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Hepatocyte-specific clusterin overexpression attenuates diet-induced nonalcoholic steatohepatitis

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ABSTRACTS

Clusterin is a multifunctional glycoprotein that plays important roles and is up-regulated in liver diseases such as hepatitis and hepatocellular carcinoma. However, little is known about the significance of clusterin in the pathogenesis of non-alcoholic steatohepatitis (NASH). The aim of this study is to examine the role of clusterin in progression of steatohepatitis in mice fed a methionine and choline deficient (MCD) diet. We generated hepatocyte-specific clusterin overexpression (hCLU-tg) mice, and hCLU-tg mice showed lower levels of hepatic triglycerides, less infiltration of macrophages and reduction of TNF-α, activation of Nrf-2 than wild-type littermates fed the MCD diet. Also, sustained clusterin expression in liver ameliorated hepatic fibrogenesis by reducing the activation of hepatic stellate cells by MCD diet. Sustained expression of clusterin in liver functioned as a preconditioning stimulus and prevented MCD diet-induced severe steatohepatitis injury via Nrf2 activation. These results demonstrate a novel function of clusterin as an immune preconditioning regulator in various inflammatory diseases including steatohepatitis.

1. Introduction

Clusterin is a disulfide-linked heterodimeric glycoprotein of 75–80 kDa originally isolated from ram rete testis fluid [1,2]. Since then, it has been reported to be ubiquitously expressed in a variety of tissues and found in almost all body fluids at low levels under normal physiological conditions [2,3]. In addition, clusterin has been shown to be significantly up-regulated in diverse tissues and/or fluids undergoing patho-physiological disturbances such as ischemia, inflammation [4,5]. In particular, clusterin is highly induced during the acute phase of pancreatitis, and protects cells from inflammation of the exocrine pancreas by reducing the expression of pro-inflammatory cytokines such as TNF-α and also suppressing NF-κB activation [6]. However, it was later demonstrated that clusterin conferred resistance to TNF-α mediated cell death in breast cancer cells by activating the NF-κB pathway [7]. In addition, other studies demonstrated that clusterin prevented cell death induced by oxidative stress [8]. In sum, while these findings draw attention to a potentially protective association of clusterin in modulating oxidative and inflammatory diseases, the effect of clusterin on regulation of the NF-κB pathway is unclear and needs to be further refined.

Non-alcoholic fatty liver disease (NAFLD), is a type of chronic liver disease associated with metabolic diseases such as obesity, diabetes. NAFLD can be classified into simple steatosis and NASH. The pathological feature of simple steatosis is that the total fat content accounts for over 5% of the liver due to excessive fat deposition. However, the pathological feature of NASH is oxidative stress and inflammation. During the progression to NASH, oxidative stress resulting from elevated intracellular reactive oxygen species (ROS) is a major contributing factor that can further aggravate inflammation characterized by the activation of macrophages [9]. In addition, it has been shown that the toll-like receptor 4 (TLR4) signaling pathway, is critical in the pathogenesis of NASH, and deficiency of TLR4 signaling reduces hepatic damage and inflammation in MCD diet-induced NASH [10,11].

In a previous study, we demonstrated that TLR4 signaling is required for clusterin-induced TNF-α in macrophages [12]. We have also reported that clusterin functions as a cytokine with chemotactic activity, and induces migration of macrophages and NF-κB activity [13,14]. In contrast to the anti-oxidative and anti-inflammatory effect of clusterin studied *in vivo* [4–6,15], our

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in vitro studies suggested that clusterin induced both oxidative stress and pro-inflammatory cytokines in macrophages through the activation of TLR4 signaling pathway [12–14,16]. In this study, we generated transgenic mice to constitutively express clusterin in liver (hCLU-tg), and examined its controversial role in an experimentally-induced oxidative and inflammatory liver disease in mice.

The present study shows that clusterin is up-regulated during pathogenesis resulting in NASH induced by the MCD diet, and that sustained expression of clusterin in liver prevents MCD diet-induced severe steatohepatitis injury through the reduction of pro-inflammatory cytokines via TRL4.

2. Materials and methods

2.1. Animals and dietary experiments

All animal experiments were performed under the guidelines of the Institutional Animal Care and Use Committee of Korea University (KUIACUC-2015-257). 6 week-old male mice were fed a MCD diet (#518810 Dyets, Inc) or control diet for 3 weeks.

2.2. RT-PCR and western blot analysis

RT-PCR and Western blot were performed as described previously [14,16].

2.3. Blood analysis

Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and triglyceride were measured using their assay kits (#K752-100-1, K753-100-1, K622-100-1 Biovision, USA) according to manufacturer's instruction.

2.4. Immunohistochemistry

Liver tissue was fixed in 10% NBF solution for 48 h at room temperature and embedded in paraffin. For immuno-staining, the tissue sections were incubated with primary antibody for overnight at 4 °C. Subsequently, tissue sections were incubated with secondary antibody for 2 h at room temperature and then incubated with ABC solution for 1 hr at room temperature.

2.5. Statistical analysis

All data are presented as mean ± standard error mean (SEM) and analyzed by one-way analysis of variance (ANOVA). $P < 0.05$ was considered significant.

3. Results

3.1. Up-regulation of clusterin in livers of mice on MCD diet

We found that clusterin mRNA and protein level increased 2-fold in the liver of mice with steatohepatitis induced by MCD diet (Fig. 1A and B). Based on these results, we performed immunohistochemistry to examine which cells express clusterin. As shown in Fig. 1C, weak immunoreactivity against clusterin was detected only in hepatocytes in the liver of mice fed with control diet. However, clusterin immunoreactivity was greatly increased in hepatocytes of mice fed with MCD diet. In addition, double immunolabelling revealed that clusterin was expressed not only in hepatocytes, but also in hepatic macrophages (co-localized in cells stained with F4/80 antibody) and activated stellate cells (co-localized with α-smooth muscle actin-positive cells) as shown in Fig. 1D and E, respectively. Interestingly, we also found that the higher level of clusterin expression at an early time point (i.e., first week of MCD feeding) was related to a less worsening of liver histology and lower levels of serum ALT (Fig. S1A–B).

Sustained overexpression of clusterin diminished hepatic steatosis and liver damage in an MCD diet-induced NASH model.

In this experiment, we found that the degree of hepatic steatosis was significantly lower in hCLU-tg mice than wild-type mice fed with MCD diet (Fig. 2A). Also, we found that serum ALT and AST levels of hCLU-tg mice was less than that of wild-type mice fed with MCD diet (Fig. 2B). In addition, we measured triglyceride in liver tissues to quantitatively determine the degree of lipid accumulation. Similar to the histologic results shown in Fig. 2A, the amount of triglyceride in mice fed MCD diet showed a significant increase compared to mice fed with control diet, whereas its level in hCLU-tg mice was significantly lower than that of wild-type mice (Fig. 2C). The body weight of mice fed the MCD diet was significantly reduced compared to mice fed with control diet, which was similar to the results of previous experiments [17]. However, there was no difference in body weight between hCLU-tg and wild-type mice fed with MCD diet or control diet (Fig. 2D).

3.2. Clusterin ameliorates oxidative stress and hepatic inflammation induced by MCD diet

To examine the effect of clusterin on oxidative stress induced by MCD diet, we immunostained for 4-hydroxy-2-nonenal (4-HNE). We found that the immuno-reactivity against 4-HNE was significantly lower in hCLU-tg mice than wild-type mice fed with MCD diet (Fig. 3A). To examine whether NF-κB activation is involved in the protective role of clusterin against oxidative stress in hCLU-tg mice, liver immunostaining for NF-κB was performed. As shown in Fig. 3B, the immune-reactivity for NF-κB showed a significant increase in the liver of hCLU-tg mice fed with control diet compared to wild-type mice. However, MCD diet provided to hCLU-tg mice slightly reduced the NF-κB immuno-reactivity as compared to the mice fed with control diet, while the NF-κB expression in wild-type mice was highly up-regulated by MCD diet, which was in line with prior findings [18,19]. Next, we measured nuclear factor-erythroid 2-related factor 2 (Nrf2), which is activated during oxidative stress [20], thereby inhibiting the activity of NF-κB and inflammation [17,21]. Our results revealed that MCD diet feeding increases the levels of Nrf2 protein and heme oxygenase-1 (HO-1) mRNA which is one of Nrf2's main targets in the liver of wild-type mice. The sustained overexpression of clusterin further enhanced the expression of Nrf2 and HO-1 in the liver of hCLU-tg mice fed with MCD diet (Fig. 3C and D).

To further examine the role of clusterin in MCD diet-induced inflammation, F4/80 immunostaining was performed to evaluate macrophage recruitment and activation. We found that the immuno-reactivity against F4/80 was significantly lower in hCLU-tg mice than wild-type mice fed with MCD diet (Fig. 3E). Also, we observed that the expression of TLR4 and its downstream molecules in the liver of hCLU-tg mice than wild-type mice fed with MCD diet (Fig. 3F). Interestingly, we also noticed that the degree of clusterin expression was appreciably similar to the pattern of F4/80 immunostaining, in such a way that clusterin expression in the liver of hCLU-tg mice fed with MCD diet was less than that of wild-type by both immunohistochemistry (Fig. 3G) and western blot (Fig. 3H), although clusterin was upregulated in the liver of hCLU-tg fed with control diet compared to wild-type mice.

Hepatic clusterin expression attenuates the activation of hepatic stellate cells and liver fibrosis.

We found that the number of α-SMA positive stellate cells in hCLU-tg mice was significantly lower than wild-type mice fed with

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