Children with rare diseases of neutrophil granulocytes: from therapeutic orphans to pioneers of individualized medicine

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Neutrophil granulocytes are the most abundant immune cells in the blood yet the pathways orchestrating their differentiation and biological function remain incompletely understood. Studying (ultra-) rare patients with monogenetic defects of neutrophil granulocytes may open new horizons to understand basic principles of hematopoiesis and innate immunity. Here, recent insights into genetic factors controlling myelopoiesis and their more general role in biology will be presented in a clinical perspective. Advances in supportive care, first and foremost the use of recombinant human granulocyte-colony stimulating factor, has made a substantial difference for the quality of life and life expectancy of patients with congenital neutropenia (CN). Up to date, the only definitive cure can be provided by transplantation of allogeneic hematopoietic stem cells. The elucidation of the underlying molecular factors contributing to defective differentiation and function of neutrophil granulocytes nurtures new ideas of targeted individualized therapies.

Learning Objectives

- To understand the genetic heterogeneity of diseases associated with severe CN
- To translate knowledge on molecular pathomechanisms into novel targeted therapies

Neutrophil granulocytes and severe congenital neutropenia (SCN)

Neutrophil granulocytes were first identified by Paul Ehrlich: he described 3 different types of granulocytes, basophilic, neutrophilic, and eosinophilic polymorphonuclear cells.[1] Independently, Elie Metchnikoff discovered phagocytosis in starfish larvae and thus unraveled a principal functional capacity, which also characterizes neutrophil granulocytes.[2] Diseases related to dysfunction of neutrophil granulocytes were discovered around the turn of the 19th/20th century, when Philip King Brown first described a patient with lethal pharyngitis due to agranulocytosis.[3] Similar cases were reported thereafter,[4] many associated with the medical use of aminopyrines becoming available as a drug at that time. The first report of inherited neutrophil deficiency was published by the Swedish pediatrician Rolf Kostmann in 1950.[5]

SCN is defined as a condition with absolute neutrophil count of $<500/\mu L$, associated with invasive bacterial infections such as omphalitis, skin infections, pneumonia, or septicemia. Characteristically, sites of infection lack formation of pus. SCN must be distinguished from more common immune-mediated neutropenia, which usually is not associated with severe infections. Rolf Kostmann originally described "agranulocytosis infantilis hereditaria," now renamed SCN, as an autosomal recessive trait characterized by severe neutropenia and "maturation arrest" in the bone marrow (BM).[6,7] Clinical manifestations included omphalitis, skin infections, otitis, tonsillitis, abscesses, or sepsis. Children died in the first year of life. The use of antibiotics and recombinant human granulocyte colony-stimulating factor (G-CSF) has made a marked difference in the quality of life for patients with Kostmann disease. A recent review by Karl Welte discusses the development and clinical application of filgrastim, lenograstim, and other biosimilar G-CSFs.[8]

Thus, therapy with G-CSF has become a standard of care for many patients with SCN of various genetic subtypes and has greatly improved survival of affected patients.

Nevertheless, many patients continue to suffer from incomplete control of their disease, either related to nonhematopoietic symptoms, to insufficient immunity due to unresponsiveness of G-CSF therapy, or to secondary clonal hematopoietic disorders in association with somatic mutations.[9]

Patients with malignant clonal evolutions and patients nonresponsive to G-CSF are candidates for transplantation of allogeneic hematopoietic stem cells (HSCs). Fioredda et al have recently analyzed clinical data from the European Society for Blood and Marrow Transplantation to assess the outcome of 136 patients with SCN after allogeneic stem cell transplantation (SCT).[10] Overall survival after 3 years was 82%, and transplant-related mortality was 17%. A multivariate analysis showed that transplants performed in more recent years, in patients <10 years, and using fully-matched HLA donors, had a better outcome. Although these results document that SCN patients can be cured by allogeneic SCT with increasingly better results, morbidity and mortality of this approach remain considerable and require that patients to whom a transplant is offered must be carefully selected.[10]

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Table 1. Monogenetic defects associated with CN and novel therapeutic perspectives

Disease	MIM #	Gene	Inheritance	Predisposition to MDS/AML	Novel therapeutic ideas
SCN1	202700	*ELANE*	AD	+	NE inhibitors? Proteasome inhibitor?
SCN2	600871	*GFI1*	AD	+	Histone modifiers?
SCN3	605998	*HAX1*	AR	+	Gene replacement in HSC?
SCN4	611045	*G6PC3*	AR	?	?
SCN5	610035	*VPS45A*	AR	?	?
SCN6	616012	*JAGN1*	AR	+	GM-CSF?
SCN7	138971	*CSF3R*	AR	—	GM-CSF?
WHIM	193670	*CXCR4*	AD	—	Plerixafor Genetic recombination?

AD, autosomal dominant; AML, acute myeloid leukemia; AR, autosomal recessive; MDS, myelodysplastic syndrome; MIM, Mendelian inheritance in man.

In view of these limitations, alternative therapeutic approaches are warranted. Deeper knowledge on precise molecular pathomechanisms may provide clues toward the design of new therapeutic strategies. However, at this point in time, none of these perspectives have become clinical reality and it must be stated clearly that much more work has be to done before these ideas might even be tested in a clinical setting.

Genetic factors contributing to congenital neutropenia (CN)

Whereas the genetic etiology and pathomechanisms of SCN have remained elusive for a long time since the original description of the disease, in recent years a better molecular understanding of CN has been reached (Table 1). These insights have also stimulated a search toward the development of targeted therapies.

SCN1 (*ELANE*)

In their landmark studies, David Dale and Marshall Horwitz identified monoallelic mutations in the gene encoding neutrophil elastase (NE) (*ELANE*), both in patients with cyclic neutropenia and in patients with SCN1.[11] *ELANE*-mutated patients represent the largest subgroup of patients with SCN in the Western world.[12] NE belongs to the class of serine proteases, and is expressed exclusively in mature myelomonocytic cells and their committed immature precursor cells. Cells expressing a mutated *ELANE* allele induce an unfolded protein response (UPR); ie, a physiological rescue mechanism to prevent toxic effects by improperly folded proteins.

The UPR signal cascade is initiated by 3 endoplasmic reticulum (ER)-localized protein sensors: inositol-requiring 1α, double-stranded RNA-dependent protein kinase-like ER kinase, and activating transcription factor 6. In case these rescue mechanisms cannot prevent undue ER stress, cells undergo apoptosis. Neutrophils from patients with mutations in *ELANE* show increased signs of ER stress and apoptosis,[8,13,14] suggesting that ER stress is critically involved in the pathophysiology of SCN associated with mutations in *ELANE*.

The use of new in vitro models, such as induced pluripotent stem (iPS)-derived differentiation of neutrophil granulocytes, has facilitated investigations of *ELANE*-mutated neutrophils. *ELANE* point mutations result in promyelocyte death and differentiation arrest, associated with neutrophil elastase mislocalization and activation of the UPR/ER stress in iPS-derived neutrophil granulocytes.[15] Sivelestat, a pharmacologic inhibitor of NE currently used in the treatment of acute respiratory failure, has been shown in vitro to correct dysgranulopoiesis by restoring normal intracellular NE localization in primary granules, to ameliorate UPR/ER stress, and to promote promyelocyte survival and differentiation.[15]

Another mechanism for *ELANE*-mediated neutropenia, at least in a subset of patients, has recently been proposed by Tidwell et al.[16] When the canonical initiation codon is mutated, downstream ATGs are used as initiators for translation, leading to truncated variants of NE, which are no longer routed to the ER and granules but instead remain in the cytoplasm.

Various genetic subtypes of SCN, including *ELANE*, may be related to decreased expression of transcription factor lymphoid enhancer-binding factor 1 (*LEF-1*) in myeloid progenitor cells.[17] In vitro data that show that bertezumib, the first member of proteasome inhibitors and currently licensed for therapeutic use in multiple myeloma, inhibits STAT5-dependent degradation of LEF-1. Bortezomib also reverses the defective G-CSF–triggered granulocytic differentiation of $CD34^+$ cells from CN patients and increased LEF-1 protein levels.[18] Whether proteasome inhibitors may be interesting pharmacologic agents for selected patients with CN remains to be shown in further studies.

SCN2 (growth factor independent-1 [*GFI1*])

GFI1 is a zinc finger transcription factor and acts as a transcriptional repressor by recruiting histone-modifying enzymes to promoters and enhancers of target genes. Similar to mutations in *ELANE, GFI1* mutations are inherited in an autosomal dominant pattern. Five distinct mutations have been identified in individuals with CN.[19]

These mutants generate dominant-negative variants of GFI1 and thus interfere with transcriptional networks on multiple target genes and regulatory RNAs. As a consequence, patients show not only a severe maturation arrest of myeloid cells, but also aberrations in monocytoid and lymphoid cells. No new therapeutic concepts have been developed.

SCN3 (HCLS1-associated protein X-1 [*HAX1*])

In view of the dominant inheritance patterns of *ELANE* and *GFI1* mutant alleles, these factors could be excluded as causative genes in the original "Kostmann pedigree." When loss-of-function mutations in the gene encoding *HAX1* were discovered, a more than 50-year-old enigma on the etiology of Kostmann disease has been solved.[20] The term "Kostmann disease" is now reserved for SCN patients with mutations in *HAX1*.

HS1-associated protein X1 (ie, HAX1) was originally discovered as a protein interaction partner of HCLS1, a kinase involved in B-cell receptor signal transduction.[21] HAX1 predominantly localizes to mitochondria, but can also be found at the nuclear membrane and the

ER. Translocation from mitochondria to the plasma membrane has been documented in the presence of various HAX1-binding partners. In *Hax1*-deficient mice, early cell death in the neuronal and lymphocytic system was found, suggesting a specific antiapoptotic role for HAX1. Interestingly, neutrophil granulocytes appear normal in *Hax1*-deficient mice, pointing toward species-specific differences between mice and humans.

A genotype–phenotype association linking the presence of neurologic dysfunction to the lack of expression of isoform B, preferentially expressed in neuronal cells, has been postulated.[22] Mutations exclusively affecting isoform A are associated with CN, whereas mutations affecting both isoforms A and B result in a combined hematologic and neurologic disease.[22,23]

Several explanations on the function of the HAX1 protein have been proposed. HAX1 stabilizes the mitochondrial membrane potential ($\Delta\Psi_m$) in neutrophils.[20] Biochemical studies in the *Hax1*-knockout mice have also shown a role for HAX1 in maintaining viability. The mitochondrial proteins high-temperature–related A2 (HTRA2, also known as Omi) and presenelin-associated, rhomboid-like are interaction partners of HAX1.[24] HAX1 may thus facilitate the processing of HTRA2 by presenelin-associated, rhomboid-like protein. Upon processing of HTRA2, the mature enzyme is released and displays its antiapoptotic function. HAX1 is also subject to cleavage by granzyme B.[25] Whereas the *N*-terminal cleavage product localizes to mitochondria and mediates depolarization, the C-terminal cleavage product can be found in the cytosol.[25]

HAX1 may interact with X-linked inhibitor of apoptosis, preventing polyubiquitination of X-linked inhibitor of apoptosis 1 and thus act to maintain viability.[26]

In addition to a prominent role in protecting against cell death, HAX1 has also been reported to be involved in multiple other cellular functions, such as calcium homeostasis, cell motility and cytoskeletal rearrangement, and messenger RNA processing.[27] To date, it remains challenging to unify these diverse functions of HAX1 in a simple concept.

HAX1 is also involved in B-cell neoplasms, as recently shown by Baumann et al. The group analyzed patients with mantle cell lymphomas and found frequent mutations in the gene encoding the orphan F-box protein FBXO25, a protein mediating phosphorylation-dependent ubiquitination.[28] Mechanistically, HAX1 is being directed toward degradation by FBXO25 via the proapoptotic protein kinase C δ. In the absence of FBXO25, this axis is perturbed and HAX1 accumulates in lymphoma cells. HAX1 may thus represent a novel target for patients with mantle cell lymphomas. HAX1 has also been highlighted as a biomarker in cancer patients, and expression of HAX1 in colorectal cancer is associated with malignant progression and poor prognosis.[29]

From a therapeutic point of view, G-CSF remains the first treatment choice for patients with *HAX1* deficiency. Most patients respond to this therapy with a normalization of peripheral neutrophil counts. However, like in *ELANE*-mutated hematopoiesis, there is an increased long-term risk of myelodysplastic syndrome/acute myeloid leukemia development.

HAX1 deficiency can, in principle, be corrected by transgenic expression of HAX1. Because *Hax1*-deficient mice are not neutropenic, other models, such as iPS-derived neutrophils, must be used to test this approach. In fact, lentivirus-mediated *HAX1* gene transfer into patient-derived induced pluripotent stem cells has been shown to reverse disease-related aberrations of granulopoiesis.[30] HSC gene therapy might become a future option for those *HAX1*-deficient patients who do not suffer from neurologic deterioration, pending further preclinical and clinical studies that are balancing the potential risks and benefits. No curative approach for the neurologic symptoms, ranging from mild cognitive impairment to severe epilepsy and progressive neurologic deterioration, is currently in sight.

SCN4 (*G6PC3*)

Neutrophil granulocytes critically depend on glucose metabolism, as highlighted by human defects in *G6PC3*, a ubiquitously expressed homolog of glucose-6-phosphatase.[31] *G6PC3* deficiency causes CN in conjunction with variable structural defects or the cardiovascular and urogenital system, as well as inner ear deafness and growth failure. The complexity of the clinical picture of this disease is increasing. Intermittent thrombocytopenia, bone deformities, immune dysregulation associated with colitis,[32] and other symptoms have been described.[33,34] *G6PC3* deficiency is usually highly responsive to moderate doses of G-CSF and the evolution of malignant clonal disorders appears to be exceptionally rare. Therefore, the urgency to develop alternatives to the standard of G-CSF therapy is less in comparison with other variants of SCN.

SCN5 (vacuolar protein sorting 45A [*VPS45A*])

In 2013, a new syndrome associating refractory CN, neutrophil dysfunction, progressive BM fibrosis, and nephromegaly was described.[35] The disease is caused by biallelic mutations in *VPS45A*, a regulator of endosomal function. Specifically, VPS45 controls the assembly of the soluble *N*-ethylmaleimide–sensitive factor attachment protein receptor complex. VPS45 plays a role in protein and membrane trafficking through the endosomal system, and defective VPS45 function leads to defective neutrophil function such as perturbed maturation, decreased motility, and increased apoptosis. Several pathways are affected in VPS45-dependent lysosomal dysfunction, such as the β1 integrin expression on the cell surface. *VPS45*-deficient patients do not respond to G-CSF. In several patients, a curative attempt of allogeneic SCT has been frustrating, but a recent report documents that this approach has been successfully applied at least in 1 patient.[36]

SCN6 (Jagunal homolog 1 [*JAGN1*])

A recent asset to the growing spectrum of CN disorders are patients with loss-of-function mutations in *JAGN1*.[37] Using homozygosity mapping and exome sequencing, 9 distinct homozygous mutations were identified in a subgroup of patients with SCN. Granulocytes with biallelic mutations in *JAGN1* are characterized by ultrastructural defects, a paucity of granules, aberrant *N*-glycosylation of multiple proteins, and increased incidence of apoptosis. JAGN1 participates in the secretory pathway. Located in the ER, JAGN1 is critical for membrane/protein transport from the ER to the Golgi. In the absence of JAGN1, many glycoproteins are not properly glycosylated, including the G-CSF receptor CSF3R. Therefore, aberrant cell surface expression of CSF3R leads to refractoriness of *JAGN1*-deficient patients to G-CSF.

Mice carrying a hematopoietic lineage-specific deletion of *Jagn1* (encoding JAGN1) also show defects in neutrophil granulocytes.[38] They cannot mount an efficient neutrophil-dependent immune response to the human fungal pathogen *Candida albicans*. In contrast to therapy with G-CSF, treatment with granulocyte-macrophage colony-stimulating factor (GM-CSF) protected mutant mice from

increased weight loss and accelerated mortality after *C albicans* challenge. Of note, GM-CSF also restored the defective fungicidal activity of BM cells from *JAGN1*-deficient SCN patients. Studies are under way to determine whether *JAGN1*-mutant patients may respond to GM-CSF therapy in vivo.

SCN7 (*CSF3R*)

When early studies revealed mutations in the gene encoding the G-CSF receptor (*CSF3R*) in patients with SCN, some have postulated that dysfunctional G-CSF receptor underlies the phenotype of CN. However, subsequent studies have clearly shown that this hypothesis was not correct, because mutations in *CSF3R* were somatically acquired and not inherited.[5] Many years later, in 2014, the first 2 families with recessively inherited loss-of-function mutations in *CSF3R* were described.[39] Despite peripheral neutropenia, all patients had morphologic evidence of full myeloid cell maturation in BM. None of the patients responded to treatment with recombinant human G-CSF. In an unrelated study, Klimiankou et al reported a third patient with biallelic mutations in *CSF3R* who responded to GM-CSF,[40] which deserves to be tested as an alternative mode of therapy for this rare subgroup of SCN patients. Three affected children in one of these families carried a homozygous missense mutation (NM_000760.3:c.922C>T, NP_000751.1:p.Arg308Cys), which resulted in perturbed *N*-glycosylation and aberrant localization of the G-CSF receptor to the cell surface. Of note, this mutation in close proximity to the conserved WSXWS motif in class I cytokine receptors, is reminiscent of mutations seen in patients with loss-of-function mutations in other class I cytokine receptors such as the growth hormone receptor or interleukin 21 receptor.[39] Ongoing studies address the question whether pharmacologic chaperones are capable of redirecting mutated receptors to the cell surface in an attempt to (partially) compensate for the effects of improper folding and glycosylation.

Warts, hypogammaglobulinemia, infections, and myelokathexis (WHIM) syndrome (*CXCR4*)

WHIM syndrome represents a primary immunodeficiency disorder associating warts, hypogammaglobulinemia, infections, and myelokathexis. Patients may come to clinical attention because of documentation of a significant decrease of neutrophil granulocytes in the peripheral blood. In contrast to patients with SCN, WHIM syndrome is characterized by marked cellular hyperplasia of granulocytic cells in the BM. These cells show cytoplasmic vacuolization, hypersegmented and chromatin hypercondensation (a phenotype termed "myelokathexis"), and is consistent with defective release and trafficking of neutrophil granulocytes. In addition to the myeloid lineage, B cells and T cells are equally affected. WHIM syndrome is caused by gain-of-function mutations in the G-protein–coupled chemokine receptor CXCR4. CXCR4 is a G-protein–coupled receptor that is activated upon cognate binding to stromal-derived factor (SDF1 or CXCL12). As a consequence, hetero-trimeric Gi proteins are activated, and a signal cascade involving downstream molecules such as AKT and extracellular signal-regulated kinases 1/2 triggers hematopoietic stem homing to the BM and retention of neutrophil granulocytes.

An attractive option for a targeted therapy became available when plerixafor, a potent CXCR4 inhibitor originally identified because of its anti-HIV activity, entered the pharmacologic scene as release agent for HSCs. By virtue of interfering with the SDF1-CXCR4 axis, plerixafor, in conjunction with G-CSF, releases HSCs into the peripheral blood. Early reports have shown that plerixafor reversed leukopenia in WHIM patients.[41,42] More recently, a phase 1 clinical trial of long-term, low-dose therapy of WHIM syndrome patients with plerixafor showed lack of toxicity and durably increased circulating leukocytes. The concept of targeted CXCR4 inhibition in WHIM patients warrants further clinical studies.

Recently, McDermott et al have reported on a serendipitous observation with therapeutic implications for WHIM syndrome patients. Chromothripsis, large-scale chromosomal recombination events associated with cancer, may rarely be associated with loss of dominant negative alleles and thus have beneficial effects. The authors describe the case of a WHIM syndrome patient with a deletion of the disease allele, *CXCR4(R334X)*, as well as other genes from one copy of chromosome 2 in an HSC, which was found to repopulate the myeloid but not the lymphoid lineage. This recombination event led to a spontaneous cure of the immunodeficiency.[43]

In conclusion, the molecular diversity of genetic diseases associated with immunodeficiency related to paucity and dysfunction of neutrophil granulocytes continues to increase. Novel insights into disease mechanisms are not only relevant for genetic diagnosis and counseling of patients, but also have the potential to develop new ideas for targeted therapies.

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