

Granulocyte Colony-Stimulating Factor Receptor Signaling Defects from Neutropenia to Leukemia

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*O man, what is good,
And what the Lord requires of you:
Only to do justice
And to love goodness
And to walk modestly with your God.*

Micah 6:8

Beginning a project on neutrophil priming, I attended a Gordon Conference on phagocytes held in a New Hampshire secondary school during the 1987 summer recess. Serendipitously, Yaacov was my roommate. To me, a fresh post-doctoral fellow, it was exciting to live and eat with much more accomplished scientists. But, academic medicine can be as politically raucous and full of machinations as a session in the Knesset and friendships can last as long as some coalitions. Yet, Yaacov remained a gentle and warm colleague, a welcome face at the annual American Society of Hematology meeting. He lived the words of the prophet Micah.

The prodigious production of neutrophils is carefully controlled through cytokines, the most critical being granulocyte colony-stimulating factor. Mice rendered deficient through gene ablation of either G-CSF or its receptor suffer from profound neutropenia. The importance of G-CSF in supporting neutrophil numbers and quality is found in the widespread use of its recombinant form, filgrastim.

The role of G-CSF in normal hematopoiesis suggests that its disturbance might be pathologic. Just as much of Yaacov's research focused on the mis-firing of neutrophil activation in familial Mediterranean fever, disturbed control of neutrophil production results in disease states of severe deficiency or leukemia. This brief review will thus highlight the pathophysiology of G-CSF receptor signaling [Figure 1].

Uncommonly, some children are born with profound neutropenia (<200 neutrophils/l) and quickly develop life-threatening infections. Severe chronic neutropenia, also known as Kostmann's syndrome, is a hematopoietic disorder characterized by maturation arrest of neutrophil precursors at the level of promyelocytes or myelocytes in the bone marrow [1]. Even with broad-spectrum antibiotics, morbidity and mortality remained very high until the

introduction of filgrastim in the late 1980s [2]. While almost all children will respond with normalization of their neutrophil counts, disappearance of recurrent bacterial and viral infection, and marked improvement in their quality of life, a few will not respond at all. These children can be treated with allogeneic hematopoietic progenitor cell transplantation. Approximately 10% of all children with severe chronic neutropenia will develop either myelodysplastic syndrome or acute myeloid leukemia [3]. How could a disease evolve from profound deficiency of neutrophils to leukemic excess? Is there a role for altered G-CSF receptor signaling? Does pharmacologic administration of G-CSF promote leukemogenesis?

Although G-CSF plays a central role in neutrophil production, studies have failed to demonstrate a defect in G-CSF levels, G-CSF receptor expression, or immediate post-receptor responses. Much interest has recently centered on the neutrophil elastase (*ELA2*) gene. Mutations of neutrophil elastase have been found in all

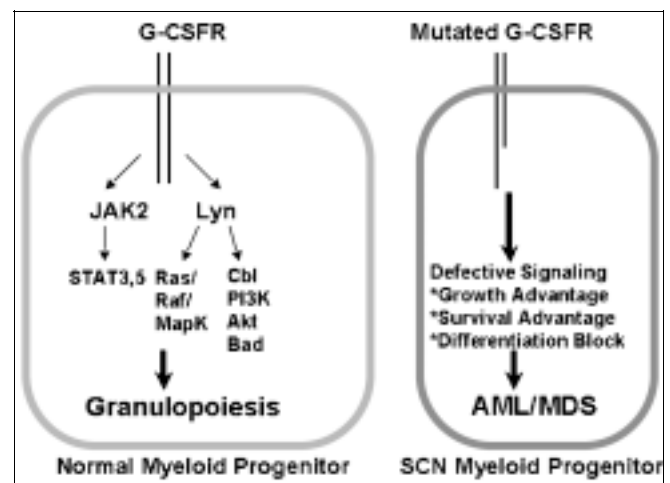


Figure 1. How G-CSF receptor contributes to myeloid leukemia. In normal myeloid progenitor cells, G-CSF receptor proceeds with activation of Jak2 and Lyn protein tyrosine kinases and the subsequent recruitment of downstream signaling pathways involving, but not limited to, STAT3 and STAT5, Ras/Raf/MAP Kinase, and Cbl/PI 3-Kinase, Akt, and Bad phosphorylation. In a myeloid progenitor cell from patients with severe chronic neutropenia (SCN), myelopoiesis is altered. In about 10% of patients, myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML) will develop. Most of these affected individuals will have a mutated G-CSF receptor, which results in one chain being shorter than the other. Consequently, growth advantage, survival advantage, and differentiation block occur from aberrant downstream signaling. Within a myeloid progenitor cell already genetically unstable, additional mutations occur. Eventually, AML develops.

G-CSF = granulocyte colony-stimulating factor

patients with cyclic neutropenia. Approximately 75% of patients with SCN have been found to contain a mutation in the neutrophil elastase gene [4,5]. Unlike SCN, patients with cyclic neutropenia can produce adequate numbers of neutrophils. However, the neutrophil count decreases with a 21 day periodicity. Unlike SCN, the disease presents in adulthood. A variety of mutations has been found in neutrophil elastase, without any consistent effect on elastase activity itself. Furthermore, neutrophil elastase-deficient mice have been generated, and these do not suffer from neutropenia. The genetic cause(s) for SCN remains unknown.

The G-CSF receptor is a member of the hematopoietin/cytokine receptor superfamily. It is characterized as a single transmembrane protein, which forms a homodimer through disulfide linkages. While seven alternative spliced isoforms have been isolated, only class I and class IV G-CSF receptor mRNA isoforms are detected at significant levels in myeloid cells. The class I receptor consists of 813 amino acids and four potential tyrosine phosphorylation sites. The G-CSF receptor contains several structural domains. The extracellular amino-terminal domain is immunoglobulin-like followed by the cytokine receptor homologous region. The CRH domain contains the WSDWS motif found in all cytokine receptors and the cysteine residues required for disulfide linkage and homodimerization. G-CSF binds to the tertiary structure formed by the immunoglobulin and CRH domains. Three fibronectin type III domains are found toward the plasma membrane. The fibronectin-like repeats are required for receptor stability and signal transduction [6]. The cytoplasmic domain of G-CSF receptor can be divided into a membrane proximal domain that contains two conserved regions known as box 1 and box 2, and a membrane distal domain that contains a conserved box 3 as well as the four tyrosine residues [7].

Functional mapping studies have shown that the carboxy-terminal 87 amino acids of class I G-CSF receptor are crucial to the maturational signaling function, whereas the membrane proximal 96 amino acids are sufficient to drive proliferation. In lieu of the carboxy-terminal differentiation domain, the class IV G-CSF receptor isoform contains 34 amino acids of novel peptide sequence, thus leaving the proliferative capacity of this isoform intact. In one report, about 50% of AML samples, showed an aberrant class IV/class I G-CSF receptor mRNA ratio, when compared to normal CD34+ bone marrow cells. It is hypothesized that this increased expression of class IV G-CSF receptor uncouples proliferative and maturational G-CSF receptor signaling pathways in AML cells [8].

The G-CSF receptor has no intrinsic tyrosine kinase activity, but rapid changes in protein phosphotyrosine content occur upon ligand-receptor binding. Non-receptor protein tyrosine kinases, either Jak2 or Lyn, are recruited and activated [9]. These protein tyrosine kinases then trigger a variety of pathways, involving the STAT family of transcription factors, adaptor molecules such as Cbl, CrkL and Shc, and secondary effectors, such as phosphatidylinositol

3-kinase. Counter-regulatory mechanisms, involving the tyrosine phosphatases SHP-1 and SHP-2 or lipid phosphatases such as SHIP, are eventually recruited [10,11]. Since these signaling molecules are commonly activated upon engagement of a wide range of cytokine receptors, pathophysiologic defects may be found either in the receptor itself or downstream of it.

Two patients with SCN have been described with genomic mutations involving the G-CSF receptor. Interestingly, both of these individuals had mutations in the extracellular domain and failed to respond to filgrastim. Touw's laboratory [12] described a child with a point mutation that substitutes a histidine for proline at codon position 206. We have described a girl with a 182 bp deletion occurring in her G-CSF receptor gene at codon position 295 in the WSDWS motif and which led to an out-of-frame premature stop codon.

Almost all children with SCN do not have a germline mutation in the G-CSF receptor, but most of the approximately 10% who develop MDS or AML acquire a nonsense mutation of that receptor. The premature stop codon occurs in the carboxy-terminal domain of G-CSF receptor and results in the formation of truncated receptor [13,14]. One group reported an 18 year old patient with SCN who received G-CSF treatment since 1989 and developed AML in 1998 [15]. They found four distinct variants of mutated G-CSF receptor genes: one variant with the mutation at nt 2363, one with the mutation at nt 2414, one with both mutations, and another with a nonsense mutation at nt 2390. The increasing number of mutations documents the instability of the G-CSF receptor gene. Not all who have acquired the mutation go on to develop AML, and not all who develop AML possess a mutation in their G-CSF receptor. Additional genetic changes, such as Ras mutations, partial or complete loss of chromosome 7 and trisomy 21, occur during this malignant transformation. Altogether, these data suggest that a truncated G-CSF receptor confers a growth advantage in a genetically unstable myeloid precursor cell.

Expression in murine myeloid cells lines of the truncated G-CSF receptor showed enhanced proliferation and defective differentiative signaling. However, when the cDNA for the truncated G-CSF receptor was introduced into the mouse germline ("knock-in"), neither profound neutropenia nor MDS/AML developed [16,17]. Depending on the strain of mice, hyperproliferative myeloid progenitors did occur. In response to G-CSF treatment *in vivo*, mutant mice developed peripheral neutrophil counts that significantly exceeded those in mice not carrying the G-CSF receptor mutation. The absolute numbers of G-CSF-responsive progenitors in the bone marrow of the mutant mice were increased, and these progenitor cells exhibited increased proliferative responses to G-CSF and a defect in the induction of apoptosis. The observation that mice carrying targeted G-CSF receptor mutations do not develop MDS/AML implicates a requirement for additional oncogenic events, such as those listed above, or suggests that human granulopoiesis cannot be totally mimicked in the mouse. One

SCN = severe chronic neutropenia
CRH = cytokine receptor homologous
AML = acute myeloid leukemia

MDS = myelodysplastic syndrome

other possibility is that pharmacologic doses of G-CSF promote leukemogenesis [18].

A mutant G-CSF receptor form from patients with SCN/AML showed resistance to apoptosis and prolonged cell survival and affected Akt [19]. A downstream target of phosphatidylinositol 3-kinase, the serine kinase Akt phosphorylates a variety of substrates critical for survival [20]. G-CSF stimulation of cells expressing the G-CSF receptor truncation mutant induced sustained activation of Akt and prolonged phosphorylation of pro-apoptotic protein Bad. Bad promotes cell death through heterodimerization with the anti-apoptotic proteins Bcl-2 and Bcl-X_L. When serine phosphorylated Bad is sequestered in the cytosol by 14-3-3 proteins, it cannot bind to Bcl-2 and Bcl-X_L. Other pathways are likely affected, and the composite of these disturbances leads to advantages in growth and survival as well as defects in differentiation and apoptosis. In addition, ligand-induced internalization and receptor down-regulation is defective in cells expressing the truncated G-CSF receptor.

While it is ironic that a child born with severe deficiency of neutrophils has a high risk of developing myeloid leukemia, this scenario is no different from familial Mediterranean fever. As Yaacov and others showed, there is inappropriate inflammation resulting from deregulated neutrophil function [21]. These entities – severe chronic neutropenia and familial Mediterranean fever – demonstrate the critical and salubrious importance of homeostasis within the neutrophil compartment.

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There is one spectacle grander than the sea, that is the sky; there is one spectacle grander than the sky, that is the interior of the soul.

Victor Hugo, 19th century French poet, novelist and dramatist, best known for his works The Hunchback of Notre Dame and Les Misérables