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Research Article

Increased Expression of Hepatic Endothelin1- during Fibrosis Progression in Non-alcoholic Fatty Liver Disease

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Abstract

Non-alcoholic fatty liver disease (NAFLD) ranges from simple steatosis, steatohepatitis (NASH) to fibrosis and cirrhosis. The mechanism by which NASH progresses to fibrosis has not been fully realized. Endothlein-1 (ET-1) is a strong profibrogenic factor which may play a role in the fibrosis progression of NAFLD, particularly after the development of NASH. This study explored the time-course relationship between hepatic ET-1 and steatosis and fibrosis progression in NAFLD. SD rats received methionine and choline deficient (MCD) diet for 9 weeks (W9) and 14 weeks (W14) were used. The control rats were fed by re-supplemented MCD control diet. The blood and liver were harvested for biochemistry, histology and gene expressions studies. The degree of steatosis and fibrosis were analyzed by computer software. In MCD rats, ALT, AST, and liver weights increased significantly. Hepatocyte steatosis was observed at W9 and it continued to accumulate at W14. The degree of hepatic fibrosis was minimal initially but increased rapidly after W9 and became significantly greater than control at W14. Both ppET-1 and ECE-1 mRNA expressions were significantly elevated at W14. The expression of ET-1 receptor subtype ET-A was not different between two groups while ET-B expression was significantly higher in MCD rats. During the progression of NAFLD, hepatic ET-1 gene expression increased when the fibrosis became significant. Inhibition of ET-1 could be a potential therapeutic strategy for the management of NAFLD and deserves further investigation.

Keywords: Endothelin-1; Fibrosis; Steatohepatitis

Abbreviation ET1: Endothelin-1; ECE1: Endothelin Converting Enzyme Type I; MCD: Methionine and Choline Deficient; NASH: Nonalcoholic Steatohepatitis; NAFLD: Nonalcoholic Fatty Liver Disease

Introduction

Non-alcoholic fatty liver diseases (NAFLD) are the most common liver disease in Western regions and with rising prevalence in Asia-Pacific region in recent years [1,2]. NAFLD is a wide spectrum disease ranging from simple steatosis (fatty liver), steatohepatitis (NASH) to liver fibrosis and cirrhosis [3,4]. Significant liver damage could be noticed in the stage of NASH. The important point is that NASH is not always having a benign outcome.

It has been estimated that about one-third of the NAFLD patients would developed NASH [5]. For patients in early

stage of NASH, about 9% to 20% will progress to advanced fibrosis and then cirrhosis in 5-10 years [6,7]. In addition, NASH is associated with an increased risk of developing hepatocellular carcinoma and higher mortality rate than patients with simple steatosis [2,8]. The pathogenesis of NASH has been explained by "two-hit" theory in which hepatic steatosis is the first hit and the subsequent liver injuries are the second hit [9]. Recently, a "multiple parallel hit" hypothesis has been proposed [10]. Multiple hit theory described that insulin resistance and free fatty acid accumulation as initial insults leading to metabolic disturbance and hepatocyte vulnerability [10].

Currently, it is demonstrated that chronic liver injury progress into fibrosis needs the activation of hepatic stellate cell (HSC) [11-13]. HSC is the key cellular element involved in hepatic fibrogenesis [14-16]. Endothelin-1 (ET-1) is a potent mitogen stimulating HSC activation, proliferation and contraction to promote hepatic fibrogenesis [8]. ET-1 is also a mediator that is elevated in conditions such as insulin resistance, hyperglycemia, oxidative stress, and endothelial cell dysfunction [17-19]. Recently, it has been found that the serum levels of ET-1 were significantly higher in NASH patients and those with more fibrosis [20]. The purposes of this study aimed at exploring the time-course relationship of ET-1 expression during the fibrosis progression of NAFLD. Until now, the exact mechanisms or the factors that make NAFLD in progression have not been completely revealed [3,21].

Materials and Methods

Animal models of NASH

Sprague-Dawley rats Rat were housed in a standard facility according to the animal care rules. Rodents were fed with methionine and choline deficient (MCD) diet to the 9th to 14th weeks (W9 and W14, n=5~6 respectively, MCD group) to induce NASH [11]. In this way, the rodent liver showed characterized by significant steatosis, higher hepatic triglyceride concentration, lobular inflammation and fibrosis. Rats fed with MCD re-supplemented control diet for the same period of time (W9 and W14) were used as control groups (n=6 respectively, Control group). All animal were kept in a temperature- and humidity-controlled environment in a 12:12-h light /dark cycle. The animals were handled according the guideline of NIH and approved by the IACC of Taipei Veterans General Hospital (IACC 2011-120).

Determination of serum and liver biochemistry

The serum level of aspartate aminotransferase (AST) and alanine aminotransferase were measured by standard laboratory method in an automatic chemistry analyzer in Taipei Veterans General Hospital.

Portal pressure measurement:

The portal pressures were measured in W9 MCD and control rats (n=3 in each group) by catheterization method [22]. Briefly, immediately after induction of anesthesia, laparotomy is performed and intestines displaced laterally and the portal vein was exposed. A 23 gauge IV catheter is introduced into portal vein, secured with glue and connected to perfusion system and digital pressure recorder. After stabilization, changing flow rate at regular interval was given and the portal pressure was recorded for 1 min continuously.

RNA isolation and mRNA quantification:

The total RNA was extracted from stellate cells or whole liver using TRIZOL reagent (Invitrogen, Carlsbad, CA). The concentration of RNA was determined spectrophotometrically and 1ug of RNA were used in synthesizing cDNA. Real-time PCR was performed according to the manufacture's recommendation by using Bio-RAD thermocycler. Amplification reaction was performed using a SYBR Green PCR Master Mix (Applied Bios stems). All samples were analyzed in triplicate. GAPDH were used as internal control. The primers used in this study are listed in Table 1.

Table 1. Primers used in Real-Time PCR

ppET-1	F ACTTCTGCCACCTGGACATC	200kb
	R GCTCGGAGTTCTTTGTCTGC	
ETA	F TTCCCTCTTCACTTA AGCCGA	201kb
	R GCAACAGAGGCATGACTGAAA	
ETB	F GCCACCCACTAAGACCTCCT	204kb
	R ATGCCTAGCACGAACACGAG	
ECE-1	F GGACTTCTTCAGCTACGCCTG	201kb
	R CTAGTTTCGTTCATACACGCACG	
GAPDH	F TGCACCACCAACTGCTTAGC	87kb
	R GGCATGGACTGTGGTCATGAG	

ppET-1: Prepro-ET-1; F: forward; R: reverse.

Histological and immunohistochemical study

The liver specimens were preserved in 4% buffered Para formaldehyde and dehydrated in a graded alcohol series. Following xyline treatment, the liver specimens were embedded in paraffin blocks and cut into 5-um thick sections and were placed on plain glass microscopic slides. The liver sections were stained with haematoxylin and eosin (HE) stain or Masson Trichom staining for fibrosis degree evaluation under light microscope. The degrees of steatosis and fibrosis were evaluated using computerized image analyzing software (Microcam, M&T OPTICS, Taiwan) [23].

Statistical Analysis

The results are expressed as mean±SEM. Statistical analysis was performed by using an independent Student t test or one- and two-way ANOVA with Tukey post hoc test when appropriate. A p value <0.05 was considered statistically significant.

Results

Body weight, liver ratio, liver biochemistry and portal hypertension

The body weights of rats in the control group increased gradually to levels significant higher than the MCD-fed rats (p>0.05, Figure 1). The liver ratio, calculated by dividing the liver weight to body weight, increased obviously at W9 (p<0.05) and maintained at similarly high levels at W14. For the control group, the ratio at W9 kept at the same level as at the beginning. It increased slightly at W14 but the difference was not significant (p>0.05). The levels of ALT showed significant increase at W9 and W14 as compared to the control groups (p<0.05). For AST levels, it increased significantly at W9 and W14 (p<0.05) and were higher than ALT. We measured the portal pressure in W9 MCD and control groups (Figure 2). The results showed that the portal pressures were significantly higher in MCD rats (12.6±1.7mmHg) when compared with those of the control rats (8.3±1.2mmHg, p<0.05). This indicated the presence of portal hypertension.

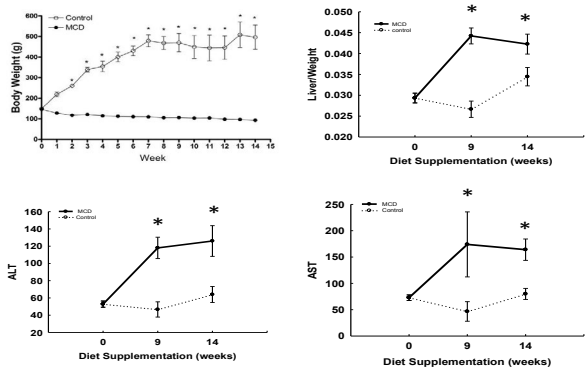

Figure 1. The changes of body weight, liver ratio and liver biochemistry in control and MCD-fed rats. The body weight of MCD control group increased significantly while the body weight of the MCD groups decreased slightly then stabilized around 100g (left upper). Liver ratio (liver weight/body weight) increased significantly from 9 weeks and remain high at 14 weeks in MCD-fed rats (right upper). Serum ALT increased significantly in MCD group when compared with the control group. Serum AST significantly increased at 9 weeks and 14 weeks after MCD diet. (* p<0.05 vs. control)

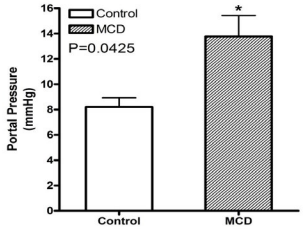

Figure 2. The inter-observer union (yellow colorwash) is defined as the volume encompassing the contours of all observers in an imaging modality. The inter-observer intersection (orange colorwash) is defined as the volume common to all observers in one imaging modality. The volume

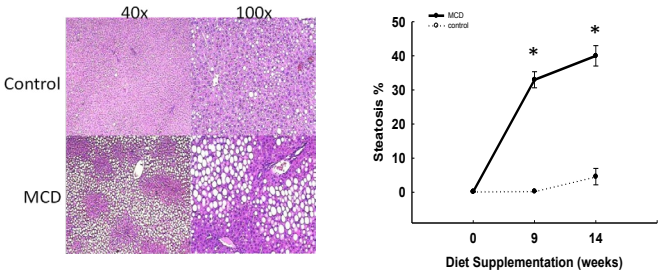

Figure 3. The steatosis of liver in control and MCD-fed rats. (Left) Representative pictures of H.E. stained liver sections after control diet and MCD diet. The hepatocytes are characterized by significant macrovesicular steatosis, higher hepatic triglyceride concentration and lobular inflammation. (Right) The degree of steatosis was calculated by computer software. Significant increased steatosis was noted at 9 weeks after MCD diet

Liver steatosis and liver fibrosis

The H.E. stain showed increased fatty deposition in hepatocyte after MCD diet. In the control group, only small amount of steatosis can be detected under higher power field (100x). In contrast, obvious steatosis could be demonstrated in the MCD group. Under high power field, it appeared mostly as macrovesicular type (Figure 4). The degrees of steatosis were significantly higher in the liver of MCD-fed rats at W9 and W14 when compared with the control group (p<0.05). For fibrosis evaluation, the Masson Trichome stain was used. Increased collagen fiber deposition was noted from W9 to W14. Compared with the control group, the difference become significant at W14 (p<0.05, Figure 4).

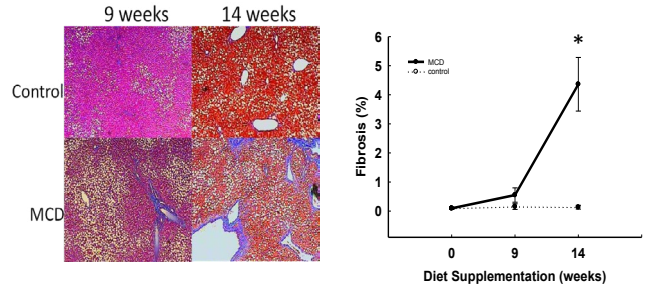

Figure 4. The fibrosis of liver in control and MCD-fed rats. (Left) Representative pictures of Masson Trichrome stained liver sections after control diet and MCD diet. In addition to the steatosis and inflammatory change, large amount of extracellular matrix depositions were noted at 9 weeks and 14 weeks (blue color). (Right) The degrees of fibrosis in both groups that calculated by computer software according to time. Significant increased fibrosis was noted at 14 weeks after MCD diet.

The expression of Endothelin-1 synthesis related gene

The ppET-1 mRNA expression in MCD group increased moderately at W9 and was significantly higher than the control group at W14 (* p<0.05 vs. control, Figure 5). The ECE-1 mRNA expressions were at similar levels at W9 but was significantly increased at W14 in the MCD group (* p<0.05 vs. control). The expressions of ETA receptor were not different between two groups while increased expression of ETB receptor was found in MCD rats in either W9 or W14 (* p<0.05 vs. control, Figure 5).

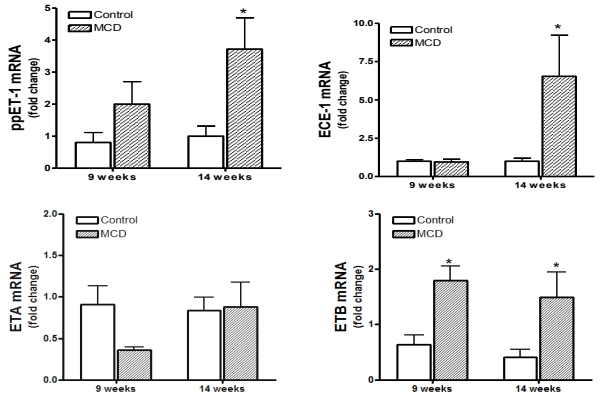

Figure 5. The ppET-1, ECE-1 and ET receptor mRNA expression in control and MCD-fed rats at 9 weeks and 14 weeks. The ppET-1 mRNA expressions were increased gradually and significantly higher at 14 weeks in MCD group while ECE-1 mRNA expression was similar at 9 weeks and significantly increased at 14 weeks in MCD group (* $p<0.05$ vs. control). The expressions of ETA receptor were not different between two groups while increased expression of ETB receptor was found during fibrosis progression in MCD rats.

Discussion

In the present study, there were increased liver ratio and persistent hepatic necro-inflammation in MCD rats after W9. Hepatic steatosis developed quickly in about 30% to 40% of hepatocytes and was distributed majorly in macrovesicular pattern. The degree of fibrosis was minimal before W9; however it increased rapidly and became prominent thereafter. Meanwhile, increased expression of ET-1 and ECE-1 were observed and significantly higher than control at W14. These histological changes were similar to the disease course happened in NASH. The sequential changes of steatosis and fibrosis allowed us to investigate two important conditions; there were "steatohepatitis without fibrosis" at early stage (W9) and "steatohepatitis with fibrosis" at late stage (W14).

The mechanisms of MCD to induce steatosis and NASH were through increased fatty acid uptake and decreased VLDL secretion [24]. Steatosis has been recognized as a risk factor for the progression of fibrosis in several different type of liver diseases such as alcoholic liver disease, chronic hepatitis C and hemochromatosis [25-28]. In vitro study demonstrated that lipid accumulation in hepatocyte induced the release of profibrogenic factors of HSC and accelerated their activation and proliferation [29]. In addition, hepatocyte steatosis could enhance HSC's resistance to apoptosis and expression of profibrogenic genes including TGF-β, tissue inhibitor of metallo-proteinase-1 (TIMP-1) and others. All these effects either directly or indirectly caused by steatosis would further promote the progression of liver fibrosis. In MCD-induced NASH, we found that ET-1 expression increased moderately in early stage (W9). Though the degree of fibrosis was minimal in early stage, portal pressure had already been elevated. In late stage of NASH (W14), high ET-1 expression and severe fibrosis were observed at the same time. ET-1 can be released from several different types of cells in the fibrotic liver [30-32]. It exerts a contractile effect on HSC and may play an important regulatory role of portal pressure. ET-1 is not only a vasoconstrictor but a strong mitogen and activator to HSC [33,34]. These characters render ET-1 one of the major contributors to portal hypertension and liver fibrosis [28,34]. In a previous cohort study of patients with NAFLD, HSC activation score was significantly increased in patients with fibrosis progression versus patients in whom no fibrosis progression was observed [35]. In addition, it had been reported that NASH patients with grade IV fibrosis have approximately 1.5 fold higher ET-1 levels than patients with grade I fibrosis [20]. These results suggest that ET-1 may participate in the pathogenesis and progression of fibrosis in NASH.

In this study, the expressions of ETA receptor were not different while increased expression of ETB receptor was found in MCD rats. ET-1 can bind to either ETA or ETB receptors. Activated HSC display a high number of Endothelin receptors [14]. ETA receptor mediated the contractile response and ETB receptor mediated the antiproliferative effect of ET-1 [36]. In normal liver, ETB was predominantly expressed on sinusoidal endothelial cells and HSCs, while ETA was scantily expressed [37]. In contrast in the cirrhotic liver tissue, ETB was over-expressed, particularly on HSCs, while ETA expression was increased but remained low [37]. Since ETB mediated growth inhibition, the up-regulation of ETB receptor had been regarded as a positive feedback phenomenon that would amplify the anti-proliferative effect of ET-1 on activated HSC [36].

According to multiple hit theory, insulin resistance is one of the key events underlying the pathogenesis of NASH. ET-1 may regulate insulin and glucose metabolism. It has been reported that intraperitoneal injection of ET-1 induced insulin resistance in conscious rats [38]. Chronic ET-1 administration leads to decreased insulin-stimulated glucose transport and whole-body insulin resistance as well as impaired insulin signaling [39,40]. In patients with metabolic syndrome, plasma triglycerides and glycosylated hemoglobin are independently correlated with ET-1 concentration [41]. On the other side, insulin may modulate ET-1 synthesis. Cell culture studies have shown that increased ET-1 gene expression and release after exposure to insulin [42,43]. Administration of insulin increased plasma ET-1 levels in healthy subjects and patients with non-insulin-dependent diabetes mellitus or obesity [44-46]. In NASH patients, the plasma ET-1 level was 2-3 folds higher than that in patients having steatosis [20]. These studies indicate there is a close interaction between ET-1 and insulin, while imbalance of this regulation may results in metabolic dysfunction. In the present study, we did not investigate the contributory role of hepatic insulin resistance in fibrosis progression of NASH. However, there was an animal study demonstrated that knock out of ET-1 in vascular endothelial cells preventing high-salt induced insulin resistance [47]. Another study reported that ET-1 antagonists may provide an effective means of improving glucose tolerance in obese humans [18]. More prospective studies are needed to assess whether ET-1

antagonism could improve insulin resistance and attenuate the progression of fibrosis in NASH.

Conclusion

In conclusion, we had observed an increased expression of ET-1 paralleled with the progression of fibrosis in the MCD model of NAFLD. Inhibition of ET-1 could be a potential therapeutic strategy for the management of NAFLD, which deserves further investigation in the future.

Acknowledgements

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