

Notch signaling maintains proliferation and survival of the HL60 human promyelocytic leukemia cell line and promotes the phosphorylation of the Rb protein

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Received: 1 September 2009 / Accepted: 3 February 2010 / Published online: 16 February 2010
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Abstract The Notch signaling pathway has been implicated in the development of several leukemia and lymphoma. In order to investigate the relationship between Notch signaling and acute myeloid leukemia (AML), in this study, we expressed a recombinant Notch ligand protein, the DSL domain of the human Jagged1 fused with GST (GST-Jag1). GST-Jag1 could activate Notch signaling in the human promyelocytic leukemia cell line HL60, as shown by a reporter assay and the induced expression of Notch effector gene *Hes1* and *Hes5*. However, GST-Jag1 had no effect on the proliferation and survival of HL60 cells. HL60 cells expressed both Notch ligands and receptors, and had a potential of reciprocal stimulation of Notch signaling between cells. We, therefore, blocked

Notch signaling in cultured HL60 cells using a γ -secretase inhibitor (GSI). We found that GSI inhibited the proliferation of HL60 cells significantly by blocking the cell-cycle progression in the G1 phase. Furthermore, GSI induced remarkably apoptosis of HL60 cells. These changes in GSI-treated HL60 cells correlated with the down-regulation of c-Myc and Bcl2, and the low phosphorylation of the Rb protein. These results suggested that reciprocal Notch signaling might be necessary for the proliferation and survival of AML cells, possibly through the maintenance of the expression of c-Myc and Bcl2, as well as the phosphorylation of the Rb protein.

Keywords Notch · Jagged1 · γ -Secretase inhibitor · Acute myeloid leukemia · HL60 cells · Cell proliferation · Apoptosis

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Introduction

Notch receptors are a family of highly conserved type I transmembrane proteins that transduce signals involved in the control of normal and abnormal cell differentiation, proliferation, and apoptosis [1–3]. Active Notch signaling is triggered by the interaction of Notch receptors (Notch 1–4 in mammals) with their ligands (Delta 1, 2, and 4 and Jagged 1 and 2), resulting in the cleavage and release of the intracellular domain of Notch (NICD) by a membrane-associated protease complex involving γ -secretase [4]. The NICD then translocates into the nucleus to form a complex with the DNA-binding protein RBP-J [5] and the transcriptional coactivators of the Mastermind family [6]. This protein complex transactivates the transcription of downstream genes such as the *Hes* family basic helix–loop–helix genes [7].

Notch receptors and ligands are expressed in many populations of hematopoietic cells and bone marrow (BM) stromal cells, and have been demonstrated to play critical roles in the development and differentiation of various hematopoietic lineages. On the other hand, deregulated expression of Notch pathway elements or abnormal activation of Notch signaling can lead to hematopoietic malignancies. For example, a rare chromosomal translocation event, t(7;9)(q34;q34.3), has been identified as an etiological factor in the development of T-cell acute lymphoblastic leukemia (T-ALL) and lymphoma. The chromosomal translocation juxtaposes the genomic region encoding NICD of the human Notch1 to the T-cell receptor (TCR) β enhancer, leading to aberrant expression of N1ICD (TAN1, for translocation-associated Notch homologue) in T-lineage cells [8]. In addition to the t(7;9) translocation [9], activating mutations in Notch1 independent of t(7;9) have been identified in more than 50% of human T-ALL [10], and lethally irradiated mice that received transplants of BM cells transduced with N1ICD develop T-ALL with 100% penetration [11].

In contrast to T-ALL, a definite role for Notch signaling in the development of acute myeloblastic leukemia (AML) is less clear. AML is characterized by an excessive self-renewal capacity and a block of differentiation of dysregulated hematopoietic progenitors. It has been shown that Jagged1 plays a critical role in myeloid differentiation as its forced expression inhibits differentiation following the stimulation with GM-CSF [12]. In another study [13], it was shown that Jagged1 promoted differentiation of 32D cells; probably due to that the role of Notch signaling is very cell context dependent. Tohda [14] reported that Notch1 and Jagged1 were highly expressed in AML cell lines and primary AML cells. γ -secretase inhibitor (GSI), which blocks Notch signaling, suppresses the proliferation of AML cell lines in a dose-dependent manner, through induction of apoptosis [15]. However, there were contradictory results as well. Tohda et al. [16] have also found that Delta-like1 suppresses growth and induces differentiation in human AML cell line OCI/AML-6. The same group has also shown that Notch ligands, Delta-like 1 or Jagged1, did not promote the self-renewal capacity of any of the AML cells examined and instead tended to induce differentiation under the conditions used [17].

In order to further investigate the relationship between Notch signaling and AML, we observed the role of Notch signaling in the human promyelocytic leukemia cell line, HL60. We found that GSI inhibited the proliferation of HL60 cells significantly, although recombinant Notch ligand Jagged1 did not influence the proliferation of HL60 cells. Furthermore, GSI inhibited cell-cycle progression and could induce apoptosis of HL60 cells, which was correlated closely with down-regulation of c-Myc and

Bcl2, and the hypo-phosphorylation of the retinoblastoma (Rb) protein. These results suggest that Notch signaling is necessary for the proliferation of HL60 AML cells.

Materials and methods

Cell culture

HL60 cells and HeLa cells were cultured in RPMI1640 medium and Dulbecco's modified Eagle's medium (DMEM), respectively, supplemented with 10% fetal calf serum (FCS, Sigma Chemical Co., St. Louis, MO), 2 mM glutamine, and antibiotics in a humidified chamber with 5% CO₂ and at 37°C. For treatment with recombinant Notch ligands or GSI, cells (3×10^4) were cultured in 48-well culture plates with a recombinant Jagged1, GSI, or dimethyl sulfoxide (DMSO) as a control. Cells were harvested at indicated time points, and cell numbers were counted with the trypan blue exclusion assay. For morphological observations, treated and untreated cells were stained by Wright–Giemsa staining, and examined under a light microscope.

Expression of a soluble Jagged1 protein in *E. coli*

Total-RNA isolated from normal human BM was reverse transcribed with oligo(dT)₂₀ as a primer, using the ReverTra Ace™ system following the manufacturer's instructions (TOYOBO, Osaka, Japan). The human Jagged1 cDNA was amplified by PCR with the first-strand cDNA as a template, using the specific primers and LA Taq polymerase (Takara Biotechnology, Dalian, China) under the following conditions: 94°C, 30 s; 61°C, 4 min; and 72°C, 30 s, for 40 cycles. The DSL (for Delta-Serrate-Lag2) domain was then amplified and was inserted in frame into pGEX4T-1, to construct pGEX-Jag1. *E. coli* BL21 (DE3) transformed with pGEX4T-1 or pGEX-Jag1 was inoculated in 5 ml of Luria–Bertani (LB) medium supplemented with ampicillin (100 $\mu\text{g ml}^{-1}$), and was cultured at 37°C with shaking (210 rev min⁻¹) overnight. The bacterial suspension (0.5 ml) was then transferred into 50-ml fresh LB medium in a 250-ml shake flask, and was cultured further until OD₆₀₀ reached 0.8. Expression of recombinant protein was induced by addition of isopropyl- β -D-thiogalactoside (IPTG) to 0.5 mM. The induction was permitted for 24 h at 30°C. Bacteria were harvested and disrupted by sonication, and analyzed by SDS-12% polyacrylamide gel electrophoresis (PAGE). The recombinant protein was purified from bacterial lysates with glutathione Sepharose 4B (eBiosciences, San Diego, CA), according to the manufacturer's instructions.

Reporter assay

HeLa cells were transiently co-transfected with pGa981-6 [18] and pRL-TK using Lipofectamine 2000™ according to the manufacturer's protocol (Promega). Cells were cultured in the presence of different concentrations of expressed proteins. Cells were harvested 48 h after the transfection, and were assayed for the luciferase activity, which was normalized using the activity of the Renilla luciferase. Each set of experiments was performed in triplicate and repeated for three times.

RT-PCR

Total RNA from HL60 cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the procedure provided by the supplier. Reverse transcription was carried out as described above. PCR was run for 35 cycles with cDNA from 1 µg of total RNA as a template. The primers used for the amplification of target genes are listed in Table 1. The amplified fragments were analyzed by electrophoresis with 2% agarose gel.

Flow cytometry

For cell-cycle analysis, cells were fixed in ice-cold ethanol (70% vol/vol), and were stained with propidium iodide (PI) solution (25 g/ml PI, 180 U/mL RNase, 0.1% Triton X-100, and 30 mg/ml polyethylene glycol in 4 mM citrate buffer, pH 7.8). The DNA content was determined using a FACSCalibur™ flow cytometer (Becton–Dickinson Immunocytometry Systems, San Jose, CA). Cell-cycle distribution was analyzed using the ModFit LT software (Verity Software House, Topsham, ME).

Western blot analysis

The concentration for each antibody was titrated according to manufacturer's instructions. Whole-cell extracts were prepared by lysing cells with the RIPA buffer (50 mM Tris–HCl, pH7.9, 150 mM NaCl, 0.5 mM EDTA, and 0.5% Nonidet P-40), containing protease inhibitor PMSF (0.1 mM). The protein concentration in the cell extracts was determined using the BCA Protein Assay reagents (Pierce). Total proteins were separated by SDS-10% PAGE, and were electroblotted onto polyvinylidene difluoride membrane. Western blots were performed using rabbit antibodies

Table 1 Primers used for RT-PCR analysis

Genes	Primers	Size of targets (bp)
Notch1	5'-CCGCCTTTGTGCTTCTGT	490
	5'-TCCTCCTCTCCTCGCTGT	
Notch2	5'-GCAGCCAAGATCCTGTTA	328
	5'-ACTTCTTCTCCTACTACCCT	
Notch3	5'-GAGTGGCGACCTCACTTACGA	478
	5'-GCCGTTACCCGATTTGT	
Notch4	5'-CGCTCACCAACGTAACCA	388
	5'-TCCGTATCTTCTCGCATT	
Delta-like1	5'-CGGGATCCCTCCACACAGATTCTCCTG	297
	5'-CGGAATTCGATCGGCTCTGTGCAGTAG	
Delta-like4	5'-GACGACAGTAGCGGCGGGGGGC	375
	5'-GCAATATTCCCAGTCCAACCGGGCA	
Jagged1	5'-CGCGGATCCGTGACCTGTGATGACTACTAC	153
	5'-CCGGAATTCACATTCGGGGCCCATCC	
Hes1	5'-ATCACACAGGATCCGGAGCT	300
	5'-TGACACTGGCTGGGGTAGC	
Hes5	5'-CGACCGCATCAACAGCAG	203
	5'-GCAGGCACCACGAGTAGC	
β-actin	5'-TGGATTCTGTGTCATCCATGAAAC	345
	5'-TAAAACGCAGCTCAGTAACAGTCCG	
c-Myc	5'-GTGGTCTTCCCCTACCCTCTCA	355
	5'-GCTGCGTAGTTGTGCTGATGTG	
GAPDH	5'-GGAGTCCACTGGCGTCTTACC-3'	592
	5'-GAGGAGTGGGTGTCGCTGTTG-3'	

against Bcl-2, the Rb protein, c-Myc (Santa Cruz Biotechnology), or monoclonal antibodies to β -actin, the His tag, or GST (Sigma) at appropriate dilutions, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit IgG, Promega; or goat anti-mouse IgG, Boster, Shen Zhen, China). Blots were developed using an enhanced chemiluminescence kit (NEN).

Statistical analysis

The significance of difference was determined by Student's *t*-test, with $P < 0.05$ regarded as statistically significant.

Results

Blocking notch signaling with GSI inhibited the proliferation of HL60 cells

In order to examine the relationship between Notch signaling and HL60 cells, we first carried out an RT-PCR analysis with primers specifically targeting Notch receptors and ligands, with GAPDH as an internal control. As shown in Fig. 1a, while the mRNA for Notch 1, 3, and Delta-like1 was hardly detectable, Notch2, 4, Delta-like4, and Jagged1 were all expressed in HL60 cells. Jagged2 expression was not detected (data not shown). Notch2 and Jagged1 were relatively highly expressed, suggesting that this pair of Notch receptor and ligand might play a role in HL60 cells. Hes1 and Hes5, the main downstream molecules of the Notch pathway, were also expressed in normally cultured HL60 cells (see later), suggesting that Notch signaling might be activated in these cells, likely due to reciprocal cell–cell interactions between cultured cells.

In order to examine roles of Notch signaling in the proliferation of HL60 cells, we treated cultured cells with GSI, which inhibits the cleavage of Notch receptors and thus blocks Notch signaling. The result showed that compared with DMSO, the GSI treatment significantly inhibited the proliferation of HL60 cells (Fig. 1b). We also examined the proliferation of HL60 cells in the presence of different concentrations of GSI. The results showed that the inhibition effect of GSI on HL60 proliferation was in a dose-dependent manner, with an almost complete inhibition of cell proliferation at 100 μ M (Fig. 1c). DMSO also mildly inhibited HL60 cell proliferation. These results suggested that Notch signaling was necessary for the proliferation of HL60 cells.

Generation of a soluble recombinant Jagged1 protein

In order to examine whether Notch activation might modulate the proliferation of HL60 cells, we produced the

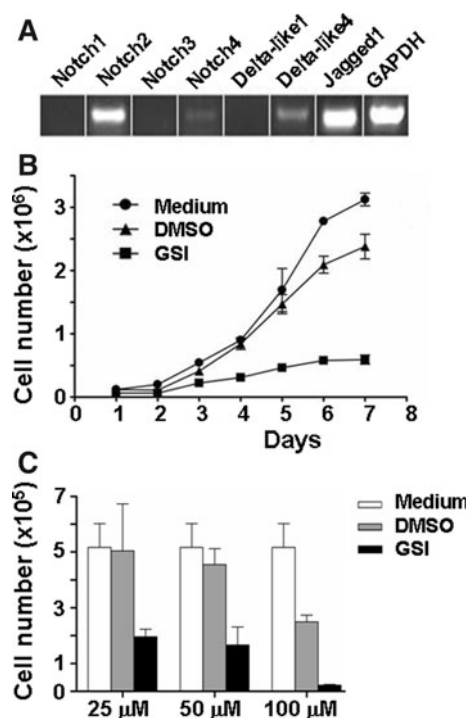


Fig. 1 GSI inhibits the proliferation of HL60 cells. **a** Expression of Notch receptors and ligands in untreated HL60 cells, as assessed by RT-PCR, with GAPDH as an internal control. **b** Growth inhibition of HL-60 by GSI. HL60 cells were cultured with GSI (100 μ M) or DMSO. Cell number was counted on different days of the culture. * $P < 0.05$. **c** GSI inhibits HL60 growth in a dose-dependent manner. Cells were cultured with different concentrations of GSI or DMSO for 48 h, and were counted as in (b)

DSL domain of human Jagged1, which is highly expressed by HL60 cells. The DSL domains of Notch ligands are highly conserved and appear to be both necessary and sufficient to bind to the extracellular domain of Notch [12, 19]. The DSL domain (amino acids 186–230) of human Jagged1 was cloned by RT-PCR from normal human BM cells and was inserted in frame into the pGEX4T-1 to generate a fusion protein (GST-Jag1). The fusion protein and the control GST protein were produced in *E. coli*, and was purified using the glutathione affinity chromatography (Fig. 2a). The purified proteins were estimated to be more than 95% pure by Coomassie-blue staining after SDS-PAGE, and were confirmed by western blotting using an anti-GST antibody (Fig. 2b).

In order to verify that the recombinant GST-Jag1 protein could activate Notch signaling, we transiently transfected HeLa cells with a reporter construct, pGa981-6, which has six RBP-J-binding sites in front of a basic promoter driving a firefly luciferase gene. The transfected HeLa cells were incubated with GST or GST-Jag1, in the absence or presence of GSI. The luciferase activity in cell lysates was examined 48 h after the transfection. We found that compared with GST, GST-Jag1 showed a dose-dependent

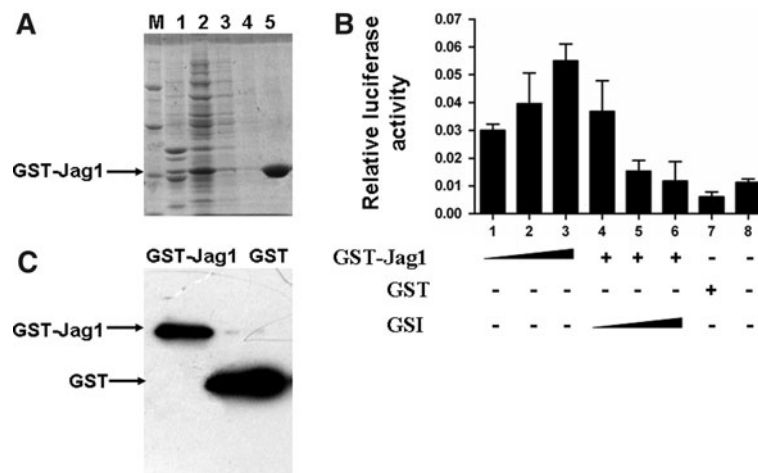


Fig. 2 Expression of GST-Jag1 in *E. coli*. **a** SDS-PAGE. M, molecular weight marker (from top, Kd); lane 1, inclusion bodies from *E. coli* BL21/pGEX-Jag1 induced with IPTG; lane 2, supernatant from *E. coli* BL21/pGEX-Jag1 induced by IPTG; lanes 3 and 4, washings of the glutathione Sepharose 4B; lane 5, purified GST-Jag1. **b** Western blotting. Purified GST and GST-Jag1 were run by SDS-PAGE, electroblotted, and were probed using an anti-GST antibody.

c Reporter assay. HeLa cells were transiently transfected with pGa981-6 and phRL-TK, and were cultured in the presence of GST (10 μ g/ml), GST-Jag1 (0, 5, 10 μ g/ml for lanes 1–3; 10 μ g/ml for lanes 4–6), and GSI (25, 50, 100 μ M for lanes 4–6). Cells were harvested 48 h after the transfection, and cell lysates were tested for luciferase activity by chemoluminescence

transactivation of the reporter gene expression (Fig. 2c). The transactivation was inhibited by GSI, suggesting that it was dependent on Notch activation.

Activating Notch signaling by GST-Jag1 did not promote cell-cycle progression of HL60 cells

We have shown that GSI, which blocks Notch signaling, suppressed the growth of HL60 cells. In order to further investigate the mechanism of the GSI-mediated growth inhibition, and the effect of Notch activation on HL60 cells, we performed cell-cycle analysis of HL60 cells cultured in the presence or absence of GSI or GST-Jag1. The result (Fig. 3a) showed that treatment with GSI for 48 h induced significant cell-cycle arrest at the G1/G0 phase. This treatment also induced a large fraction of cells with hypoploid DNA content, suggesting that GSI treatment induced apoptosis of HL60 cells. This was further confirmed by morphological observations of GSI-treated HL60 cells. When treated with GSI, HL60 cells had significant morphological changes manifested by staining with Wright–Giemsa, with concentrated cytoplasm, shrunk cell bodies, as well as condensed and fragmented nuclei (Fig. 3b). These data indicated that blocking of Notch signaling significantly induced cell-cycle arrest and apoptosis of HL60 cells. GST-Jag1, although being able to activate Notch signaling, did not alter the cell-cycle progression and survival of HL60 cells. Therefore, Notch signaling was essential, but probably not sufficient, for cell-cycle progression and survival of HL60 cells. However, it is also possible that the cells express high levels of

Jagged1 and, therefore, Notch signaling is active in HL60 cells already through cell–cell contacts, and possibly cells proliferate at maximum.

The molecular mechanism of notch signaling involved in the growth of HL60 cells

In order to access the mechanisms underlying the cell-cycle and survival regulation of HL60 cells by Notch signaling, we examined the expression of molecules related with cell-cycle progression and apoptosis. We first looked at the expression of direct downstream molecules of Notch signaling, Hes1 and Hes5, in HL60 cells treated with GST-Jag1 or GSI. RT–PCR analysis revealed that normally cultured HL60 cells expressed low levels of Hes1 and Hes5 mRNA, indicating a basal Notch signaling. GST-Jag1, but not GST stimulation, enhanced the expression of both Hes1 and Hes5, suggesting that Notch pathway was activated by GST-Jag1 in HL60 cells (Fig. 4a). Consistent results were obtained at the protein level using western blot (Fig. 4b). GSI, on the other hand, completely blocked Hes1 and Hes5 expression in HL60 cells, to a level lower than those in untreated cells (Fig. 4a, b). c-Myc was down-regulated in response to GSI (Fig. 4c). We also examined the expression of Bcl-2 and the phosphorylation of Rb protein at the protein level by western blotting. The results showed that the level of Bcl-2 down-regulated after treatment with GSI. Moreover, the level of phosphorylated Rb protein (Rb-p) reduced greatly in GSI-treated HL60 cells (Fig. 4d), consistent with cell-cycle arrest in these cells.

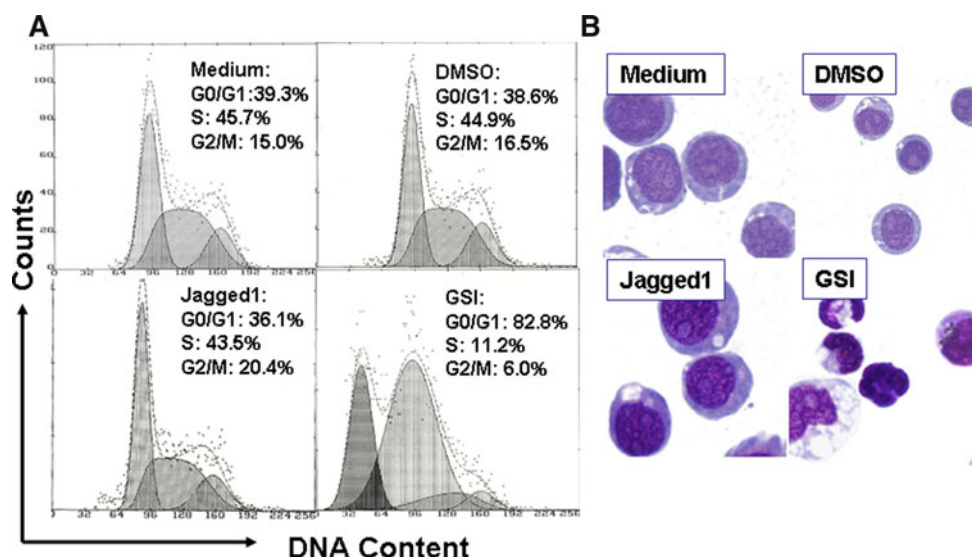


Fig. 3 GSI induces cell-cycle arrest and apoptosis in HL60 cells. **a** Cell-cycle analysis. HL60 cells cultured under different conditions for 48 h were permeated and stained with PI, and DNA content was analyzed by FACS. Cells in different phases of cell cycle were estimated and shown. Data represented three independent experiments.

b Giemsa–Wright staining. HL60 cells cultured under different conditions for 48 h were stained by Giemsa–Wright staining. Images are from one representative out of three separate observations. GSI and GST-Jag1 were used at the concentrations of 100 μ M and 10 μ g/ml, respectively

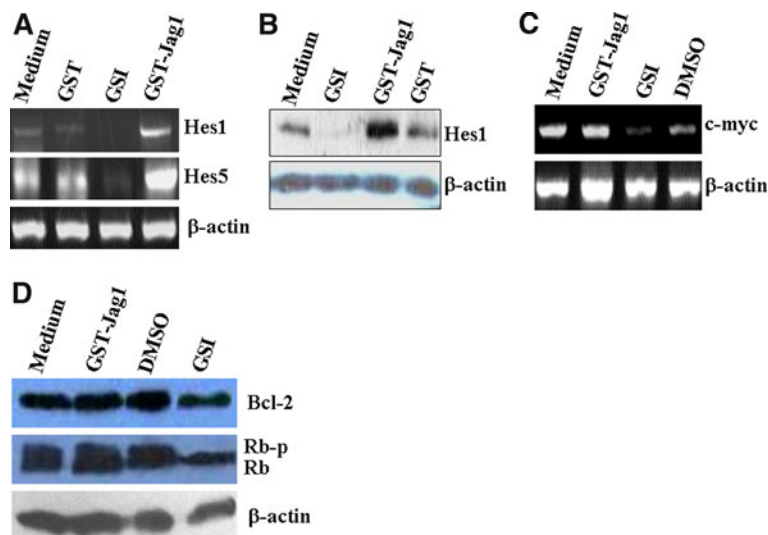


Fig. 4 Gene expression analysis. HL60 cells were treated as indicated, and expression of the indicated genes was assessed by RT–PCR or western blotting. **a** RT–PCR of Hes1 and Hes5, with β -actin as an internal control. **b** Western blotting of Hes1, with β -actin as an internal control. **c** RT–PCR of c-myc, with β -actin as an

internal control. **d** Western blotting of Bcl-2 and Rb protein, with β -actin as an internal control. RT–PCR and western blotting were performed at least twice with similar results. GSI and GST-Jag1 were used at the concentrations of 100 μ M and 10 μ g/ml, respectively

Discussion

Deregulated Notch activation has been reported in a growing number of human malignant diseases. However, the relationship between Notch signaling and AML is still vague. In this study, we investigated the potential role of Notch signaling and AML using HL60, a human promyelocytic leukemia cell line, as a model. We found that

Notch2 and Jagged1 were highly expressed in HL60, suggesting that Jagged1 might play a role in the development and/or progress of myeloid leukemia [14, 17, 20]. In cultured HL60 cells, cell–cell interaction might stimulate Notch activation reciprocally, because Hes1 and Hes5 mRNA could be detected in these cells, and GSI treatment significantly blocked Hes1 and Hes5 expression in HL60 cells.

In order to access the role of Notch signaling in HL60 cells, we employed GSI and a truncated human Jagged1, which contained the DSL domain. This domain was expressed as a GST-fusion protein (GST-Jag1), and was shown to be able to activate Notch signaling, by both a reporter assay and the induction of the Notch downstream genes. Li [12] expressed a truncated extracellular domain of Jagged1 and a DSL peptide, and found that these soluble forms of Jagged1 were capable of activating Notch in FLN-32D cells. In addition, in *C. elegans*, truncated forms of LAG-2 and APX-1 as well as DSL peptides activated rather than antagonized Notch signaling [21], consistent with our observations. This was in contrast to some reports showing that a soluble form of Jagged1 played a dominant negative role in Notch signaling [22, 23], while the immobilized form could activate Notch signaling [24]. The difference may lie in EGF-like repeats, which interact with EGF-like receptors with different affinity [25, 26].

Treatment of HL60 cells with GSI blocked Notch signaling. However, these treatments did not lead to consistent changes in cell proliferation and apoptosis. GSI, which blocked Notch activation in HL60 cells, inhibited the proliferation, and induced apoptosis of HL60 cells with a high efficiency. Further activation of Notch did not lead to changes in either cell-cycle progression or apoptosis in HL60 cells. These results suggest that Notch signaling is necessary for proliferation and survival of HL60 cells. Our study suggested that Notch signaling inhibitors such as GSI could affect the progression of AML, and might be a potentially useful strategy for anti-leukemia therapy.

Our preliminary molecular investigation suggested that c-Myc, Bcl2, and Rb protein might be involved in cell-cycle arrest and apoptosis in HL60 cells treated with GSI. c-Myc has been reported to up-regulate the expression of positive cell-cycle regulators, including cyclin D1, cyclin D2, and CDK4, and down-regulate the expression of p21^{Cip1} and p27^{Kip1}, which was also shown to be a direct target of Notch activation [27, 28]. The Rb protein operates in the midst of the cell-cycle clock apparatus. Its main role is to act as a signal transducer connecting the cell-cycle clock with the transcriptional machinery. In this role, the Rb protein allows the clock to control the expression of genes that mediate cell-cycle progression. Phosphorylation causes the inactivation of the growth inhibitory functions of the Rb protein [29]. Our results suggested that the GSI-induced dephosphorylation of Rb protein coincided with the cell-cycle arrest in HL60 cells.

APL cells are extremely sensitive to all-*trans* retinoic acid (ATRA), which induces APL cell differentiation into mature granulocytes and results in cell apoptosis. Treatment of APL patients with ATRA in addition to chemotherapy yields a high rate of complete remission and long-term survival [30]. Murata-Ohsawa [31] examined the

effects of another Notch ligand, Delta1 protein, on the ATRA-induced differentiation, growth suppression, and apoptosis in primary APL cells and NB4 cells, which is also a human promyelocytic leukemia cell line. They found that RA plus Delta-1 treatment reduced the RA-induced apoptosis. It will be interesting to see whether GSI might have any impact on ATRA-induced differentiation of AML cells.

Acknowledgments We thank Ms Hui Wang for English editing of the manuscript. This study was supported by grants from the National Natural Science Foundation of China (30370598, 30425015, 30400079, 30600544), the PCSIRT program (IRT0459) of the Ministry of Education of China, and the Ministry of Science and Technology of China (2006AA02A111, 2009CB521706).

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