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ORIGINAL ARTICLE

Knockdown of the Shwachman-Diamond syndrome gene, *SBDS*, induces galectin-1 expression and impairs cell growth

Masafumi Yamaguchi[1] · Yukihiro Sera[1] · Hanae Toga-Yamaguchi[1] · Hirokazu Kanegane[2] · Yusuke Iguchi[1] · Kingo Fujimura[3]

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Abstract

Shwachman-Diamond syndrome (SDS) is an autosomal recessive disorder characterized by exocrine pancreatic insufficiency and bone marrow failure. The depletion of SBDS protein by RNA interference has been shown to cause inhibition of cell proliferation in several cell lines. However, the precise mechanism by which the loss of SBDS leads to inhibition of cell growth remains unknown. To evaluate the impaired growth of SBDS-knockdown cells, we analyzed Epstein-Barr virus-transformed lymphoblast cells (LCLs) derived from two patients with SDS (c. 183_184TA > CT and c. 258 + 2 T > C). After 3 days of culture, the growth of LCL-SDS cell lines was considerably less than that of control donor cells. By annealing control primer-based GeneFishing PCR screening, we found that galectin-1 (Gal-1) mRNA expression was elevated in LCL-SDS cells. Western blot analysis showed that the level of Gal-1 protein expression was also increased in LCL-SDS cells as well as in SBDS-knockdown 32Dcl3 murine myeloid cells. We confirmed that recombinant Gal-1 inhibited the proliferation of both LCL-control and LCL-SDS cells and induced apoptosis (as determined by annexin V-positive staining). These results suggest that the overexpression of Gal-1 contributes to abnormal cell growth in SBDS-deficient cells.

Keywords Bone marrow failure · Neutrophil · Neutropenia · Maturation · SBDS

Introduction

Shwachman-Diamond syndrome (SDS; OMIM 260400) is a rare autosomal recessive disorder characterized by bone marrow failure and exocrine pancreatic dysfunction [1]. Neutropenia is the most common hematological manifestation, and patients have an increased risk of leukemia (≥ 15–25%) [2]. Patients with SDS also have non-hematologic manifestations such as pancreatic dysfunction, short stature, skeletal abnormalities, eczema, developmental delay, and liver abnormalities [3–5].

SDS results from variants in the Shwachman-Bodian-Diamond syndrome gene (*SBDS*). In ~90% of cases, SDS results from by biallelic mutations in the Shwachman-Bodian-Diamond syndrome gene (*SBDS*). Approximately 10% of individuals with SDS lack mutations in the *SBDS gene*. Among them, biallelic mutation in the *EFL1 gene* has shown clinical features of SDS.

SBDS is highly conserved and its orthologues are found in diverse species, ranging from archaea to vertebrate animals. However, little is known about the exact function of the SBDS protein. Through studies in yeast, archaea, and bone marrow cells, it has been postulated that SBDS functions in RNA metabolism and ribosome biogenesis [6]. Recently, it was reported that SBDS is involved in the release of eukaryotic initiation factor 6 (eIF6) from the 60S ribosomal subunit for translation initiation through association with GTPase elongation factor-like 1 (EFL1) [7, 8]. Furthermore, diverse alternate functions of SBDS in chemotaxis, mitotic spindle stabilization, Fas ligand-induced apoptosis, cellular stress responses, and monocyte migration have been observed in

✉ Masafumi Yamaguchi
m-yamagu@hirokoku-u.ac.jp

[1] Laboratory of Physiological Chemistry, Hiroshima International University, 5-1-1 Hirokoshingai, Kure-Shi, Hiroshima 737-0112, Japan

[2] Department of Child Health and Development, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45, Yushima, BUnkyo-ku, Tokyo 113-8519, Japan

[3] Department of Nursing, Yasuda Women's University, 6-13-1 Yasuhigashi, Asaminami-ku, Hiroshima 731-0153, Japan

mammalian cells [9–13]. However, it remains unclear how variants in *SBDS* manifest as disease in particular organs and phenotypes.

A mouse model with *SBDS* depletion was used to elucidate the cellular function of SBDS. Mice homozygote for null alleles of *SBDS* exhibit early embryonic lethality [14]. Knockdown of SBDS protein in murine hematopoietic cells induces defects in granulocytic differentiation, myeloid progenitor generation, and short-term hematopoietic engraftment [15, 16]. Our group and others also reported that depletion of SBDS leads to cell growth inhibition [12, 15, 17], which might be influenced by reactive oxygen species (ROS) production or osteoprotegin and vascular endothelial growth factor A production [12, 17]. However, a growth curve showed that *SBDS*- knockdown 32Dcl3 cells could divide similarly as control cells until 48 h after dilution [15]. Although the mechanism underlying growth inhibition is unclear, we hypothesized that inhibitory metabolites might accumulate in the cell culture medium. To clarify the cell proliferation abnormalities in *SBDS*-depleted cells, we examined SDS patient-derived cells.

Materials and methods

Cell lines

Epstein-Barr virus-transformed B-lymphoblastoid cell lines (LCLs) from SDS patients were established after written informed consent was obtained [18]. The human LCL cell lines LCL-control (LCL-C), 277-LCL (RVB2283), the murine myeloid cell line 32Dcl3, and the murine hematopoiesis myelocytic leukemia cell line WEHI-3b were provided by the RIKEN BioResource Research Center through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan. LCL cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS). LCL cell lines were passaged every 2 days by transferring 1×10^6 cells into 10 mL fresh medium. 32Dcl3 cells were cultured in Iscove's Modified Dulbecco's Medium supplemented with 10% FCS and 10% WEHI-3b conditioned medium. SBDS-knockdown 32Dcl3 cells were established as previously described [15]. HL-60 cells were cultured in RPMI1640 medium supplemented with 10% FCS. The cell proliferation assay was performed with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA).

Annealing control primer-based GeneFishing PCR

Differentially expressed genes in LCL-C and LCL-SDS cells were screened with the annealing control primer (ACP)-based PCR method using the GeneFishing DEG Kits (Seegene, Seoul, South Korea). Equal amounts (2 μg) of pooled total RNA were used as the RT-PCR template. The PCR fragments were cloned into the pT7Blue Vector (Merck, Darmstadt, Germany) and sequenced. The identified genes were confirmed by reverse transcription-polymerase chain reaction (RT-PCR) using primer sets. The first-strand cDNA was normalized with the GAPDH gene and used as a template. A primer set for LGALS1 (Gal-1) was designed as follows: forward, 5′-GACGCTAAGAGCTTCGTGCT-3′; reverse 5′-ATTCGTATCCATCTGGCAGC-3′.

Generation of Gal-1 constructs and production of recombinant proteins

To produce His-tagged human Gal-1, cDNA was generated by PCR (forward, 5′-CATATGGCTTGTGGTCTGGTCGC-3′; reverse 5′-CTCGAGGTCAAAGGCCACACATTTGATCTTG-3′). The fragment encoding Gal-1 was ligated into pET30 (Novagen, Madison, WI, USA), which encodes a His-tag at the N-terminus of Gal-1. The expression of His-tagged Gal-1 in *Escherichia coli* strain BL21/DE3 was induced by 1 mM IPTG at 37 °C for 3 h. His-tagged Gal-1 was purified from the bacterial lysate using Ni-charged Chelating Sepharose (GE Healthcare Life Sciences, Pittsburgh, PA, USA). After elution from the resin with 200 mM imidazole, the eluate was dialyzed against phosphate-buffered saline (PBS) at 4 °C. His-tagged Gal-1 was further purified with lactose-agarose (Seikagaku Corporation, Tokyo, Japan), and the eluate was dialyzed against PBS at 4 °C and stored at −80 °C.

Detection of Gal-1 protein

To detect Gal-1 protein in LCL-cells, cells were attached to poly-L-lysine coated slide grass and fixed in 4% paraformaldehyde for 10 min. Then the cells were permeabilized with 0.2% Triton X-100 for 2 min. After washing with phosphate-buffered saline (PBS), cells were incubated with anti-Gal-1 antibody (H-45: sc-28248) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as previously described [18]. Its epitope is the amino-terminal 45 amino acids. Cells were viewed using the Fluoview FV300 confocal laser scanning microscope (Olympus, Tokyo, Japan). All experiments were repeated three times independently and the data were reproducible.

Apoptosis assays

LCL-cells were cultured in the presence or absence of 10 μM Gal-1 protein at 37 °C for 8 h. Annexin V staining of LCL-cells was performed to detect apoptotic cells as previously described [15]. The results are reported as the percentage of annexin V-positive cells and 7-aminoactinomycin D (7AAD)-negative cells, which represented cells in the early

stages of apoptosis. The apoptosis assay was also performed LCL-SDS cells at 4 day culture.

Flow cytometry

Gal-1 was biotinylated by incubating with 2 mM EX-link Sulfo NHS-LC-Biotin (sulfosuccinimidyl-6-(biotinamido) hexanoate) (Pierce Biotechnology, Rockford, IL, USA). Cells were resuspended in PBS containing 1% bovine serum albumin and then incubated with biotinylated Gal-1 in the presence or absence of 100 mM lactose for 30 min at 4 °C. Next, cells were washed with PBS followed by incubation with avidin-APC (BD Pharmingen, San Jose, CA, USA) for 30 min at 4 °C. Fluorescence-activated cell sorting analysis was performed with the BD FACSCalibur Flow Cytometer (BD Biosciences).

Western blotting

Cells were lysed with lysis buffer (50 mM Tris–HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1% Triton X-100, 1 mM PMSF, 2 µM leupeptin, and 2 µM pepstatin A). Lysates were centrifuged at 15,000 g for 15 min at 4 °C. Proteins were separated with 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to Immobilon-P PVDF membranes. Gal-1 protein was detected with the anti-galactin-1 antibody as described above.

Statistical analysis

The data are expressed as the mean ± SD. p values were calculated using student's t-test. A p value of < 0.05 was used as the criterion for statistical significance.

Results

Impaired growth of SDS patient-derived LCL-cells

We previously reported that *SBDS*-knockdown 32Dcl3 cells showed growth failure [15]. To clarify the mechanism underlying the abnormal cell growth, we established LCL-cells from two SDS patients (c.183_184TA > CT and c.258 + 2 T > C) [18]. After 3 days of culture, the growth of both LCL-SDS cell lines was considerably lower than that of LCL-C cells (Fig. 1). LCL-SDS cells did not divide after day 3 under the condition in which LCL-C cells divided until day 5. However, when LCL-SDS cells at the 5 day were diluted by initial cell densty, the cells became able to reproliferate with similar grouth curve as shown Fig. 1 (data not shown). These results suggest that some inhibitory metabolites accumulated during the culture.

Overexpression of Gal-1 protein in LCL-SDS cells

To identify differentially expressed mRNAs between LCL-C and LCL-SDS cells, we performed ACP-based PCR using GeneFishing DEG Kits, and the expressed genes were cloned and sequenced. We have identified 11 candidate genes (Supplementary Table 1). Two transcripts representing LGALS1 (Gal-1) were upregulated in the LCL-SDS cells (Fig. 2A), which was confirmed with semi-quantitative RT-PCR (Fig. 2B). By adjusting for GAPDH expression levels, the expression of Gal-1 was found to be increased by 1.17 ± 0.072 -fold in LCL-SDS cells ($p < 0.05$). Western blot analysis showed that Gal-1 protein was upregulated in LCL-SDS cells (Fig. 3A). To determine if the decreased SBDS level induced Gal-1 expression, we analyzed *SBDS*-knockdown 32Dcl3 cells, which

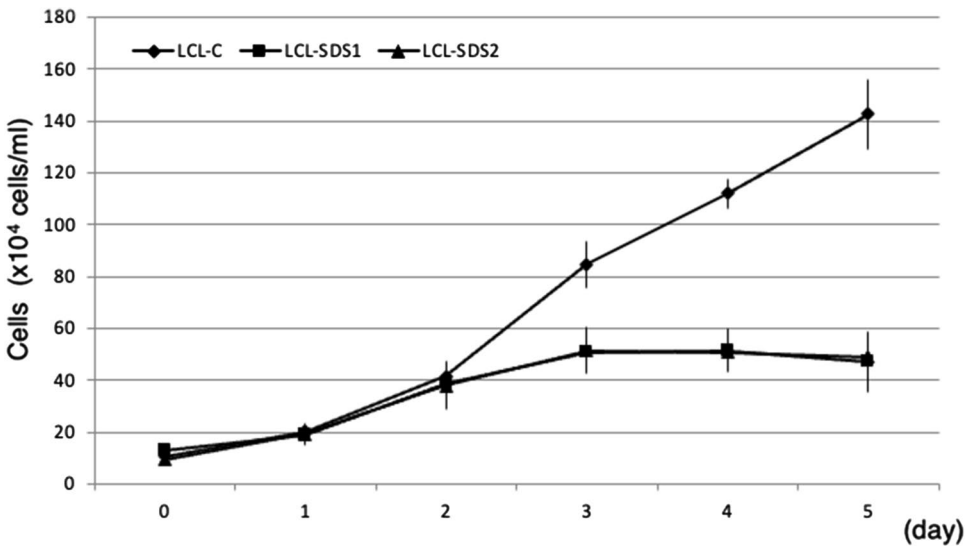

Fig. 1 Cell growth abnormalities in SDS patient-derived LCL-cells. LCL-cells were cultured at a density of 1×10^5 cells/mL in RPMI 1640/10% FCS medium, and viable cells were counted. Values shown are the average of three separate experiments ± standard deviation of the mean

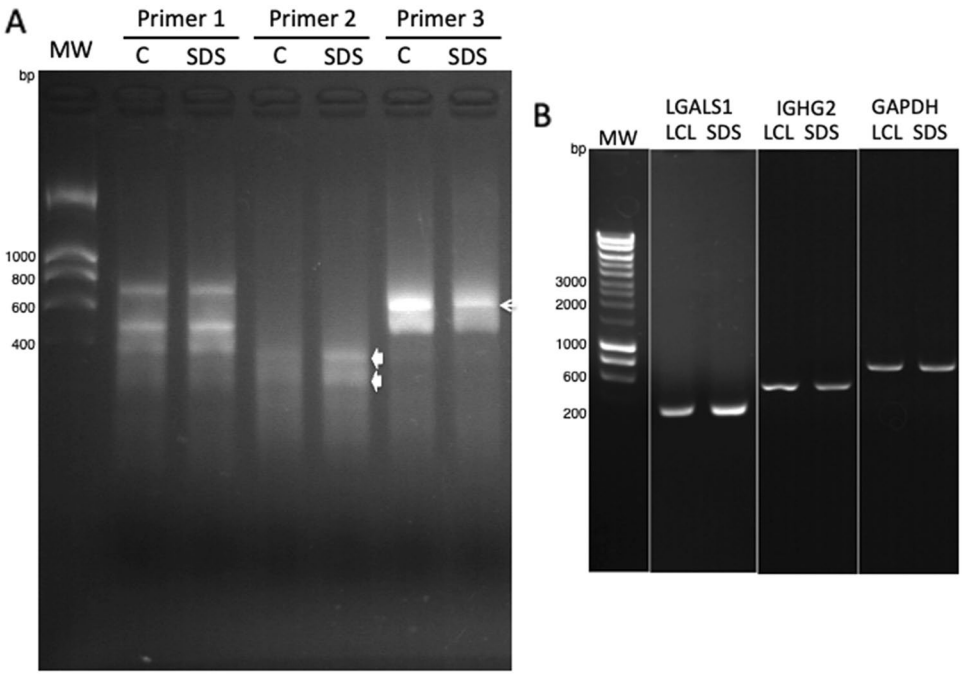

Fig. 2 Screening of altered gene expression in LCL-SDS cells. **A** Representative PCR band patterns of genes that were differentially expressed between LCL-C and LCL-SDS cells using ACPs and pooled RNA samples. Equal amounts (2 μg) of pooled total RNA were used as the RT-PCR template. The PCR products were separated by electrophoresis on 2% agarose gels. The thick arrows indicate increase expression of genes, while the thin arrow indicates drecreased expression. **B** Semiquantitative PCR products obtained with gene-specific primers. LGALS1, Gal-1; IGHG2, immunoglobulin heavy constant gamma 2; and GAPDH as the internal control

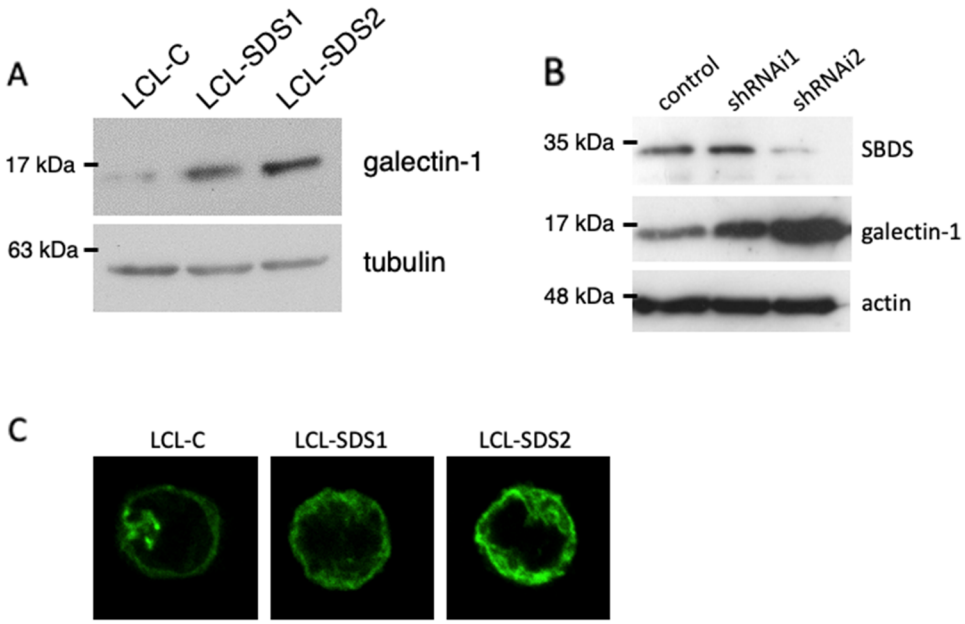

Fig. 3 Overexpression of Gal-1 in SBDS-depleted cells. **A** Lysates of LCL-C and LCL-SDS cells were subjected to Western blot analysis. The membrane was probed for Gal-1 and tubulin. **B** The lysate from 32Dcl3 cells and *SBDS*-knockdown cells were subjected to Western blot analysis to detect Gal-1. **C** LCL-C and LCL-SDS cells were fixed in 4% paraformaldehyde in PBS and permeabilied with 0.2% Triton X-100. Then cells were incubated with anti-Gal-1 antibody, followed by incubation with FITC-conjugated anti-rabbit antibody. The images are representation of at least 3 independent experiments

were established by *SBDS* short hairpin RNA (shRNA) transfection. Gal-1 protein was also overexpressed in the SBDS-knockdown cells (Fig. 3B). These results indicate that Gal-1 expression is induced by the decreased expression of SBDS. Then we analyzed the expression and subcellular localization of Gal-1 using confocal microscopy. Accumulation of Gal-1 around plamsa membranes suggests high expression of Gal-1 protein in LCL-SDS cells (Fig. 3C). Although Gal-1 does not have a canonical signal sequence, Gal-1 is localized to the plasma membrane [19], suggesting that a portion of Gal-1 is secreted in the medium and then binds to the membranes. To assess whether Gal-1 secreted from the cells is present in the medium or associated with the membranes, the medium was harvested from cells after 5 days of culture and passed through a lactose-agarose column to isolate Gal-1. However, Gal-1 was not detected in the eluate from the column (data not shown). Almost Gal-1 secreted seems to be bound to the membranes.

Gal-1 binds to the LCL cell surface and inhibits cell proliferation

To assess the effect of Gal-1 on LCL cell proliferation, we determined whether Gal-1 protein binds to the cell surface. Recombinant human Gal-1 (rhGal-1) was purified as described in the Materials and methods. The activity of purified protein was demonstrated by binding to lactose-agarose (data not shown). SDS-PAGE analysis of the purified fraction demonstrated that the protein was homogeneous and migrated with a molecular mass of 15 kDa (data not shown). These results indicated that the protein used in this assay was active and pure. As shown in Fig. 4A, rhGal-1 bound to both LCL-C and LCL-SDS cells, and the interaction was inhibited by lactose. These results suggest that Gal-1 binds to β-galactosides specifically on the cell surface. Next, we analyzed whether Gal-1 inhibits LCL cell proliferation. When rhGal-1 was added to LCL cell culture and cultured 3 days, rhGal-1 inhibited both LCL-C and LCL-SDS cell proliferation in a dose-dependent manner (Fig. 4B). We also assessed the effect of rhGal-1 on 32Dcl3 and HL-60 human leukemia cells, and found that rhGal-1 inhibited both cell lines, suggesting Gal-1 is related to impired cell growtn of even control cells.

As Gal-1 induces apoptosis in some leukocytes, we assessed whether rhGal-1 could induce apoptosis by staining cells with annexin V. The apoptosis assay was perfomrd after LCL-SDS and LCL-C cells were inbubated with rhGal-1 for 8 h. As shown in Fig. 5, we found that 15% of Gal-1-treated LCL-C cells underwent apoptosis compared to 5% of untreated cells; LCL-SDS cells exhibited

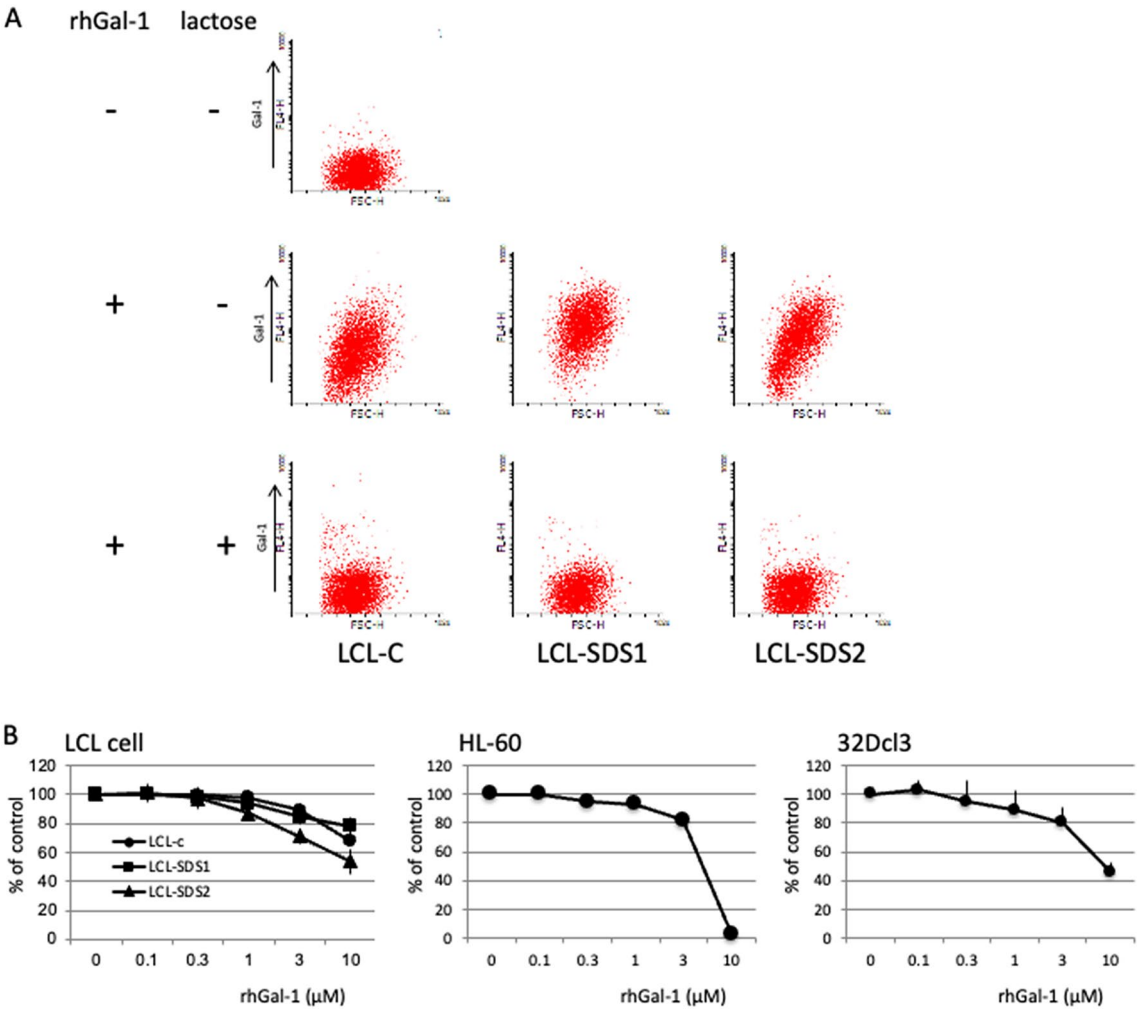

Fig. 4 rhGal-1 inhibited LCL proliferation. **A** Gal-1 bound to the cell surface. Biotinylated rhGal-1 protein was incubated with LCL-cells in the presence or absence of 100 mM lactose, and then cells were treated with avidin-APC. **B** rhGal-1 inhibited cell proliferation. rhGal-1 protein was expressed in *Escherichia coli* and purified. LCL, 32Dcl3, and HL-60 cells were cultured with rhGal-1 at the indicated concentration for 3 days, and the cell proliferation assay was performed

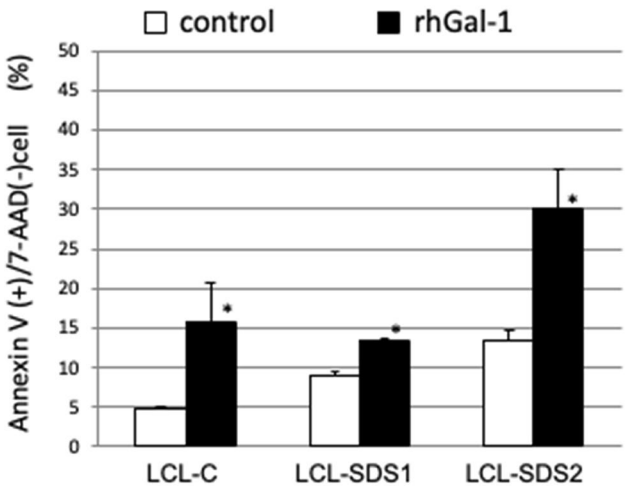

Fig. 5 rhGal-1 induced apoptosis in LCL-cells. Apoptosis in rhGal-treated LCL-cells. LCL-C and LCL-SDS1 and 2 cells were cultured with 10 μM rhGal-1 protein at 37 °C for 8 h. Apoptosis was assessed using Annexin V and 7AAD staining and flow cytometry. Values shown are the averages of three separate experiments ± standard deviation of the mean. $*p < 0.05$

the same tendency. These results indicate that Gal-1 is an apoptotic inducer in both LCL-C and LCL-SDS cells.

Gal-1 indices cell aggregation and apoptosis

Cell proliferation was inhibited in LCL-SDS cells (Fig. 1) and apoptosis of LCL-SDS cells seems to be induced compared to that LCL-C cells at 8 h culture (Fig. 5). In addition, rhGal-1 added in the medium inhibited cell proliferation and induced apoptosis (Figs 4B and 5). Considering these observations, we investigated how the cell condition is different between LCL-SDS and LCL-C cells in culture. As shown in Fig. 6A, LCL-SDS cells showed the tendency to aggregate compared to LCL-C cells. The aggregation was progress over days. In addition, rhGal-1 added in the medium induced the aggregation (Fig. 6B). As Gal-1 involves cell–cell interaction, the increased Gal-1 on LCL-SDS cells might causes cell aggregation through between Gal-1, and Gal-1 with N-type sugar chains on the cell surface.

As rhGal-1 inhibited cell proliferarion as (Fig. 4B), we investigated whether apoptosis on LCL-SDS cells increase compared to LCL-C cells. Early apoptosis was substantially increased in LCL-SDS cells at 4 day culture (Fig. 7).

Discussion

SDS is characterized by bone marrow failure, and it has been suggested that variants of *SBDS* in patients with SDS are loss of function. Knockdown of SBDS in hematopoietic cells results not only in a defect in granulocytic differentiation but also impaired cell growth [15]. Although SBDS has many proposed functions including chemotaxis, mitotic spindle stabilization, Fas ligand-induced apoptosis, cellular stress responses, and monocyte migration, the mechanism of growth defect remains unclear [9–13]. It is reported that inbalance of the ribosome maturation may yieled stress via p53 pathway to induce cell apoptosis and cell cycle arrest [20]. In this study, we analyzed SDS patient-derived cells to evaluate impaired cell growth after SBDS depletion.

We found that the growth rate of LCL-SDS cells was similar to that of control cells until day 2 but considerably decreased on day 3, and the cell number did not increase after day 3 (Fig. 1). However, the cells divided again after dilution, similar to control cells. SBDS deficiency leads to increased ROS levels, causes apoptosis, and reduces cell growth [12]. Because ROS oxidize bioactive molecules, such as thiol and render them inactive, dilution of the cells still have oxidative damage. It is unlikely that ROS is responsible for cell growth inhibition. We hypothesized that a humoral factor or some gene products accumulate in and outside of cells and inhibit cell growth. The following lines of evidence show that Gal-1 is involved in cell growth inhibition. Gal-1 is a member of a family of carbohydrate-binding lectins with an affinity for β-galactosides and is present both inside and outside cells [19]. Gal-1 is also differentially expressed in various normal and pathological tissues [21]. Even though Gal-1 lacks recognizable secretion signal sequences, it is secreted and binds to the extracellular side of cell membranes as well as the extracellular matrices of various normal and neoplastic tissues [19]. In our experiments, Gal-1 protein clearly accumulated in both the membrane and intracellular area of LCL-SDS cells (Fig. 3C); however, Gal-1 was not detected in the culture medium of LCL-SDS cells (data not shown). Because Gal-1 binds to its ligand with a Kd ranging from 109 nM to 3 μM [22, 23], Gal-1 secreted from LCL-SDS cells is likely bound to the cell surface. This is supported by data showing that almost all rhGal-1 bound to LCL-cells when the cells were incubated with 1 μM rhGal-1 (Fig. 4A).

It has remained unknown how Gal-1 inhibits the growth of SBDS-deficient cells. As Gal-1 modulates cell–cell and cell–matrix interactions, it might act as an autocrine factor that regulates cell proliferation. Many factors regulate cell turnover, including members of the tumor necrosis factor (TNF) and galectin families. TNF family members including Fas affect leukocyte contraction through the induction of apoptosis. Mononuclear cells from SDS patients highly express the Fas antigen and increase apoptosis [10]. Similarly, several galectin family members including galectin 3 and 9, also induce leukocyte removal [24, 25]. Gal-1 has antiproliferative effects on activated T cells, and causes them to undergo apoptosis in many conditions [26–28]. Gal-1 also inhibited 32Dcl3 and HL-60 cell proliferation

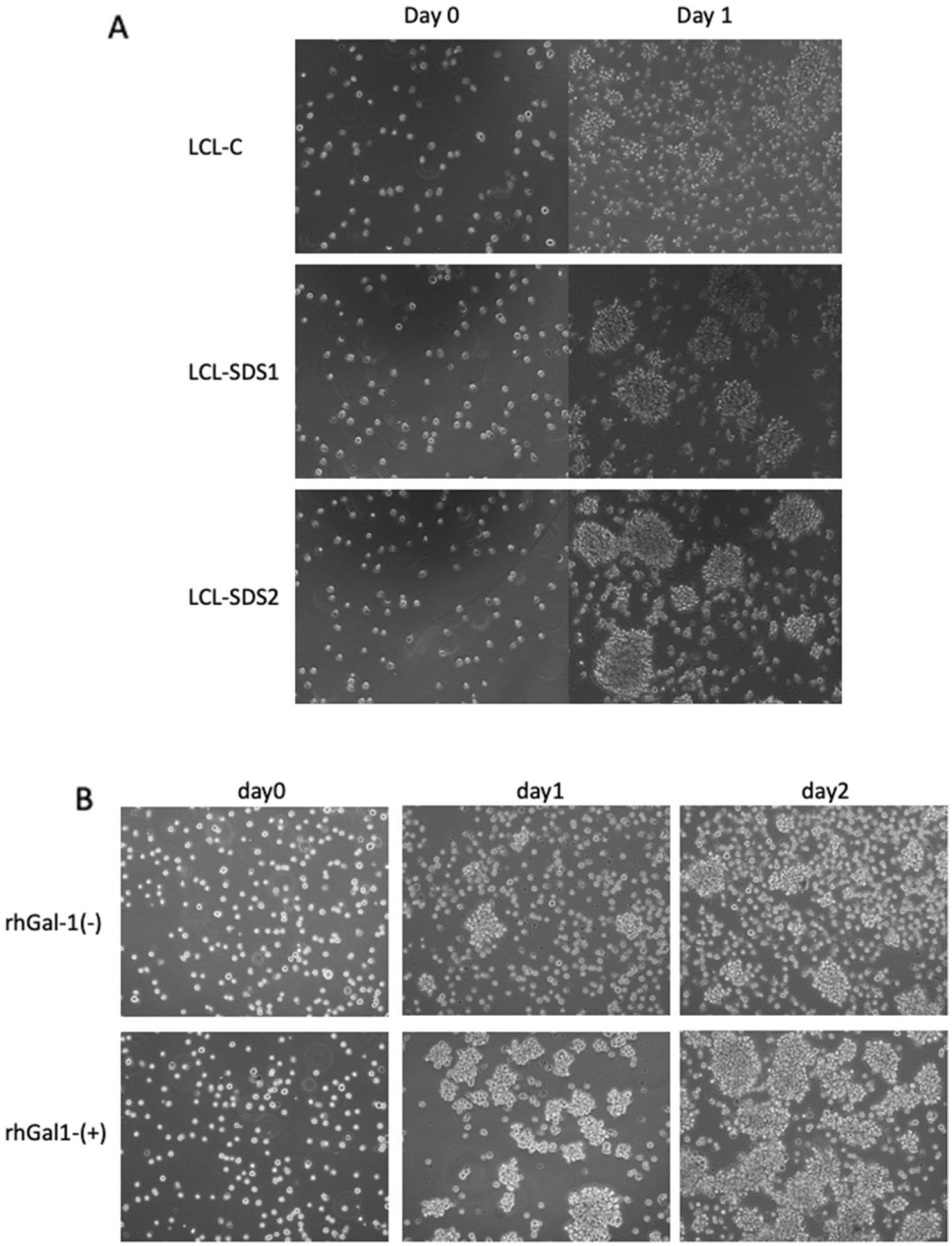

Fig. 6 Aggregation of LCL-SDS cells increased compared to LCL-C cells during cell culture. **A** LCL-cells were cultured at a density of 1×10^5 cells/mL in RPMI 1640/10% FCS medium and incubated at 37 °C for 1 day. **B** LCL-C cells were cultured with 5 μM rhGal-1 protein at 37 °C for 2 days. The images are representation of at least 3 independent experiments

in a dose-dependent manner (Fig. 4B). Gal-1 also delays cell cycle progression through the G_1/S phase transition in HT-29α5 cells [29]. However, we found that the cell cycle were few different between LCL-C and LCL-SDS cells (Supplementary Fig. 2). Our data also showed that rhGal-1 induced apoptosis in LCL-C cells (Fig. 5). In addition, it has been shown that Gal-1 can mediate homotypic cell aggregation of human melanoma cells in a carbohydrate-dependent manner [21]. In this study, LCL-SDS cells aggregated in cell culture (Fig. 6A), which was apparently induced by overexpressed Gal-1. Actually, aggregation of LCL-C cells and 32Dcl3 cells was induced by the addition of rhGal-1 (Fig. 6B and Supplementary Fig. 1). Gal-1 is known to form dimer and oligomer on cell surface. Based on these observations, we have thought increased Gal-1 on LCL-SDS cells involves in cell aggregation through the interaction between Gal-1, and Gal-1 and sugar chains on adjunct cells. Cell aggregation via Gal-1 might cause apoptosis of the cells, which affect cell proliferation. The apoptotic cells seem to be removed by dilution and pipetting and also reduce of cell–cell interaction and then the cells start to reproliferate.

Various physicochemical agents are able to modulate the expression of Gal-1 [19]. It has been reported that Gal-1 is overexpressed in classical Hodgkin lymphoma in an activator protein 1-dependent manner [30, 31]. In both LCL-SDS cells and *SBDS* shRNA knockdown 32Dcl3 cells, the

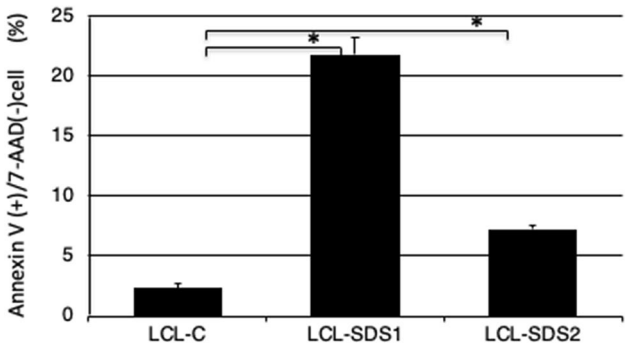

Fig. 7 Apoptosis of LCL-SDS cells increased compared to LCL-C cells during cell culture. LCL-cells were cultured at a density of 1×10^5 cells/mL in RPMI 1640/10% FCS medium and incubated at 37 °C for 4 days. Apoptosis was assessed using Annexin V and 7AAD staining and flow cytometry. Values shown are the averages of three separate experiments ± standard deviation of the mean. $^*p<0.05$

depletion of SBDS led to cellular changes that triggered induction of the *Gal-1* gene (Fig. 3). On the other hand, the immunoglobulin heavy constant gamma 2 (*IGHG2*) gene was downregulated in LCL-SDS cells (Fig. 2B). LCL-SDS cells have been established from peripheral bold B cells derived from SDS patients. The low expression of *IGHG2* mRNA might reflect clinical symptom of SDS patients[32].

In summary, we have demonstrated that Gal-1 protein is overexpressed in both SDS patient-derived cells and *SBDS*-knockdown cells. This highly expressed Gal-1 is partially involved in impaired cell growth. Overexpression of Gal-1 in the primary cells from SDS patients and understanding the significance of Gal-1 in the pathophysiology of SDS are subjects in future studies.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12185-024-03709-z.

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Data availability The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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