論文の内容の要旨

Elucidation of Pathophysiological Mechanisms in Wiskott Aldrich Syndrome Using Patient-Derived Induced Pluripotent Stem Cells

(患者由来人工多能性幹細胞を用いたウィスコットオルドリッチ症候群における病態解明研究)

ハラジ　アミルホセイニ　モジガン
Mozhgan Khalaj Amirhosseini

Wiskott Aldrich syndrome (WAS) is an X-linked disease, which is caused by mutations in the gene encoding the WAS protein (WASp). WASp expression is restricted to the hematopoietic cell lineages and plays significant role in a variety of hematopoietic cells such as T cells, B cells, dendritic cells, monocytes and Platelets regarding their development and function. This disease is characterized by thrombocytopenia, immunodeficiency and eczema. As thrombocytopenia (Platelet count of below 150 x 10^9 per liter) is the most typical feature of this disease, often exposing patients to a significant risk of life-threatening hemorrhage, it has been the main subject of study for many researchers. Based on the severity of symptoms of patients, they categories into 5 groups. The milder phenotype of the disease is called XLT and the sever type is named WAS. Mice models does not phenocopy the disease features properly as they appear to have almost normal platelet count. Because of limitations in disease modeling, precise mechanisms of the platelet abnormality remain to be elucidated. Recently, human pluripotent stem cell

(including human embryonic stem cells [hESC] and induced pluripotent stem cell [iPSC]) have gave a unique opportunity to the scientist to work on understanding the pathophysiology of genetic diseases. iPSCs are patient-derived cells which harbor the patient cell's defects and provide us infinitely proliferation *in vitro*, therefor they can be used as a useful model providing important information about microthrombocytopenia in WAS/XLT patients. Here we established induced pluripotent stem cell (iPSC) lines from $CD34^+$ cells of peripheral blood by utilizing Sendai virus harboring four Yamanaka factors (SOX2, KLF4, OCT3/4 and c-MYC) from two XLT and one WAS patients (each harboring different mutations) as disease models to address the issues. Characterization of iPSC lines revealed that iPSC clones retained typical characterization of pluripotent stem cells. We then confirmed that these disease-specific iPSCs retained gene mutations characteristic to each patient. Further we used this iPSCs to produce megakaryocyte and platelet upon differentiation to hematopoietic lineages. In brief, iPSCs were co-cultured with C3H10T1/2 cells in the presence of 20 ng/ml VEGF to generate hematopoietic progenitor cells. The progenitor cells fraction which were double positive for CD34 and CD41 were sorted using FACS analysis and further cultured for 9 more days in the presence of a cytokine cocktail including 100 ng/ml TPO and 50 ng/ml SCF to produce mature megakaryocytes and platelets. Using our differentiation culture system, we demonstrated that numbers of both megakaryocytes (MK)s and platelets (PLT)s - showing double positive CD41 and CD42 cell surface markers- obtainable from the same number of hematopoietic progenitor cells are significantly smaller in both XLT- and WAS-iPSCs comparing to those from healthy-iPSCs. In addition we demonstrated that the number of proplatelet bearing cells obtained from same numbers of progenitor cells is significantly reduced in patient samples

comparing to the healthy counterpart. Estimation of platelet numbers released by each megakaryocytes showed that both patient-iPSCs and healthy-iPSCs generate comparable numbers of platelets and platelet production ability of megakaryocytes is not affected by WASp deficiency. Hence, we suggested that the observed defects in platelet number of patient samples were mainly due to insufficient production of proplatelet-bearing cells, but not to impaired platelet production per MK. Previous studies have suggested that WASp has important role in hematopoietic cell proliferation and differentiation. We assessed colony formation ability of both XLT- and WAS-iPSCs using MethoCult H4434 semisolid medium differentiation kit. In this assay hematopoietic progenitor cells (HPC; CD 41^- , CD 235^- , CD 34^+, CD 43^+, CD 45^+) obtained from SAC structure in the day 14 of iPSC differentiation were used. Interestingly our data revealed that the ability of patient-HPCs for differentiation to the hematopoietic lineage is affected by WASp deficiency and it varies between patient samples and depends on the distinct type of mutation. Later we sought for a possible role of WASp in the cytokine mediated cell signaling in the process of differentiation of progenitor cells to megakaryocytes. We analyzed phosphorylation of downstream molecules of TPO receptor (MPL) as TPO is the main applied cytokine which is essential for megakaryocyte differentiation. At first we tested the expression level of MPL using FACS and could not detect any significant different between healthy samples and patient samples. Based on the FACS analysis data we suggest that WASp in involved in TPO signaling pathway since a dull phosphorylation of downstream molecules of MPL was seen for WASp null progenitor cells ($CD34^+$)comparing to healthy counterpart upon stimulation with 100 ng/ml TPO for 10 minutes. Lentiviral-mediated gene transfer led to appearance of WASp expression in patient iPSC-derived MKs. Although the expression

level of WASp did not reach the normal level that was seen in control-iPSC-MKs; it seemed to be sufficient to significantly increase yields of platelets and megakaryocytes comparing to the control vector after gene transfer. The low expression level of WASp after gene therapy comparing to the physiological expression of the protein in healthy sample maybe the reason which we could not get as high number of platelets as healthy samples after gene addition. This may suggest the necessity of a distinct expression pattern and level of WASp in $CD34^+$ progenitor cells for complete reconstitution of platelet number. The low expression level of WASp in megakaryocytes after gene transfer may be consistent with a recently published clinical trial's data reporting dull expression level of WASp in the platelets after gene therapy while other hematopoietic cells such as T cells expressed almost normal level of WASp.

In conclusion our data propose that reduced number of platelet which was observed in both XLT and WAS patients is due to lessen proplatelet bearing cells and WASp deficiency does not interrupt the ability of each megakaryocytes to shed platelets and besides, less numbers of proplatelet bearing cells is attributed by defective TPO signaling pathway in WASp deficient progenitor cells. Although further investigation is necessary, these results indicate the utility of iPSC-based disease modeling for WAS. More detailed analysis is required to understand the mechanism underlying insufficient WASp expression in megakaryocytes after gene transfer and consequently discern how critically expression levels of WASp will affect the efficacy in platelet number recovery after gene transfer.