SEVERE CONGENITAL NEUTROPENIA: GENETICS AND PATHOGENESIS

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ABBREVIATIONS: ANC, absolute neutrophil count; ELA2, elastase gene; AML, acute myeloid leukemia; MDS, myelodysplasia; G-CSF, granulocyte-colony stimulating factor; PI, propidium iodide; RT-PCR, reverse transcriptase; EGFP, enhanced green fluorescence protein; FBS, fetal bovine serum; NE, neutrophil elastase; SCN, severe congenital neutropenia; FACS, fluorescent activated cell sorter; SCNIR, Severe Chronic Neutropenia International Registry

ABSTRACT

Severe congenital neutropenia (SCN) is a rare hematological disease characterized by a selective decrease in circulating neutrophils, bone marrow maturation arrest at the promyelocyte stage, and occurrence of infections. Studies of SCN patients revealed impaired proliferative characteristics and accelerated apoptosis of bone marrow progenitor cells. Sequencing analysis indicated a heterozygous deletion or substitution mutations in the neutrophil elastase (NE gene) in many patients. Expression of NE mutants but not normal NE accelerated apoptosis of human HL-60 progenitor cells similar to that seen with actual patient cells. Our data indicate that impaired survival of bone marrow myeloid progenitor cells arises from expression of mutant NE which contributes to neutropenia in SCN. In an unusual clinical set of studies during which SCN developed in five children from four families impregnated by the same sperm donor, we identified the responsible donor by employing genomic DNA studies.

Introduction

Neutrophils play a critical role in the acute inflammatory response and host-defense against bacterial infections. A deficiency of these cells predisposes to infection chiefly by organisms present on body surfaces. The risk of infection is greatest with severe neutropenia defined by an absolute blood count (ANC) less then 0.5×10^9/L. Severe chronic

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neutropenia, lasting for more than a few months to years can be caused by congenital marrow and intrinsic myeloid defects and acquired disorders (1).

Neutropenic patients are usually infected by organisms of their endogenous flora, the resident bacteria of the mouth and oropharynx, gastrointestinal tract, and skin. The susceptibility to bacterial infections even in the presence of severe neutropenia varies considerably, especially in those patients with severe chronic idiopathic neutropenia. On the other hand, gingivitis and mouth ulcerations are probably the most common problems initially encountered by patients with severe congenital neutropenia because of the role of neutrophils in protecting the oral mucosa from bacterial infusion. Thus, patients with severe congenital neutropenia usually present in the first year of life with stomatitis, gingivitis, perirectal inflammation or cellulitis. Abscesses, pneumonia and septicemia may also occur. Clinically signs of infections are attenuated by the presence of neutropenia, but fever then serves as a marker of inflammation and regularly develops when infection occurs. These patients are not predisposed to parasitic, viral or fungal infections. Fungal infections are uncommon except as a complication of prolonged antibiotic therapy. The most common pathogens encountered are *Staphylococcus aureus* and gram-negative organisms.

Severe congenital neutropenia or Kostmann syndrome was originally described in 1956 as an autosomal recessive disorder characterized by severe neutropenia and recurrent bacterial infections (2). Kostmann reported neutropenia accompanied by a promyelocytic maturation arrest in the bone marrow in an inbred family from northern Sweden. Subsequently, severe congenital neutropenia has been found to be genetically heterogeneous with most cases arising sporadically and clinically similar to those originally reported by Kostmann (3–5). The original cases that Kostmann reported are also similar to cases transmitted with autosomal dominant pattern of inheritance (6), which are now associated with appearance of heterozygous mutations in the gene for neutrophil elastase (ELA2 gene) (7,8).

Affected individuals with severe congenital neutropenia are usually children who have an ANC often less then 0.2×10^9/L. These patients prior to the availability of myeloid growth factors usually died from severe bacterial infections in early childhood (9). In the early 1990's, clinical trials demonstrated that patients' blood neutrophil counts could be increased and the frequency of fevers and infections decreased with long-term treatment with the use of re-

combinant granulocyte-colony stimulating factor (G-CSF) (10). This finding led to the identification of many more cases that were previously unrecognized and intensified investigations into possible disease mechanisms (11). Following the excellent clinical response of these patients to G-CSF, the Severe Chronic Neutropenia International Registry (SCNIR) was established in 1994 to facilitate research among its other goals.

Successful treatment of severe congenital neutropenia with recombinant G-CSF suggested the likelihood of abnormal G-CSF receptor (G-CSFR) function as the basis of some of the severe congenital neutropenia cases (12). Later it was appreciated that G-CSFR mutations represented acquired, non-inheritable, somatic events of the bone marrow occurring as severe congenital neutropenia progresses to myelodysplasia syndrome (MDS) and/or acute myeloid leukemia (AML) (13). The acquired somatic mutations of the G-CSFR were subsequently found in the cytoplasmic domain of the G-CSFR gene in almost all of these patients. The myelodysplastic syndrome and AML may also show acquired monosomy 7, trisomy 21, and *ras* mutations (11).

Heterogynous mutations in the ELA2 gene have been detected in most patients with severe congenital neutropenia and cyclic neutropenia (8). It has been proposed that the ELA2 mutations may predispose patients with severe congenital neutropenia to leukemogenesis because MDS or AML primarily arises in the subset of severe congenital neutropenia patient whose illness is associated with the ELA2 mutation (8). In contrast, patients with cyclic neutropenia, who do not have a sustained arrest in myeloid development, are largely not at risk to develop MDS/AML (11).

To further our understanding of the cellular and molecular mechanisms of severe congenital neutropenia and its leukemic evolution, we performed mutational analysis of the neutrophil elastase (NE) and G-CSFR genes and investigated the survival characteristics of bone marrow progenitor cells from severe congenital neutropenic patients including several who evolved to leukemia. We also reported data demonstrating that expression of NE mutants, but not normal neutrophil elastase in human myeloid cell lines results in accelerated apoptosis, which appears to replicate the poor cell survival observed in primary bone marrow progenitors cell in severe congenital neutropenia.

Through the efforts of the SCNIR we noted several cases of severe congenital neutropenia associated with the identical mutation in gene for NE. Further investigations revealed a common parent for these

patients, who were either conceived through *in vitro* fertilization or donor insemination. These observations led to investigations supporting an autosomal dominant inheritance.

PATIENTS AND METHODS

Patients

Twelve severe congenital neutropenia patients, including five affected individuals who developed AML and eleven normal volunteers gave informed consent to participate in the studies. All patients had severe congenital neutropenia with an ANC less then 0.2×10^9/ML at diagnosis. All patients at the time of bone marrow aspiration, except for patient number 4, were not receiving G-CSF. Blood samples of bone marrow aspirates were obtained by standard techniques. The number of blasts in the bone marrow of the leukemic patients ranged between 14% to 70% at the time of the bone marrow aspiration. The five severe congenital neutropenic patients who developed MDS/AML subsequently underwent allogeneic matched or mismatched stem cell transplantation. The patients, 4, 9, and 12 were in complete remission one year after transplantation; whereas patients 10 and 11 relapsed and subsequently died of leukemia.

Purification of Bone Marrow Progenitors Cells

Bone marrow mononuclear cells were isolated from bone marrow aspirates as previously described (14). Commercially available monoclonal antibodies to human CD34, CD35, and CD15 cell surface antigen and immunomagnetic beads from Miltenyi Biotech, Inc. (Auburn, CA) were sequentially employed to isolate and purify corresponding bone marrow hematopoietic cell populations according to the manufacturer's recommendation. The resultant cells from patients or healthy donors were further fractionated into $CD34^+$ early progenitors, $CD33^+/CD34^-$ myeloid progenitors, and $CD15^+/CD33^-/CD34^-$ bone marrow granulocyte precursors subpopulations. The purity of each of the subpopulations exceeded 96% as tested by FACS analysis.

Apoptosis Assays

The apoptotic rate of bone marrow-derived cells subpopulations was assessed by flow cytometry using an Annexin V and PI (Propidium

Iodide) binding, which permits detection of cells in early and late stages of apoptosis. For performance of these studies 3 to 20 $\times$ 10^4 freshly isolated cells where cultured overnight at 37°C in RPMI (Bio-Whittaker, Walkersville, MD) supplemented with 10% autologous serum and labeled with fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI according to manufacturer's recommendations (R & D Systems, Minneapolis, MN). The samples were then analyzed by two-color flow cytometry using CellQuest Analysis (Becton-Dickinson, Mountain View, CA) or Multi Plus (Phoenix Flow System, Inc., San Diego, CA) software programs. A minimum of 10,000 events was counted per sample. The results are reported as the percentage of Annexin V^+ cells, thereby reflecting the relative proportion of apoptotic cells in both early and late stages of apoptosis.

SEQUENCING THE GENE FOR NEUTROPHIL ELASTASE OR G-CSF RECEPTOR

Genomic DNA with isolated either from bone marrow or peripheral blood mononuclear cells using proteinase K solution as previously described (15). Total RNA was isolated from bone marrow mononuclear cells of patients or healthy donors using RNeasy Kit (Qiagen, Inc., Valencia, CA) and reversed transcribed for 60 min. at 45°C using Superscript II (GIBCO, BRL, Grand Island, NY). Amplification of genomic DNA or cDNA with neutrophil elastase or G-CSF receptor-specific primers was performed as previously described (7). The resultant PCR-amplified products were directly sequenced in both directions using a Big Dye terminator chemistry and an ABI/PE Biosystems 3700 machine (Perkin-Elmer Applied Biosystems, Foster City, CA). The neutrophil elastase direct sequencing analysis was performed on all five exons of the gene for neutrophil elastase using specific sense and antisense primers. All identified mutations were confirmed by repeat sequencing from a freshly prepared PCR product.

CELL LINES

The human U937 promyelomonocytic leukemia cell line was purchased from ATCC, (Manassas, VA). The human HL-60 leukemia cell line was kindly provided by Dr. Steven Collins (Fred Hutchinson Cancer Research Center, Seattle, WA). Cells were maintained at 37°C in a CO_2– incubator RMPI supplement with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD).

cDNA CONSTRUCTS

In order to clone neutrophil elastase, cDNA was amplified by reversed transcriptase polymerase chain reaction (RT-PCR) using total RNA from human U937 cells and NE-specific primers containing BamHI and EcoR1 restriction sites. The cDNA was cloned into PcDNA3.1 expression vector under control of CMV promoter (Innitrogen, Carlsbad, CA). Neutrophil elastase cDNA's carrying P110L, del145-152, or G185R mutations were obtained by site-directed mutagenesis using mutant NE-specific primers. To verify the preservation of an open reading frame in absence of PCR-induced artifacts, each construct was sequenced across the entire coding region.

EXPRESSION AND SURVIVAL ASSAYS

The transfection experiments were carried out by employing 10 mcg of plasmid DNA containing normal or mutant NE cDNA following electroporation of 5×10^6 HL-60 cells. The transfection efficiency was monitored by employing 5 mcg of pEGFP-c3 plasmid that directed the expression of enhanced green florescent protein (EGFP) under control of CMV promoter (Clontech Laboratories, Inc., Palo Alto, CA), which was cotransfected with each NE construct. Cells were transfected with pEGFP-C3 plasmid or mock-vector as additional controls. Forty-eight hours after transfection, an aliquot of the cells cultured in RMPI supplemented with 10% FBS was incubated for an additional 20 hours in serum-deprived medium at 37°C, labeled with PE-conjugated annexin V (Pharmingen, San Diego, CA), and analyzed by flow-cytometry. At least 20,000 events were counted per sample. The cells positive for EGFP expression were analyzed using Multi-Plus or CelQuest software (Becton-Dickenson, San Diego, CA). Results were reported as the percentage of Annexin V^+ cells. The results are based on transfection experiments repeated at least three times.

WESTERN BLOT ANALYSIS

Aliquots of transfected cells following a 48-hours incubation period were lysed in the presence of a cocktail of serine protease inhibitors containing 5mM PMSF and 10 mcg/ml of aprotinin and leupeptin. The cell lysates were separated under reducing conditions on a 9% polyacrylamide gel, and were blotted onto nitrocellulose membranes followed by blocking with 5% nonfat milk and probing with rabbit NE specific polyclonal antibody followed by secondary goat anti-rabbit

antibody conjugated with horseradish peroxidase (Research Diagnostics, Flanders, NJ). The protein bands were visualized using chemoluminescence substrate (Nen Life Science Products, Boston, MA) according to manufacturer's recommendations. To control for loading, a commercially labeled monoclonal antibody to beta-actin (Sigma, St. Louis, MO) was employed.

PATIENTS PARTICIPATING IN INHERITANCE STUDIES

Five children developmentally normal at birth were recognized to have severe congenital neutropenia by the Severe Chronic Neutropenia International Registry. Further analysis of the clinical information by a physician at the University of Michigan indicated that all five children were conceived using the same sperm donor. The same sperm donor was utilized by four different families to impregnate mothers by either donor insemination or *in vitro* fertilization. Three sets of twins and a single child were conceived in the five families. Collectively, one set of twins and one child each from the other two families conceiving twins and a single child from another family were all found to have severe congenital neutropenia. The sperm donor was not available for analysis. Alternative methods were employed to determine whether the sperm donor was responsible for transmitting severe congenital neutropenia. DNA isolated from peripheral blood leukocytes following informed consent was used to sequence the gene for NE in the affected children and their mothers' as detailed above.

MICROSATELLITE MARKERS

PCR was performed with genomic DNA from mothers and affected children using a set of twenty-two fluorescently labeled microsatellite PCR primers on chromosomes 14 and 19 to establish linkage of the paternal allele to ELA2 found on chromosome 19. The PCR products were analyzed using AB1 3700 sequencers and AB1 Gene Mapper software as previously described (8).

STATISTICAL ANALYSIS

To establish statistical significance, the students T-test and the standard Anova test (Graph Bad Prism 2.01, Graph Bad Software, Inc., San Diego, CA) was used. Differences were considered significant if $p < 0.05$ resulted.

RESULTS

Molecular Analysis of the ELA2 Gene

Human NE, like other serine proteases is synthesized as a precursor protein composed of two amino- and carboxy-terminal domains flanking the mature NE enzyme (16). We analyzed the sequence of all of the exons with their intron-exon boundaries, including the sequence of the mature enzyme and its prodomains in twelve unrelated patients, as well as the mothers and affected children conceived from a single sperm donor. A heterozygous mutation was found in eight of twelve patients in the unrelated patients. The ELA2 analysis of the affected children derived from the single sperm donor will be discussed below. As noted in Table 1, all eight identified ELA2 mutations were different. All of the eight distinct mutations observed in this study affected residues of the mature NE protein. All of the eight mutations were located in the region encompassing Exons 2 to 5, which encodes the mature NE protein; five were missense mutations resulting in amino acid substitutions. There was in-frame deletion in exon 4 and another nonsense mutation in exon 5 leading to a deletion. One mutation was located in the donor's splice site of intron 4 at positions $^{+}1$. The splicing defect resulted in the activation of a cryptic donor's splice site 30 nucleotides upstream leading to an in-frame deletion of the last four residues of exon 4 (7).

TABLE 1
Summary of ELA2 molecular studies of patients with SCN with or without AML

Patient	Diagnosis	Location	Nucleotide Change*	Protein Level†	Mutation Type
1	SCN	Exon 4	534 C>T	P11OL	Missense
2	SCN	—	Negative	Negative	NA
3	SCN	—	Negative	Negative	NA
4	SCN/AML	Exon 2	1897T>C	131T	Missense
5	SCN	Exon 5	4953C>A	C194ter	Nonsense
6	SCN	Exon 4	4638-4661del	V145-C152del	In-frame Deletion
7	SCN	Exon 4	4495C>T	S97	Missense
8	SCN	—	Negative	Negative	NA
9	SCN/AML	Exon 5	4924G>A	G185R	Missense
10	SCN/AML	—	Negative	Negative	NA
11	SCN/AML	Intron 4	IVS4+1G>A	V161-F170del	In-frame Deletion
12	SCN/AML	Exon 2	1913T>A	V36D	Missense

SCN, Severe Congenital Neutropenia; AML, Acute Myelogenous Leukemia; * indicates nucleotide nomenclature according to genomic sequence GenBank Y00477; + indicates protein nomenclature after considering the lie of the mature NE or the first amino acid; NA, not analyzed.

SURVIVAL CHARACTERISTICS OF BONE MARROW HEMATOPOIETIC PROGENITORS IN SEVERE CONGENITAL NEUTROPENIA

To test the hypothesis that accelerated apoptosis of myeloid progenitor cells is responsible for under production of neutrophils in severe congenital neutropenia, an apoptosis detection method based on flow cytometry analysis of cells labeled with FITC-annexin V and PI was employed. The viability of freshly isolated bone marrow-derived $CD34^+$, $CD33^+$, and $CD15^+$ from seven severe congenital neutropenia patients was not significantly different from control values (control $CD34^+$ cells, 10% ± 3%; patients' $CD34^+$ cells, 15% ± 2%, $P > 0.05$). Following overnight culture in the presence of 10% autologous serum, the proportion of apoptotic cells was significantly increased in severe congenital neutropenia patients; whereas, no substantial changes were observed in control subpopulations (Table 2). The flow cytometry analysis demonstrated that 53% ± 5% of $CD34^+$ cells, 68% ± 15% of $CD33^+/CD34^-$ cells, and 60% ± 13% of $CD15^+/CD33^-/CD34^-$ cells were apoptotic in samples from SCN patient compared to 22% ± 5%, 10% ± 4%, and 15% ± 5% in respective control subpopulations (Table 2, $p < 0.05$). Only patient four, who later evolved to develop AML, was receiving G-CSF treatment at the time of the study.

LEUKEMIC EVOLUTION

Bone marrow samples from four severe congenital neutropenic, who developed AML were also analyzed (Table 1, patients 9, 10, 11, and 12). Apoptosis analytic data of bone marrow progenitor's cells from two of

TABLE 2

Apoptosis of myeloid progenitor subpopulations from SCN patients and healthy controls as determined by flow cytometry after 24 hours in culture

Subjects	$CD34^+$ Cells (%)	$CD33^+$ Cells (%)	$CD15^+$ Cells (%)
Controls (n = 11)	22 ± 5	10 ± 4	15 ± 5
Patient 1	48	63	50
Patient 2	53	78	73
Patient 3	50	76	57
Patient 4 (on G-CSF)	46	37	38
Patient 5	61	79	72
Patient 7	55	80	62
Patient 8	56	65	69
SCN Patients (n = 7)	53 ± 5	68 ± 15	60 ± 13
Patient 9	4	9	78
Patient 10	4	14	61

Patients 9 and 10 had SCN/AML.

these patients (patients 9 and 10) were available for study. Similar to the severe congenital neutropenic patients without leukemia, the percentage of apoptotic cells and freshly purified $CD34^+$ and $CD33^+$ cells subpopulations from both severe congenital neutropenia/AML patients examined did not differ from controls (data not shown). However, compared to severe congenital neutropenia patients without leukemia (Table 2), the percentage of CD34+ and CD33+ cells undergoing apoptosis during overnight culture differed in cells obtained from the severe congenital neutropenia/AML patients (4% apoptosis in severe congenital neutropenia/AML $CD34^+$ cells versus 53% ± 5% apoptosis in severe congenital neutropenia $CD34^+$ cells; 9% and 14% apoptosis in severe congenital neutropenia/AML $CD33^+$ cells versus 68% ± 15% apoptosis in severe congenital neutropenia $CD33^+$ cells). On the other hand, following overnight culture, the percentage of apoptotic cells in more differentiated $CD15^+$ cells from severe congenital neutropenia/AML patients was similar to that of $CD15^+$ cells obtained from severe congenital neutropenia patients without leukemia, (78% and 61% apoptosis in severe congenital neutropenia/AML $CD15^+$ versus 60% ± 13% in severe congenital neutropenia $CD15^+$ cells).

MOLECULAR STUDIES

G-CSF Receptor

Following the sequencing analysis of the cytoplasmic domain of G-CSF receptor, a novel missense mutation in one of the severe congenital neutropenic/AML patients (patient 4, Table 3) was noted. A Pro residue to Ala substitution was noted immediately outside of the proliferative domain of the G-CSF receptor. Sequencing analysis of PCR-amplified genomic DNA isolated from a smear of bone marrow cells from a patient 4 obtained more than ten years prior to G-CSF treatment revealed a substitution mutation in the NE gene and no mutation in the G-CSF receptor gene (data not shown). The analysis of genomic DNA from other severe congenital neutropenia/AML patients following sequencing revealed one novel (patient 9) and two previously reported mutations (patients 10 and 12) in the intracellular domain of the G-CSF receptor gene. Analysis of purified cell populations from other patients was not available for this study. None of the seven severe congenital neutropenia patients not manifesting leukemia or in eleven healthy controlled donors had mutations of the G-CSF receptor.

TABLE 3
Summary of G-CSFR mutations and cellular studies of patients with SCN with or without AML

Patient	Diagnosis	G-CSFR Mutation	Cell Survival	Blasts
1	SCN	Negative	Impaired	N/A
2*	SCN	Negative	Impaired	N/A
3	SCN	Negative	Impaired	N/A
4	SCN/AML	Positive	Impaired	18%
5*	SCN	Negative	Impaired	N/A
6	SCN	Negative	NA	N/A
7	SCN	Negative	Impaired	N/A
8	SCN	Negative	Impaired*	N/A
9	SCN/AML	Positive	Impaired*	53%
10	SCN/AML	Positive	Impaired*	63%
11	SCN/AML	Positive	N/A	70%
12	SCN/AML	Positive	N/A	14%

SCN, Severe Congenital Neutropenia; AML, Acute Myelogenous Leukemia; * indicates impaired survival of $CD15^+$ cells only; NA, not analyzed.

EXPRESSION AND CELL SURVIVAL ASSAYS

The effect of NE expression on apoptosis of the human HL-60 cells transfected with normal or mutant NE and EGFP constructs was determined following labeling cells with PE-conjugated annexin V and then analyzing them by flow cytometry. The transfected cells were analyzed by flow-cytometry as described in METHODS. As noted in Table 4, flow-cytometric analysis following gating on GFP+ cells co-transfected with either normal elastase or EGFP revealed that 22% ± 3% of PE-annexin V^+ cells were apoptotic compared to HL-60 cells transfected with EGFP alone, which similarly had a low proportion of apoptotic cells (19% ± 2%, Table 4). Following transfection with three different mutant NE constructs, approximately 40–50% of cells underwent apoptosis as determined by FACS analysis of PE-annexin V-

TABLE 4
Apoptosis of HL-60 leukemia cells transfected with normal or different mutant neutrophil elastase cDNA

Transfected	(%) Apoptosis
EGFP	19% ± 2%
Wt NE	22% ± 3%
mut NE G185R	45% ± 4%
mut NE P110L	47% ± 5%
mut NE del 145–152	41% ± 5%

EGFP, enhanced green fluorescent protein; NE, Neutrophil elastase; mut, Mutant

labeled $EGFP^+$ cells (Table 4). The effect of mutant NE was established by performing three independent experiments with 45% ± 4% apoptosis occurring in G185R-transfected cells, 47% ± 5% in P110L, and 41% ± 5% in d145-152 mutNE-transfected cells. Western blot analysis of HL-60 cell lysates with NE-specific polyclonal antibody revealed that the level of NE expression was comparable in cells transfected finding normal mutant NE (data not shown here, but found in Reference 17). The level of NE expression as determined by Western blot was elevated with respect to the physiological level of NE expression observed in EGFP or mock-transfected HL-60 cells (12).

MOLECULAR ANALYSIS OF THE FOUR FAMILIES EFFECTED WITH FIVE EFFECTED CHILDREN

Sequencing analysis of PCR-amplified genomic DNA from five affected children and their respective mothers' from the four different families revealed the identical mutation in ELA2 in the effected patients, but none of the mothers (Table 5). Sequence analysis of ELA2 indicated that all of the five children had the same nucleotide gene at nucleotide 4534 C to T leading to amino acid change, S97L. To further establish paternity linkage mapping determination of twenty-two microsatellite markers on chromosome 14 and 19 were done and confirmed that all affected children possessed one of the two paternal alleles. For an 11 centimorgan region immediately adjacent to the NE gene on chromosome 19, all affected children shared a single paternal

TABLE 5
Molecular studies in five children affected with SCN conceived by in vitro *or donor insemination*

Family Studies	NE Mutation*	Genotyped Alleles	Inferred Paternal Alleles
Mother 1	None	A/?	
Patient 1	S97L	A/B	B
Mother 2	None	CD	
Patient 2	S97L	BC	B
Mother 3	None	AA	
Patient 3A	S97L	A/B	B
Patient 3B	S97L	A/B	B
Mother 4	None	A/E	
Patient 4	S97L	A/B	B

SCN, Severe Congenital Neutropenia; (*) indicates neutrophil elastase (NE) mutation and its amino acid position; The inferred paternal allele is based on microsatellite marker results for Marker D195886 employed on chromosome 19 using the affected children and their mother's cDNA.

allele, and 4 or 5 affected children shared a single paternal allele for a 20 centimorgan region adjacent to the ELA2 gene.

DISCUSSION

To accomplish the clinical studies cited in this report, data were provided by the Severe Chronic Neutropenia International Registry (SCNIR). As of July 1st, 2005 the Registry had data on 935 patients enrolled in North America. Enrolled patients had three documented absolute neutrophil counts $<0.5 \times 10^9$/L in the three months prior to enrollment. Each patient also had a bone marrow aspirate showing a selective decrease in cells in the neutrophil lineage without an excess of myeloblasts or dysmorphic features to suggest the diagnosis of MDS, a cytogenetic evaluation, and a history of infections. If the diagnosis of cyclic neutropenia was suspected, it was confirmed by monitoring the ANC three times per week for at least six weeks. Inclusion criteria including drug or chemotherapy-induced neutropenia, thrombocytopenia, MDS, aplastic anemia, known HIV infection, or other hematological diseases, and known immune diseases such as rheumatoid arthritis, systemic lupus erythematosus, and autoimmune neutropenia. At the time of enrollment all data from the referring physicians were reviewed by physician members of the Registry Advisory Board. Patients were enrolled in three categories: congenital, cyclic, and idiopathic neutropenia. The diagnosis of MDS and AML were made by the patients' personal physicians and reported to the Registry and reported to the Registry by these physicians. Table 6 summarizes the Registry patient population by diagnostic category, age, sex, and race/ethnicity. The heading, "congenital" includes patients with Shwachman-Diamond syndrome (N = 23), Glycogen Storage disease 1b (N = 23), Myelokathexis (N = 7), and Barth syndrome (N = 7). Congenital patients were primarily pediatric (61.7% of total), and there was no sex predominance. Cyclic patients were evenly distributed among the adult and female populations, as were the idiopathic patients. Patients in all three categories were largely of a Caucasian background. Within the severe congenital neutropenia group, 42 (11%) progressed to MDS/AML, of whom all were confined to the Kostmann form of neutropenia.

Based on data provided to the Registry we selected twelve patients with congenital neutropenia who could be available for research studies at the University of Washington. The twelve patients had molecular analysis of the ELA2 gene and eight were found to have gene mutations. The results show that the mutational spectrum encompassed the coding region of the mature enzyme. Mutations located in

TABLE 6
Demographics of neutropenic patients in the SCNIR

Category	Congenital n = 366 (%)	Cyclic n = 175 (%)	Idiopathic n = 355 (%)	Autoimmune n = 39 (%)	Total n = 935 (%)
Age (years)					
Mean	15.6	28.8	31.8	13.6	24.12
Standard Deviation	10.9	19.4	21.7	14.9	19.0
Median	13.6	23.1	27.7	7.9	17.8
Range	0.3–66.8	2.2–82.2	1.2–88.7	2.1–77.8	0.3–88.7
Age Category					
Pediatric (<18 yrs)	226 (61.7)	66 (37.7)	126 (35.5)	31 (79.5)	449 (48.0)
Adult (≥18 yrs)	140 (38.3)	109 (62.3)	229 (64.5)	8 (20.5)	486 (52.0)
Sex					
Male	198 (54.1)	81 (46.3)	106 (29.9)	14 (35.9)	399 (42.7)
Female	168 (45.9)	94 (53.7)	249 (70.1)	25 (64.1)	536 (57.3)
Race					
White	270 (73.8)	157 (89.7)	328 (92.4)	35 (89.7)	790 (84.5)
Black	32 (8.8)	3 (1.7)	9 (2.6)	1 (2.6)	45 (4.8)
Asian	18 (4.9)	2 (1.2)	3 (0.8)	0 (0)	23 (2.5)
Hispanic	45 (12.3)	12 (6.9)	15 (4.2)	2 (5.1)	74 (7.9)
Native American	1 (0.2)	1 (0.5)	0 (0)	1 (2.6)	3 (0.3)

sequences and coding the mature enzyme mainly consisted of missense substitutions (63%); or changes resulting in a truncated protein with either a splicing defect, in-frame deletion, or nonsense mutation. In a more extensive study others have reported a large proportion of missense mutations (8).

The association of the mutant NE gene with severe congenital neutropenia suggested that NE may contribute to an abnormal survival of bone marrow progenitor cells in severe congenital neutropenia. To test whether the lack of neutrophils in severe congenital neutropenia is due to impaired survival of myeloid progenitor cells, we examined bone marrow-derived subpopulation hematopoietic cells from the patients and healthy volunteers. The viability of freshly isolated myeloid cells from severe congenital neutropenias remained similar to corresponding cell populations isolated from healthy controls. On the other hand, following overnight cultures in the presence of 10% autologous serum, the patients' myeloid progenitor cells underwent substantial apoptosis compared with responding control cells subpopulations. Impaired survival of bone marrow progenitor cells has been observed in other congenital disorders of neutropenia including myelokathexis (18), cyclic neutropenia (19), Shwachman-Diamond syndrome (20), and Glycogen Storage disease Type 1b (21). The data from the latter studies together with our findings suggest that cell loss by intramedullary

apoptosis is a common cellular mechanism underlying several disorders causing severe neutropenia.

Constitutional heterozygous ELA2 mutations are present in DNA extracted from the peripheral blood of 50 to 84% of severe congenital neutropenia cases (7,8). Mutations causing severe congenital neutropenia are generally distinct from those responsible for cyclic neutropenia (7,8). The genotypes and phenotypes can overlap; however, in the mutation P110L appears roughly to be distributed equally among both cyclic neutropenia and severe congenital neutropenia populations (8). Genetic data supporting the role of ELA2 mutation in the pathogenesis of severe congenital neutropenia have been confirmed independently in several laboratories (22,23). Our own studies tested the hypothesis that expression of mutant elastase is sufficient to induce accelerated apoptosis of myeloid progenitor cells. Several mutants or intact NE were coexpressed with EGFP in human promyelocytic HL-60 cells. FACS analysis of $EGFP^+$ revealed an approximate twofold rate of apoptosis in cells transfected with mutant NE compared with normal and NE EGFP-transfected cells. Western blot analysis demonstrated comparable levels of NE expression in these cells indicating that mutant NE expression is sufficient for triggering apoptotic cell death in human myeloid progenitor cells. Subsequently Massulo and colleagues performed retroviral expression with a strongly leukemia-associated severe congenital neutropenia mutation, ELA2G185 in HL-60 progenitor cells (23). They observed that the mutant enzyme accelerated apoptosis of the HL-60 cells induced to differentiate with dimethyl sulfoxide. They corroborated the findings of subcellular mislocalization of the mutant neutrophil elastase to membranes rather than primary granules where it is normally found. Furthermore, the expression of the G185R mutant altered the subcellular distribution and expression of the adaptor protein 3 (AP3) complex. The AP3 complex normally transports vacuolar cargo proteins from the trans-Golgi apparatus to the endosome and mutations of the β-subunit of the AP3 complex appear to underlie the pathogenesis of neutropenia in canine cyclic neutropenia (22). Normally AP3 conveys neutrophil elastase to granules and the absence of AP3 in canine cyclic neutropenia may cause neutropenia by mislocalizing neutrophil elastase to membranes. Thus, NE mutations in patients possible cause neutropenia by abnormal protein trafficking and accelerated apoptosis of differentiating myeloid cells as suggested by our own and other studies.

Some ELA mutations are particularly severe. G185R occurred in four severe congenital neutropenia patients known in the French Neutropenia Registry and among our own samples each of whom developed

MDS or AML (8). Analysis of bone marrow progenitor cells from two severe congenital neutropenia patients who evolved to AML (patients 9 and 10), and who had the G185R mutation, demonstrated normal survival characteristics in primitive $CD34^+$ and $CD33^+/CD34^-$ populations, but not in more differentiated $CD15^+/CD33^-/CD34^-$ neutrophil precursors. The differences in apoptotic profile of myeloid progenitor cells in severe congenital neutropenia before and during the leukemia evolution suggests that additional mutation(s) may likely be involved in the pathogenesis of severe congenital neutropenia to MDS/AML and likely override the proapoptotic signals triggered by mutant NE.

The G-CSF receptor mutation analysis in this study revealed substitution or truncation mutations in the cytoplasmic domain of the receptor in all five SCN/AML patients and none of the seven SCN patients examined. The G-CSF receptor mutations represent acquired noninheritable somatic events in the bone marrow accumulating as severe congenital neutropenia progresses to MDS/AML. Although the mutations do not always occur in AML and they also appear in the absence of neoplasia (24). The evolution to leukemia in severe congenital neutropenia appears to be a multi-step process through the acquisition in some patients of monosomy 7, trisomy 21, and *ras* mutations (25).

We had the unusual opportunity to investigate an unusual series of cases of severe congenital neutropenia occurring with the same sperm donor was utilized by four different families to pregnant mothers by other donor insemination or *in vitro* fertilization. Three sets of twins and a single child were conceived in the four families. Collectively one set of twins and one child each from the other two families conceiving twins and the single child were all found to have severe congenital neutropenia. At the University of Michigan the physician responsible for managing patients with neutropenia recognized three of the involved families possibly utilized the same sperm donor to conceive the five affected children. This led to the molecular studies to test whether the five effected children shared the same sperm donor. Since detailed genetic analysis of the parental DNA and the clinical history of the sperm donor were not available for investigation, genetic studies were performed on the mothers' and their effected children. The evidence indicated a single common parent of origin was likely based on the results of the sequencing of the neutrophil elastase gene and the microsatellite markers. The linkage mapping analysis of the markers established that all affected children possessed the paternal allele containing ELA2 and supported the notion of an autosomal dominant

form of inheritance. Presumably, the father of the affected children in our study was healthy as assessed by the sperm bank. If indeed he was, he may have been a germ line mosaic for the affected children's ELA2 mutation. Another family has been described where such a mosaicism is found (26). One quarter of the DNA from the father's T-cells contained the mutant gene; whereas, less than 5% of his neutrophils' were generated from bone marrow stem cells containing the mutated elastase gene. The observation in this family strongly supported the notion that the mutant elastase impaired the development of promyelocytes into neutrophils because of likely accelerated apoptosis of the bone marrow progenitor cells. The few cases previously reported supported autosomal dominant inheritance of severe congenital neutropenia, but none of the other reported studies had as many affected children with severe congenital neutropenia associated with the ELA2 mutation as observed in the current report (8,27).

In summary, the Registry has served as a source for basic investigation of the pathogenetic mechanisms of severe congenital neutropenia. Neutrophil elastase is a proteinase synthesized early in the neutrophil development by $CD34^+$ precursor cells. As these cells mature to the level of promyelocytes, neutrophil elastase is normally packaged in the primary granule together with myeloperoxidase, proteinase-3, and several other enzymes (28). Our studies along with the others suggest that the mutant enzyme causes accelerated apoptosis in neutrophil precursors and interrupts the pathway of neutrophil differentiation. Ongoing investigations are directed towards understanding the linkage between the mutations of neutrophil elastase and the predisposition to leukemia in severe congenital neutropenia.

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DISCUSSION

Vercelotti, Minneapolis: Larry, The patients who get treated with growth factor, do they have more leukemogenesis if they have the mutation? Or do they need two mutations? What can you tell us about those individuals who get leukemia?

Boxer, Ann Arbor: It turns out, Greg, that severe congenital neutropenia is a preleukemic syndrome. The evidence for that resides in the French registry for this particular disorder. There have been a half-dozen children not treated with growth factors who develop leukemia. What we believe is that the growth factor, G-CSF, in itself does not cause leukemia, but once the conversion takes place, there is a multi-step process that allows for better growth of the malignant cell. The conversion occurs independently of the ELA2 mutation.

Tweardy, Houston: I enjoyed the talk a lot. The questions I have are two-fold. You showed nicely that mixed expression, if you will, of the mutant and wild-type ELA2 protein leads to apoptosis of the cells. Are there any thoughts about how that happens? What is it that the ELA2 mutant does to cause apoptosis of the cell?

Boxer: The exact mechanism, David, is not known. There're some studies by Marshall Horowitz that indicate that the mutant gene is improperly sequestered in lysosomes, but, rather, is transported to membranes; and in another study from Germany just presented a year ago at the Hematology meetings, it turns out the mutant ELA2 has decreased proteolytic activity compared to wild-type ELA2 and is found in the cytoplasm. It is likely that this improper localization of ELA2 damages the cell, thereby leading to apoptosis.

Tweardy: The second question relates to the more rapid turnover of progenitor cells that express this mutant due to their increased apoptosis. Does that lead to a predisposition to leukemia? What are your thoughts about that?

Boxer: That would be one supposition, coupled with the prolonged survival of the precursors cells.

Falk, Chapel Hill: So there are knock-out mice that do not have elastase or proteinase genes, since elastase and proteinase 3 are right next door to each other, and we thought we were going to find all sorts of fun abnormalities. In fact, the mice are all from studies that can look awfully normal. So it can't just be the absence of elastase. Secondly, we have also shown that elastase causes apoptosis by cleaving NFκB just upstream from a caspase site. What do you think is funny about this mutated elastase that you have found? Is it doing something different to NFκB, because that's where normal elastase effects an apoptotic event?

Boxer: I would make the following comment. First of all, you proved that the mouse is not a man, and I believe investigators at Washington University in St. Louis made the same observations. Dan Link at Washington University made a transgenic mouse expressing a mutant elastase and found nothing wrong with myelopoiesis. So there are clearly other factors. As I mentioned, in congenital neutropenia, only 40%–70% of patients have been identified to have the mutant gene. In terms of NFκB activation, Andrew Aprikyan, a collaborator of mine from the University of Washington, has looked at NFκB expression and has found it to be normal. So right now we really don't have a mechanism.