Hematological abnormalities in Shwachman Diamond syndrome: lack of genotype-phenotype relationship

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Condensed title: Neutrophil defects related to genotype in SDS

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Abstract

Shwachman-Diamond syndrome (SDS) is an autosomal-recessive disorder characterized by short stature, exocrine pancreatic insufficiency, and hematological defects. The causative SBDS gene was sequenced in 20 of 23 unrelated patients with clinical SDS. Mutations in the SBDS gene were found in 75%, being identical in 11 patients. Hematological parameters for all three lineages were determined over time such as absolute neutrophil counts (ANC), granulocyte functions, and erythroid and myeloid colony formation (BFU-E and CFU-GM) from hematopoietic progenitor cells, HbF%, and platelet counts. Persistent neutropenia was present in 43% in the absence of apoptosis and unrelated to chemotaxis defects (in 65%) or infection rate. Irrespective the ANC in vivo, abnormal CFU-GM was observed in all SDS patients tested (14 of 14), whereas BFU-E was less often affected (9 of 14). Cytogenetic aberrations occurred in 5 of 19 patients in the absence of myelodysplasia. One child died during allogeneic bone marrow transplantation.

In conclusion, neutropenia and defective chemotaxis did not result in severe clinical infection in SDS. CFU-GMs were impaired in all patients tested. From the SBDS sequence data, we conclude that in genetically proven SDS patients a genotype-phenotype relationship in SDS does not exist in clinical and hematological terms.
Introduction

Shwachman-Diamond syndrome (SDS; OMIM 260400) is an autosomal-recessively inherited disorder showing a wide variety of abnormalities and symptoms. It is mainly characterized by short stature, exocrine pancreatic insufficiency, and bone marrow dysfunction. Several studies have shown that, with advancing age, 40% to 60% of patients becomes pancreatic sufficient. Elevated liver enzymes and hepatomegaly have been observed in the first years of life with subsequent improvement over the years.

Intermittent neutropenia is the most common hematologic finding in SDS. Hematologic manifestations other than neutropenia include anemia, raised fetal hemoglobin (HbF) levels, thrombocytopenia, and, finally, aplastic anemia. Granulocyte colony-stimulating factor (G-CSF) has been used in some SDS subjects with severe neutropenia but is not generally advised because of the potential risk of acute myeloid leukemia.

Growth retardation is the third typical manifestation: weight and length are already deficient at birth and remain decreased over time. A broad spectrum of skeletal abnormalities has been observed, of which metaphyseal dysplasia of ribs and long bones is the most common symptom. Several additional, less frequently observed clinical features such as heart failure or psychomotor retardation have been described.

Although the genetic basis of this rare disease has recently been described, no unifying pathogenic mechanism(s) has yet been shown to be responsible for SDS. Indirect lines of evidence from orthologs such as YLR022c, indicate that the SBDS gene may encode an RNA-processing enzyme. 75% of the alleles in SDS were the result of a gene conversion confined to a short segment of about 240 base pairs (derived from an inactive pseudogene). Of the affected individuals about 90% carry one and 60% carry two converted alleles. Alleles from affected individuals without conversion mutations had other changes in the coding region of SBDS that led to frameshift and missense changes.
Neutrophils are produced in large numbers every day in the bone marrow, being predisposed to demise by apoptosis, a process that prevents the cytotoxic contents from the neutrophil granules to be released into the surrounding tissues and facilitates the harmless elimination of these cells by tissue macrophages. Aging of normal neutrophils is accompanied by a progressive loss of functions, such as adherence, chemotaxis, and respiratory burst. We have studied the functional characteristics of neutrophils in other syndromes with concomitant neutropenia, and other have reported that in Glycogen Storage Disease type 1-b (GSD1b) the functional defects of circulating neutrophils were largely due to premature apoptosis. In SDS, apoptosis has been noticed before in cultures of hematopoietic stem cells. However, apoptosis may be eminent at early stages only, whereas the mature circulating neutrophils are not affected and still functional.

To investigate the functional characteristics of neutrophils in SDS more closely, we sequenced the SBDS gene in a group of patients clinically diagnosed with SDS. We investigated long-term hematological parameters, colony-forming potential of committed hematopoietic progenitor cells, cytogenetic abnormalities of BM cells, and a variety of characteristic granulocyte functions, as well as signs of early neutrophil apoptosis, in order to establish a genotype-phenotype relationship in these patients.
Methods and Materials

SDS patient definition and inclusion

Patients were defined by the major criteria. 1. early gastrointestinal symptoms (fat malabsorption and/or unexplained decrease in serum levels of fat-soluble vitamins, low fecal levels of elastase and/or chymotrypsin in the presence of normal sweat tests to exclude cystic fibrosis; 2. hematological findings (unexplained anemia, low neutrophil and/or platelet number); 3. radiologic findings compatible with Shwachman-Diamond syndrome \(^{17}\), and 4. growth failure. For a diagnosis of SDS, evidence of pancreatic exocrine dysfunction is obligatory at an early age. The presence of two major criteria made the diagnosis probable, and of three criteria definite –irrespective of the genetic analysis of the SBDS gene. Minor features were scored but not considered for the diagnosis. These consisted of an unexplained hepatitis and/or hepatomegaly, skin manifestations, psychomotor skills and behavioral difficulties. The study was approved by the institutional medical ethical committee in accordance with the standards laid down in the 1964 declaration of Helsinki.

Molecular studies

Genomic DNA from peripheral mononuclear cells and fibroblasts from the patient were extracted by standard methods. All coding exons of the SBDS gene were amplified in separate PCRs. Sequencing was performed with the PE dye terminator kit and products were analyzed on an automated sequencer (ABI3100, Applied Biosystems, USA). The genomic DNA was tested after written consent was obtained from the parent(s) and the patient (when >12 years of age). There were three refusals.
**Hematologic studies**

Morphologic analysis demonstrated hypocellular BM in smears, in some patients confirmed by BM biopsies. No excess of collagen or signs of fibrosis, disturbed bone marrow stroma development or disorganized hematopoiesis was observed. Absolute numbers of progenitor B cells (CD19⁺, CD10⁺, CD24⁺), T cells (CD2⁺, CD3⁺, CD4⁺, CD8⁺), NK cells (CD3⁻, CD16⁺, CD56⁺), and myeloid cells (CD15⁺, CD14⁺, CD16⁺, CD65⁺) were determined by standard FACScan procedures. Colony-forming units of the erythroid and granulocyte/macrophage progenitors (BFU-E and CFU-GM) were determined in a 10- to 14-day semisolid culture and compared to normal age-matched values.

**Cytogenetic analysis**

Freshly obtained bone marrow sample were collected, cultured (without stimulation) in RPMI-1640 supplemented with 15% foetal calf serum for 24 hours and harvested according to standard protocols. PHA-stimulated peripheral blood lymphocytes were cultured for 72 hours. Metaphase chromosomes were analysed with a routine Q-banding method (QFA) and abnormal clones were defined according to the International System for Human Cytogenetic Nomenclature ¹⁸.

**Neutrophil purification and functional testing**

Heparinized venous blood was collected from healthy donors and from SDS patients, after obtaining informed consent. Granulocytes were isolated as described ¹⁴,¹⁵. Purity was always >95%. In some experiments whole leukocyte preparations were used from which the erythrocytes were lysed by ice-cold isotonic NH₄Cl solution ¹⁴,¹⁵. Neutrophil migration was assessed by means of the Fluoroblok inserts (Falcon; Becton Dickinson, San Jose, CA). Cells (5 x 10⁶/mL) were labeled with calcein-AM (1 μM final
concentration; Molecular Probes, Leiden, Netherlands) for 30 min at 37°C, washed twice, and resuspended in HEPES buffer at a concentration of 2 x 10^6/mL. Chemoattractant solution (PAF, IL-8 and C5a, all at 10 nM) or medium alone (0.8 mL/well) were placed in a 24-well plate, and 0.3 mL of cell suspension was delivered to the inserts (3 µm pore size) and placed in the 24-well plate. Cell migration was assessed by measuring fluorescence in the lower compartment at 2.5-min intervals for 45 min with the HTS7000+ plate reader (Perkin Elmer, Norwalk, CT). Maximal slope of migration was estimated over a 10-min interval.

NADPH-oxidase activity was assessed as hydrogen peroxide production determined by an Amplex Red kit (Molecular Probes). Neutrophils (1 x 10^6/mL) were stimulated with 1 µM fMLP, 10 ng/mL serum-treated zymosan (STZ), or 100 ng/mL PMA, in the presence of Amplex Red (0.5 µM) and Horseradish Peroxidase (1 U/mL). Fluorescence was measured at 30-sec intervals for 20 min with the HTS7000+ plate reader. Maximal slope of H_2O_2 release was assessed over a 2-min interval.

Annexin-V binding, mitochondrial staining and morphology was performed exactly as described 19,20.

Morphology was determined after Giemsa staining of cytospin preparations. Apoptotic morphology was defined as the presence of condensed nuclei and simultaneous loss of the polysegmented nuclear appearance.

**Statistics**

Statistical analysis was performed with the SPSS package for windows, version 10.0 (SPSS Inc.). For normally distributed data the student’s t-test was used to compare group means, otherwise the Mann-Whitney U test was applied. A two-sided *p*-value of < 0.05 was considered statistically significant.
Results and Discussion

The SBDS gene was sequenced in all but three cases clinically diagnosed as SDS. These latter three patients had gastrointestinal features as well as hematological findings compatible with SDS (Table 1). Other causes had been excluded. In the other 20 patients, genetic abnormalities were found in the majority of patients. There were only a limited number of mutations present in our cohort. One mutation had not been reported until recently in Japanese SDS patients as the 292-295 del AAAG \(^{21}\), annotated here as the 297-300 del AAGA [E99fs] mutation in exon 3, resulting in a frame shift and premature stop codon. The other is a unique point mutation at codon 250 T>C [C84R], that is predicted to result in a single amino acid substitution at a highly conserved position. This change was absent in DNA from more than 100 healthy controls, excluding the possibility of a common polymorphism.

Normal SBDS genes were found in 5 patients (25%), comparable with other patient cohorts described thus far \(^{9,21,22}\). In one patient we found only one mutation (patient # 8).

Growth failure resulted in an extremely short stature in some patients, but was never disproportionate of nature. As indicated, radiological investigations were performed in all patients, including chest, spine and hips, upper and lower limbs including hands and feet. In 15 of the 23 patients (65%), defects were established (Table 1). The lack of abnormalities in some of the patients may be related to the age of radiologic scoring \(^{17}\).

Hepatitis and/or hepatomegaly were commonly present, not necessarily combined. Hepatitis was limited to increased levels of liver enzymes that were moderately increased (up to 300 U/mL) and in all patients gradually decreased over a variable period of months to years. When liver biopsies were performed during diagnostic procedures at the time SDS had not yet been considered, histological findings of steatosis were observed without features of inflammation or fibrosis.
Elastase (and chymotrypsin) were tested in fecal samples. In all patients these were under the cut-off of 200 µg per g feces, irrespective of the substitution of pancreatic enzymes or the patient’s age at fecal sampling, even though some clinical resolution of the pancreatic insufficiency has been described in SDS 3. Gastrointestinal symptoms varied between patients and within the individual patient. For instance, the patient with congenital aplastic anemia (patient # 7) initially had signs of diarrhea (passing of loose stools >4 times a day) and developed eczematous skin lesions after birth suspect of hyperreactivity toward cow-milk protein. Symptoms did not disappear upon hydrolyzed formula feeding. After several months the eczema-like skin lesions resolved spontaneously—as more often observed in the SDS patients mentioned here—and her stools became so compact that the girl suffered from constipation for several weeks.

The number of red cells and thrombocytes did not fluctuate much apart from periods of mild infections (patients # 4, 5, 7, 14, 15). Some of the patients had low platelet counts for a long time without major bleedings except for the patient with aplastic anemia. Some had a mild anemia most often normocytic without abnormal levels of iron or iron-binding capacity of plasma, folic acid or vitamin B12 levels. Four patients had a macrocytic anemia and an increased HbF% on Hb-electrophoresis (patients # 2, 4, 5 and 14). As reported before, the patient with congenital anemia was on epopoietin but had a normocytic anemia without any increments in HbF compared to age controls 23.

Hematological variation was observed in their blood counts (Table 1). As known from previous reports, the neutrophil counts may fluctuate in SDS 1-4. We classified the patients into 3 groups according to their ANC (serious neutropenia with <600 PMN/µL, moderate
neutropenia with 600-1200 PMN/µL, and no relevant neutropenia with >1200 PMN/µL). Neutrophil counts were normal in 5 and moderately affected in 8 patients. Some of the patients became seriously neutropenic during infections only. Neutropenia and circulating myeloid progenitor cells disappeared spontaneously at the time the infection resolved (patients # 8, 14, 15). In 10 patients a severe chronic neutropenia for more than 6 months was present (patients # 1, 4, 5, 7, 13, 16, 18, 20, 21, 22). One patient was put on G-CSF (patient # 7; up to 30 µg per kg, 3 times a week) because of repeated infections due to congenital aplastic anemia 23. The patient’s response to G-CSF did not result in a significant rise in circulating neutrophil numbers, although her clinical condition improved considerably. Apart from this single case, we may conclude that the rate of infection was not strongly increased in our cohort.

We tested both neutrophil numbers and functions in 20 patients with SDS (Table 1). Directed cell motility (chemotaxis) of neutrophils can be disturbed in SDS 3,7,24,25. We tested chemotaxis towards a variety of neutrophil-specific stimuli, i.e. C5a, IL-8, or PAF. In 13 out of 20 patients motility was diminished (65%) (Table 1). The activity toward different chemoattractants showed individual variation but as a patient group were defective toward all three stimuli (Fig. 1A). In general, the variation in motility became more evident with the less potent stimulus. The rank order of potency in the chemotaxis assay used is as follows: C5a > PAF > IL-8. Also in controls, the IL-8-mediated chemotaxis is particularly prone to variation, due to minor illnesses such as common cold or unknown subclinical events. Due to this variation, we may explain that the individual patient not always differed with all 3 stimuli used from the controls of that day (taking 70% as cut-off as in Table 1). In a dose-response study over a log3 range in 3 of the SDS patients with an abnormal chemotaxis response we found similar bell-shaped curves as well as the same optimal concentration for the induction of motility in the patients’ neutrophils compared with control cells (data not shown).
Chemotaxis defects did not correlate with ANC scores (Table 1; $p = 0.8$), which may indicate that there is no altered motility due to the presence of younger and more immature neutrophils in these patients.

The reason for the neutropenia was investigated by the determination of Annexin-V binding to freshly isolated neutrophils or whole blood samples from these SDS patients. In contrast to our observations in GSD1b or Barth syndrome $^{14,15}$, no enhanced Annexin-V binding or any other feature of premature apoptosis was detected in the freshly purified SDS neutrophils, as tested by mitochondrial staining, Bax translocation or morphology (data not shown). NADPH-oxidase activity was preserved in SDS neutrophils of all patients, when tested with a variety of stimuli. Similar findings were made for phagocytosis and killing of $S$ aureus or $E$ coli (data not shown). Apart from the lack of Annexin-V binding to the circulating blood cells in SDS, a normal NADPH oxidase activity upon activation by serum-treated zymosan (STZ; Figure 1B) or the phorbol ester PMA, and phagocytosis also exclude a death-prone cell type in this disease. We therefore believe that the myeloid defect or neutropenia in SDS patients is unrelated to enhanced cell death or peripheral clearance of the circulating neutrophils.

Neutropenia may be the consequence of bone marrow failure. Overt failure at birth was observed in a single of our SDS patients only $^{23}$. Although transfusion-dependent aplastic anemia has been reported at later age during childhood and adolescence $^{6,7}$, we did not observe any features of imminent bone marrow failure in any of the other SDS patients. Colony formation tests (CFU-GM and BFU-E) were performed in 14 patients. BM cells uniformly showed a defective maturation and outgrowth of committed progenitor cells in the hematopoietic lineages tested (Figure 1C), irrespective of the blood cell and differential counts in the circulation (Table 1).
Abnormal hematopoietic progenitor as well stromal cell function has been previously reported in SDS \(^{16,26}\), which would indicate that the in vitro culture conditions used remain poor estimations of in vivo BM functionality. On the other hand, the results demonstrate intrinsic growth defects, where CFU-GM from pediatric controls or children with benign autoimmune neutropenia of childhood show normal or a slightly increased outgrowth (Figure 1C).

Although some of the BM smears showed hypoplasia, dysplastic features were not present. We observed abnormal cytogenetic findings in 5 out of 19 SDS patients (Table 1). In one case a characteristic isochromosome 7, i(7)(q10), was noted (patient # 1). This abnormality seems to be unrelated to myelodysplasia or malignancy, as discussed by us and others before \(^{27,28}\). Another patient is closely followed because of increased HbF\% as possible sign of marrow failure or dysplasia. BM smears and biopsies repeatedly excluded myelodysplasia. BM cells were tested 46,XY [22] / 45 (−7)[3] (patient # 14). During the last year, the blood cell count and differentials have normalized (apart from persisting neutropenia) and HbF\% decreased spontaneously. Variability in cytogenetic abnormalities is an intriguing aspect in SDS. Smith et al \(^{29}\) reported the transient appearance of a 20q-deletion in a SDS patient with i(7)(q10) as a non-random secondary change in SDS. As described here, we observed del(20q) as the sole feature in the BM of two patients in the absence of i(7)(q10) (patients #15 and #20). In the adolescent patient the cytogenetic anomaly disappeared in 2 years. Aplasia, malignant transformation or progressive disease remained absent during the recent 5 years of follow-up in this patient. Another patient had a mosaic 46,XY / 46,XY t(7;10) abnormality in his blood cells (patient #23); its relevance warrants further study for the potential involvement of the regulation of the SBDS gene or neighbouring sequences.
In conclusion, a lack of concordance in hematologic findings among affected siblings and the large variability within a family was noticed before ⁴. At that time the gene responsible for SDS was not yet identified. Using sequence data from 20 out of 23 SDS patients, we may infer that SDS-like syndromes exist without mutations in the \textit{SBDS} coding sequence as was suggested previously ⁹,²¹,²². In the genetically proven SDS patients only a small series of mutations make up the genetic defect. Even though our conclusions are based on a relatively small number of patients, we may infer from the genetic homogeneity in our study that a clear genotype-phenotype relationship in SDS does not exist in terms of clinical and hematological parameters.

\textbf{Acknowledgments}

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References


Legends.

**Figure 1.** Myeloid defects in SDS. *A:* Granulocyte chemotaxis toward rC5a. *B:* NADPH oxidase activity upon addition of serum-treated zymosan (STZ). Both tests compare purified neutrophils from SDS patients and controls (n=40). *C:* BFU-E and CFU-GM of BM samples obtained SDS patients compared to healthy pediatric controls (n=20) and children with benign autoimmune neutropenia of childhood (AINP; n=6).

The data are summarized from measurements performed over 5 years of study follow-up. Each patient is represented once. Some have been tested more often, in that case the mean of the experiments is taken. Results are the average of standard triplicates. Significance is marked with a cross when p<0.05, one asterisk when p<0.001, and two asterisks when p<0.0001.
Figure 1A
Figure 1B

![Bar graph showing H$_2$O$_2$ production in control and SDS treatments with STZ and none conditions.](image-url)
Figure 1C

The bar chart shows the comparison of colonies per 10.5 NBC between Control, SDS, and AINP conditions. The y-axis represents the number of colonies per 10.5 NBC, ranging from 0 to 1000. The x-axis categorizes the samples as Control, SDS, and AINP. The bars indicate the counts of BFU-E (black) and CFU-GM (gray) colonies. The asterisks (*) and (**) denote statistical significance compared to the control group.
Table 1. Patient characteristics

<table>
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<th>Patient</th>
<th>Age (y)</th>
<th>SBDS Mutation</th>
<th>Clinical Symptoms</th>
<th>BFU-E&lt;sup&gt;+&lt;/sup&gt; &amp; CFU-GM</th>
<th>Infections&lt;sup&gt;+&lt;/sup&gt;</th>
<th>Hb (g/dL) &amp; HbF (%)</th>
<th>Plts&lt;sup&gt;+&lt;/sup&gt; (10&lt;sup&gt;9&lt;/sup&gt;/L)</th>
<th>ANC&lt;sup&gt;+&lt;/sup&gt; (uL)</th>
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<td>Coxa vara Femora</td>
<td>+</td>
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<sup>1</sup> Sex: F, female; M, male.
<sup>2</sup> Chemotaxis: C5a, IL-8, PAF.
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<td>#18</td>
<td>F</td>
<td>4</td>
<td>183-184 TA&gt;CT [K62X]</td>
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<td>IVS2 (258)+2T&gt;C [C84fs]</td>
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<tr>
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<td>25</td>
<td>250 T&gt;C [C84R]</td>
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<td>IVS2 (258)+2T&gt;C [C84fs]</td>
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<td>IVS2 (258)+2T&gt;C [C84fs]</td>
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<tr>
<td>#23</td>
<td>M</td>
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<td>No mutation</td>
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</table>
1 M=male; F=female

2 Elastase expressed in µg per g of feces (N<200 µg per g).

3 Infections are defined as follows: −, not present; ±, only at early age; and +, invasive or recurrent infections requiring antibiotics

4 Normal BFU-E values: 295 ± 100.8 colonies per 10^5 nuclear cells, CFU-GM values 492 ± 120.2 colonies per 10^5 nuclear cells, age-matched

5 A = abnormal (see text), N = normal findings

6 ANC, absolute neutrophil counts (low <600 cells per µL, normal >1200 per µL)

7 Chemotaxis is expressed as percentage of the mean maximal slope (cells/min) of two age-matched controls measured on the same day (<70% of the mean of the day as well as <70% of the mean of 40 historic controls is considered abnormal); blanks without stimuli were not subtracted (see Figure 1A).

8 G-CSF, 30 µg/kg every other day
Hematological abnormalities in Shwachman Diamond syndrome: lack of genotype-phenotype relationship

Taco W Kuijpers, Mariel Alders, Anton T Tool, Clemens Mellink, Dirk Roos and Raoul C Hennekam