

論文の内容の要旨
Thesis Summary

論文題目

Optimization of gene therapy strategies for chronic granulomatous disease using patient autologous iPS cells

(患者由来人工多能性幹細胞を用いた遺伝子治療の至適化戦略)

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Introduction

Chronic granulomatous disease (CGD) is a monogenetic hematological disorder caused by a defect with the enzyme NADPH oxidase. The most common form of this disease is attributed to gp91phox deficiency, which is encoded by the CYBB gene found on the X chromosome (XCGD). Consequently neutrophil (NEU) functionality in the production of reactive oxygen species (ROS) is compromised thus rendering patients vulnerable to potentially life threatening infections. The definitive treatment for XCGD is hematopoietic stem cell gene therapy (SCGT) where an HLA-matching donor cannot be found. In this scenario, integrating viral vectors are commonly used to induce expression of a therapeutic transgene in autologous hematopoietic stem cells (HSCs). This is infused back into the patient with the hope of achieving long-term sustained expression of a defective protein in all hematopoietic lineages and to restore cell functionality.

Unfortunately, XCGD appears to be unique compared to other primary immune deficiencies (PIDs) in that the appearance of functionally restored cells in the peripheral blood (PB) following hematopoietic SCGT occurs only for a transient period. This is despite similarities in vector design and conditioning regimen in treating patients of other PIDs. It is possible that this clinical observation is related to the ectopic constitutive expression of gp91phox due to the use of ubiquitous promoters within integrating vectors. This may lead to perturbations in ROS production in NEUs with potentially detrimental consequences. However, this hypothesis has yet to be investigated under formal experimental settings.

Hematopoiesis is a process that occurs largely in the bone marrow with only mature blood cells released into the blood stream. Therefore it is necessary to utilize an *in vitro* modeling system in order to study the differentiation process intensely. In terms of physiological relevance, the preferred choice would be to use CD34⁺ cells from XCGD patients. For reasons of practicality however, these cells can be difficult to obtain repeatedly especially those from patients suffering from a specific disorder. These limitations may be overcome through the utilization of induced pluripotent stem cells (iPSCs). Cells differentiated from patient autologous iPSCs could recapitulate the disease phenotype at either a molecular or morphological level. This system can be controlled by cytokines and is amenable to genetic modification, allowing for the precise analysis of cellular functions and its attributing factors.

In this project, the main objective is to establish the use of patient autologous iPSCs as a reliable modeling system for XCGD *in vitro*. This will be utilized to identify and evaluate an element of the underlying pathophysiology that may be unique to XCGD. More specifically, the potential occurrence of ectopic gp91phox expression and the consequential effects shall be investigated. A method of using digital droplet PCR

(ddPCR) technology will also be applied to estimate the average copy numbers in transduced cells, which will permit a more accurate correlation between transgene expression and functional recovery. It is hoped that the data generated may eventually contribute towards the optimization of gene therapy strategies for CGD and improve clinical outcomes.

Materials and methods

To generate iPSCs, peripheral blood (PB) CD34⁺ cells from a X-CGD patient and from a healthy donor were reprogrammed to pluripotency using a Sendai virus vector expressing Oct4/Sox2/Klf4/c-Myc. Reprogrammed cells were characterized by demonstrating alkaline phosphatase (ALP) activity and expressed the surface pluripotency markers SSEA-4, Tra-1-60 and Tra-1-81. Expression of pluripotency genes was confirmed by RT-PCR. Chromosome karyotyping of X-CGD iPS cells and sequencing analysis to confirm the location of the point mutation in the *CYBB* gene was also carried out.

Neutrophil differentiation was induced using a combination of vascular endothelial growth factor (VEGF) and granulocyte colony-stimulating factor (G-CSF). Cell maturation status was determined morphologically through histochemical staining and the expression of neutrophil specific antigens. The expression of gp91phox together with that of p47phox and p67phox was determined by intracellular staining in flow cytometry analyses. Neutrophil functionality was determined by detecting reactive oxygen species (ROS) production as indicated using the dihydrorhodamine (DHR) flow cytometry assay following phorbol 12-myristate 13-acetate (PMA) stimulation.

Self-inactivating alpharetroviral vectors were used to insert the gp91^{phox} transgene the expression of which was driven by the ubiquitous EFS or SFFV promoters. The original vector design was modified to include a puromycin resistance gene to allow for the selection of transgene positive cells. Δ LNFR (truncated NGFR) was selected as an additional selection marker for transgene positive cells where puromycin selection pressure had to be lifted.

The PLB-985 (PLB) myeloid leukemic cell line was induced to undergo differentiation using dimethyl sulfoxide (DMSO). The XCGD form (PLB-XCGD) lacked gp91 protein expression through targeted disruption by homologous recombination of the *CYBB* gene. Wild type (WT) PLB cells or those containing only a single vector insertion served as the controls.

Vector copy number (VCN) was estimated using the QX200™ Droplet Digital™ PCR System as per manufacturer's instructions. Genomic DNA was extracted from iPSCs or PLB cells using the NucleoSpin® Tissue XS kit. Target primer-probes were designed to recognize sequences within the puromycin resistance gene.

The expression of ER-stress markers was determined by intracellular staining. Cell viability was assessed using the PE Annexin V Apoptosis Detection Kit I as per the manufacturer's instructions.

Results

Patient autologous iPSCs were successfully generated and could be fully characterised to display typical features of pluripotent stem cells. NEUs differentiated from control iPSCs were functionally and phenotypically comparable to PB NEUs. Most importantly, NEUs differentiated from XCGD iPSCs showed a complete absence of oxidase activity thus indicating that the disease phenotype has been successfully recapitulated.

In terms of cellular recovery, the expression of gp91phox and ROS production in NEUs differentiated from transduced XCGD-iPSCs was incomplete. This remained the case even when the SFFV promoter was used, which is known to have stronger activity than the EFS promoter at driving transgene expression. By using the ddPCR method, it could be determined that in some gp91phox-transduced XCGD-iPSCs, the estimate average VCN was as high as 14 copies. Furthermore, there appeared to be a negative correlation between an increase in gp91phox expression and that of the intracellular NADPH oxidase subunits p47phox and p67phox.

It was found that “ectopic” gp91phox expression could only be detected in the (CD64^{dull}CD15^{dull}) developing fraction of NEUs differentiated from gp91phox transduced XCGD iPSCs. This corresponded with non-physiological ROS production. Concomitantly, there appeared to be raised expression of ER stress markers, suggesting that these cells have an increased propensity for undergoing apoptosis. Indeed, developing NEUs ectopically expressing gp91phox appeared to show more prominent levels of cell death irrespective of a XCGD or healthy background.

Conclusions

Control iPSC-derived neutrophils were demonstrated to be characteristically similar to PB NEUs thus validating the reliability of this system in modelling XCGD. The use of the surface markers CD64 and CD15 allowed for the distinction between developing and mature NEUs within the same differentiation culture.

The observation of incomplete cellular recovery on a per cell basis compared with the wild type control may be attributed to incomplete gp91phox expression. It is possible that this is due to susceptibility of the promoters to silencing. However, for the assessment of cellular recovery, puromycin selection pressure was sustained throughout the differentiation process to select only for transgene positive cells. Therefore promoter silencing alone may not fully explain these observations. Alternatively, insufficient numbers of provirus insertions may also be a possibility. However, using the ddPCR method, it could be confirmed that all transduced iPSCs have an estimated VCN of at least 1 with some as high as 7.16 (EFS promoter MOI 10) and 13.75 (SFFV promoter MOI 10) respectively.

Although GRP78 and pEIF2a were found to show raised expression, this was not the case for all the ER stress markers tested. In addition, the response between cells with either an healthy or XCGD genetic background appears to be different so it cannot be concluded that this is the main underlying mechanism that leads to cell death but it does not preclude the possibility of non-physiological ROS exerting a detrimental effect.

Future plans

It would be important to validate the findings from this study using human CD34⁺ cells from patients or perhaps the mouse equivalent. Bisulphite sequencing of the promoter regions may indicate the extent of methylation that has occurred. Should the same findings remain consistent then the next step would be to find ways of antagonizing the detrimental effects secondary ectopic gp91phox expression. This may be aimed at a more specific target such as using chemical agents to reduce the levels of ER-stress or administer a more generalized ROS scavenger such as N-acetylcysteine (NAC). If this method of cellular protection is to be adopted then further determination of the precise cause of cell death plus timing of drug administration will be necessary.

Alternatively regulated transgene expression may be another possibility. Recently a dual-regulatory strategy combining transcriptional and post-transcriptional regulation in which the use of a lentiviral vector with a myeloid specific promoter and two micro RNA 126 “sponge” sequences were shown to effectively restrict gp91phox expression to the myeloid compartment with minimal leakage. In the context of this study, this method of lineage-restricted expression of gp91phox may eliminate some of the impediment imposed upon developing cells that can differentiate into mature NEUs. There is also the additional benefit of sparing the HSC compartment to minimize any genotoxic risks, making it a promising approach to improve XCGD gene therapy in the future. Taken together, it is hoped that further study using this iPSC modeling system may yet yield additional therapeutic targets the resolution of which could lead to even greater improvements in CGD clinical outcomes.