Colony Stimulating Factors: Regulation of Production *

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Introduction

Life span of mature blood cells is ephemeral, requiring hematopoiesis throughout life. A complex network of hematopoietic progenitor cells and cytokines maintain an enormous daily production of granulocytes, monocytes, erythrocytes, platelets, and lymphocytes. This population of hematopoietic cells must be able to respond rapidly to changing needs such as bleeding, infections, cancer, or exposure to cytotoxic agents. Colony stimulating factors (CSFs) are a family of glycoproteins that promote growth and differentiation of hematopoietic progenitor cells and also enhance the function of the mature blood cells. production of CSFs is under tight control since either their over- or underproduction will result in dysregulation of hematopoiesis. Proliferation of hematopoietic progenitor cells require the continuous presence of these factors.

A variety of cells including nonhematopoietic cells such as fibroblasts, endothelial cells, and smooth muscle cells are capable of producing many kinds of CSFs. This chapter will describe cells that make CSFs and the mechanisms involved in this production.

Mesenchymal Cells

Mesenchymal cells originate from either mesoderm or ectoderm. Three major cells that compose the mesenchymal tissues include fibroblasts, vascular endothelial cells, and smooth muscle cells. Fibroblasts provide the scaffolding required for cellular organization; this extracellular matrix is required for tissue cohesion [39, 40]. These cells are not functionally effete; they produce CSFs and a variety of cytokines. They play a major role in the response to tissue injury, being the primary cells involved in tissue repair. These cells respond to interleukin-1 (IL-1) and tumor necrosis factor (TNF) by proliferation, synthesis, and assembly of collagen [18]. Fibroblasts in the bone marrow may function as part of the microenvironment [15, 21].

Pluznik and Sachs originally showed that fibroblasts could produce CSF [36]; somewhat more recently endothelial cells and smooth muscle cells were found to be capable of stimulating granulopoiesis [25, 35]. Studies have not shown definitely that, in vivo, these cells constitutively make CSF. Our preliminary studies suggest that embryonic and adult lung fibroblasts produce very low amounts. Sustained myelopoiesis in long-term culture of bone marrow cells requires the presence of stromal cells composed of a complex network of cell types, including fibroblasts and endothelial cells [16].

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These cells produce low levels of hematopoietic growth factors.

Physiological Stimulators of CSF Production

Bagby et al. [4, 5] initially noted that macrophages exposed to lipopolysaccharide produced factors that stimulated both endothelial cells and fibroblasts to produce CSFs. Several years later, we [31] found that TNF-a, one of the products of macrophages, was able to stimulate fibroblasts, endothelial cells, and smooth muscle cells to produce CSFs. At the same time, IL-1 was noted to increase synthesis of CSFs in the same cells [6, 8, 27, 45]. Further studies have shown that mesenchymal cells cultured with either TNF or IL-1b produced macrophage-CSF (M-CSF) [1, 24], as well as IL-1b and IL-6 [2, 28, 42]. We have noted that messenger ribonucleic acid (mRNA) for each of these growth factors is produced in a coordinate fashion after mesenchymal cells are stimulated by either TNF or IL-1b. Lymphotoxin, which is produced by activated lymphocytes and has peptide homology to TNF, can also stimulate mesenchymal cells to produce CSFs, although the potency of this cytokine may be less than TNF (Fig. 1) [1, 9].

TNF-a and IL-1b are made in abundant amounts by activated macrophages [17, 34]; lymphotoxin is mostly synthesized by activated lymphocytes. A number of conditions, including bacterial invasion, are known to stimulate these cells to synthesize TNF, IL-1, and lymphotoxin, which can enhance CSF production by mesenchymal cells. This inter-

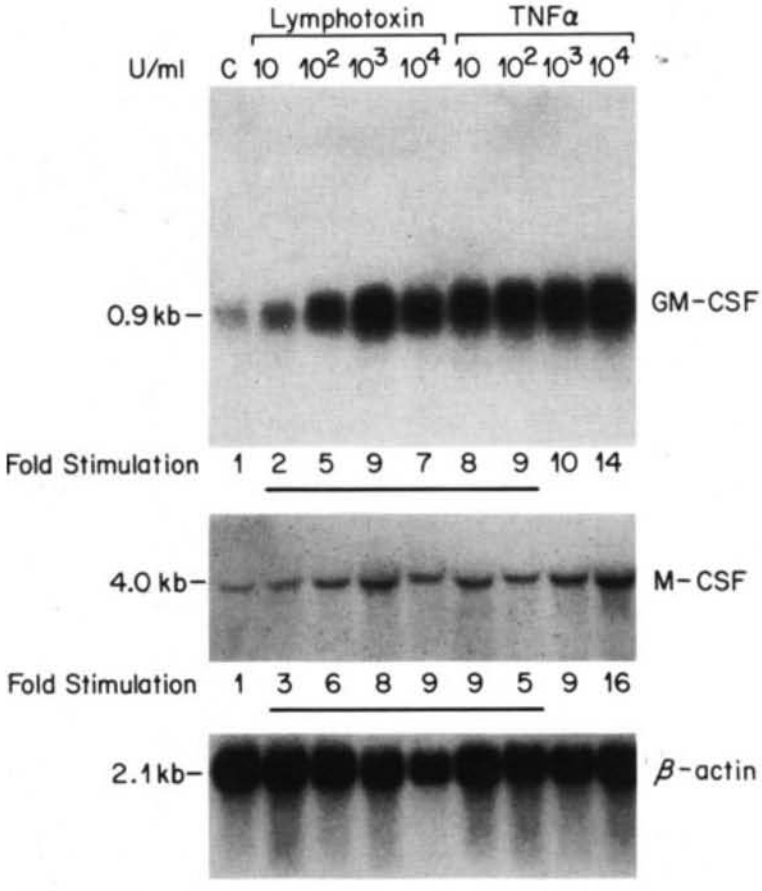

Fig. 1. Dose-dependent effect of lymphotoxin and TNF on levels of GM- and M-CSF mRNAs in human lung fibroblasts. Fibroblasts were cultured with lymphotoxin or TNF for 8 h. Cytoplasmic RNA (15 μg per lane) was prepared and analyzed by formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane. Hybridization was with ^{32}P-labeled GM-CSF and M-CSF cDNA and β-actin DNA. Fold stimulation of levels of CSF mRNAs as compared to levels in untreated cells was equalized for levels of β-actin

communication of cells results in a cascade of synthesis of cytokine in the region of inflammation, such as sites of bacterial and viral infections, rheumatoid arthritis, and some collagen vascular disorders. Steady state hematopoiesis in the bone marrow perhaps is in part regulated by the constant, short-range production of cytokines synthesized by mesenchymal cells, macrophages, and lymphocytes.

CSF: Regulation of Synthesis

Mesenchymal cells have detectable levels of CSF mRNA within 30–60 min of exposure to TNF [27]. This stimulation by TNF can occur in the absence of new protein synthesis [27]. Previous studies have shown that protein kinase C activators can increase accumulation of CSFs in fibroblasts (Fig. 2) [1, 27]. Depletion of protein kinase C activity by prolonged exposure of fibroblasts to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) blocks the accumulation of GM-CSF RNA by TPA but does not affect the accumulation of this RNA induced by TNF [41]. This result suggests that the effect of TNF is independent from protein kinase C activation.

Further studies of TNF showed that it can cause the alkalinization of mesenchymal cell; this probably occurs through stimulation of the Na^+/H^+ antiporter [43]. Amiloride blocks this alkalinization, but does not block accumulation of GM-CSF mRNA. Taken together, our experi-

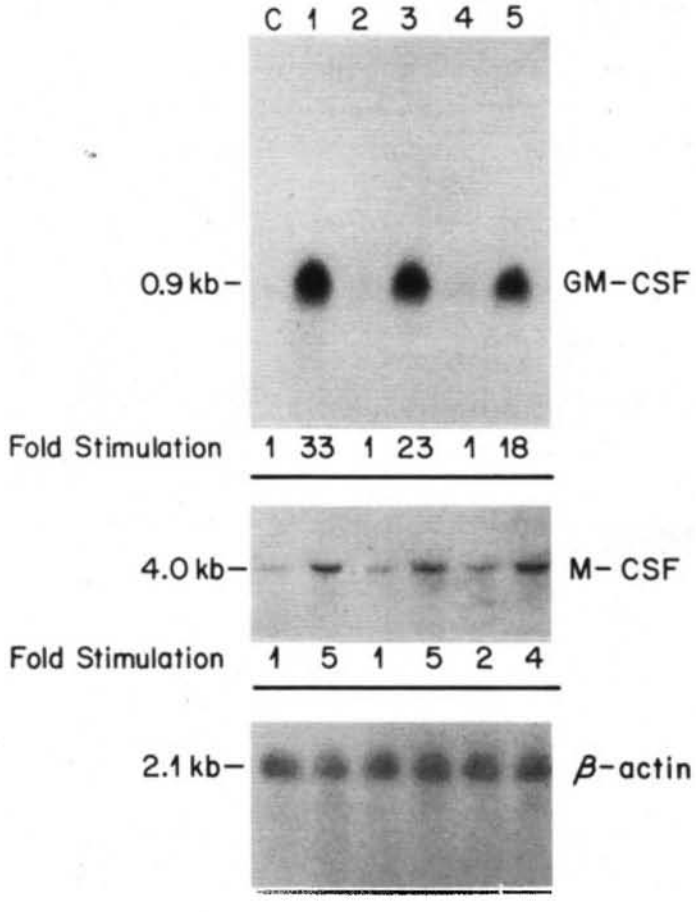

Fig. 2. Effect of various derivatives of phorbol ester on expression of GM- and M-CSF studied by northern analysis. TPA (*lane 1*), PDD (*lane 3*), and PDB (*lane 5*) are phorbol esters that are potent activators of protein kinase C; their derivatives, 4-*O*-methyl TPA (*lane 2*) and 4-alpha-PDD (*lane 4*) are unable to activate protein kinase C. Fibroblasts were exposed to each compound (50 nM) for 2 h and levels for CSFs mRNA were measured. Fold stimulation of levels of mRNA as compared to levels in untreated cells was calculated as described in Fig. 1. Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; PDD, phorbol 12,13-didecanoate; PDB, phorbol 12,13-dibutyrate

ments provide strong evidence that TNF does not mediate its action through either PKC or Na^+/H^+ antiporter.

Using an array of agonist and antagonist, we found that those agents that increase levels of intracellular Ca^{2+} and K^+ also increase levels of CSF mRNA [43]. Increase of K^+ levels may stimulate the Ca^{2+}/K^+ pump causing increased levels of cytosolic Ca^{2+}. In addition, we found that NaF in the presence of Al^{3+} is a potent stimulator of levels of CSF mRNA. This stimulation cannot be blocked by pertussis toxin suggesting that NaF/Al^{3+} may be enhancing the activity of G-binding proteins that are insensitive to the action of pertussis toxin. This observation is consistent with preliminary data suggesting that transformation of mesenchymal cells by transfection of activated H-*ras* can lead to their increased expression of GM-CSF mRNA.

Some tumors are able to synthesize CSF constitutively and patients with these tumors often have peripheral blood leukocytosis. We examined cell lines from tumors that produced CSFs; these tumors were associated with leukocytosis in the patients (H. Ross and H. P. Koeffler, in preparation). Cells of each expressed high levels GM-, G-, and M-CSF mRNAs as well as IL-1 and IL-6 mRNAs. Furthermore, the stability of mRNA coding for each of these growth factors was 10 to 20-fold greater than that in nontransformed cells. The tumors have well-defined oncogene alterations that may be closely associated with inappropriate stability of normally transiently expressed genes.

Monocytes/*Macrophages*

Macrophages are pivotal in inflammation and immunity. In the 1970's, monocytes/macrophages were found to produce CSF [12, 20]. Further studies have found that human monocytes/macrophages from many tissues produce predominantly G- and M-CSF, as well as IL-1, IL-6, and TNF, but synthesize very little GM-CSF. However, other studies found that human monocytes/macrophages accumulate GM-CSF when exposed to lipopolysaccharide, fetal calf serum, or thioglycollate, or when cells phagocytose and adhere in the presence of fibronectin [38]. Resting macrophages produce little CSF, but their synthesis of CSF markedly increase with activation after exposure to a variety of physiologically relevant agents including TNF, interferon-r (IFN-r), GM-CSF, IL-3, IL-1, and endotoxin. Besides producing M-CSF, IL-1, and TNF, these cells have receptors for cytokines, suggesting that under certain circumstances these cells might develop an autocrine stimulation which might foster inflammation. This inflammation may be either salutary (e.g., bacterial infections) or detrimental (e.g., rheumatoid arthritis). Nuclear run-on transcription assay and half-life studies showed that the induction of G- and M-CSF genes is due to mRNA stabilization [19].

Granulocytes

Granulocytes share a number of common properties with monocytes, including phagocytic activity, similar membrane receptors, and a common progenitor cell. They are relatively short-lived, nondividing cells, which often are considered to have little biosynthetic capacity. However, as early as 1948, granulocytes were known to release endogenous pyrogen [7]. Recent studies have demonstrated that granulocytes can be induced to accumulate mRNA coding for G- and M-CSF, IL-1, and TNF after exposure to GM-CSF [30]. These cells also produce a number of other proteins such as plasminogen activator [22], FOS [23], and IL-1 [29]. These findings suggest that neutrophils may be involved in the regulation of hematopoietic growth factors.

Lymphocytes

Cline and Golde [13] first showed that human lymphocytes in vitro produce sig-

nificant CSF; these cells especially synthesize large amounts when stimulated with lectin or antigenic stimulation. CSF can be synthesized by both $CD4^+$ and $CD8^+$ lymphocytes; the former are the most potent producers of cytokines. T lymphocytes can produce all the interleukins and GM-CSF. The cells lack the ability to secrete G- and M-CSF suggesting that transregulatory proteins may be different in mesenchymal cells and T lymphocytes, and those that regulate G- and M-CSF production possibly are different from those that control GM-CSF. A recent study showed that mRNA for M-CSF can accumulate in natural killer cells stimulated with IL-2 and CD16 ligands [14]. Only T lymphocytes secrete IL-3 in the human system [44].

The in vivo importance of production of CSFs by T lymphocytes is unclear. They are present in small but significant numbers in bone marrow, allowing them to interact closely with hematopoietic progenitor cells by releasing growth factors. A role of lymphocytes in regulating normal hematopoiesis is indirectly suggested by alternation of hematopoiesis with alternation of subsets of lymphocytes. Nevertheless, children with congenital deficiencies of T lymphocytes appear to have fairly normal myeloid hematopoiesis, suggesting that other sources of CSFs can compensate for a lack of T lymphocytes.

Comparison of Production of CSF by Mesenchymal Cells, T Lymphocytes, and Macrophages

In the resting state, both mesenchymal cells and macrophages transcribe cytokines, but do not accumulate these mRNAs (Table 1). With stimulation, cytokine mRNA accumulates in macrophages and mesenchymal cells as well as in T lymphocytes. Maximal mRNA accumulation occurs after 2–8 h of stimulation in all three cell types. The constellation of cytokines produced by each of these cells differs. For example, G- and M-CSF mRNA can be synthesized by mesenchymal cells and macrophage, but not by T lymphocytes; GM-CSF mRNA is produced predominantly by T lymphocytes and mesenchymal cells, but little is synthesized by human macrophages. Many of the same signals of CSF production are operative in two or three of the cell types including IL-1, TNF, agents that increase intracellular calcium levels, endotoxin, and stimulators of protein kinase C. T lymphocytes are unique for several reasons. Studies suggest that they require two signals for CSF production instead of one, such as lectin plus phorbol ester, or calcium ionophore plus phorbol ester. In contrast, only one is probably required for macrophages and mesenchymal cells. T lymphocytes are also unique in another manner; these cells can be stimulated by special antigens, in the presence of an antigen presenting cell, to produce CSFs.

CSF: Regulation of Gene

We constructed a promoter-reporter gene construct containing various regions of the GM-CSF gene 5' to the start site of transcription. These were transfected into fibroblasts and stimulated with either TNF or IL-1. These constructs showed no enhancement of reporter-gene activity. In contrast, protein kinase C activators markedly increased levels of the reporter-gene [33]. These are consistent with our notion that TNF and IL-1 do not have a major effect on transcription of CSF but modulate post-transcriptionally levels of CSFs. On the other hand, protein kinase C activators stimulate both transcription as well as stabilization of these CSF mRNAs in each of the cell types. Promoter sequences encompassed by -53 to the start site for transcription of the GM-CSF gene are required to stimulate transcription by protein kinase C activators in mensenchymal cells and lymphocytes [11].

RNA of most cytokines including GM-CSF have a short half-life. Stabilization

Table 1. Regulation of CSF

Variables	Mesenchymal cells	T lymphocytes	Macrophages
CSF produced in activated state	G-, M-, GM-CSF, IL-1, IL-6	GM-CSF, All interleukins	G-, M-CSF
CSF produced in resting state	Transcription, little accumulation	No transcription	Transcription, little accumulation
Maximal level of CSF mRNA after activation	4–8 h	4–8 h	2–8 h
Mechanism of enhanced accumulation of CSF mRNA			
Stimulator			
GM-CSF, IL-3	–	–	Stabilization
TNF, IL-1	Stabilization	–	Stabilization
PKC stimulator		Increased stability and increased transcription	
GM-CSF mRNA half-life			
Resting	< 0.25 h	–	?
Activated (TNF or mitogen)	0.6 h	0.6 h	?
TPA or CHX	> 4 h	> 2 h	?
Signal pathway for CSF synthesis	One signal	Two signal	?
Signals	TNF, IL-1	IL-1	TNF, IL-1
	PKC	PKC	PKC
	Ca^{2+}	Ca^{2+}	?
	NaF	?	?
		Specific antigens	
Critical region for GM-CSF expression	DNA sequences from −53 to +1		

CHX: cycloheximide; *PKC:* protein kinase C.

of short-lived mRNAs play a pivotal role in the accumulation of cytokines in each of the cell types. AU-rich sequences have been found in the 3' untranslated regions of most of the genes coding for these short-lived cytokines and oncogenes [10, 37]. We found that TNF, IL-1 b, phorbol diesters, NaF, and cycloheximide enhance expression of GM-CSF RNA through stabilization (Fig. 3) [1, 27, 41]. We transfected into fibroblasts constructs containing an AT-rich sequence from GM-CSF gene placed into the 3' untranslated region of the reported gene [3]. These transfected cells were stimulated with TPA, cycloheximide, NaF, TNF, or IL-1 b. TPA, NaF, and CHX required an AT-rich sequence for stabilization of the reporter gene. On the other hand, a reporter gene containing the AT-rich sequence does not respond to either TNF or IL-1 b. These experiments suggest that TNF and IL-1 b stabilize GM-CSF RNA independent of these AU sequences and that different mechanisms are used by various agents to stabilize GM-CSF RNA.

Conclusion

Hematopoietic cells are produced and destroyed continuously under precise control. This chapter begins to illustrate

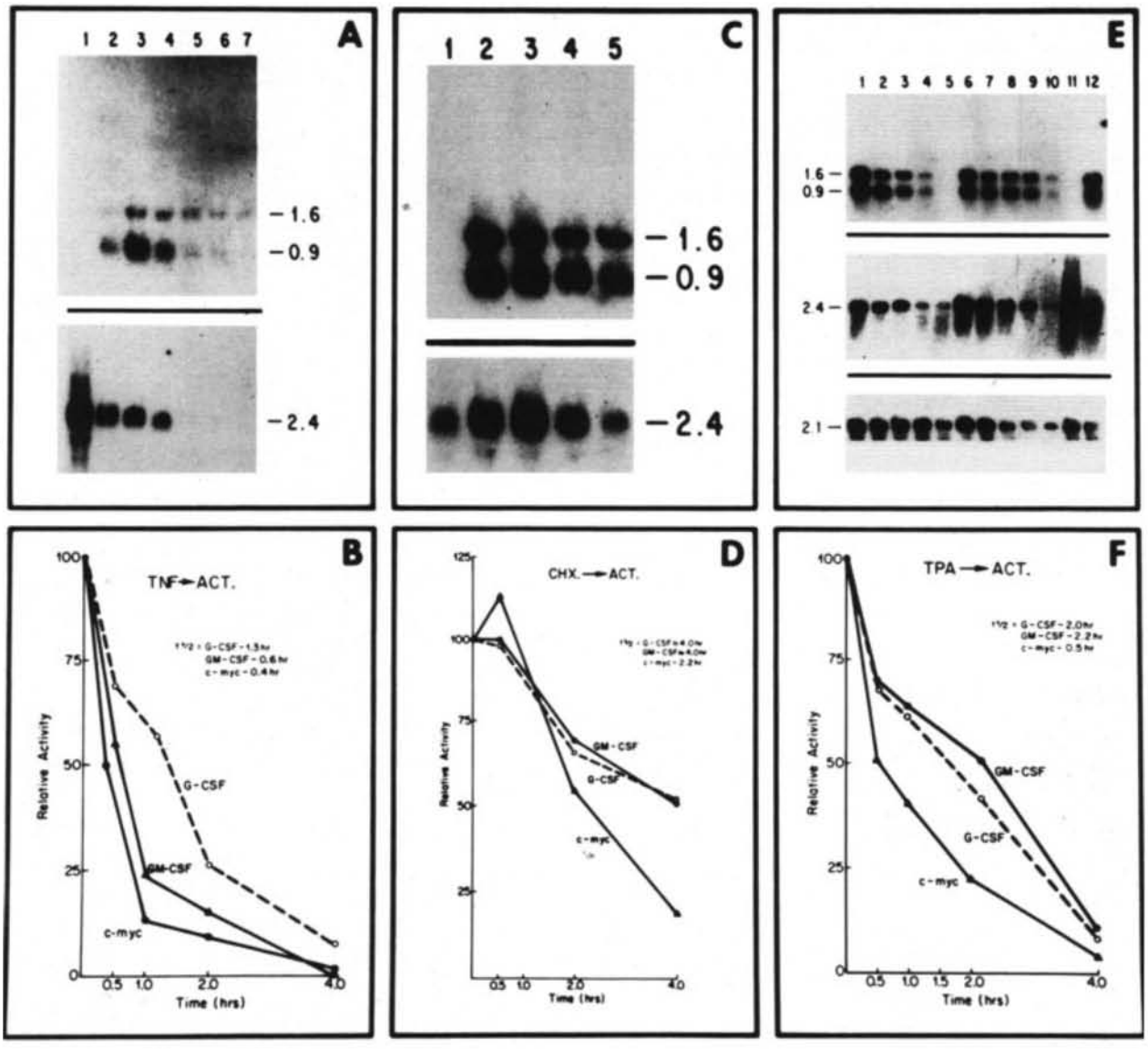

Fig. 3A–F. Stability of steady state of G- and GM-CSF and *c-myc* mRNA in fibroblasts and ability of TNF and TPA to stimulate accumulation of these mRNAs in absence of protein synthesis. **A, B** Stability of steady state of G- and GM-CSF and *c-myc* mRNA in fibroblasts exposed to TNF. Cells were cultured with TNF (25 ng/ml per 4 h) and then actinomycin D (ACT) (5 μg/ml) was added to the culture for various durations. Total RNA (10 μg per lane) was analyzed by RNA blotting. Hybridization was with GM-CSF cDNA (0.9 kb band of hybridization) and the G-CSF oligonucleotide (1.6 kb band) and with the *c-myc* (exon II) probe (2.4 kb). Lanes: *1*, HL-60 (negative control); *2*, control fibroblasts; *3*, fibroblasts exposed to TNF alone;*4*, *5*, *6*, and *7*, cells exposed to TNF and also to ACT for 0.5, 1, 2, and 4 h, respectively. Intensity of hybridization was determined by densitometory. **C, D** Stability of steady state of G- and GM-CSF and *c-myc* mRNA in fibroblasts exposed to CHX. Cells were cultured with CHX (20 μg/ml per 4 h) and then ACT was also added for various durations. RNA was analyzed and intensity of hybridization was determined by densitometory as described in **A** and **B**. Lanes: *1*, control fibroblasts; *2*, cells exposed to CHX alone; *3*, *4*, and *5*, CHX and ACT for 1, 2, and 4 h, respectively. **E, F** Ability of TNF and TPA to stimulate accumulation of G- and GM-CSF and *c-myc* mRNAs in absence of protein synthesis, and stability of steady state of these mRNAs in fibroblasts exposed to TPA. Lanes: *1*, pretreated with CHX (0.5 h) and then cultured with CHX and TPA for 4 h; *2*, pretreated with CHX (0.5 h) and then cultured with CHX and TNF for 4 h; *3*, CHX (4.5 h); *4*, TNF (4.5 h); *5*, untreated control cells; *6*, TPA (50 nM, 4 h); *7* to *10*, TPA (50 n*M*, 4 h) and ACT for 0.5, 1.0, 2.0, and 4 h, respectively; *11*, HL-60 cells; *12*, Lu-CSF-1 (positive control). Abbreviations: *CHX*: cycloheximide; *TPA*: 12-*O*-tetradecanoylphorbol 13-acetate

the complexity of cytokine-mediated communication pathways in induction of hematopoietic growth factors. Regulation of induction of hematopoietic growth factors reflects an integrated network of bioregulator molecules. Some act directly on the hematopoietic progenitor cells; other affect the accessory cell; and some have both direct and indirect affects on the hematopoietic cells. Many hematopoietic growth factor genes have been cloned and their products have been expressed in mammalian cells. Use of these clones has provided the opportunity to evaluate the regulation of expression of CSFs and the evaluation of their affects on target cells.

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