Original Article

Comparison of Serum High–Molecular Weight (HMW) Adiponectin With Total Adiponectin Concentrations in Type 2 Diabetic Patients With Coronary Artery Disease Using a Novel Enzyme-Linked Immunosorbent Assay to Detect HMW Adiponectin

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Adiponectin (Acrp30), an adipocyte-derived protein, exists in serum as a trimer, a hexamer, and a high–molecular weight (HMW) form, including 12–18 subunits. Because HMW adiponectin may be biologically active, we measured it in serum using a novel enzyme-linked immunosorbent assay (ELISA) confirmed by gel filtration chromatography that the ELISA detected mainly adiponectin with 12–18 subunits, and we compared HMW with total adiponectin concentration in patients with type 2 diabetes. We next investigated the relationship between serum HMW and coronary artery disease (CAD) in 280 consecutive type 2 diabetic patients, including 59 patients with angiographically confirmed CAD. Total adiponectin was measured in serum by a commercially available ELISA. Like serum total adiponectin, HMW adiponectin correlated positively with HDL cholesterol and negatively with triglyceride, insulin sensitivity, creatinine clearance, and circulating inflammatory markers. Total and HMW adiponectin were significantly higher in women than in men, as was the HMW-to-total adiponectin ratio. Serum HMW and the HMW-to-total adiponectin ratio were significantly lower in men with than without CAD ($P < 0.05$, respectively). In women, the ratio, but neither total nor HMW adiponectin, tended to be lower when CAD was present. In conclusion, determination of HMW adiponectin, especially relative to total serum adiponectin, is useful for evaluating CAD in type 2 diabetic patients. *Diabetes* 55:1954–1960, 2006

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CAD, coronary artery disease; CCr, creatinine clearance; ELISA, enzyme-linked immunosorbent assay; HOMA-IR, homeostasis model assessment of insulin resistance; HMW, high molecular weight; LMW, low molecular weight; MMW, medium molecular weight.

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Adiponectin, also known as Acrp30 (1), AdipoQ (2), gelatin-binding protein of 28 kDa (GBP28) (3), and apM1 (4), is an adipocyte-specific protein that enhances insulin sensitivity and promotes lipid metabolism (5,6). Adiponectin circulates in plasma as three forms: a trimer (low–molecular weight [LMW]), a hexamer (trimer-dimer) of medium–molecular weight (MMW), and a larger multimeric high–molecular weight (HMW) form (3,7–9). Previous studies reported that HMW adiponectin may be the active form of this protein, because after treatment with thiazolidinedione, changes in serum HMW adiponectin, but not total adiponectin, were associated with improvement in hepatic insulin sensitivity (10,11). Another study reported that HMW adiponectin suppressed apoptosis in cultured endothelial cells, suggesting a vasculoprotective property of HMW adiponectin (12). Waki et al. (9) demonstrated that only HWM adiponectin can induce activation of AMP-activated protein kinase in muscle. On the other hand, Tsao et al. (8) proposed that HMW adiponectin represented a precursor pool that can be activated, with the cleaved form, i.e., LMW adiponectin, being responsible for the effect on AMP kinase activity. Thus, biological activities among these isoforms are a matter of controversy, although most investigators now appear to believe that HMW adiponectin has more biological activity than LMW or MMW adiponectin.

Paradoxically, serum concentrations of total adiponectin are reduced in obesity (13), type 2 diabetes (14), and coronary artery disease (CAD) (15). One might suspect that low serum concentrations of HMW adiponectin might be associated more strongly with CAD than low total adiponectin. Unfortunately, although total adiponectin can be measured readily using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (13), HMW determinations have been difficult and only semiquantitative, requiring size fractionation by velocity sedimentation, followed by SDS-PAGE, and then Western blotting (10,12,16–18). This semiquantitative method is time consuming, labor intensive, and ill suited to the study of large

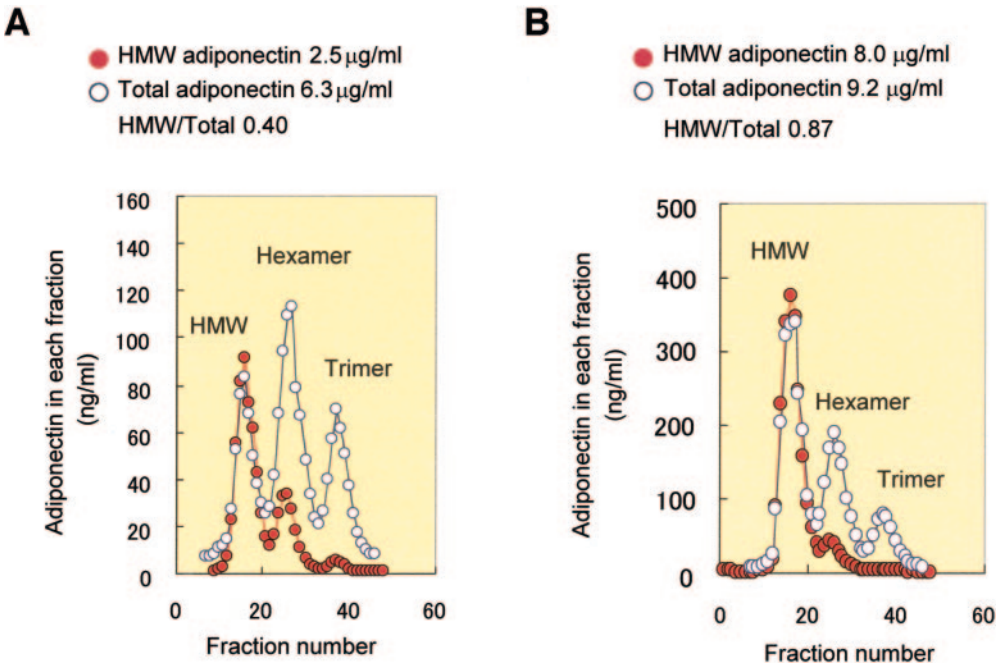

FIG. 1. Elution profiles of adiponectin in serum from two subjects (*A* and *B*) determined by ELISA for total and HMW adiponectin. Serum was fractionated by gel filtration chromatography, and concentrations of adiponectin in each fraction were measured by both ELISA systems. Open circles indicate concentrations of adiponectin determined by the total adiponectin ELISA system, whereas red closed circles indicate concentrations of adiponectin determined by our novel HMW adiponectin ELISA system. Top parts of panels show serum concentrations of total (open circles) and HMW adiponectin (red closed circles) in each individual as determined by the two ELISA systems and HMW-to-total adiponectin ratios.

subject cohorts. Such investigators require a high-throughput assay such as ELISA.

A monoclonal antibody against human HMW adiponectin, IH7, was developed by members of our group of collaborators (3) using GBP28, an HMW adiponectin, as the antigen. With IH7 as both the capturing and detecting antibody, we devised a novel sandwich ELISA able to measure HMW adiponectin specifically in serum (19). We presently used this novel HMW adiponectin-specific ELISA to analyze sera from type 2 diabetic patients. We simultaneously measured serum total adiponectin with the commercial ELISA kit and compared clinical significance of serum HMW adiponectin with that of serum total adiponectin and that of a ratio of HMW to total adiponectin. We furthermore investigated the relationships between serum HMW, total adiponectin, or the ratio and CAD confirmed by coronary angiography in patients with type 2 diabetes. This report is believed to be the first to compare clinical implications of serum total adiponectin with those of HMW adiponectin in type 2 diabetes. Serum HMW adiponectin concentrations, especially expressed relative to those of total adiponectin, proved to be closely related to CAD in diabetic patients than results of simple total adiponectin determinations.

RESEARCH DESIGN AND METHODS

We studied 280 consecutive type 2 diabetic patients (121 women and 159 men) who had been referred to the outpatient diabetes clinic at Dokkyo Medical University Hospital for improvement of glycemic control.

Diabetic patients were assigned to one of three groups according to urinary albumin excretion in a 24-h collection: normoalbuminuria, with urinary albumin excretion <30 mg/24 h; microalbuminuria, 30–299 mg/24 h; or macroalbuminuria, >300 mg/24 h. As an index of glomerular filtration rate, creatinine clearance (CCr) was determined using the same 24-h urine collection.

This study of 280 diabetic patients included 59 who also had CAD. All patients with CAD underwent coronary angiography, which confirmed significant atherosclerotic CAD by demonstrating coronary artery stenosis causing >75% luminal obstruction in at least one of the three major coronary arteries. Two experienced invasive cardiologists, who were blinded to the patients' adiponectin measurements, evaluated the angiography. We excluded patients with acute coronary syndromes such as acute myocardial infarction or those with symptomatic heart failure from this study.

Among the diabetic patients, 133 had hypertension, defined as systolic blood pressure exceeding 140 mmHg and/or diastolic blood pressure exceeding 90 mmHg or alternatively as treatment with one or more antihypertensive agents, including angiotensin-converting enzyme inhibitors (n = 28), β-blocker (n = 21), calcium channel blockers (n = 73), or angiotensin receptor blockers (n = 29).

As a control group, 52 nondiabetic subjects (23 women and 29 men) were selected to match the overall age and sex distribution of the diabetic group. Their age was 60.5 ± 10.0 years and their BMI was 24.3 ± 3.3 kg/m^2. The investigations were conducted according to the principle outlined in the Declaration of Helsinki. All patients gave informed consent. The study was approved by the local ethics committee.

Total and HMW adiponectin measurements. Venous blood was obtained between 6:00 and 8:00 A.M. after an overnight fast. Serum was separated after centrifugation at 2,500*g* for 15 min; the supernatant was stored at −70°C until use. Total serum adiponectin concentration was measured by a sandwich ELISA (Otsuka Pharmaceuticals, Tokyo, Japan) as previously described (13). In brief, after boiling serum samples in SDS buffer for 5 min to convert all adiponectin to a monomeric form, samples were analyzed with the ELISA system to determine total adiponectin in serum. Intra- and interassay coefficients of variation (CVs) were 4.06 and 4.69%, respectively.

Serum HMW adiponectin concentration was measured using our novel sandwich ELISA based on a monoclonal antibody to human HMW adiponectin (Fujirebio, Tokyo, Japan). GBP28, the target antigen, is an HMW adiponectin purified from human serum with Gelatin-Cellulofine (Seikagaku, Tokyo, Japan) (3). The monoclonal antibody IH7, raised against human GBP28, was used as the capture antibody, whereas horseradish peroxidase-conjugated IH7 Fab (POD-IH7) was used as the detecting antibody, and GBP28 was used as the standard (19). In brief, the monoclonal antibody IH7 was prepared by conventional methods. HMW adiponectin (GBP28) was purified from human plasma as described previously (3). The plasma supplemented with 5 mmol/l EDTA was applied to the gelatin-Cellulofine column equilibrated with 10 mmol/l Tris-HCl, pH 7.4, and 150 mmol/l NaCl (Tris-buffered saline). The fractions containing HMW adiponectin were applied to HiPrep 16/60 Sephacryl 300-HR column (Pierce, Rockford, IL) equilibrated with Tris-buffered saline containing 1 mmol/l EDTA. The purity was checked by SDS-PAGE under the conditions with or without reduction by 2-mercaptoethanol and/or with or without boiling for 3 min by Coomassie Brilliant Blue staining (Kanto Chemical, Tokyo, Japan) (3). BALB/c mouse (SLC, Shizuoka, Japan) were immunized with HMW adiponectin in complete Freund's adjuvant. Spleen cells, which were removed from the mice, were fused with the P3U1 myeloma cell line in the presence of polyethylene glycol (PEG-40). The specificity of IH7 was verified using SDS-PAGE and Western blotting under the conditions with or without reduction by 2-mercaptoethanol and/or with or without boiling for 3 min. The IH7 monoclonal antibody recognized mainly HMW adiponectin under the condition without heat denature (19).

This sandwich ELISA measuring essentially only HMW adiponectin in sera

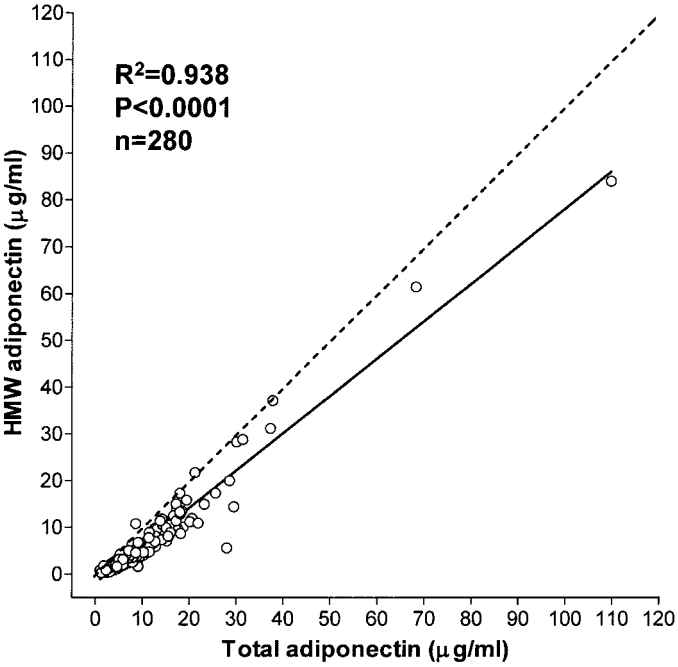

FIG. 2. Correlation between serum total and HMW adiponectin concentrations in patients with type 2 diabetes.

is now available as a kit from Fujirebio. This sandwich HMW ELISA kit is composed of the same monoclonal antibody IH7 as solid-phase and horseradish peroxidase–conjugated detection antibody. It is therefore presumed that this novel HMW adiponectin ELISA kit can measure specifically HMW adiponectin. In brief, 96 wells of a microtiterplate were coated with anti-HMW adiponectin monoclonal antibody (IH7). One hundred microliters of serum samples diluted 1:441 was placed in each of the 96 wells. IH7 conjugated with horseradish peroxidase was used as the detecting antibody. Contents of wells were incubated for 30 min with tetramethylbenzipine. After the reaction was stopped, the absorbance was measured at 450 nm. Using the same antibody as both capturing and detecting antibody, this sandwich ELISA system could specifically measure HMW adiponectin in sera. HMW adiponectin concentrations of a working standard were determined by human HMW adiponectin purified by affinity for gelatin-Cellulofine (3).

To confirm that this novel ELISA measures only HMW adiponectin in sera, we compared the two ELISA methods regarding serum adiponectin concentration in each fraction after fractionation of sera by gel filtration chromatography. In brief, serum samples were filtered through 0.44-μm pore membranes. The serum samples were applied to a HiLoad 16/60Superdex 200 column in fast protein liquid chromatograph (Amersham Bioscience, Piscataway, NJ), which equilibrated with 50 mmol/l Tris-HCl (pH 8.0) buffer solution containing 100 mmol/l NaCl, 1 mmol/l EDTA, and 0.05% sodium azide at 4°C. The samples then were eluted with the same buffer solution at a speed of 0.7 ml/min. Fractions were collected, and concentrations of adiponectin in each fraction were determined by both ELISA methods. The assay was validated by determination of sensitivity (lower detection limit), working range, linearity, accuracy (recovery test), and reproducibility.

Serum concentrations of high-sensitivity C-reactive protein (CRP) were determined by an immunonephelometric assay (N-High-sensitivity CRP; Dade Behring, Marburg, Germany), for which intra- and interassay CVs were 1.72 and 2.80%, respectively. Plasma insulin concentrations were determined by radioimmunoassay. Insulin resistance was evaluated by homeostasis model assessment (HOMA-IR), calculated as fasting plasma insulin (μU/ml) × fasting plasma glucose (mmol/l)/22.5.

Statistical analysis. Data are presented as means ± SD or median and interquartile range. Normally distributed data were analyzed by Student's unpaired *t* test. Nonparametric data were compared between groups by the Mann-Whitney *U* test. Correlations were determined by linear regression analysis or multivariate analysis. Logarithmic transformations of high-sensitivity CRP and HOMA-IR were used to render the distribution normal for parametric tests. Significance of differences between groups in prevalence of categorical characteristics was analyzed by a χ^2 test. A *P* value below 0.05 was accepted as indicating statistical significance.

RESULTS

Figure 1 presents elution profiles for serum adiponectin determined in two individuals by both total and HMW adiponectin ELISA systems. Our specific ELISA system did not measure adiponectin in the trimer to hexamer range in aliquots of each fraction obtained by gel filtration chromatography (Fig. 1*A* and *B*), whereas total adiponectin ELISA system measured all isoforms.

For the validation of our novel sandwich ELISA, the sensitivity and the upper limit of the working range of HMW adiponectin concentration was 0.18–22.05 μg/ml. The dilution curve was close to linear, confirming parallelism between the serum samples (data not shown). The recovery tests ranged from 98.4 to 99.2%. Intra- and interassay CVs were 2.4 to 3.0% and 4.2 to 5.1%, respectively.

We found a close positive correlation between serum HMW and total adiponectin concentrations in all diabetic patients ($r = 0.969$, $P < 0.0001$; Fig. 2). Values for HMW adiponectin determined by our ELISA were all below those of total adiponectin in each individual.

As shown in Table 1, simple linear regression showed positive correlation of serum HMW adiponectin with duration of diabetes ($P < 0.05$), HDL cholesterol ($P < 0.0001$), and fibrinogen ($P < 0.01$) and negative correlation

TABLE 1
Linear regression analysis of relationships between HMW adiponectin, total adiponectin, and the HMW-to-total adiponectin ratio to characteristics of patients with type 2 diabetes

	HMW		Total		Ratio	
Variable	*r*	*P* value	*r*	*P* value	*r*	*P* value
Age (years)	0.1008	0.0933	0.1229	0.0405	0.0259	0.6675
BMI (kg/m^2)	−0.1346	0.0247	−0.1467	0.0144	−0.0505	0.4015
Diabetes duration (years)	0.1400	0.0204	0.1343	0.0260	0.2254	0.0002
FPG (mmol/l)	0.0657	0.2753	0.0711	0.2376	0.0413	0.4933
A1C (%)	−0.0193	0.7513	−0.0107	0.8598	0.0049	0.9359
LDL cholesterol (mmol/l)	−0.0609	0.3116	−0.0711	0.2368	0.0731	0.2243
Triglyceride (mmol/l)	−0.1596	0.0077	−0.1790	0.0027	−0.1381	0.0213
HDL cholesterol (mmol/l)	0.2814	<0.0001	0.2879	<0.0001	0.1522	0.0070
(ln)HOMA-IR	−0.2179	0.0010	−0.2252	0.0007	−0.1566	0.0093
CCr (ml/min)	−0.3100	<0.0001	−0.3277	<0.0001	−0.1921	0.0014
hs-CRP ($\log_{10}$ng/dl)	−0.1616	0.0074	−0.2010	0.0008	−0.0502	0.4075
Fibrinogen (mg/dl)	0.1624	0.0066	0.1452	0.0211	0.2636	<0.0001

FPG, fasting plasma glucose; hs-CRP, high-sensitivity CRP.

TABLE 2
Characteristics and laboratory data for diabetic patients grouped according to sex and presence of CAD

	Women			Men		
	No CAD	CAD	*P* value	No CAD	CAD	*P* value
n	103	18		118	41	
Age (years)	59.6 ± 11.8	67.6 ± 7.7	0.0064	58.1 ± 11.6	63.4 ± 9.4	0.0091
BMI (kg/m^2)	25.4 ± 5.5	25.2 ± 3.4	0.9197	23.9 ± 3.7	25.1 ± 2.5	0.0621
Diabetes duration (years)	11.3 ± 7.7	11.1 ± 7.6	0.9133	10.7 ± 7.9	9.6 ± 7.4	0.4583
Fasting plasma glucose (mmol/l)	10.1 ± 3.3	9.2 ± 3.0	0.2605	9.6 ± 3.0	9.1 ± 7.4	0.3816
A1C (%)	9.8 ± 1.8	8.1 ± 1.9	0.0005	9.5 ± 1.9	8.1 ± 1.6	0.0001
LDL cholesterol (mmol/l)	3.19 ± 0.93	3.14 ± 0.61	0.8330	3.15 ± 0.85	2.94 ± 0.67	0.1608
Triglyceride (mmol/l)	2.46 ± 1.55	2.40 ± 0.91	0.8821	2.34 (1.57–3.15)	2.32 (1.61–2.58)	0.4253
HDL cholesterol (mmol/l)	1.31 ± 0.39	1.21 ± 0.27	0.3413	1.19 ± 0.35	1.16 ± 0.35	0.6383
Creatinine clearance (ml/min)	78.5 ± 35.6	67.8 ± 26.6	0.2661	80.6 ± 45.9	89.0 ± 31.9	0.2772
Total adiponectin (μg/ml)	7.9 (5.4–11.7)	9.4 (8.2–14.9)	0.0684	6.0 (4.4–9.3)	5.6 (3.4–7.3)	0.0849
HMW adiponectin (μg/ml)	4.3 (2.8–7.0)	5.3 (3.7–8.0)	0.3684	2.9 (1.8–5.0)	2.2 (1.2–3.6)	0.0345
Non-HMW adiponectin (μg/ml)	3.1 (2.2–4.2)	5.1 (3.9–6.4)	0.0005	3.0 (2.3–4.3)	2.9 (2.1–3.8)	0.2780
hs-CRP ($\log_{10}$ng/ml)	2.89 ± 0.57	3.06 ± 0.60	0.2450	2.79 ± 0.49	2.98 ± 0.50	0.0343
Hypertension	51 (49.5)	14 (77.8)	<0.05	44 (37.3)	24 (58.5)	<0.05
Nephropathy (nor/micro/macro)	43/32/28	9/4/5	NS	47/33/38	25/10/6	NS
Current smoking	15 (14.6)	4 (22.2)	NS	71 (60.2)	27 (65.9)	NS
Treatment (diet/OHA/insulin)	8/80/15	6/7/5	NS	12/89/17	7/30/4	NS

Data are means ± SD, median (interquartile range), or *n* (%). hs-CRP, high-sensitivity CRP; nor, normoalbuminuria; micro, microalbuminuria; mac, macroalbuminuria; OHA, oral hypoglycemic agent.

with BMI ($P < 0.05$), triglyceride ($P < 0.01$), HOMA-IR ($P < 0.01$), CCr ($P < 00001$), and high-sensitivity CRP ($P < 0.01$). Total adiponectin in serum correlated positively with age ($P < 0.05$), duration of diabetes ($P < 0.05$), HDL cholesterol ($P < 0.0001$), and fibrinogen ($P < 0.05$) and correlated negatively with BMI ($P < 0.05$), triglyceride ($P < 0.01$), HOMA-IR ($P < 0.001$), CCr ($P < 0.0001$), and high-sensitivity CRP ($P < 0.001$). The HMW-to-total adiponectin ratio correlated positively with duration of diabetes ($P < 0,001$), HDL cholesterol ($P < 0.001$), and fibrinogen ($P < 0.0001$) and negatively with triglyceride ($P < 0.05$), HOMA-IR ($P < 0.01$), and CCr ($P < 0.01$).

In nondiabetic women, serum total and HWM adiponectin concentrations were 9.8 (7.3–12.8) and 6.4 μg/ml (3.8–8.6), respectively. The HMW-to-total adiponectin ratio was 0.59 ± 0.10. In nondiabetic men, serum total and HWM adiponectin concentrations were 7.6 (4.9–8.3) and 3.4 μg/ml (2.1–5.2), respectively. The HMW-to-total adiponectin ratio was 0.52 ± 0.17.

Both total and HMW serum adiponectin concentrations were significantly higher in women than in men (8.20 [5.45–12.2] vs. 5.95 μg/ml [4.20–9.10], $P < 0.0001$; 4.40 [2.80–7.60] vs. 2.80 μg/ml [1.70–4.60], $P < 0.0001$). Furthermore, the HMW-to-total adiponectin ratio was significantly higher in women than men (0.58 ± 0.17 vs. 0.49 ± 0.14, $P < 0.0001$). However, we found no significant difference in serum non-HMW (total minus HMW) adiponectin between women and men (3.4 [2.3–4.7] vs. 3.0

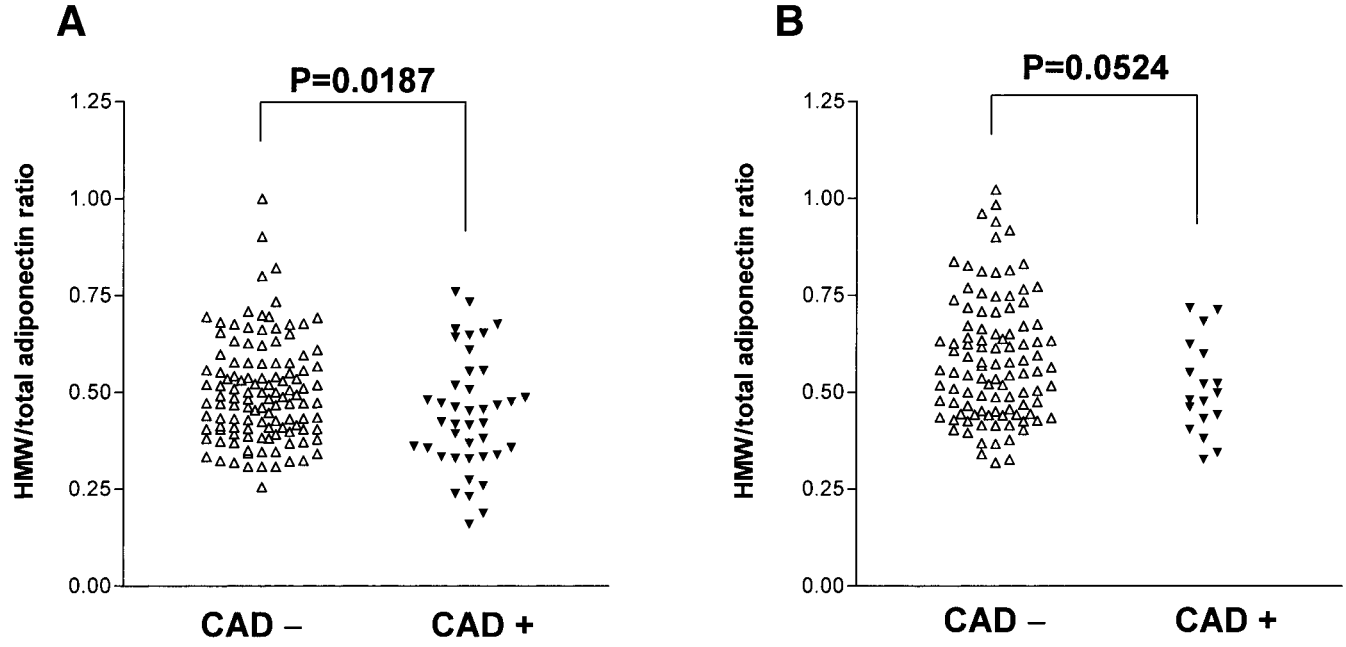

FIG. 3. HMW-to-total adiponectin ratios compared between diabetic patients (*A*, men; *B*, women) with and without CAD.

μg/ml [2.3–4.3], $P = 0.3792$). CAD was significantly more prevalent in men than women (25.8 vs. 14.9%, $P < 0.05$).

Considering sex differences in the prevalence of CAD, total and HMW adiponectin total-to-HMW adiponectin ratio, we investigated the relationship between CAD and adiponectin separately for each sex. Women with CAD were older than those without CAD. HbA_{1c} (A1C) was significantly lower in women with than in those without CAD. Although we found no significant difference in total adiponectin or HMW adiponectin concentration between diabetic women with and without CAD (Table 2), the HMW-to-total adiponectin ratio tended to be lower in diabetic women with than without CAD (0.51 ± 0.12 vs. 0.59 ± 0.17, $P = 0.0524$; Fig. 3*B*). Hypertension was more prevalent in diabetic women with CAD than in those without CAD ($P < 0.05$; Table 2).

Men with CAD were older than those without CAD. A1C also was significantly lower in men with than without CAD. Serum high-sensitivity CRP was significantly higher in diabetic men with than without CAD. Although we found no significant difference in serum total adiponectin between men with and without CAD, serum HMW adiponectin was significantly lower in men with than without CAD ($P < 0.05$; Table 2). Furthermore, the HMW-to-total adiponectin ratio was significantly lower in men with than without CAD (0.44 ± 0.15 vs. 0.50 ± 0.13, $P = 0.0187$; Fig. 3*A*). However, we found no significant difference in serum non-HMW adiponectin between men with than without CAD (Table 2). Hypertension was more prevalent in men with than without CAD ($P < 0.05$; Table 2).

To investigate whether HMW-to-total adiponectin ratio is an independent factor for CAD, we performed multiple logistic regression analysis controlling age and sex. The HMW-to-total adiponectin was an independent determinant of CAD in patients with type 2 diabetes (partial coefficient −0.1269, $P = 0.0106$; Table 3). Both sex and age were independently associated with CAD (partial coefficient 0.0828, $P = 0.0474$; partial coefficient 0.1804, $P = 0.0008$, respectively).

TABLE 3
Multiple logistic regression analysis of CAD

Variable	β	*P* value	Wald χ^2
Sex (men)	0.0828	0.0474	3.9312
Age	0.1804	0.0008	11.1619
HMW-to-total adiponectin ratio	−0.1269	0.0106	6.5382

The partial coefficient is indicated as β.

DISCUSSION

This study is the first to compare serum concentrations of HMW adiponectin in patients with type 2 diabetes measured by our novel sandwich ELISA with total adiponectin concentrations. When each fraction obtained by gel filtration chromatography was analyzed by this ELISA system, the method was HMW specific and did not measure trimer or hexamer adiponectin. Simple linear regression analysis demonstrated a very strong positive correlation between serum total and HMW adiponectin in diabetic patients, suggesting that it can accurately measure adiponectin concentrations in sera. Linear regression analysis confirmed that each HMW adiponectin result obtained by this ELISA was below that for total adiponectin in each individual, supporting specificity of the HMW ELISA. Thus, we now can reliably measure serum concentrations of HMW adiponectin. Previous studies have suggested that different isoforms of adiponectin have different biological activities (7–9). In particular, the ratio of HMW to total adiponectin may be a particularly sensitive marker of the biological activity of adiponectin, because changes in the ratio correlated with changes in sensitivity after treatment with thiazolidinedione (10,11). Moreover, the ratio was significantly lower in patients with CAD than in healthy subjects (12).

Accurate serum determinations of HMW adiponectin are important in assessing relationships between adiponectin and diabetes or atherosclerosis. Previously, determinations of HMW adiponectin in plasma were only semiquantitative and required size fractionation by velocity sedimentation followed by SDS-PAGE and Western blotting (10,12,16–18). The time and effort involved in such assays essentially precluded their use in the clinical study of large cohorts. In contrast, our method for measuring HMW adiponectin is an ELISA system appropriate for high throughput and does not even require pretreatment of samples, unlike the commonly used ELISA for total adiponectin, which uses heat denature and reduction in SDS buffer to convert all adiponectin in samples to monomeric form (13).

As described above, we noted a very strong correlation between serum HMW and total adiponectin ($r = 0.969$), in agreement with a recent study in offspring whose parents both had type 2 diabetes using velocity sedimentation with Western blotting ($r = 0.72$ between total adiponectin and the HMW-to-total adiponectin ratio) (20). This suggests that total and HMW adiponectin may be regulated synchronously. The present study demonstrated that both total and HMW adiponectin were related to BMI, blood lipids, including HDL cholesterol, HOMA-IR, and renal function. These results are in agreement with previous studies of total adiponectin (13,14,21,22). In a previous study, we reported that in a model that explained 61.7% of variation of serum total adiponectin, sex, A1C, HDL cholesterol, and renal function were independent determinants of total adiponectin in sera from patients with type 2 diabetes (23). The present study in diabetic patients demonstrated that serum HMW adiponectin similarly was associated with clinical variables shown to be related to total adiponectin. Thus, the kidneys and the liver are the main elimination sites for circulating adiponectin, including HMW adiponectin (23–25).

We confirmed that serum total and HMW adiponectin were significantly higher in diabetic women than men, suggesting that some adiponectin variability is sex related (7,9). Furthermore, we found a higher ratio of HMW to total adiponectin in women than in men. Pajvani et al. (7) found that female mice also had higher HMW concentrations in serum than males. Furthermore, in humans, Waki et al. (9) found the HMW form, but not MMW or LMW forms, to be significantly less abundant in men than women. A number of previous studies have demonstrated higher serum concentrations of total adiponectin in women than in men (13,23,26). The sex difference in total adiponectin concentration may reflect mainly the higher concentrations of HWM adiponectin in women. We also found no significant difference in serum non-HMW adiponectin, i.e., LMW and MMW adiponectin, between women and men. Recent studies have demonstrated that testosterone selectively reduces the HMW form of adi-

ponectin by inhibiting its secretion by adipocytes (27,28). Such suppression of HMW adiponectin might contribute to the sexual dimorphism of adiponectin oligomeric complex distribution and partly explain higher risk for insulin resistance and atherosclerosis in men than in women. Accordingly, suppression of HMW adiponectin in men may contribute to their higher cardiovascular risk. The present study also showed a greater prevalence of CAD in men.

The present study demonstrated that in diabetic men, serum HMW adiponectin and the HMW-to-total adiponectin ratio were significantly lower in those with than without CAD. On the other hand, in diabetic women, the ratio of HMW to total adiponectin also was lower when CAD was present, although falling short of statistical significance. These results suggest that a low fraction of serum adiponectin in the HMW form is associated more strongly than low total adiponectin with CAD in diabetic patients, especially men. One small previous study of nondiabetic subjects reported a significantly lower percentage of HMW adiponectin in sera from eight patients with CAD than in eight healthy subjects (12). The present study confirmed in a relatively larger number of diabetic subjects that low serum HMW adiponectin and the amount relative to total adiponectin were associated closely with CAD.

However, we found a significant difference in age between diabetic patients with and without CAD. Advancing age, a nonreversible factor, is a major risk factor for atherosclerotic vascular disease, irrespective of diabetes. Unlike in the men, we could not find a significant difference in the HMW-to-total adiponectin ratio between women with and without CAD. It is well established that men have higher risk for CAD than women. We therefore performed multiple logistic regression analysis controlling age and sex to investigate whether HMW-to-total adiponectin ratio is an independent factor for CAD. We found that the HMW-to-total adiponectin ratio was an independent predictor of CAD in patients with type 2 diabetes.

Mechanisms responsible for the low HWM-to-total adiponectin ratio in diabetic patients with CAD remain unclear. When members of our group used gelatin-affinity chromatography to purify HMW adiponectin (GBP28) in serum, only the 440-kDa multimer, representing the HMW isoform, bound to the gelatin column (3). This suggested that HMW adiponectin has higher affinity for collagen than LMW or MMW isoforms. In an in vitro study, adiponectin bound specifically to collagens I, III, and IV, which are present mainly in the vascular intima (29). Adiponectin contains three domains: an NH_2-terminal signal sequence, a collagen domain, and a COOH-terminal globular domain. Thus, adiponectin possesses structural similarity to collagens VIII, X, and C1q, which interact with extracellular matrix proteins (30). Recently, Ouchi et al. (31) identified adiponectin in the catheter-injured rat vascular wall but not in intact vascular wall, suggesting that decreases in plasma adiponectin might correlate with the severity of atherosclerotic vascular disease. We suspect that circulating HMW adiponectin binds specifically to exposed collagen in the injured endothelium of the vascular wall in patients with CAD, a removal resulting in a decrease of serum HMW adiponectin. Because the HMW-to-total adiponectin ratio showed a particularly strong relationship with CAD, measuring both serum total and HMW adiponectin to calculate the ratio is preferable to measuring total or HMW adiponectin alone.

The present study clearly has several limitations. The major limitation is the cross-sectional nature of the design. Because a causal relationship cannot be proven by cross-sectional data, a prospective study should be undertaken to confirm causality between HMW-to-total adiponectin ratio and development of CAD. Another limitation is the possibility of selection bias or ascertainment bias. Only subjects with symptoms suggestive of CAD underwent coronary angiography. It is therefore possible that some CAD-affected patients buried among the control subjects classified as non-CAD, because silent (asymptomatic) myocardial ischemia is common in patients with diabetes. This may reduce the difference in HMW-to-total adiponectin ratio between diabetic women with and without CAD.

In conclusion, we can specifically quantitate serum concentrations of HMW adiponectin using our novel ELISA system. Such measurement of serum HMW concentration, especially as a ratio to total adiponectin, is more useful clinically for evaluating CAD in patients with type 2 diabetes than simply measuring total serum adiponectin.

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