NF-κB activation induced by Notch ligand stimulation in acute myeloid leukemia cells

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Abstract. There are conflicting reports regarding the effects of Notch activation on nuclear factor-κB (NF-κB) activity. The relationships are cell type-dependent and have not been fully elucidated. We examined the effects of Notch activation induced by a recombinant Notch ligand, Delta-like1 (Dll1), on the NF-κB activity in two acute myeloid leukemia (AML) cell lines. We found that Delta1-induced Notch activation activated the NF-κB pathway in THP-1 cells. Regarding the possible mechanisms, Dll1 stimulation increased the mRNA and protein expression levels of some components of the NF-κB pathway and induced phosphorylation of IKKα/ß, IκB and RelA proteins after 24 or 48 h of stimulation. Since the phosphorylation required a long time, it did not appear to be caused by physical interactions between Notch and NF-κB proteins, but rather by indirect effects. One possible mechanism for the indirect effects was the observed induction of IL-1ß expression by Dll1 stimulation. On the other hand, Notch activation did not affect NF-κB activity in TMD7 cells. RelA was phosphorylated without stimulation, indicating that NF-κB was constitutively activated in TMD7 cells. To the best of our knowledge, this is the first study to investigate AML cells and use a recombinant Notch ligand to activate Notch. The present findings lead to better understanding of Notch functions, which have not been fully elucidated.

Introduction

Notch signaling regulates cell growth during normal hematopoiesis and in various hematological malignancies (1,2). Binding of Notch ligands to Notch protein results in release of the intracellular fragment of Notch (ICN). This fragment translocates to the nucleus and induces the transcription of several genes, including *HES1*. The resulting signals regulate the self-renewal, proliferation and differentiation of stem cells, depending on the cellular context. The downstream aspects of the signaling pathway are not fully understood.

Nuclear factor-κB (NF-κB) is the collective term for a small family of transcription factors that play important roles in inflammation, hematopoiesis and malignancies (3). The effects of Notch signaling on NF-κB activity have been investigated by some research groups and conflicting data have been reported. Some researchers found that Notch stimulated NF-κB activity (4,5) while others showed that Notch inhibited it (6). The effects in the individual contexts were reviewed by Osipo *et al* (7). Regarding the issue of how Notch signals regulate NF-κB, two types of mechanisms have been proposed, namely transcriptional regulation of NF-κB members by Notch and physical interactions between Notch and NF-κB (7).

We previously reported that Notch has various effects on the growth of acute myeloid leukemia (AML) cells (8). In the present study, we examined the effects of Notch activation induced by a recombinant Notch ligand, Delta-like1 (Dll1), on the NF-κB activity in two AML cell lines. To the best of our knowledge, this is the first study investigating AML cells using a recombinant Notch ligand to activate Notch. In general, cells constitutively expressing ICN have been used as Notch-activating models in most previous studies (6). Our use of a Notch ligand and AML cells has brought about some interesting findings regarding the interactions between Notch and NF-κB.

Materials and methods

Cells and reagents. Two AML cell lines exhibiting Dll1-responsive growth, namely TMD7 (9) and THP-1 (supplied by the Japanese Cancer Research Resources Bank, Japan), were used. The recombinant Notch ligand protein, Dll1, was synthesized by Dr S. Sakano (Asahi Kasei Corporation, Japan), as described previously (10). Briefly, partial cDNAs encoding the extracellular domain of Dll1 were fused to a sequence of human IgG_1 Fc. The fusion gene was inserted into an expression vector and electroporated into Chinese hamster ovary cells. The chimeric proteins were purified from conditioned media. We previously showed that Dll1 stimulation releases the ICN using immunoblot analysis. Dll1 stimulation promotes the growth of TMD7 cells (9). In contrast, it suppresses the growth of THP-1 cells and induces their macrophage-like differentiation (11).

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Microarray analysis. The recombinant Dll1 protein and human IgG-Fc (Athens Research, USA) as a control were coated in the wells (3 μg/well) of 24-well culture plates (Becton-Dickinson Labware, USA), as described previously (10). The cells were cultured in the wells for 24 h, before the total RNA was extracted. Cy5-labeled and Cy3-labeled cDNAs were synthesized from each RNA sample using a Fluorescence Labeling Core kit (Takara, Japan). The labeled cDNA samples were hybridized to an IntelliGene Human Cytokine CHIP version 3.0 (Takara) and scanned using an Affymetrix 428 scanner (Affymetrix, USA). The data were analyzed using ImaGene version 5 and GeneSight 4 software (BioDiscovery Inc., USA). We performed Lowess normalization and color-swap analysis to verify the reproducibility.

Quantitative RT-PCR. First-strand cDNAs were synthesized from RNA extracts from cells treated with Dll1 for 6 and 24 h. Quantitative PCR was performed using QuantiTect primers (Qiagen, Germany), a FastStart DNA Master SYBR-Green I kit and a LightCycler (Roche Diagnostics, Germany). The expression level of each mRNA was normalized by the corresponding ß-actin mRNA level, which was measured concurrently. We repeated the analyses more than twice to verify the reproducibility.

Immunoblotting. To examine the effects of Notch activation on the amount and activity of NF-κB protein, we performed immunoblot analyses. Cells were harvested and lysed before and after stimulation with Dll1. Cell lysates ($1x10^6$ cells/lane) were separated by SDS-PAGE, transferred to membranes and immunoblotted with antibodies against IKKα, IKKß, phospho-IKKα/ß, IκB, phospho-IκB, NF-κB1, RelA (p65), phospho-RelA (p65), NF-κB2 (Cell Signaling Technology, USA) and α-tubulin (Abcam, USA). Immunoreactive bands were detected with an ECL kit (Amersham Biosciences, USA). We repeated the experiments at least twice to verify the reproducibility.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from cells treated with Dll1 for 2 days and control cells were incubated with a biotin-labeled NF-κB oligonucleotide (GGGACTTTCC) in the binding buffer of a Lightshift Chemiluminescent EMSA kit (Pierce, USA). For specific competition, a 100-fold excess of unlabeled probe was added. The samples were separated in 5% polyacrylamide gels and transferred to membranes. The bands were visualized on X-ray films by chemiluminescence.

Results

Effects of Notch on NF-κB mRNA. Quantitative RT-PCR analyses revealed that Dll1-induced Notch activation increased the mRNA expression levels of *NFKB1*, *NFKB2* and *RELB* in THP-1 cells (Fig. 1). The expression of *RELA* mRNA was not significantly increased until 24 h after stimulation. In TMD7 cells, the mRNA expression levels of *NKFB1*, *RELA* and *RELB* were not significantly affected by Dll1 stimulation. The mRNA expression level of *NFKB2* was decreased.

Effects of Notch on NF-κB proteins. Immunoblot analyses revealed that Dll1-induced Notch activation activated the

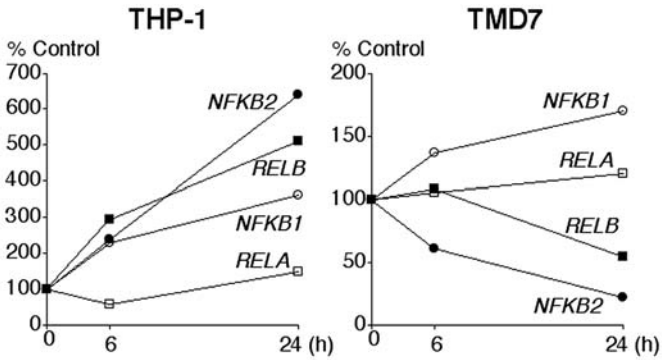

Figure 1. Quantitative RT-PCR analyses of NF-κB family genes in cells stimulated with Dll1 for 6 or 24 h. The expression level of each mRNA was normalized by the corresponding ß-actin mRNA level and compared with the level in control cells as 100%.

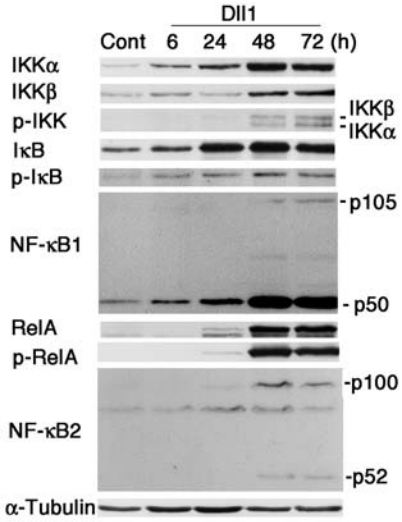

Figure 2. Expression and phosphorylation of NF-κB family proteins in Dll1-stimulated THP-1 cells. Cells stimulated with Dll1 for the indicated times were harvested and lysed. The lysates (10^6 cells/lane) were separated by SDS-PAGE, transferred to membranes and immunoblotted with the indicated antibodies.

NF-κB pathway in THP-1 cells (Fig. 2). Specifically, the amounts of IKKα and IKKß as well as the phosphorylation of IKKα/ß were upregulated after stimulation. The amount of IκB and its phosphorylation were also increased after stimulation. The amounts of NF-κB1 (p105/p50), RelA (p65) and phosphorylation of RelA were also increased. The amount of NF-κB2 (p100) was slightly increased. A band for p52, a processed form of p100, was recognized after stimulation. We also performed immunoblot analyses for these proteins at earlier times after stimulation. At 5, 15, 30 and 60 min after Dll1 stimulation, the amounts and phosphorylation levels of these proteins were not altered compared with the control cells (data not shown). In TMD7 cells (Fig. 3), we found that the NF-κB pathway was constitutively activated. In other words, RelA was phosphorylated without Dll1 stimulation. Phosphorylation of RelA was not significantly affected by Dll1 stimulation. Phosphorylation of IKKα/ß was below the detectable level. NF-κB2 (p100/p52) was not expressed (data not shown).

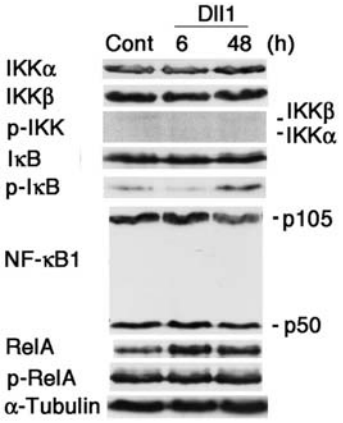

Figure 3. Expression and phosphorylation of NF-κB family proteins in Dll1-stimulated TMD7 cells. Immunoblotting was performed as described in the legend to Fig. 2.

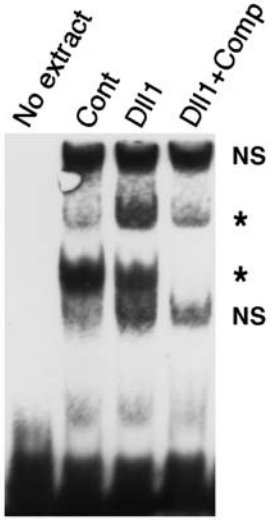

Figure 4. EMSA to detect binding of nuclear NF-κB factors to a consensus NF-κB oligonucleotide. Nuclear extracts from THP-1 cells stimulated with Dll1 for 2 days and control cells were used. The rightmost lane shows competition of the specific binding with a 100-fold excess of unlabeled probe. Asterisks indicate specific nuclear complexes. NS indicates non-specific binding.

Effects of Notch on NF-κB binding to DNA. To clarify the NF-κB activation by Dll1 stimulation in THP-1 cells, we performed EMSAs. As shown in Fig. 4, Dll1 stimulation increased the intensities of the bands for the DNA-bound NF-κB complex (upper asterisk). One band (lower asterisk) was not affected by Dll1 stimulation, suggesting constitutive NF-κB activation that was independent of Notch. Addition of unlabeled probe diminished these two bands, indicating that they were specific complexes.

Gene expression profile associated with Notch activation. As shown in Fig. 2, Dll1 stimulation activated NF-κB after 24 or 48 h. As a typical example, TNF-α stimulation activates NF-κB within several minutes (12). Therefore, the NF-κB activation by Dll1 appeared to occur in an indirect manner. To obtain clues regarding its mechanism, we examined the gene expression profile after Dll1 stimulation using a cDNA microarray. Table I shows representative genes whose expression levels were changed in THP-1 cells following Dll1 stimulation. Upregulation of the IL-1ß gene was recognized and its signaling is known to activate the NF-κB pathway. In TMD7 cells, IL-1ß gene expression was not significantly affected by Dll1 (data not shown). We verified the reproducibility of these results using another cDNA microarray, Cancer CHIP ver 3.0 (Takara) (data not shown).

Table I. Representative genes whose expression levels were changed in THP-1 cells following Dll1 stimulation.[a]

Gene	Change
MMP9	4.9
IL-1ß	4.0
M-CSF-R	3.0
IL-8	2.5
TNFSF10	2.1
M-CSF	1.4
Cyclin D2	-1.6
MYC	-2.0
MYB	-2.0

[a]Values are shown as $\log_2$ (signal in Dll1-stimulated cells/signal in control cells).

Discussion

In the present study, we have shown that Notch activation induced by recombinant Dll1 protein activated the NF-κB pathway in THP-1 cells. Regarding the possible mechanisms, we found that Dll1 stimulation increased the mRNA and protein expression levels of components of NF-κB in both the canonical and non-canonical pathways. These findings are compatible with the mechanism reported in ICN-induced cells (13). We also found that Dll1 stimulation induced phosphorylation of IKKα/ß, IκB and RelA proteins. Since the phosphorylation required 24 or 48 h, we considered that it was not due to direct effects, such as physical interactions between ICN and IKK (13), but due to indirect effects of Notch signaling. As a possible mechanism for the indirect effects, we showed that some cytokines, such as IL-1ß, produced by Dll1 stimulation activated the NF-κB pathway, based on microarray analyses. However, we cannot confirm this hypothesis because the expression of such cytokines may be the result of NF-κB activation, rather than the cause.

On the other hand, Notch activation did not affect NF-κB activity in the TMD7 cell line. Specifically, Dll1 stimulation did not significantly affect the mRNA expression levels and amounts or phosphorylation of proteins in the NF-κB pathway. Therefore, the effects of Notch activation on NF-κB are dependent on the cellular context, even among different AML cell lines. As shown in Fig. 3, RelA was phosphorylated without stimulation in TMD7 cells. This means that NF-κB was constitutively activated, as reported in a study using primary AML cells (14). Notch was constitutively activated

in TMD7 cells, although the activity is weaker than that in *NOTCH1*-mutated T-lymphoblastic leukemia cells. TMD7 cells do not have any mutations in the *NOTCH1* gene. Since TMD7 cells express Jagged1 protein (15), Notch may be activated in a cell-autonomous manner. Constitutive activation of Notch is one of the possible causes of the constitutive activation of NF-κB, as observed in Dll1-stimulated THP-1 cells.

The cross-talk between Notch and NF-κB is complex and has not been fully elucidated (7). We clarified that Dll1-induced Notch activation activated NF-κB signaling in THP-1 cells. The next problem to be solved is the role of the NF-κB activation induced by Notch in THP-1 cells. This will lead to better understanding of Notch functions, which have also not been fully elucidated.

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