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研究課題名（和文）非アルコール性脂肪性肝炎の病態形成におけるサイトグロビンの関与

研究課題名（英文）Loss of Cytoglobin Exacerbates Liver Fibrosis and Cancer Development in Steatohepatitis by Activating the Oxidative Stress Pathaway

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研究成果の概要（和文）：Cygb-/-マウスにCDAA投与後１６週間までの観察では野生型に比較してCygb-/-マウスで炎症、線維化反応の増強が観察された。３２週間まで観察を継続するとCygb-/-マウスでは肝臓が萎縮しており高度の線維化が生じ、同時に腫瘍形成が確認された。Cygb-/-マウスはCDAAモデルにおいても易発がん性を呈することが改めて確認された。Cygb-/-マウス肝ではラジカル反応が強く生じていることが示されCygbの存在が肝臓を生理的状況を維持するのに不可欠である事が示された。myeroperoxidase陽性の細胞は浸潤した好中球であることや肝臓内では抗酸化ストレス物質が減弱することが示された。

研究成果の概要（英文）：Cytoglobin was discovered in 2001 as a protein expressed uniquely in hepatic stellate cell.The present study clarifies the role of Cygb in steatohepatitis induced by a choline-deficient amino acid-defined dietin mice.CDAA treatment for 8 weeks induced prominent inflammation and fibrosis in Cygb-/- mice,which was inhibited by macrophage deletion.Suprisingly,at 32 weeks,despite no tumor formation in the WT mice,all Cygb-/- mice developed liver cancer,which was ameliorated by N-acetylcysteine treatment.Altered expression of 31 genes involved in the metabolism of reactive oxygen species was notable in Cygb-/- mice.Moreover,primary untreated-HSCs isolated from Cygb-/- mice showed a pre-activated condition characterised by augmented ROS and cytokine production.In human NASH livers,the expression of CYGB was declined in a negative correlation with increased NASH acore.A similar decline in CYGB protein and mRNA expression likely contributes to the development of human NASH and liver cancer.

研究分野：肝臓病学

キーワード：遺伝子　ｻｲﾄｸﾞﾛﾋﾞﾝ

１．研究開始当初の背景

Non-alcoholic steatohepatitis (NASH), an increasingly recognised obesity-related liver disease, is characterised by hepatocyte steatosis accompanied by a fibro-inflammatory reaction (Day and James 1998, Pessayre, Berson et al. 2001). Several studies have shown that NASH patients are at risk for progression to cirrhosis, the most common risk factor for hepatocellular carcinoma (HCC) (Pessayre, Berson et al. 2001, Starley, Calcagno et al. 2010). Compared to what is known about the pathogenesis of hepatitis virus-induced HCC, insight into NASH-associated HCC remains immature. Cytoglobin (CYGB) was originally discovered in rat HSCs in 2001 (Kawada, Kristensen et al. 2001), and is the fourth globin to be discovered in mammals (Burmester, Ebner et al. 2002, Sawai, Kawada et al. 2003). CYGB is present in fibroblasts that store vitamin A in the visceral organs, including the liver and pancreas (Nakatani, Okuyama et al. 2004). CYGB facilitates oxygen (O_2) diffusion through tissues, scavenges nitric oxide (NO) and other ROS, has a protective function during oxidative stress (Sugimoto, Makino et al. 2004), and suppresses tumourigenesis (Presneau, Dewar et al. 2005, McRonald, Liloglou et al. 2006, Xinarianos, McRonald et al. 2006, Shaw, Omar et al. 2009). We previously showed that *Cygb*-deficient (*Cygb*$^{-/-}$) mice exhibit susceptibility to cancer development in the liver and lung with diethylnitrosamine (DEN) administration (Thuy le et al. 2011). Therefore, the absence of CYGB likely promotes a carcinogenic process in the presence of liver disease.

２．研究の目的

The present study aims to clarify the role of *Cygb* in steatohepatitis induced by a choline-deficient amino acid-defined diet (CDAA) in mice.

３．研究の方法

(1) *Human Tissues and Specimens*

Human NASH specimens (n = 15), used for immunohistochemistry of CYGB, were obtained from patients in Osaka City University Hospital who were diagnosed with NASH according to Matteoni's classification[17]. Intact human specimens (n = 3) of non-tumor lesion were obtained from patients who had metastasis liver tumors or cholangiocarcinoma treated by surgical resection. HCC tissues and noncancerous liver tissues were obtained from 9 patients without hepatitis virus B or C infection, who had undergone a hepatectomy at the Osaka City University Hospital. The specimens were routinely processed, formalin fixed, and paraffin-embedded. A portion of tissues were frozen and stored at -80°C without fixation. RNA were extracted from them by the acid guanidinium thiocyanate-phenol-chloroform method as described in our previous study[18]. All patients gave written informed consent to participate in this study in accordance with the ethical guidelines of the 1975 Declaration of Helsinki, and according to the process approved by the ethical committee of Osaka City University, Graduate School of Medicine.

(2) *Mice and Diet*

C57BL/6 *Cygb* conventional knockout mice were generated in our laboratory as described previously[15]. C57BL/6 mice (WT) were purchased from SLC (Shizuoka, Japan).
For the NASH model, 78 *Cygb*$^{-/-}$ and 77 WT mice were used, including males and females. Eight-week-old mice were fed CDAA (Catalogue #518753, Dyets, Bethlehem, PA, USA) or a control diet, choline-supplied amino acid-defined diet (CSAA, Catalogue #518754,

Dyets) with n = 5–14 per group. Mice were fed these diets continuously for 8, 16, or 32 weeks. To investigate tissue hypoxia, 1 h before sacrifice, some mice were injected intraperitoneally with Hydroxyprobe-1 solution at a dose of 60 mg/kg body weight using the Hydroxyprobe-1 Omni Kit (Hydroxyprobe, Burlington, MA, USA) according to the manufacturer's protocol.

In the macrophage-depletion experiment, a sub-group of 20 mice were divided into four groups. A short 8-week protocol on the CDAA diet followed with macrophage deletion in the final week was used to examine the early events of NASH. At the 7th week of CDAA feeding, Kupffer cell depletion was induced by injecting 200 μL liposomal clodronate (FormuMax Scientific, Palo Alto, CA, USA) into the mouse tail vein, according to the manufacturer's protocol. Control mice were injected with the same amount of plain control liposomes. Mice were continuously fed the CDAA diet and sacrificed 1 week after injection.

For N-acetylcysteine (NAC) treatment, a total of 53 *Cygb*$^{-/-}$ and WT mice, divided into 6 groups, n = 5–13 per group, were fed the CDAA diet together with 0.1 mM NAC (Sigma-Aldrich, St. Louis, MO, USA) in the drinking water for 2, 8, or 32 weeks starting at 8 weeks of age. NAC was prepared as a 0.5 M stock in sterile water once a month, aliquoted, and stored at –30°C in the dark. Sterile drinking water was freshly made from the stock and changed twice a week. Animal care and procedures were approved by the Osaka City University Animal Care and Use Committee as set forth in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

(3) Histology, Immunohistochemistry and Immunofluorescence Analysis

Hematoxylin and eosin, immunohistochemistry and immunofluorescence analysis were performed as previously described (Thuy le et al. 2011). The primary antibodies used for mouse and human samples including CYGB antibodies which were generated by our laboratory were described. Pathological severity of NAFLD was assessed using criteria described by Kleiner et al. Liver fibrosis area in each samples and CYGB-positive cells in each human sample were quantified.

(4) *DHE Assay*

To examine the oxidative stress condition induced by CDAA diet and by the absence of CYGB, primary HSCs cultured as described below or freshly prepared frozen liver sections which were warm up at 37°C for 2 h were incubated with 2 μmol/L dihydroethidium (DHE) (Invitrogen, Eugene, Oregon) in PBS for 30 min at 37°C. Then, they were counterstained with DAPI and observed under fluorescent microscopy.

(5) *Hydroxyproline Assay*

Hydroxyproline content of the liver was measured by a spectrophotometric assay by using Hydroxyproline Assay Kit (BioVision, Milpitas, CA) according to the assay protocol.

(6) ALT Measurement

Alanine aminotransferase (ALT) activity (UV test at 37°C) was measured in serum using a commercially available kit (Wako, Osaka, Japan) according to manufacturer's protocol.

(7) *Quantitative Real-time PCR*

Total RNA was extracted from cells and liver tissues using the miRNeasy Mini Kit (Qiagen, Valencia, CA). cDNAs were synthesised using total RNA, a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) and oligo(dT)$_{12\text{-}18}$ primers according to the manufacturer's instructions. Gene expression was measured by

real-time PCR using the cDNAs, SYBR qPCR Mix Reagents (Toyobo) and gene-specific oligonucleotide primers (Table 2) with an ABI Prism 7500 Fast Real-Time PCR System (Applied Biosystems, Foster, CA). The *Gapdh* level was used to normalize the relative abundance of mRNAs.

(8) *Gene Expression Profile for Specific Pathway*

The Mouse Oxidative Stress and Antioxidant Defense RT² *Profiler*™ PCR Array from SA Biosciences (Frederick, Maryland; cat # PAMM-065) was performed to examine the expression of 84 genes related to oxidative stress according to manufacturer's protocol.

(9) *Immunoblot Analysis*

Protein samples (10 to 40 μg) were subjected to SDS-PAGE and transferred to Immobilon P membranes (Millipore Corp, Bedford, MA). After blocking, membranes were probed with primary antibodies against CYGB (1: 500) from our laboratory (Table 1), AKT (1:1000; Cell Signaling, Danvers, MA), phosphorylated AKT (1:500; Cell Signaling), BCL-2 (1:1000; Cell Signaling), extracellular signal–regulated kinase (ERK;1:500; Cell Signaling), phosphorylated ERK (1:1000; Cell Signaling), CYCLIN D1 (1:5000; Cell Signaling), phosphorylated SMAD3 (1:1000; Abcam, Cambridge, MA), total SMAD3 (1:1000; Abcam), heme oxygenase-1 (HO-1; 1:1000; Cosmo Bio Co. Ltd, Japan), Myeloperoxidase (MPO, 1:1000, Abcam), α Smooth muscle actin (αSma, 1:1000; Abcam) or GAPDH (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were then incubated with horseradish peroxidase–conjugated secondary antibodies at 1: 2000 dilutions. Immunoreactive bands were visualized using the ECL detecting reagent (GE Healthcare UK Ltd, Buckinghamshire, UK), and documented with the Fujifilm Image Reader LAS-3000 (Fujifilm, Tokyo, Japan) coupled with image analysis software (Multi Gauge, Fujifilm).

(10) *Cells*

HSCs were isolated from WT ($HSC^{Cygb\text{-}wild}$) and *Cygb*$^{-/-}$ ($HSC^{Cygb\text{-}null}$) mice using the pronase-collagenase digestion method as previously described [21] and were cultured on uncoated-plastic dishes (BD Falcon, Franklin Lake, NY) or glass chamber slides (Thermo Fisher Scientific, Waltham, MA) in DMEM (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen, Carlsbad, CA) and antibiotics (100 U/ml penicillin, and 100 μg/ml streptomycin). $HSC^{Cygb\text{-}wild}$ and $HSC^{Cygb\text{-}null}$ cells were harvested at day 1, 4, 7 for RNA, protein extractions or for immunofluorescence, Oil Red O staining.

(11) *siRNA Transient Transfection*

siRNA *Cygb* (si*Cygb*) or the siRNA negative control (siNC) (Ambion, Austin, TX) were transfected into $HSC^{Cygb\text{-}wild}$ using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) at a final concentration of 50 nM/L as previously described [22]. After 24 h, the culture medium was changed to fresh DMEM (Sigma-Aldrich) supplemented with 10% FBS (Invitrogen) and antibiotic. Then, after 72 h the cells were collected for total RNA extraction or after 96 h they were collected for protein extraction and for double immunofluorescence of αSMA and HO-1.

(12) Recombinant Human CYGB Treatment

Primary $HSC^{Cygb\text{-}null}$ were isolated from *Cygb*$^{-/-}$ mice and cultured on uncoated-plastic dishes. After 24 h, the culture medium was supplemented with 100 μg/mL of recombinant human CYGB[10]. And after 72 h, the cells were subjected for mRNA and protein analysis of αSMA and CYGB expression.

(13) Statistical Analysis

All data are expressed as the mean ± standard error of the mean. Two groups were compared using an unpaired Student *t* test (two-tailed). *P* values less than 0.05 were considered statistically significant.

４．研究成果

The present study clarifies the role of *Cygb* in steatohepatitis induced by a choline-deficient amino acid-defined diet (CDAA) in mice. CDAA treatment for 8 weeks induced prominent inflammation and fibrosis in *Cygb*$^{-/-}$ mice, which was inhibited by macrophage deletion. Surprisingly, at 32 weeks, despite no tumor formation in the WT mice, all *Cygb*$^{-/-}$ mice developed liver cancer, which was ameliorated by N-acetylcysteine treatment. Altered expression of 31 genes involved in the metabolism of reactive oxygen species was notable in *Cygb*$^{-/-}$ mice. Moreover, primary untreated-HSCs isolated from *Cygb*$^{-/-}$ mice showed a pre-activated condition characterised by augmented ROS and cytokine production. In human NASH livers, the expression of CYGB was declined in a negative correlation with increased NASH score. A similar decline in CYGB protein and mRNA expression was observed in human HCC regions. Therefore, a decline of CYGB expression likely contributes to the development of human NASH and liver cancer.

５．主な発表論文等

〔雑誌論文〕（計 3 件）

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〔学会発表〕（計5件）

① (Poster presentation) Thuy le TT. Protective Role of Cytoglobin in Liver Inflammation and Fibrogenesis in Mouse Steatohepatitis Model. APASL 2016, February 20-24, **グランドプリンスホテル高輪(東京都港区)**

② (Selected oral presentation) Thuy le TT. Loss of Cytoglobin Exacerbates Liver Fibrosis and Cancer Development in Steatohepatitis through the Activation of Oxidative Stress Pathway. AASLD, November 13-17, 2015, **サンフランシスコ(アメリカ)**

③ (Presidential choice oral presentation) Thuy le TT. Loss of Cygb accelerates liver fibrosis and cancer development despite of its etiology. ISHSR, November 11-13, 2015, **アシロマ(アメリカ)**

④ (Oral presentation) Thuy le TT. Absence of Cytoglobin, a Specific Marker of Hepatic Stellate Cells, Promotes Multiple Organ Abnormality in Aged Mice via Increased Nitric Oxide. Japan Society of Hepatic Sinusoidal Research, 2015, October 30-31, **秋田市にぎわい交流館 AU(秋田県秋田市)**

⑤ (Oral presentation) Thuy le TT. Cytoglobin Deficiency Promotes Liver Cancer Development from Hepatosteatosis through Activation of the Oxidative Stress Pathway. 51th JSH meeting, May 21-22, 2015, **ホテル日航熊本(熊本県熊本市)**

〔図書〕（計 2 件）

① Le Thi Thanh Thuy, Hoang Hai, and Norifumi Kawada. Studies on Hepatic Disorders. Oxidative Stress in Applied Basic Research and Clinical Practice. Chapter 18: Antioxidant Approach to the Therapy of Chronic Liver Diseases. Springer International Publishing Switzerland 2015, 518 (389-413).

② Le Thi Thanh Thuy, Tuong Thi Van Thuy, Hoang Hai, and Norifumi Kawada. Liver Pathophysiology: Therapies and Antioxidants. Chapter 17. Role of Oxidative and Nitrosative Stress in Hepatic Fibrosis. Elsevier publishing group, in press.

〔産業財産権〕

○出願状況（計 0 件）

○取得状況（計 0 件）

〔その他〕

ホームページ等

http://www.med.osaka-cu.ac.jp/liver/index.html

６．研究組織

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