論文の内容の要旨

論文題目　NF-κB/TNF-α positive feedback loop with active proteasome machinery is important for myeloid leukemia initiating cell capacity

(NF-κB/TNF-αによるポジティブフィードバック回路と高いプロテアソーム活性の協調が骨髄性白血病幹細胞の機能維持に重要な役割を担う)

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Acute myeloid leukemia (AML) is an aggressive hematologic malignancy arising from leukemia initiating cells (LIC). Although intensive chemotherapy is initially effective in most cases, the surviving LIC clones repopulate the disease and cause subsequent relapse. Another problem is that AML is comprised of highly heterogeneous groups with different cytogenetic and molecular abnormalities, which makes it difficult to establish a broadly effective therapeutic strategy against the disease. To establish widely applicable therapeutic strategies, it is crucially important to elucidate the mechanisms universally involved in LIC. Nuclear factor-kappa B (NF-κB) is a transcription factor initially discovered in B cells. Besides controlling various aspects of immune responses, it is now also recognized as an important regulator of cell survival, proliferation and differentiation and its constitutive activation has been reported in a variety of malignancies. Since constitutive NF-κB pathway activation has been reported in different types of AML cells, it is one of the promising candidates which are universally involved in the LIC phenotype. However, the mechanism of activation and its significance in leukemia progression have not been studied well. Although several gene mutations found in hematologic malignancies have been reported to be associated with enhanced NF-κB signal, these findings do not fully explain why the activation of NF-κB is observed in a number of different types of leukemia. It is more intriguing as well as reasonable to consider that the NF-κB activation arises from the signaling pathways which are commonly involved in LIC. In this study, I aimed to explore NF-κB pathway activity and its role in LIC using various myeloid leukemia mouse models including MLL-ENL, MOZ-TIF2, and BCR-ABL/Nup98-HoxA9 leukemias. Furthermore, I aimed to clarify the mechanism of activation in LIC.

First, I determined LIC-enriched fractions and those which barely contain LIC by limiting dilution transplantation assay. Leukemic granulocyte-monocyte progenitors (L-GMP) in MLL-ENL and MOZ-TIF2 leukemia mice and lineage$^-$ Sca-1$^+$ fraction in BCR-ABL/Nup98-HoxA9 leukemia mice were highly enriched for LIC, while lineage$^-$ c-Kit$^-$ cells in MLL-ENL and MOZ-TIF2 leukemia mice and

lineage$^+$ cells in BCR-ABL/Nup98-HoxA9 leukemia mice barely contained LIC, which were defined as non-LIC fractions. In immunofluorescence staining, each type of LIC displayed prominent nuclear translocation of NF-κB subunit p65. On the other hand, p65 was localized mainly in the cytoplasm in normal hematopoietic stem cells (HSC), GMP, and, interestingly, non-LIC fractions in the bone marrow cells of the leukemia mice. To further test NF-κB transcription activity in LIC, I investigated the expression profiles of a subset of genes regulated by NF-κB pathway. I first utilized two sets of published gene expression microarray data, which compared the expression profiles of MOZ-TIF2 L-GMP, MLL-AF9 and HoxA9-Meis1 L-GMP with those of normal hematopoietic stem or progenitor cells (HSPC). The expression profiles of previously identified NF-κB target genes were assessed by gene-set enrichment analysis (GSEA), which showed that L-GMP had increased expression levels of NF-κB target genes, compared with normal HSPC in both of the expression microarray data. When I compared the expression profiles of the same gene set in CD34$^+$ CD38$^-$ human AML cells with the equivalent population in normal bone marrow cells, which corresponded to HSC fraction, similar tendency was also seen. These data were also validated in real-time PCR assay. Furthermore, the level of p65 phosphorylation, which is important for enhancing its transcription activity, was significantly increased in LIC compared with normal cells. These results suggest that NF-κB pathway is selectively activated in LIC.

In order to study the mechanism of activation, I analyzed gene expression profiles of LIC and normal HSC in murine and human AMLs using the published microarray data. After narrowing down genes commonly upregulated in LIC of different types of murine leukemia models, I further selected 19 genes whose expression is also elevated in human AML CD34$^+$ CD38$^-$ cells. Amongst the 19 genes with commonly elevated expression in LIC, I focused on TNF-α, one of the major activators of the NF-κB pathway. Consistent with these results, the culture media conditioned by LIC had higher TNF-α levels than those of normal cells. In all of the three types of leukemia mice, bone marrow extracellular fluid included higher TNF-α than that of control mice. Then I studied the relationship between constitutive NF-κB activation and autonomous TNFα secretion in LIC. LIC retained NF-κB activity even after serum-free culture. Surprisingly, TNF-α blockage by the neutralizing antibody significantly attenuated p65 nuclear localization in LIC, indicating that LIC maintain their NF-κB activity by autocrine TNF-α signaling. To explore the effect of autocrine TNF-α secretion on leukemia progression, I designed shRNAs targeting murine TNF-α, transduced them into freshly isolated MLL-ENL L-GMP, and compared their colony-forming capacity and leukemia progression in in vivo transplantation model. Knockdown of TNF-α expression significantly reduced the colony-forming capacity of L-GMP and resulted in significant inhibition of leukemia progression when they were transplanted into sublethally irradiated mice. The reconstituted leukemia cells with knockdown of TNF-α also had significant decrease in S/G2/M cell cycle phases. These results demonstrate that autocrine TNF-α secretion in LIC is significantly associated with leukemia progression both in vitro and in vivo. I also investigated the

influence of direct NF-κB pathway inhibition on leukemia progression by transducing leukemia cells with a retroviral vector expressing a dominant-negative form of IκBα (super-repressor: IκB-SR) or a control vector. They were transplanted into recipient mice and characteristics of the repopulating cells were examined. First, I confirmed that p65 was almost completely sequestered in the cytoplasm in LIC with IκB-SR and the expression levels of NF-κB target genes, including TNF-α, were significantly decreased. Then, we explored the phenotype of these cells. Introduction of IκB-SR significantly decreased a proportion of cells in the S/G2/M phase of the cell cycle and resulted in a significant growth delay of those cells in liquid culture. More over, the leukemia cells with IκB-SR had reduced colony-forming capacity, while transduction of IκB-SR into normal HSC exerted no significant influence on their colony formation, warranting that NF-κB pathway inhibition confers a marginal influence on normal hematopoiesis. Finally, I transplanted leukemia cells with IκB-SR into sublethally irradiated mice, which showed remarkable delay in leukemia progression. Collectively, these findings clearly demonstrate that enhanced NF-κB activity in LIC has a critical role for leukemia progression and its inhibition severely attenuates its proliferating ability. Since TNF-α secreted from LIC contributed to the maintenance of NF-κB activation in LIC, and the introduction of IκB-SR significantly suppressed the expression level of TNF-α in LIC, they formed NF-κB/TNF-α positive feedback loop.

We also addressed the mechanism of the difference in NF-κB activity between LIC and non-LIC. Notably, LIC had decreased protein levels of IκBα compared with non-LIC in spite of rather increased tendency of the mRNA expression levels, indicating that the differences of IκBα protein levels are caused by its predominant degradation in LIC. As both LIC and non-LIC are similarly exposed to high levels of TNF-α within leukemic bone marrow, I considered that there would be differences in the response to the stimulus and sequentially examined the downstream signals. I first tested if there might exist a difference in the expression levels of TNF-α receptors between LIC and non-LIC. The expression patterns of TNF receptor I and II were, however, almost similar in LIC and non-LIC although they varied between leukemia models. I next investigated the phosphorylation capacity of IκB kinase (IKK) by examining the ratio of phosphorylated IκBα to total IκBα after treatment with proteasome inhibitor MG132. Contrary to my expectation, similar accumulation of phosphorylated form of IκBα was seen in both LIC and non-LIC, indicating that there was no significant difference in IKK activity between them. Then, I investigated the proteasome activity in LIC and non-LIC because it is required for degradation of phosphorylated form of IκBα. Interestingly, I found that LIC had distinctly enhanced proteasome activity than non-LIC. In addition, the expression of several genes encoding proteasome subunits was elevated in LIC compared with non-LIC. The same propensity was seen in human AML $CD34^{+}CD38^{-}$ cells versus $CD34^{-}$ cells in the analysis of microarray expression data. Furthermore, the administration of proteasome inhibitor bortezomib to leukemic mice selectively killed LIC fraction and prolonged survival in the in vivo transplantation model. These results indicate that enhanced proteasomal degradation of IκBα could lead to selectively high NF-κB activity of LIC. From these findings, I

reasoned that the attenuation of NF-κB activity might be related with the transition from LIC to non-LIC. To test this hypothesis, I transduced MLL-ENL leukemia cells with a retrovirus encoding shRNA against IκBα and transplanted them into sublethally irradiated mice. I first confirmed that MLL-ENL leukemia cells with shRNA-mediated knockdown of IκBα (MLL-ENL-IκBα^{KD}) showed decreased IκBα protein level in the cytoplasm and the increased nuclear p65 protein level, showing that NF-κB signal is enhanced due to the reduction of its cytoplasmic holder. In accordance with this, MLL-ENL-IκBα^{KD} cells had significantly higher ability of TNF-α secretion than control cells, reflecting activated NF-κB/TNF-α signaling loop. Interestingly, in the analysis of mice reconstituted with the IκBα-downregulated leukemic cells, the bone marrow mononuclear cells of MLL-ENL-IκBα^{KD} mice showed a marked increase in immature Gr-1^{low} c-Kit^{high} population. They also showed increased colony-forming cell capacity and enhanced LIC frequency as determined by in vivo limiting dilution serial transplantation assay. These data indicated that the transition from LIC to non-LIC might be associated with the attenuation of NF-κB activity due to inefficient degradation of IκBα.

Finally, I investigated the NF-κB/TNF-α positive feedback signaling in human AML LIC. In the analysis of a total of eight patients with AML and one healthy donor, I found that LIC had mostly increased nuclear translocation of p65 and secretory potentials of TNF-α, compared with normal HSC. Interestingly, when I plotted the two parameters, significant positive correlation was demonstrated ($p=0.014$), which was well consistent with my hypothesis of the positive feedback loop between NF-κB and TNF-α in LIC. Furthermore, I compared p65 intensity between LIC and non-LIC in two patients. When the p65 intensity was compared between LIC and non-LIC, predominant p65 nuclear translocation was selectively seen in LIC of both patients, which is also consistent with the data obtained in murine AML models.

In summary, these findings elucidate that NF-κB/TNF-α signaling in LIC, under support of the proteasome activity, has a critical role for both maintenance and propagation of LIC and provide a widely applicable approach for targeting LIC in myeloid leukemias.