Increment and impairment of adiponectin in renal failure

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Aims

Patients with chronic renal failure are at high risk of cardiovascular diseases. Previous studies in healthy population showed that hypoadiponectinemia was associated with high cardiovascular disease risk. However, plasma adiponectin (APN) levels are increased in renal dysfunction. Therefore, the clinical significance of plasma APN level in patients with moderate renal dysfunction is controversial. The aim of this study was to determine the change of plasma APN levels in a mouse model of renal failure and the loss of vasculo-protective function of APN in the presence of high cystatin C levels.

Methods and results

Subtotal (5/6) nephrectomy was performed in APN-knockout (KO) mice and wild-type (WT) mice. The procedure in WT mice resulted in the significant increase of plasma APN and cystatin C levels. The clearance rate of APN was measured by injecting plasma from WT mice into KO mice. The clearance rate was significantly decreased in subtotal nephrectomized KO mice compared with sham-operated KO mice. Adiponectin protein and mRNA levels in adipose tissue were similar to subtotal nephrectomized and sham-operated mice. In cultured endothelial cells, at a high concentration corresponding to renal failure, cystatin C abolished the suppressive effects of APN on tumour necrosis factor α-induced expression of monocyte adhesion molecules.

Conclusion

Plasma APN increases in chronic renal failure, at least in part due to low clearance rate. High concentrations of cystatin C abolish the vasculo-protective effect of APN.

Keywords

Adiponectin • Renal failure • Clearance rate • Cystatin C • Atherosclerosis

1. Introduction

Obesity, defined as excess fat accumulation, is a common cause of cardiovascular morbidity and mortality in industrialized countries. Excess body fat, especially abdominal visceral fat accumulation, is frequently accompanied by diabetes mellitus, dyslipidemia, and hypertension, and could result in atherosclerotic vascular diseases. Adipose tissue secretes various bioactive molecules that may directly contribute to the obesity-related diseases. Among them, adiponectin (APN) is an adipocyte-specific plasma protein, which is abundantly present in human plasma. We reported previously that physiological concentrations of human recombinant APN suppress tumour necrosis factor-α (TNF-α)-induced endothelial adhesion molecule expression, transformation from macrophages to foam cells, and proliferation of vascular smooth muscle cell. Hypoadiponectinemia has been observed in patients with obesity, diabetes mellitus, and hypertension. Initial prospective study in healthy population suggested that high APN concentration was associated with lower cardiovascular risk. However, recent prospective studies showed that hyperadiponectinemia is associated with mortality in patients with chronic kidney disease (CKD). These findings raise the possibility of another prognostic implication of APN concentration in specific populations. With regard to renal disease, plasma APN levels are markedly increased in patients with end-stage renal disease (ESRD) compared with healthy subjects, although a significantly lower rate of cardiovascular disease events was registered in the hyperadiponectinemia-ESRD group than the hypoadiponectinemia-ESRD group. The mechanism of APN increment in renal failure has not been clarified.

In vitro studies indicated that APN binds to cystatin C, although mutual effects of these proteins were not investigated. Cystatin C is an inhibitor of the cathepsin family, which is known to degrade elastin and collagen, the two major extracellular matrix constituents.

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of the vascular wall. Serum cystatin C levels are elevated in patients with renal failure, and high cystatin C concentrations provide stronger association with the risk of cardiovascular events and death than either creatinine level or estimated glomerular filtration rate.

In the present study, we confirmed the elevation of plasma APN in a mouse model of renal failure and loss of the vasculo-protective function of APN in the presence of high cystatin C concentrations corresponding to renal failure.

2. Methods

2.1 Animals and animal treatment

Male wild-type (WT) mice in a C57BL/6J background were obtained from Clea Japan (Tokyo, Japan). Adiponectin-knockout mice were generated and backcrossed as described previously. All surgical procedures were carried out under anesthesia induced by intraperitoneal pentobarbital (30 mg/kg body weight; Sigma St Louis, MO, USA). Subtotal (5/6) nephrectomy was performed in 12-week-old mice as described previously. Sham-operated mice were anesthetized and subjected to the same surgical protocol except for subtotal nephrectomy. At 1 month after operation, mice were anesthetized, blood was collected, and epididymal white adipose tissue (WAT), subcutaneous WAT, and mesenteric WAT were dissected out and frozen in liquid nitrogen. Subtotal nephrectomy (partial kidney loss model) and bilateral total nephrectomy (total kidney loss model) were performed as described previously. Briefly, for bilateral total nephrectomy, mice were anesthetized with intraperitoneal pentobarbital, a midline incision was made, and both kidneys were removed from a flank incision, with preservation of the adrenal gland. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Osaka University School of Medicine. This study also conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2 Laboratory methods

Blood samples were collected in capillary tubes coated with heparin (Chase Scientific Glass Inc., TN, USA). Plasma APN concentrations and protein amounts in WAT were measured by enzyme-linked immunosorbent assay (ELISA) kit (Otsuka Pharmaceutical Co. Tokushima, Japan). Plasma creatinine and cystatin C levels were measured in a commercial laboratory. Mice were placed in metabolic cages to collect 24 h urinary samples, which were used for the measurement of creatinine clearance, as described previously.

2.3 Clearance rate of APN

We injected 10 μL/g body weight of WT mice plasma intravenously into APN-KO mice through the cervical vein (Figure 2A, C). In the experiment of Figure 2B, the APN concentrations of WT mice plasma were measured both in subtotal nephrectomized WT mice and sham-operated WT mice before injection, adjusted the final concentration of APN with phosphate buffered saline, and then injected into APN-KO mice. In the experiment with cystatin C, 300 μL of plasma from WT mice was pre-incubated with 3 μg of mouse recombinant cystatin C (R&D systems Inc., MN, USA) at 37°C for 60 min, and then injected the adjusted amount of APN as above. Venous blood samples (10 μL each) were taken from the tail vein at the indicated time after plasma injection to measure the clearance rate of APN. Plasma APN levels were measured by ELISA kit (Otsuka). In order to determine the accuracy of the measurement of APN in diluted samples, we diluted mouse plasma and measured APN concentration by ELISA kit (Otsuka). Linear correlation was confirmed between 1:10 to 1:10 000 dilution of mouse plasma (Supplementary Figure). Plasma disappearance curves were plotted semilogarithmically as percent of the initial plasma APN level at 1 min after injection.

2.4 Cell culture

Human umbilical vein endothelial cells (HUVECs) (Kurabo, Osaka, Japan) were maintained in Humeda-EG2 medium (Kurabo), and cells from passages 4–6 were used for experiments. Human recombinant APN was prepared as described previously.

2.5 Effects of cystatin C on TNF-α-induced expression of adhesion molecules in HUVECs

Human umbilical vein endothelial cells were incubated for 18 h in HuMedia-EB2 medium (Kurabo) containing 2% foetal calf serum in the presence or absence of APN or human recombinant cystatin C (BioVendor Laboratory Medicine, Brno, Czech Republic) at the indicated concentrations, then exposed to human recombinant TNF-α (R&D systems) at a final concentration of 10 ng/mL for 4 h.

2.6 Western blot analysis of oligomeric APN complexes

Total RNA was extracted from HUVECs or mouse adipose tissue or mouse aorta by RNA-STAT kit (Tel-Test Inc., Friendswood, TX, USA) according to the protocol supplied by the manufacturer, and the cDNA was synthesized using the ThermoScript RT–PCR system (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed on a Light Cycler using the FastStart DNA Master SYBR Green I (Roche Diagnostics, Manheim, Germany) according to the protocol provided by the manufacturer. The sequences of the primers used for real-time PCR were as follows: mouse APN, 5'-GATGGCAGAGATGGCACTCC-3' and 5'-CTTTGCCAGTGCTGCGCTAT-3'; mouse 36B4, 5'-GCTTCCAAGCAGATGCAAGCA-3' and 5'-CCGGATGTGAGGACAGCAG-3'; human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5'-TGGTCTCCTCTGTAATCCCAAC and 5'-GTTAGGGTTCTCTCTCTTCCT-3'; human vascular cell adhesion molecule-1 (VCAM-1), 5'-GGGGTTTCTCTGTGCGGGA-3' and 5'-AACAGAGGTTAGTACCCCG-3'; human inter-cellular adhesion molecule-1 (ICAM-1), 5'-GCCGCCAAGCTTATACACAA-3' and 5'-CAATCCTCTCTGCAATCG-3'; and human E-selectin, 5'-TTCCGGGAAAGATCAACATGA-3' and 5'-CTTTGCCAGTGCTGCGCTAT-3'.

2.7 Quantification of mRNA levels

Data are presented as mean ± SEM. Differences between groups were evaluated by the unpaired Student’s t-test or analysis of variance followed by Fisher’s protected least significant difference test. A probability value of less than 0.05 denoted the presence of a statistically significant difference. All calculations were performed using SPSS for Windows, version 11.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1 Elevated plasma APN levels in WT mice after subtotal nephrectomy

Plasma APN levels increased significantly in WT mice at 1 month after subtotal nephrectomy, but not in WT mice at 1 month after...
3.2 Low clearance rate in subtotal nephrectomized mice

We injected plasma from WT mice into subtotal nephrectomized APN-KO mice and sham-operated APN-KO mice at 1 month after operation (Figure 2A). The initial APN concentration was 1.52 ± 0.09 μg/mL in subtotal nephrectomized APN-KO mice and 1.51 ± 0.09 μg/mL in sham-operated APN-KO mice. The plasma disappearance curve of APN showed two exponential components: a rapid phase (~3 h) and a slow phase (3–24 h). Plasma APN levels in the slow phase were significantly higher in subtotal nephrectomized APN-KO mice than in sham-operated APN-KO mice. These results are indicative of low APN clearance rate in subtotal nephrectomized APN-KO mice. We also analysed the isoforms of APN in APN-KO mice at 24 h after WT plasma injection by western blotting. Although the total APN immunoreactive mass was increased in subtotal nephrectomized APN-KO mice, no difference was detected in isoform patterns between subtotal nephrectomized and sham-operated APN-KO mice (Figure 2A inset).

In the next series of experiments, plasma from WT mice with subtotal nephrectomy was injected into APN-KO mice (Figure 2B). The initial APN concentration was 1.37 ± 0.14 μg/mL in APN-KO mice injected plasma from subtotal nephrectomized WT mice and 1.34 ± 0.07 μg/mL in APN-KO mice injected plasma from sham-operated WT mice. The injection of plasma from subtotal nephrectomized WT mice resulted in a significant decrease in clearance rate compared with the injection of plasma from sham-operated WT mice.

Next, plasma obtained from sham-operated WT mice was pre-incubated with recombinant cystatin C as described in Section 2, and then injected into APN-KO mice. At 1 min after the injection, plasma APN and cystatin C concentrations were 1.35 ± 0.09 μg/mL and 0.15 ± 0.02 mg/L, respectively, in APN-KO mice. Plasma APN levels were significantly higher at 12 and 24 h after the injection when cystatin C-treated WT plasma was injected compared with the injection of vehicle-treated WT mice plasma (Figure 2B).

In another experiment, plasma from WT mice was injected into APN-KO mice immediately after bilateral nephrectomy (total kidney loss), subtotal nephrectomy (partial kidney loss), or sham operation (Figure 2C). The initial APN concentrations were similar to the three groups (1.32 ± 0.09 μg/mL in APN-KO mice with total kidney

sham-operation (Figure 1A). Body weight was similar to the subtotal nephrectomized WT mice (26.1 ± 0.4 g) and sham-operated WT mice (26.8 ± 0.5 g) at 1 month after operation. Plasma creatinine levels were significantly higher and creatinine clearance was significantly lower in subtotal nephrectomized WT mice than sham-operated WT mice (Figure 1B, C). Plasma cystatin C levels were significantly higher in subtotal nephrectomized WT mice than sham-operated WT mice (Figure 1D).

![Figure 1](image-url)
loss, 1.41 ± 0.08 μg/m in APN-KO mice with partial kidney loss, and 1.35 ± 0.08 μg/m in sham-operated APN-KO mice). Compared with sham operation, plasma APN levels in the slow phase were significantly higher both in the total and partial kidney loss models. The clearance rate of APN tended to be lower in the total kidney loss model compared with that of the partial kidney loss model.

### 3.3 No change in APN protein and mRNA levels in WAT after subtotal nephrectomy

The concentrations of APN protein (in μg/mg tissue) and mRNA levels in epididymal WAT, subcutaneous WAT, and mesenteric WAT were not different between subtotal nephrectomized mice and sham-operated mice at 1 month after operation (Figure 3).

### 3.4 Cystatin C abrogates the suppressive effects of APN on TNF-α-induced expression of adhesion molecules in HUVECs

As described above, plasma APN levels and cystatin C levels are elevated in patients with renal failure, and previous reports suggested that APN binds to cystatin C. Accordingly, we examined the mutual effects of these proteins on vascular inflammation (Figure 4). Pretreatment of HUVECs with APN significantly suppressed the TNF-α-induced expression levels of VCAM-1, ICAM-1, and E-selectin in these cells. Co-treatment with cystatin C dose-dependently

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**Figure 2** Plasma disappearance curves of APN after intravenous injection of plasma from WT mice into APN-KO mice. (A) Injection of WT mice plasma into subtotal nephrectomized APN-KO mice (nephrectomy: solid circles, n = 4) or sham-operated APN-KO mice (Sham: open circles, n = 4). Inset: SDS-PAGE separation of the multimeric complexes of plasma APN in a non-reducing and non-heating condition in sham-operated and subtotal nephrectomized APN-KO mice at 24 h after WT plasma injection. (B) Injection of plasma from subtotal nephrectomized mice (renal failure plasma: solid circles, n = 4), sham-operated mice containing cystatin C (sham plasma + cystatin C: dotted circles, n = 4), or sham-operated mice (sham plasma: open circles, n = 4) into APN-KO mice. (C) Injection of WT normal plasma into APN-KO mice with bilateral nephrectomy (total kidney loss: solid circles, n = 5), subtotal nephrectomy (partial kidney loss: dotted circles, n = 5), or sham operation (sham operation: open circles, n = 5). *P < 0.05 compared with sham-operated mice (A and C) and plasma from sham-operated mice (B). **P < 0.001 compared with sham operation. Data are mean ± SEM.

**Figure 3** Adiponectin protein concentrations (in μg/mg tissue) and APN mRNA levels in epididymal WAT, subcutaneous WAT, and mesenteric WAT at 1 month after subtotal nephrectomy or sham operation. Data are mean ± SEM of nine mice in each group. The results of APN mRNA levels were normalized to 36B4.
abrogated the suppressive effects of APN on TNF-α-induced expression of these adhesion molecules in HUVECs, although cystatin C alone had no such effect.

4. Discussion

The major findings of the present study were the following: (1) Plasma APN levels were significantly increased in the mice model of chronic renal failure. (2) The clearance rate of APN was significantly low in the mice model of renal failure. Injection of plasma from mice with renal failure also reduced the clearance rate of APN. (3) Subtotal nephrectomy did not alter APN protein and mRNA levels in WAT. (4) The suppressive effects of APN on TNF-α-induced expression of adhesion molecules in HUVECs were abrogated by cystatin C in a dose-dependent manner.

Our experiments in a mouse model of ESRD showed the presence of hyperadiponectinemia at 1 month after subtotal nephrectomy. The increase in plasma APN is likely due to low clearance rate, because no differences in APN protein and mRNA levels in WAT were observed between subtotal nephrectomized mice and sham-operated mice. Previous studies demonstrated the participation of the kidney in the clearance of renin in the bilateral nephrectomy model. We used the same kidney loss model to investigate the role of the kidney in the clearance of APN. In the present study, the mRNA levels of Adipo-R1 and T-cadherin were decreased in the remnant kidney (data not shown). The impairment of kidney-related degradation

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and accumulation of various uremic toxins including cystatin C may be involved in the impaired degradation of plasma APN. However, the full mechanism for the increment of APN remains to be clarified.

We and others reported that APN plays a protective role by reducing albuminuria through a direct effect on podocyte function and modulation of inflammation and oxidative stress. However, several clinical studies reported that hyperadiponectinemia is a predictor of renal disease progression. The results of the present study suggest that increment of APN concentration associated with the low clearance rate may explain, at least in part, these controversial results.

Cystatin C, which inhibits collagen- and elastin-degrading cysteine proteases of the cathepsin family, is produced by virtually all types of cells. Cystatin C is freely filtered by renal glomeruli and then metabolized by the proximal tubules, and elevated serum cystatin C level is a marker of renal dysfunction. The amount of cystatin C is reduced in human atherosclerotic lesions, suggesting the involvement of an imbalance between cystatin C and cysteine proteases, such as cathepsins, in atherosclerosis. Sukhova et al. reported that cystatin C- and apo-E-double deficient mice showed increased fragmentation of the lamina elastica of blood vessel tunica media, together with increased smooth muscle cells, leading to aortic dilatation. Bengttson et al. reported that after 25 weeks of atherogenic diet, CysC+/-/ApoE+/- mice had larger subvalvular plaques with high macrophage content. These studies suggest that cystatin C has anti-atherosclerogenic effects. On the other hand, elevated cystatin C level is a risk marker of cardiovascular disease. Shlipak et al. reported that high cystatin C concentration is a stronger predictor of cardiovascular events in elderly patients than serum creatinine and creatinine-based estimated glomerular filtration rate.

Masase et al. reported previously that APN is bound to cystatin C in vitro. We confirmed the binding of APN and cystatin C in vitro (data not shown). Furthermore, we found that high concentrations of cystatin C abrogated the suppressive effects of APN on TNF-α-induced expression of various adhesion molecules, including VCAM-1, ICAM-1, and E-selectin in a dose-dependent manner. Cystatin C may inhibit the suppressive effects of APN on TNF-α-induced endothelial inflammation through IκB-α-NF-κB pathway. Adhesion of monocytes to the endothelium is an important initial step of atherosclerosis. The presence of pathologically high systemic cystatin C levels may accelerate atherosclerosis through impairment of the anti-atherosclerotic function of APN, even though physiological concentrations of cystatin C play an important role in modulating vascular cysteine protease system.

In the current study, plasma cystatin C concentrations were 0.08 ± 0.03 mg/L in mice at 1 month after subtotal nephrectomy. Song et al. reported that serum cystatin C concentrations were 0.28 ± 0.09 mg/L at 24 h in mice after subtotal nephrectomy, which was acute renal failure model. On the other hand, serum cystatin C concentrations in humans were 1.0—10 mg/L, which are almost 10—100 times higher than mouse serum cystatin C concentration. Accordingly, we performed the in vitro experiments of HUVEC with human recombinant cystatin C at the human plasma levels. In HUVEC study, the human plasma levels of cystatin C abrogated the suppressive effects of APN on TNF-α-induced expression of adhesion molecules. However, 1 mg/L of cystatin C, which is higher than mouse plasma level, had little effect.

In the early stages of CKD, serum creatinine levels are lower than 1.5 mg/dL, and previous studies showed a high prevalence of coronary artery disease in male patients with hyperadiponectinemia, independent of well-known risk factors. In addition, we reported that low APN level was a predictor of cardiovascular disease, including recurrent ischaemic heart disease, after adjustment for the stage of CKD. On the other hand, hyperadiponectinemia in renal failure is reported to be associated with increased cardiovascular disease risk. Therefore, in advanced CKD stage, the greater part of elevated APN might be inactivated by co- elevated cystatin C.

We and others suggested that the ratio of the isoforms of APN was important for the prevention of atherosclerosis. von Eyنان et al. reported that atorvastatin therapy was associated with significant changes in APN multimer distribution without affecting total APN levels in patients with Type 2 diabetes that may represent the effect of atorvastatin for prevention of cardiovascular disease. However, no significant isoform differences were observed between sham-operated and subtotal nephrectomized mice (data not shown).

In conclusion, the high plasma APN level in the mice renal failure model was due to a low clearance rate, which is probably associated with high cystatin C levels. High concentrations of cystatin C abrogate the anti-atherosclerotic effects of APN, suggesting that measurements of both APN and cystatin C levels might be helpful in evaluating the risk of cardiovascular disease in patients with renal failure.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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References
Adiponectin in renal failure


