at 1 h after stimulation and the level remained elevated up to 4 h. In cells pretreated with forskolin before IL-1 stimulation (Fig. 5, panels C & D closed diamonds), the kinetics of IκBα mRNA levels looked similar to ones in cells treated with IL-1 only. But IκBα mRNA levels rose significantly higher than IL-1 alone and the up-regulation was maintained up to 8 h. Notice that IL-1 was added 1 h after the initial exposure of the cells to forskolin, the cells were in contact with forskolin for 2 h when they were harvested. At that time point, maximal IκBα mRNA accumulation induced by each of IL-1 or forskolin alone were reached (Fig. 5, panels A & B). This result essentially explains the finding others and we have previously observed that DB-cAMP or forskolin had to be added to the cells 30 to 120 min prior to the stimuli, such as IL-1 or LPS, in order to provide maximal inhibition of NF-κB activation.

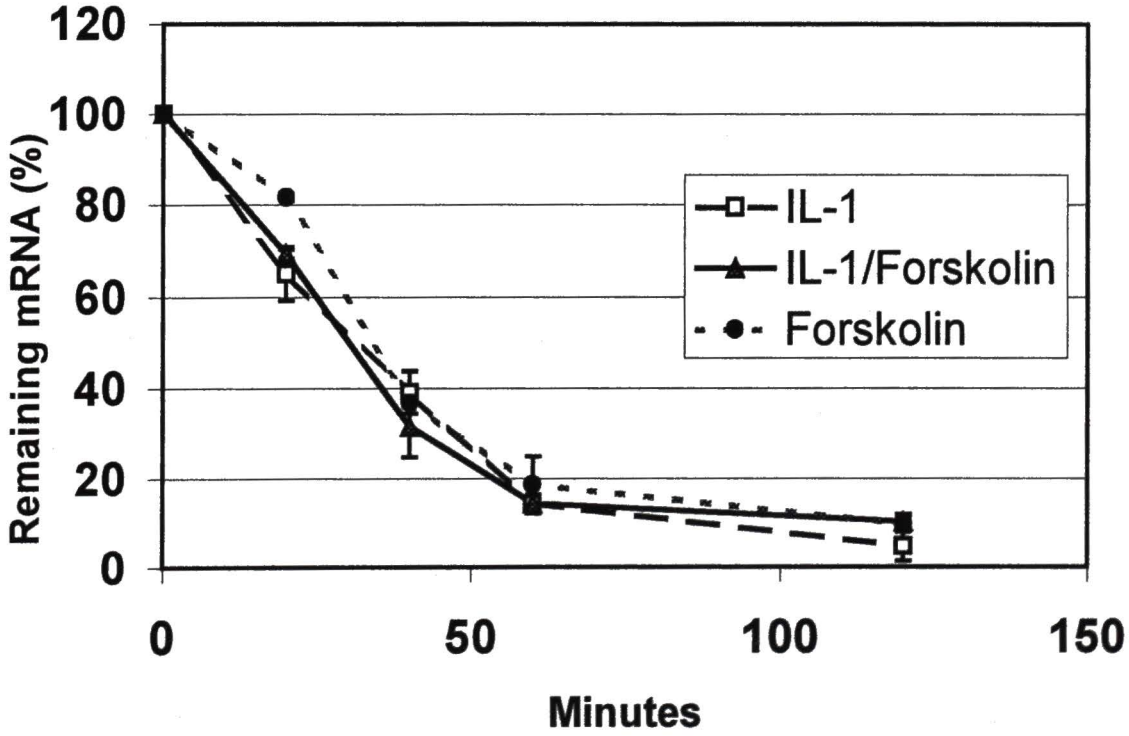
After peak accumulation of IκBα mRNA (60 min when IL-1 was added or 120 min in forskolin alone), 5 μg per ml of actinomycin D was added to inhibit RNA synthesis.

**Figure 6. Forskolin did not stabilize IκB mRNA message.** After MIA PaCa-2 cells were treated with IL-1 with/without forskolin pre-incubation for 1 h, cells were harvested at 20, 40, 60 and 120 minutes after 5 μg/ml actinomycin D addition. Total cellular RNA was extracted, northern blots were hybridized with a cDNA probe for IκBα. The chart represent percentage of IκB mRNA signal intensity at specified time points comparing to that before actinomycin D was added (means ± S.E.M. of three independent experiments).

**A.**


min
0
20
40
60
120
IL-1
IL-1/Forsk.
Forskolin
GAPDH

**B.**


120
100
80
60
40
20
0
Remaining mRNA (%)
0
50
100
150
Minutes
IL-1
IL-1/Forskolin
Forskolin

Those peak levels of IκBα mRNA were considered 100%. Then cells were collected to isolate total RNA and assayed for IκBα mRNA by northern blots at 20, 40, 60 and 120 min after RNA synthesis was halted by actinomycin D. The chart (Fig. 6, panel B) represents remaining IκBα mRNA at those time points in percentage of the peak levels achieved before actinomycin D was added. IκBα mRNA decayed rapidly regardless of the treatment that induced the expression. Apparent half-lives of the IκBα mRNA were approximately 30 min. Forskolin either alone or in combination with IL-1 did not stabilize IκBα mRNA messages. Since it did not change the rate of IκBα mRNA decay, these sets of experiments demonstrated the capability of forskolin to induce IκBα mRNA synthesis and in turn up-regulate cytosolic IκBα protein levels.

# DISCUSSION

BAY11-7082 inhibits IκBα phosphorylation and thus avoids the ubiquitin and proteasome-mediated degradation [19]. Similarly, cell permeable proteasome inhibitor II directly interferes with proteasome activity. Acting at consecutively different steps of the same pathway, both inhibitors exhibited similar results in antagonizing NF-κB activation. Both most likely resulted in high level of cytosolic NF-κB-sequestering IκB. Our transfection and EMSA experiments compliment each other to suggest that modulation of IκBα phosphorylation and the increase in availability of cytosolic IκBα protein antagonize transcriptional activation of M-CSF gene in the similar manner to intracellular cAMP elevation.

Parallel resemblance is observed between the way that arsenite blocks TNFα-induced transcription of IL-8 (Roussel et al., 2000) and the way in which cAMP attenuates IL-1 stimulated M-CSF transcription we reported earlier (Kamthong et al., 2000). Arsenite blocks the induced transactivation of NF-κB but not AP-1. Similarly, cAMP antagonizes NF-κB activation without any effect on AP-1. In addition, arsenite has been shown to prevent NF-κB activation by directly blocking IKK activity and inhibiting the inducible phosphorylation and degradation of IκBα. This sprang the question whether cAMP possesses the ability to interfere with activation of NIK/IKK axis. The kinase activity of IKKβ, which is the most downstream protein kinase in this axis, was assessed. The results above indicate that intracellular cAMP elevation does not modulate the activation of NIK/IKK.

At least three ARE-like repeats, which normally subject immediate-early mRNAs to rapid degradation, are present in the 3' untranslated region of IκB (MAD-3) RNA (Haskill et al., 1991). Many short-lived mRNAs associated with transcription and inflammation possess several repetitive AU-rich sequence elements (AREs) in their 3' noncoding region (Gillis and Malter, 1991). Stabilization of these molecules predestined to be rapid decaying is a possible way to up-regulate their expressions. But it is shown above that forskolin did not affect IκBα mRNA decaying rates, more likely it induced IκBα transcription to increase the availability of the IκBα mRNAs.

The presence of several NF-κB binding sites in the promoter region of IκBα gene (Siebenist et al., 1994) likely leads to speculation that any stimuli that activate NF-κB would inevitably induce IκBα mRNA synthesis. The fact that transcriptional rate of IκBα was increased at least threefold within 30 min after LPS was added to adherent human donor monocytes (Lofquist et al., 1995) supports this notion, more or less similar to what we observed here. Rapid induction of its own inhibitor IκBα mRNA by NF-κB, futile it might sound, is conferred to be the machinery to restore the off signal after NF-κB activation. Enhancement of IκBα mRNA transcription by cAMP could arguably keep the off signal longer, hence renders its ability to antagonize NF-κB activation. Likewise, synthetic glucocorticoid dexamethasone also induces the transcription of IκBα, cuases the newly released NF-κB to quickly reassociate with newly synthesized IκBα, and thus mediates its immunosuppression (Scheinman et al., 1995). Induction of IκB synthesis is just one mechanism among other several other signaling events bestowing modulation at

the level and duration of NF-κB activity, such as phosphorylation of NF-κB, hyperphosphorylation of IKK subunits and processing of NF-κB precursors. This mechanism, commanded by ubiquitous second messenger cAMP through the well regulated cAMP-dependent protein kinase, potentially provides signal transduction cross talks to modulate an NF-κB signaling.

Altogether, we provided evidence suggesting that the attenuation by cAMP in the IL-1 stimulated M-CSF expression is not by the inhibition of IκB kinase activity. But rather by inducing the up-regulation of IκB expression which eventually traps NF-κB in the cytosolic NF-κB/IκB complex and attenuates M-CSF expression.

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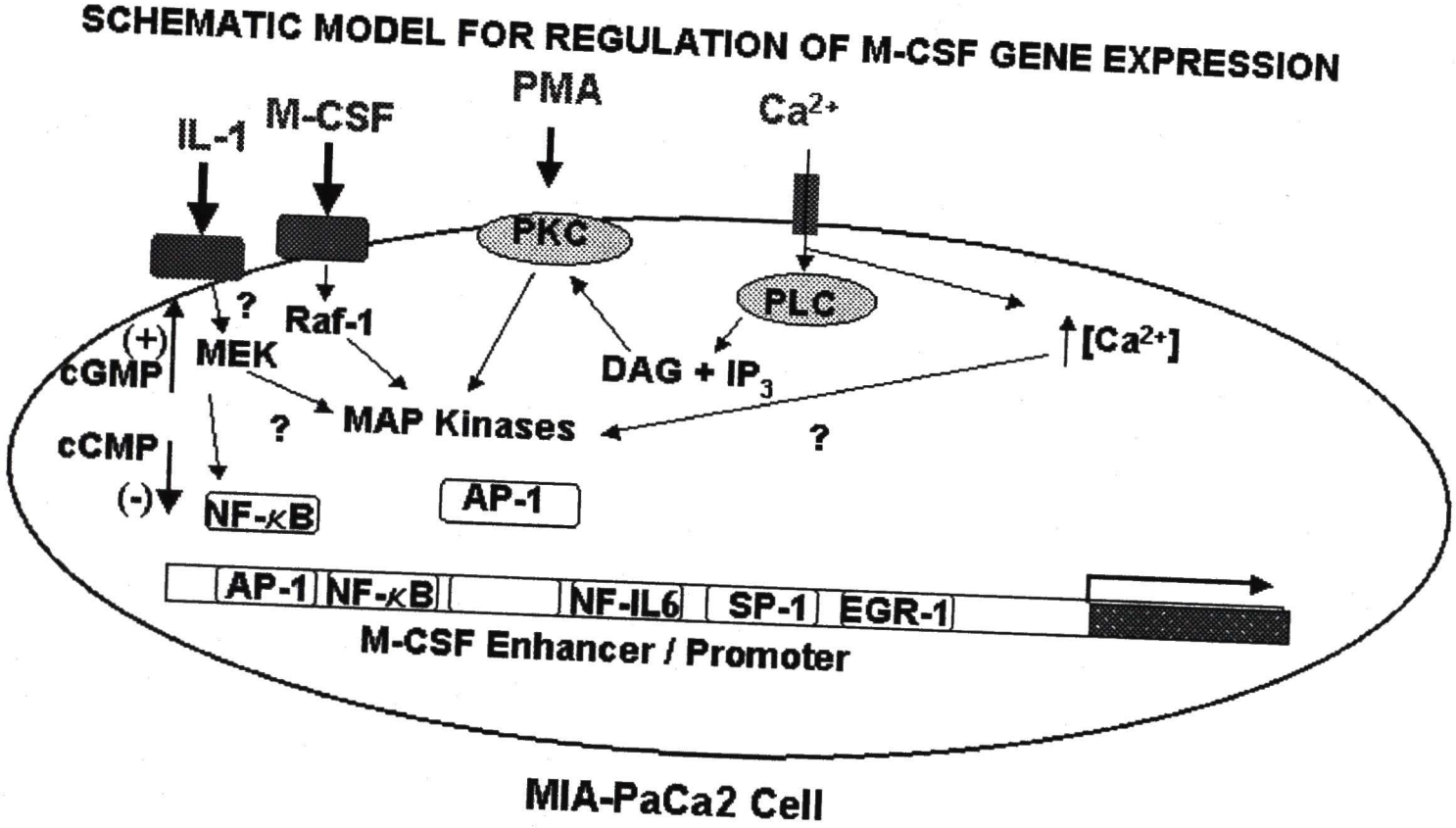
# CHAPTER V

## CONCLUSION AND FUTURE DIRECTION

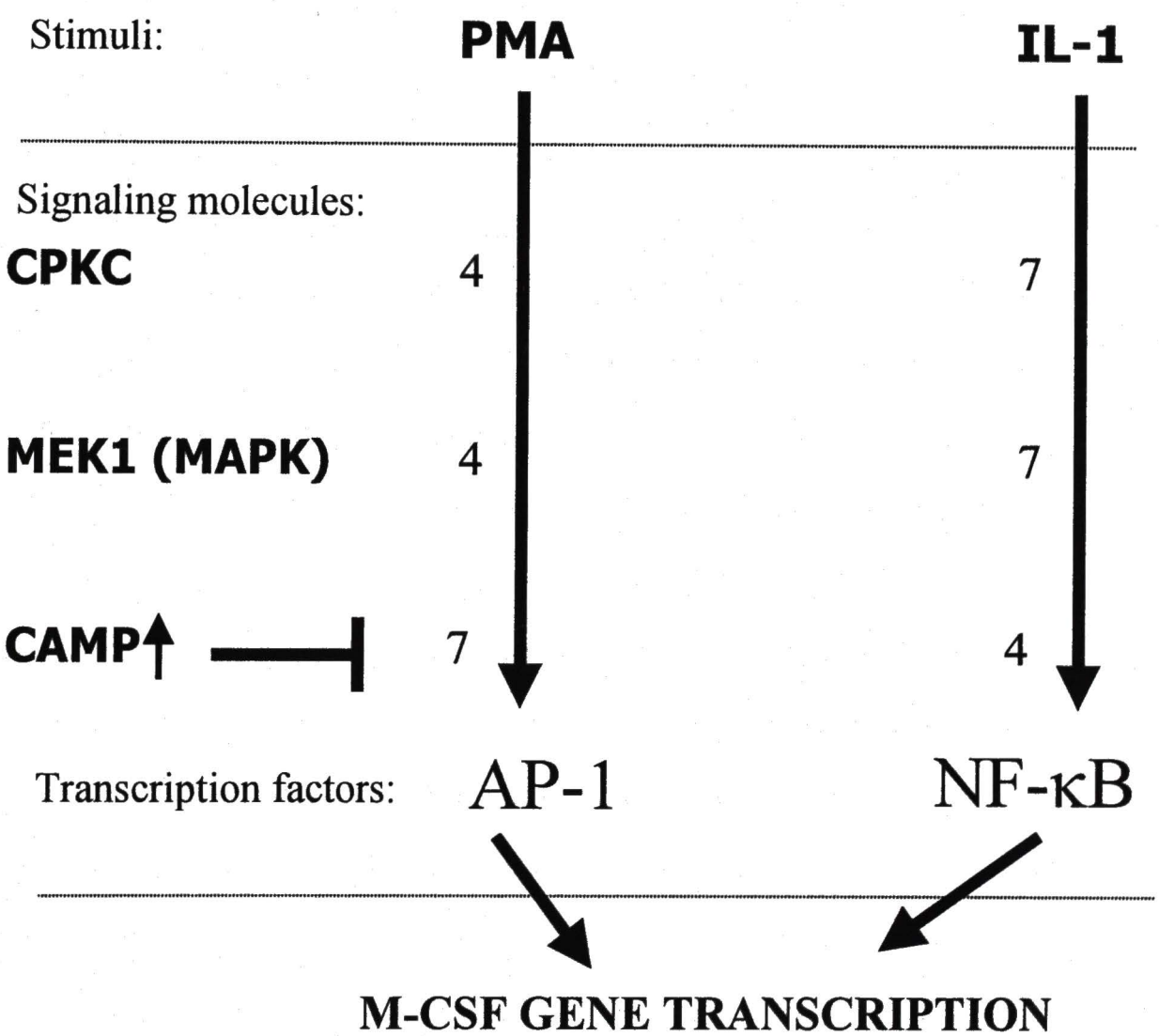
This study focuses on intracellular events regulating the expression of human M-CSF gene in response to the extracellular stimuli. The model depicted in figure 1 summarizes the stimuli previously known to increase human M-CSF gene expression as well as the potential signaling pathways involved. These stimuli may be categorized into two groups: first, growth or proliferative stimuli such as growth factors like M-CSF, GM-CSF; second stress-induced or pro-inflammatory stimuli such as IL-1, IL-6, TNF, and IL-10. Proliferative stimuli are usually associated with the activation of PLC and DAG, which is the reason PMA, an analog of DAG, is commonly used as experimental stimulus to investigate this pathway. This pathway is important in growth, development, and cancer cell proliferation. For the latter group, IL-1 is used as a representative of pro-inflammatory stimuli that are implicated in the development of chronic degenerative diseases. The transcription factors AP-1 and NF-κB have been independently reported to be involved in M-CSF gene expression (Harrington *et al.* 1991, Yamada et al. 1991). Among all of the signaling molecules previously investigated in this lab and others, intracellular elevation of the second messenger cAMP is the only signal demonstrated to negatively affect M-CSF expression, and prior to this study, the mechanism was unknown. For this reason, the investigation into the mechanistic action of cAMP on the activation of AP-1 and NF-κB has been pursued.

**Figure 1. Schematic model showing stimuli and their potential signaling molecules involved in human M- CSF gene transcription.** IL-1, TNF, phorbol ester, endotoxin (LPS), cGMP and calcium/calcium ionophores have previously been shown to stimulate the expression of M-CSF in variety of M-CSF producing cells. Most signaling pathways downstream to the cell surface receptors remained unclear until the recent elucidation of TNF/IL-1 signaling pathways involving the activation of transcription factor NF-κB. The other known central pathway is via MAP kinase cascade activation, which involves the activation of Fos, Jun, and the transcription factor AP-1. Despite the presence of potential transcription binding sites for several transcription factors on the M-CSF gene promoter region, experimental evidence demonstrates only direct involvement of AP-1 and NF-κB.


SCHEMATIC MODEL FOR REGULATION OF M-CSF GENE EXPRESSION
IL-1
M-CSF
PMA
Ca2+
PKC
PLC
?
Raf-1
(+)
cGMP
MEK
DAG + IP3
[Ca2+]
MAP Kinases
?
?
cCMP
(-)
NF-κB
AP-1
AP-1
NF-κB
NF-IL6
SP-1
EGR-1
M-CSF Enhancer / Promoter
MIA-PaCa2 Cell

From the evidence in this investigation, relationship of extracellular stimuli and their main signaling pathways leading to human M-CSF gene transcription in human pancreatic cancer cell line MIA-PaCa-2 is shown in figure 2. The current study revealed that IL-1-mediated human M-CSF gene transcription in MIA PaCa-2 indeed depends on NF-κB activation. The signal transduction pathway of IL-1-induced M-CSF transcription was found not to involve activation of conventional protein kinase C or mitogen-activated protein kinase cascades. This is in contrast to the mechanism employed by PMA to induce human M-CSF which is mediated via the activation of conventional protein kinase C, mitogen-activated protein kinase cascade and AP-1 activation. The finding that PMA does not activate NF-κB in MIA PaCa-2 cell line, and that the inhibitor of IκB phosphorylation -- BAY11-7082 does not affect PMA-induced M-CSF gene transcription, lead to the conclusion that PMA- and IL-1-induced M-CSF transcription are distinct and independent of each other. This finding is rather unique and unexpected. Normally, in prototypic T-cell lymphocytes, either PMA or IL-1 would activate interconnected signaling pathways leading to the activation of both AP-1 and NF-κB (Ninomiya-Tsuji *et al.* 1999). This in turn would cooperatively drive the transcription of target genes. The likely explanation for this observation is that this is cell line-specific property. Aberrant changes in the transformed or cancer cells may contribute to the diversion of these signaling pathways. As M-CSF also functions as a survival signal to the cells, it would be more beneficial for the cancer cells to be able to produce M-CSF upon exposure to one stimulus via an independent signaling pathway instead of having to rely on cooperative signals from more than one stimulus. If this were a common feature

**Figure 2. Diagram summarizing signaling pathways involved in PMA- and IL-1-induced human M-CSF gene transcription in pancreatic cancer cell line MIA PaCa-2.**


Stimuli:
PMA
IL-1
Signaling molecules:
CPKC
4
7
MEK1 (MAPK)
4
7
CAMP↑
7
4
Transcription factors:
AP-1
NF-κB
M-CSF GENE TRANSCRIPTION

of pancreatic cancer cells, raising intracellular cAMP would not be an effective approach to decrease M-CSF expression or retard the cell growth. Whether this property is common for any cancer cells still remains to be investigated. However, the observed sensitivity of IL-1-induced M-CSF expression to intracellular cAMP elevation is a common finding in every cell line investigated in this lab, which include pancreatic cancer cell MIA PaCa-2, human lung fibroblast CCL202, and primary human umbilical vein endothelial cells (Ku 1990). This suggests that cAMP elevation may be the key to hinder unwanted M-CSF expression driven by pro-inflammatory cytokines. Our finding, from the experiment using relative RT-PCR to detect expression of a minor 1.6 kb M-CSF RNA transcript, showing that its expression is more sensitive to the negative effect of cAMP than the major 4.0 kb transcript (Chapter II, p. 45), further supports this idea. The minor M-CSF RNA transcript encodes the membrane-associated M-CSF, which is believed to play a major role in the microenvironment during pathophysiological development of atherosclerosis (Flanagan and Lader 1998). An alternative spliced minor M-CSF RNA transcript lacks of the sequence encoding exon 6 that contains proteolytic cleavage sites, thus, rendering its expression on the cell surface stably (Rettenmier *et al.* 1988). This makes it function in the microenvironment as an autocrine or paracrine signaling molecule for longer period of time.

This also suggests that the mechanism by which intracellular cAMP elevation antagonizes IL-1-induced M-CSF gene transcription involves an induction of IκBα gene expression. The induction of IκBα gene expression has also been reported as a mechanism mediating the inhibition of IL-6 expression caused by agonists of peroxisome

proliferator-activated receptor-α (PPARα) (Delerive *et al.* 2000). This is significant because IL-6, like M-CSF, is a protein believed to have a significant role in promoting chronic inflammation, a hallmark of degenerative diseases such as atherosclerosis. Hence this implies a potential relationship between intracellular cAMP elevation and the activation of PPARα, a protein that belongs to the nuclear receptor superfamily. Direct protein-protein interactions between PPARα and AP-1 and NF-κB proteins have been reported as mechanisms of pro-inflammatory gene transrepression (De Bosscher *et al.* 1999). These interactions could trap the transcription factors or interfere with their DNA-binding ability, thereby, repressing the transcription of the target genes. A future experiments using the cellular transfection of M-CSF promoter/reporter gene, could provide a clue whether PPARα agonists, such as fenofibrate or Wy-14643, has any effect on the human M-CSF gene expression. A member of this group, bezafibrate, was demonstrated to retard the progression of focal coronary atherosclerotic lesions in a clinical trial (Ericsson 1998). This PPARα agonist also induced the expression of IκBα gene. It would therefore be expected to have inhibitory effect on human M-CSF expression. If this relationship could be substantiated, these drugs may provide benefit in prevention of the diseases related to deleterious effect of M-CSF.

The finding that IκBα mRNA appeared to be synergistically induced by cAMP elevation in presence of IL-1 (Chapter IV, Fig. 5) is further similar to the outcomes of fibrate experiments. Basal NF-κB binding activity present in vascular smooth muscle cells was not increased by PPARα agonist, but conversely was lowered (Delerive *et al.* 2000). This observation is also similar to the observation from our experiments with

cAMP treatment that there was no change in basal NF-κB activity when MIA PaCa-2 cells were exposed to DB-cAMP or forskolin. These indicate that neither intracellular cAMP elevation nor PPARα activator activates IκBα transcription in an NF-κB – dependent manner. This suggests that PPARα agonist and cAMP use a distinct signaling pathway to regulate IκBα gene induction.

We have demonstrated that intracellular cAMP elevation by either DB-cAMP or forskolin causes the up-regulation of steady-state IκBα mRNA. Forskolin has also been reported to increase the rate of IκBα mRNA transcription in experiments using a nuclear run-on technique to measure the rate of transcription (Mustafa and Olson 1998). However, there is no available information about how cAMP or PPARα agonist directly induces IκBα gene expression. So far, an NF-κB-dependent pathway is the only mechanism demonstrated to up-regulate IκBα gene induction (Le Bail *et al.* 1993). An analysis using a computer-assisted motif search of the sequence of 5'-flanking region of the IκBα gene up to 2 kb nucleotides upstream to the transcription starting site reveals that there is neither a cAMP-response element (CRE) nor a specific binding site for PPARα. Therefore, it is possible that cAMP or PPARα indirectly activates IκBα gene transcription through interaction with other DNA binding proteins, or via the induction of intermediary genes. Hence, a functional analysis of the promoter sequences of the IκBα gene by promoter/reporter gene assays is required to understand this intricate regulation. This assay would identify the promoter/enhancer region of the IκBα gene that is

responsive to cAMP or PPARα activation and, consequently, lead to the identification of their mechanistic partners.

The induction of IκBα gene, however, is only one possible mechanism to antagonize NF-κB –dependent M-CSF transcription. RhoB, a novel Ras-related small GTPase, has been reported to change the basal DNA binding activity of the transcription factor NF-κB in overexpression experiments (Fritz and Kaina 2000). Furthermore, RhoB also prevents a decrease in IκBα protein level after treatment of cells with NF-κB activating stimuli such as ionizing radiation, UV light, and TNFα ( Fritz and Kaina 2001). In the same study, it was suggested that RhoB prevents proteasomal degradation of IκBα. In addition, NF-κB activation was hindered by RhoB overexpression without any consequence on IKK activity (Li and Karin 1998). Impediment of the NF-κB activation, independent of the IKK activity, is similar to our observation that cAMP interfered with NF-κB activation without any effect on IKK activation (Chapter IV, Fig. 4). Moreover, RhoB represses NF-κB signaling without any effect on MAP kinase activation by PMA (Li and Karin 1998). Previously, other members of the Rho family GTPases RhoA, Rac and Cdc42 were shown to activate NF-κB via stimulation of IκBα phosphorylation (Perona *et al.* 1997). This suggests that multiple Rho-regulating pathways may be involved in NF-κB –dependent M-CSF transcription. Up-regulation of RhoB expression is a probable alternate mechanism to antagonize NF-κB –dependent M-CSF transcription. It would be of interest in light of these observations to ascertain

whether there occurs a mechanistic interaction between intracellular cAMP elevation and RhoB.

In this investigation, PMA-induced M-CSF transcription was sensitive to the PKC inhibitor Gö 6850, indicating the involvement of either classical PKC (α, βI, βII, γ) or novel PKC (δ, ε). Given that different PKC isoforms may serve diverse functional roles in cells depending on their distinct regulatory properties and tissue distribution, further investigation into which PKC isoform plays major role in M-CSF transcription is necessary. Temporal changes in cellular levels of PKC isoforms when the cells are treated with specific stimuli can be established using isoform-specific antibodies in Western blots. PKC isoform-specific peptide inhibitor or anti-sense oligodeoxynucleotides may be utilized in promoter/reporter gene assays as performed in this investigation to selectively deplete the individual PKC isoform, thus, identifying whether and how it functions in M-CSF transcription.

M-CSF activates osteoclastogenesis and causes the loss of bone matrix. It also enhances the life span of macrophage-derived foam cells so that their continued presence becomes deleterious to the blood vessel wall (Flanagan and Lader 1998). Unwanted expression seems to be the cause of diseases related to M-CSF expression, such as osteoporosis and atherosclerosis. The work of this dissertation demonstrates that induction of IκBα gene is a mechanism by which intracellular cAMP elevation attenuates NF-κB activation, and exerts its negative effect on M-CSF gene expression. Thus, it substantiates the ability of cAMP to down-regulate M-CSF transcription. Several findings in this dissertation also lead to future directions to elucidate the fundamental

knowledge of pathophysiologic states linked to abnormal or unwanted M-CSF gene expression. The complete understanding of this mechanism is anticipated to reveal novel strategies to interrupt M-CSF expression and potential therapeutic strategies on the treatment of these diseases.

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