SBDS-deficient cells undergo accelerated apoptosis through the Fas-pathway

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The online version of this article contains a supplemental appendix.

ABSTRACT

Background
Shwachman-Diamond syndrome is an inherited multisystem disorder characterized by bone marrow and pancreatic dysfunction as well as metaphyseal dysostosis. Ninety percent of the patients have mutations in the Shwachman-Bodian-Diamond syndrome gene (*SBDS*). The relationship between *SBDS* and cell survival is unknown. In this study we investigated whether deficiency of the SBDS protein can cause increased apoptosis and, if so, what pathways are involved in this process.

Design and Methods
To determine whether accelerated apoptosis of Shwachman-Diamond syndrome cells is caused by a deficiency in SBDS we generated two *SBDS*-knockdown cell clones. We then evaluated, *Fas* expression and levels of the intracellular proteins, BAX, BCL-2 and BCL-X_L and determined the effects of apoptosis inhibitors. Using oligonucleotide-microarrays we also analyzed apoptosis-related gene expression in Shwachman-Diamond syndrome marrow cells.

Results
We found that knocking down *SBDS* by short interfering hairpin RNA in HeLa cells resulted in a prominent increase in cell death. The mechanism for the accelerated apoptosis was related to marked hypersensitivity to Fas stimulation, and increased Fas expression. In contrast, there was no increase in the expression ratio of the pro-apoptotic factor, BAX, to the pro-survival factors, BCL2 and BCL-X_L in the *SBDS*-knockdown cells and in the patients' marrow cells. Furthermore, inhibition of Fas and caspase 8, but not caspase 9, significantly improved the defective cell growth phenotype.

Conclusions
Our work provides new data about the pro-survival properties of SBDS, whose inhibition results in accelerated apoptosis through the Fas pathway.

Key words: *SBDS*, apoptosis, Fas, Shwachman-Diamond syndrome, shRNA.

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Introduction

Shwachman-Diamond syndrome (SDS) is an inherited multisystem disorder characterized mainly by reduced cell mass in the bone marrow and exocrine pancreas as well as short stature.[1-3] It has been recently found that mutations in the Shwachman-Bodian-Diamond syndrome gene (*SBDS*) cause 90% of the cases of SDS.[4,5] This gene is highly conserved, and encodes a protein (SBDS) with a predicted length of 250 amino acids.

The function of SBDS is unknown, but it has been suggested to play a role in ribosomal biogenesis.[6,7] Based on the characteristic apoptotic phenotype of primary SDS cells[8] and similar to other genes related to ribosomal biogenesis,[9-13] *SBDS* might also be involved in protection from apoptosis. Indeed, genes related to other inherited marrow failure syndromes have been shown to be multifunctional, and are sometimes involved in protection from apoptosis.[14-16] The haploid spores of *Saccharomyces cerevisiae* lacking the SBDS ortholog, YRL022c, manifest growth defects. Using proteome chips, YLR022c was shown to bind the phospholipids PI2P2,[17] which suppress apoptosis by inhibition of caspases 3, 8, and 9.[18]

We have previously shown that bone marrow cells from SDS patients undergo accelerated apoptosis and are hypersensitive to Fas activation.[8] Furthermore, Fas expression on marrow cells from SDS patients was significantly higher than on cells from normal controls.[8] The difference in Fas expression between SDS patients and controls was significant for both $CD34^+$ and $CD34^-$ cells. This might explain the lower numbers of $CD34^+$ cells in SDS and the reduced ability of these $CD34^+$ cells to generate hematopoietic colonies of all lineages *in vitro*.[19] Despite this demonstration of accelerated apoptosis of SDS marrow cells and increased Fas expression, it is unknown whether SBDS plays a mechanistic role in these abnormalities. We, therefore, investigated whether deficiency of SBDS can cause increased apoptosis and, if so, whether the Fas pathway or BAX/BCL2/$BCLX_L$ pathways are involved in this process. We also conducted a comprehensive analysis of apoptosis-related gene expression in SDS bone marrow mononuclear cells.

Design and Methods

Short interfering hairpin RNA expression cassettes, plasmids and generation of SBDS-knockdown HeLa cells

To determine whether endogenous *SBDS* has a causative role in protecting cells from apoptosis, we generated two short interfering hairpin RNA (shRNA) consisting of nucleotide 585-603 of the *SBDS* open reading frame (shSBDS-1) and nucleotide 137-156 after the open reading frame (shSBDS-3). A shRNA expression cassette with a U6 promotor-sense-loop-antisense DNA sequence as well as a scrambled sequence control were synthesized by polymerase chain reaction (PCR) amplification and cloned into a pSEC/Neo plasmid (Ambion). Stable shRNA-expressing cell lines were generated by transfecting HeLa cells (ATCC, Manassas, VA, USA) with the various vectors and selecting cells with geneticin (Invitrogen). According to the shRNA expression construct, we termed the lines HeLa/shSBDS-1, HeLa/shSBDS-3 and HeLa/shSCR. The cells were subcultured twice weekly in Dulbecco's modified Eagles medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS) and geneticin. We chose HeLa cells as a model, because they have been used extensively to study protein function by shRNA,[20] they express Fas and can be induced to undergo apoptosis.[21,22]

Bone marrow samples

Bone marrow aspirate samples were collected into preservative-free heparinized syringes. Marrow mononuclear cells were separated using Ficoll-Hypaque as previously described,[19] and used fresh. The studies were approved by the institutional Research Ethics Board, and informed written consent was obtained from patients, controls, or their legal guardians prior to sample collection. Patients were diagnosed as having SDS based on institutional clinical criteria,[2] which included clear evidence of both hematologic and exocrine pancreatic dysfunction. In seven of the nine patients tested, the diagnosis was supplemented by the finding of *SBDS* mutations (*Online Supplementary Data, Table 1*). No patient had evidence of malignant transformation at the time of collection of the bone marrow samples. Hematologically healthy donors of bone marrow for transplantation served as controls.

Generation of lymphoblastoid cell lines

Lymphoblastoid cell lines from SDS patients who were heterozygous for the common mutations 258+2T>C/258+2T>C+183-184TA>CT and had undetectable levels of SBDS protein by western blotting, and from normal controls were established and maintained as we previously described.[23]

Cell viability assay

SBDS-knockdown and control cells were plated in 96-well plates in triplicate wells. Cell viability was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (ATCC, Manassas, VA, USA) according to the manufacturer's instructions at different time points after plating depending on the specific experiment. Absorbance was measured at 570 nm in a microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Cells were assayed daily to evaluate their growth. In order to determine cytotoxicity in response to Fas stimulation, the cells were incubated overnight, treated with various concentrations of activating mouse anti-human Fas IgM antibody (Clone CH-11, Immunotech, Marseille, France), and assayed 48 hours later.

Determination of apoptosis from DNA content

To evaluate cell DNA content, $5x10^5$ *SBDS*-knockdown and control cells were washed with phosphate-buffered saline (PBS), fixed with 70% ethanol, re-washed and incubated at 4°C for an additional 4 hours with propidium iodide, RNase A and triton-X-100. The DNA content was then analyzed by flow cytometry and the sub-G_1 cell population, which represents apoptotic cells, was determined.

Flow cytometric analysis of Fas expression

To determine Fas expression, 5×10^5 cells were washed and re-suspended in 50 µL of cold PBS. After incubation at 4°C for 30 minutes with either a phycoerythrin-Cy5-conjugated anti-human CD95 IgG1 antibody (BD Pharmingen) or an isotype control (BD Pharmingen), cells were washed twice, re-suspended in PBS, fixed with 1% paraformaldehyde and analyzed by flow cytometry as previously described.[8]

Western blotting

To determine the levels of the intracellular proteins, BAX, BCL-2 and BCL-X_L, we performed western blotting as previously described.[23] A rabbit monoclonal antihuman BAX antibody (BD Pharmingen), a rabbit monoclonal antihuman BCL-2 antibody (BD Pharmingen) and a mouse anti-human BCL-X_L (a gift from Dr. J. C. Reed, Burham Institute) were used as the first antibodies, and donkey anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Biosciences Uppsala Sweden) was used as the second antibody. Antibody-reactive proteins were detected using enhanced chemiluminescence reagents (Amersham).

For detection of SBDS, membranes were probed with a chicken-anti human SBDS, which we generated against the C-terminus of the protein by immunization of the animals with a peptide containing the SBDS amino acids 233 to 250. The antibody clearly recognized the endogenous protein, and its specificity after affinity purification was demonstrated by western blotting following transfection with a plasmid containing a *GFP-SBDS* fusion gene (*Online Supplementary Data, Figure 1*), and by a negative signal in lymphoblastoid cells from patients bearing bi-allelic *SBDS* mutations (Figure 1). A horseradish peroxidase-conjugated rabbit anti-chicken IgY (Pierce Biotechnology, Beckford, IL, USA) was used as a secondary antibody. Band intensity was quantified by a laser scanning densitometer (SynGene, Frederick, MD, USA).[23]

Effect of apoptosis inhibitors on cell growth

SBDS-knockdown and control cells were plated in quadruplet (2×10^3 cells per well) with either 50 µM of caspase 8 inhibitor (Z-IETD-FMK, Trevigen, Gaithersburg, MD, USA), caspase 9 inhibitor (Z-LEHD-FMK, Trevigen) or 100 ng/mL blocking anti-Fas antibody (clone ZB4, Immunotech). After incubation, cell growth was analyzed by the MTT cytotoxicity assay, as described above.

Oligonucleotide microarray

For comprehensive analysis of apoptosis-related gene expression in SDS marrow cells, RNA was extracted from post-Ficoll marrow mononuclear cells and analyzed by the oligonucleotide HG_U133_ Plus 2.0 GeneChip array (Affymetrix Inc.) as previously described (*See also Online Supplementary Data*).[24]

Multiplex PCR

Post-Ficoll marrow mononuclear cells were incubated in Iscove's medium with 10% FBS. Duplicate cultures were irradiated at 15 Gy. After 48 hours, non-adherent cells were harvested, and total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) as previously described.[24] The RNA was reverse transcribed using the Advantage RT-for-PCR kit (Clontech, Palo Alto, USA) and oligo dT primers according to the manufacturer's instructions. cDNA product aliquots underwent multiplex PCR of the *BAX*, *BCL-2*, *BCL-XL*, *BCL-Xs* and *GAPDH* genes using the cytoXpress Human Apoptosis Gene VII kit containing multiple primer pairs specific for these genes (Biosource International, Camarillo, CA, USA). An aliquot of the completed PCR reaction was fractionated on 1% agarose gel by electrophoresis, autoradiographed, and scanned by a laser scanning densitometer (SynGene, Frederick, MD, USA).

Statistics

Means and standard deviations are used to describe the results concerning apoptotic cells and gene expression assessed by semiquantitative multiplex PCR. Student's t-test was used to determine the statistical significance of differences between samples. For the oligonucleotide microarray experiments, data were analyzed as previously described[24] and moderated T-statistic values were generated (*See also Online Supplementary Data*).

Results

Establishing SBDS-knockdown cells

To study whether the accelerated apoptosis of SDS cells is caused by a deficiency in SBDS, we generated two shRNA-mediated *SBDS*-knockdown cell clones. The inhibition efficiency of the cloned shSBDS was confirmed by determining protein expression using western blotting. For the present study, which was aimed to study the effect of SBDS deficiency on apoptosis, we selected two representative clones with marked decreases in the levels of SBDS. HeLa/shSBDS-3 does not express detectable levels of SBDS protein, while HeLa/shSBDS-1 expresses 8% of the levels expressed by HeLa/shSCR and HeLa/WT (Figure 1A). These cells enable the effect of SBDS-deficiency to be evaluated since they mimic the SBDS levels in cells from most SDS patients,[25] and are consistent with the autosomal recessive inheritance of the disease.

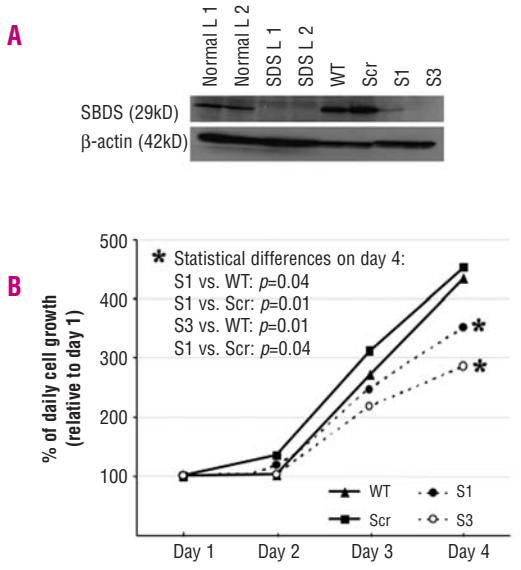

Figure 1. Protein expression in *SBDS*-knockdown cells and cell growth characteristics. A. HeLa cells were stably transfected with pSEC/Neo vectors carrying either shSBDS-1 or shSBDS-3 cassettes to downregulate *SBDS* or a shSCR scrambled RNA control cassette. Western blotting of cells after transfection showed that SBDS protein was undetectable in HeLa/shSBDS-3 cells and reduced (8% compared to the level in wild type cells) in HeLa/shSBDS-1 cells. Lymphoblastoid cell lines from patients and controls were also generated and SBDS protein expression was evaluated by western blotting. Thirty micrograms of protein were loaded for HeLa cells and 50 μg for lymphoblastoid cells. B. HeLa cells (2×10^3 cells/well) were plated in 96-well plates and analyzed daily for cell growth using an MTT assay. The absorbance readings at 560 nm were normalized to blank wells and expressed relative to the values obtained from the same lines on day 1. The means of six independent experiments are presented. The asterix indicates statistically significant differences between the knockdown and the control cells. The results of the analyses of the differences between the MTT readings on day 4 are shown within the graph. (L, lymphoblastoid cell line 1; L2, lymphoblastoid cell line 2; WT, wild type control; Scr, scrabbled control; S1, HeLa/shSBDS-1; S3, HeLa/shSBDS-3).

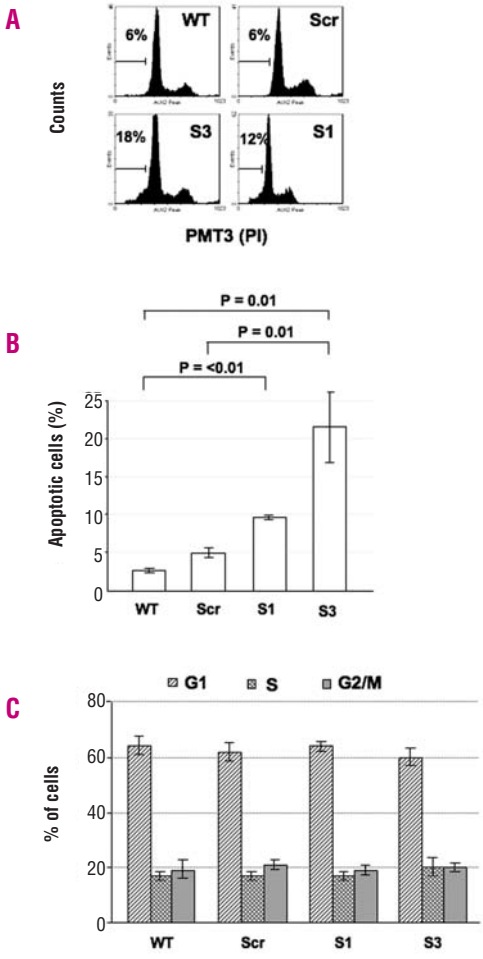

Figure 2. Spontaneous apoptosis and cell cycle analysis of *SBDS*-knockdown cells. A. Representative DNA content analysis of HeLa/shSBDS-1 & 3 and control cells. Cells were incubated for 24 hours at a concentration of 5×10^5/mL. Adherent and not adherent cells were then collected, stained with propidium iodide and analyzed for the percentage of pre-G_1/G_0 cells, which represent apoptotic cells. B. Mean rates (±SD) of spontaneous apoptosis in *SBDS*-knockdown cells and controls. Cells were assayed by DNA content as described above, and the mean of five independent experiments are shown. C. Analysis of the cell cycle phases of *SBDS*-knockdown cells and controls. Cells were assayed for DNA content as described above and the means of ten independent experiments (±SD) of the various cell cycle phases are shown. (WT, HeLa/WT; Scr, HeLa/shSCR; S-1, HeLa/shSBDS-1; S3, HeLa/shSBDS-3).

SBDS-knockdown cells are characterized by decreased cell growth and accelerated apoptosis

We first studied the growth rate of the *SBDS*-knockdown cells. We plated 2×10^3 *SBDS*-knockdown or control cells per well in 96-well plates and analyzed cell density daily by an MTT assay. The combined results of six separate experiments showed slower growth of HeLa/shSBDS-1 and HeLa/shSBDS-3 cells compared to HeLa/WT and HeLa/shSCR cells (Figure 1B).

To determine whether the decreased rate of cell growth is due to accelerated apoptosis, we analyzed the DNA content of the cells using propidium iodide staining and flow cytometry. The results showed a marked increase in apoptotic cells (sub-G_1 cell population) in unstimulated HeLa/shSBDS-3 cells (21.9±4.9%) compared to HeLa/shSCR (6.2±1.3%) and HeLa/WT cells 3.8±0.2% (Figure 2A, 2B). HeLa/shSBDS-1 cells demonstrated an intermediate defect with mean spontaneous apoptosis of 8.1±0.29%. Accelerated apoptosis was also seen after staining the cells with annexin V and propidium iodide (*Figure 2, Online Supplementary Data*).

In the light of a previous report of G_1 arrest in YRL022c-depleted yeast cells, we examined the cell cycle distribution of the *SBDS*-knockdown cells. In contrast to the effect in yeast cells, knocking down the human *SBDS* in HeLa cells did not cause abnormalities in cell cycle distribution (Figure 2C). The mean percentages of HeLa/shSBDS-1 cells in G_1, S and G_2/M in ten different experiments were 64%, 17% and 19%, respectively; the corresponding percentages for HeLa/shSBDS-3 cells were 60%, 20% and 20%. These distributions over the cell cycle were not statistically different from those of HeLa/WT and HeLa/shSCR cells.

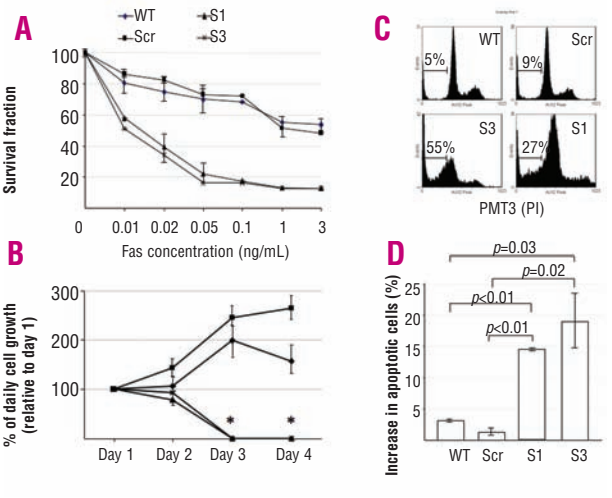

Figure 3. Fas-induced apoptosis in *SBDS*-knockdown cells. A. To determine sensitivity to Fas stimulation, 5×10^3 cells/well of HeLa/shSBDS-1 and HeLa/shSBDS-3 cells were incubated with increasing concentrations of an activating anti-Fas antibody (CH-11) for 48 hours, and cell viability was measured by the MTT assay. The means (±SD) of three different experiments are presented. The results were normalized to blank wells and expressed relative to the values obtained from the same cell type without treatment. B. HeLa cells (5×10^3 cells/well) were plated in 96-well plates with or without CH-11 and analyzed daily for cell growth using an MTT assay. The absorbance readings at 560nm were normalized to blank wells and expressed relative to the values obtained from the same lines on day 1. The means (±SD) of four different experiments are presented. The asterix signifies $p<0.01$. C. Representative DNA content analysis of *SBDS*-knockdown cells and controls after CH-11 stimulation. Cells were plated at a concentration of 5×10^5/mL for 24 hours with or without CH-11. Adherent and non-adherent cells were then collected, stained with propidium iodide and analyzed for the percentage of pre-G_1/G_0 cells, which represent apoptotic cells. D. Fas-induced apoptosis in *SBDS*-knockdown cells and controls. Cells were assayed by DNA content as described in the legend to Figure 2C and the means (±SD) of five independent experiments are show (WT, HeLa/WT; Scr, HeLa/shSCR; S-1, HeLa/shSBDS-1; S3, HeLa/shSBDS-3; PI, propidium iodide).

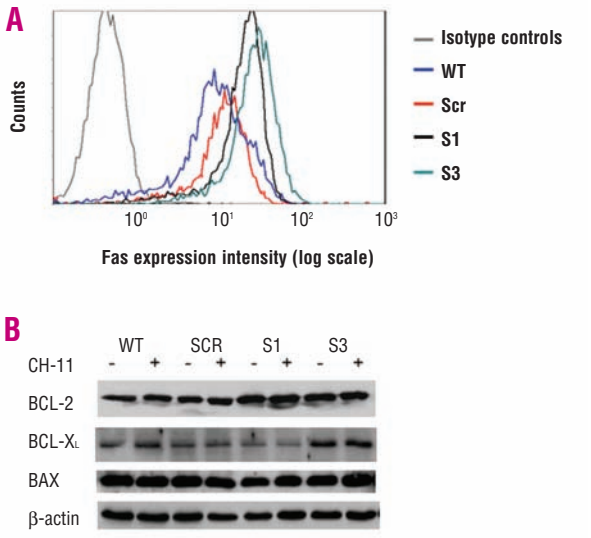

Figure 4. Protein expression in SBDS-knockdown cells. A. Exponentially growing cells were incubated for 24 hours and then harvested, stained with a PE-Cy5 conjugated anti-Fas antibody and analyzed by flow cytometry. B. Exponentially growing cells were plated for 6 hours either with or without CH-11 and then analyzed for the expression of BAX, BCL-2 and BCL-X_L by western blotting (WT, HeLa/WT; Scr, HeLa/shSCR; S-1, HeLa/shSBDS-1; S3, HeLa/shSBDS-3).

SBDS-knockdown cells are hypersensitive to Fas stimulation

Based on our previous observation in SDS marrow cells,[8] we hypothesized that knocking down *SBDS* would result in hypersensitivity to Fas stimulation.

To test this hypothesis, *SBDS*-knockdown and control cells were plated in 96-well plates in triplicate wells (5×10^3 cells/well) and incubated overnight, then treated with increasing concentrations of activating mouse anti-human Fas IgM antibody (Clone CH-11, Immunotech, Marseille, France). After 48 hours, cell viability was determined by the MTT assay. Interestingly, HeLa/shSBDS-1 and HeLa/shSBDS-3 showed marked hypersensitivity to activating anti-Fas antibody (Figure 3A, 3B).

To study whether the cell growth inhibition by CH-11 might be due to increased apoptosis, we analyzed DNA content after incubation of SBDS-knockdown cells and controls with 0.02 mg/mL CH-11. The results showed significantly higher increments in apoptotic cells in HeLa/shSBDS-3 cells (19%±5.0%) compared to those in HeLa/shSCR (0.7%±0.5%; p=0.01) and HeLa/WT (2.5±0.3%; p=0.01) cells. The increment in HeLa/shSBDS-1 cells was slightly lower than that in HeLa/shSBDS-3 cells, but was still pronounced and significantly higher than that in HeLa/WT ($p<0.01$) and HeLa/shSCR ($p<0.01$) cells (Figure 3C, 3D). An increase in cell death after stimulation with CH-11 was also seen after staining the cells with annexin V and propidium iodide (*Figure 2, Online Supplementary Data*).

Fas protein expression in SBDS-knockdown cells

We have previously reported that Fas expression on marrow cells (both $CD34^+$ and $CD34^-$) from SDS patients is higher than that on cells from normal controls.[8] To determine whether the hypersensitivity of the *SBDS*-knockdown cells to Fas stimulation is due to increased Fas expression, we analyzed the cells for surface binding of Cy5-conjugated anti-Fas antibody by flow cytometry. We found pronounced increases in cell surface Fas expression in the HeLa/shSBDS-1 and HeLa/shSBDS-3 compared to in wild-type and HeLa/shSCR cells (Figure 4A).

BAX, BCL-2 and BCL-X_L protein expression in SBDS-knockdown cells

BAX/BCL-2/BCL-X_L signaling is another important pathway of cell death in many physiological and disease-related conditions. We, therefore, determined whether the BAX/BCL-2/BCL-X_L pathway is also activated and contributes to the accelerated apoptosis in SBDS-deficient cells. To study the expression of BAX, BCL-2 and BCL-X_L at the protein level, we performed western blotting of lysates from *SBDS*-knockdown and control HeLa

cells. The results did not show a trend to expression of pro-apoptotic proteins. BAX was not overexpressed in the *SBDS*-knockdown cells. BCL-2 and BCL-X_L were not downregulated (Figure 4B). BCL-XL was, however, upregulated in HeLa/shSBDS-S3 cells, which might represent a compensatory mechanism.

Fas pathway inhibitors improved the cell growth of SBDS-knockdown cells

To determine whether activation of the Fas-signaling pathway plays a role in the reduced cell growth in *SBDS*-deficient cells, we tried to rescue the *SBDS*-knockdown cells by suppressing the Fas-pathway using a blocking anti-Fas antibody (ZB4). Treating the *SBDS*-knockdown cells with this antibody did, indeed, significantly improve cell growth (Figure 5A). After ZB4 treatment, the difference in cell growth between the two *SBDS*-knockdown cells and the HeLa/WT and HeLa/shSCR control cells became statistically insignificant (p value between 0.40 to 0.82).

To compare the relative contributions of the Fas pathway and the BAX/BCL-2/BCL-X_L pathway to the reduced cell growth in the *SBDS*-knockdown cells, we treated the cells with inhibitors of caspase 8 and 9. Treatment of *SBDS*-knockdown cells with a caspase 8 inhibitor, but not with a caspase 9 inhibitor, resulted in a significant improvement in cell growth (Figure 5B).

Gene expression in marrow mononuclear cells

The cellular pathways in which Sbds functions are unknown. If Sbds plays a role in protecting cells from apoptosis, we could expect to find dysregulation of apoptosis-related gene expression in SDS, which might be directly or indirectly caused by Sbds-deficiency. We, therefore, conducted oligonucleotide microarray analy-

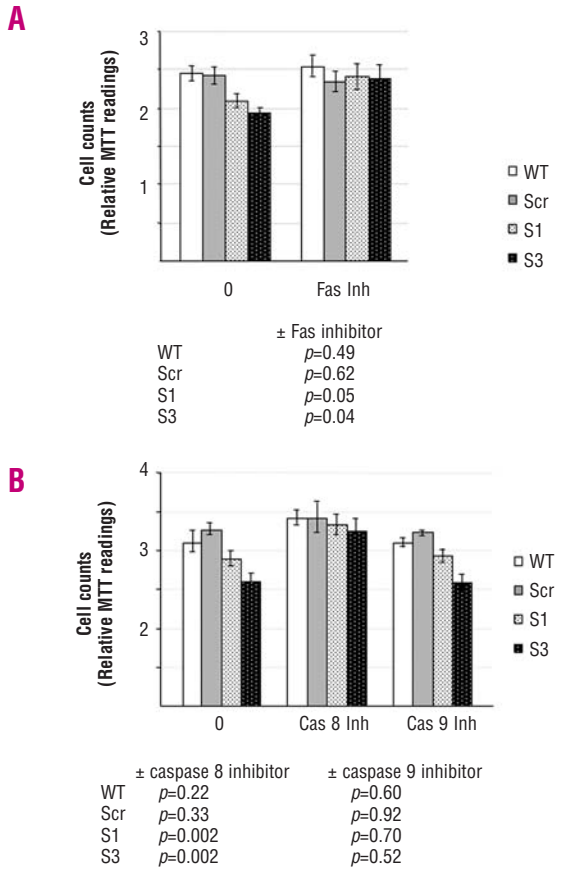

Figure 5. Effect of apoptosis inhibitors on cell growth. *SBDS*-knockdown cells and controls were incubated for 5 days either with or without an inhibitor, and analyzed by the MTT assay. No apoptosis inducers were added in these experiments. The average readings from six experiments (±SEM) are presented. A. The cells were incubated with or without 100 ng/mL blocking anti-Fas antibody, ZB4. B. Cell were prepared with or without a caspase 8 inhibitor or a caspase 9 inhibitor (Cas, caspase; Inh, inhibitor; WT, HeLa/WT; Scr, HeLa/shSCR; S-1, HeLa/shSBDS-1; S3, HeLa/shSBDS-3).

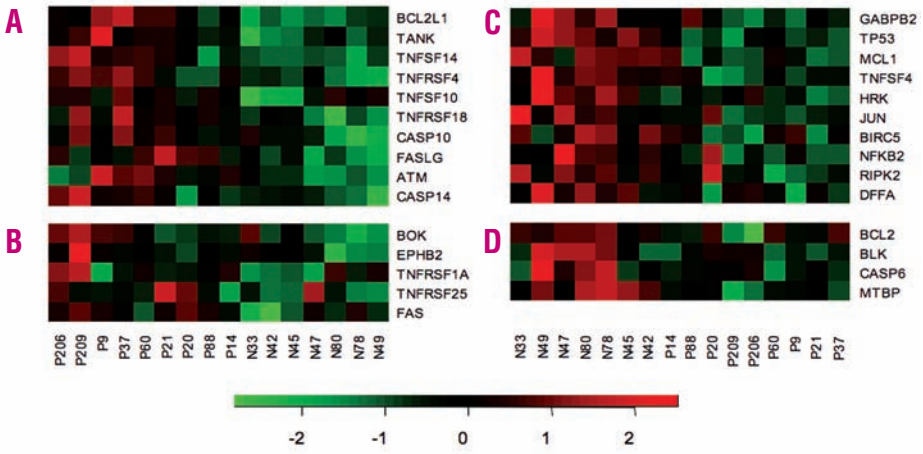

Figure 6. Apoptosis-related genes in bone marrow mononuclear cells. RNA was extracted from freshly obtained cells from patients and healthy control subjects after Ficoll-Hipaque separation. Gene expression was analyzed by oligonucleotide microarray using the Affymetrix HG_U133_Plus 2.0 GeneChip. The green color represents downregulation, and the red color represents upregulation. The gene symbols are indicated on the right, and the patients' numbers (starting with the letter P) or the normal control numbers (starting with the letter N) are indicated on the bottom of the figure. A. Prominently downregulated genes in SDS marrow cells (T value of <-2.3). B. Modestly downregulated genes in SDS marrow cells (T value between -1.9 to -2.3). C. Prominently upregulated genes in SDS marrow cells (T value of >2.3). D. Modestly upregulated genes in SDS marrow cells (T value between 1.9 to 2.3).

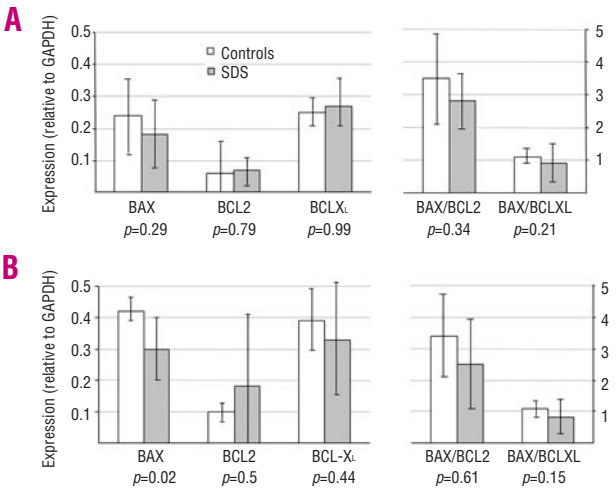

Figure 7. *BCL-2*, *BAX* and *BCL-X*$_L$ gene expression in bone marrow mononuclear cells. Total RNA from bone marrow mononuclear cells from five SDS patients and seven controls was reverse transcribed, and underwent semiquantitative multiplex PCR. The mean (±SEM) relative band intensity values normalized against *GAPDH* band intensity are shown. A. Without radiation. B. Cells were irradiated with 15 Gy and incubated for 24 hours.

sis of apoptosis-related gene expression in marrow mononuclear cells from nine SDS patients (five of whom were also included in the multiplex PCR experiments) and seven age-matched controls. All the patients enrolled in this study had normal percentages of the myeloid (46.4±6.8% standard deviation, SD), erythroid (31.1±7.3% SD), lymphoid (19.5±5.5% SD) and other cell lineages (3.0±1.3% SD). Furthermore, no patient had maturation arrest of the myeloid or erythroid series. We used a whole population of marrow mononuclear cells since we have previously shown that all cell lineages, (myeloid, erythroid and lymphoid) are quantitatively and qualitatively affected in SDS and since purification of enough RNA from a single lineage at one maturation stage in SDS is impossible. Some mild to moderate changes in gene expression might escape detection by this approach, but those genes with more prominent differential expression will probably be recognized.

Strikingly, among 90 apoptosis-related genes obtained from *http://www.superarray.com/gene_array_product/html/hs-002.html*, ten genes were markedly upregulated, with a significant T value of more than 2.3, and an additional five were mildly upregulated, as indicated by T values between 1.9 to 2.3 (Figure 6 and *Online Supplementary Data Table 2*). In addition, 11 genes were markedly downregulated with a T value of less than -2.3, and an additional three were mildly downregulated, having, T values between -1.9 to -2.3 (Figure 6 *and Online Supplementary Data, Table 3*).

FAS mRNA expression in the microarray experiments showed only mild upregulation (T=1.95), which might suggest that the predominant regulation of Fas expression by *SBDS* is at the post-transcriptional level. The Fas-signaling related genes, *FADD, CASP8, CASP3* and the Fas-signaling pathway inhibitors, *XIAP, C-FLIP* and *ERK* were normally expressed. It is interesting that several other members of the tumor necrosis factor superfamily were upregulated, including *TNFSF6, TNFSF10* and *TNFSF14*. The tumor necrosis factor receptor superfamily members, *TNFRSF4* and *TNFRSF18*, were also prominently upregulated. However, the significance of the upregulation of these genes remains to be clarified, since the inhibitor of the tumor necrosis factor signaling pathway, *TANK*, was also upregulated.

In this study we were specifically interested to determine whether BAX/BCL-2/BCL-X also play a role in the accelerated apoptosis of SBDS-deficient cells. Therefore, in addition to microarray expression analysis, we studied the expression of the genes encoding for these proteins in non-adherent bone marrow mononuclear cells by semiquantitative multiplex PCR. This was particularly important since studying the levels of the proteins by western blotting did not show a trend to pro-apoptotic expression. *BAX* expression in the microarray experiments was unchanged. In the multiplex PCR experiment, the mean *BAX* band intensity from SDS cells after normalization to *GAPDH* was not statistically different from that of cells from healthy controls (0.183 vs. 0.239, p=0.27) (Figure 7A). *BCL-2* expression showed modest downregulation, as assessed by microarray (T=-2.02); however, in the multiplex PCR experiment mean *BCL-2* band intensity normalized against *GAPDH* was not statistically different in SDS patients compared to controls (0.066 vs. 0.059, p=0.79) and, more importantly, the *BAX/BCL-2* ratio in SDS was 2.8 vs. 3.5 in healthy controls (p=0.36) (Figure 7A). *BCL-X* gives rise to two isoforms: *BCL-X*$_L$, which promotes cell proliferation, and *BCL-X*$_S$, which stimulates apoptosis. In the microarray experiments total *BCL-X* mRNA, was upregulated. This upregulation seemed, however, to be due to overexpression of the pro-survival isoform, *BCL-X*$_L$, since in the multiplex PCR experiments, *BCL-X*$_L$ was upregulated and the pro-apoptosis isoform, *BCL-X*$_S$, was undetected in both patients and controls (Figure 7A). Further, by multiplex PCR, the *BAX/BCL-X*$_L$ ratio in the group of patients (0.9) was not higher than that of the healthy controls (1.1) (p=0.21).

Interestingly, also when irradiation was used to induce apoptosis, the expression of *BAX, BCL-2* and *BCL-X*$_L$ did not show a pro-apoptosis trend. Furthermore, *BAX* expression in irradiated SDS cells showed a pro-survival trend, and was significantly (p=0.02) less expressed in these cells than in cells from healthy controls (Figure 7B).

Discussion

In the present study we showed that suppression of the *SBDS* gene by shRNA caused a pronounced

decrease in cell growth and an increase in apoptosis. The reduced cell growth and the accelerated apoptosis in SBDS-deficient cells were mediated predominantly through the Fas pathway, since blocking the Fas-signaling pathway at the Fas and caspase 8 levels significantly improved the defective cell growth phenotype of the *SBDS*-knockdown HeLa cells to levels close to those of control cells. Furthermore, *SBDS*-knockdown cells were hypersensitive to Fas stimulation and overexpressed Fas on their surface. In contrast to Fas, the expression ratios of the pro-apoptosis protein, Bax, to the anti-apoptosis proteins, BCL-2 and BCL-X_L in the SBDS-deficient cells were not in favor of apoptosis, and inhibition of this pathway by a caspase 9 inhibitor did not improve cell growth. Since the current data are in agreement with those on primary SDS cells, and since the scrambled RNA control HeLa cells had similar properties to the wild type cells in our experiments, but differed from the *SBDS*-knockdown cells, the results are unlikely to be related to inherent anomalies of the HeLa cells.

The *SBDS*-knockdown cell lines used in this study had severe reduction in SBDS levels and similar phenotype. It is, however, noteworthy that HeLa/sh-SBDS-3 cells, in which SBDS could not be detected by western blotting, had a more severe phenotype than HeLa/shSBDS-1, which expressed 8% SBDS, with lower spontaneous cell growth, and higher rates of spontaneous and Fas-induced apoptosis. These results suggest that SBDS is a pro-survival protein, and this property is likely related to the cellular level of the protein. If this is the case, how can it be explained that our HeLa/shSBDS-3 cells are viable? It is possible that *SBDS*-knockdown in HeLa/shSBDS-3 cells is not complete, and residual SBDS levels below the detection sensitivity of western blotting are present, consistent with the finding of embryonic lethality of the *SBDS* knockout mice model.[26] Indeed, we were also able to generate lymphoblastoid cell lines from patients, which survive and can be cultured for at least 3 months despite having similarly low SBDS protein levels as assessed by western blotting (Figure 1A). It is also possible that *SBDS*-knockdown results in compensatory changes, for example overexpression of BCL-X_L, as we showed in our SBDS-deficient cell model.

The mechanism for Fas upregulation in *SBDS*-deficient cells is unclear. One possibility is increased transcriptional activity. In the microarray experiments, Fas expression was only modestly increased. Expression of Fas is stimulated by the transcription factors p53,[27] NFkB (NFkB1 and NFkB2),[28] AP-1 (c-Jun and c-Fos)[29] and GABP,[29] which can be activated by tumor necrosis factor-α,[30] interferon-γ,[31] protein kinase C[32] or CD30 signaling pathways. In our microarray analysis there was no increase in mRNA levels of the transcription factors involved in Fas expression. These results on gene expression of Fas and its transcriptional activators have to be further confirmed and quantified by quantitative methods; nevertheless, the findings do not suggest that an increase in FAS mRNA levels is a potential mechanism for mediating Fas cell surface overexpression in SDS. Post-transcriptional and post-translational modifications of Fas may, however, occur in SBDS-deficient cells, leading to increased Fas cell surface expression (*KW, unpublished data*). It is interesting that several ligands and receptors which belong to the tumor necrosis factor superfamily were upregulated in SDS, including *TNFSF6, TNFSF10, TNFSF14, TNFRSF4* and *TNFRSF18*. Future studies should investigate the expression of these genes, and their encoded proteins, and explore the mechanisms for their dysregulation, as well as the role of these abnormalities in disease manifestations and progression.

The experiments in this study focused on the function of SBDS in apoptosis, and demonstrated a protective role of SBDS on cell death. Several studies have suggested that *SBDS* has a role in RNA processing or ribosomal biogenesis.[6,7] However, it has been well established that the nucleolus and ribosomal-related proteins are involved in cell cycling and apoptosis.[9-13] Furthermore, as demonstrated for genes associated with other inherited marrow failure syndromes, such as *FANCC*, *ELA2* and *RPS19*, the inherited marrow failure syndrome gene products might be multifunctional, and can be involved in several basic cellular pathways including apoptosis.[14-16] Future studies should focus on the link between SBDS-deficiency and Fas hypersensitivity at the biochemical level. Hypersensitivity to Fas stimulation in SBDS-deficient cells might be related to direct loss of inhibition of the Fas pathway. Alternatively, SBDS-deficiency may result in activation of proteins, such as p53, which are regulated by ribosomal proteins[33,34] and are known to influence the Fas pathway at the transcriptional[27] and post-transcriptional[35] levels. Furthermore, based on our previous data[8,19] and the results herein, it is possible that apoptosis through the Fas pathway is at least in part responsible for the reduced number of SDS marrow cells, exocrine pancreatic cells and short stature. Thus, inhibitors of the Fas pathway may be studied in preclinical models for their ability to correct the abnormally reduced cell growth phenotype in SDS.

Authorship and Disclosures

PR and KIW designed and performed the research, analyzed data, and wrote the paper. CA and HW designed and performed the research and analyzed data. JB performed the research, analyzed data, and wrote the paper. AS designed the research and wrote the paper. YD designed the research, analyzed data, and wrote the paper. The authors reported no potential conflicts of interest.

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