Crosstalk between high-molecular-weight adiponectin and T-cadherin during liver fibrosis development in rats

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Received July 20, 2007; Accepted August 28, 2007

Abstract. Adiponectin, a circulating adipocyte-derived secretory protein, reportedly plays an important role in liver fibrosis development, although the biological role of adiponectin in liver fibrogenesis is still controversial. Adiponectin is present in the serum as three oligometric complexes; namely, high-, middle-, and low-molecular weight (HMW, MMW, and LMW, respectively). Adiponectin exerts different biological activities in an oligomerization-dependent manner. The aim of our current study was to examine the alteration of each isoform of adiponectin and its receptors (AdipoR1, AdipoR2, and T-cadherin) during the choline-deficient L-amino acid-defined (CDAA) diet-induced rat liver fibrosis development. We also elucidated the methylation status of all receptors. The serum level of total adiponectin significantly increased during the liver fibrosis development. Among the three isoforms, only HMW adiponectin was significantly up-regulated whereas MMW and LMW were not. The expression of T-cadherin, which exclusively binds with HMW adiponectin, was significantly augmented as well. The AdipoR2 expression was markedly decreased and showed no marked difference from that of AdipoR1. No obvious methylation change was observed in all three receptors, suggesting that another mechanism is involved in the alteration of receptor gene expression. Collectively, since the specific ligand and receptor were augmented together, crosstalk between HMW adiponectin and T-cadherin may play an important role during liver fibrosis development in rats.

Introduction

Adiponectin (also known as ACRP30, GBP28, and AdipoQ) is a hormone secreted exclusively by adipocytes and reportedly plays important roles in the regulation of glucose and lipid metabolism. Adiponectin concentrations are reduced in obese and insulin-resistant human subjects and animal models, making it a promising approach for the treatment of obesity-mediated diseases (1-3). In the liver, initial studies of adiponectin mainly focused on non-alcoholic steatohepatitis (NASH), which is frequently associated with diabetes mellitus, insulin resistance, and obesity (4,5). In NASH patients, circulating adiponectin reportedly decreases (6,7). Adiponectin administration alleviates non-alcoholic fatty liver disease in mice, and liver fibrosis is accelerated in adiponectin knockout (KO) mice, indicating the protective effect of adiponectin against liver fibrosis development (8). On the other hand, contradictory findings have been noticed in patients with liver cirrhosis of diverse etiology. Circulating adiponectin reportedly increases in proportion to the severity of human liver cirrhosis (7,9-12).

Adiponectin exists in many multimer complexes in the plasma, and combines via its collagen domain to create 3 major oligometric forms; namely, LMW trimer, MMW hexamer, and HMW 12-to 18-mer adiponectin (13,14). It has been suggested that adiponectin exerts several different biological activities in an oligomerization-dependent manner (15). Two receptors for adiponectin were initially defined and designated as AdipoR1/R2. AdipoR1 is ubiquitously expressed, whereas AdipoR2 is predominantly expressed in the liver. AdipoR1 and AdipoR2 synergistically mediate anti-diabetic, insulin-sensitive signals. In addition, T-cadherin has been recently identified as an adiponectin receptor (16,17). T-cadherin characteristically binds only to HMW adiponectin although its physiological function is still not fully understood (18). The expression of each isoform of adiponectin and receptor during liver fibrosis has not yet been examined.

In the current study, we examined the total and individual oligomerized adiponectin during liver fibrosis development in rats. We also examined the expression and methylation status of all types of receptors.

Materials and methods

Animals and animal treatment. Male Fisher 344 rats, aged 6 weeks, were purchased from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). They were housed in stainless-steel, mesh cages under controlled conditions of temperature (23±3˚C)

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Key words: adiponectin, T-cadherin, liver fibrosis, hepatic stellate cells

and relative humidity (50±20%), with 10-15 air changes per h and light illumination for 12 h each day. The animals were allowed access to food and tap water *ad libitum* throughout the acclimatization and experimental periods. The choline-deficient L-amino acid-defined (CDAA) diet and its control, a choline-supplemented, L-amino acid-defined (CSAA) diet, with the previously described composition, were obtained from Dyets Inc. (Bethlehem, PA, USA). The CDAA diet model exhibits pathological sequences similar to those of human liver disease; namely, hepatocellular necrosis, fibrotic change, cirrhosis, and finally hepatocellular carcinoma (19,20). The experimental period in all experiments was 16 weeks. Each group consisted of 10 rats. All animal procedures were performed according to approved protocol and in accordance with the recommendations for the proper care and use of laboratory animals.

Adiponectin measurement. The total serum adiponectin levels were determined using the adiponectin enzyme-linked immunosorbent assay (ELISA) kit (AdipoGen, Seoul, Korea) according to the manufacturer's instructions. To measure each isoform of adiponectin, SDS-PAGE was performed according to the standard Laemmli's method under non-reducing and non-heat-denaturing conditions. For non-reducing conditions, 2-mercaptoethanol was excluded from the sample buffer. For non-heat denaturation, the heat denaturation at 95˚C for 5 min was also excluded (14). The proteins separated by SDS-PAGE were transferred to PVDF membranes. The membranes were blocked with PBS-T (PBS, 0.05% Tween-20) containing 10% skim milk and were then incubated with 1:5000 diluted adiponectin antibody (ALX-804-515, Alexis Biochemicals, San Diego, CA, USA) in PBS-T containing 5% skim milk for 1 h at room temperature. After washing, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse antibody (1:5000) for 1 h at room temperature and were then washed thoroughly. The membranes were exposed to X-ray film (Kodak Film, Rochester, NY, USA) using ECL Western blotting detection reagent (Amersham Biosciences, Uppsala, Sweden).

Adiponectin receptor expression. A quantitative real-time RT-PCR method was employed to elucidate the adiponectin receptor expression. The mRNA expression of AdipoR1, AdipoR2, and T-cadherin was estimated by the One-Step SYBR RT-PCR (Perfect Real Time) kit (Takara Bio, Tokyo, Japan). Relative gene expression was measured using ß-actin as an internal control. The PCR reaction was performed in triplicate in MicroAmp 96-well reaction plates (Applied Biosystems, Foster City, CA, USA); amplification was carried out in the ABI PRISM 7700 Sequence Detector (Applied Biosystems). The amplification conditions were 15 min at 42˚C, 2 min at 95˚C, followed by 40 cycles of 5 sec at 95˚C, 10 sec at 60˚C, and 34 sec at 72˚C. The results were analyzed using the Sequence Detector 1.9 software. The sequence of the primers were as follows: AdipoR1-forward, 5'-CTT CTA CTG CTC CCC ACA GC-3' and reverse, 5'-TCC CAG GAA CAC TCC TGC TC-3'; AdipoR2-forward, 5'-ATT TGG AGC CCA GTT TAG AG-3' and reverse, 5'-CGG AAA GAA GGC ATA GGA-3'; T-cadherin-forward, 5'-TCG GGT CTG TCA CTA TCA AC-3' and reverse, 5'-TGA GGT CTC AAG CCC ATA C-3'; ß actin-forward, 5'-ATC GCT GAC AGG ATG CAG AA-3' and reverse, 5'-TAG AGC CAC CAA TCC ACA CAG-3'.

Methylation status of the receptors. We used the methylation-specific PCR (MSP) to examine the methylation status of the receptors. Methylation analysis for adiponectin receptor genes was performed by MSP of sodium bisulfite-treated DNA as described previously (21). Briefly, genomic DNA was digested by restriction enzyme into shorter fragments and PCR amplified followed by chemical treatment with sodium bisulfite. To confirm the specificity, genomic DNA from CSAA-treated liver was artificially methylated by SssI methylase and used as a positive control of the primers for the methylated sequence. All primer sets for the unmethylated sequence were able to amplify the PCR product from DNA of the CSAA-treated liver. The primers were custom-synthesized by Invitrogen (Invitrogen, Eugene, OR, USA). The PCR conditions including primer sequences were as follows: AdipoR1: U-F 5'-TTATTATGTGTTTAGTGTGTG TTT-3', U-R 5'-CTAAAAACAACCTACAACCA-3', M-F 5'-TACGCGTTTAGTGTGCGTTC-3', M-R 5'-CTCTAAAA ACGACCTACGACCG-3'; AdipoR2: U-F 5'-AGTTGGTT GTTGTTTTATTGT-3', U-R 5'-CCTCATACAATACACA TCACA-3', M-F 5'-GAGTTGGTCGTCGTTTTATCGC-3', M-R 5'-CTCGTACGATACGCGTCACG-3'; and T-cadherin: U-F 5'-TGTGTGTGAATGTAAATGTT-3', U-R 5'-CCAA ATCTATCTACACAACA-3', M-F 5'-GCGTGTGTGAATG TAAACGTC-3', M-R 5'-CCGAATCTATCTACGCGACG-3', U-F 5'-TGTGTGTGAATGTAAATGTT, U-R 5'-CCAAAT CTATCTACACAACA-3'; M 60, U55 30 cycles.

Statistical analysis. The differences between groups were analyzed using the Student's t-test for independent samples. Statistical significance was inferred at a two-tailed p-value <0.05. The quantitative data were expressed as the mean ± SEM.

Results

Serum adiponectin levels and multimer formation of adiponectin. The CDAA treatment resulted in a marked liver fibrosis development with fatty accumulation as reported previously (5,20). The total adiponectin levels in the serum stepwise increased in the CDAA-treated group along with liver fibrosis development, and became significantly higher at 16 weeks as compared with the CSAA-treated group (Fig. 1). We next examined which type of isoform increased during the liver fibrosis development. Representative features of immunoblotting under non-reducing and non-denaturing conditions are shown in Fig. 2. Among the three isoforms, only HMW adiponectin significantly increased whereas MMW and LMW did not. These results indicated that HMW adiponectin exclusively was up-regulated during the CDAA-induced liver fibrosis development.

Expression levels of adiponectin receptors in the liver. Because serum adiponectin, especially HMW, increased in the CDAA-treated group, we next examined the expression

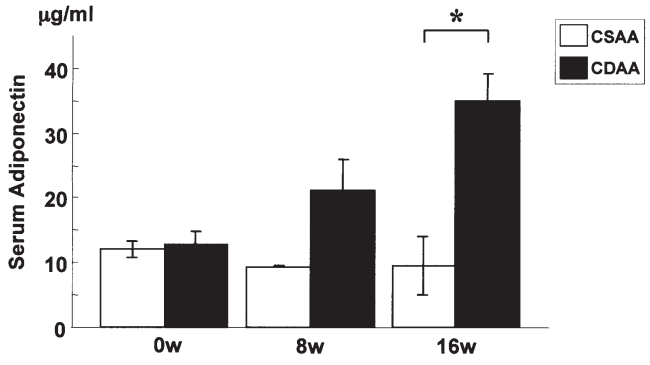

Figure 1. The serum adiponectin level during the liver fibrosis development as measured by ELISA. The total adiponectin levels in the serum stepwise increased in the CDAA-treated group along with liver fibrosis development, and became significantly higher at 16 weeks as compared with the CSAA-treated group. A white box indicates the CSAA-treated liver and a black box indicates CDAA. The data represent the mean ± SEM (n=10). *Statistically significant differences between the indicated experimental groups ($p<0.01$).

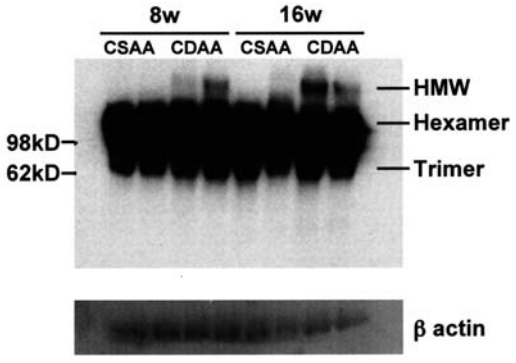

Figure 2. Representative features of immunoblotting under non-reducing and non-heat-denaturing SDS-PAGE separate multimer species of adiponectin. All three different molecular weight isoforms (HMW, MMW, and LMW) of adiponectin were detected in the liver of the CDAA-treated rats. Among the three isoforms, only HMW adiponectin significantly increased in the liver of CDAA-treated rats, whereas MMW and LMW did not.

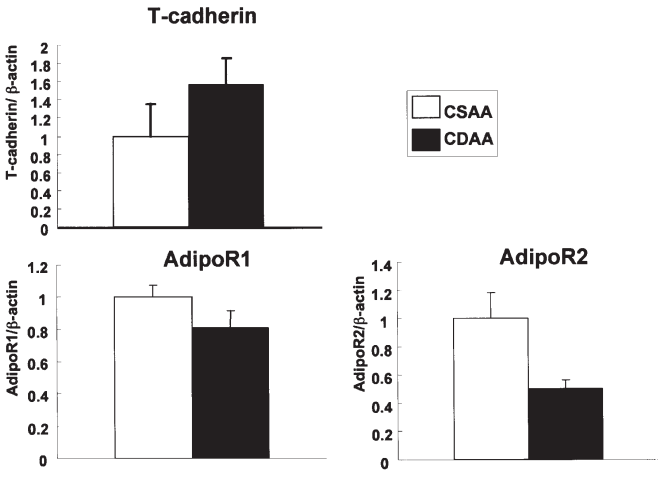

Figure 3. The expression level of adiponectin receptors in the liver. The expression of T-cadherin, which is known to exclusively bind to HMW adiponectin, significantly increased in the liver of the CDAA-treated rats. On the contrary, the expression of AdipoR2 decreased in the liver of the CDAA-treated rats as compared with the CSAA-treated rats, and AdipoR1 remained at the control level. The relative gene expression was measured by an image analysis system as described in Materials and methods. A white box indicates the CSAA-treated liver and a black box indicates CDAA. The data represent the mean ± SEM (n=10). *Statistically significant differences between the indicated experimental groups ($p<0.01$).

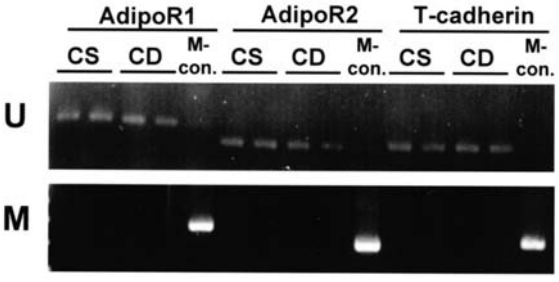

Figure 4. Methylation status of adiponectin receptors. T-cadherin was not methylated in the liver of the CDAA- or CSAA-treated rats. Similarly, methylation of AdipoR1 or AdipoR2 was not observed. The methylation status of adiponectin receptors was analyzed by methylation-specific PCR (MSP). CS, CSAA-treated group; CD, CDAA-treated group; M-con, normal hepatic DNA methylated by SssI methylase; U, PCR product with unmethylated sequence-specific primers; and M, PCR product with methylated sequence-specific PCR.

levels of adiponectin receptors by real-time PCR. As shown in Fig. 3, the expression of T-cadherin, which is known to bind exclusively to HMW adiponectin, was significantly increased in the liver of the CDAA-treated rats. In contrast, the expression of AdipoR2 was suppressed in the liver of the CDAA-treated rats as compared with the CSAA-treated rats, and AdipoR1 remained at the control level. Collectively, HMW adiponectin and T-cadherin interaction was only up-regulated during the CDAA-induced liver fibrosis development. We examined whether T-cadherin was expressed in hepatic stellate cells (HSC), which play a pivotal role in liver fibrosis development. There was no expression of T-cadherin in the activated or HSC (data not shown).

Methylation status of adiponectin receptors. Since the methylation status of genes reportedly sometimes influence the alteration of gene expression *in vivo*, we performed the MSP amplification to elucidate the mechanism of regulation of the adiponectin receptor expression (21). T-cadherin was not methylated in the liver of CDAA- or CSAA-treated animals (Fig. 4). Similarly, methylation of AdipoR1 or AdipoR2 was not observed, indicating that another mechanism other than hypomethylation or hypermethylation may be involved in the regulation of gene expression of all adiponectin receptors.

Discussion

In the current study, we found that serum adiponectin, exclusively HMW, and its receptor T-cadherin were significantly up-regulated during the CDAA-induced liver fibrosis development. We also observed that AdipoR2 was

down-regulated in the fibrotic liver, and that methylation was not involved in the gene regulation of the receptors.

Several investigators have noted that serum adiponectin increases in the advanced fibrotic liver (7,9,11,12). Furthermore, it has been reported that serum adiponectin increases in advancing liver fibrosis and declines with reduction of fibrosis in chronic hepatitis B as well as with HCV infection, suggesting that adiponectin positively regulates liver fibrosis development (9). The mechanistic insight of elevation of adiponectin in the fibrotic liver is still obscure. It has been reported that biliary excretion is involved in the clearance of adiponectin (12,22). The increase in the serum adiponectin level in patients with cirrhosis can be partly explained by impaired biliary secretion, which leads to accumulation of adiponectin in the circulation and its deposition in cirrhotic livers.

Among the three isoforms of adiponectin, we observed that only HMW adiponectin significantly increased whereas the other two did not. It has been reported that each isoform is responsible for different signals. In human monocytes, HMW adiponectin induces interleukin (IL)-6 secretion, while LMW adiponectin reduces IL-6 secretion (23,24). In endothelial cells (EC), inverse effects on cell growth have been observed between the HMW and LMW isoforms. HMW adiponectin exerted an anti-apoptotic effect whereas the recombinant LMW adiponectin induced apoptosis through caspase-3 activation (25,26). Furthermore, it has been reported that HMW but not LMW binds to PDGF-BB, which is known as the most potent proliferating stimulus for HSC (27,28). The coordination of these biological effects may, at least partly, contribute to the promotion of liver fibrosis development.

Along with the up-regulation of HMW adiponectin, T-cadherin, which exclusively binds to HMW adiponectin, markedly increased in the CDAA-treated fibrotic liver. Although we did not identify any localization of T-cadherin in the fibrotic liver, no expression was observed in HSC *in vitro*. It has been reported that T-cadherin is mainly expressed in EC, which are the main targets of neovascularization (29). It is now known that angiogenesis plays an important role in many physiological and pathological events (30,31). We previously reported that angiogenesis plays a pivotal role in the development of liver fibrosis (32). As described above, HMW adiponectin exerted an anti-apoptotic effect on EC. Moreover, it was shown that HMW adiponectin activates NF-κB, which is known to activate angiogenesis in EC (33). We previously reported that neovascularization stepwise increased during the CDAA-induced liver fibrogenesis (5). Together, neovascularization mediated by HMW adiponectin and T-cadherin plays a certain role in CDAA-induced liver fibrosis development through activation of NF-κB, and prevents apoptosis of EC.

In contrast to the promoting effects of adiponectin on liver fibrosis, several studies have revealed an anti-fibrotic effect of adiponectin in the liver of NASH. In contrast to virus-originated liver cirrhosis, adiponectin levels decreased in NASH (6,7). Adiponectin KO mice demonstrated marked liver fibrosis development as compared with control mice, and in cultured HSC, adiponectin suppressed the PDGF-BB-induced proliferation and migration (8). This KO mouse also showed an anti-fibrotic effect in the CDAA-induced model (34). The exact reason for these discrepancies is not fully understood at this time. Since NASH is closely associated with insulin resistance, which has been suggested to play certain biological roles in liver fibrosis development, some differences in the host clinical background may be involved with the antagonistic effect against adiponectin during liver fibrogenesis. Furthermore, the adiponectin KO mouse lacks all isoforms of adiponectin, and the recombinant LMW adiponectin has been used for the *in vitro* HSC experiment. Similar to the diverse effects on EC, LMW and HMW adiponectin may exert different biological effects on HSC. Further studies to elucidate the exact mechanistic insights of the above mentioned discrepancy and the gene regulation of adiponectin receptors are required in the future.

In summary, serum adiponectin, especially the HMW isoform, significantly increased during the CDAA-induced liver fibrosis development along with the augmentation of T-cadherin, a specific receptor of HMW adiponectin. Since the specific ligand and receptor were augmented together, crosstalk between HMW adiponectin and T-cadherin may play an important role during liver fibrosis development in rats.

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