human adiponectin mutations, which are associated with diabetes and hypoadiponectinemia, did not form HMW multimers [14], raising the possibility that each multimer may have distinct functions. However, the precise components of each multimer have not been fully determined.

In this study, we describe selective purification and characterization of multimer species of adiponectin from human plasma by affinity column using anti-adiponectin and gelatin, and gel filtration.

Materials and methods

Purification of human adiponectin multimers. Fig. 1 shows the process of purification of multimer adiponectin from human plasma. Human plasma (3 L from a pool of 250 L Advanced BioServices, L.L.C. according to the principles laid out by FDA, informed consent for research use was obtained from each subject) was applied to a column (80 mL) consisting of CNBr-Sepharose4B resin with rabbit anti-mouse adiponectin globular domain antibody [4]. After washed with 100 mM Tris–HCl (pH 8.5) containing 0.5 M NaCl and 100 mM acetate-Na (pH 5.0), proteins were eluted with 100 mM glycine–HCl (pH 2.5). The eluant was collected (10 mL) into tubes containing 2 M Tris–HCl pH 8.0 (1 mL) for neutralization. The pooled fraction was applied to a Protein-A Sepharose column for removing immunoglobulins. The non-binding fraction was dialyzed against 20 mM sodium phosphate (pH 7.0).

Separation of purified human adiponectin multimers. The purified fraction was applied to a gelatin-celullofine column (25 mL, equilibrated with 20 mM sodium phosphate, pH 7.0) and eluted stepwise with 20 mM sodium phosphate (pH 7.0) containing 100, 200, and 500 mM NaCl. The fractions eluted with 500 mM NaCl was further purified by gel filtration chromatography. The fractions eluted with 100 mM NaCl were applied to a column (25 mL) that combined the CNBr-Sepharose4B resin with goat anti-human albumin antibody (Capple). Washed with PBS, proteins were eluted with 100 mM glycine–HCl (pH 2.5). The eluant was collected (5 mL) into tubes containing 2 M Tris–HCl pH 8.0 (0.5 mL) for neutralization and was purified by gel filtration chromatography. Non-binding fractions were separated by gel filtration chromatography. All purifications were performed at 4 °C except for the gel filtration chromatography.

Gel filtration chromatography. Proteins were applied to HiPrep 26/60 Sephacryl S-300 HR (GE Healthcare) column equilibrated with PBS and eluted at room temperature in the same buffer at a flow rate of 1.5 mL/min. Eluted proteins were monitored at 280 nm.

Western blotting analysis. Native PAGE and SDS–PAGE were performed according to the standard methods of Davis [18] and Laemmli [19], respectively. For immunoblotting, proteins separated by native PAGE or SDS–PAGE were transferred to polyvinylidene difluoride membranes (Millipore). Following transfer, the membranes were blocked with 5% skim milk in PBS. The membranes were reacted with goat anti-adiponectin antibody and then reacted with HRP-conjugated rabbit anti-goat IgG using a Vector ABC kit according to the manufacturer's instruction. The membranes were stained with diaminobenzidine. In this study, native PAGE was performed 2–15% and detected with CBB staining.

N-terminal amino acid analysis. For amino-terminal sequencing, the sample was transferred to a Sequiblot PVDF membrane (Bio-Rad) after SDS–PAGE resolution. The band was visualized by Coomassie Brilliant Blue R-250 (Bio-Rad) staining, excised, and subjected to Edman degradation N-terminal analysis [20].

C2C12 cell culture. Induction of myogenic differentiation was carried out according to Ref. [9]. In brief, the mouse C2C12 myoblasts were grown in 90% DMEM and 10% (v/v) FBS. When the cells were 80% confluent, the myoblasts were induced to differentiate into myotubes by replacing the medium with a low serum differentiation medium (97.5% DMEM, 2.5% (v/v) horse serum), which was changed daily. By day 5, the cells had differentiated into multinucleated contracting myotubes.

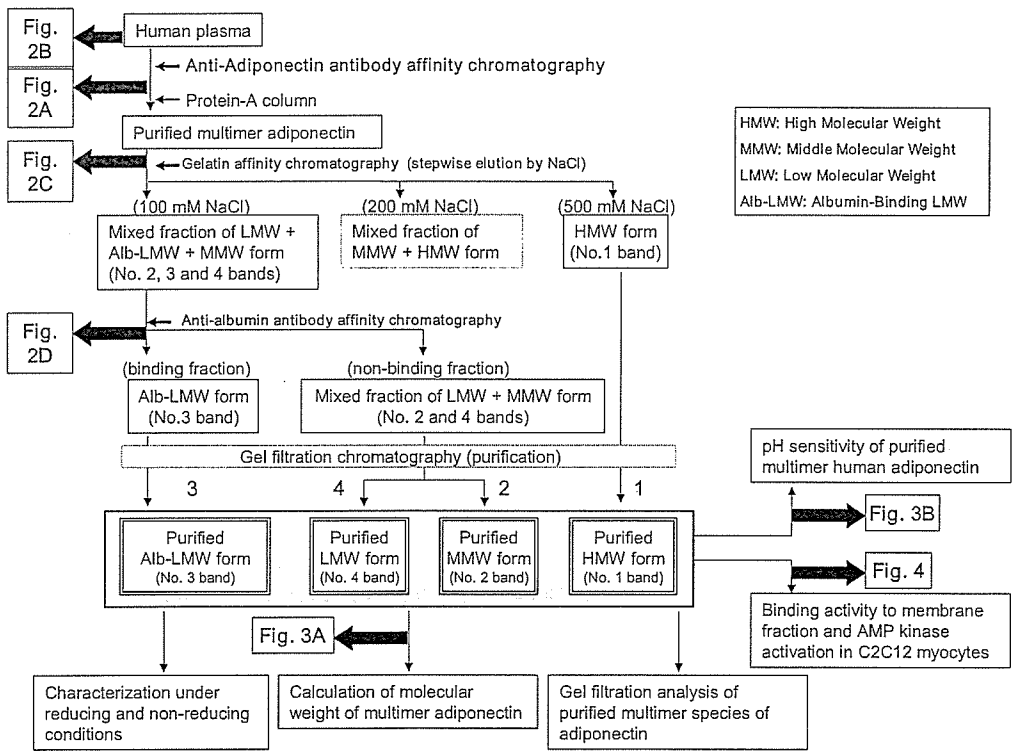

Fig. 1. Selective purification schema of human adiponectin multimers. This shows the process of purification of multimer adiponectin from human plasma and our process of analysis. No. 1–4 bands are match to Fig. 2A. Each figure number is matched to each process.

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Binding activity of multimer adiponectin to membrane fraction. Each purified multimer adiponectin was ^{125}I-labeled at Tyr by IODO-beads (Pierce) in the presence of Na^{125}I (2000 Ci/mmol, GE healthcare science) according to the manufacturer's protocol. Membrane fractions of myocytes were purified by sucrose gradient ultracentrifugation and incubated at 4 °C for 1 h with binding buffer (ice-cold PBS/10% skim milk) containing the designated concentrations of [^{125}I]adiponectin (5000 cpm/ng of protein) plus unlabeled competitors of adiponectin multimers purified from human plasma. The binding equilibrium was judged to have been established when the binding assay was conducted at 4 °C after 2 h. The membrane fractions were then washed three times with ice-cold PBS, and the membrane-bound radioactivity was determined using a γ-counter. Nonspecific binding was determined using a 200-fold excess of unlabeled adiponectin. Specific binding was calculated by subtracting the nonspecific binding from the total binding. The values presented under "Results" represent an average of triplicate determinations of three experiments.

Phosphorylation of AMPK by adiponectin. The AMPK pathway was analyzed according to Ref. [14]. Briefly, after the cells had been incubated in serum-free DMEM medium for 6 h, DMEM containing 1 or 10 μg/ml of each purified multimer adiponectin was added to the well and incubated for 5 min at 37 °C. The reaction was stopped with liquid nitrogen and the cells were lysed and homogenized by a sonicator in lysis buffer (25 mM Tris–HCl, pH 7.4, 10 mM Na_3VO_4, 10 mM sodium pyrophosphate, 100 mM NaF, 10 mM EDTA, 10 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, and 1% Nonidet P-40). The lysate was centrifuged and the protein concentration was assayed using BCA protein assay reagent (Pierce). The same amount of lysate protein was applied to SDS–PAGE under reducing and heat-denaturing conditions, blotted onto PVDF membranes and immunostained with anti-phosphorylated AMPK. NIH-Image was used for band quantification.

Results

Selective purification of human multimer adiponectin and analysis by native PAGE and SDS–PAGE

The purified multimer species of adiponectin from human plasma were obtained from affinity chromatography of anti-adiponectin antibody, separated by native PAGE. Four different bands were detected as protein bands of adiponectin and were defined as No. 1–4 bands (Fig. 2A) except for a band around 70 kDa which was not detected with anti-adiponectin antibody among CBB stained bands (data not shown). Hereafter No. 1–4 bands mean each numbered bands of Fig. 2A.

In the same way, human plasma was separated by native PAGE. Among four bands of adiponectin, three bands, No. 1–3 bands, were identified clearly as three different isoforms of adiponectin (Fig. 2B). No. 4 band was also identified as the fourth isoform of adiponectin with anti-adiponectin antibody, but much lesser extent (Fig. 2B).

To analyze the component of each multimers, the gels of bands corresponding to each form of adiponectin (No. 1–4 bands) were eluted using an electro-eluter (model 422 Bio-Rad) and separated by 10–20% SDS–PAGE and detected with CBB staining. Adiponectin has been reported having a disulfide bond through an amino-terminal cysteine and thus could be detected as a dimer and a monomer under nonreducing and heat-denaturing conditions [14]. No. 1 and 2 bands were converted to the 60 kDa dimers and No. 4 band was converted to the 60 kDa dimers and the 30 kDa monomers under nonreducing and heat-denaturing conditions (data not shown). Interestingly, the double bands No. 3 were converted to the 30 kDa monomers and a 67 kDa protein under reducing and heat-denaturing conditions. This 67 kDa band was identified as human albumin using N-terminal amino acid analysis (from N-terminal, D-A-H-K-S, it was the same as human albumin). The double bands No. 3 were also converted to dimers and a 90–100 kDa band under nonreducing and heat-denaturing conditions. This 90–100 kDa band was thought to

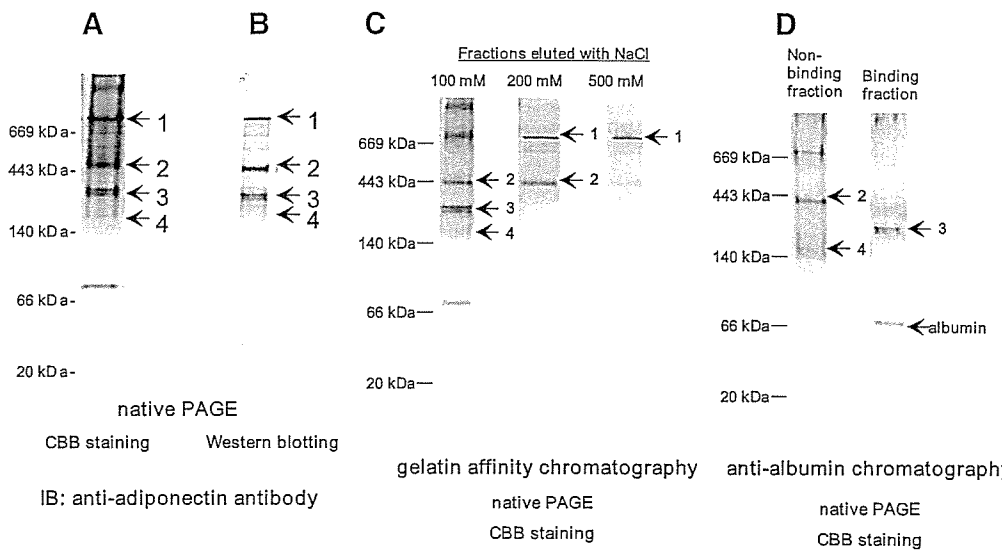

Fig. 2. (A) Separation of multimer species of adiponectin from human plasma. Multimer species of adiponectin were obtained, and four different bands were detected (numbered arrows). (B) Western blot analysis of multimer species of adiponectin from human plasma. (C) Eluted fractions from gelatin affinity chromatography with NaCl. No. 1 band was purified in this step with 500 mM NaCl solution. (D) Non-binding fraction and binding fraction from human anti-albumin antibody affinity chromatography. Elute with 100 mM (C) was applied onto anti-albumin antibody affinity chromatography. No. 3 band was purified as binding fraction.

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be albumin-binding monomers based on their molecular weight (data not shown).

To purify each adiponectin multimers species, we applied binding fractions of anti-adiponectin affinity column onto gelatin affinity chromatography followed by stepwise NaCl elution (Fig. 2C). The eluted fractions with 100 mM NaCl and 200 mM elution were contained three bands (No. 2–4 bands) and two bands (No. 1 and 2 bands), respectively. No. 1 band was purified in this step with 500 mM NaCl elution (Fig. 2C). To separate the albumin-binding adiponectin (No. 3 band), the eluted fraction with 100 mM NaCl was applied onto anti-albumin antibody affinity chromatography. No. 3 band was separated using anti-albumin antibody affinity chromatography (Fig. 2D). No. 2 and 4 bands, which exist in non-binding fraction of anti-albumin chromatography (Fig. 2D) were separated. Each elution was separated and purified by gel filtration chromatography. Finally, we obtained each selective purified fractions of human adiponectin multimers and separated these fractions by native PAGE (data not shown).

Calculation of molecular weight of multimer adiponectin from human plasma

To determine these molecular weights accurately, purified multimer species of adiponectin were cross-linked with bis(sulfosuccinimidyl) suberate (BS^3, Pierce) as described previously [1] and analyzed by SDS–PAGE and Western blotting. From the results for cross-linking with BS^3, No. 2, 3, and 4 bands became 200–250, 150 and 100 kDa and corresponded to the hexamer, trimer and one albumin molecule, and trimer, respectively. The molecular weight of No. 1 band seemed to be too large to determine the molecular weights by this method (Fig. 3A). We defined adiponectin form No. 1 band as HMW, No. 2 band as MMW, No. 3 band as Alb-LMW and No. 4 band as LMW. Fur-

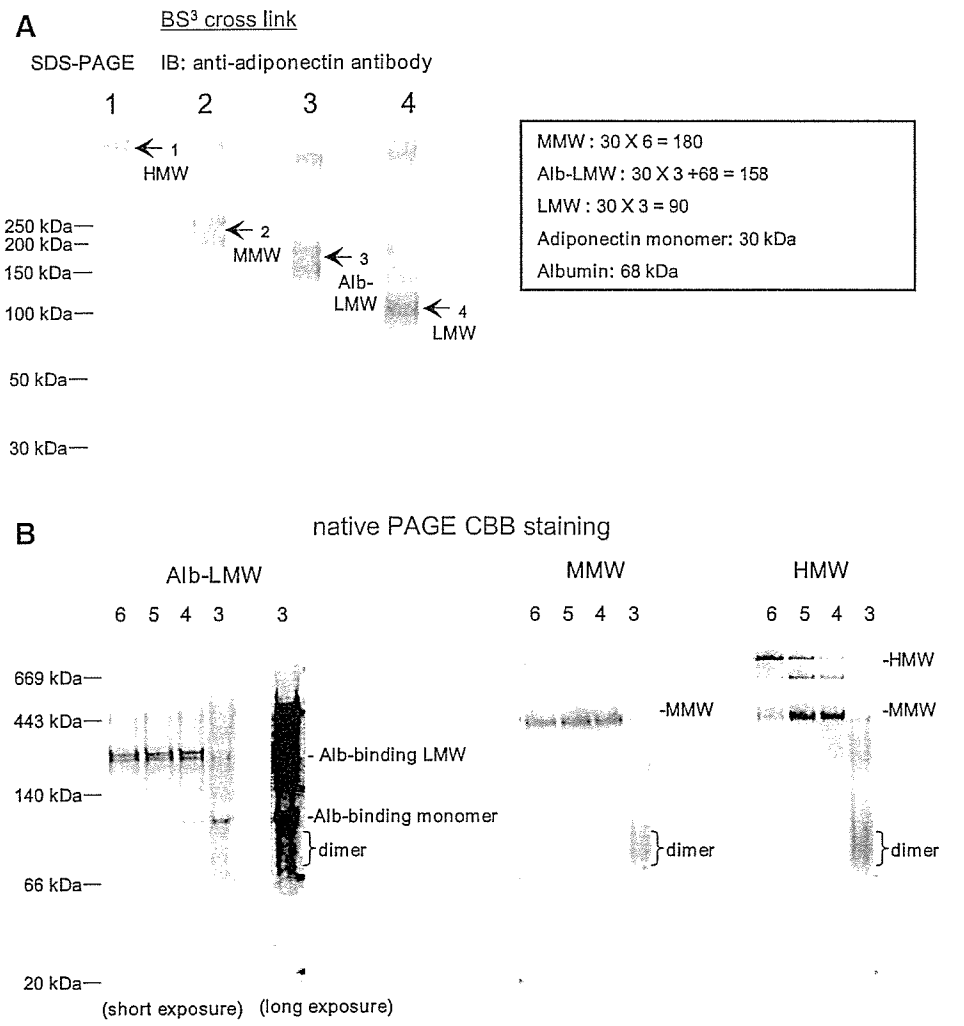

Fig. 3. (A) Calculation of molecular weight of multimer adiponectin from human plasma. Purified multimer species of adiponectin were cross-linked with BS^3. The molecular weight of adiponectin monomer is about 30 kDa, albumin is about 68 kDa. (B) pH sensitivity of purified multimer human adiponectin. At pH 3.0, Alb-LMW disrupted to albumin-binding monomers and dimers. Dimers were detected under long exposure.

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thermore, we majored each purified multimer adiponectin by gel filtration. It is suggested that Alb-LMW is difficult to separate from MMW by gel filtration because of their similar peak (data not shown).

Influence of pH on adiponectin multimer formation from human plasma

Low pH denaturation is reported to result in trimer dissociation and produce dimers and monomers [4]. Purified human adiponectin was analyzed by native PAGE after treating each purified multimer for 30 min at 37 °C in 100 mM citrate-Na, pH 6.0–3.0 (Fig. 3B). At pH 3.0, Alb-LMW adiponectin was disrupted to dimers and albumin-binding monomers. MMW and HMW adiponectin were disrupted to dimers. Alb-LMW and MMW maintained their multimer forms above pH 4.0 (Fig. 3B, left and mid panel), but purified HMW shifted to MMW together with an intermediate structure at pH 5.0–4.0 (Fig. 3B, right panel). These results suggest that HMW consists of some MMW, and its size is at least 12 mer or more.

Binding of multimer adiponectin to membrane fractions and AMPK activation

To examine whether each multimer form of adiponectin might have different biological characteristics, we measured the binding activities of HMW, MMW and LMW to the membrane fractions of C2C12 myocytes which express AdipoR1 abundantly but express much less AdipoR2 [9]. HMW had the highest binding activity towards membrane fractions (Fig. 4A). We next studied which multimer forms of adiponectin could activate AMPK most potently in C2C12 myocytes and found that HMW was the most potent form (Fig. 4B). This result is consistent with the observation that HMW had the highest binding activity.

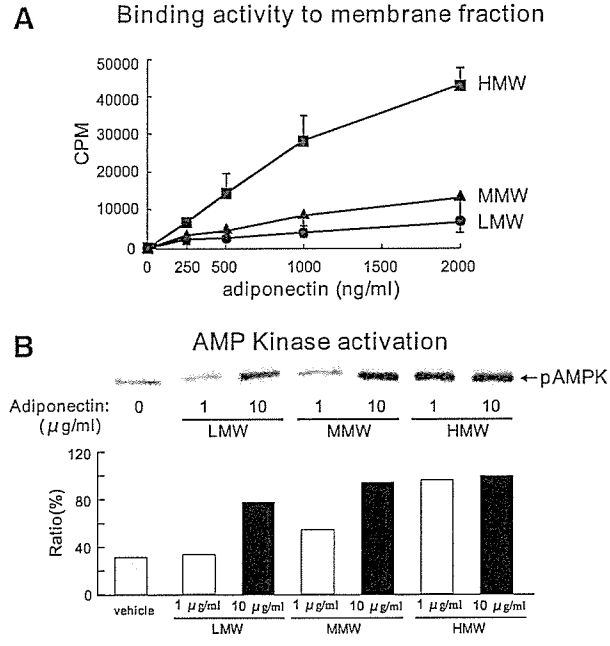

Fig. 4. (A) Binding activity to membrane fraction of HMW, MMW, and LMW. Binding of ^{125}I-labeled HMW, MMW and LMW to membrane fractions of C2C12 myocytes which express AdipoR1 abundantly but AdipoR2 much less so [9]. (B) AMPK activation by HMW, MMW, and LMW. Representative data from one of three independent experiments is shown. Vertical axis of this graph is performed HMW 10 μg/ml phosphorylation to be 100%. Black column is 10 μg/ml adiponectin, white column is 1 μg/ml adiponectin and grey column is viecle. HMW increased AMPK phosphorylation more potently than MMW and LMW.

Discussion

We could purify adiponectin multimers while maintaining their structures in human plasma for the first time by using anti-adiponectin affinity chromatography. Moreover, we could determine the structures, molecular weights and the functions of adiponectin multimers from human plasma. Previously, adiponectin was shown to form HMW, MMW, and LMW, using recombinant adiponectin expressed in *Escherichia coli* or mammalian cells. We revealed that the human plasma also contains these HMW, MMW and LMW. Moreover, we identified a protein which interacted with adiponectin, and found that a substantial amount of the adiponectin shared disulfide bonds with albumin. Thus our detailed analysis revealed that human adiponectin in plasma is composed of a trimer (LMW), albumin-binding trimer (Alb-LMW), hexamer (MMW) and 12 mer or larger multimers (HMW).

We found that the HMW consists of some MMW based on the pH sensitivity data (Fig. 3B), and that the MMW consists of two LMW linked by a disulfide bond between cysteine residues at the N-terminus [14]. All multimers are converted to trimers by reduction of disulfide bonds between the trimers. On the other hand, heat-denaturation results in trimer dissociation and produces dimers and monomers. Adiponectin is converted to a 28 kDa monomer by both treatments. By contrast, C39 S mutant adiponectin, which lacks the amino-terminal cysteine, exists exclusively as a trimer and is converted to a monomer by heat-denaturation [14]. Tsao found that assembly of adiponectin multimers depends upon formation of N-terminal disulfide bonds [16]. In this study, Alb-LMW was converted to albumin and a trimer under reducing conditions (data not shown). Thus we thought that the cysteine residue of the N-terminus of LMW binds albumin forming Alb-LMW. Whether the cysteine residue on the trimer that is not associated with albumin is free or binds with small thiols is currently under investigation. We believe that the trimer form of adiponectin secreted into the bloodstream combines with albumin through SH-bases in plasma. This was also seen in murine plasma (data not shown), suggesting that it might be common in mammals. Considering them, it is thought that Alb-LMW may have influence on the formation of multimer structures, and may have effects on functional activities and the degradation of adiponectin multimers. In particular, when measuring the amount of adiponectin multimers using anti-adiponectin antibodies,

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since there is the possibility of interference of albumin-binding trimer with anti-adiponectin antibodies, it is necessary to care this interference when establishing the assay system for measuring adiponectin multimers. In addition, preliminary experiments revealed that Alb-LMW had almost the same binding activity as LMW and could activate AMPK to almost the same extent as LMW (data not shown). Whether the function of Alb-LMW would differ from that of LMW or the amount of Alb-LMW would change during different metabolic conditions remains to be clarified.

In our preliminary measurement of adiponectin multimers concentration in normal human plasma, HMW is 2–5 μg/ml in male and 2–9 μg/ml in female, MMW is 2–5 μg/ml and 2–8 μg/ml, and Alb-LMW (including LMW) is 1–4 μg/ml both. Following these results we selected stimulation concentration of adiponectin multimers. Previously, it is shown that recombinant HMW activates AMPK most potently in human umbilical vein endothelial cells and specifically confers the vascular protective activities of adiponectin, but they used recombinant adiponectin [21]. In this study, we showed the different binding activity to the membrane fractions and different ability of activation of AMPK phosphorylation of each adiponectin multimers purified from human plasma. HMW had the highest binding activity and activated AMPK most potently. We showed that adiponectin increased glucose uptake via AdipoR1 and AMPK in C2C12 myocytes. These results raise the possibility that blood levels of HMW may be more closely correlated with insulin sensitivity than total adiponectin levels. This is indeed the case [22–25], thus the measurement of HMW may be of important clinical value.

Acknowledgments

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Measurement of the High-Molecular Weight Form of Adiponectin in Plasma Is Useful for the Prediction of Insulin Resistance and Metabolic Syndrome

KAZUO HARA, MD, PHD[1,2]
MOMOKO HORIKOSHI, MD, PHD[1,2]
TOSHIMASA YAMAUCHI, MD, PHD[1,2]
HIROKAZU YAGO[3]
OSAMU MIYAZAKI[3]
HIROYUKI EBINUMA[3]
YASUSHI IMAI, MD, PHD[4,5]
RYOZO NAGAI, MD, PHD[4,5]
TAKASHI KADOWAKI, MD, PHD[1,2]

OBJECTIVE — The high–molecular weight (HMW) form of adiponectin, an adipocyte-derived insulin-sensitizing hormone, has been reported to be the most active form of this hormone. We investigated whether measurement of plasma HMW adiponectin levels, using our newly developed enzyme-linked immunosorbent assay system for selective measurement of human HMW adiponectin level, may be useful for the prediction of insulin resistance and metabolic syndrome.

RESEARCH DESIGN AND METHODS — A total of 298 patients admitted for diabetes treatment or coronary angiography served as study subjects. Receiver operator characteristic (ROC) curves for the HMW ratio (HMWR; ratio of plasma level of HMW adiponectin to that of total adiponectin) and plasma total adiponectin levels were plotted to predict the presence of insulin resistance and metabolic syndrome.

RESULTS — The area under the ROC curve (AUC) of the HMWR values to predict the presence of insulin resistance was significantly larger than that of plasma total adiponectin level in total subjects (0.713 [95% CI 0.620–0.805] vs. 0.615 [0.522–0.708], $P = 0.0160$). The AUC for the HMWR values to predict the presence of metabolic syndrome was significantly larger than that for plasma total adiponectin levels in men (0.806 [0.747–0.865] vs. 0.730 [0.660–0.800], $P = 0.0025$) and in women (0.743 [0.659–0.828] vs. 0.637 [0.532–0.742], $P = 0.0458$).

CONCLUSIONS — The HMWR value has better predictive power for the prediction of insulin resistance and metabolic syndrome than plasma total adiponectin level.

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Adiponectin (also known as ACRP30, GBP28, and AdipoQ) is a hormone secreted exclusively by adipocytes (1–4). Adiponectin replenishment has been found to ameliorate the abnormalities of metabolic syndrome, including insulin resistance, hyperglycemia, and dyslipidemia, in a murine model of obesity-linked metabolic syndrome associated with decreased adiponectin levels (5). Adiponectin-deficient mice (6,7) have been demonstrated to show features of metabolic syndrome, such as insulin resistance, glucose intolerance, dyslipidemia, and hypertension. In humans, decreased plasma adiponectin levels have been demonstrated in patients with obesity, diabetes, and coronary artery disease (8–10), all of which are linked to insulin resistance. Moreover, the degree of hypoadiponectinemia has been reported to be correlated with the degree of insulin resistance (11,12), and hypoadiponectinemia has been shown to be closely associated with the clinical phenotype of metabolic syndrome (13,14). The gene encoding adiponectin (*APM1*) has been mapped to chromosome 3q27, which has been reported to be linked to type 2 diabetes and metabolic syndrome by genome-wide scans in Japanese (15), American, (16), and French-Caucasian (17) populations. A single nucleotide polymorphism in the adiponectin gene was shown to be associated with hypoadiponectinemia, insulin resistance, and increased risk of type 2 diabetes (18,19), indicating that adiponectin may play a crucial role in the regulation of insulin sensitivity and glucose and lipid metabolism and that reduced plasma adiponectin levels caused by genetic and environmental factors may lead to the development of insulin resistance, type 2 diabetes, and metabolic syndrome (20). Indeed, a recent study demonstrated that individuals with high plasma adiponectin levels had a substantially lower relative risk of developing type 2 diabetes, even after adjustment for conventional risk factors, such as BMI (21,22).

We have reported that adiponectin forms multimers and is present in the serum as a trimer, hexamer, or as a high–molecular weight (HMW) form (23). The HMW isoform binds most avidly to its receptors and stimulates AMP-activated protein kinase, one of the key molecules mediating the metabolic actions of adiponectin (Y. Hada, T.Y., H. Waki, K.H.,

From the [1]Department of Metabolic Diseases, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; [2]Core Research for Evolutional Science and Technology, Japan Science and Technology, Tokyo, Japan; the [3]Diagnostics Research Laboratories, Daiichi Pure Chemicals, Ibaraki, Japan; the [4]Department of Cardiovascular Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; and the [5]Department of Clinical Bioinformatics, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

Address correspondence and reprint requests to Dr. Takashi Kadowaki, Department of Metabolic Diseases, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan. E-mail: kadowaki-3im@h.u-tokyo.ac.jp.

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Abbreviations: AUC, area under the curve; HMW, high molecular weight; HMWR, HMW ratio; HOMA-IR, homeostasis model assessment of insulin resistance; IDF, International Diabetes Federation; ROC, receiver operator characteristic.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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H.Y., O.M., H.E., T.K., unpublished observations). Mutations in the adiponectin gene that cause impaired multimerization and decreased plasma HMW adiponectin levels have been found to be associated with insulin resistance and type 2 diabetes (23), suggesting that alterations in plasma HMW adiponectin level may be more relevant in the prediction of insulin resistance than those in plasma total adiponectin levels. Indeed, a recent study has shown that the ratio of the plasma level of HMW adiponectin to that of total adiponectin level (HMWR) is significantly more useful for monitoring the improvement of insulin sensitivity in response to thiazolidinediones in cases of type 2 diabetes (24). The HMWR value has been also shown, by oral glucose tolerance tests, to be more significantly inversely correlated with 2-h glucose levels than total adiponectin level (25). However, in this study, the adiponectin multimers were separated by velocity sedimentation/gel filtration and quantified HMW adiponectin level by Western blotting. In the present study, we investigated the clinical usefulness of measurement of plasma HMW adiponectin level using a newly developed method, as compared with that of plasma total adiponectin level by analyzing the sensitivity or specificity of total adiponectin levels, HMW adiponectin levels, and HMWR values for the prediction of insulin resistance and metabolic syndrome. This study is the first to demonstrate the clinical usefulness of measuring HMW adiponectin levels in making precise prediction of insulin resistance and metabolic syndrome.

RESEARCH DESIGN AND METHODS

— The subjects of this study were 298 patients admitted to Tokyo University Hospital for the treatment of diabetes or coronary angiography. The present study was conducted according to the principles outlined in the Declaration of Helsinki. Informed consent was obtained from each of the study subjects. The protocol of the study was approved by the ethics review committee of Tokyo University School of Medicine.

Height, weight, hip, waist, fasting plasma glucose, serum insulin, serum total cholesterol, triglyceride, HDL cholesterol, and LDL cholesterol levels were measured the morning after an overnight fast. We modified the diagnostic criteria of the National Cholesterol Education Program Adult Treatment Panel III for metabolic syndrome (26) by adopting a cutoff level for the waist circumference of 85 cm in men and 90 cm in women, in accordance with the recommendations for the Japanese population by the International Diabetes Federation (IDF) (27). The homeostasis model assessment of insulin resistance (HOMA-IR) (28) was determined in 171 subjects (110 men and 61 women); however, it could not be assessed in subjects being treated with insulin or in some of the subjects admitted for coronary angiography. We defined subjects with a HOMA-IR of >2.5 as having insulin resistance. This cutoff has been adopted in the Japanese guideline for the treatment of diabetes. We analyzed the relationship between total adiponectin levels or HMWR values and severity and extent of coronary atherosclerosis using the score by Gensini (29). Subjects with a history of percutaneous coronary intervention were excluded from this analysis. Because thiazolidinediones have been reported to exhibit direct antirestenotic effects in the vasculature (30) and have protective effects against coronary artery disease (31), after the prescription of thiazolidinediones was incorporated into the model in addition to the conventional risk factors for atherosclerosis, stepwise regression analysis was done to analyze the relationship between HMWR values and Gensini score. The measurement method for plasma HMW adiponectin level is described elsewhere (H.E., O.M., H.Y., K.H., T.Y., T.K., unpublished observations) and coefficients of variations of the assay were 5.3% for total adiponectin and 3.3% for HMW adiponectin level.

Statistical analyses

The values of the clinical parameters were expressed as means ± SD. All statistical analyses were performed using JMP for Windows software (version 4.0; SAS Institute, Cary, NC). The significance of differences in plasma total adiponectin levels, HMW adiponectin levels, and HMWR values was analyzed by ANOVA. ROC curves were plotted and compared using the Stata software (College Station, TX). P values <0.05 were considered to denote statistical significance.

RESULTS

— As shown in online appendix Table 1 (available at http://care.diabetesjournals.org), there is no difference in the proportion of the subjects who took at least one of the drugs potentially affecting plasma adiponectin levels, such as thiazolidinediones, biguanide, ACE inhibitors, and angiotensin receptor blockers, between subjects with and without metabolic syndrome (68 [42.5%] vs. 54 [39.1%], $P = 0.686$).

Correlations between patient characteristics and plasma total adiponectin and HMW adiponectin levels and HMWR values

Women had higher plasma total adiponectin levels (5.59 ± 0.31 vs. 4.55 ± 0.22 μg/ml, $P = 0.0069$), HMW adiponectin levels (2.19 ± 0.14 vs. 1.54 ± 0.11 μg/ml, $P = 0.0003$), and HMWR values (35.9 ± 1.1 vs. 29.9 ± 0.8%, $P = 0.00001$) than men. There was an inverse correlation between BMI and plasma total adiponectin levels ($r = -0.29$, $P = 0.0001$), HMW adiponectin levels ($r = -0.27$, $P = 0.0001$), and HMWR values ($r = -0.13$, $P = 0.012$). In multivariate analysis taking into account BMI, sex, and the interaction between sex and BMI, sex and BMI were independently correlated with plasma total adiponectin levels ($P = 0.0001$ and 0.0025, respectively), plasma HMW adiponectin levels ($P = 0.0001$ and 0.0001, respectively), and HMWR values ($P = 0.0075$ and 0.0001, respectively). However, since there was no interaction between sex and BMI for these three different measurements of plasma adiponectin ($P = 0.29$, 0.23, and 0.34, respectively), the inverse correlation between BMI and plasma total adiponectin level, plasma HMW adiponectin levels, or HMWR values were not affected by sex. Plasma total adiponectin level, plasma HMW adiponectin levels, and HMWR values did not vary with age in either sex (data not shown).

HMWR value predicted insulin resistance more precisely than plasma total adiponectin level

Insulin resistance is closely linked with metabolic syndrome and is often observed even in the early stage of metabolic syndrome (32). We investigated the correlation between insulin resistance and each of the three different measurements of plasma adiponectin. The total adiponectin level was inversely correlated with the HOMA-IR ($P = 0.0280$) in 171 men and women, consistent with the results of previous studies (11,12); the HMW adiponectin levels ($P = 0.0035$) and HMWR values ($P = 0.0008$) were also inversely correlated with HOMA-IR. In 110 men, there was a significant inverse correlation between HOMA-IR and

plasma HMW adiponectin levels (P = 0.0083) and HMWR values (P = 0.0010), while there was only a tendency toward inverse correlation between HOMA-IR and plasma total adiponectin levels (P = 0.0613). In 61 women, tendencies were found for decreased plasma total adiponectin levels and for HMW adiponectin levels or HMWR values to be associated with increased HOMA-IR values. HMWR values were significantly and inversely correlated with HOMA-IR, even after adjusting for age, sex, and BMI (P = 0.0304) or adjusting for BMI alone (P = 0.0389) in 171 men and women, suggesting that correlation between HMWR values and HOMA-IR was independent of the association between BMI and HOMA-IR. In contrast, the statistically significant association between total adiponectin and HOMA-IR disappeared after adjusting for age, sex, and BMI (P = 0.227) or adjusting for BMI alone (P = 0.249) in 171 men and women. These results are again consistent with the superiority of HMWR values over total adiponectin level to predict the presence of insulin resistance. We then plotted ROC curves to compare the power of plasma total adiponectin level and HMWR value to predict the presence of insulin resistance (Fig. 1). The AUC of HMWR values was significantly larger than that of plasma total adiponectin levels (0.713 [95% CI 0.620–0.805] vs. 0.615 [0.522–0.708], P = 0.0160) (Fig. 1), suggesting that the HMWR value had better predictive power for the prediction of insulin resistance than plasma total adiponectin level. When a cutoff value of 35% was used, the HMWR value predicted the presence of insulin resistance with a sensitivity of 72% and specificity of 66%. On the other hand, at a cutoff level of 4.2 μg/ml, plasma total adiponectin diagnosed insulin resistance with a sensitivity of 56% and specificity of 63%. When stratified according to sex, there was a significant difference between the AUCs for plasma total adiponectin levels and HMWR values in men (0.713 [0.605–0.821], P = 0.048 vs. 0.624 [0.514–0.733], P = 0.048), while we could detect no such difference in women (0.794 vs. 0.665, P = 0.09).

Correlation between the number of risk factors and plasma total adiponectin levels, HMW adiponectin levels, or HMWR values

We then investigated the association between the number of risk factors defining the prediction of metabolic syndrome (see RESEARCH DESIGN AND METHODS) in the subjects and plasma total adiponectin levels, HMW adiponectin levels, and HMWR values. The plasma total adiponectin levels (P = 0.0001), HMW adiponectin levels (P = 0.0001), and HMWR values (P = 0.0001) decreased as the number of risk factors present increased; this tendency was observed irrespective of sex (data not shown).

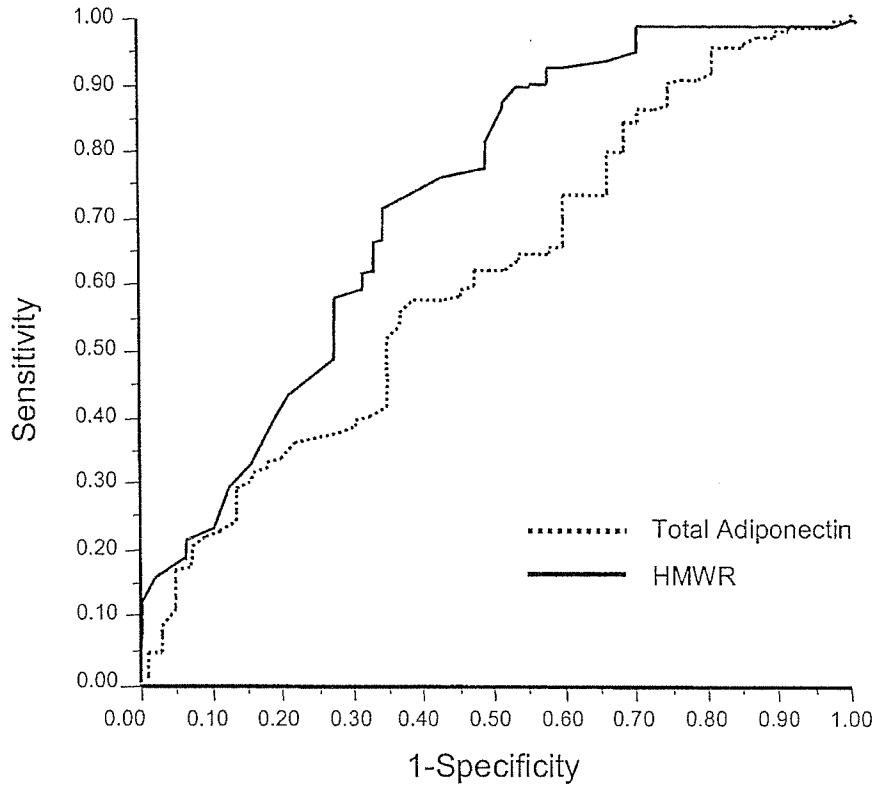

Figure 1—*ROC curves of plasma total adiponectin levels and HMWR values for the prediction of insulin resistance, defined as a HOMA-IR index >2.5 (n = 171). The AUC for the HMWR values was significantly larger than that for plasma total adiponectin levels (0.713 [95% CI 0.620–0.805] vs. 0.615 [0.522–0.708], P = 0.0160).*

ROC curves for models to predict the presence of metabolic syndrome

We performed an ROC analysis to quantify the power of the HMWR values to make a prediction of metabolic syndrome. ROC curves for plasma total adiponectin levels and HMWR values to discriminate between subjects with and without metabolic syndrome were plotted for total subjects (data not shown), men (Fig. 2*A*), and women (Fig. 2*B*). The areas under the curve for plasma total adiponectin level and HMWR values were then compared to determine whether the HMWR value had a better predictive power for the prediction of metabolic syndrome than plasma total adiponectin level. The AUC for the HMWR values was significantly larger than that for plasma total adiponectin levels in all subjects (0.780 [95% CI 0.736–0.824] vs. 0.692 [0.632–0.751], P = 0.039) (data not shown), in men (0.806 [0.747–0.865] vs. 0.730 [0.660–0.800], P = 0.0025) (Fig. 2*A*), and in women (0.743 [0.659–0.828] vs. 0.637 [0.532–0.742], P = 0.0458) (Fig. 2*B*). When a cutoff value of 32.0% for men and 40.2% for women was adopted to maximize the sensitivity (%) plus specificity (%), HMWR value predicted the presence of metabolic syndrome with a sensitivity of 83.2% and specificity of 62.0% in men and a sensitivity of 77.4% and specificity of 55.0% in women.

We have undertaken the subgroup analyses in both diabetic and nondiabetic subjects. In both groups, AUCs for the HMWR values to predict metabolic syndrome were larger than those for total adiponectin levels (type 2 diabetic group: 0.815 [95% CI 0.755–0.875] vs. 0.676 [0.616–0.736], P = 0.0013; nondiabetic group: 0.843 [0.743–0.943] vs. 0.751 [0.651–0.851], P = 0.00475).

We have also plotted ROC curves for

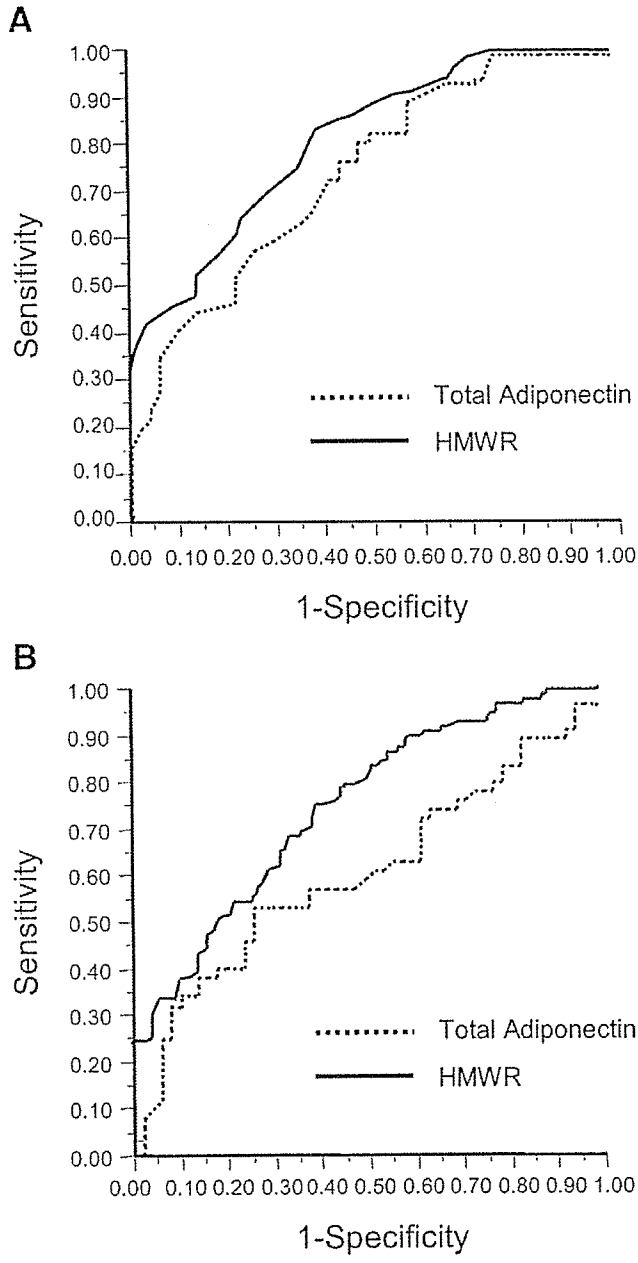

Figure 2—*ROC curves of plasma total adiponectin levels and HMWR values for prediction of metabolic syndrome in men* (A) (n = *193*) *and women* (B) (n = *105*). *Prediction of metabolic syndrome was based on the criteria of the National Cholesterol Education Program Adult Treatment Panel III for prediction of metabolic syndrome. The AUC for the HMWR values was significantly larger than for total adiponectin in both men (0.806 [95% CI 0.747–0.865] vs. 0.730 [0.660–0.800],* P = *0.0025) and women (0.743 [0.659–0.828] vs. 0.637 [0.532–0.742],* P = *0.0458).*

total adiponectin levels and HMWR values to predict metabolic syndrome defined by newly announced IDF criteria (27). Indeed, HMWR values were more predictive of metabolic syndrome defined by IDF than total adiponectin levels in total subjects (0.733 [95% CI 0.693–0.773] vs. 0.680 [0.640–0.720], P = 0.0037).

HMWR value predicts the presence or absence of metabolic syndrome independently of plasma total adiponectin level

The HMWR values varied substantially even among subjects with similar total adiponectin levels in plasma. The present ROC analysis suggested the possibility that the HMWR value may be useful for predicting the presence of metabolic syndrome among subjects with similar plasma levels of total adiponectin. We examined whether the HMWR values were different between subjects with and without metabolic syndrome after stratifying the subjects into quartiles of plasma total adiponectin levels. HMWR values were significantly lower in all the quartiles of total adiponectin in subjects with metabolic syndrome than in those without, suggesting that the HMWR value may be useful for the prediction of metabolic syndrome, irrespective of plasma total adiponectin level.

The relationship between HMWR value and the severity of coronary artery disease

We analyzed the relationship between total adiponectin levels or HMWR values and severity and extent of coronary atherosclerosis using the score by Gensini (29). Gensini scores were significantly associated with age (P = 0.0022), prescription of thiazolidinediones (P = 0.0443), and HMWR values (P = 0.0437). Subjects with lower HMWR value had higher Gensini score, suggesting that HMWR value might be associated with the onset and development of coronary artery disease independently of age and prescription of thiazolidinediones. Total adiponectin level was not correlated with Gensini score before and after adjustment for the conventional risk factors for atherosclerosis and prescription of thiazolidinediones.

CONCLUSIONS— Prediction of metabolic syndrome, defined by the presence of a cluster of metabolic abnormalities, including impaired glucose metabolism, high BMI and abdominal fat distribution, dyslipidemia, and hypertension, is very important because of its association with the subsequent development of type 2 diabetes and cardiovascular disease (28). Because of the epidemic of obesity and a sedentary lifestyle worldwide, metabolic syndrome is becoming increasingly commonly recognized. According to the National Cholesterol Education Program (NCEP) definition, roughly one-fourth of middle-aged men and women in the U.S. have metabolic syndrome (33). Development of a method for convenient prediction of metabolic syndrome in daily clinical practice presents a major challenge for physicians and public health policy makers facing

the epidemic of obesity and a sedentary lifestyle.

There is a mounting body of evidence to suggest that adiponectin is an insulin-sensitizing hormone and that the plasma level of this hormone is the best predictor of the subsequent development of type 2 diabetes among the various plasma biomarkers (34). Recently, however, it has been reported that adiponectin forms a wide range of multimers in plasma and that mutations in the adiponectin gene that inhibit the formation of HMW adiponectin are closely associated with the subsequent development of type 2 diabetes. Therefore, it was considered that HMW adiponectin value might be an attractive biomarker for the prediction of insulin resistance and metabolic syndrome. Indeed, the present study demonstrated that the AUC of HMWR values was significantly larger than that of plasma total adiponectin levels and that the sensitivity of the HMWR value for predicting the presence of metabolic syndrome reached 80%. Thus, this study is the first to demonstrate that HMWR value is more closely associated with insulin resistance and the presence of metabolic syndrome than plasma total adiponectin level.

The present study provided evidence of the usefulness of a newly developed method of measurement of plasma HMW adiponectin level as a convenient and sensitive biomarker for the prediction of insulin resistance and metabolic syndrome.

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A novel ELISA system for selective measurement of human adiponectin multimers by using proteases

Hiroyuki Ebinuma [a,*], Osamu Miyazaki [a], Hirokazu Yago [a], Kazuo Hara [b], Toshimasa Yamauchi [c], Takashi Kadowaki [c]

[a] *Diagnostics Research Laboratories, Daiichi Pure Chemicals Co. Ltd., 3-3-1, Koyodai, Ryugasaki, Ibaraki 301-0852, Japan*
[b] *Department of Clinical Bioinformatics, Graduate School of Medicine, University of Tokyo 113-8655, Japan*
[c] *Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo 113-8655, Japan*

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Abstract

Background: Adiponectin, an antiatherogenic adipocyte-derived protein exists in human blood as multiple isoforms—trimeric low molecular weight (LMW), albumin-binding LMW (Alb-LMW), hexameric middle molecular weight (MMW), and high molecular weight (HMW) forms. We developed a novel ELISA system to detect total human adiponectin and the selective level of each adiponectin multimer for investigating the distribution of these levels in human blood.

Methods: Two monoclonal antibodies that were raised against human adiponectin were used to construct a sandwich ELISA to measure adiponectin levels. Adiponectin multimers were selectively measured after sample pretreatment with two proteases that specifically digested the trimeric forms or both the hexameric and trimeric forms.

Results: The ELISA had a dynamic range of 0.075–4.8 ng/ml. Intraassay variations (CV) were 5.3% (total adiponectin), 4.1% (MMW+HMW), and 3.3% (HMW). Comparison of the results of ELISA and quantitative western blot analysis of multimeric adiponectin in serum samples revealed good correlation (LMW+Alb-LMW, $r=0.873$; MMW, $r=0.907$; HMW, $r=0.950$). Each of the three forms of adiponectin multimer levels closely correlated with total adiponectin levels in healthy subjects.

Conclusions: This ELISA system can be used to further investigate the physiological roles of human adiponectin multimers.

Keywords: Adiponectin; Multimers; ELISA; Protease; Monoclonal antibody

1. Introduction

It has been reported that a decrease in adiponectin (also known as apM1, Acrp30, GBP28, and AdipoQ), a hormone secreted by adipocytes [1–4], is related to the metabolic syndrome, including type 2 diabetes [5–7], obesity [8], hypertension, and arteriosclerosis [9,10]. Adiponectin is known to possess a multimeric structure, and gel filtration and velocity gradient techniques have been used to analyze these multimers [11–13]. Waki et al. [14] reported a simple method to separate adiponectin multimers by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing and non-heat-denaturing conditions. According to the reports on the biological activity of adiponectin multimers, AMP-activated protein kinase (AMPK) activity varies among the multimeric forms of adiponectin [13–15]. Recently, it has been shown that high molecular weight (HMW) adiponectin is an active form and its ratio to total adiponectin is closely correlated with insulin sensitivity. Based on these findings, the importance of the selective measurement of adiponectin multimers has been suggested [15–17]. However, since the structure of adiponectin in human blood has not been fully elucidated and no method has been developed that can determine the individual multimers of adiponectin with high sensitivity and accuracy, clinical studies on the relationship between adiponectin multimers and various disorders have not made much progress.

Abbreviations: ELISA, enzyme-linked immunosorbent assay; LMW, low molecular weight; Alb-LMW, albumin-binding low molecular weight; MMW, middle molecular weight; HMW, high molecular weight; PBS, 20 mmol/l phosphate-buffered saline (pH 7.2); MAb, monoclonal antibody; SDS, sodium dodecyl sulfate.

* Corresponding author. Tel.: +81 297 62 6425; fax: +81 297 62 8635.
E-mail address: ebinuma@daiichichem.co.jp (H. Ebinuma).

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More recently, we revealed the structure of adiponectin by the selective separation of each adiponectin multimer from the human plasma and confirmed the presence of an albumin-binding trimer in addition to the known trimeric, hexameric, and HMW forms [Hada et al. personal communication].

In the present study, we first prepared monoclonal antibodies (MAbs) and developed a sandwich ELISA for measuring human adiponectin. We also combined a sample pretreatment method in which adiponectin multimers were converted to a dimeric form by using acid buffer containing SDS. Further, we unexpectedly detected 2 kinds of proteases that are capable of selectively digesting the adiponectin multimers in the presence of various serum proteins. Based on these findings, we developed an improved ELISA system for the selective measurement of adiponectin multimers using these proteases. Here, we report the development of this improved ELISA and the validation of the pretreatment with proteases for selective digestion. Using this ELISA system, we selectively measured the levels of each adiponectin multimer in the serum of healthy subjects and investigated the distribution of these adiponectin levels in human blood as a preliminary study.

2. Materials and methods

2.1. Proteases

Proteinase K from *Tritirachium album* [E.C. 3.4.21.64], a recombinant, was obtained from Roche Diagnostics. Protease A "Amano" from *Aspergillus oryzae* [E.C. 3.4.24.39] was obtained from Amano Enzyme.

2.2. Preparation of serum samples

Serum samples were obtained from healthy volunteers (27 males and 20 females) after obtaining their informed consent.

2.3. Purified human adiponectin

Purified forms of the multimers [low molecular weight (LMW, trimer), Alb-LMW (albumin-binding trimer), middle molecular weight (MMW, hexamer), and HMW (≥octadecamer)] were prepared as described previously [Hada et al. personal communication]. After the purified HMW adiponectin was converted to the dimeric form with SDS sample buffer [100 mmol/l sodium citrate (pH 3.0) containing 2% SDS], it was used as the calibrator. The adiponectin protein content was quantified by Lowry's method (Bio-Rad Laboratories) using bovine serum albumin (BSA) as the standard.

2.4. Preparation of adiponectin-free plasma

Adiponectin-free plasma was prepared as described previously [Hada et al. personal communication]. In brief, human plasma was applied to a column of CNBr-Sepharose 4B resin combined with rabbit anti-mouse adiponectin globular domain antibody. The nonbinding fractions were used.

2.5. Preparation of monoclonal antibodies

Purified human adiponectin multimers emulsified with complete Freund's adjuvant (volume ratio of 1:1, 0.1 mg/0.1 ml; Gibco) were subcutaneously injected into BALB/c mice 4 times at 2-week intervals. Two days after the final injection of the antigen, the spleen cells were extracted and fused with P3U1 murine myeloma cells at a ratio of 6:1 in the presence of 50% polyethylene glycol 1540 (Wako Chemicals). The fused cells (2.5×10^6/ml) were suspended in hypoxanthine–aminopterin–thymidine medium and seeded into 96-well plates (Corning), followed by incubation in 5% CO_2 at 37 °C for 2 weeks.

To select the clones producing anti-adiponectin MAbs, the hybridoma cell supernatant was added to a microplate (Nunc) coated with goat anti-mouse IgG (FMC) antibody (Jackson ImmunoResearch) and incubated overnight at 4 °C. After washing the plates, the SDS-treated purified adiponectin was added to the plates and incubated at room temperature for 1 h. These plates were washed again, the rabbit anti-mouse adiponectin globular domain antibody was added to the plates, and they were incubated at room temperature for 1 h. Another step of washing was carried out, the horseradish peroxidase-conjugated goat anti-rabbit IgG (Biosource) was added to the plates, and they were incubated at room temperature for 1 h. After washing the plates, the peroxidase activity was measured as absorbance at 492 nm by using *o*-phenylenediamine in citrate buffer (pH 5.0) containing hydrogen peroxide as the substrate and 11 MAbs were detected. Based on the preliminary sandwich assays involving combinations of these antibodies, 2 (64404 and 64405) were selected as highly sensitive to adiponectin. One MAb (64404) was then conjugated with Sulfo-NHS-LC-Biotin (Pierce) according to the manufacturer's instructions.

2.6. Western blotting analysis

Native PAGE was performed according to the standard method of Davis [18]. For immunoblotting, the proteins that were separated by native PAGE were transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was then blocked with 5% skim milk in phosphate-buffered saline (PBS), reacted with goat anti-adiponectin antibody (GT), and subsequently reacted with horseradish peroxidase-conjugated rabbit anti-goat IgG using a VECTASTAIN ABC kit (Vector Laboratories) according to the manufacturer's instructions. Next, the membrane was stained with diaminobenzidine. The bands were scanned with an IMAGE CAPTOR DPE-HA201 (Daiichi Pure Chemicals) and quantified by using 1D Image Analysis Software (Kodak).

2.7. ELISA for the determination of total adiponectin and selective measurement of adiponectin multimers (selective ELISA)

2.7.1. Sample pretreatment

For the total adiponectin assay, the samples (10 μl) were pretreated with 100 μl of 50 mmol/l Tris–HCl (pH 8.0) followed by the addition of 400 μl of SDS sample buffer. For the selective assay of HMW adiponectin, the samples (10 μl) were pretreated with

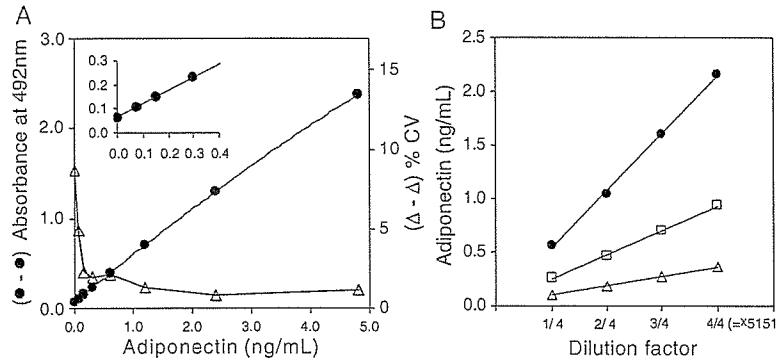

Fig. 1. Evaluation of the ELISA for human adiponectin. (A) Typical calibration curve (●) and imprecision profile (△) for the ELISA obtained using adiponectin purified from human plasma. The imprecision was assessed based on six replicates of the calibration curves. (B) Linearity of the dilution curves for human serum samples. After the three human serum samples containing 1.82 (△), 4.70 (□), and 10.8 (●) μg/ml of total adiponectin were treated according to the sample pretreatment procedure outlined for the total adiponectin assay, the samples were further diluted serially with BSA-PBST.

100 μl of 50 mmol/l Tris–HCl (pH 8.0) containing 7.5 U/ml of proteinase K for 20 min at 37 °C, and 400 μl of SDS sample buffer was subsequently added. For the selective assay of MMW+HMW adiponectin, the samples (10 μl) were pretreated with 100 μl of 50 mmol/l Tris–HCl (pH 8.0) containing 1.0 mg/ml Protease A "Amano" for 20 min at 37 °C, and 400 μl of SDS sample buffer was then added. These pretreated samples were further diluted 101-fold with BSA-PBST (PBS containing 1% BSA and 0.05% Tween 20).

2.7.2. Sandwich ELISA

The MAb (64405; 5 μg/ml in PBS) was incubated in a 96-well ELISA plate (Nunc) overnight at 4 °C. The plate was rinsed with PBST, 100 μl of BSA-PBST was then added, and the plate was incubated at room temperature for 1 h. The diluted samples or the calibrator (50 μl/well) was then placed into the wells and incubated for 1 h at room temperature. The plate was subsequently rinsed and reacted with the biotinylated MAb (64404) for 1 h at room temperature. Next, the plate was rinsed and reacted with horseradish peroxidase-conjugated streptavidin (Pierce) for 30 min at room temperature. The plate was then rinsed and the peroxidase activity was measured as absorbance at 492 nm by using *o*-phenylenediamine in citrate buffer (pH 5.0) containing hydrogen peroxide as the substrate. The MMW adiponectin concentration was calculated by subtracting the HMW adiponectin concentration from the combined MMW and HMW adiponectin concentration. Further, the LMW+Alb-LMW adiponectin concentration was calculated by subtracting the combined MMW and HMW adiponectin concentration from the total adiponectin concentration.

2.8. Evaluation of protease for selective digestion

To confirm the selectivity of the protease digestion, sample solutions were prepared by adding each of the purified form of adiponectin to adiponectin-free plasma at a concentration of 5 μg/ml, and the optimum conditions for selective digestion were investigated using commercially available proteases (proteinase K and Protease A "Amano"). After treatment with the proteases, adiponectin concentration in each solution was measured by ELISA as mentioned above. In addition, each sample was separated by native PAGE and analyzed by western blotting.

3. Results

3.1. Characterization of MAbs (64404 and 64405)

The reactivity of the selected MAbs was tested by western blotting before and after pretreatment of human serum with SDS under nonreducing or reducing conditions. MAbs 64404 and

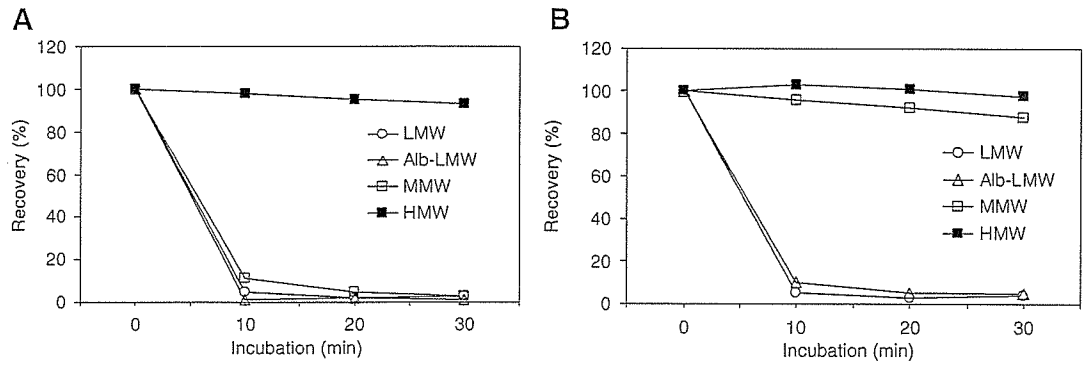

Fig. 2. Effect of selective digestion by proteases. Separated multimers of adiponectin were spiked into adiponectin-free human plasma samples at a concentration of 5 μg/ml. Each sample was then digested with 7.5 U/ml of proteinase K (A) or 1.0 mg/ml of Protease A "Amano" (B) at 37 °C for 10–30 min.

Fig. 3. Selectivity of digestion by proteases. After human serum was treated by Protease A "Amano" or proteinase K according to sample pretreatment procedure, without SDS sample buffer, 2 μl of the pretreated samples were separated by native PAGE and analyzed by western blotting.

64405 reacted with not only the intact multimeric forms separated by native PAGE but also the denatured dimeric or monomeric forms that were separated by SDS-PAGE; they did not crossreact with mouse and rat adiponectins. Therefore, these antibodies might recognize the collagen domain of human adiponectin, particularly, the N-terminal region, because this region exhibits low homology across different species [13].

3.2. Calibration curve, detection limit, and dilution test for the total adiponectin ELISA

The adiponectin preparation was used for calibration to generate a dose response curve for the adiponectin ELISA. A typical calibration curve is shown in Fig. 1A along with the coefficient of variation (CV) of each calibration sample used to obtain 6 independent calibration curves. The CV of each calibration sample at concentrations of 0.075–4.8 ng/ml was <10%. The detection limit was 0.038 ng/ml ($P<0.001$, by Student's t test vs. the blank sample). A dilution test showed that the curve obtained by plotting the serial dilutions of the human serum samples was parallel to the calibration curve (Fig. 1B).

3.3. Validation of pretreatment with proteases for selective digestion

We examined the optimum conditions required for the selective digestion of adiponectin multimers by using 2 commercially available proteases (proteinase K and Protease A "Amano"). The recovery rate (%) was calculated as the ratio of the adiponectin level after digestion to the adiponectin level without protease treatment. It was revealed that 7.5 U/ml proteinase K digested all forms of adiponectin other than the HMW form on incubation at 37 °C for 10–30 min (Fig. 2A). Further, 1.0 mg/ml Protease A "Amano" digested the trimeric

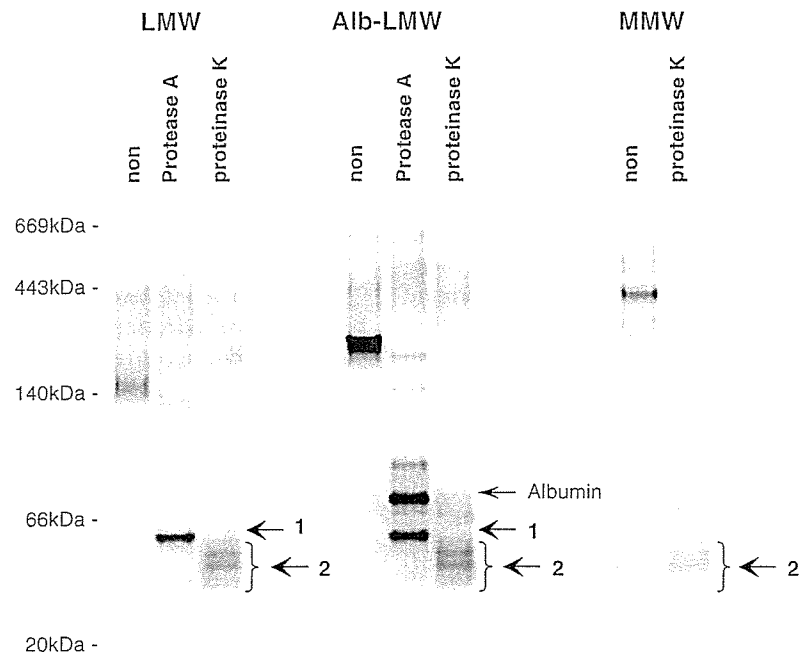

Fig. 4. Identification of the protease-digested product. Purified LMW, Alb-LMW, and MMW forms of adiponectin (2 μg) were incubated at 37 °C for 20 min in the presence of Protease A "Amano" (2 μg) or proteinase K (0.02 U). Each treated sample was analyzed by native PAGE and CBB staining. The proteins digested with protease are denoted by numbered arrows.

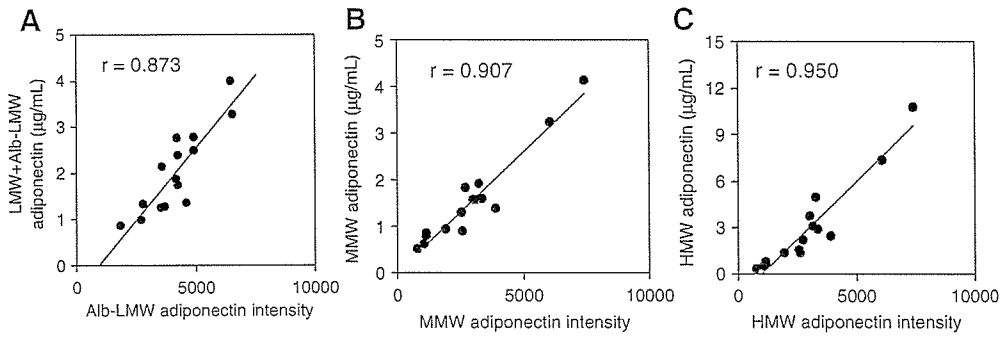

Fig. 5. Comparison of the selective adiponectin multimer ELISA and densitometry. The concentration of each adiponectin multimer in human serum ($n=16$) was determined by ELISA and quantified by densitometry.

(LMW and Alb-LMW) forms of adiponectin on incubation at 37 °C for 10–30 min (Fig. 2B). We used native PAGE and western blotting to confirm that adiponectin multimers in human serum had been selectively digested by the proteases.

It was observed that all forms of adiponectin other than the HMW form disappeared after digestion by proteinase K, while the trimeric (LMW and Alb-LMW) forms of adiponectin disappeared after digestion by Protease A "Amano" (Fig. 3).

3.4. Identification of the protease-digested product

To reveal the influence of the protease-digested product in sandwich ELISA, we analyzed the cleavage site of Protease A "Amano" and proteinase K using the purified form of each adiponectin multimer. Native PAGE analysis identified a common protein band (band 1) digested by Protease A "Amano" from the purified trimeric (LMW and Alb-LMW) forms of adiponectin, and four common types of protein bands (band 2) were digested by proteinase K from the purified trimeric and MMW forms of adiponectin (Fig. 4). To analyze the N-terminal amino acid sequence of these proteins (bands 1 and 2), each protein band was eluted and separated by reducing SDS-PAGE and detected by CBB staining. It was observed that only two types of peptide bands of size approximately 10 kDa were obtained from the protein present in bands 1 and 2. Since N-terminal amino acid sequences (GR*GE and YVYRS) of 2 peptides match amino acid residues 99–103 and 109–113 of human adiponectin, the digested products (bands 1 and 2) were identified as partial globular domains that lacked a small portion of the C-terminal region. In addition, western blotting confirmed that MAbs 64404 and 64405 did not react with these digested products. This result suggested that these proteases digested the collagen domain of human adiponectin; this domain is possibly recognized by these MAbs.

3.5. Comparison of western blotting and selective ELISA

In this study, the serum concentrations of total adiponectin, HMW adiponectin, and HMW+MMW adiponectin were determined in 16 serum samples obtained from healthy volunteers, and the concentrations of MMW and LMW+Alb-LMW adiponectins were calculated using a formula. In a separate experiment, the intensity of the three clearly stained bands (LMW form was excluded because its detection was not possible) obtained from western blotting was individually determined by densitometry and compared with the data obtained after performing ELISA. Since a strong positive correlation was obtained between the data obtained using the 2 methods (Fig. 5A–C), the accuracy of the selective ELISA for identifying the adiponectin multimers by using proteases for selective digestion was confirmed.

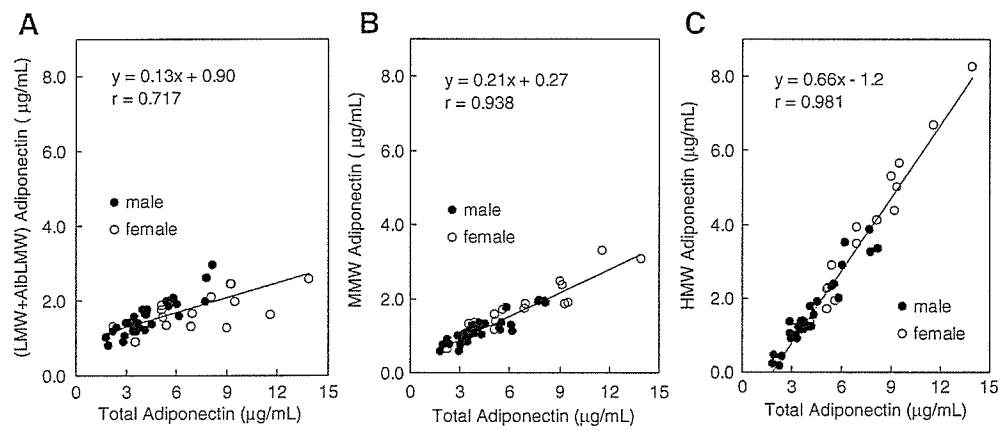

Fig. 6. Relationship between total adiponectin and adiponectin multimers in 47 healthy subjects.

3.6. Precision of the total adiponectin and selective ELISAs

Human serum samples having two different concentrations of adiponectin were consecutively measured eight times. The CV was 5.4% and 5.3% at concentrations of 3.98 and 9.22 μg/ml, respectively. The human serum samples were also analyzed after treatment with proteinase K or Protease A "Amano"; the CV was 3.3% (4.53 μg/ml; HMW) and 4.1% (6.60 μg/ml; HMW+MMW), respectively.

3.7. Serum adiponectin multimer levels in healthy subjects

Multimer levels of the three forms of adiponectin were closely correlated with the total adiponectin levels in 47 healthy subjects (Fig. 6A–C). There was no significant difference in the mean level of the trimeric forms of adiponectin levels between the male and female subjects (1.54±0.50 vs. 1.68±0.46 μg/ml), whereas the mean of the total, HMW, and MMW adiponectin levels were significantly higher in females than in males (6.62±3.04 vs. 4.30±1.76 μg/ml, $P<0.005$; 3.24±2.13 vs. 1.62±1.02 μg/ml, $P<0.005$; 1.70±0.68 vs. 1.15±0.38 μg/ml, $P<0.001$, by Student's t test).

4. Discussion

It has been reported that the 3 main forms of adiponectin, i.e., the trimer (LMW), hexamer (MMW), and HMW, are secreted by the adipocytes [13–16]. We have recently found that the separation of multimers by native PAGE is extremely useful for the analysis of the adiponectin structure [Hada et al. personal communication]. Using this method, we have confirmed that the 4 main multimers of adiponectin, including the albumin-binding trimer (Alb-LMW), are present in the human blood. Pajvani et al. [16] and Tonelli et al. [17] confirmed the close correlation between the ratio of HMW to total adiponectin and insulin sensitivity. In addition, Kobayashi et al. [15] recently reported a possibility that the ratio of the percentage of each form of adiponectin to total adiponectin was changing in coronary artery disease (CAD) patients or obese subjects. These findings suggest that it is clinically important to determine not only the total adiponectin level but also the level of each adiponectin multimer separately.

In the present study, we constructed a novel ELISA system designed for clinical studies on human adiponectin multimers by using MAbs and proteases. The prepared MAbs reacted with the various forms of human adiponectin (intact multimers, dimeric or monomerics) that do not crossreact with mouse and rat adiponectins. These prepared MAbs did not react with the protease-digested products that had a partial globular domain, which lacked a small portion of the C-terminal region. Pajvani et al. [11] reported that trypsin could completely digest LMW adiponectin but not the MMW and HMW forms. We investigated the use of various commercially available proteases in order to detect proteases that exhibited selectivity to adiponectin multimers in the presence of proteins such as albumin, which are present in a high concentration in blood. We found two proteases that selectively digested adiponectin multimers and developed a selective assay method that used these proteases for pretreatment. Protease A "Amano" and proteinase K were selected as the proteases that are capable of selectively digesting the trimeric forms, and both the hexameric and trimeric forms, respectively. In this system, while the total adiponectin and HMW levels are calculated directly, the LMW+Alb-LMW and MMW levels are calculated indirectly.

As a preliminary study using this ELISA system, we investigated the distribution of each multimer of serum adiponectin in healthy subjects. Unexpectedly, a positive correlation was observed between the total adiponectin levels and each of the three forms of adiponectin multimer levels. Arita et al. [8] showed that the total adiponectin levels in females were higher than those in the males.

In our study, no significant difference was observed between the males and females with regard to the levels of the trimeric forms, whereas the total, HMW, and MMW adiponectin levels in females were significantly higher than those in males; further, we observed that the total adiponectin levels depended mostly on the HMW adiponectin levels in both males and females. These findings suggest that the level of secretion of the HMW form from an adipocyte decreases in subjects with low level of adiponectin. Therefore, further studies are needed to clarify the level of each adiponectin multimer in various diseases.

In conclusion, we developed a novel ELISA system for determining the levels of total adiponectin and each of the adiponectin multimer by using proteases for selective digestion. This system can be used to further investigate the physiological roles of adiponectin multimers in humans.

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A Proposal for the Cutoff Point of Waist Circumference for the Diagnosis of Metabolic Syndrome in the Japanese Population

KAZUO HARA, MD, PHD[1,2,3]
YUMI MATSUSHITA, PHD[1,3]
MOMOKO HORIKOSHI, MD, PHD[1]
NOBUO YOSHIIKE, MD, PHD[3]
TETSUJI YOKOYAMA, PHD[4]
HEIZO TANAKA, MD, PHD[5]
TAKASHI KADOWAKI, MD, PHD[1,2,3]

Over the past 2 decades, there has been a dramatic increase in the number of subjects with the metabolic syndrome in Japan as well as in Western countries. Because subjects with the metabolic syndrome have an elevated risk of development of type 2 diabetes and cardiovascular diseases (1–3), there is an urgent need to establish strategies to prevent an epidemic of this syndrome. In particular, a practical and sensitive screening system must be established to detect the metabolic syndrome. At present, there are two internationally recognized definitions of the metabolic syndrome, namely those of the World Health Organization (4) and the National Cholesterol Education Program's Adult Treatment Panel III (NCEP III) (5). In an attempt to establish a unified definition for the metabolic syndrome, the International Diabetes Federation (IDF) has very recently announced a new definition of the metabolic syndrome that is expected to be suitable for use in clinical practice worldwide (6). The IDF defines metabolic syndrome as the presence of central obesity plus any two of the following four factors (raised triglyceride level, reduced HDL cholesterol, raised blood pressure, and raised fasting plasma glucose). The IDF recommended that the cutoff level used for the waist circumference to define central obesity should be different among different ethnic groups (7). In fact, the new IDF definition has proposed ethnicity-specific cutoff values for waist circumference, namely, 94 and 80 cm for European men and women, respectively, and 85 and 90 cm for Japanese men and women, respectively. Nonetheless, the IDF has strongly recommended that more extensive investigations should be performed before suitable cutoff levels are established for use in clinical practice (7).

In this study, we investigated the relationship between the cutoff values used for the waist circumference to define central obesity and rates of detection of subjects having multiple risk factors of the metabolic syndrome and attempted to determine the most suitable cutoff level of waist circumference for the diagnosis of metabolic syndrome in a community-based cohort.

RESEARCH DESIGN AND METHODS

Among subjects who enrolled themselves for a routine health examination in Shibata, Niigata Prefecture, from 2000 to 2001, we invited 692 subjects ranging in age from 30 to 80 years (408 men and 284 women) to participate in this study. Well-trained interviewers recorded the histories of drug usage for hyperlipidemia, hypertension, and diabetes from all participants. The waist circumference was measured midway between the lowest rib and the iliac crest with a flexible anthropometric tape (8). The present study was approved by the ethics review committee of the University of Tokyo, and written informed consent was obtained from all of the subjects.

Definition of the state of risk-factor clustering

In this study, subjects with two or more of the following four risk factors of the criteria of the NCEP III were defined as having multiple risk factors: *1*) triglycerides ≥150 mg/dl, 2) HDL cholesterol <40 mg/dl in men and <50 mg/dl in women, 3) systolic blood pressure ≥130 mmHg or diastolic blood pressure ≥85 mmHg, 4) fasting plasma glucose ≥110 mg/dl (impaired fasting glucose). Subjects with a history of hyperlipidemia, hypertension, or diabetes were deemed as having the respective risk factors, regardless of the biochemical values.

Statistical analyses

The receiver operator characteristic (ROC) curve for waist circumference to predict the presence of two or more risk factors of the metabolic syndrome, as defined by the NCEP III (except for waist circumstance), was plotted using JMP for Windows, Version 4.00 (SAS Institute, Cary, NC).

RESULTS

The mean age of the study subjects was 52.3 ± 9.0 years for men and 53.5 ± 9.0 years for women. The mean waist circumference was 83.5 ± 7.8 cm in men and 74.3 ± 7.6 cm in women. The prevalence of high serum triglycerides was 30.4% in men and 9.9% in women. The prevalence of low serum HDL cholesterol was 6.6% in men and 9.2% in women. The prevalence of subjects with high blood pressure as defined

From the [1]Department of Metabolic Diseases, Graduate School of Medicine, University of Tokyo, Tokyo, Japan; [2]Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Tokyo, Japan; the [3]National Institute of Health and Nutrition, Tokyo, Japan; the [4]National Institute of Public Health, Saitama, Japan; and [5]Seitoku College, Tokyo, Japan.

Address correspondence and reprint requests to Dr. Takashi Kadowaki, University of Tokyo, Graduate School of Medicine, Department of Metabolic Diseases, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. E-mail: kadowaki-3im@h.u-tokyo.ac.jp .

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K.H. and Y.M. contributed equally to this work.

Abbreviations: IDF, International Diabetes Federation; NCEP III, National Cholesterol Education Program's Adult Treatment Panel III; ROC, receiver operator characteristic.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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