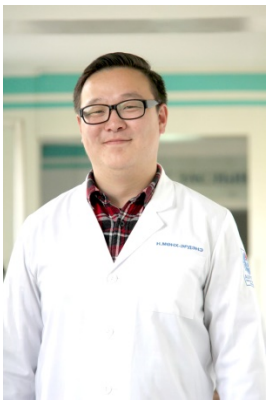
平成29年度 技術交流助成【日 本 留 学】 最 終 報 告



令和 2 年 3 月 27 日

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研究テーマ

Molecular mechanisms of drug resistance of leukemia cells through bone marrow niche cross-talk and its evaluation method

はじめに

A substantial proportion of AML patients with the genetic mutation *FLT3*-ITD are associated with a high relapse risk after hematological remission with intensive chemotherapy. Previously, increased resistance to Ara-C of the *FLT3*-ITD-positive leukemia cells has been reported by our previous researchers. Theoretically, bone marrow microenvironment and its components such as extracellular matrix (ECM), stromal cells, and growth factors could promote the cell survival despite toxic insults including chemotherapy. Interactions of these would contribute to the refractoriness to the chemotherapy. Among the intracellular signals underlying the resistance to Ara-C, transforming growth factor beta (TGFβ) is known as one of the key regulators of leukemia-stroma interactions and it differently stimulates secretion of growth factors, production of ECM components and expression of integrin receptors in various cell types including stromal cells, leukemia cells and other tumor cells. Our current study was aimed to identify the molecular mechanisms of this phenomenon through the cross-talk of bone marrow microenvironment and its components such as ECM. Also, I extended our previous observations and focused on an involvement of TGFβ pathway on resistance to Ara-C of the *FLT3*-ITD-positive leukemia cells via the components of the ECM. Following results were presented in the 66th Annual Meeting of Japanese Society of Laboratory Medicine held at Okayama in November 2019.

方法

□ Cell lines, reagents and ECM materials

Cultured cells used in this study were endogenously-*FLT3*-ITD-positive leukemia cells MOLM-14 and MV4:11 ((ATCC, Manassas, VA, USA) heterozygous and homozygous, respectively), and exogenously-*FLT3*-ITD-positive leukemia K562 cells (ATCC, Manassas, VA, USA) transfected with pMY-puro-*FLT3*-ITD (K562/*FLT3*-ITD) together with pMY-puro plasmid transfected K562 (K562/Mock) cells. The cells were cultured in RPMI 1640 (Wako, Osaka, Japan) medium with 10% fetal bovine serum (FBS; Corning, USA), 1% penicillin/streptomycin (PC/SM; Sigma-Aldrich, St. Louis, MO, USA) at 37^0C and 5% CO_2.

Human fibronectin (FN) and mouse collagen type IV (COL4) coated plates (Corning® Biocoat™, NY, USA) were used for cell culture experiments.

□ Cell viability assay and cytotoxic agents

An MTT assay was performed as previously described. In the presence of FN or COL4, 1×10^4 cells were cultured in 200µl RPMI 1640 medium containing 10% FBS and 1% PC/SM, in FN or COL4 coated 96-well plates for 2 hours, and then exposed to various concentrations of the cytotoxic agents at 37^0C for 70 hours, in a total of 72 hours. The 50% inhibitory concentration for cellular growth (IC_{50}) was determined as compared to the untreated controls and the value was adjusted by negative controls. As cytotoxic agents, Ara-C, crenolanib, idarubicin, doxorubicin and vincristine were used.

Stock solution of 5 mM Ara-C, 2 mM doxorubicin (DOX) and 5 mM vincristine (VCR) were prepared with normal saline and that of 100 mM crenolanib and 2 mM idarubicin (IDR) were dissolved with DMSO and ethanol, respectively and then diluted by normal saline.

□ Exposure to *FLT3*-ITD inhibitor

Crenolanib, type I, second generation FLT3 inhibitor was used for *FLT3*-ITD inhibition. Cells were cultured in the exposure to crenolanib at IC20 concentration for 24 or 72 hours. Expressions of various genes and resistance to Ara-C on K562/Mock, K562/*FLT3*-ITD were examined after exposure to crenolanib.

□ Total RNA extraction, RT-PCR and real-time PCR

Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan), and cDNA was generated from 500ng of RNA using the M-MLV Reverse Transcriptase (Promega, Madison, Wisconsin, USA). A PCR was performed using TaKaRa Ex Taq® DNA Polymerase (Takara Bio Inc., Otsu Japan), and a real-time PCR was performed with SYBR Green II (Takara Bio, Otsu, Japan) on StepOnePlus Real-time PCR system (Thermofisher, USA)

研究結果

1. Enhanced resistance to Ara-C of K562/*FLT3*-ITD, MV4:11 and MOLM14 cells in the presence of FN

An MTT assay was performed to determine IC_{50} of Ara-C and other cytotoxic agents. IC_{50} of Ara-C in K562/FLT3-ITD cells was found higher by 9-folds compared to K562/Mock cells in the absence of ECM. Also, IC_{50} of Ara-C in K562/*FLT3*-ITD, MV4:11 and MOLM14 were increased in the presence of FN, but not of other cytotoxic agents after incubating in the presence of FN.

2. Overexpression of the *TGFB1* and *SMAD2* genes in K562/*FLT3*-ITD cells in the presence of FN

Expressions of the SMAD dependent TGFβ-related genes were measured by real-time PCR. Expression of *TGFBR1* was increased in the K562/*FLT3*-ITD, in the absence of ECM. Also, expressions of *TGFB1* and *SMAD2* genes were increased in *FLT3*-ITD-positive leukemia cells in the presence of FN.

3. No significant change in expression of the TGFβ-related genes in the presence of ECM, after exposure to crenolanib

Expressions of same genes were checked in the K562/*FLT3*-ITD and K562/Mock cells in the presence of FN, COL4 or w/o ECM after exposure to crenolanib. Expressions of the *TGFB1, TGFBR1, TGFBR1,* and *SMAD2* were not changed significantly in K562/*FLT3*-ITD after exposure to crenolanib, in the presence or absence of ECM compared to K562/Mock cells.

まとめ

Based on obtained results we concluded as follows:

1. FN as a component of ECM in the BM microenvironment induces resistance to Ara-C of the *FLT3*-ITD-positive leukemia cells.
2. There are no collateral resistances of the *FLT3*-ITD-positive leukemia to other cytotoxic drugs cells, in the presence of ECM.
3. Expressions of genes both the ligand and receptors of TGFβ were found to be upregulated in the presence of FN or w/o ECM.

In summary, these results would suggest that TGFβ pathway has an important role on enhanced resistance to Ara-C of the *FLT3*-ITD-positive leukemia cells in the presence of FN, implicating its possible involvement in a high relapse rate of the AML after chemotherapy.

We need to continue the study for further clarifications. For this purpose, following studies would be needed:

1. To study expressions of TGFβ related genes at the protein level by Western blotting.
2. To compare gene profiles and activity of the TGFβ related major signaling pathway such as CXCR4-SDF1α interactions in *FLT3*-ITD-positive leukemia cells in the presence of ECM.
3. To study expressions of TGFβ related genes after co-culture with bone marrow stromal cells.

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留学先での生活