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Cytoglobin Deficiency Promotes Liver Cancer Development from Hepatosteatosis through Activation of the Oxidative Stress Pathway

Le Thi Thanh Thuy,* Yoshinari Matsumoto,*† Tuong Thi Van Thuy,* Hoang Hai,* Maito Suoh,‡ Yuka Urahara,* Hiroyuki Motoyama,* Hideki Fujii,* Akihiro Tamori,* Shoji Kubo,§ Shigekazu Takemura,§ Takashi Morita,¶ Katsutoshi Yoshizato,*‖ and Norifumi Kawada*

From the Departments of Hepatology, Medical Education and General Practice,‡ Hepato-Biliary-Pancreatic Surgery,§ and Molecular Genetics,¶ Graduate School of Medicine, and the Department of Medical Nutrition,† Graduate School of Human Life Science, Osaka City University, Osaka; and the PhoenixBio Co. Ltd.,‖ Hiroshima, Japan*

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Address correspondence to Norifumi Kawada, M.D., Ph.D., Department of Hepatology, Graduate School of Medicine, Osaka City University, 1-4-3 Asahimachi, Abeno, Osaka 545-8585, Japan. E-mail: kawadanori@med.osaka-cu.ac.jp.

This study was conducted to clarify the role of cytoglobin (Cygb), a globin expressed in hepatic stellate cells (HSCs), in the development of liver fibrosis and cancer in nonalcoholic steatohepatitis (NASH). Cygb expression was assessed in patients with NASH and hepatocellular carcinoma. Mouse NASH model was generated in *Cygb*-deficient ($Cygb^{-/-}$) or wild-type (WT) mice by giving a choline-deficient amino acid–defined diet and, in some of them, macrophage deletion and N-acetyl cysteine treatment were used. Primary-cultured mouse HSCs isolated from WT ($HSCs^{Cygb-wild}$) or $Cygb^{-/-}$ ($HSCs^{Cygb-null}$) mice were characterized. As results, the expression of CYGB was reduced in patients with NASH and hepatocellular carcinoma. Choline-deficient amino acid 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-acetyl cysteine treatment. Altered expression of 31 genes involved in the metabolism of reactive oxygen species was notable in $Cygb^{-/-}$ mice. Both $HSCs^{Cygb-null}$ and *Cygb* siRNA-transfected-$HSCs^{Cygb-wild}$ exhibited the preactivation condition. Our findings provide important insights into the role that *Cygb*, expressed in HSCs during liver fibrosis, plays in cancer development with NASH. *(Am J Pathol 2015, 185: 1045–1060; http://dx.doi.org/10.1016/j.ajpath.2014.12.017)*

Nonalcoholic steatohepatitis (NASH), an increasingly recognized obesity-related liver disease, is characterized by hepatocyte steatosis accompanied by a fibroinflammatory reaction.[1,2] Several studies have shown that NASH patients are at risk for progression to cirrhosis, the most common risk factor for hepatocellular carcinoma (HCC).[1,3] Compared to what is known about the pathogenesis of hepatitis virus–induced HCC, insight into NASH-associated HCC remains immature.

Currently, it is thought that the liver develops NASH via several pathological steps. Hepatocytes undergo degeneration characterized by the accumulation of fatty acids, which are excessively oxidized in the cellular organelles, including mitochondria. During this process, reactive oxygen species (ROS) are produced and trigger oxidative stress, leading to cell and tissue damage.[1] Hepatic macrophages consisting of resident Kupffer cells and infiltrating bone marrow–derived macrophages produce inflammatory mediators, such as tumor necrosis factor α (TNF-α), IL-6, IL-1β, and ROS.[4,5] These mediators further stimulate hepatocyte steatosis and initiate the activation of hepatic stellate cells (HSCs). Finally, the persistent secretion of ROS and mediators from these cells induces the development of advanced fibrosis.

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Cytoglobin (Cygb) was originally discovered in rat HSCs in 2001,[6] and is the fourth globin to be discovered in mammals.[7,8] CYGB is present in fibroblasts that store vitamin A in the visceral organs, including the liver and pancreas.[9] CYGB facilitates oxygen (O_2) diffusion through tissues, scavenges nitric oxide (NO) and other ROS, has a protective function during oxidative stress,[10] and suppresses tumorigenesis.[11–14] We previously showed that *Cygb*-deficient ($Cygb^{-/-}$) mice exhibit susceptibility to cancer development in the liver and lung with diethylnitrosamine administration.[15] Therefore, the absence of CYGB likely promotes a carcinogenic process in the presence of liver disease.

The present study clarifies the role of *Cygb* in steatohepatitis induced by a choline-deficient amino acid–defined diet (CDAA) in mice. The CDAA diet is a useful model to investigate NASH because it induces fibrosis, systemic insulin resistance, and steatohepatitis, which are compatible to the pathophysiology of human NASH. The administration of CDAA to C57BL/6 wild-type (WT) mice was reported to induce defined liver fibrosis not earlier than 22 weeks, and HCC nodules at a late time point, 84 weeks.[16] Herein, we showed $Cygb^{-/-}$ mice fed a CDAA diet, leading to a severe NASH condition and a 100% incidence of HCC at an early time point, 32 weeks. Moreover, primary untreated HSCs isolated from $Cygb^{-/-}$ mice showed a preactivated condition characterized by augmented ROS and cytokine production.

Materials and Methods

Human Tissues and Specimens

Human NASH specimens ($n = 15$), used for immunohistochemistry (IHC) of CYGB, were obtained from patients in Osaka City University Hospital (Osaka, Japan), who were diagnosed with NASH according to the classification of Matteoni et al.[17] Intact human specimens ($n = 3$) of nontumor lesions were obtained from patients who had metastasis liver tumors or cholangiocarcinoma treated by surgical resection. HCC tissues and noncancerous liver tissues were obtained from nine patients without hepatitis virus B or C infection, who had undergone a hepatectomy at the Osaka City University Hospital. They were patients with almost intact liver ($n = 2$), fatty liver ($n = 1$), liver fibrosis by undetermined etiology ($n = 1$), NASH ($n = 1$), and alcoholism ($n = 4$). The specimens were routinely processed, formalin fixed, and paraffin embedded. A portion of tissues was frozen and stored at −80°C without fixation. RNAs 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.

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 (catalog 518753; Dyets, Bethlehem, PA) or a control diet, choline-supplied amino acid–defined diet (CSAA; catalog 518754; Dyets) with $n = 5$ to 14 per group. The CSAA control diet induces simple steatosis, but neither inflammation nor fibrosis, in WT mice[16] (Supplemental Figure S1). Mice were fed these diets continuously for 8, 16, or 32 weeks. To investigate tissue hypoxia, 1 hour before sacrifice, some mice were injected i.p. with hydroxyprobe-1 solution at a dose of 60 mg/kg body weight using the hydroxyprobe-1 Omni Kit (Hydroxyprobe, Burlington, MA), according to the manufacturer's protocol.

In the macrophage-depletion experiment, a subgroup 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, which was used to examine the early events of NASH. At the seventh week of CDAA feeding, Kupffer cell depletion was induced by injecting 200 μL liposomal clodronate (FormuMax Scientific, Palo Alto, CA) 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-acetyl cysteine (NAC) treatment, a total of 53 $Cygb^{-/-}$ and WT mice, divided into six groups ($n = 5$ to 13 per group), were fed the CDAA diet, together with 0.1 mmol/L NAC (Sigma-Aldrich, St. Louis, MO) in the drinking water for 2, 8, or 32 weeks, starting at 8 weeks of age. NAC was prepared as a 0.5 mol/L 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 NIH *Guide for the Care and Use of Laboratory Animals*.[19]

Histological, IHC, and Immunofluorescence Analysis

Hematoxylin and eosin, IHC, and immunofluorescence analyses were performed as previously described.[15] The primary antibodies used for mouse and human samples, including CYGB antibodies, were generated by our laboratory[6,15,20] and are described in Table 1. Pathological severity of nonalcoholic fatty liver disease was assessed using previously described criteria.[21] To quantify liver fibrosis, sections (5 μm thick) were stained with Picrosirius red (Sigma-Aldrich) and counterstained with Fast Green (Sigma-Aldrich). Collagen stained with Sirius Red was quantitated in the sections that were randomly chosen (<100 magnifications, 10 to 20 fields each from sample)