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COMMENTARY

HEMOPOIETIC COLONY STIMULATING FACTORS

A PHYSIOLOGICAL AND PHARMACOLOGICAL ROLE IN FIGHTING INFECTION?

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The hemopoietic system generates more than 120 million new myeloid cells per minute in the adult. This massive cell turnover can be greatly modified by microbial infection of the animal. Interest in the factors that may account for the regulation of hemopoiesis led to the study of colony stimulating factors (CSFs). The observation that certain culture fluids caused bone marrow cells to form colonies in soft agar yielded both assays for and sources of the factors [1, 2]. What became apparent was that the variation in types and numbers of colonies formed depended on the source of the CSF. Colonies were either mixtures of blood cell types (erythrocytes, E; neutrophils, N; monocytes, M; and eosinophils, E_o) or predominantly one of these cell types. Isolation and purification of the factors which gave rise to these different colony types proceeded slowly due to their very low concentration in all known sources and the frequent presence of more than a single CSF.

The identities of four CSFs effecting growth of myeloid colonies from bone marrow cultures were established before the application of molecular cloning techniques [reviewed in Ref. 3]. All were shown to be relatively small glycoproteins of high specific biological activity. Multi-CSF (or interleukin-3, IL-3), GM-CSF, G-CSF, and M-CSF (or CSF-1) were characterized by their abilities to produce from bone marrow cultures colonies predominantly of the types implied by their names. The CSFs demonstrated an apparent hierarchy in being able to stimulate either the lineages of several myeloid cells or, selectively, a single lineage *in vitro*. The progenitor of all the lineages is believed to be a pluripotent stem cell. Normally found in small numbers in the bone marrow cell population, the stem cell is capable of reconstituting hemopoiesis in a lethally irradiated animal. However, neither singly nor in combination do the CSFs support the growth *in vitro* of a multipotential stem cell [reviewed in Refs. 4 and 5]. The model which evolved from these findings suggests that the CSFs are *in vivo* modifiers of hempoietic cell growth and differentiation.

Isolation of genes encoding the CSFs

The low concentration of the CSFs in native sources made the powerful gene isolation and protein production techniques of recombinant DNA technology attractive. The interest that the factors generated in their therapeutic potential to modulate myeloid cell growth mandated the rapid application of the technology. The result was that, in a period of less than 4 years, reports of the isolation of genes that encode each of the four CSFs from both mice and humans appeared (Table 1). The availability of complete sequence information for the different factors revealed both expected and unexpected features of the molecules.

The degree of sequence homology observed between murine and human CSFs reflects their abilities to act across species. Low sequence homology between murine and human GM-CSF is consistent with the inability of the proteins to generate *in vitro* bone marrow colonies with cells from the opposite species. Nonetheless, both molecules appear to generate nearly the same spectrum of myeloid colonies *in vitro* with cells from their own species [6, 7]. Greater homology is found at the level of deduced amino acid sequence between the human and murine

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Table 1. Molecularly cloned and characterized murine and human CSFs

Name	Protein encoded*		Homology between murine and human proteins (%)	Activity of human CSF on murine cells
	Murine	Human		
Multi-CSF (IL-3)	166 (134)	152 (133)	29	–
GM-CSF	144 (127)	141 (124)	50	–
G-CSF	208 (178)	204 (174)	72	+
M-CSF (CSF-1)	552 (520)	554 (522) [256 (224)]	70	+

* Number of residues encoded (residues remaining after signal peptide removal).

proteins for both M-CSF and G-CSF (Table 1). The high degree of similarity between murine and human M-CSF and G-CSF is consistent with the ability of the human proteins to act on murine cells [8, 9].

Despite the homologies that can be found between the same CSF protein from different species, nearly none are apparent between different CSFs. Their common functions as hemopoietic growth factors is not reflected in a common structural sequence. Rather, the CSFs appear to have divergent ancestral genes suggesting different functions. In contrast to the structural coding sequences, regulatory elements for GM-CSF and other lymphokines may be common. For example, untranslated 3′ sequences which appear responsible for the short half-life of GM-CSF mRNA [10] and 5′ genomic sequences regulating transcription initiation [11] are common to other lymphokines [12, 13].

M-CSF

Molecular characterization of M-CSF revealed unexpected additional differences between it and the other CSFs. Previously, the homodimeric structure of M-CSF, unique compared to the other CSFs, had been shown [14]. However, the isolation of the human gene encoding M-CSF showed at least two different mRNAs encoding different sized proteins, one encoding a subset of the larger protein [15, 16]. Furthermore, both of the human plus the murine mRNAs [17] encode larger M-CSF proteins than found extracellularly. The recognition of a putative transmembrane sequence in the proteins encoded by all three messages suggests that M-CSF is cleaved from a membrane bound form [18]. This putative proteolytic processing plus the homodimeric structure of M-CSF set it apart from the other three CSFs. IL-3, GM-CSF and G-CSF are all single chain glycoproteins which show no proform between their primary coding sequence and their extracellular proteins.

Additional significant differences between M-CSF and the other CSFs are the cells which produce M-CSF and its regulation. *In vitro* fibroblasts as well as stromal cell lines produce M-CSF constitutively [19, 20]. In contrast, G-CSF and GM-CSF production is inducible in stromal cells [21, 22]. Macrophage and monocytes also produce G-CSF and GM-CSF when induced [23, 24]. Syntheses of IL-3 and GM-CSF have been demonstrated in stimulated or activated T-cells [25, 26]. IL-3 is believed to be produced exclusively by activated T-cells.

These differences in cell-types producing M-CSF and the constitutive versus inducible nature of its production set it apart from the other CSFs. Furthermore, the recent demonstration of the unique appearance early in embryogenesis of M-CSF [27] as well as its association with placenta suggest a developmental role for M-CSF not expected for the other CSFs. These data imply a unique and as yet poorly defined role for M-CSF, different from that of IL-3, GM-CSF and G-CSF.

Do IL-3, GM-CSF and G-CSF have a role in fighting infection?

Recently, several protein factors involved in B-cell regulation have been molecularly cloned from activated T-cells [28]. These factors demonstrate one mechanism by which activated T-cells play a central role in coordinating immune response using soluble factors. Since IL-3 and GM-CSF are also produced by activated T-cells, a role for these factors in the immune response can be suggested. A model suggesting the interplay of the CSFs and immune cells is shown in Fig. 1. The production of factors which directly or indirectly lead to myeloid cell development as a part of an immune network is illustrated. The model suggests that one result of T-cell activation is the induction of CSFs which, in turn, act on myeloid cell development. Not only do the immune cells produce CSFs in response to microbial antigens, but at least one cell type is also activated by CSFs. The role of macrophages as presenting cells in immune reactions has been reported to be enhanced by GM-CSF treatment [29].

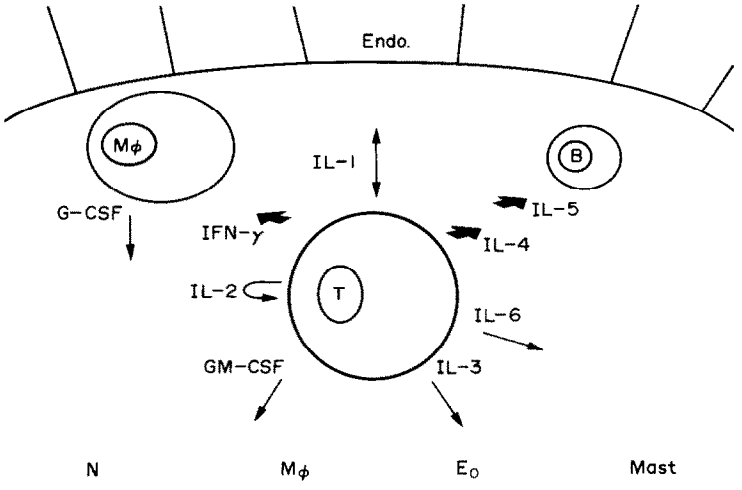

Fig. 1. Schematic model of the interactions between cells involved in fighting infections and the soluble factors that interact with them. Cells: Endo. = endothelial cells, Mϕ = macrophages, T = T-cells, B = B-cells, N = neutrophils, E_0 = eosinophils, and Mast = mast cells. Factors are as described in the text plus IL = interleukin, IFN-γ = gamma interferon.

Considering the increased numbers of macrophages, neutrophils, and/or eosinophils found in animals infected by different micro-organisms, the production of the factors regulating their growth and differentiation would appear necessary following infection. *In vitro* GM-CSF has been shown to inhibit neutrophil mobility and enhance their phagocytosis of bacteria [30, 31]. Circulating G-CSF has been found in animals treated with bacterial endotoxin [32]. In these animals the presence of GM-CSF is more controversial, while IL-3 has not been identified [reviewed in Ref. 33]. The limited demonstration of circulating CSFs may be explained by the local production and utilization of the glycoproteins. Consistent with a local role is the short half-life of less than 10 min for exogenous GM-CSF placed in the circulation [34].

An important role that these observations suggest for IL-3, GM-CSF and G-CSF is to increase locally the number and activation of myeloid cells in response to microbial infection. By recognizing the foreign antigen, T-cells would act as one of the screens to indicate the need for a response. The release of IL-3 and GM-CSF in varying amounts would ensure the generation and activation of the appropriate myeloid cells needed to participate in the killing and clearance of the infecting organism. Other cell types such as endothelial cells respond to secondary mediators secreted by activated macrophages (e.g. IL-1 or TNF) to produce GM-CSF and G-CSF [35]. The model suggests that the induction of CSFs would be part of a network response to microbial infection where production of IL-3, GM-CSF, and G-CSF increases the numbers of, and activates, myeloid cells. This model does not exclude the growth and maturation role for different CSFs in a hierarchy of myeloid lineage development. Rather, it suggests a close link between production of CSFs in response to infection and myeloid cell development. Such a scheme differs from a primary role for IL-3, GM-CSF, and G-CSF in maintaining normal hemopoiesis.

Production of recombinant CSFs for pharmacological evaluation

Following isolation and characterization of the genes encoding CSFs, means have been sought to provide sufficient quantities of their protein products to evaluate their pharmacological potentials. Purification of CSFs from native sources for clinical application has been achieved for M-CSF [36] and G-CSF [37]. However, the extremely low concentrations of the CSFs in most native sources have severely limited this approach for large scale production. Instead, a number of groups have used recombinant technology to produce large quantities of the different CSFs. As an example, recombinant production of GM-CSF has been reported in *Escherichia coli* [7, 38–40], yeast [41–42] and animal cells [44]. While all these organisms produce biologically active GM-CSF, the amounts produced per liter of culture generally decline 10-fold from *E. coli* to yeast and from yeast to animal cells.

In addition to the amount of proteins produced, two other important considerations for recombinant proteins are the state of glycosylation and the amount of native versus denatured protein isolated. The GM-CSF sequence contains signals for glycosylation that result in sugar side chain additions in both animal cells and yeast but not *E. coli*. *A priori*, animal cell glycosylation would be expected to be most like the native modifications. For GM-CSF as for other CSFs, the function of the glycosylation remains unknown. However, the range of both *in vitro* and *in vivo* biological activities of the *E. coli* made and glycosylated GM-CSF are the same [6, 7, 39, 45]. What role the sugar side chains may play in the animal remains unidentified.

A more puzzling result arose when we attempted to determine specific activity of recombinant GM-CSF made in different organisms. This was important in order to estimate the portion of the protein isolated from recombinant *E. coli* having the native conformation. Unexpectedly, the *E. coli* produced GM-CSF had a specific activity 10- to 20-fold greater than the animal cell or yeast produced protein [39, 46]. Furthermore, removal of the sugar side chains of the yeast or animal cell produced GM-CSF resulted in an increase in the specific activity of the previously glycosylated recombinant proteins compared to that of the non-glycosylated GM-CSF. The most likely explanation for these results is the generation of an inappropriate glycosylation on recombinant GM-CSF that interferes with receptor–ligand interaction. However, these differences have not prevented the consideration of potential therapeutic effects of recombinant GM-CSF made in all three cell types.

Clinical application of CSFs

Since CSFs are postulated to modulate myeloid cell development, a logical clinical application is the restoration of myeloid cell numbers in immune-suppressed patients. The testing of this hypothesis in experimental animals and humans is well advanced. Both GM-CSF and G-CSF appear capable of increasing myeloid-cell numbers in normal and drug-induced myeloid-cell suppressed animals [47, 48]. Similarly, patients with suppressed myeloid-cell numbers due to AIDS or drug-induced suppression respond to GM-CSF and G-CSF, respectively, by increasing their numbers of circulating myeloid cells [49, 50]. An additional application of CSFs for myeloid cell stimulation may be in bone marrow transplantations. The period of neutropenia following bone marrow transplants was shown to decrease with GM-CSF treatment [51, 52]. In contrast, human IL-3 in normal primates has shown very little ability to alter myeloid cell numbers. In combination, low concentrations of IL-3 and GM-CSF, however, have been reported to synergistically increase the number of circulating myeloid cells in normal primates [53]. It would appear premature to guess which of the CSFs—or combinations of CSFs—might be most beneficial for the reversal of myeloid suppression due to different causes.

Initially, an important concern in the pharmacological use of the CSFs was the potential for precursor or stem cell depletion as development of end cells increased. Although precursor pools were reduced somewhat in experiments using mice and GM-CSF [45], no evidence for important changes in

bone marrow cellularity has been found in patients treated with GM-CSF. Equally important, no toxic or serious other effects have been reported for dosages of G-CSF or GM-CSF sufficient to reverse myeloid suppression. These results suggest a potentially useful clinical role for GM-CSF and G-CSF in the reversal of myeloid cell suppression whether it is due to disease, drug-induced, or a result of bone marrow transplantation.

Future areas for development

Amongst the CSFs, the application of at least GM-CSF and G-CSF in the clinic appears highly probable. An important remaining question is the amount of protection against infection that the administration of these CSFs will confer. Reports indicating a reduced infection rate due to CSF use have begun to appear. For example GM-CSF has been shown to protect neutropenic mice from microbial infection [54]. If appropriate schedules and dosages for humans can be found to protect against infection as well as prevent myeloid suppression, the successful development of CSFs is highly probable. The generation of an effective and safe medicine from investigations of the regulation of hemopoiesis will stand as a major accomplishment.

The nature, however, of the physiological regulation of normal hemopoiesis remains unclear. The factor(s) which might control normal development and renewal of the stem cell population may well be different than those already known. The role of M-CSF in embryonic hemopoiesis and thereafter is well worth understanding. Once the factor(s) responsible for stem cell renewal is identified, complete regeneration of the hemopoietic system may be possible. In this scheme, a few stem cells taken early in life would be maintained cryogenically for expansion whenever necessary under the influence of the appropriate factor(s). *De novo* regeneration of the hemopoietic system might allow a more aggressive treatment of most cancers and significantly reduce morbidity due to leukemias. Unraveling the means by which normal hemopoiesis is regulated would appear to be the next important task.

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