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研究課題名（和文）Development of a novel therapy for sickle cell disease by Nrf2 activation

研究課題名（英文）Development of a novel therapy for sickle cell disease by Nrf2 activation

研究代表者
Ｋｅｌｅｋｕ　Ｎａｄｉｎｅ（Keleku-Lukwete, Nadine）

東北大学・医学系研究科・助教

研究者番号：４０７８１７６１

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研究成果の概要（和文）：鎌状赤血球症はグロビン遺伝子の変異により、赤血球が鎌状に変形することで、酸化ストレスを惹起し、様々な組織に障害をもたらす疾患である。以前に私たちは、全身でNrf2を活性化させることにより、鎌状赤血球症の症状を改善できることを明らかにした。そこで本研究では、Nrf2活性化による症状改善における責任細胞の同定を目的とした。炎症細胞及び血管内皮細胞特異的にNrf2を活性化させたマウスを解析したところ、両マウスにおいて鎌状赤血球症の症状である肝障害と炎症の改善がみられた。このことから、炎症細胞及び血管内皮細胞におけるNrf2の活性化が重要であると結論づけられた。

研究成果の概要（英文）：Our research project was aimed to understand the mechanisms of specific-cell response to the activation of Nrf2 and how they contribute to the amelioration of the sickle cell disease (SCD) phenotype. We studied the function of Nrf2 in myeloid and endothelial cells and it influence to the surrounding organs by using mutant mice harboring the globin mutation, Keap1 deletion in either myeloid cells (SCD::Keap1F/F::LysM-Cre mice) or endothelial cells (SCD: Keap1F/F::Tie1-Cre mice). We collected peritoneal macrophages and cultured primary pulmonary endothelial cells for the cell specificity experiments; and some tissues we collected and to perform histological and hematological analysis; biochemistry; IMS and LC-mass; and genes expression (using PCR and RNA- sequencing). All the results are summarized in the manuscript that we submitted to Blood Advances journal for publication which is currently under revision.

研究分野：　医化学分野

キーワード：　sickle cell disease　Nrf2　Keap1 knockout　Myeloid cells　endothelial cells

１．　研究開始当初の背景

Sickle cell disease is a hematological blood disorder of the red blood cells. It is characterized by sickling of RBC responsible of reducing the blood flow and favoring vaso-occlusion under low oxygen supply conditions. The deformed RBCs are subjected to chronic hemolysis, which generates substantial amount of free heme and iron ions in the bloodstream. These pro-oxidants factors can trigger activation of endothelial cells and vaso-occlusion that will evolve to ischemia-reperfusion injuries and organ damages.

In our approach we speculate that hemolysis and subsequent generated reactive oxygen species (ROS) contribute in most of the damages found in SCD mice. We previously showed that genetic activation and CDDO-Im administration suppress inflammation and organ damages in SCD mice.

The transcription factor nuclear factor erythroid-derived 2-like 2 (Nrf2) mediates the gene expression of a variety of detoxifying and antioxidative enzymes/proteins. Nrf2 activity is mainly controlled by Keap1, which mediates Nrf2 degradation through the proteasome pathway during unstressed conditions. By contrast, upon exposure to oxidative or xenobiotic stressors, Keap1activity of is inactivated, and newly generated Nrf2 accumulates within cells. Nrf2 efficiently translocates in the nucleus and activates the transcription of target genes. We aimed to assess the myeloid cells and endothelial cells –specific activation of Nrf2, knowing that these cells participate in the pathological process in SCD.

２．　研究の目的

In this study, we intended to dissect the specific-cell response to the activation of NRF2 in SCD mice. SCD is a complex disorder in which more than one single cell type participates to the progression of the pathology. During hemolysis crisis, endogenous mechanism scavenging the buy product of RBC breakdown can in the early times operate to reduce the burst of free heme and iron ions from the bloodstream. Later with the recurrence of the hemolysis these mechanisms are often overloaded. We hypothesized that using the KEAP1-NRF2 pathway in specific cell that participate in the pathology of SCD would first reinforce cell defense mechanism against ROS and contribute to the understanding of cell specific response to NRF2 activation in the context of SCD.

３．研究の方法

To activate NRF2 in specific cells, we conditionally deleted *Keap1* gene in myeloid linage and endothelial cells of SCD mice. To that end, we crossed SCD mice with the Keap1flox mice and obtained the SCD::Keap1F/F mice. These mice did not show a particular phenotypic difference with the SCD mice. We next breed the SCD::Keap1 F/F mice with either LysM-Cre or Tie1-Cre mice with express the lysozyme and Tie1 promoter in myeloid and endothelial cells respectively. After many backcrossing, we generated the SCD::Keap1F/F as control,

SCD::Keap1::Lysm-Cre and SCD::Keap1::Tie1-Cre mice. To confirm the deletion of Keap1, we asses the recombination degree of Keap1 in peritoneal macrophages of SCD::Keap1::Lysm-Cre mice and endothelial cells of SCD::Keap1::Tie1-Cre mice, in addition the recombination PCR was also performed in lung, liver kidney and spleen of both three genotypes. We also confirmed the activation of Nrf2 in cells and organs by measuring the expression of the messenger RNA of Nqo1.We performed the measurements of hematological parameters and heme, bilirubins, ALT in plasma. Among complications of SCD, avascular necrosis of organs and inflammatory damages are frequently reported, we examined liver histology, lungs and kidney sections. Heme trigger oxidative damages when it accumulates in cells, to assess the effect of Nrf2 on the accumulation of heme in organs, we performed imaging mass spectrophotometry (IMScope) of heme in liver and spleen. Additionally, LC-mass was performed the measure the glutathione activity as key of the antioxidant mechanism. To determine the function of Nrf2 in the vascular endothelium, we measure the permeability of cells junctions by the level of vascular leakage in of the skin, lung and liver. With the hypothesis that each cell responds differently to Nrf2 activation, we cultured primary pulmonary endothelial cells (PPECs), collected from 6-10 days old puppies. PPECs were used to examine the expression patterns of genes known to be dependent or independent of Nrf2 activation, by performing RNA-sequencing analysis.

４．研究成果

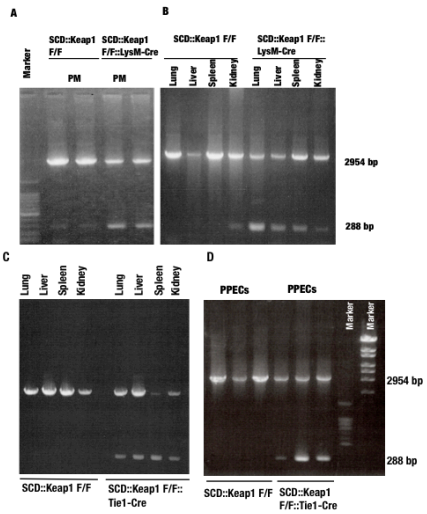

FIG 1. Deletion of Keap1 up regulated Nrf2 genes expression

(1) Deletion of Keap1 up regulated Nrf2 genes expression

To confirm the deletion of Keap1, we collected genomic DNA from peritoneal macrophages, endothelial cells and from different organs and performed PCR. The deletion was confirmed with the presence of the 288bp band in SCD::Keap1::LysM-Cre mice and SCD::Keap1::Tie1-Cre (Figure 1)

Keap1 deletion was responsible of the upregulation of the mRNA expression of Nqo1 in peritoneal macrophages and mRNA of Nqo1 and Ho1 in liver, lung, Kidney and aorta of SCD::Keap1::LysM-Cre mice (Figure 2)

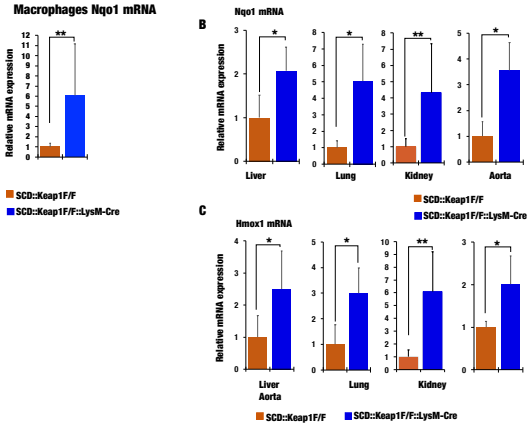

FIG 2. Deletion of Keap1 up regulated Nrf2 genes expression

(2) Nrf2 activation in myeloid cells of SCD::Keap1::LysM-Cre mice attenuates the inflammation

To examine the effect of Nrf2 activation in myeloid cells, we analyzed lungs sections. The H&E stained sections showed infiltration of myeloid inflammatory cells, edema and congestion. Furthermore, the F4/80 staining shows infiltration of macrophages in SCD::Keap1 F/F mice in comparison with the SCD::KEap1F/F::LysM-Cre mice which showed lower inflammatory process and low expression of TNFα and IL-1β mRNA. SCD::KEap1F/F::LysM-Cre mice liver analysis revealed decreased lesions of necrosis and decreased level of plasma ALT, one of the liver damages marker (Figure 3).

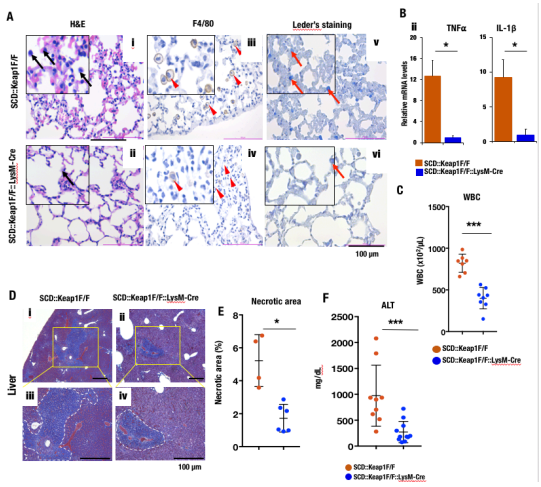

FIG 3. Nrf2 activation in SCD::LysM-Cre mice suppress organ damages

(3) Nrf2 activation in SCD::Keap1F/F::LysM-Cre mice improves heme metabolism.

Chronic hemolysis can facilitate intra cellular accumulation of free heme. The IMScope analysis revealed that heme accumulation in the livers of SCD::KEap1F/F::LysM-Cre mice were lower than that of SCD::KEap1F/F mice. We measure plasma levels of free heme, total and indirect bilirubin was significantly reduced compared to control mice (Figure 4).

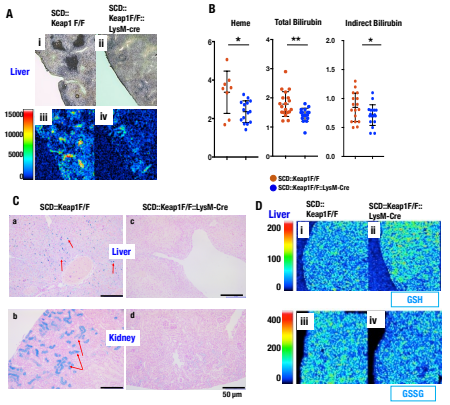

FIG 4. Nrf2 activation in SCD::LysM-Cre mice mitigates heme metabolism

(4) Nrf2 activation in endothelial cells protect organs and the vascular endothelium function

Vascular endothelium is involved in the pathology of SCD. During hemolysis endothelial cells are activated and interactions with other cells promotes vaso-occlusion and impaired vascular function. The induction of Nrf2 lowers the expression of adhesion molecules *V-cam1* and *P-selectin*. Furthermore, injection of Evans blue to assess the integrity of the vessels revealed fewer leaking vessels of the skin, liver and lung (Figure 5).

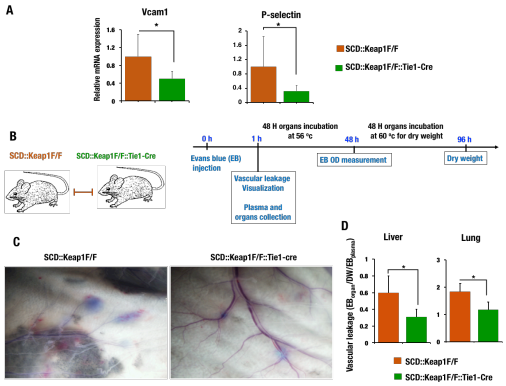

FIG 5. Nrf2 activation in endothelial cells of SCD::Tie1-Cre mice protect the vascular endothelium

(5) Primary pulmonary endothelial cells express Nrf2 target genes

To identify the molecular basis of how Nrf2 benefits ECs in SCD model mice, we measured the expression of Nqo1 mRNA. RNA-sequencing analysis revealed that 879 genes were significantly upregulated in SCD::Keap1F/F::Tie-Cre cells compared with those in SCD::Keap1F/F cells, and 898 genes were significantly downregulated in SCD::Keap1F/F cells compared with those in SCD::Keap1 F/F::Tie-Cre cells. The overall analysis revealed an upregulation of most of Nrf2 dependent pathways (Figure 6). These results unequivocally demonstrate that in order to protect organs/tissues from SCD pathology, Nrf2 activation is required in both myeloid-lineage cells and ECs in a distinct but overlapping manner.

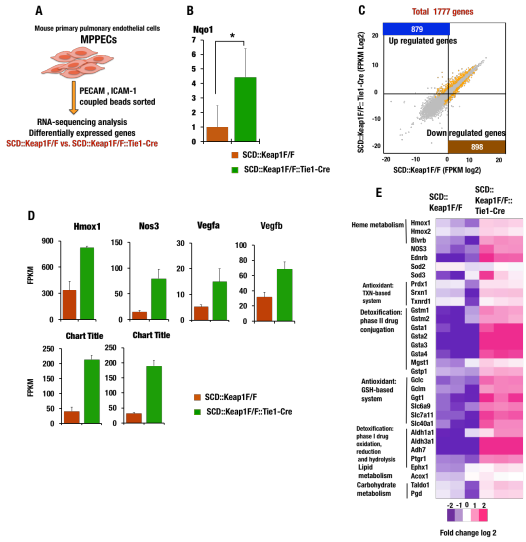

FIG 6. Primary pulmonary endothelial cells of SCD::Keap1::Tie1-Cre mice show upregulation of Nrf2 target genes and others pathway

５．主な発表論文等

（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　3　件）

1. **Keleku-Lukwete N**, Suzuki M, Yamamoto M. An Overview of the Advantages of KEAP1-NRF2 System Activation During Inflammatory Disease Treatment. ***Antioxid. Redox Signal***, in press, 2018. 査読有 doi: 10.1089/ars.2017.7358.

2. Katayama S, Suzuki M, Yamaoka A, **Keleku-Lukwete N**, Katsuoka F, Otsuki A, Kure S, Engel JD, Yamamoto M. GATA2 haploinsufficiency accelerates EVI1-driven leukomogenesis. ***Blood***, 130; 908-919. 2017. 査読有
doi: 10.1182/blood-2016-12-756767.

3. Tsuchida K, Tsujita T, Hayashi M, Ojima A, **Keleku-Lukwete N**, Katsuoka F, Otsuki A, Kikuchi H, Oshima Y, Suzuki M,

Yamamoto M. Halofuginone enhances the chemo-sensitivity of cancer cells by suppressing NRF2 accumulation. ***Free Radic Biol Med.*** 103; 236-247. 2017. 査読有
doi: 10.1016/j.freeradbiomed.2016.12.041.

〔学会発表〕（計　1　件）

1. **Keleku-Lukwete N,** Panda H, Naganuma E, Suzuki M, Yamamoto M. Insight mechanisms underlying the importance of Nrf2 in sickle cell disease. ***The 89th annual meeting of the Japanese Biochemical society***, Sendai, Japan, September 25-27, 2016

６．研究組織

(1)研究代表者

Nadine Keleku-Lukwete（Nadine Keleku-Lukwete）

東北大学・大学院医学系研究科・助教

研究者番号：40781761