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研究課題名（和文）Protective role of CYGB in prevention of liver fibrosis development in vitro and in vivo

研究課題名（英文）Protective role of CYGB in prevention of liver fibrosis development in vitro and in vivo

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研究成果の概要（和文）：Cygb欠損、Cygb過剰発現マウス、および組換えヒトCYGBタンパク質を我々の研究室で作製した。 抗線維症および癌におけるCygbの役割は、以下のような肝障害のさまざまなモデルで調べられた. 我々は、Cygbの非存在下では、Cygb-KOマウスにおいて肝臓の損傷が拡大することを見出した。 対照的に、Cygbの過剰発現は、異なる病因によって引き起こされる肝障害からマウスを保護した。本発明者らはrhCYGBタンパク質を産生し、rhCYGBが抗酸化活性およびペルオキシダーゼ活性を有することを見出した。 ＴＡＡ処置マウスにおけるｒｈＣＹＧＢ投与は、肝損傷、炎症および線維症発生を抑制した。

研究成果の学術的意義や社会的意義
Cygbの機能の継続的な解明、特に肝臓、最大の外分泌腺および内分泌腺の障害へのその寄与、ならびに線維症および腫瘍形成の予防におけるその役割は、極めて重要である。 HSCにおけるCygbの存在とその影響を理解することは、そのプロテアチンの特徴に関連した多くの展開する謎を持つ特別な細胞であり、依然として挑戦的です。さらに、HSCに対するCygb依存性の効果は、肝細胞の機能および表現型に間接的に影響を及ぼし得ると推測される。我々のCygb欠損マウスおよびCygb-過剰発現マウスは、線維症発生中のHSCにおけるCYGBの関与を研究し、その作用機序を探究するためのユニークで価値のあるモデルであろう。

研究成果の概要（英文）：Cygb deficient mice, Cygb overexpressing mice, and recombinant human CYGB protein were generated in our laboratory. The role of Cygb in anti-fibrosis and cancer were examined in different models of liver injuries including (1) chemical induced liver fibrosis using thioacetamide (TAA) treatment for 10 weeks; (2) diet induced non-alcoholic fatty liver diseases (NASH) using choline deficient amino acid define (CDAA) diet for 16 weeks. We found that in the absence of Cygb liver injuries were magnified in Cygb-KO mice. In contrast, Cygb overexpression protected mice from liver damage induced by different etiologies. Furthermore, we produced rhCYGB protein and found that rhCYGB possess antioxidant and peroxidase activities. In vivo model, rhCYGB administration in TAA-treated mice inhibited liver injuries, inflammation and fibrosis development. Thus, CYGB with ROS scavenger could play an important role for preventing from liver fibrosis.

研究分野：医歯薬学

キーワード：Cytoglobin　Hepatic stellate cells　Liver Fibrosis

１．研究開始当初の背景

Cygb was originally identified by our group in 2001 as an upregulated protein of rat hepatic stellate cells under pro-fibrotic conditions, named stellate cell activation-associated protein (STAP) (J Biol Chem 2001;276:25318, Accession number NM_130744), and was later found to be the fourth globin in mammals and given its current name. Cygb may facilitate diffusion of oxygen through tissues, scavenge nitric oxide (NO) or other reactive oxygen species, or serve a protective function during oxidative stress (J Mol Biol 2004, 339:873; J Biol Chem 2010;285:23850). However, there are many unclear points in which how Cygb involved in the pathologic of liver diseases. To study the biological function of Cygb at tissue level, we ourselves first generated Cygb-deficient (KO) mice and observed that, after treatment with DEN, KO mice showed high incidence of tumor development in the liver and lungs (Am J Pathol 2011;179:1050-60). On the other hand, we futher showed the crucial role of Cygb in the development of liver cancers emerged from hepatosteatosis in mice administrated choline-deficient amino acid-defined (CDAA) diet or control diet (choline-supplied amino acid-defined) for 8–32 weeks. CDAA treatment induced prominent inflammation and fibrosis in KO mice at 8 weeks, and exacerbation of steatohepatitis and fibrosis at 16 weeks. Surprisingly, at 32 weeks, while there was no tumour in WT mice, all of the KO mice developed liver cancer (Am J Pathol 2015;185:1045-1060). In parallel, during maintaining and propagating our KO mice, we uncovered the formation of age-dependent-multiple organ abnormalities in 82 (71.3%) out of 115 mice, including heart hypertrophy, tumors in the lung, liver, ovary, small intestine, and lymphatic organs, significantly different from 5.8% in WT (Scientific reports. 2016May5;6:24990). Interestingly, serum and urine analysis demonstrated that concentration of nitric oxide metabolites significantly increased in KO mice compared to WT counterparts. Thus, presence of Cygb in pericytes of all organs serves an important function in maintaining homeostasis of NO and antioxidant system.

２．研究の目的

We aim to assess the effect of Cygb-overexpressing in HSCs on the development of liver fibrosis; explore the mechanism action of Cygb including its impact on the oxidative stress and anti-oxidant system which in turn inhibits HSC activation and prevents fibrosis formation; produce and examine the therapeutic approach of human recombinant CYGB in preventing liver fibrosis.

３．研究の方法

(1) ***Mouse model for fibrosis induction***: We recently created a fluorescent protein reporter-transgenic mouse line, Cygb-mCherry (TG), in which both Cygb and mCherry specifically over-expressed in HSCs in the liver as well as in pericytes of all organs. These mice can be used as a tool to study HSC in the pathophysiology of liver diseases. C57BL/6 mice (WT) were purchased from SLC (Shizuoka, Japan).

Bile duct ligation model (BDL)*:* WT, KO, and TG mice at 9-10 weeks old are performed BDL or sham operation for acute phase (24, 48, and 72 hours), and chronic phase (1-3 weeks) with n = 5–10 per group.

TAA induced fibrosis model: Cygb-TG and WT mice, 10 mice per group, were given an i.p. injection of an escalating dose of TAA twice a week for 10 weeks. Mice were sacrificed 2 days

after the last TAA application. Healthy controls were given only an adequate saline solution by i.p. injection.

Metabolic liver injury model: Choline-deficient L-amino acid-defined diet (CDAA) and its control diet (choline-supplied L-amino acid-defined diet, CSAA) are administrated to WT, KO, and TG mice for 16 weeks.

(2) ***Necropsy***: All mice under different models of liver diseases were sacrificed at the end of treatment. At necropsy, mice were weighed, anaesthetized, and examined for grossly visible lesions in whole organs. All tissues were collected, weighed (in the case of liver), and examined for macroscopic lesions. For RNA, protein and biochemistry examinations, 20-30 mg of tissue was stored at -80°C until analysis.

(3) ***Histology, Immunohistochemistry and Immunofluorescence Analysis***: H&E staining, immunohistochemistry and immunofluorescence analysis were performed as described previously. Polyclonal antibodies against CYGB were generated in our laboratory. For quantification of liver fibrosis, 5-μm-thick sections were stained with PicroSirius Red and counterstained with Fast Green dye (SiR-FG). Each section was imaged separately at 100 times magnification by a BZ-X700 microscope and merged into whole- lobe pictures by using BZ-X Analyser software. Percentages of SiR-FG- positive areas per corresponding lobe area were calculated.

(4) ***Assay:*** Hydroxyproline**,** Alanine aminotransferase (ALT) activity was measured according to the assay protocol.

(5) ***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 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.

(6) ***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, AKT, phosphorylated AKT , BCL-2 , extracellular signal–regulated kinase, phosphorylated ERK, CYCLIN D1, phosphorylated SMAD3, total SMAD3, heme oxygenase-1, Myeloperoxidase, α Smooth muscle actin or GAPDH. Immunoreactive bands were visualized using the ECL detecting reagent and documented with the Fujifilm Image Reader LAS-3000 coupled with image analysis software.

(7) ***Generation of recombinant human CYGB protein and application****:* rhCYGB protein is produced according to Kawada et al., 2001 (J Biol Chem 2001;276:25318-23), and Gardner et al 2010 (J Biol Chem 2010;285:23850-7). Protein is isolated by chromatography, measured concentration. CYGB protein is further purify and heme assay together with its peroxidase activity are assessed.CYGB protein is also assessed in vivo using 10 week-TAA treated WT. Untreated controls and rhCYGB treated at 2 mg/kg BW for the last 2 or 5 weeks before terminal

of TAA treatment.

(8) ***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

(1) ***Liver injuries and fibrosis were amplified in KO mice but blocked in TG mice***. In BDL model, compared to WT, fibrosis was robustly developed in KO mice indicated by marked increase expression of α-SMA, collagen 1a1, and Sirius-red positive area. TG mice showed suppression of these factors up to 55% compared to WT mice (Figure 1). Oxidative stress were markedly increased in KO but inhibited in TG livers. Of note, silencing Cygb or ROS inducer in primary HSCs isolated from WT mice promoted their activation and collagen production. Interestingly, these effects were inhibited in HSCs isolated from TG mice. Microarray analysis from 1 week of BDL in all WT, KO, TG livers indicated CYGB regulated oxidative stress pathway including Nox-1, antioxidant protein 1, N-acetyltransferase 8, and myeloperoxidase is responsible for BDL-induced liver fibrosis. In TAA model, TG mice significantly ameliorated liver inflammation and fibrosis compared to WT ones (Figure 2).

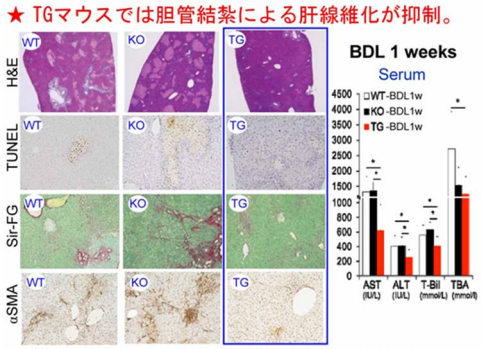

Figure 1. Overexpression of Cygb suppressed liver injuries and fibrosis in BDL mice.

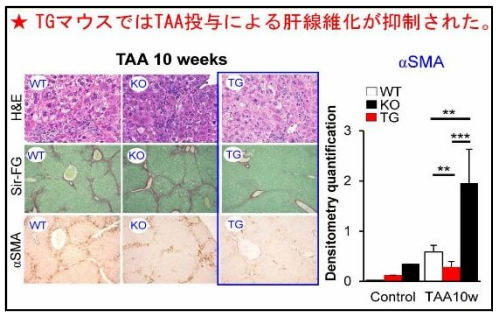

Figure 2. Overexpression of Cygb suppressed liver fibrosis in TAA mice.

CDAA treatment for 16 weeks induced strongest steatosis and liver fibrosis in KO mice, lesser in WT and at least in TG mice.

(2) ***Generation of human recombinant CYGB protein and its application in prevent fibrosis development in vitro.***

For *in vitro* experiments, we use hepatic stellate cells HHSteC cell line which show the most relevant to primary human HSCs, and also the well-known HSCs cell line LX-2. We also use primary human HSCs in collaboration with UCL University, London. His-CYGB treated HHsteCs showed time and dose dependently decreased αSMA and collagen expression at both protein and mRNA levels (Figure 3).

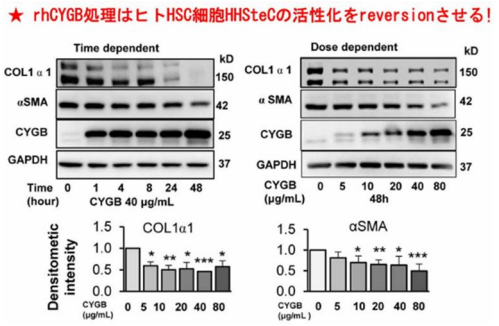

Figure 3. rhCYGB suppressed HSCs activation and fibrosis productions

These results indicated that rhCYGB may inhibit HSCs activation and suppress fibrosis development in vitro.

In conclusion: We have showed that in the absence of Cygb, KO mice suffered severe liver injuries and fibrosis development under different etiologies such as cholestasis, fibrosis or hepatosteatosis induced liver diseases. Importantly, KO mice showed increase oxidative stress condition but reduced antioxidant capacity. Interestingly, when we overexpressed Cygb in TG mice, these above phenotypes were markedly reversed. TG mice clearly exhibited the increase antioxidant function which suppressed liver injuries and fibrosis. Importantly, rhCYGB treatment reversed activated HSCs to close to quiescent phenotype and suppressed collagen production. Thus, CYGB would be the potential therapeutic target for liver diseases.

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