

Transition of Cleaved Notch1 and Gene Expression Changes in Myeloblastic Leukemia Cells Stimulated with Notch Ligands

LU FU¹, KEN-ICHI KATSUBE² and SHUJI TOHDA¹

Departments of ¹Laboratory Medicine and ²Oral Pathology,
Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-Ku, Tokyo 113-8519, Japan

Abstract. *Background:* Notch activation by ligand stimulation regulates the growth of acute myeloid leukemia (AML) cells. However, the molecular mechanisms underlying this have not been fully elucidated. *Materials and Methods:* Two AML cell lines, THP-1 and TMD7, and three Notch ligands, Jagged1, Dll1 and Dll4, were used. The effects of the ligands on cell growth, cleavage of Notch1, and the expression of various genes were examined by cell culture, immunoblotting, and quantitative RT-PCR, respectively. *Results:* Ligand stimulation suppressed the growth of the THP-1 cells but promoted that of the TMD7 cells. Immunoblots showed two cleaved Notch1 bands, which became intense with different peak time from the start of stimulation. Ligand stimulation changed the expression levels of various genes related to cell proliferation. Some genes changed in opposite directions in the two cell lines. The three ligands had similar effects on gene expression. *Conclusion:* The time course for Notch1 cleavage following ligand stimulation and the effects of stimulation on gene expression are shown.

Notch signaling regulates self-renewal and differentiation of stem cells. In bone marrow, Notch protein on hematopoietic stem cells is activated by Notch ligands such as Jagged and Delta-like (Dll) proteins on stromal cells. The binding of Notch ligands to Notch protein causes cleavage of the transmembrane subunit (NTM) at S2 and S3 sites, and releases an intracellular fragment of Notch (ICN). ICN is translocated to the nucleus where it induces expression of *HES1* (Hairy and enhancer of split 1) and other genes (1).

Notch activation is also involved in the growth of leukemia cells. More than half of acute T-cell lymphoblastic

leukemia (T-ALL) cases have mutations in the *NOTCH1* gene. These mutations cause constitutive activation of Notch and abnormal growth (2, 3). Down-stream of Notch signaling, direct Notch1 mediated activation of *c-MYC* is required to maintain leukemic growth (4, 5).

We have previously reported that Notch activation by Notch ligand stimulation had various effects on the growth of acute myeloid leukemia (AML) cells (6-8). However, the precise molecular mechanisms underlying these effects of ligand stimulation in AML cells are unknown. For example, the time course for cleavage of ICN after ligand stimulation has not been reported. The mRNA expression profile after stimulation is not fully known. The differences in the effects of five different ligands, Jagged1, Jagged2, Dll1, Dll3 and Dll4 have not been elucidated.

In this study, the effects of three recombinant Notch ligands, Jagged1, Dll1 and Dll4, on the cleavage of Notch1 protein and the expression of various genes in AML cells was examined using immunoblotting and quantitative RT-PCR. It is important to understand the molecular mechanisms underlying Notch signaling because Notch represents a potential molecular target for therapy against leukemias.

Materials and Methods

Cells and reagents. Two AML cell lines known to grow in response to the Notch ligands, TMD7 (8) and THP-1 (supplied by the Japanese Cancer Research Resources Bank, Tokyo, Japan) were used. The recombinant Notch ligand proteins Jagged1, Dll1 and Dll4 were synthesized by Dr. S. Sakano (Asahi Kasei Corporation, Fuji, Japan) as described previously (9,10). Briefly, partial cDNAs encoding the extracellular domain of the Notch ligands were fused to a sequence of human IgG₁-Fc. The fusion gene was inserted into an expression vector and electroporated into Chinese hamster ovary cells. The resulting chimeric proteins were purified from conditioned media.

Proliferation assay. The effects of Notch ligands on cell growth were examined by counting cell numbers in culture. Briefly, 1×10^4 cells were cultured in 0.1 ml of α -MEM containing 10% FCS, in 96-well culture plates coated with 1.5 μ g/well of recombinant Jagged1, Dll1, Dll4, or human IgG-Fc (Athens Research, Athens,

Correspondence to: Shuji Tohda, MD, Department of Laboratory Medicine, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-Ku, Tokyo 113-8519, Japan. Tel: +81 358035334, Fax: +81 358035629, e-mail: tohda.mlab@tmd.ac.jp

Key Words: Notch, leukemia, Jagged, Delta-like.

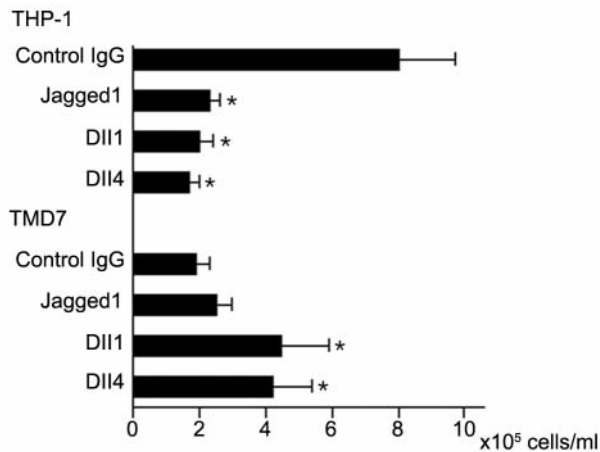


Figure 1. Effects of Notch ligands Jagged1, Dll1 and Dll4 on the growth of THP-1 and TMD7 cells. The mean cell number per well from quadruplicate cultures after 5 days is shown. * $p < 0.05$, significantly different from the control.

GA, USA) as a control. After 5 days, the cells were harvested and cell numbers were counted by the trypan blue method. The mean cell number per well from quadruplicate cultures are calculated. Student's *t*-test was used to determine statistical significance.

Immunoblotting. To examine the time course for the cleavage of Notch1 protein by ligand stimulation, immunoblotting was performed using an anti-Notch1 antibody (Ab) raised against the carboxy-terminus (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an anti-cleaved Notch1 (Val1744) Ab (Cell Signaling Technology, Beverly, MA, USA) to selectively detect the ICN. After being cultured in 24-well culture plates coated with 5 μ g/well of Notch ligands (Jagged1, Dll1, Dll4) or control IgG-Fc for 0.5, 3, 6, 24 or 48 hours, the cells were harvested and lysed. The lysates of 10^6 cells/lane were subjected to SDS-PAGE and immunoblotted with the first and second antibodies. Immunoreactive bands were detected using an ECL kit (GE Healthcare, Piscataway, NJ, USA), using an anti- α -tubulin Ab as loading control.

Quantitative RT-PCR. To identify the genes whose expression levels were altered by ligand stimulation, cDNA microarray analysis was performed using RNA extracted from cells stimulated by the ligands and control cells (data not shown). To quantitatively examine changes in the expression levels of the selected genes in the cells stimulated with Notch ligands, real-time RT-PCR was performed. The total RNA was extracted from cells cultured with the ligands (Jagged1, Dll1, Dll4) or human IgG-Fc for 6 or 24 hours. First-strand cDNA was synthesized and quantitative PCR was performed using a LightCycler FastStart DNA Master SYBR Green I kit, LightCycler primer sets, and LightCycler ST300 (Roche Diagnostics, Mannheim, Germany). QuantiTect primer sets (QIAGEN, Hilden, Germany) were used for *c-MYC*, *p27^{Kip1}* (*CDKN1B*; cyclin-dependent kinase inhibitor 1B) and *p18^{INK4c}* (*CDKN2C*). The expression level of each mRNA was normalized to the β -actin mRNA level, which was measured concurrently. The analysis was repeated more than twice to verify the reproducibility of the results.

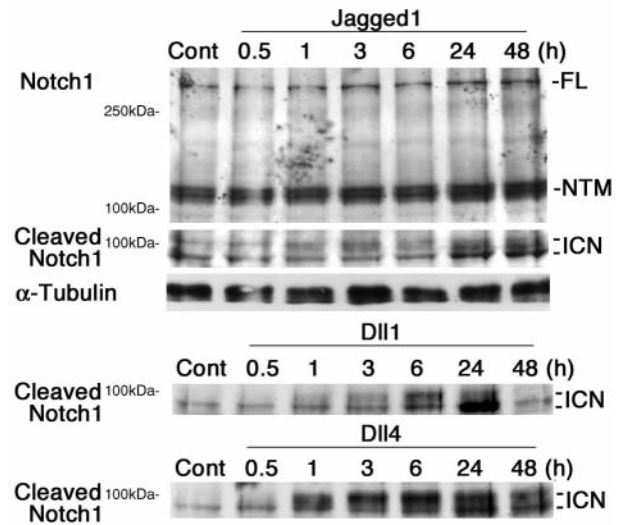


Figure 2. Notch1 proteins in TMD7 cells stimulated with Notch ligands, Jagged1, Dll1 or Dll4. Cell lysates (10^6 cells/lane) were subjected to SDS-PAGE and immunoblotted with anti-Notch1 antibody (Ab), anti-cleaved Notch1 Ab, and anti- α -tubulin Ab. FL, full-length; NTM, Notch transmembrane fragment; ICN, intracellular fragment of Notch.

Results

Effects of Notch ligands on cell growth. The effects of the Notch ligands on the growth of THP-1 and TMD7 cells are shown in Figure 1. Stimulation with Jagged1, Dll1 or Dll4 suppressed the growth of the THP-1 cells. On the other hand, growth of the TMD7 cells was promoted by Dll1 and Dll4 stimulation. The growth promotion induced by Jagged1 was not statistically significant.

Notch1 protein after Notch ligand stimulation. Figure 2 shows the immunoblot analysis for the TMD7 cells. In the immunoblots with the anti-Notch1 Ab, a full-length band (300 kDa) and NTM band (120 kDa) were observed. The densities of these two bands did not significantly change during the time-course of Jagged1 stimulation (upper panel). In the immunoblots with the anti-cleaved Notch1 Ab (the second panel), two bands (100 kDa and 90 kDa) were recognized as ICN bands. These ICN bands were detected with high intensity after one hour stimulation. From one to 24 hours of Jagged1 stimulation, the 100 kDa band increased in intensity. Then, from 24 to 48 hours, the 90 kDa band increased in intensity. The transition of the intensities of the two ICN bands after Dll1 and Dll4 stimulation was similar to that after Jagged1 stimulation (lower panels). The THP-1 cells stimulated with the Notch ligands produced the same findings, namely, two bands that emerged in the immunoblots with the anti-cleaved Notch1 Ab (data not shown).

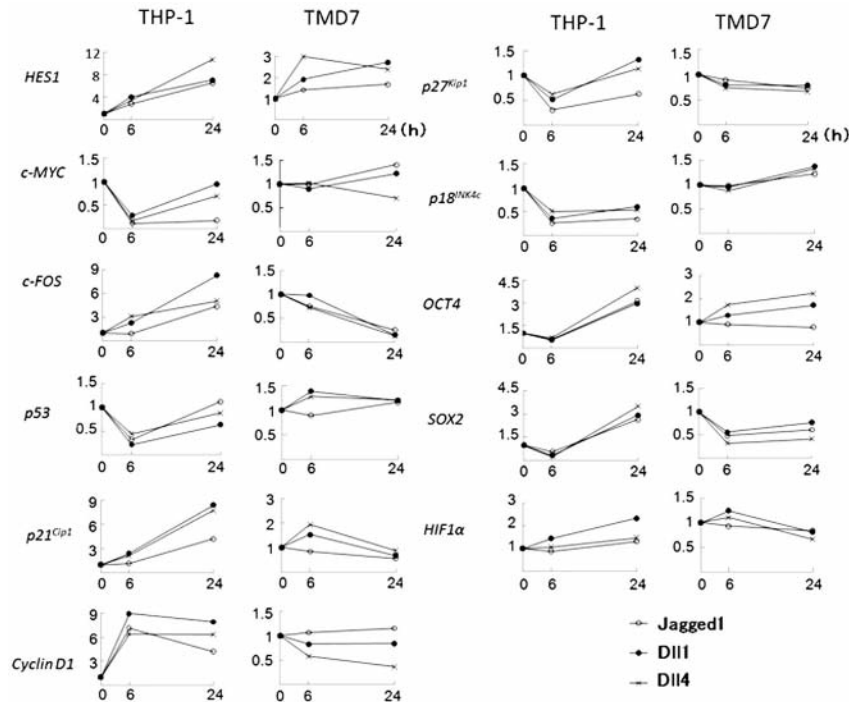


Figure 3. Expression of mRNA in THP-1 and TMD7 cells stimulated with Notch ligands, Jagged1, Dll1 or Dll4. The expression level of each gene after quantitative RT-PCR was normalized to the β -actin mRNA expression level and shown as fold change compared to the level in the control (IgG-treated) cells.

Gene expression after Notch ligand stimulation. Changes in the expression levels of various genes caused by ligand-induced Notch activation were examined by quantitative RT-PCR analyses (Figure 3). The expression of *HES1*, a well-known target gene of the Notch signal, was increased by stimulation with all three of the ligands in both cell lines. The increase induced by Jagged1 stimulation was slight (1.7-fold) at 24 hours in the TMD7 cells. On the whole, all three of the ligands showed similar tendencies in terms of the changes in gene expression they induced in each cell line.

Regarding the expression of genes related to cell proliferation, ligand stimulation down-regulated *c-MYC* expression at six hours and then Dll1 completely and Dll4 partially restored it by 24 hours in the THP-1 cells. The expression of *c-FOS* was up-regulated in the THP-1 cells, but was down-regulated in the TMD7 cells. Regarding the expression of cell cycle-related genes in the THP-1 cells, *p53*, *p27Kip1* and *p18INK4c* were down-regulated at six hours and the levels of *p53* and *p27Kip1* were restored in the Dll1 and Dll4 cultures by 24 hours. The expression of *p21Cip1* was gradually up-regulated and the expression of *Cyclin D1* was rapidly up-regulated. The changes in the expression levels of these genes in the TMD7 cells were slight. Regarding genes concerned with "stemness", the expression levels of *OCT4* and *SOX2* in the THP-1 cells were down-regulated at six hours and then up-regulated at 24 hours.

Discussion

Notch activation by ligand stimulation resulted in diverse effects on the short-term growth of AML cells, suppressing the growth of THP-1 cells while promoting the growth of TMD7 cells. To investigate the underlying molecular mechanisms, the changes in gene expression following ligand stimulation were examined. *HES1* was up-regulated in both cell lines. The changes in the expression levels of some genes differed between the THP-1 and TMD7 cells. For example, *c-FOS* was up-regulated in the THP-1 cells while it was down-regulated in the TMD7 cells. The possible mechanisms by which Notch activation suppresses the growth of THP-1 cells include the down-regulation of *c-MYC* and up-regulation of *p21*. However, the up-regulation of *Cyclin D1* and down-regulation of *p53* are inconsistent with growth suppression. The precise mechanisms remain to be determined.

Two types of time-sequential changes in gene expression levels were found (Figure 3). In one type, changes were observed after six hours of stimulation, but were restored to the control levels after 24 hours (for example, *p53* expression in the THP-1 cells). In the other type, the expression levels gradually changed up to 24 hours of stimulation (for example, *c-FOS* expression in both the THP-1 and TMD7 cells). It remains to be clarified whether these changes were due to a

direct effect of the ICN or indirect effects through the up-regulation of direct target genes such as *HES1*.

Regarding the cell cycle, it has been reported that Notch activation accelerated G₁/S cell cycle transition (11-13), possibly by up-regulating SKP2 (S-phase kinase-associated protein 2), which degraded *p27^{Kip1}* and *p21^{Cip1}* (11). The regulation of *Cyclin D1*, *CDK2* and *AKT* expression by Notch (12) and regulation of the expression of Cyclin D3, CDK2 and CDK6 proteins by Notch have also been reported (13). The present results partly differed from these reports. For example, *p21* expression was up-regulated by Notch activation in the THP-1 cells in the present study. The cause of this discrepancy remains to be determined.

The transition and molecular size of the ICN after ligand stimulation was also interesting. The immunoblots showed that two ICN bands with different molecular weights became intense with different peak time from the start of stimulation. As a possible mechanism for the emergence of these two bands, we surmised that the 90 kDa band was ICN and the 100 kDa band was phosphorylated ICN, because it has been reported that cleaved Notch2 fragments are phosphorylated after ligand binding (14). To verify this, the same immunoblotting experiments were performed using phosphatase-treated lysates. However, the two bands did not unite at 90 kDa (data not shown) and thus this hypothesis could not be proved. Another possible mechanism is the existence of an additional cleavage site in the NTM, such as the S4 site (15). However, this theory cannot explain the existence of two ICN bands because the S4 site is located on the N-terminal side of the S3 site. Therefore, the reasons why two bands emerged could not be elucidated.

Here, new findings regarding changes in the expression levels of various genes and cleaved Notch1 protein after Notch ligand stimulation are described. However, the precise mechanisms underlying these changes and their significance are still to be resolved. The Notch system is a promising pathway to target for therapies against leukemia. Therefore, a detailed understanding of the molecular mechanism associated with Notch activation is needed.

Acknowledgements

We thank Associate Professor T. Kubota, Dr. M. Itoh and Mr. Y. Okuhashi for their technical assistance. We also thank Dr. S. Sakano and Professor N. Nara for helpful discussion. This study was supported in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science.

References

- Suzuki T and Chiba S: Notch signaling in hematopoietic stem cells. *Int J Hematol* 82: 285-294, 2005.
- Weng AP, Ferrando AA, Lee W, Morris IV JP, Silverman LB, Sanchez-Irizarry C, Blacklow SC, Look AT and Aster JC: Activating mutations of NOTCH1 in human T-cell acute lymphoblastic leukemia. *Science* 306: 269-271, 2004.
- Leong KG and Karsan A: Recent insights into the role of Notch signaling in tumorigenesis. *Blood* 107: 2223-2233, 2006.
- Sharma VM, Calvo JA, Draheim KM, Cunningham LA, Hermance N, Beverly L, Krishnamoorthy V, Bhasin M, Capobianco AJ and Kelliher MA: Notch1 contributes to mouse T-cell leukemia by directly inducing the expression of c-myc. *Mol Cell Biol* 26: 8022-8031, 2006.
- Palomero T, Lim WK, Odom DT, Sulis ML, Real PJ, Margolin A, Barnes KC, O'Neil J, Neuberg D, Weng AP, Aster JC, Sigaux F, Soulier J, Look AT, Young RA, Califano A and Ferrando AA: NOTCH1 directly regulates c-MYC and activates a feed forward loop transcriptional network promoting leukemic cell growth. *Proc Natl Acad Sci* 103: 18261-18266, 2006.
- Murata-Ohsawa M, Tohda S and Nara N: Cellular analysis of growth suppression induced by the Notch ligands, Delta-1 and Jagged-1 in two myeloid leukemia cell lines. *Int J Mol Med* 14: 223-226, 2004.
- Tohda S, Kogoshi H, Murakami N, Sakano S and Nara N: Diverse effects of the Notch ligands Jagged1 and Delta1 on the growth and differentiation of primary acute myeloblastic leukemia cells. *Exp Hematol* 33: 558-563, 2005.
- Tohda S, Sakano S, Ohsawa M, Murakami N and Nara N: A novel cell line derived from *de novo* acute myeloblastic leukaemia with trilineage myelodysplasia which proliferates in response to a Notch ligand, Delta-1 protein. *Br J Haematol* 117: 373-378, 2002.
- Karanu FN, Murdoch B, Gallacher L, Wu DM, Koremoto M, Sakano S and Bhatia M: The Notch ligand Jagged-1 represents a novel growth factor of human hematopoietic stem cells. *J Exp Med* 192: 1365-1372, 2000.
- Karanu FN, Murdoch B, Miyabayashi T, Ohno M, Koremoto M, Gallacher L, Wu DM, Itoh A, Sakano S and Bhatia M: Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. *Blood* 97: 1960-1967, 2001.
- Sarmiento LM, Huang H, Limon A, Gordon W, Fernandes J, Tavares MJ, Miele L, Cardoso AA, Classon M and Carlesso N: Notch1 modulates timing of G1-S progression by inducing SKP2 transcription and *p27^{Kip1}* degradation. *J Exp Med* 202: 157-168, 2002.
- Guo D, Ye J, Dai J, Li L, Chen F, Ma D and Ji C: Notch-1 regulates Akt signaling pathway and the expression of cell cycle regulatory proteins cyclin D1, CDK2 and p21 in T-ALL cell lines. *Leuk Res* 33: 678-685, 2009.
- Joshi I, Minter LM, Telfer J, Demarest RM, Capobianco AJ, Aster JC, Sicinski P, Fauq A, Golde TE and Osborne BA: Notch signaling mediates G1/S cell-cycle progression in T cells via cyclin D3 and its dependent kinases. *Blood* 113: 1689-1698, 2009.
- Shimizu K, Chiba S, Hosoya N, Kumano K, Saito T, Kurokawa M, Kanda Y, Hamada Y and Hirai H: Binding of Delta1, Jagged1, and Jagged2 to Notch2 rapidly induces cleavage, nuclear translocation, and hyperphosphorylation of Notch2. *Mol Cell Biol* 20: 6913-6922, 2000.
- Okochi M, Fukumori A, Jiang J, Itoh N, Kimura R, Steiner H, Haass C, Tagami S and Takeda M: Secretion of the Notch-1 A beta-like peptide during Notch signaling. *J Biol Chem* 281: 7890-7898, 2006.

Received April 28, 2009

Revised July 24, 2009

Accepted August 12, 2009