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The role of the granulocyte colony-stimulating factor receptor (G-CSF-R) in disease

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1. ABSTRACT

Granulocyte colony-stimulating factor (G-CSF) is a key regulator of granulopoiesis via stimulation of a specific cell-surface receptor, the G-CSF-R, found on hematopoietic progenitor cells as well as neutrophilic granulocytes. It is perhaps not surprising, therefore, that mutations of the G-CSF-R has been implicated in several clinical settings that affect granulocytic differentiation, particularly severe congenital neutropenia, myelodysplastic syndrome and acute myeloid leukemia. However, other studies suggest that signalling via the G-CSF-R is also involved in a range of other malignancies. This review focuses on the molecular mechanisms through which the G-CSF-R contributes to disease.

2. INTRODUCTION

2.1. G-CSF and its receptor

Neutrophilic granulocytes are white blood cells that play an essential role against infection, especially of a bacterial or fungal nature. These cells are generated from bone marrow stem cells via intermediate myeloid progenitors that expand in number and differentiate in response to external signals. G-CSF plays a crucial role in the production and function of neutrophilic granulocytes (1-3). It is able to mobilize various precursor cells, stimulate the proliferation and differentiation of cells along the neutrophilic lineage, as well as activate the functions of mature neutrophils (4-6). The various biological effects of G-CSF are mediated through a specific cell surface

receptor, the G-CSF-R, a member of the hematopoietin receptor superfamily that binds as a homo-oligomeric complex to its ligand (7-10). The G-CSF-R, like other hematopoietin receptors, lacks intrinsic tyrosine kinase activity but activates several associated cytoplasmic tyrosine kinases (2,7). These include Janus tyrosine kinases (Jaks), particularly Jak1 and Jak2 (11-14), and members of the Src kinase family, particularly, Lyn and Hck (15-19). Key downstream pathways are the signal transducer and activator of transcription (STAT) proteins, especially STAT3 and STAT5 (12,14,20-23), the Ras-MAPK pathway (24-26), the PI 3-kinase-Akt pathway (19,27,28). These are negatively regulated by members of the SOCS family (29-31), as well as various phosphatases (27,30-32).

2.2. Neutropenia and other relevant disorders

Neutropenias represent a series of potentially life-threatening disorders characterised by a reduction in circulating neutrophils. Since neutrophils play a major role in host defense against bacteria, neutropenia patients suffer from frequent episodes of opportunistic bacterial infections (33). Severe congenital neutropenia (SCN) is a heterogeneous group of disorders characterized by a severe decrease in the number of blood neutrophils ($<0.5\times10^9$/l), and a maturation arrest of bone marrow progenitor cells mainly at the promyelocyte/myeloid stage (34,35). Although SCN was originally described as an autosomal recessive disorder in Swedish families, this form is now recognized as a separate syndrome, Kostmann's neutropenia, which produces even lower neutrophil counts ($<0.2\times10^9$/l) (36). Instead, SCN exists in both sporadic and autosomal dominant forms. A major clinical concern for SCN patients is their increased risk of developing myelodysplastic syndrome (MDS) and/or acute myeloid leukemia (AML) with poor prognosis for survival (37,38). The incidence of progression to leukemia among SCN patients is at least 7 %, but possibly as high as 15 % (39,40).

2.3. G-CSF therapy

Since G-CSF plays a crucial role in the stimulation of granulopoiesis (3,8), this cytokine has been widely used in the treatment of SCN (40). Although myeloid progenitor cells from SCN patients frequently show reduced responsiveness to G-CSF (41,42), treatment with pharmacological doses of G-CSF are able to restore the neutrophil count in the majority of SCN patients (33), leading to a concomitant reduction in infection-related events (33,42-44). It has also been employed in other neutropenic conditions, including those associated with chemotherapy (45-47). However, the ability of G-CSF to mobilize hematopoietic stem cells (HSCs) has seen it extensively used in the harvesting of HSCs from the periphery, thereby obviating the need for traditional bone marrow transplantations in many instances (48,49).

3. DIRECT ROLE OF G-CSF-R MUTATIONS IN MYELOID DISORDERS

A considerable number of independent mutations in the gene encoding the G-CSF-R, designated *CSF3R*, have been described. These mutations fall into a number of distinctive classes that relate to the type of mutation as well as their biological and clinical consequences. Mostly these relate to perturbations of the myeloid lineage, as might be expected.

3.1. "Hyperresponsive" intracellular truncations

By far the most studied clinical abnormalities of the *CSF3R* gene are a series of acquired nonsense mutations identified in a subset of SCN patients (50,51). These mutations truncate between 82 and 98 amino acids from the carboxyl-terminus of the receptor (Figure 1), a region implicated in maturation induction and growth arrest (52,53). Such truncated receptors show normal affinity for G-CSF (52). However, when expressed in myeloid cell lines these truncated receptors transduce a strong growth signal but fail to induce maturation (50). Co-expression of wild-type and truncated receptors has revealed that receptors truncated at their C-terminus act in a dominant-negative manner over wild-type receptors to enhance proliferation at the expense of maturation (50).

3.1.1. Clinical details

The role of truncated G-CSF-Rs in neutropenia appears to be modest. Only around 20% of SCN patients harbor such truncating *CSF3R* mutations, and these are only represented in a proportion of transcripts in the bone marrow, often a relative minor percentage (54). These levels may remain constant for several years, or even disappear spontaneously (55). In addition, mutations have been found to appear after the onset of neutropenia (56).

However, SCN patients carrying truncating G-CSF-R mutations show a strong predisposition to both MDS and AML (57) (as well as in more than one case of ALL (58,59)). Indeed in SCN patients progressing to AML, the most common mutations identified are in the *CSF3R* gene (82%), followed by Ras mutations (~50%) and monosomy 7 (60). It has been suggested that this is a result of an underlying genetic instability (33), although it is unclear what might cause this. What is clear is that when *CSF3R* mutations are present, 100% of blasts in these patients carry the mutation (50,60). However, since mutations are not always seen in AML and can spontaneously disappear (55), progression to leukemia does not seem to be inevitable. However, even the most skeptical concede that at the very least truncating G-CSF-R mutations may confer a survival advantage to HSCs that leads to the common involvement of such mutations in MDS/AML (54).

There have been considerable discussions regarding the possible role of G-CSF administration in the selective expansion of G-CSF-R mutant clones. However, the data are complex and the conclusions controversial. In one study there was no statistically significant relationship between the age of onset of MDS/AML and G-CSF dose or duration of therapy (60). Another study of 101 SCN patients determined that the risk of leukemia increased with the degree of G-CSF exposure (61). However, higher doses may be reflective of a more severe disease and so a naturally higher propensity to MDS/AML. Moreover, Kostmann's patients developed AML prior to the advent of

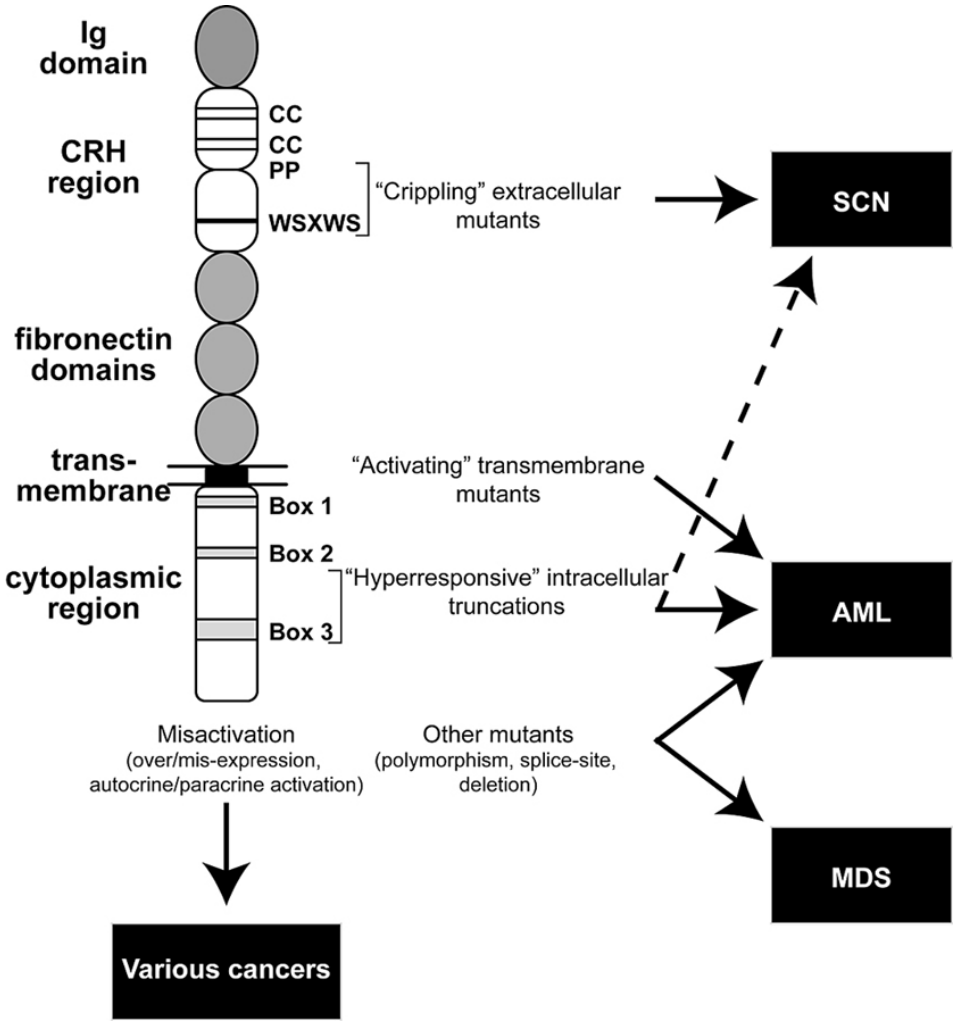

Figure 1. G-CSF-R perturbations in disease. Schematic representation of the mature G-CSF-R, showing important subdomains and residues conserved among members of the hematopoietin receptor superfamily. The relative positions of various classes of mutation are indicated along with the respective clinical manifestations of these and other G-CSF-R perturbations. Abbreviations: Ig – immunoglobulin-like; CRH – cytokine receptor homology; SCN – severe congenital neutropenia; MDS – myelodysplastic syndrome; AML – acute myeloid leukemia).

G-CSF therapy. One study has reported an SCN patient that progressed to CMML in the absence of G-CSF treatment, who expressed a truncated G-CSF-R (as well as mutant Ras and monosomy 7) (62). Thus it is possible that the mutant receptor form may have a selective advantage in the absence of treatment, perhaps due to the elevated G-CSF levels seen in SCN patients as a result of their neutropenia (60). G-CSF therapy may then accelerate the propensity of AML in these patients. This is consistent with the analysis of SCN patient that initially possessed no *CSF3R* mutation but, following treatment with G-CSF, developed AML with a truncating *CSF3R* mutation. These blasts decreased to undetectable levels when G-CSF was withheld in the absence of chemotherapy, although the G-CSF-R mutation could still be detected (63). In another patient there was a step-wise increase in the number of independent *CSF3R* mutations, which correlated with transformation to AML (64). Others have argued that these mutations merely act as a "bystander" (40), correcting the neutropenia and prolonging survival, allowing time for malignant transformation to occur. More work is needed to resolve these conflicting conclusions.

3.1.2. Mouse models

To assess the contribution of C-terminal G-CSF-R truncations to the pathogenesis of SCN and AML, several groups have sought to recapitulate the clinical situation in mice. We showed that mice with a targeted "knock-in" truncating mutation possessed reduced basal levels of circulating neutrophils (65). Heterozygote animals showed intermediate levels of peripheral neutrophils, suggesting the presence of a wild-type receptor was unable to fully compensate for the mutation (65). These results are supported by another study that generated mice transgenically expressing a truncated human G-CSF-R, which exhibited peripheral neutrophil counts one-third of

normal and impaired resistance to bacterial infection (66). These data are in agreement with the hypothesis that truncated G-CSF-R proteins interfere with the function of wild type G-CSF-R in a dominant-negative manner (50). In each case, there was an increased percentage of immature myeloid cells in the respective mice, with these cells showing a maturation defect in vitro (66,67). In contrast, another study analysing an independently targeted receptor truncation in mice failed to show basal neutropenia, although in this case the truncated from of the receptor appeared to be overexpressed, perhaps counteracting an intrinsic neutropenia (68). However, all three studies showed a hyper-responsiveness to G-CSF, such that mice administered exogenous G-CSF showed elevated neutrophil counts compared to wild type controls (65,66,68), due to increased proliferation of myeloid progenitors (66,67).

Together these studies suggest that the C-terminus of G-CSF-R exerts a differential effect on neutrophil production in vivo. Firstly, truncation of the G-CSF-R can give rise to neutropenia under basal conditions, even in the presence of a full-length receptor, suggesting that truncating *CSF3R* mutations can indeed contribute to the etiology of SCN. However, the neutropenia seen in the mouse studies was not as severe as in SCN. One possibility is that this difference is related to differences between mice and man. A more likely explanation is that the *CSF3R* mutation may not be the initial cause of severe neutropenia, but rather that other genetic defects are responsible for the SCN phenotype, such as *ELA2* (69,70), *GFI1* (71) and WASP (72). However, the expansion of a population of cells with an acquired *CSF3R* truncation mutation – possibly due to G-CSF treatment – could then further exacerbate the neutropenic condition. Secondly, the strong hyperproliferative function of the truncated G-CSF-R in vivo provides a compelling indication of how this type of mutation once present in neutropenia patients may contribute to their frequent progression to MDS/AML. Notably, expression of the truncated receptor in mice is not by itself leukemogenic, since no spontaneous leukemias have been reported in mice hetero- or homozygous for the mutation (65,68). Apparently other genetic defects are required for cells to become transformed. However, the *CSF3R* mutation clearly contributes to a potentially pre-leukemic state.

3.1.3. Molecular mechanisms

The results from mouse and cell line models indicate that truncating G-CSF-R mutations act in a dominant-negative manner to exert three effects on responsive cells, specifically: (i) decreased differentiation; (ii) increased sensitivity to ligand and, perhaps most importantly, (iii) enhanced proliferation. This is largely consistent with observations in SCN/AML patients, since truncating *CSF3R* mutations affect just a single allele (50,53,57), with the mutations associated with a block in differentiation (neutropenia) and susceptibility to unrestrained proliferation (clonal expansion, myelodysplasia and leukemia). We are beginning to understand the molecular basis for these effects.

The inability of truncated receptors to mediate differentiation strongly suggests that an important signaling pathway activated via the C-terminal region of the receptor has been removed by the truncation. One candidate is STAT3, which is prominently activated by G-CSF (11,20), and has been shown to be involved in both macrophage and neutrophilic differentiation and survival (73-76). G-CSF induced activation of STAT3 depends on the recruitment of STAT3 to the G-CSF-R via binding of STAT3 SH2 domains to multiple phosphotyrosines of the activated G-CSF-R (23,75,77), including those located in the C-terminal region. Using bone marrow cells from mice harboring a targeted G-CSF-R truncation (*gcsfr-Δ715*) (65), we have shown that STAT3 activation from the truncated G-CSF-R is reduced, even at saturating G-CSF concentrations. In addition, there is an altered dose-response of STAT3 activation, such that at lower G-CSF concentrations the STAT3 deficiency is even more pronounced, a result confirmed in myeloid 32D cells (67,78). Given the relative dose-response properties, this would seem to be primarily due to loss of the Y744-dependent route of STAT3 activation. However, while STAT3 has been shown to be a vital factor in G-CSF-dependent differentiation (74,76), more recent studies have that STAT3 deficiency results in neutrophilia (79). Thus, rather than contributing to the basal neutropenia, defective STAT3 activation more likely contributes to the G-CSF-induced neutrophilia seen in *gcsfr-Δ715* mice (65).

Cells expressing truncated G-CSF-R receptors are hypersensitive to G-CSF (50,80). We have shown that these cells exhibit an altered dose-response of STAT3 activation compared to STAT5 activation in both cell lines and mice, such that the ratio of STAT3:STAT5 is drastically reduced at low concentrations of ligand (78). There is now considerable evidence that STAT5 contributes to proliferative responses to G-CSF (81,82), while STAT3 activation is inhibitory (74,76,79). Therefore, the reduced STAT3:STAT5 ratio in cells with truncated receptors at low G-CSF concentrations may shift the balance of toward proliferation, providing a plausible explanation for the hypersensitivity of these cells to G-CSF (50,52).

Truncated G-CSF-Rs lead to hyperproliferation in response to G-CSF in both mice and myeloid cell lines, with truncated receptors acting dominantly over wild-type receptors (50,65). Furthermore, SCN patients with mutation of a single *CSF3R* allele show clonal expansion of the mutant population and are predisposed to AML, suggesting an equivalent effect in these patients. Several groups have identified molecular mechanism(s) that help explain this dominant hyperproliferative function of truncated G-CSF-Rs.

Compared to wild-type receptors, truncated receptors showed prolonged activation, due to a much slower “off-rate” (67,78,83). This appears to be the result of several independent mechanisms. The first is a defective internalization of truncated receptors, which act in a dominant-negative manner over wild-type receptors in this regard (67,78,80). This is due to the combined loss of a

conserved di-leucine containing motif in Box 3 (31,78,80), and a less well defined motif located between residues 756 and 769 (31). However, other studies have shown that the receptor truncation interferes with several negative pathways. This includes the loss of recruitment sites for two members of the SOCS family, CIS (at Y729 and Y744) (30) and SOCS3 (at Y744) (31), the latter exacerbated by a 60% reduction in *SOCS3* transcripts caused by the decrease in STAT3 activation by truncated receptors (31). In addition, the docking sites for the receptor-associated tyrosine phosphatases, SHP-1 (at an undefined site in the C-terminus) (32) and SHP-2 (at Y744) (31) are lost, as are those for the inositol phosphatase, SHIP (at Y764 and Y744?) (30).

Consistent in all of these studies is that each mechanism impacts on the length of receptor activation, and particular of STAT5 (31,32,67,78,83), and pathways downstream of PI 3-K, such as Akt (28,84). Several laboratories have now shown that constitutive activation of STAT5 plays a key role in myeloid cell proliferation, including malignancy (82,85,86). Indeed, we have shown that dominant-negative STAT5 strongly inhibits the hyperproliferative function of truncated G-CSF-Rs (ACW et al., unpublished). Others have also shown that PI 3-K, MAPK and STAT3 play supporting role(s) in proliferation and/or survival (84,87), consistent with our observations (ACW et al., unpublished).

3.2. "Crippling" extracellular mutants

Around 10% of SCN patients do not respond to normal G-CSF treatment. In several of these patients mutations have been identified in the extracellular domain of the G-CSF-R that appear to be responsible. These mutations have in common the property of not only being defective themselves, but also crippling co-expressed wild-type receptor. The first of these mutations, Pro206His, converts a highly conserved proline that is part of a proline-rich "hinge" motif located between the N- and C-terminal "barrels" of the cytokine receptor homologous domain (88). When expressed in myeloid cells this mutant receptor was defective in both G-CSF-mediated proliferation and survival, which correlated with greatly diminished activation of the receptor complex, and altered dose-response properties. The mutant receptor showed a normal K_d of ligand binding, but a reduction in the number of binding sites per receptor, suggesting that the mutation perturbed the architecture of the ligand/receptor complex with severe consequences for intracellular signal transduction. It also suppressed the activity of co-expressed wild-type receptors in a dominant-negative manner (88). The second mutation, Δ322, represented a 182 bp deletion of the *CSF3R* gene in the region encoding the extracellular domain, commencing within the WSxWS motif. The resulting change in reading frame lead to a receptor that possessed around half of its normal extracellular sequence, followed by a novel sequence and a premature stop. This severely truncated receptor also acted in a dominant-negative manner to suppress wild-type responses (89). The third mutation, Δ319, resulted from a similar 191 bp deletion, extending 9 bp further upstream, producing a slightly more truncated receptor. This product was found to constitutively heterodimerized with the wild-type receptor, thereby affecting its trafficking and function (10). Finally, an extracellular mutation has been reported in a case of chronic idiopathic neutropenia, again involving a frameshift that truncated the intracellular domain of the receptor, although in this case it was after the fibronectin type III domains. This receptor was unable to signal in response to ligand. Interestingly, the patient went on to develop acute myeloid/natural killer cell leukemia, although whether the *CSF3R* mutation played a role in the latter was not determined (90). Collectively, this class of extracellular "crippling" mutations are consistent with studies showing that disruption of the *Gcsfr* gene in mice resulted in severely reduced neutrophil numbers (8,9). They also serve to further suggest a role of *CSF3R* mutations and aberrant G-CSF signaling in the etiology of SCN.

3.3. "Activating" transmembrane mutants

A study of 555 *de novo* AML patients revealed that two possessed activating Thr617Asn mutations in the transmembrane domain of the receptor. This mutation lead to growth factor-independent growth in Ba/F3 cells, including phosphorylation of the receptor, JAK2, STAT3 and ERK, apparently due to stabilisation of transmembrane helix-helix interactions in the absence of ligand (91). This class of mutation has parallels in the GM-CSF-R system (92).

3.4. Other mutants

Three other *CSF3R* mutations have been identified in MDS and *de novo* AML. The first is a three nucleotide deletion that changes Asn630Arg631 to Lys 630 in MDS. This leads to prolonged activation of signalling (93). Another is a SNP in the intracellular region, Glu785Lys, seen in 6% of the population, which shows a highly significant correlation with the development of high-risk MDS (94). Although the mechanism of action remains unknown, the receptor appears functional, but leads to a reduction in colony formation. Finally, the blasts of a *de novo* AML patient showed high expression of a new *CSF3R* splice variant, termed SD, in which the carboxyl terminus was altered due to a change in the reading frame, caused by a single base change adjacent to a cryptic splice-donor site involved in the alternative RNA splicing. (95). This variant was unable to transduce proliferation and maturation signals in murine cell systems. Furthermore, the primary AML blast cells of the patient failed to respond to G-CSF in proliferation assays *in vitro*, while the responsiveness to IL-3 or GM-CSF was maintained.

4. INDIRECT INVOLVEMENT OF THE G-CSF-R IN DISEASE

A number of studies have also described a role for altered G-CSF-R signalling as a contributing factor to a range of hematological malignancies. For example, expression of the *CSF3R* gene is increased by two oncogenic fusions, directly in the case of *E2A-Pbx1* (96), or indirectly (via C/EBPε) by *AML1-MTG8* (97). In the latter case, this lead to increased G-CSF-dependent proliferation (97). In addition proliferative responses of leukemia cells from CML in blast crisis or BCR-ABL-positive ALL are

frequently stimulated by G-CSF (98). G-CSF-R is also highly expressed in acute promyelocytic leukemias (APL), with APL cells predominantly proliferating in response to G-CSF treatment (99). It has also been reported that AML cells show a tendency for significantly increased levels of a normally minor *CSF3R* transcript, class IV (100), which encodes a maturation-deficient receptor form (95). The authors argue that the altered balance of class IV to normal (class I) receptors might contribute to AML, although only indirectly affecting proliferation, via its effects on maturation. These observations collectively suggest a direct role for G-CSF-R signalling in the perturbations of cell growth control observed in leukemogenic transformation. Interesting, the response of APL cells to G-CSF has been used to sensitise the cells to cell-cycle-dependent agents, as a therapeutic strategy (99), highlighting the importance of understanding the role played by cytokines and growth factors in disease.

The G-CSF-R also appears to play a role in malignant states of a non-hematological nature. Both G-CSF and its receptor are frequently expressed in ovarian cancers– possibly in a truncated form – leading to the potential involvement of autocrine and paracrine loops in over 90% of primary ovarian carcinomas (101,102). Expression of G-CSF and G-CSF-R appears to be an early event during malignant transformation in some bladder cancers (103). In addition, dysplastic and squamous cell carcinomas have been shown to exhibit higher G-CSF-R expression than normal controls (104,105). Expression of G-CSF was also increased in SCC (105), although in this case expression of G-CSF-R rather than its ligand correlated with poor prognosis, including survival and chance of relapse (106), suggesting paracrine activation is important. While the mode of action of G-CSF-R in these cases remains unclear, it is known to regulate expression of MMP-2 in head and neck carcinoma cell lines (107) and increase expression of β-integrin in bladder cancers (103), thereby contributing to adhesion and tissue invasion (103,107). This is of considerable clinical interest as G-CSF is used clinically to overcome neutropenic periods during chemotherapy for a range of cancers (45-47).

Finally, mutation of the gene encoding neutrophil elastase (NE), *ELA-2*, has been identified as an important mediator of both CN and SCN (69,70,108). However, there is considerable evidence that such mutations exert their effects, at least in part, via the G-CSF-R. Indeed, both G-CSF-R (109) and its ligand (109,110) are targets of NE. G-CSF is rapidly cleaved and rendered inactive by the enzyme (110), while expression of NE reduces surface expression of the receptor (109), leading to compromised G-CSF-stimulated viability and proliferative responses (109). Consistent with the "crippling" G-CSF-R mutations, these negative effects on G-CSF-R signaling could be a principal mediator of the neutropenia. Some have also argued that the mutant NE leads to reduced survival of cells of the neutrophil lineage, which can then be compensated by truncating G-CSF-R mutations that restore normal survival (40).

5. CONCLUSIONS

G-CSF therapy has proven to be an effective treatment in a range of life-threatening conditions. However, it is clear that altered signaling from the G-CSF-R – caused by mutation or misexpression – also contributes to several disorders. Importantly, many of these are malignant conditions in clinical settings where G-CSF may be administered. This is not to say that G-CSF treatment should be stopped in these settings. However, in each case, appropriate analysis of *CSF3R* mutations or misexpression is recommended to ensure that this information is factored into the judicious consideration of the most beneficial therapeutic option.

6. ACKNOWLEDGMENTS

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