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Expression, Purification, and Characterization of a Novel Soluble Form of Human Delta-like-1

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Abstract The notch signaling pathway plays an important role in inhibiting cell differentiation and enhancing the repopulation capability of hematopoietic stem/progenitor cells. In this study, we developed rhDSL, a novel soluble form of Notch ligand Delta-like-1, which contains the DSL domain and the N-terminal sequence of the ligand, and investigated its function in ex vivo expansion of human umbilical cord blood (UCB)-primitive hematopoietic cells. The coding sequence for rhDSL was cloned into a pQE30 vector, and the recombinant rhDSL, fused with a 6× His tag, was expressed in *Escherichia coli* as inclusion bodies after isopropyl β-D-thiogalactoside induction. After renaturing by dilutions, the protein was purified through anion exchange followed by affinity chromatography. The purity of rhDSL protein was more than 99% with very low endotoxin. In combination with human c-kit ligand, the effect of rhDSL on ex vivo expansion of UCB $CD34^+$ cells was found to be optimal at 1.5 μg/ml of rhDSL. The rhDSL protein might therefore be a potential supplement for the expansion of UCB-primitive hematopoietic cells.

Keywords Notch signaling · rhDSL · Expression · Purification · HS/PCs

Introduction

Notch signaling pathway is an evolutionarily conserved mechanism that has been identified in both invertebrate and vertebrate species [1, 2]. Notch signals exchanged between

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neighboring cells through Notch receptors and ligands can amplify and consolidate molecular differences, which eventually dictate cell fates and influence organ formation and morphogenesis [2–4].

Notch ligands are single-pass transmembrane proteins expressed on the cell surface. Two Notch ligands, Delta and Serrate, have been identified in Drosophila, and one Notch ligand, Lag2, has been identified in *Caenorhabditis elegans* [5]. In mammals, Delta homologues are termed "Delta" or "Delta-like" and Serrate homologues are called "Serrate" or "Jagged" [6]. The extracellular domain of Notch ligands consists of multiple EGF-like repeats and a conserved N-terminal DSL (Delta, Serrate, and LAG-2) domain. The DSL domain is vital for the binding of the Notch receptor and consecutive receptor activation [7].

The widespread expression of Notch family members by hematopoietic cells including progenitors has led to speculations about their role in hematopoiesis. Hematopoietic cells and stromal cells express Notch receptors and their ligands, and Notch signaling affects the survival, proliferation, and fates of precursors at various stages of hematopoietic development including hematopoietic stem cell renewal and differentiation [8]. They are also relevant in the decision whether common lymphoid precursors undergo T- or B-cell differentiation, or whether monocytes differentiate into macrophages or dendritic cells [6, 8]. Notch ligands, especially human Delta-like-1, have been expressed and purified by a number of research groups in order to investigate the function of Notch signal in the hematopoietic system. The immobilized Delta-like-1 extracellular domain has been identified to inhibit myeloid and lymphoid differentiation and promote the repopulation of $CD34^+$ cells from human umbilical cord blood (UCB) [9, 10]. $hDll1^{NDSL}$ consisting of the DSL domain and the N-terminal sequence of Delta-like-1 inhibits cell differentiation and promotes the proliferation of mouse progenitor cells in culture [11].

Delta-like-1 is a homologue of the Drosophila Delta and is composed of 723 amino acids with one DSL and eight EGF-like repeats [12]. Due to the very large number of disulfide bonds associated with multiple EGF repeats, the Delta-like-1 extracellular domain was expressed in eukaryotic cell systems [13]. However, Varnum-Finney et al. [14] reported immobilization on plastic surface is required for this derivate to activate Notch signaling in vitro. The immobilized ligands might then be adequately "concentrated" and thus sufficiently cluster receptors to induce proteolysis and Notch activation [14]. As an immobilized form, the derivate is more difficult to handle than the soluble form during cell culture, and its advanced application in vivo might be limited.

Others, however, showed that soluble forms of Notch ligands could induce Notch activation in oligodendrocytes and hematopoietic cells [10, 15–17]. Several hypotheses exist to explain these controversial results: firstly, the differences in the structures of soluble ligands and the different responsiveness of various cell systems used may have led to such discrepancies [13]; secondly, Delta was found to mediate cell-to-cell homeotypic adhesion suggesting the existence of a Delta–Delta interaction in addition to ligand–receptor interactions [13]. Soluble ligands at low dose may sequester other endogenous ligand molecules and reduce the concentration of free ligands available for Notch binding. Once endogenous Notch ligands are saturated, a pure agonist effect would be observed at higher doses, since exogenous ligands could be set free to interact with Notch [13].

For example, a truncated human Delta-like-1, $hDll1^{NDSL}$ (containing the DSL domain and the N-terminal sequence of Delta-like-1) of about 39 kDa size and fused with a glutathione S-transferase (GST) tag, was produced in *Escherichia coli* and used as a soluble form in cell cultures [10, 18]. Unlike the Delta-like-1 extracellular domain, this derivate is shorter and could be produced in bacterial hosts in the disulfide-bound form [10, 13, 18]. The Delta-like-1 derivates expressed by *E. coli* are more economic than those expressed in eukaryotic cell systems. The 26-kDa GST tag of $hDll1^{NDSL}$ consists of 220 amino acids,

which could potentially influence the complete protein structure and therefore requires additional proteolytic cleavage. This additional step can diminish protein recovery [19–21]. Another drawback is the immunogenicity of the GST tag [21]. Therefore, we developed a novel, soluble derivate with a simpler structure and hypothesized that this could function like other derivates [9–11] on hematopoietic stem/progenitor cells (HS/PCs).

Materials and Methods

Construction of rhDSL Expression Vector

For bacterial expression, the vector pQE30 (Qiagen, Hilden, Germany) coding for an N-terminal detection and purification epitope (6× His-tagged) was used. The fragment encoding the rhDSL region, including the DSL domain and its adjacent N-terminal 50 amino acids (aa 127–225) of human Delta-like-1 (Gene Bank: AF196571), was amplified by polymerase chain reaction (PCR) from pGEX-hDSL (previously cloned in our lab) and was subcloned into a pQE30 vector to create pQE30-hDSL [11]. PCR primers were synthesized by Sangon (Shanghai, China) with a forward primer containing an engineered *Bam*HI site (underlined): 5′-CG GGATCC CTC CAC ACA GAT TCT CCT G-3′, and a reverse primer containing an engineered *Hin*dIII site (underlined): 5′-CG AAGCTT TTA GAT CGG CTC TGT GCA GTA G-3′. An engineered translation stop condon "TAA" was included in the reverse primer.

After DNA denaturation for 5 min at 94 °C, amplification consisting of 45 s at 95 °C, 45 s at 55 °C, and 1 min at 72°C was performed for 26 cycles. The purified PCR product and pQE30 vector were digested with *Bam*HI and *Hin*dIII (Fermentas, MD, USA), gel-purified, and ligated with T4-DNA ligase (Toyobo, Osaka, Japan) overnight (16 °C). *E. coli* DH5α (Tiangen, Beijing, China) was chemically transformed with the recombinant pQE30-hDSL vector (Fig. 1) and cultured at 37 °C in Luria–Bertani (LB) agar with ampicillin (100 μg/ml) for the selection of positive transformants. The pQE30-hDSL was further

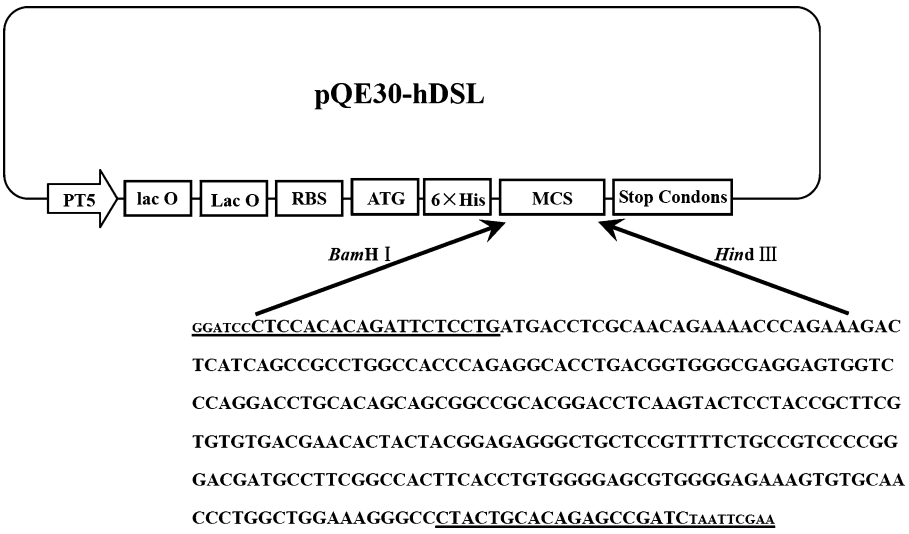

Fig. 1 Plasmid map of pQE30-hDSL. Primer sequences are *underlined*. The forward primer contained a *Bam*HI site, the reverse primer contained a *Hin*dIII site and "TAA" as an engineered translation stop condon

analyzed by restriction enzyme digestion and finally confirmed by DNA sequencing (Sangon).

Expression of rhDSL

A single transformed DH5α colony was inoculated into 20 ml LB medium supplemented with ampicillin (100 μg/ml) and cultured with 250 rpm overnight at 37°C. Ten milliliters of the above culture was transferred to fresh 500 ml LB and cultured with 250 rpm shaking at 37 °C to the optical density of 0.8–1.0 at 600 nm. Isopropyl β-D-thiogalactoside (IPTG; Sangon, 500 mM, 1 ml) was added to induce protein production for 4 h at 37 °C. One milliliter of IPTG-induced and uninduced samples was collected for analyzing the expression of rhDSL using 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining R-250 (Dingguo, Beijing, China) [22].

Preparation of Inclusion Bodies

IPTG-induced bacteria were harvested by centrifugation (4 °C, 5,000 rpm for 20 min) and washed with phosphate-buffered saline (PBS, pH 7.4). Cell pellets were re-suspended in lysis buffer (PBS, 1 mM ethylenediamine tetraacetic acid (EDTA), 5 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4). After sonication and centrifugation [23], the undissolved inclusion bodies were washed with pellet wash buffer (PBS, 1% Triton X-100, 10 mM EDTA, 50 mM NaCl, pH 7.4) and centrifuged at 4°C with 15,000 rpm for 5 min. One gram of inclusion bodies was then solubilized in 9 ml denaturation buffer (8 M urea, 50 mM Tris–HCl, 1 mM EDTA, 50 mM NaCl, 0.1 mM PMSF, pH 8.5) and shaken slightly at 37 °C for 1 h. Non-dissolved debris was removed after centrifugation at 4 °C with 10,000 rpm for 15 min, and dissolved inclusion bodies in the supernatant were further subjected to renaturation.

Refolding and Purification of rhDSL

The denatured protein was renatured using dilutions. Dissolved inclusion bodies were added dropwise into a 10-fold volume of refolding buffer (50 mM NaH_2PO_4, 0.1 mM PMSF, pH 10.7) under vigorous stirring. The pH value of the solution was monitored and maintained at pH 10.7 throughout the procedure. Then, an equal volume of dilution buffer (20 mM Tris–HCl, 0.1 mM PMSF, pH 8.0) was added into the refolding buffer and the final pH of the refolded protein solution was adjusted to 9.1 and stored at 4 °C for about 24 h.

The refolded protein solution was centrifuged at 4 °C with 18,000 rpm for 30 min. The aqueous phase was loaded onto a Q Sepharose™ Fast Flow column (Q Sepharose FF, Amersham, NJ, USA) in a AKTA Purifier System (AKTA Purifier-10, Pharmacia, Uppsala, Sweden) and pre-equilibrated with wash buffer (20 mM Tris–HCl, pH 9.1). The column was washed with wash buffer, and column-bound proteins were eluted by slowly increasing ionic strength from 0 to 1 M NaCl elution buffer (20 mM Tris–HCl, pH 9.1). The rhDSL protein containing fractions were identified by 15% SDS-PAGE and protein staining.

The eluted protein was further purified using His-Select™ Nickel Affinity Gel (Sigma, NY, USA) and the manual gravity-flow purification [24]. The column was equilibrated with His-Tag binding buffer (0.05 M imidazole, 0.5 M NaCl, 20 mM Tris, pH 8.5). The protein samples were combined with imidazole buffer (0.4 M imidazole, 20 mM Tris, pH 8.5) up to a final imidazole concentration of 0.05 M, and the pH value of the mixture was set to 8.5. Then, the protein samples were loaded onto the column, and the column was washed with

His-Tag binding buffer and His-Tag wash buffer (0.06 M imidazole, 0.5 M NaCl, 20 mM Tris, pH 8.5). Column-bound recombinant proteins were eluted with His-Tag elution buffer (0.2 M imidazole, 0.5 M NaCl, 20 mM Tris, pH 8.5). The purified protein was identified by SDS-PAGE and Western blot and analyzed by the Bradford method [25].

Silver Staining and Western Blot Assay

To further verify the purity of the protein, 1 μg rhDSL was loaded upon SDS-PAGE and were visualized by silver staining using a Fast Silver Stain Kit (Beyotime, Haimen, China). For Western blot, proteins separated by SDS-PAGE were transferred to polyvinylidene fluoride membranes [22]. The filters were then incubated with a purified, polyclonal rabbit anti-6×His tag antibody (PTGlab, Chicago, USA), and counterstained with the horseradish peroxidase-conjugated, goat anti-rabbit IgG (PTGlab). Colorization occurred after the application of diaminobenzidine.

Endotoxin Detection

Endotoxin content in purified protein fractions was measured by end-point chromogenic assays using a quantitative Chromogenic End-point Tachypleus Amebocyte Lysate (TAL) Kit (Chinese Horseshoe Crab Reagent Manufactory, Xiamen, China) [26]. UV absorbance was detected at 545 nm. According to the established standard curve ($A_{545\ nm}$=endotoxin content×1.0086+0.023, R=0.993), the endotoxin content of the purified protein sample was analyzed as EU per microgram by the use of definite volumes of standard solutions and reagents provided in the kit.

In Vitro Bioactivity Assay of rhDSL

Bioactivity of rhDSL was identified by the expression of the *HES-1*, a target gene for Notch signaling [27]. C2 myoblast cells (1.3×10^6) were plated in 10-cm diameter dishes. After 24 h, rhDSL protein (1.5 μg/ml) was added and the cells were grown in DMEM (Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, UT, USA) for another 17 h. Total RNA was isolated from cultured cells with Trizol (Invitrogen, CA, USA), and cDNA was synthesized with oligo (dT) 18 primers using ThermoScript RT-PCR system (Toyobo) in a 20-μl reaction volume containing 2 μg total RNA. Primers (Sangong) of *HES-1* were as follows: *HES-1* forward primer, 5′-GAA AGA TAG CTC CCG GCAT-3′; *HES-1* reverse primer, 5′-GTC ACC TCG TTC ATG CAC T-3′. The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) gene was used as an endogenous control with the following primers (Sangong): forward primer, 5′-GGG CTC ATG ACC ACA GTC-3′; reverse primer, 5′-GTT CAG CTC TGG GAT GAC-3′. RT was performed at 46 °C for 1 h, followed by heat inactivation at 95 °C for 5 min. PCR amplification was carried out 1 min at 95 °C, 15 s at 52 °C, and 15 s at 72 °C for 40 cycles.

Isolation of Human UCB $CD34^+$ Cells

Human UCB was collected from full-term deliveries according to the institutional guidelines of the International Peace Maternity & Children Health Hospital, Shanghai, China. UCB was 1:2 diluted with cold PBS (pH 7.4), before mononuclear cells (MNCs) were separated by density gradient centrifugation using Ficoll solution (ρ= 1.077±0.002 g/ml, Huajing Biological High Technology Ltd, Shanghai, China). MNCs

were washed twice with PBS and resuspended in 600 μl PBS buffer containing 2 mM EDTA and 0.5% BSA. $CD34^+$ cells were isolated using magnetically conjugated mouse anti-human CD34 beads (Miltenyi Biotec, Bergisch Gladbach, Germany) followed by separation on MS columns (Miltenyi Biotec) according to the manufacturer's instructions [28]. The purity of the $CD34^+$ cells was 98.39% as determined by fluorescence-activated cell sorting (FACS).

Ex Vivo Expansion of UCB $CD34^+$ Cells

The freshly isolated UCB $CD34^+$ cells were cultured in a 24-well plate at a concentration of 8.5×10^4 cells in 1 ml Dulbecco's modified Eagle's medium/Nutrient F-12 (DMEM/F-12; Gibco) medium supplemented with 15% FBS, 2 mM L-glutamine (Sigma), 50 μg/ml penicillin (Invitrogen), and 50 U/ml streptomycin (Invitrogen) [29]. Different concentrations of rhDSL in combination with human c-kit ligand (KL, 20 ng/ml, Peprotech, NJ, USA) were added to the culture fluids. The cells were incubated for 2.5, 4.5, and 6.5 days at 37 °C, 5% CO_2 and saturated moisture. Then the total numbers of cells, $CD34^+$ cells, and $CD34^+$ $CD38^-$ cells were assessed.

Flow Cytometry

After incubation for 2.5 days, the cultured $CD34^+$ cells were washed with 4 °C PBS containing 2% FBS. The cells were resuspended in 0.1 ml of 2% FBS/PBS with 1 μl FcR-blocking Reagent (eBioscience, CA, USA). Antibodies were fluorescein isothiocyanate (FITC)-conjugated mouse anti-human CD34 (Caltag, Buckingham, UK) and phycoerythrin (PE)-conjugated mouse anti-human CD38 (Caltag) [11, 30]. One microliter of antibodies was added to each sample. After 30 min of incubation, the cells were washed once and suspended in 2% FBS/PBS for FACS analysis. FITC- or PE-conjugated isotype-matched antibodies were used as controls. Flow cytometry analysis was performed using a FACSCalibur cell analyzer (Becton-Dickinson, NJ, USA).

Progenitor Assay

After incubation for 2.5 days, colony-forming cells were determined by colony-forming unit (CFU) assays [31]. Cells were plated in 1 ml of Iscove's modified Dulbecco's medium (IMDM, Gibco) containing 1.08% (*w*/*v*) methylcellulose, 30% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol (Amresco, OH, USA), and 0.024 mM monothioglycerol (Sigma) plus cytokines including 10 ng KL, 10 ng granulocyte–macrophage colony-stimulation factor (GM-CSF), 10 ng IL-3, and 3 U erythropoietin (all from Peprotech). Triplicate cultures were incubated for 14 days at 37 °C in a fully humidified 5% CO_2 atmosphere. Colonies including total CFU, CFU-Mix (mixed colony-forming unit), burst-forming unit—erythroid (BFU-E), colony-forming unit—erythroid (CFU-E), colony-forming cell—granulocyte macrophage (CFU-GM), and high proliferation potential colony-forming cell (HPP-CFC, diameter >0.5 mm) were counted.

Statistical Analysis

Data are presented as mean ± SE. Analysis of variance (ANOVA) with follow-up LSD tests using SPSS version 11.5 (SPSS, IL, USA) was performed. Differences between data sets with p values <0.05 were considered significant, p values <0.01 as highly significant.

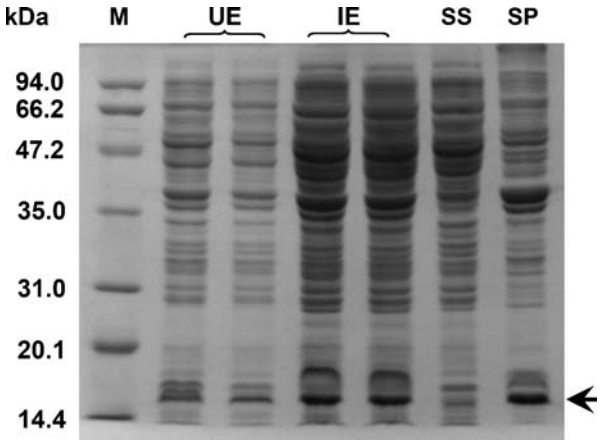

Fig. 2 Expression of rhDSL in *E. coli* DH5α. rhDSL was expressed in *E. coli* induced by IPTG and the inclusion bodies were obtained by ultrasonication. *Lane M*, low M_w protein markers. *Lane UE*, uninduced *E. coli* cell lysate. *Lane IE*, *E. coli* lysate after IPTG induction. *Lane SS*, supernatant of *E. coli* lysate after ultrasonication and centrifugation of the induced *E. coli*. *Lane SP*, the pellet fraction after the ultrasonication and centrifugation of the induced *E. coli*. The *arrow* stands for rhDSL at 15 kDa

Results

Expression and Purification of rhDSL

A protein of M_w ~15 kDa was induced by IPTG in the *E. coli* harboring pQE30-hDSL (Fig. 2, lane IE). After sonication, the soluble cell lysate (Fig. 2, lane SS) and inclusion bodies (Fig. 2, lane SP) were analyzed. The inclusion bodies contained mainly the rhDSL accounting for 26.2% of the total protein in the inclusion bodies measured by densitometry scanning (Table 1).

As the isoelectric point (p*I*) value of rhDSL is around 6.0 (estimated by Sci Ed Central software, Scientific & Educational Software, NC, USA), the anion exchange chromatography with buffer pH above this point was used for purification. The protein-binding condition was optimized to 20 mM Tris–HCl (pH 9.1) after three independent preliminary absorption/elution experiments (data not shown). The purity of rhDSL was about 73.9% (Table 1) estimated by densitometry scanning of the stained protein gel after purified by Q Sepharose™ Fast Flow anion exchange chromatography (Fig. 3a, lane EL1). This eluted protein was further purified by His-Select™ Nickel affinity chromatography and analyzed by SDS-PAGE and Coomassie staining (Fig. 3b, lane EL2). No contaminating proteins were observed in the final preparation of rhDSL (Fig. 3b, lane EL2). In order to increase the detection sensitivity, silver staining was performed to further determine the purity of the purified rhDSL. Except the band corresponding to the rhDSL, no other bands were observed (Fig. 3c, lane SEL2). As the sensitivity of protein silver staining is 0.3 ng and

Table 1 The relative yield of rhDSL protein from the *E. coli* expression system.

Purification steps	Total protein (mg)	rhDSL purity (%)	Total rhDSL (mg)	Relative protein yield (%)
Cell lysate by sonication	350	26.2	91.7	100
Denaturation with 8 M urea	90	29.4	26.6	29
Renaturation by dilutions	27.7	56.2	15.6	17
Q Sepharose FF eluate	6.7	73.9	4.0	5.4
His-Select™ Nickel affinity gel eluate	2.3	>99	2.3	2.5

Results are calculated from 1,000 ml cultures of *E. coli* expressing the rhDSL protein. Total protein was estimated using the Bradford method

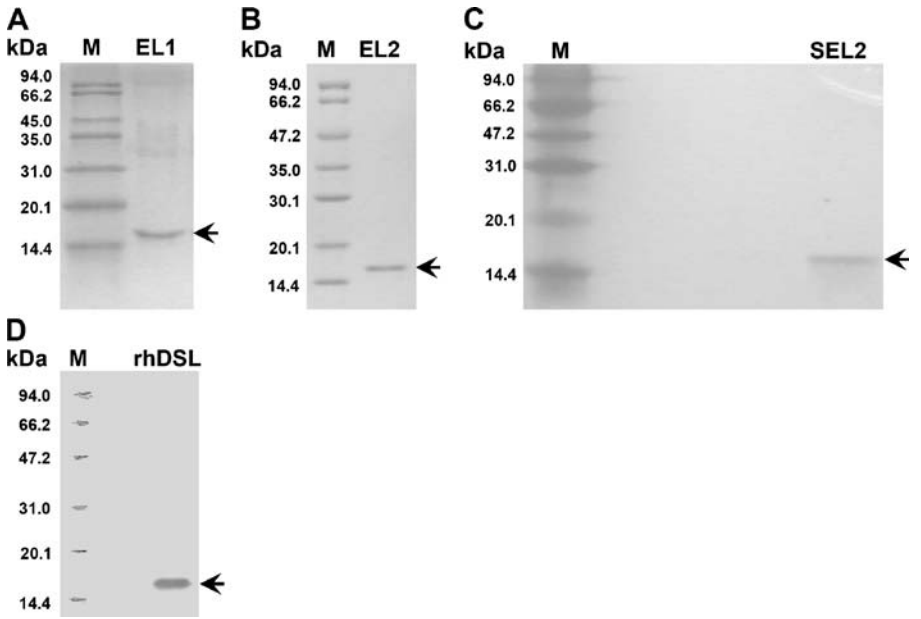

Fig. 3 Purification of rhDSL. **a** SDS-PAGE of the eluate from Q Sepharose™ Fast Flow; the purity of rhDSL was around 73.9% assayed by BandScan 5.0 software. *Lane EL1*, the eluated protein. **b** SDS-PAGE of the purified rhDSL elution from HIS-Select™ Nickel Affinity Gel column following Q Sepharose™ Fast Flow. *Lane EL2*, the eluted protein. **c** One microgram of protein after the two-step chromatographic purification analyzed by silver staining. No contaminant proteins were observed. Therefore, the purity of rhDSL was more than 99%. *Lane SEL2*, rhDSL protein obtained after two-step chromatographic purification. **d** Western blot analysis of purified rhDSL protein with anti-6× His antibody. *Lane M*, low M_w protein markers (drawn by pencil after staining of the filter by Ponceau red). *Lane rhDSL*, purified rhDSL protein. The *arrow* stands for rhDSL

500 ng rhDSL was loaded, the purity of rhDSL is expected to be more than 99%. Western blot assays showed one band corresponding to the size of rhDSL and demonstrated that the purified protein was rhDSL (Fig. 3d, lane rhDSL).

The stained protein bands from each purification step were assayed using BandScan 5.0 software (Glyko Inc., CA, USA). The intensity of each band was transformed into digital data, and the rhDSL purity was calculated. The final rhDSL yield was 2.5%. Cell lysate after sonication was used as reference (Table 1). The final purified protein was also examined for endotoxin contamination. According to the established standard curve, the endotoxin content of rhDSL was around 0.117 EU/μg, which was far below the acceptable level (1 EU/μg), according to the recombinant protein standard of R&D Systems, Inc. (MN, USA), and met the requirements for further ex vivo and in vivo studies.

In Vitro Bioactivity Assay of rhDSL

After the activation of Notch signaling, the transcription of target genes such as *HES* (Hairy/Enhancer of Split) is initiated and ultimately followed by Notch signaling [27]. C2 myoblast cells, which express at least the three Notch homologues Notch-1, Notch-2 and Notch-3, were used to analyze the bioactivity of the Notch ligands or their derivates by detecting the *HES-1* expression level [27]. In our experiment, *HES-1* mRNA was clearly detected in the C2 cells after 17 h of stimulation with rhDSL, whereas a faint signal was

obtained in the PBS treated cells (Fig. 4). The results suggested that the rhDSL is biologically potent in the activation of Notch.

Expansion of UCB-Primitive Hematopoietic $CD34^+$ Cells by Purified rhDSL

Previous in vitro studies showed that KL, in combination with IL-3 or other cytokines, increased the number of BFU-E, CFU-GM, and CFU-Mix, which suggested that KL may act on more primitive cells capable of directly generating colony-forming cells [32]. In our study, KL was used in the culture system as a basal cytokine, as Lin et al. [33] reported GST-hDll1^{NDSL} could not promote the expansion of hematopoietic progenitor $CD34^+$ cells by itself, unless it was combined with other cytokines such as KL and GM-CSF. Therefore, we analyzed the influence of the novel rhDSL on hematopoiesis only in combination with KL. Compared to KL alone, the combination of KL and 1.5 μg/ml rhDSL significantly promoted the proliferation of total cells and $CD34^+$ cells leading to expansion folds of 1.68 and 1.57 versus 1.03 and 0.97 folds ($p<0.05$) after 2.5 days (Table 2, Fig. 5a, b). As the culture time extended to 4.5 days, all three dosages of rhDSL (0, 0.5, 1.5, and 4.5 μg/ml) augmented the expansion effect of KL leading to 2.49, 2.66, and 2.49 more total cells and 1.17, 1.27, and 1.27 more $CD34^+$ cells, respectively. Under KL alone, the total number of cells and $CD34^+$ cells showed a 1.95- and 0.94-fold expansion, respectively (Fig. 5b, c). After 6.5 days, the proliferation of total cells and $CD34^+$ cells was lower at rhDSL of 1.5 and 4.5 μg/ml than in the KL control (Fig. 5a, b). The number of the $CD34^+$ $CD38^-$ cells (a population highly enriched for stem cells) decreased with the increase of culture time (Fig. 5c).

Not only $CD34^+$ cell numbers, but also colony formation was positively affected (Table 3). The rhDSL of 1.5 μg/ml significantly improved the total numbers of colonies compared to KL alone leading to 1.35 times more total CFU, 2.20 times more HPP-CFC, and 1.39 times more CFU-GM ($p<0.01$). Again, 1.5 μg/ml was found to be the optimal rhDSL concentration for the expansion of UCB-derived progenitors. No differences in the numbers of CFU-Mix, BFU-E, CFU-E, and plating efficiencies were observed between rhDSL treated groups and KL controls.

Discussion

In the present study, we developed rhDSL as a novel soluble form of human Notch ligand Delta-like-1. Similar to the GST-tagged hDll1^{NDSL}, the rhDSL contained the DSL domain

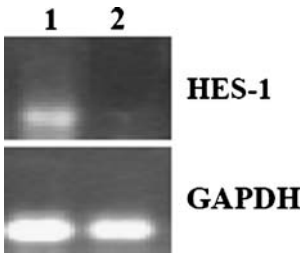

Fig. 4 Endogenous *HES-1* expression. Endogenous *HES-1* expression was increased in C2 cells cultured with rhDSL (1.5 μg/ml). The *HES-1* RNA of the C2 cells was analyzed by RT-PCR. The signal of *HES-1* was detected at the position of 189 bp and *GAPDH* (endogenous control) at the position of 159 bp. *Lane 1*, C2 cells culture with rhDSL. *Lane 2*, C2 cells culture with the PBS control

Table 2 rhDSL promotes the expansion of UCB hematopoietic progenitor cells.

rhDSL (μg/ml)	Total cells ($\times 10^4$)	$CD34^+$ cells ($\times 10^4$)	$CD34^+$ $CD38^-$ cells ($\times 10^3$)
0	8.8±0.41	8.1±0.34	4.5±1.4
0.5	9.1±0.76	8.5±0.67	4.4±0.63
1.5	14.3±0.69*	13.2±0.69*	6.7±0.10
4.5	10.3±0.08	9.6±0.29	4.5±0.33

UCB $CD34^+$ cells were cultured for 2.5 days with KL (20 ng/ml) and with graded concentrations of rhDLS. After 2.5 days of culture, the numbers of total cells, $CD34^+$ and $CD34^+$ $CD38^-$ immature hematopoietic cells were determined, and cells were transferred to semisolid medium for methylcellulose assays. Shown are mean values ±SE

*$p<0.01$, highly significant difference compared to KL alone

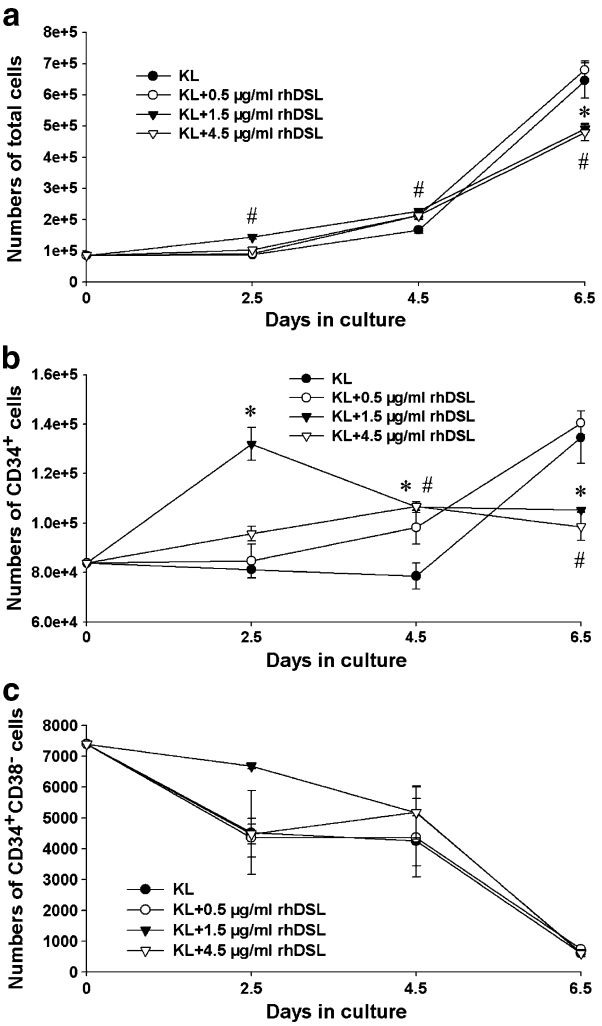

Fig. 5 Progenitor cell expansion. After culturing for 2.5, 4.5, and 6.5 days, UCB $CD34^+$ cells, expanded by various concentrations of rhDSL (0, 0.5, 1.5, and 4.5 μg/ml) combined with human KL (20 ng/ml), were collected and the numbers of total cells (**a**), $CD34^+$ (**b**), and $CD34^+$ $CD38^-$ (**c**) immature hematopoietic cells were counted. *$p<0.05$, significant difference compared to KL alone; #$p<0.01$, highly significant difference compared to KL alone

Table 3 rhDSL promotes the expansion of UCB hematopoietic colony-forming cells.

rhDSL (μg/ml)	Total CFU ($\times 10^4$)	HPP-CFU ($\times 10^3$)	CFU-Mix ($\times 10^2$)	CFU-GM ($\times 10^4$)	BFU-E ($\times 10^2$)	CFU-E ($\times 10^2$)	Plating efficiency (%)
0	2.3±0.05	1.0±0.25	6.8±1.5	2.1±0.04	2.2±0.57	6.0±0.79	25.7±1.8
0.5	2.5±0.10	1.2±0.20	8.1±0.36	2.3±0.07	4.0±0.76	6.3±2.1	27.4±1.2
1.5	3.1±0.16**	2.2±0.08**	9.2±1.4	2.9±0.16**	3.8±1.1	7.1±1.6	21.7±1.9
4.5	2.7±0.09*	1.8±0.22*	6.6±0.71	2.5±0.07*	3.7±0.89	8.7±1.4	25.9±1.0

UCB $CD34^+$ cells were cultured for 2.5 days with KL (20 ng/ml) and with graded concentrations of rhDSL. After 2.5 days in culture, the cells were transferred to semisolid medium for methylcellulose assays. Numbers of total CFU, HPP-CFC, CFU-Mix, CFU-GM, BFU-E, and CFU-E were counted after 14 days. The plating efficiency (percent) is also shown. Data are expressed as mean results ±SE

*$p<0.05$, significant difference compared to KL alone; **$p<0.01$, highly significant difference compared to KL alone

and the N-terminal sequence of the ligand, while it was fused to a much smaller tag of 6× His (0.9 kDa). This tag is widely used to facilitate the purification of proteins. In contrast to the GST tag, the 6× His tails have almost no effect at most circumstances on the overall structure and biological functions of proteins and do not need to be cleaved from the fusion proteins such as the Nef protein [19–21]. The designed rhDSL, contained little endotoxin, was successfully obtained from bacterial inclusion bodies and was highly purified through anion exchange chromatography followed by affinity chromatography (>99%). Compared to previous reports on purification of Delta-like-1 derivates which were expressed in eukaryotic cell systems and used as a immobilized form or fused with a big GST tag [10, 12, 15], our method has several advantages: (1) it is cost effective since the rhDSL is expressed in *E. coli*; (2) it is easy to produce; (3) the fusion with a 6× His tag conserved the bioreactive structure; and (4) as a soluble form, the rhDSL could be used conveniently for research in vitro and even in vivo.

Since endotoxin could tightly bind to Q Sepharose [34, 35], the crude rhDSL protein was firstly purified through the anion exchange chromatography to remove endotoxin before performing the affinity chromatography. This two-step chromatographic purification resulted in rhDSL protein of high purity and a very low endotoxin level. The predicted molecular weight of the rhDSL protein is 12.4 kDa, while the protein showed an unexpectedly decreased mobility stopping at ~15 kDa position on SDS-PAGE. The slightly different results may be ascribed to the more concentrated polyacryl gels (15%). A similar phenomenon was also observed in the electrophoresis analysis of other chemokines such as human recombinant MIG (monokine induced by INF-γ) [23].

The effect of the novel rhDSL on hematopoietic progenitor expansion was further investigated. The highest expansion was obtained at a concentration of 1.5 μg/ml. A higher concentration of 4.5 μg/ml was less effective. Delaney et al. [9] also reported that the enhanced generation of $CD34^+$ cells was only obtained at lower concentrations of Delta-like-1, while higher concentrations of the ligand could result in apoptosis of $CD34^+$ progenitors. In this study, UCB-primitive hematopoietic $CD34^+$ cells cultured for 2.5 days in KL and rhDSL significantly increased CFU-GM colony formation compared to KL alone. This implies that rhDSL synergizes with KL in HS/PCs expansion driving the progenitor cells into the granulocyte and macrophage lineage.

Han et al. [11] showed that the optimal concentration of GST-hDll1^{NDSL} in the inhibition of myeloid differentiation is 0.3–0.4 μM. Our present study found that 1.5 μg/ml (0.12 μM) of rhDSL is the optimal concentration. Thus, the biological efficiency of rhDSL in the

present study is about three times higher than GST-hDll1^{NDSL}, suggesting that the GST tag may affect the activity of the protein. Moreover, the in vivo activity of GST-tagged protein may further be compromised due to the immunogenicity of GST. However, the smaller 6× His tag is usually considered to lower immunogenic which is an advantage over GST tag.

As each cell type requires unique culture conditions containing specific cytokines and growth factors, the stem cell maintenance in vitro may be difficult. Here, we preliminarily identified the effect of the novel rhDSL on hematopoietic stem/progenitor cells expansion and optimized its concentration, which offers a basis for the combination of Notch signaling with other pathways such as Wnt. The activation of the Notch signaling pathway in vivo has been advanced with a main focus on gene therapy or transgenic animals by enforcing the expression of Notch ligands or an active form of Notch receptors, or inhibiting the enzymes involved in Notch signaling, such as Fringe and γ-secretase, to reduce the maturity of Notch receptor or the responsiveness of Notch receptor to its ligands [36–39]. Due to its higher similarity with the wild-type counterpart and its positive influence on hematopoietic stem/progenitor cells in vitro, this novel rhDSL might offer a potential alternative for the advanced research of the Notch signaling pathway in vivo.

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