

Review

# The Role of Apoptosis in the Pathophysiology of Chronic Neutropenias Associated with Bone Marrow Failure

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## ABSTRACT

Chronic neutropenia syndromes associated with bone marrow (BM) failure comprise distinct congenital and acquired hematologic disorders with varying degree of neutropenia due to decreased or ineffective BM neutrophil production. Recent evidence suggests that defective granulocytopoiesis in these neutropenia states is a consequence of accelerated apoptotic cell death of BM myeloid progenitor cells and/or their differentiated progeny. Inherited or spontaneously appearing mutations in the *ELA2* gene encoding for neutrophil elastase have been implicated in the accelerated apoptotic process of the BM myeloid cells in patients with cyclic and severe congenital neutropenia. A disturbed balance between pro-apoptotic and anti-apoptotic intracellular or membrane molecules such as down-regulation of the *bcl-2* family members or up-regulation of the death receptor Fas, have been implicated in neutropenia associated with myelokathexis, Shwachman-Diamond syndrome and acquired chronic idiopathic neutropenia of adult. In this review we summarize the available evidence suggesting that abnormally increased apoptosis and impaired proliferative and differentiating properties of neutrophil progenitor and precursor cells represent a common pathogenetic mechanism for impaired granulocytopoiesis in both acquired idiopathic and congenital neutropenia states. The underlying distinct cellular and molecular abnormalities and the role of the BM microenvironment are extensively analysed.

## INTRODUCTION

Neutrophil granulocytopoiesis is the process of proliferation, differentiation and expansion of bone marrow (BM) hematopoietic progenitor and precursor cells into functional mature neutrophils. Mechanisms promoting and regulating the multi-step process of normal granulocytopoiesis in vivo remain still largely speculative. Local regulatory events include hematopoietic-to-stromal cell interactions, activation of colony-stimulating factors and their cognate receptors, and up-regulation of specific transcriptional factors that rescue myeloid cells from apoptosis and direct myeloid development to neutrophil differentiation.<sup>1,2</sup> A regulatory negative feedback effect of peripheral blood neutrophils on BM granulocytopoiesis has also been proposed.<sup>3-5</sup>

While the exact mechanisms that govern normal granulocytic development are still not well understood, there have recently been remarkable advances in the identification of the cellular, genetic and molecular mechanisms implicated in the pathophysiology of chronic neutropenia syndromes associated with BM failure. These neutropenia states include distinct congenital and acquired hematologic disorders with varying degree of neutropenia due to decreased or ineffective BM neutrophil production. The most severe cases comprise patients with congenital disorders of granulocytopoiesis<sup>6-8</sup> while acquired idiopathic cases may display neutropenia of varying severity.<sup>9,10</sup>

A series of studies have shown that these neutropenia syndromes display overlapping pathophysiologic features and there is strong evidence suggesting that accelerated apoptosis of BM granulocytic progenitor cells is the central mechanism responsible for the underlying faulty granulocytopoiesis.<sup>11</sup> Mutations in the gene encoding for neutrophil elastase or other constituents of neutrophil granules,<sup>5,12-15</sup> downregulation of anti-apoptotic molecules such as the *bcl-2* family members in the granulocytic progenitors<sup>7</sup> or upregulation of the pro-apoptotic death receptor Fas as a result of an inhibitory BM microenvironment effect on granulocytopoiesis<sup>16,17</sup> are recently proposed apoptosis-triggering mechanisms in patients with congenital or acquired neutropenias.

In this review, we summarise the recently described cellular and molecular abnormalities implicated in the pathogenesis of these neutropenia syndromes. By elucidating the pathogenetic mechanisms underlying these specific disorders we may clarify further the mechanisms that govern normal myelopoiesis.

## CLINICAL SYNOPSIS OF CHRONIC NEUTROPENIA SYNDROMES

As mentioned above, chronic neutropenia syndromes associated with BM failure comprise congenital and acquired disorders of neutrophil production. The congenital neutropenias are rare hematological diseases usually characterized by extremely low levels of circulating neutrophils predisposing patients to recurrent severe infections, oropharyngeal ulcers, gingivitis, malaise, and fever.<sup>15,18-20</sup> They may occur both sporadically and as autosomal inherited disorders and comprise distinct disease entities such as cyclic neutropenia (CN, also known as cyclic hematopoiesis),<sup>6,21</sup> severe congenital neutropenia (SCN, also known as Kostmann infantile agranulocytosis),<sup>20,22</sup> Schwachman-Diamond syndrome (SDS)<sup>8,23</sup> and myelokathexis.<sup>7,24</sup> They are commonly characterized by impaired formation and reduced delivery of neutrophils from the BM to the periphery but display distinctive BM morphological features. Cyclic neutropenia is typically characterized by waves of myeloid cell formation in the BM resulting in periodic oscillations in the number of circulating neutrophils<sup>25</sup> while SCN is a static neutropenia characterized by invariable maturation arrest of BM myeloid cells at the (pro)myelocyte stage of differentiation.<sup>19</sup> A varying degree of BM hypoplasia is also found in SDS.<sup>16</sup> Myelokathexis, unlike the other forms of congenital neutropenias, is characterized by BM hyperplasia of granulocytic series with degenerative changes and hypersegmentation of mature neutrophils resulting in increased intramedullary cell death.<sup>7</sup>

The most common form of acquired chronic neutropenia associated with BM failure is chronic idiopathic neutropenia (CIN) of adults.<sup>9,10,17,26</sup> The disorder can be identified among the other types of acquired chronic neutropenias by the absence of phasic variations in neutrophil counts, lack of clinical and laboratory evidence for any underlying systemic disease, absence of any drug relationship and negative tests for anti-neutrophil antibodies.<sup>10,27</sup> It displays a female predominance and an HLA class II genetic predisposition.<sup>28,29</sup> Unlike the above congenital neutropenia states, CIN is characterized by low incidence of severe infections.<sup>30</sup> Bone marrow examination reveals a varying degree of myeloid hypoplasia affecting mainly the postmitotic, maturing pool of the granulocytic series and mild dysplastic features of the erythroid and megakaryocyte lineages.<sup>31,32</sup>

Longitudinal studies have shown that patients with BM failure-associated neutropenia syndromes display increased risk of developing myelodysplasia or acute myeloid leukaemia (AML).<sup>33,34</sup> Such evolution occurs mainly in patients with SCN and SDS while no risk has been recognized for patients with CN. The malignant transformation in SCN is associated with mutations of the gene encoding for the granulocyte colony stimulating factor receptor (G-CSF-R),<sup>35-39</sup> the oncogene *Ras* and monosomy of chromosome 7,<sup>40</sup> while overexpression of p53 protein has been reported to precede the clonal evolution in SDS.<sup>41</sup> Although a relationship between the widely used G-CSF treatment in these patients and evolution to AML has not been entirely excluded,<sup>42-45</sup> inferential evidence based mainly on clinical data from the Severe Chronic Neutropenia International Registry (SCNIR), suggests that G-CSF accelerates rather than induces a malignant propensity.<sup>19,33,34,45,46</sup> Consistent with this assumption are recent reports suggesting that patients with CIN, never treated with G-CSF, display also a tendency for malignant myeloid transformation.<sup>47,48</sup> It is, therefore, likely that progression into AML is a part of the natural history of the BM failure-associated neutropenia syndromes. Presumably, the increased compensatory influx of cells from the stem cell compartment in response to the impaired neutrophil production may result in a stem cell population more vulnerable to leukemic.<sup>15</sup>

## CELLULAR AND MOLECULAR STUDIES

A common cellular defect in the above neutropenia states is the varying degree of maturation arrest of the myeloid development suggesting increased intramedullary cell death due to an intrinsic cell defect and/or to a faulty marrow microenvironment. Since apoptosis has been emerged as a central mechanism responsible for the ineffective hematopoiesis in acquired and inherited BM failure syndromes such as myelodysplastic syndromes,<sup>49,50</sup> Diamond-Blackfan<sup>51</sup> and Fanconi<sup>52,53</sup> anemia, a number of studies have probed the role of apoptosis in the pathophysiology of BM failure-associated neutropenia disorders.

**Cyclic Neutropenia.** By analyzing a mathematical model of white blood cell production, Mackey et al have shown that a moderate increase of apoptosis rate in the hematopoietic stem cell compartment might be responsible for the oscillations of hematopoiesis in CN resulting in periodic fluctuations in neutrophil counts.<sup>25,54</sup> The proposed model was focused on giving a plausible explanation for the induction of cyclic hematopoiesis without probing deeper into the underlying intrinsic, local or peripheral defect(s) triggering stem cell apoptosis. Subsequent studies by Aprikyan et al who analysed the survival characteristics of BM myeloid progenitor and precursor cells in CN by means of electron microscopy, culture assays and flow cytometry, have shown accelerated apoptotic cell death in all stages of granulocytic differentiation from the early CD34<sup>+</sup> progenitor cells to the myeloid-committed CD33<sup>+</sup> and the more mature CD15<sup>+</sup> precursor cells, regardless of the stage of the neutropenia cycle.<sup>55</sup>

The identification of mutations in the *ELA2* gene encoding for neutrophil elastase in all cases of CN has recently provided new insights in the pathophysiology of the disease.<sup>12,56,57</sup> Neutrophil elastase is a 218-amino acid chymotryptic serine esterase synthesized early in the myeloid development, during the myeloblast to promyelocyte transition, and is processed through the Golgi apparatus before packing in the active form in the primary azurophil cytoplasmic granules.<sup>58</sup> It is released from neutrophils at sites of inflammation to inhibit matrix proteins, clotting and complement factors and is mainly degraded by the serpine  $\alpha_1$ -antitrypsin.<sup>59</sup> The *ELA2* gene is located in chromosome 19p13.3 and consists of five exons and four introns, spanning approximately 5000 base pairs of genomic DNA.<sup>13</sup> Mutational analysis in inherited and sporadic cases of CN has shown heterozygous substitution, deletion or insertion mutations clustered in intron 4 and exon 5, suggesting a dominant-negative effect of mutant neutrophil elastase product.<sup>13,56</sup> Although the mechanism by which the mutant enzyme contributes to the pathophysiology of CN remains still elusive, it has been suggested that the mutant elastase display resistance to serine protease inhibitors and/or alterations in its substrate specificity and might, thus, be more active in degrading vital intracellular or BM microenvironment proteins resulting in, directly or indirectly, apoptotic death of BM hematopoietic cells.<sup>13,60</sup> The recent finding that elastase may regulate granulocytogenesis by digesting G-CSF produced by BM mononuclear cells corroborates this assumption; the resistant to inhibition mutant enzyme might affect BM granulocytic cell survival by locally destroying G-CSF and thus promoting apoptosis.<sup>4</sup> An alternate hypothesis is that mutations in the *ELA2* gene might disrupt regulatory elements required for the expression of neighbouring genes encoding for other enzymes of neutrophil granules such as azurocidin and proteinase 3, contained in the same locus and known to have a functional effect on granulocytogenesis.<sup>61,62</sup>

**Severe Congenital Neutropenia.** Impaired survival and abnormal cell cycle progression of the BM myeloid cells has been reported in patients with SCN.<sup>11,57</sup> In particular, BM derived CD34<sup>+</sup>, CD33<sup>+</sup> and CD15<sup>+</sup> cells from SCN patients display morphological characteristics of early apoptotic cells in electron microscopy and contain increased proportion of apoptotic cells in flow cytometry analysis.<sup>63</sup> Consistent with these findings is the low proportion of CD34<sup>+</sup> cells in the proliferative S-phase of the cell cycle compared to healthy controls and the impaired clonogenic potential of the CD34<sup>+</sup> cells in BM culture assays.<sup>63,64</sup> The previously reported reduced responsiveness of patient myeloid progenitor cells to cytokines including G-CSF might be responsible for the increased susceptibility of these cells to apoptosis.<sup>65-68</sup>

Recent evidence, however, suggests that a significant proportion of SCN patients harbor inherited or acquired mutations in the ELA2 gene.<sup>13,14</sup> The currently described mutations are spread throughout the entire gene and consist of amino acid missense substitutions, in-frame deletions or insertions, and protein truncating mutations of the carboxyl terminus resulting from nonsense substitutions and deletions leading to frameshifts.<sup>69</sup> Interestingly, the same mutations have been occasionally described in both SCN and CN patients.<sup>70</sup> Molecular modelling and three-dimensional analysis of the neutrophil elastase tertiary structure, however, have shown that unlikely CN in which mutations are predominantly located around the binding pocket of the active site, mutations in SCN are usually located around the glycosylation sites of the molecule and may result in abnormal processing of the mutant protein. In either case the mutant elastase seems likely to affect the survival of the granulocyte progenitor cells. In favor of this hypothesis are recent findings by Aprikyan et al demonstrating accelerated apoptosis of human HL-60 promyelocytes upon transient transfection with mutant elastase cDNA compared to HL-60 cells transfected with normal elastase cDNA.<sup>70</sup> The fact that the elastase mutants in the transfected cells have been previously identified in both SCN and CN patients confirms further the implication of ELA2 mutations in the pathophysiology of both disease states by activating the apoptotic process. The differences in the phenotype may simply reflect the differences in the relative rates of apoptosis of the early granulocyte progenitor cells in CN and SCN.<sup>15,57</sup>

**Myelokathexis.** Impaired granulocytopoiesis in patients with myelokathexis is associated with hypercellular BM with presence of bizarre hypermature neutrophils with cytoplasmic vacuoles, nuclear hypersegmentation with condensed and connected by stringy filaments lobes, features suggestive of apoptosis.<sup>71</sup> Recent evidence, however, suggests that not only BM neutrophils but also their BM progenitor and precursor cells display profound apoptotic features.<sup>7</sup> In particular, electron microscopy studies have revealed that BM myeloid-committed precursor cells from patients with myelokathexis undergo degenerative changes characterized by cytoplasmic membrane blebbing, granule aggregation, cytoplasmic vacuolisation, and intensive condensation of heterochromatin in the nucleus. In keeping with the morphological features are data from flow cytometric analysis suggesting increased proportion of apoptotic cells within the patient BM CD34<sup>+</sup>, CD33<sup>+</sup> and CD15<sup>+</sup> progenitor, early and late precursor cells, respectively, and results from clonogenic assays suggesting reduced colony forming potential of patient CD34<sup>+</sup> cells probably due to their contamination by apoptotic cells. Interestingly, it has been estimated by means of a simple mathematical model that the apoptosis rate within the CD15<sup>+</sup> precursor cell compartment is about 17 times greater in myelokathexis patients than the normals.<sup>72</sup>

The underlying molecular defect responsible for myelokathexis

remains unknown. It has been recently suggested that accelerated apoptosis of granulocytes in patients' BM is due to downregulation of the anti-apoptotic *bcl-x* gene expression in the granulocyte precursor cells.<sup>7</sup> It remains unclear, however, whether the downmodulation of *bcl-x* is a primary defect in the disease or is the consequence of alterations in other genes that are primarily involved in the pathogenesis of myelokathexis and potentially affect *bcl-x* expression in patient BM granulocyte precursor cells.

**Shwachman-Diamond Syndrome.** BM failure in this genetically undefined multisystemic disorder is associated with an intrinsic hematopoietic progenitor cell abnormality and a faulty marrow microenvironment.<sup>8,16,73</sup> Specifically, it has been shown low frequency and decreased clonogenic potential of SDS BM CD34<sup>+</sup> cells due to accelerated apoptotic cell death. Experimental evidence has demonstrated that increased propensity of hematopoietic progenitor cells to apoptosis is mainly due to Fas antigen overexpression and Fas signaling hyperactivation in patient CD34<sup>+</sup> cells. Interestingly, Fas upregulation is evident in all stages of granulocytic differentiation, from the early progenitors to the mature neutrophils, and thus seems likely to be largely involved in patients' neutropenia.<sup>16</sup> However, it remains unclear whether this abnormality is a sequel of the underlying genetic defect or is the consequence of a consistent inhibitory effect of patients' BM microenvironment.

Indeed, data from crossover long-term BM culture (LTBMC) experiments from SDS patients and healthy controls have demonstrated impaired capacity of patient stromal layers to sustain not only the autologous but also the normal hematopoiesis, suggesting a primary BM microenvironment defect in addition to the hematopoietic progenitor cell abnormality in SDS.<sup>73</sup> Although the mechanisms responsible for the defective BM microenvironment in SDS have not been extensively studied, previous investigations have shown that stromal-derived inhibitory cytokines such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and interferon- $\gamma$  (IFN $\gamma$ ) may induce Fas-mediated hematopoietic progenitor cell apoptosis in BM failure associated with aplastic anemia and autoimmune systemic diseases.<sup>74-81</sup> Since, however, a consistent stromal cell defect has not been previously reported in inherited BM failure syndromes other than SDS, further investigation is required to identify the basis of the stroma impairment in the disease and its possible relationship to the apoptotic depletion of patient hematopoietic progenitors.

**Chronic Idiopathic Neutropenia.** Impaired BM granulocytopoiesis has been proposed as a possible pathogenetic mechanism in CIN.<sup>31,82-84</sup> We have recently investigated the BM myeloid cell reserve and function at sequential stages of granulocytic development, from the early progenitors to the mature neutrophils and also the influence of BM microenvironment on myelopoiesis in patients with CIN by means of flow cytometry and *in vitro* BM cultures.<sup>17</sup> We have shown that impaired granulocytopoiesis in CIN is associated with low frequency of granulocyte committed progenitor cells, namely the CD34<sup>+</sup>/CD33<sup>+</sup> cells and granulocyte colony forming units (CFU-G), due to accelerated apoptosis in this particular cell compartment. Unlike patients with congenital neutropenias, CIN patients display normal rate of spontaneous apoptosis in the CD34<sup>+</sup>/CD33<sup>+</sup> and CD33<sup>+</sup>/CD15<sup>+</sup> mature stages of granulocytic differentiation and the peripheral blood neutrophils. Furthermore, despite the low serum elastase levels reflecting probably the low peripheral neutrophil counts, no mutations in the ELA2 elastase encoding gene were identified in CIN as anticipated, since potential elastase abnormalities seems unlikely to mediate apoptosis restricted to the CD34<sup>+</sup>/CD33<sup>+</sup> progenitor cell compartment.<sup>85</sup>

We have also shown that patient CD34<sup>+</sup>/CD33<sup>+</sup> cells display markedly increased Fas antigen expression, in comparison to normal subjects, and that the rate of apoptosis is significantly higher among the Fas<sup>+</sup> than the Fas<sup>-</sup> cells suggesting that Fas upmodulation is actively involved in the apoptotic depletion of patient granulocyte progenitors. In favor of this hypothesis is the normal Fas expression in the primitive BM CD34<sup>+</sup>/CD33<sup>-</sup> cells, the mature CD34<sup>+</sup>/CD33<sup>+</sup>, CD33<sup>-</sup>/CD15<sup>+</sup> cells and the peripheral blood neutrophils of CIN patients.

LTBMC stromal layers from the patients produce abnormally high amounts of TNF $\alpha$ , IFN $\gamma$  and Fas-Ligand and fail to support normal myelopoiesis. It has, therefore, been postulated that Fas upregulation and subsequent apoptotic depletion of granulocyte progenitors in CIN is due to overproduction of inflammatory cytokines by immune cells within the BM microenvironment. The precise origin of TNF $\alpha$ -, IFN $\gamma$ - and Fas-Ligand producing cells, however, the possible association of the BM immune dysregulation with the previously described HLA class II genetic predisposition,<sup>29</sup> and the cause for the selective inhibition of the myeloid development remain to be elucidated.

## PERSPECTIVES AND PRACTICAL ISSUES

Although many aspects on the pathophysiology of congenital and acquired idiopathic neutropenia states are still poorly understood, data from experimental studies reviewed herein provide evidence that intamedullary apoptotic death of granulocyte progenitor cells and/or their marrow progeny is a central shared feature underlying neutropenia in these disorders. A number of investigations are currently focused on identification of the molecular abnormalities underlying the cellular defect in these neutropenia syndromes and definition of their potential impact on the biochemical and signaling intracellular pathways of the granulocytic development. Recently, mutations in the *WASp* gene, ordinarily leading to the Wiskott-Aldrich syndrome characterized by thrombocytopenia and immunodeficiency, have been recognized to be causative for rare forms of SCN<sup>86</sup> while mutations in the *Gfi-1* proto-oncogene mainly involved in T-cell differentiation and lymphoma development, have been identified in isolated patients with SCN and CIN.<sup>5,87</sup> Interestingly, both genes have a putative role in neutrophil granule formation.<sup>87,88</sup> Therefore, the list of genes involved in the neutropenia process is continually extended. From the practical point of view, identification of the precise molecular and cellular events regulating impaired neutrophil production may lead to the development of new therapeutic approaches for the severe neutropenia disorders.

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