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Neutrophil biology and the next generation of myeloid growth factors

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Abstract

Neutrophils are the body's critical phagocytic cells for defense against bacterial and fungal infections. The bone marrow must produce approximately 10×10^9 neutrophils/kg/d to maintain normal blood neutrophil counts. Production of neutrophils depends on myeloid growth factors, particularly granulocyte colony-stimulating factor (G-CSF). After the original phase of development, researchers modified these growth factors to increase their size and delay their renal clearance, increase their biologic potency, and create unique molecules for business purposes. Pegylated G-CSF is a successful product of these efforts. Researchers have also tried to identify small molecules to serve as oral agents that mimic the parent molecules, but these programs have been less successful. In 2006, the European Medicines Agency established guidelines for the introduction of new biologic medicinal products claimed to be similar to reference products that had previously been granted marketing authorization in the European community. In Europe these products are called *bio-similars*. Throughout the world, new version and copied version of G-CSF and other myeloid growth factors are now appearing. Some properties of the myeloid growth factors are similar to other agents, offering opportunities for the development of alternative drugs and treatments. For example, recent research shows that hematopoietic progenitor cells can be mobilized with a chemokine receptor antagonist, chemotherapy, G-CSF, and granulocyte macrophage colony-stimulating factor. Advances in neutrophil biology coupled with better understanding and development of the myeloid growth factors offer great promise for improving the care of patients with cancer and many other disorders.

Keywords

Neutrophils; neutropenia; myeloid growth factors; granulocyte colony-stimulating factor; pegylated granulocyte colony-stimulating factor; bio-similars; follow-on proteins

Neutrophil Biology

Neutrophils (polymorphonuclear leukocytes or *polys*) are the critical cells forming the body's first line of defense against bacterial and fungal infections. They are easily identified

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in blood and tissues by the shape of their nucleus and their faintly pink cytoplasm using most tissue stains. Light, phase, and electron microscopy and fluorescence activated cell sorting analysis show the distinctive primary, secondary, and tertiary granules; the dense glycogen deposits in the cytoplasm; and the multiplicity of receptors with highly selective functions on the surface of these cells.^{1,2}

Key features of neutrophil biology are the massive rate of daily production, estimated to be approximately 10×10^9 neutrophils/kg/d; their rapid turnover in the blood; and their selective trafficking from the marrow to tissue sites of inflammation.^{3,4} Even with extremely high blood neutrophil counts (i.e., leukemoid reactions), neutrophils do not randomly infiltrate the body's tissues. However, at any inflammatory focus, large numbers of neutrophils can accumulate rapidly, form "laudable pus," and rid the body of invading pathogens.²

In response to most infections, acute neutrophilia occurs because of accelerated release of the cells from the postmitotic marrow pool, the marrow reserves. With infections, the proportion of "band" neutrophils in the blood, the glycogen content in the cytoplasm, and the cells' leukocyte alkaline phosphatase score all increase, and the primary granules stain more deeply. All of these responses are now attributable to increased levels of several cytokines, particularly the myeloid growth factor G-CSF.⁵ Administering G-CSF to healthy volunteers or patients simulates the neutrophil response to infections.⁶⁻⁹ Genetic modifications causing the absence of G-CSF and the G-CSF receptor, or the transcription factors controlling the expression of G-CSF or the G-CSF receptor, are known to cause neutropenia and an enhanced susceptibility to infections.¹⁰ G-CSF is now generally accepted as the key regulator of neutrophil production and deployment.¹⁰

Neutrophil responses are governed by several families of glycoprotein receptors on the neutrophils and their progenitors (e.g., myeloid growth factor receptors, integrins and selectins, immunoglobulin receptors, complement receptors, chemokine receptors).² The genes for these receptors and the transcription factors that regulate their expression are all critical determinants of the cell functions. Notably, neutrophil progenitors and the mature cells have receptors for G-CSF and granulocyte macrophage colony-stimulating factor (GM-CSF). On progenitors, these receptors enhance production, whereas on mature cells they enhance the metabolic burst associated with phagocytosis and the killing of microbes. Although the myeloid growth factors are now useful pharmacologic agents, they are natural endogenous regulators of the innate immune response to infections and other inflammatory conditions.^{9,10}

Myeloid Growth Factors

The myeloid growth factors were identified through their capacity to stimulate formation of colonies of hematopoietic cells with in vitro culture systems. G-CSF (locus 17q11.2q 12) is an 18-kd glycoprotein produced by many cell types in response to endotoxins, tumor necrosis factor, and other inflammatory signals. Its receptor is a homodimer expressed almost exclusively on myeloid cells. Knock-outs of G-CSF or the receptor cause neutropenia. GM-CSF (locus 5q31.1) is an 18-28 kd glycoprotein produced by T

lymphocytes, endothelial cells, and fibroblasts, but not all types of cells. Its receptor is a heterodimer expressed on neutrophils, monocytes, fibroblasts, and some other cell types. Knock-outs of GM-CSF do not cause neutropenia.

Interleukin (IL)-3 (locus 5q31.1) is a multilineage factor produced by T lymphocytes and mast cells. Its receptor is a heterodimer similar to that for GM-CSF. Deficiencies of IL-3 do not affect hematopoiesis but are associated with impaired delayed hypersensitivity. IL-5 (locus 5q31.1) is a 50- to 60-kd glycoprotein produced by T lymphocytes. Its receptor is a heterodimer and shares structural similarities to that of GM-CSF and IL-3. IL-5 plays an important role in the production and deployment of eosinophils.

Macrophage colony-stimulating factor (M-CSF; locus 1p21–p13) is a 40- to 90 kd glycoprotein produced by monocytes, macrophages, epithelial cells, and many other cell types in response to endotoxin and inflammatory stimuli. Its receptor is expressed on monocytes and macrophages and its activation promotes both proliferation and survival of these cells. Deficiencies lead to monocytopenia, decreased osteoclasts, and osteopetrosis.

Stem cell factor (SCF or kit-ligand; locus 12q22–24) is a 40-kd glycoprotein produced by fibroblasts and endothelial and stromal cells that synergizes with myeloid growth factors to promote survival and proliferation. Its receptor, kit, is widely expressed. Deficiencies in kit are well characterized and lead to anemia, pigmentation abnormalities, and infertility. Administration of SCF is associated with mast cell proliferation.

Although these are the principal myeloid growth factors, several other interleukins and growth factors are known to have various effects on early myeloid, erythroid, and lymphoid cells and their progenitors.

Myeloid Growth Factor and Their Clinical Applications

The myeloid growth factors G-CSF and GM-CSF were introduced into clinical trials in 1986.¹¹ The initial clinical trials focused on chemotherapy-induced neutropenia,^{12,13} hematopoietic recovery from bone marrow transplantation,¹⁴ and long-term treatment of severe chronic neutropenia.¹⁵ As knowledge emerged that G-CSF is more potent and better tolerated than GM-CSF, clinical research and practice have focused on this myeloid growth factor. Recombinant G-CSF is produced both in bacteria and marketed as a non-glycosylated product (e.g., Filgrastim, Amgen, Thousand Oaks, California) and as a glycosylated product made in yeast (e.g., Lenograstim, Chugai Pharmaceuticals Co., Ltd., Tokyo, Japan). Clinical indications and guidelines for the uses the myeloid growth factors are the subject of several recent reviews and guidelines.^{16,17}

Modification of the Myeloid Growth Factors

The myeloid growth factors have been modified to increase their size and delay renal clearance, increase their biologic potency, and create unique molecules for patent and business purposes. The following section presents publicly available information and the principals and problems involved in modifying these agents, focusing on G-CSF because of

its wide applications. Undoubtedly much of the information in this realm is not in the public domain.

Pegylation

Addition of polyethylene glycol (PEG) is now a widely used approach to extend the plasma half-life of therapeutic proteins and thus enhance their effectiveness.¹⁸ Pegylation generally does not cause immunogenicity. The currently marketed product, pegylated G-CSF (Pegfilgrastim, Neulasta, Amgen), is produced by adding a 20-kd polyethylene glycol moiety to the *N*-terminus of the filgrastim. This modification does not affect the *in vitro* or *in vivo* effects of the molecule, but almost completely eliminates the drug's renal clearance.¹⁹ The clearance (elimination half-life) after a single injection in patients with normal renal function is increased from approximately 3.5 hours to 20 to 30 hours, and the biologic effects last much longer.

Randomized, controlled clinical trials established the therapeutic equivalence of filgrastim (5 mcg/kg) given daily for up to 14 days; pegfilgrastim (100 mcg/kg) given as a single injection; and fixed-dose pegfilgrastim (6 mg) given as a single injection to prevent neutropenia and its complications in patients with breast cancer undergoing multiagent chemotherapy.^{20,21} In these trials, the filgrastim and pegfilgrastim were given the day after each course of chemotherapy. The adverse events associated with either form of G-CSF were equivalent.^{20–22}

Another pegylated form of G-CSF, differing by changes in the G-CSF gene sequence to create multiple new pegylation sites, has been tested in animal studies and phase I and IIa human trials (Maxy-G34, Maxygen, Redwood City, California).²³ Released results suggest a similar effect to the marketed pegylated G-CSF.

Glycopegylation of G-CSF is another variation. Although pegylation prolongs the half-life and enhances the pharmacodynamics of therapeutic proteins, it may lead to multiple isoforms. A novel strategy for site-directed pegylation leads to selective attachment of pegfilgrastim at O-glycan sites (BioGeneriX, Mannheim, Germany, and Neose, Horsham, Pennsylvania).^{24,25} Released results show similar *in vivo* effects to other forms of pegylated G-CSF.

Pegylated GM-CSF is also in development and reported to have a much longer half-life and enhanced biologic effects.²⁶

Other Modifications

G-CSF can be modified with poloxamer 407 or poloxamer 407 plus hydroxypropyl methylcellulose to make a deposited and slowly released form of G-CSF.²⁷ Studies in mice showed modified G-CSF to have a long pharmacologic and biologic effect compared with the parent drug. G-CSF has been linked to an ionic copolymer, pluronic F127, and shown to have greater effectiveness than the parent G-CSF in animal studies of progenitor cell mobilization.²⁸

Modifications to produce a recombinant G-CSF/IgG-Fc protein showed longer and greater effectiveness in rats.²⁹ Other fusion proteins (e.g., recombinant G-CSF/albumin³⁰ and recombinant SCF/IgG-Fc) also seemed to be more effective than the parent drug.³¹

Other interesting combinations have included development of a recombinant diphtheria toxin–G-CSF fusion protein, with the G-CSF serving to target the cytotoxic agent.³² A fusion protein made from the extracellular portion of the G-CSF receptor linked to IgG1-Fc produced a decoy molecule that inhibited proliferation of leukemic cells in vitro.³³ Extensive efforts were also made to engineer more effective forms of the myeloid growth factor by combining G-CSF with IL-3,³⁴ GM-CSF with IL-3,^{35,36} and G-CSF with GM-CSF.³⁷ In general, these agents initially looked promising but have proven to be immunogenic and therefore are not appropriate for full clinical development and medical applications.

Small Molecule Mimics of the Myeloid Growth Factors

Since the discovery and characterization of the myeloid growth factor receptors in the mid-1990s, investigators have been challenged to identify potentially small molecules to serve as oral agents to activate these receptors.³⁸ In 1998, Tian et al.³⁹ described small molecule cyclic peptides that functioned as G-CSF mimics. This small molecule, called *SB 247464*, activated by the G-CSF signal transduction pathways, was identified in a high-throughput assay in cultured cells. Like G-CSF, *SB 247464* induced tyrosine phosphorylation of multiple signaling proteins and stimulated formation of granulocytic colonies and increased blood neutrophil counts in mice by dimerizing the external domains of the G-CSF receptor chains.^{39,40} This concept continues to be explored⁴¹ but has not been proven to be clinically applicable.

Bio-Similar Forms of the Myeloid Growth Factors

In 2006, the European Medicines Agency (EMA) established guidelines for introducing new biologic medicinal products claimed to be similar to a reference product that was previously granted marketing authorization in the European community.⁴² In Europe these products are called *bio-similars*, whereas in Canada they are called *subsequent entry biologicals* (SEBs).⁴³ Another term used in the United States is *follow-on protein drugs*.⁴⁴ With advances in biotechnology, it was readily apparent that the same product or one very similar could be manufactured by many companies using standard methods. The rationale for introducing these similar or identical drugs is simply to expand their availability and reduce prices through competition between manufacturers. In China an estimated 20 companies are now producing G-CSF, and several companies produce G-CSF in Japan. Bio-similar forms of G-CSF and other myeloid growth factors have not yet been introduced in the United States, but have been proposed.

The EMA guidelines require that any new product have similar quality, safety, and efficacy to the referenced medicine,⁴² and that a series of nonclinical studies be performed before clinical development is initiated. These initial studies should provide a clear understanding of the physical, chemical, and biological properties of the products. In vitro investigations, such as receptor binding studies and cell-based assays, should then be used to

establish the comparability of the new and already-approved products. The guidelines then require animal studies of the pharmacodynamic effects and activities of the new agent that are relevant to proposed clinical application. Toxicity studies are critical, particularly those to determine if the antibodies are formed and whether they are neutralizing antibodies. Other studies, such as safety pharmacology, reproduction toxicology, and carcinogenicity studies, are not required for similar biologic medicinal products unless indicated through repeat dose studies.⁴²

The EMEA requirements state that clinical comparability be shown for the pharmacokinetics and pharmacodynamics of the new drug, and that the definition of comparability be established before the study begins. A trial to show clinical comparability is also necessary, with the end points also established in advance.⁴²

Bio-similars must also be shown to be safe, with an adverse effect profile at least as good as that of the previously approved drug. This requires clinical trials of a sufficient size to establish the safety profile and a pharmacovigilance program in accordance with legislation and guidelines of the European Union. A principal concern is whether a new biological product is immunogenic. Determination of immunogenicity often requires information from exposure of a sizeable population because of the intrinsic variability in these responses. A reliable assay system to detect antibodies or cellular immune responses is also required. A major reason for concern is the experience in Europe with bio-similar erythroid stimulating agents, which were found to cause drug-induced pure red cell aplasia.⁴⁵

The EMEA has produced specific guidelines for bio-similar G-CSF.⁴⁶ As outlined earlier, in vitro pharmacodynamic studies and animal (rodent) models must precede clinical investigations. The absolute neutrophil count is the relevant pharmacodynamic marker of activity and the CD34+ cell count is a required secondary end point. The recommended clinical model for showing comparability is the prophylaxis of severe neutropenia after myelotoxic chemotherapy, as was used in the pivotal trials of the currently approved forms of G-CSF.^{12,13} Bio-similar human growth hormone is now marketed in Europe.⁴⁷ In early 2008, the scientific review committee for the EMEA gave a positive opinion of G-CSF from a new manufacturer (Teva Pharmaceutical Industries Ltd.) based on the current EMEA guidelines.⁴⁸

The United States has 2 pathways of drug approval: the 1902 Biologics Control Act (BCA), passed to regulate the manufacture of biologics, and the 1906 Pure Food and Drug Act (PFDA), passed to provide basic standards for the purity and quality of drugs.^{44,49} The BCA merged with the Public Health Services Act in 1944 and the PFDA was incorporated into the Federal Food, Drug, and Cosmetic Act in 1938. Despite passage of more recent legislation, this dual-track system remains in effect in the United States and complicates approval of new protein products and consideration of bio-similars. New legislation may soon establish the pathway for study and approval of bio-similars in the United States and facilitate their entry into the U.S. market.⁴⁴

Next Generation of Agents for Progenitor Cell Mobilization

Both GM-CSF and G-CSF are approved in the United States and Europe for marketing to mobilize progenitor cells for hematopoietic transplantation. Often 2 or 3 leukaphoreses are required to collect to requisite number of CD34+ cells. The effects of G-CSF are now understood to be mediated by increased release of elastase, cathepsin G, and metalloproteinase 9 to the marrow microenvironment, resulting in cleavage and loss of function of key adherence factors on the surface of the progenitor cells.^{50,51}

G-CSF exposure also reduces transcription of SDF-1, a chemokine expressed on the surface of marrow stromal cells.⁵² Normally SDF-1 binds to its receptor, CXCR-4, on myeloid progenitor cells and thereby holds these cells in the marrow.

Recent human investigations and studies in several other species showed that a specific inhibitor of the binding of SDF-1 to CXCR-4, a drug called AMD 3100, is an effective mobilizing agent,⁵³ both as an independent agent and as an adjunct to G-CSF.⁵⁴ Clinical trials indicate that this may be a very helpful agent for mobilization in otherwise difficult situations.⁵⁵ Chemotherapy is the other principal alternative to the myeloid growth factors for progenitor cell mobilization.

Approaches to Care When Myeloid Growth Factor Therapy Fails

The clinician's dilemma is that not all patients respond to the myeloid growth factors through expanding neutrophil production and improving their peripheral neutrophil counts. This is mostly a problem of the lack of normal progenitors, either temporary because of previous exposures to myelotoxic drugs or radiation, or permanent because of a genetic abnormality, malignant transformation, or generalized marrow aplasia. For temporary conditions, cell therapies with neutrophil transfusions^{56,57} or ex vivo expanded progenitor cells⁵⁸ offer some promise, but their effectiveness has not been established in clinical trials. For the other conditions, hematopoietic transplantation is the only feasible alternative, and all of these technologies depend on uses of the myeloid growth factors to mobilize and expand their target cells.

Conclusions

The development of the myeloid growth factors, particularly G-CSF, has transformed the practice of oncology over the past 2 decades. Concurrently, many advances in neutrophil biology have occurred. This progress, coupled with innovative drug development program, provides great hope for continually improving care of patients with chemotherapy-related neutropenia and other neutrophil disorders in the decade ahead.

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