学 位 論 文 の 要 旨

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学 位 論 文 名　Internal Tandem Duplication Mutations in *FLT3* Gene Augment Chemotaxis to Cxcl12 Protein by Blocking the Down-regulation of the Rho-associated Kinase via the Cxcl12/Cxcr4 Signaling Axis

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論 文 内 容 の 要 旨

INTRODUCTION

Internal tandem duplication mutations in the *FLT3* gene (ITD-*FLT3*), which are observed in human acute myeloid leukemia (AML) stem cells, induce the refractory phenotype in patients with AML. Human AML stem cells residing in the endosteal niche of the bone marrow are relatively chemoresistant. ITD-*FLT3* enhance cell migration toward the chemokine Cxcl12, which is highly expressed in the therapy-protective bone marrow niche, providing a potential mechanism underlying the poor prognosis of ITD-*FLT3*$^+$ AML. FLT3 inhibitors fail to display significant efficacy as an anti-AML therapy. A recent report demonstrated that Rho-associated kinase (Rock) regulates the proliferation of ITD-*FLT3*$^+$ hematopoietic cells.

Cxcl12 regulates Rock activity, but its effects on Rock activity can be either stimulatory or inhibitory depending on the cell type and the time course examined. We aimed to investigate the mechanisms linking ITD-*FLT3* to increased cell migration toward Cxcl12.

MATERIALS AND METHODS

Ba/F3 cells expressing wild-type *Flt3* or ITD-*Flt3* (N51, N73, and N78) obtained from three different patients with AML were provided by Dr. DG Gilliland of Harvard Medical School. The *in vitro* migration assay was performed as described previously (Blood 105, 3117-3216; 2009). The microarray analyses were performed by Miltenyi Biotec (Auburn, CA). The genes regulated by ITD-*FLT3* and/or Cxcl12 were functionally classified based on the molecular functions and biological process defined by the Gene Ontology terms and the molecular pathways in the KEGG database. The sequences targeting *Rock1* mRNA (NM_009071) for Generation of shRNA were selected using *Bioinformatics-siRNA Designer* on the Clontech website (www.clontech.com). The shRNA oligonucleotides were cloned into the pSingle-tTS-shRNA vector containing neomycin phosphotransferase as a selectable marker (Clontech Laboratories, Mountain View, CA). The data are expressed as the means ±S.E. of the mean, and statistical significance was evaluated using Microsoft Excel (Microsoft Corp., Seattle, WA) via the two-tailed Student's *t* test.

RESULTS AND DISCUSSION

ITD-*Flt3* mutations (N51 and N78) significantly increased the migration of Ba/F3 cells toward 100 ng/ml Cxcl12 compared with wild-type $Flt3^-$ Ba/F3 cells during a 24-h period. The superior migration of ITD-*FLT3*-expressing cells was also detected in the presence of a Cxcl12 concentration of 1, 10, 50, or 500 ng/ml. Despite the enhancement in cell migration toward Cxcl12, the Cxcr4 mRNA and Cxcr4 protein surface expression levels were consistently lower in ITD-$Flt3^+$ Ba/F3 cells than in ITD-$Flt3^-$ cells. Moreover, Cxcr4 expression was further down-regulated by Cxcl12 in ITD-$Flt3^+$ cells. Because the ITD-*Flt3*-induced enhancement of cell migration toward Cxcl12 does not appear to be caused by an increase in Cxcr4 signaling, we examined whether ITD-*Flt3* qualitatively affects the pathways downstream of Cxcl12/Cxcr4 by

identifying the differentially expressed genes in ITD-*Flt3*$^{+}$ cells compared to ITD-*Flt3*$^{-}$ cells before and after migration toward Cxcl12 via mRNA microarray analysis. Classification of the expression of Cxcl12-regulated genes in ITD-*FLT3*$^{+}$ cells demonstrated that the enhanced migration of ITD-*FLT3*$^{+}$ cells toward Cxcl12 was associated with the differential expression of genes downstream of Cxcl12/Cxcr4, which are functionally distinct from those expressed in ITD-*FLT3*$^{-}$ cells but are independent of the Cxcr4 expression levels. Among these differentially regulated genes, the expression of *Rock1* in the ITD-*FLT3*$^{+}$ cells that migrated toward Cxcl12 was significantly higher than in ITD-*FLT3*$^{-}$ cells that migrated toward Cxcl12. Similar to Rock1, the expression of adducin2, a downstream effector of Rock1, was up-regulated by Cxcl12 in ITD-*Flt3*$^{+}$ (N51) cells. In ITD-*FLT3*$^{-}$ cells, Rock1 expression and phosphorylation of Mypt1, a substrate of Rho kinase, were transiently up-regulated but were subsequently down-regulated by Cxcl12. In contrast, the presence of ITD-*FLT3* blocked the Cxcl12-induced down-regulation of Rock1 and early Mypt1 dephosphorylation. Likewise, the FLT3 ligand counteracted the Cxcl12-induced down-regulation of Rock1 in ITD-*FLT3*$^{-}$ cells, which coincided with enhanced cell migration toward Cxcl12. Rock1 antagonists or *Rock1* shRNA abolished the enhanced migration of ITD-*FLT3*$^{+}$ cells toward Cxcl12. Our findings demonstrate that ITD-*FLT3* increases cell migration toward Cxcl12 by antagonizing the Cxcl12-induced down-regulation of Rock1 expression.

CONCLUSION

ITD-*FLT3* mutations modulate Cxcl12/Cxcr4 signaling pathways via a functionally distinct mechanism from that of wild-type *Flt3*, and these mutations augment chemotaxis toward Cxcl12 by blocking the down-regulation of Rock1 expression. The dual roles of Rock1 in aberrant proliferation and migration toward Cxcl12 as a functional effector of ITD-*FLT3* suggest that Rock1 plays a crucial role in the resistant phenotype by modulating the interaction between ITD-*FLT3*$^{+}$ AML cells and the bone marrow microenvironment, in addition to regulating AML cell proliferation. In this regard, antagonizing Rock1 may represent an additional approach to the treatment of patients with AML harboring ITD-*Flt3*.