

# THE MOLECULAR CONTROL OF GRANULOCYTES AND MACROPHAGES

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The clinical importance of granulocytes and macrophages comes from the fact that these cells are essential for resistance to bacterial and fungal infections. It has long been recognized that, if a patient has very low granulocyte levels (less than 1000/ $\mu$ l) either as a result of disease or following chemotherapy, there is an increased risk of severe infections that are often unresponsive to otherwise appropriate antibiotic treatment. It follows from these facts that if agents were available to increase the formation and/or function of granulocytes and macrophages, these should be valuable in the clinical management of patients with neutropenia or depleted monocytes.

Both cell types have a short lifespan and must be produced continuously throughout life by specific ancestral progenitor cells located mainly in the bone marrow. Although these two cell types differ morphologically, many share common progenitor cells so the cells can be presumed to share overlapping functions or to function in coordination with each other.

It is now recognized that the production in the bone marrow of cells of the eight major hemopoietic lineages is regulated by a group of glycoprotein regulatory molecules (now numbering at least 25) that act in concert with specialized stromal cells in the bone marrow microenvironment.

The major regulators of granulocyte and macrophage formation and function are the four glycoprotein colony stimulating factors—G-CSF, GM-CSF, M-CSF and Multi-CSF (IL-3)—although three other regulators—stem cell factor (SCF), interleukin-6 and Flk-ligand—have some capacity to stimulate granulocyte formation, SCF being the most active of these additional molecules.

## **The Development of the CSFs**

The CSFs were discovered as a result of their ability to stimulate—in semisolid cultures—the formation by progenitor cells of colonies of maturing granulocytes and macrophages. With the use of these bioassay systems, the four CSFs were first identified, then purified to homogeneity. However, even the richest tissue source of CSFs contains such low concentrations of CSFs that purification of native CSF is not a practicable source of CSF for clinical use. The size of the polypeptide core of the CSFs in humans ranges from 14,000–30,000 Da, and with the mandatory 4  $\alpha$ -helical structure of these polypeptides, synthesis of biologically-active CSF is also not a realistic possibility. As a consequence, cloning of cDNAs encoding the CSFs became of major importance and this was accomplished between 1983 and 1986. Insertion of these cDNAs into bacterial, yeast or mammalian cell expression systems resulted in satisfactory production of recombinant CSF, which proved to have identical properties to the native CSFs, and recombinant CSFs have been used subsequently for all in vivo and clinical use.

## **Properties of the CSFs In Vitro**

Like the situation with all other known hemopoietic regulators, the actions of the CSFs are not restricted to the cells of a single lineage, although some specialization is evident and is the basis for the prefix used for each CSF. Thus G-CSF is mainly a proliferative stimulus for granulocytic cells but also has actions on stem cells and some monocytes. GM-CSF is an effective proliferative stimulus for granulocytes, monocytes and eosinophils but also has actions on stem cells, dendritic cells and weaker actions on megakaryocytic and erythroid cells. M-CSF is mainly a proliferative stimulus for monocyte-macrophages but has some action on granulocytic cells and a weaker action on stem cells. Multi-CSF is the most active and diverse proliferative stimulus (at least in vitro but less so in vivo), stimulating the proliferation of granulocytes, monocytes, eosinophils, stem cells, erythroid and mast cells and B-lymphocytes.

The actions of the CSFs are mediated by dimeric membrane receptors that are specific for each CSF; most granulocyte-macrophage cells coexpress receptors for all four CSFs. The receptor for M-CSF (the *c-fms* product) is a homodimer of the classical tyrosine kinase type, activating signalling by initial transphosphorylation of its C-terminal tyrosine residues. The receptor for G-CSF is also a homodimer but of the hemopoietic receptor family—receptors lacking tyrosine kinase domains but interacting with JAK kinases and STATS to initiate signalling. The GM-CSF and Multi-CSF receptors are heterodimers comprised of a specific  $\alpha$ -chain and a common  $\beta$ -chain (also shared by the IL-5 receptor). All of these chains are members of the related hemopoietic receptor family, and the  $\beta$ -chain contains the major signalling domains, in particular a Box 1-Box 2 region required to initiate mitotic signalling and a more C-terminal domain necessary to initiate maturation and to maintain cell survival. Hemopoietic cells each express only a few hundred CSF receptors but these are highly efficient and permit CSF actions at pg to ng/ml concentrations.

CSF action is necessary for a granulocyte-macrophage precursor to divide, and there is a concentration-dependent relationship with both the cell cycle time and the total number of progeny produced by a progenitor cell. The molecular control is by activation of cyclin D1, an action resulting in shortening of the G<sub>1</sub> phase of the cell cycle and transit of cells from G<sub>0</sub>/G<sub>1</sub> to the S phase cell cycle.

In addition to CSF actions on cell division, the CSFs also influence differentiation commitment in normal and at least some myeloid leukemic cells, can initiate or enhance maturation induction and enhance the functional activity of mature granulocytes and macrophages. Influencing all these responses is an independent action of the CSFs in maintaining membrane transport integrity and preventing cell death by apoptosis.

All of the above actions are known to be direct actions mediated by specific membrane receptors on the responding cells. However, the actual response is dictated by the gene programs available or able to be activated in the responding cell.

It is characteristic of the biology of all the hemopoietic regulators that combinations of regulators elicit enhanced, commonly superadditive, responses. This feature is also present in responses to CSF combinations and particularly in combinations of stem cell factor with one of the CSFs. The precise mechanisms involved may vary with different combinations but probably include in some instances induced or enhanced receptor expression elicited by one factor for receptors for another or recruitment of

signalling intermediates by one activated receptor that had been rate-limiting for signalling from another activated receptor.

### **Actions of the CSFs In Vivo**

Injection of the CSFs either to animals or humans elicits evident increases in the production and activation of granulocytes and/or monocytes. These responses are sustained for as long as daily injections are continued but decline within 24 to 48 hours of cessation of injections. Although enhanced cell production is the ultimate basis for the increased cell numbers, in the initial phases of a response, a combination of accelerated maturation and release of cells from the marrow is responsible for the earliest changes.

Although the CSFs have short serum half-lives of 1 to 4 hours, adequate responses in humans can be achieved with a single daily subcutaneous injection of 5 to 10  $\mu\text{g}/\text{kg}$ . At this dose level, few adverse responses are encountered with the two most commonly used CSFs—G-CSF and GM-CSF—most often discomfort in marrow-containing bones. With CSFs active in stimulating macrophage function, higher doses can elicit a variety of side effects presumably due to CSF-induced macrophage production of potentially toxic products.

Clinically, CSF-induced responses are usually able to be monitored only in the blood and occasionally in the marrow. Although the qualitative range of cells responding in vivo to a CSF agrees with their known range of actions in vitro, the CSFs vary in the magnitude of the changes they induce, particularly in the peripheral blood; G-CSF elicits the most prominent response. However, in experimental animals, where responses in all tissues can be monitored, it is possible to observe prominent responses to other CSFs. In particular the spleen develops active hemopoietic tissue. The most dramatic example of this is again in response to G-CSF where the spleen becomes the major erythropoietic organ replacing production of erythroid cells in the marrow, which becomes virtually a granulopoietic tissue.

An associated dramatic CSF-induced response is the appearance in the peripheral blood of large numbers of stem and progenitor cells of all lineages. In man, this response peaks at about seven days. Analysis of the cells appearing in the peripheral blood indicates that they are not in cell cycle and are a non-random sample of cells present in marrow. This indicates a selectivity in the release process, but the mechanisms involved have yet to be identified. Harvested peripheral blood cells taken during this period have proved superior to marrow cells in repopulating ability for granulocytic and monocytic cells and greatly superior in enhancing platelet regeneration. These responses are enhanced by prior administration of SCF so that a single leukapheresis provides sufficient cells for several transplantations. CSF-induced peripheral blood stem cells are rapidly replacing marrow cells as the superior transplantation technique for most types of patients.

### **Use of the CSFs in Model Infections**

Many model systems have been used to determine whether CSF administration is protective against induced infections. The models have usually involved a suboptimal

granulocyte-macrophage population either by using newborn animals, alcohol pretreatment or more commonly leukopenic animals with marrow damage induced by chemotherapy or irradiation. In general, the administration of G-CSF or GM-CSF has been found to reduce mortality or the severity of the infections, dramatically so if CSF treatment was initiated prior to challenge with microorganisms. If CSF treatment was not initiated until after microbial challenge, protective effects were reduced and usually required the co-administration of antibiotics before becoming evident. The principles emerging from these studies indicate that CSF administration in clinical medicine should ideally be commenced *prior* to the onset of infections. It is much less effective if one follows the most common current practice of beginning CSF treatment following infections or the onset of chemotherapy-induced neutropenia. This is a suboptimal regimen that, from experimental studies, is still of value but is likely to require the co-administration of antibiotics.

### **The Role of CSFs in Hematopoiesis**

CSFs are produced in all organs by a multiplicity of cell types including stromal cells, endothelial cells, lymphocytes, fibroblasts, macrophages and possibly a wide range of epithelial cells. Production levels rise rapidly within hours of exposure to inducing agents—most commonly in the form of microbial products. However, the level of CSF production in normal health is very low. With the exception of M-CSF, levels of other CSFs are often undetectable in normal serum. This has allowed the criticism that CSFs may be primarily emergency regulators only of relevance in response to emergencies such as an acute infection. If so, other mechanisms may control the normal formation of granulocytes and monocytes under basal conditions and be the "true" regulators of these cells.

To counter these criticisms, it has been necessary to analyze a range of mouse models in which the genes encoding the CSFs or their receptors have been inactivated either by spontaneous mutations or by homologous recombination. Studies have now been made in mice lacking G-CSF, GM-CSF, M-CSF or Multi-CSF or the receptors for GM-CSF. G-CSF knockout mice exhibit a profound neutropenia and a major reduction in marrow granulocytes. M-CSF knockout mice (in the form of the spontaneous mutant *op/op* mouse with an inactivated M-CSF gene) exhibit major macrophage reductions in some, but not all, organs and failure of osteoclast formation leading to osteopetrosis and failure of teeth eruption. Administration of the appropriate CSF reverses the pathology in both types of animal. These observations confirm that G-CSF and M-CSF are major regulators of granulocyte or macrophage formation under conditions of basal hematopoiesis. The situation in mice with inactivation of the GM-CSF or GM-CSF receptor genes is more subtle in that the numbers of granulocytes and monocytes and their precursors are normal. However, the mice exhibit a profound dysfunction of alveolar macrophages leading to accumulation of surfactant and proteinaceous material, often with patchy areas of pneumonia—a disease state mimicking alveolar proteinosis in humans. Combination knockouts of GM-CSF and M-CSF accentuate both phenotypes and lead to early death from pneumonia, although some macrophages are present in many tissues. The data indicate a mandatory role for GM-CSF for normal macrophage function.

All these models suggest that additional regulators exist with some role in controlling the basal formation of granulocytes and monocytes. SCF may be such a regulator for granulocyte formation, but additional macrophage regulators remain to be discovered.

No consequences have yet been observed of inactivating the gene for Multi-CSF; however, initial information is usually incomplete in knock-out studies, and future test systems may reveal a role for Multi-CSF in normal hematopoiesis.

### **Clinical Applications of the CSFs**

The frequency of spontaneous deficiency in the production of granulocytes or monocytes is low. However, in the rare cases of congenital neutropenia, continuous administration of G-CSF has had the dramatic effect of resolving established infections and restoring such patients to a normal state with respect to susceptibility to infections. A similar outcome has been observed following the use of G-CSF or GM-CSF in patients with cyclic neutropenia. Experience over more than five years has indicated the continuing effectiveness of self-administered daily CSF injections.

In numerical terms, by far the largest numbers of patients receiving G-CSF or GM-CSF treatment have been cancer patients following chemotherapy of a type leading to marrow damage. In such patients, CSF administration immediately following chemotherapy accelerates regeneration of granulocytes and/or monocytes and reduces time of hospitalization, antibiotic use and the frequency of complicating infections. In its more extreme form of intensive chemotherapy with marrow transplantation, the use of either CSF has had a similar impact on the subsequent clinical course of the patients.

While CSF administration has proved to be a valuable support procedure, it of course has no direct impact on the eventual cure or recurrence of the cancers involved. However, the increasing use of CSF-elevated peripheral blood stem cells has permitted the introduction and successful delivery of significantly intensified chemotherapy, e.g., in breast cancer patients. Such patients will now be monitored closely to establish whether the CSF-permitted enhanced chemotherapy has resulted in improved cure rates. Either way, the use of CSF treatment or of CSF-elevated stem cells has changed perceptibly the clinical management of cancer patients with a reduction in hospitalization and more outpatient management.

In principle, the CSFs should be equally effective in the management and prevention of severe infections in other types of patient including those in intensive care units, burns patients and patients with abdominal injuries or operations. Here levels of hematopoiesis may appear normal but enhancement of granulocyte and macrophage functional activity may make the crucial difference to the outcome of the infections, regardless of antibiotic use. Such studies have yet to be widely undertaken, but it is of importance to establish the results of CSF usage in carefully controlled studies. Initial studies indicate the value of CSF treatment and if this is clearly established, it will then become appropriate for CSF treatment to be used prophylactically or in response to the development of a wide range of miscellaneous but life-threatening infections.

Most studies and clinical usage have involved the administration of single CSFs, which do not mimic normal biology and cannot achieve optimal or balanced cellular

responses. It is becoming a matter of concern that the company-based supply of single CSFs is making it difficult to shift clinical medicine to the use of complementary or enhancing combinations. Some effort is overdue to explore the likely superiority in clinical medicine of CSF combinations and CSFs combined with other available growth factors.

### **Suggested Further Reading**

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