G-CSF and its receptor in myeloid malignancy

Renée Beekman and Ivo P. Touw, Department of Hematology, Erasmus MC, Rotterdam, The Netherlands

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Correspondence:

Ivo P. Touw, PhD
Department of Hematology, Erasmus MC
Dr Molewaterplein 50, 3015 GE, Rotterdam, The Netherlands
Phone: +31-10-7043837
Fax: +31-10-7044745
E-mail: i.touw@erasusmc.nl
Summary

Granulocyte colony-stimulating factor (G-CSF) is now used in the clinic for more than two decades to treat congenital and acquired neutropenias and to reduce febrile neutropenia before or during courses of intensive cytoreductive therapy. In addition, healthy stem cell donors receive short term treatment with G-CSF for mobilization of hematopoietic stem cells. G-CSF has also been applied in priming strategies designed to enhance the sensitivity of leukemia stem cells to cytotoxic agents, in protocols aimed to induce their differentiation and accompanying growth arrest and cell death, and in severe aplastic anemia and myelodysplastic syndrome to alleviate anemia. The potential adverse effects of G-CSF administration, particularly the risk of malignant transformation, have fueled ongoing debates, some of which can only be settled in follow-up studies extending over several decades. This specifically applies to children with severe congenital neutropenia who receive life-long treatment with G-CSF and in which the high susceptibility to develop MDS and AML has now become a major clinical concern. Here, we will highlight some of the controversies and challenges regarding the clinical application of G-CSF and discuss a possible role of G-CSF in malignant transformation, particularly in neutropenia patients harboring mutations in the gene encoding the G-CSF receptor.

G-CSF and its receptor

The growth factor G-CSF, now referred to as colony-stimulating factor 3 (CSF3), is the major regulator of neutrophil production under basal conditions of hematopoiesis, as is evident from the fact that CSF3 or CSF3 receptor-deficient mice are severely neutropenic.
CSF3 is also essential for “emergency” granulopoiesis in response to bacterial infections and enhances multiple neutrophil functions. CSF3 exerts its role by inducing proliferation and survival of myeloid progenitor cells, followed by a cell cycle arrest and neutrophilic differentiation. The receptor for CSF3 (CSF3R) belongs to the cytokine receptor type I superfamily, which engage the canonical Janus kinase (Jak)/signal transducer and activator of transcription (STAT), Ras/Raf/MAPkinase and PKB/Akt pathways. When CSF3R mutants were expressed in differentiation competent factor dependent myeloid cell lines, the distal cytoplasmic region of the CSF3R of approximately 100 amino acids was crucial for CSF3-induced neutrophilic differentiation of these cells. While originally being considered as “differentiation domain”, later studies demonstrated that this C-terminal region exerts a negative role in STAT5 activation and proliferation signaling in vivo. Negative regulators of CSF3 signaling linked to the distal C-terminus of CSF3R include the protein tyrosine phosphatases SHP-1 and the suppressor of cytokine signaling (SOCS) protein SOCS3. The SOCS protein family is characterized by a so-called SOCS-box, a domain involved in the recruitment of ubiquitin (E3) ligase activity. The negative action of SOCS3 and more specifically of its SOCS-box on CSF3 signaling has been demonstrated in conditional knockout models. A mechanism for receptor downregulation has been proposed in which SOCS3 drives ubiquitination of a conserved juxtamembrane lysine residue that is important for lysosomal routing of the CSF3R. A current view is that balanced activation and subsequent attenuation of CSF3R signaling pathways, strongly depending on the kinetics of ligand-induced internalization and intracellular routing of the receptor, is important for neutrophil production, particularly during episodes of emergency granulopoiesis.
CSF3 in the treatment of AML

CSF3 as a differentiation inducing agent

Soon after Bradley and Metcalf 11 and Pluznik and Sachs 12 discovered in the mid nineteen-sixties that bone marrow progenitor cells form colonies of differentiated myeloid cells under the influence of external growth factors, it became clear that these crude growth factor preparations also stimulated the proliferation and in part differentiation of leukemic progenitors in AML 13. Once this was realized, ideas about the potential therapeutic significance of these findings rapidly evolved, which became testable in the mid nineteen-eighties when recombinant technology allowed the large scale production and purification of hematopoietic growth factors, including CSF3 14, 15. The availability of clinical grade CSF3 and GM-CSF yielded expectations for patients with severe forms of chronic neutropenia, which have proved to be realistic from the outset. Concerning the application of CSF3 in the treatment of myeloid leukemia, one line of thinking was that AML blasts would differentiate upon CSF3 exposure and thereby undergo growth arrest and cell death 16, 17. These studies provided important insights in the biology of myeloid leukemia and e.g., revealed the hierarchical nature of leukemic cell populations, consisting of leukemic stem cells, progenitors with colony forming potential in vitro (AML-CFU) and partly differentiated nonproliferative end cells 13. Since then, CSF3 has occasionally been administered to selected AML patients with the objective to induce differentiation of the leukemic cells with variable results and whether the observed therapeutic effects could be ascribed to differentiation induction remained uncertain 18. Currently the interest in further clinical development of this
concept appears to have diminished, arguably because differentiation of the leukemia “bulk” without affecting the LSCs may not lead to durable therapeutic benefits. Nonetheless, the successful implementation of all-trans retinoic acid therapy in the treatment of acute promyelocytic leukemia, serving as the key paradigm that differentiation-inducing agents combined with chemotherapeutic regimens can result in long-lasting remissions \(^{19}\), leaves the concept of differentiation induction by combinations of agents (including CSF3) open for future application in AML.

\textit{CSF3 as a chemo-sensitizer}

The use of myeloid growth factors (CSF3, GM-CSF) to activate chemo-resistant dormant leukemia stem cells (LSC) into chemo-sensitive cycling cells has been tested in multiple prospective randomized trials with variable outcome, possibly because of differences in patients groups and study design \(^{20}\). For instance, in one study beneficial effects on overall and disease free survival of standard risk AML patients was demonstrated when CSF3 was administered during induction therapy \(^{21}\), whereas others did not observe favorable responses in a similar study involving elderly AML patients \(^{22}\). More recently, the theme of chemo-sensitization of LSCs by growth factor priming has been revisited from another viewpoint, i.e., based on the ability of CSF3 and the CXCR4 antagonist AMD3100 (plerixafor) to push LSCs out of their bone marrow niches that promote self renewal and may be protective against damage by genotoxic compounds. Again, results may be variable and dependent on the subtype of AML as is illustrated by two recent studies in mouse models, one representing acute promyelocytic leukemia (APL), the other AML with high MN1 expression. In the APL model, it was shown that AMD3100
induces the mobilization of leukemic cells from their bone marrow niches into the circulation, thereby increasing their sensitivity to Ara-C or daunorubicin\textsuperscript{23}. In contrast, no chemo-sensitizing effects were seen in the AML/MN1 model\textsuperscript{24}. Despite the similarities in mobilizing activities of CSF3 and AMD3100, recent studies have shown that CSF3 and AMD3100 synergize in the mobilization of normal stem cells, suggesting that their activities are not entirely overlapping\textsuperscript{25}. These observations suggest that combinations of CSF3 and AMD3100 or other agents affecting cell migration and adhesion might be of therapeutic benefit\textsuperscript{26}.

**CSF3 and malignant transformation**

*Leukemia risk in individuals without hematological disorders*

The concern that administration of hematopoietic growth factors might accelerate or even cause leukemia has recently received major attention in the context of CSF3 treatment of healthy individuals to mobilize HSCs into the periphery. The adverse effects of CSF3 administration to peripheral stem cell donors have been evaluated in two independent studies involving more than 5000 cases with a follow up of 4-5 years\textsuperscript{27, 28}. Both studies reported no statistically significant differences in the incidence of malignancy relative to individuals not exposed to growth factor treatment. On the other hand, in a study from the RADAR project\textsuperscript{29} AML was reported in 2 out of 200 HLA-identical siblings donors for AML patients, which significantly exceeds the incidence reported in the other studies. However, irrespective of exposure to CSF3, siblings of AML patients have a 2 to 5-fold
increase in the annual incidence of leukemia, which most likely explains this discrepancy

Another context in which a possible leukemogenic effect of CSF3 has been extensively investigated is in adjuvant breast cancer therapy. A retrospective study addressed the occurrence of AML/MDS in six adjuvant breast cancer trials and showed increased rate of AML/MDS in patients treated with intensified doses of cyclophosphamide requiring CSF3 support. A different study reported a doubling in the risk of AML/MDS in a population of women aged 65 years or older treated with adjuvant chemotherapy and growth factor support for stage I-III breast cancer. Although the absolute risk of secondary leukemia was low in both studies, it was stated that the application of myeloid growth factors and possible leukemia risk should be factored into clinical decisions. However, the benefits of adjuvant chemotherapy in these patients outweighs the risk of secondary MDS or AML and given all of the unknown factors, it remains uncertain whether the weak associations found have a causal relationship to growth factor treatment. Interestingly, a recent study in an as yet small series of patients suggests that the mutational status of BRCA1 and BRCA2 genes may contribute to leukemia risk in breast cancer patients, raising the possibility that a relation between CSF3 administration and secondary MDS/AML may specifically apply to these genetically defined subgroups. Although a follow-up of 2000 stem cell donors for at least 10 years might be needed to detect a statistically significant increase in malignant transformation, the leukemia incidence associated with CSF3 administration is thus far negligible in stem cell donors and low but not yet conclusively determined in different genetic subtypes in breast cancer patients.
CSF3 treatment and malignant transformation in conditions with increased leukemia risk

CSF3, as a single growth factor or in combination with erythropoietin (EPO), has been used in MDS and severe aplastic anemia (SAA) and MDS but is not generally applied in the treatment of these conditions. In MDS, CSF3 was administered to investigate whether CSF3 would synergize with EPO to alleviate anemia and to reduce transfusion need. A collaborative study that included patients from all risk categories suggested that leukemia risk in MDS patients treated with a combination of CSF3 and EPO was not different from patients not receiving growth factor treatment. However, a complicating factor in this retrospective study is that the EPO+CSF3 treated groups were compared with untreated historical controls from a distinct cohort. In a retrospective survey among 840 SAA patients registered by the EBMT who received immunosuppressive therapy (IST) with or without CSF3, a small but significant increase in hazard (1.9) of AML/MDS was reported in the CSF3-treated group. In contrast, in a meta-analysis of 6 randomized control trials involving a total of 414 patients no statistically different risk of progression to MDS/AML between growth factor treated and control groups was noted. A similar conclusion was reached in an earlier study based in 144 patients. Strikingly, in a Japanese study, CSF3 treatment appeared to be more strongly associated with increased leukemia risk, particularly in cases refractory to IST. Why the leukemia incidence in this study differed from the European studies is unclear but may relate to a more frequent occurrence of chromosome 7 abnormalities (monosomy 7, 7q-) in the Japanese patient group. Supporting this idea, Sloand and colleagues showed that CSF3 preferentially stimulates the clonal expansion of MDS and SAA clones with monosomy 7, which was linked to an increased expression of a CSF3R isoform that lacks a major
part of the C-terminal cytoplasmic domain as a result of alternative splicing 41. On the other hand, IST unresponsive SAA patients not receiving CSF3 therapy may also develop monosomy 7 42. In summary, although the increase of leukemia risk upon CSF3 treatment of MDS and SAA patients appears to be low, a causal relationship cannot be entirely excluded. Given the limited use of CSF3 in these settings, data from prospective trials further addressing this issue will unlikely become available in the near future.

Severe congenital neutropenia (SCN)

CSF3 therapy alleviates severe neutropenia and related clinical symptoms in more than 90 % of SCN patients and is the preferred choice of treatment of SCN 43. In the pre-growth factor era, with early mortality due to opportunistic infections being the dominant complication, progression of SCN to acute leukemia was sporadically reported 44-46. Ever since the introduction of CSF3 therapy, the possibility that CSF3 treatment would increase the risk of MDS/AML development in SCN patients has been an ongoing concern. CSF3 has now been routinely administered to patients with different types of chronic neutropenia for more than two decades. These patients provide an invaluable source for studying the long-term side effects of CSF3 treatment. Since 1994, the Severe Chronic Neutropenia International Registry (SCNIR) has monitored patients with different forms of neutropenia, including SCN, cyclic neutropenia and idiopathic neutropenia 47. In 2000, the first comprehensive evaluation of the incidence of MDS/AML in SCN patients from the SCNIR was reported 48. Among 352 SCN patients monitored for an average of 6 yrs (range 0.1-11 yrs) on CSF3 treatment, 31 developed MDS/AML with a cumulative risk of 13% after 8 years of CSF3 treatment. There was no apparent relationship to duration or dose of CSF3
treatment and progression to MDS/AML. A follow-up study published in 2006 involving 374 SCN patients showed that the hazard of MDS/AML increased over time, from 2.9% per year after 6 years to 8.0% per year after 12 years on CSF3 \(^{49}\). After 10 years, the cumulative incidence for MDS/AML was 21%. This study also specifically addressed the incidence of leukemia in SCN patients relative to CSF3 responsiveness. Patients requiring more than the median dosage of CSF3 (8µg/kg/d) and nonetheless did not reach median absolute neutrophil counts after 6-18 months had a significantly increased MDS/AML incidence (40%) after 12 years compared to patients responding to lower CSF3 doses (11%) \(^{49}\). A possible explanation for these associations is that the HSC compartment in SCN patients who respond poorly to CSF3 is more damaged and therefore less susceptible to growth factors. This supports the notion that secondary leukemia in SCN arises because chronic genotoxic stress in the hematopoietic stem cell compartment leads to the acquisition of oncogenic mutations, with CSF3 possibly playing a role in the clonal expansion of (pre-)leukemic cells. However, whether CSF3 therapy had contributed to MDS/AML development could not be determined in this study \(^{49}\). Of note, patients with cyclic or idiopathic neutropenia and neutropenia patients with an underlying metabolic disorder receiving CSF3 treatment regimens comparable to SCN patients treatment do not show an increased propensity to develop MDS or AML \(^{47, 48}\). Leukemic progression of neutropenia is thus mainly confined to patients diagnosed as SCN.
CSF3R mutations and malignant transformation in SCN

Direct evidence for a possible role of CSF3 in propagating leukemic expansion comes from SCN/AML cases in which remission of leukemia occurred after termination of CSF3 treatment. However, such patients are exceptional and generally abrogation of CSF3 treatment generally has little or no effect on the leukemic burden in SCN/AML patients. The discovery that patients may harbor nonsense mutations in the CSF3R gene, resulting in the expression of truncated CSF3R proteins lacking ~100 amino acids from their C-terminal cytoplasmic domains provided a molecular indication for abnormal CSF3 signaling in SCN. Functional studies revealed that these truncated CSF3R were hampered in their ability to transduce signals required for neutrophil differentiation in murine cell line models, a characteristic associated with a possible role of CSF3R dysfunction in leukemic progression of the disease. Importantly, a later study showed that the CSF3R mutations are usually not constitutive but acquired in hematopoietic stem or progenitor cells during the course of CSF3 treatment. Another major finding of this study was that the time between the first detection of CSF3R mutations and the diagnosis of MDS/AML varied greatly. For instance, in one patient a clone with an acquired CSF3R mutation appeared just three months before AML became overt, whereas in other patients CSF3R mutant clones were already detected four years before the acquisition of monosomy 7 and disease conversion to MDS/AML. In addition, it became clear that patients may harbor multiple distinct acquired CSF3R mutations, suggestive of expansion of multiple affected clones.

The two major genetically defined subgroups of SCN prone to develop MDS/AML are patients with mutations in ELA2 and patients with mutations in the HAX1
gene. More recently, two patients with X-linked neutropenia with mutations in the WAS gene were reported in which the disease evolved to MDS/AML. In these three subtypes of SCN, leukemic progression is associated with the acquisition of CSF3R mutations and until now no differences in latencies or molecular and cytological features of the arising leukemias have been reported. In an analysis involving 145 SCN cases, CSF3R mutations were found in approximately one-third of the patients in the neutropenic phase of the disease. Of 23 patients showing signs of malignant transformation, 18 (78%) harbored CSF3R mutations, confirming that these mutations are strongly linked to leukemic predisposition. Notably, these mutations have also been detected in lymphoid cells and thus may be acquired in multipotent progenitors. In contrast to SCN, acquisition of CSF3R mutations has not been observed in patients with cyclic or idiopathic neutropenia receiving CSF3 therapy. These findings show that long-term CSF3 treatment in neutropenia patients other than SCN is not leukemogenic and further accentuate the correlation between leukemic progression of SCN and the acquisition of CSF3R mutations. However, despite all these suggestive correlations the issue whether these mutations are truly “drivers” or just “passengers” in the leukemic process cannot be settled with certainty. For instance, one critical piece of information that is still missing is whether CSF3R mutations, once detected in the neutropenic phase, are invariably present in the MDS/AML cells and not “lost” during leukemic progression, as was recently demonstrated for JAKV617F mutations in myeloproliferative disorders. So far, patients harboring clones with CSF3R mutations that progress to MDS/SCN without mutations have not been reported, but a systematic analysis is warranted to address this issue.
Molecular mechanisms responsible for leukemic progression of SCN

The critical genetic pathway(s) underlying the leukemic progression of SCN are still largely unknown. Cytogenetic abnormalities that are most frequently found in SCN/AML are chromosome 7 abnormalities (monosomy 7, 7q-) and trisomy 21. Mutations in Ras have also been detected in SCN/AML, but their frequency is still controversial. By performing mutational profiling of 14 genes previously implicated in leukemogenesis, Link and colleagues found that mutations of tyrosine kinase genes, FLT3, KIT, and JAK2, were not detected in SCN/AML and neither were other abnormalities, e.g., mutations in NPM1, CEBPA, TP53 that are common in de novo AML. As expected, mutations of CSF3R were the only regular abnormalities found in SCN/AML, again supporting the hypothesis that the mutant CSF3R may provide an "activated tyrosine kinase signal" important for leukemogenesis. Aberrant signaling from the truncated CSF3R is to a major extent driven by defective ligand-induced receptor internalization owing to the loss of a dileucine-based internalization motif and disturbed lysosomal routing due to the loss of the critical docking site for SOCS3. Prolonged CSF3-induced STAT5 activation and increased reactive oxygen species (ROS) production are two of the major consequences of CSF3R truncations, as demonstrated in vitro and in knock-in mouse models (Csf3r-D715) with patient equivalent mutations. Both of these mechanisms have been firmly implicated in cancer and may act synergistically in leukemic transformation. For instance, constitutive STAT5 activation by the mutant tyrosine kinase receptor FLT3-ITD has been suggested to drive leukemic cell growth via mechanisms involving direct transcriptional activation and chromatin remodeling. In this respect it is of note that STAT5 was indeed shown to be crucial for the selective clonal expansion...
of hematopoietic stem and progenitor cells harboring Csf3r mutations. The elevated CSF3-induced ROS levels in bone marrow cells expressing truncated CSF3R may contribute to leukemic transformation by several mechanisms: by causing DNA damage and an increasing mutation rate in the HSC compartment or by inactivation of critical phosphatases such as the lipid phosphatase PTEN and protein tyrosine phosphatases that negatively control growth factor signaling.

Despite the proposed leukemogenic role of CSF3R mutations, Csf3r-D715 mice do not spontaneously develop leukemia. This might be explained by the fact that these mice had not been systematically exposed to CSF3 treatment or that their relatively short lifespan would be prohibitive to unveil the leukemogenic nature of CSF3R mutations. Alternatively, a likely hypothesis is that the transforming abilities of CSF3R mutations become overt only in the presence of the genetic defects underlying SCN, i.e., mutations in ELA2, HAX1 or WAS. Because strains harboring SCN-derived mutations in Ela2 and mice deficient in Hax1 expression are available, this could be addressed by crossing the Csf3r-D715 allele into these mice. However, a complication is that the Ela2 and Hax1 mouse models do not copy the neutropenic phenotype found in SCN patients, suggesting that in mice the consequences of these abnormalities for granulopoiesis are less severe or even lacking.

Are CSF3R mutations useful predictors for leukemic progression of SCN?

Because most SCN patients who progress to MDS/AML have a dismal therapy outcome, it is crucial to detect signs of malignant transformation at the earliest possible stage to create the opportunity to timely consider alternative treatments, such as allogeneic stem
cell transplantation (allo-SCT)\textsuperscript{58, 76}. Regular monitoring of \textit{CSF3R} mutations has been considered to be helpful to screen for the risk of leukemic transformation\textsuperscript{58}, but when \textit{CSF3R} mutations are present in minor clones, they can easily be missed in direct sequencing protocols. Possibly, next generation sequencing technologies allowing mutation detection in smaller subsets of cells will resolve this problem. Still, the unpredictable time intervals between the first detection of \textit{CSF3R} mutations and the eventual leukemic transformation remains a major dilemma that makes a decision to opt for an allo-SCT in SCN patients who respond favorably to CSF3 treatment difficult. For that reason, the decision to transplant these patients without other additional evidence of leukemic progression (such as acquisition of monosomy 7) remains controversial and “watchful” waiting is being considered the most acceptable option, even though the success rate of treatment at a more advanced stage of malignant transformation will significantly decline\textsuperscript{77}. Nonetheless, it must be taken into account that all patients with \textit{CSF3R} mutations will eventually progress to AML\textsuperscript{77}, with time intervals varying between months, years, or even decades after the initial detection of mutant clones. A striking example of such a long latency comes from the child in whom a \textit{CSF3R} mutation was first identified\textsuperscript{53}. CSF3 treatment of this patient started in 1990 and the \textit{CSF3R} mutation was first detected in a majority of bone marrow cells in 1992\textsuperscript{53}. Chronological sampling revealed that the mutant clone persisted and gave rise to RAEBT in 2007, rapidly followed by AML harboring trisomy 21 and a mutation in \textit{RUNXI}.

Irrespective of the possible leukemogenic effects of CSF3 and \textit{CSF3R} mutations in SCN patients, the case reported above stipulates that reliable predictors of leukemic transformation allowing a timely consideration of alternative treatment are
urgently needed. Systematic sequential analysis may reveal which (epi-)genetic changes that occur early-on during the neutropenic phase of SCN may be linked to malignant transformation. For instance, SNP-CGH analysis in the above-mentioned SCN patient suggests that copy number neutral LOH, indicative of acquired uniparental disomy (UPD) in certain chromosomal regions, had already occurred in 1992, i.e., 15 years before malignant transformation (Beekman and Touw, unpublished results). Because UPD is one of the hallmarks of AML, these and other genetic modifications may give new insights in the mechanisms of leukemic progression of SCN and provide valuable indicators of leukemia risk in SCN patients, additional to reduced CSF3 responsiveness and CSF3R mutations.

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