methods

A novel enzyme-linked immunosorbent assay specific for high-molecular-weight adiponectin

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Abstract Human plasma contains at least three forms of adiponectin: a trimer, a hexamer, and a high-molecular-weight (HMW) multimer. We purified HMW adiponectin from human plasma using its affinity to gelatin and obtained monoclonal antibodies against it. On Western blot analysis, the reactivity of these monoclonal antibodies was shown to be restricted to a non-heat-denatured form of adiponectin molecules. On heating, the collagen-like domain of adiponectin molecules became denatured, and thus the trimer form could not be maintained. From these, monoclonal antibodies against HMW adiponectin were suggested to react with the intact trimer of adiponectin. With these monoclonal antibodies, we developed a sandwich ELISA system for quantifying adiponectin in human serum. Its specificity was verified by analysis of serum fractions separated by gel-filtration chromatography, and our ELISA system was found to be HMW adiponectin-specific. With this novel ELISA, the HMW adiponectin concentrations were 8.4 ± 5.5 μg/ml (mean ± SD) in healthy women and 6.2 ± 3.6 μg/ml in healthy men. Also, serum with a lower HMW adiponectin concentration was shown to have a lower HMW ratio (i.e., HMW adiponectin/total adiponectin).—Nakano, Y., S. Tajima, A. Yoshimi, H. Akiyama, M. Tsushima, T. Tanioka, T. Negoro, M. Tomita, and T. Tobe. **A novel enzyme-linked immunosorbent assay specific for high-molecular-weight adiponectin. *J. Lipid Res.* 2006. 47: 1572–1582.**

Supplementary key words high-molecular-weight multimer • monoclonal antibody • gelatin-binding protein of 28 kDa

Adiponectin is an adipocyte-specific secretory protein that is highly and specifically expressed in adipose tissue (1–3). Adiponectin includes a collagen-like domain, and in this domain, three adiponectin peptides form one stable trimer and the trimers further multimerize to form "bouquet" forms (**Fig. 1**). In human plasma, adiponectin was found to circulate as a trimer, a hexamer, and a high-molecular-weight (HMW) multimer, and we purified the HMW adiponectin of 420 kDa from human serum using gelatin-Cellulofine and previously reported it as the gelatin binding protein of 28 kDa (GBP28) in 1996 (4).

Plasma adiponectin levels are reported to be decreased in obese individuals, to be negatively correlated with visceral fat accumulation, and to be significantly lower in type 2 diabetic patients with coronary artery disease (5–7). Adiponectin mRNA levels are significantly reduced in omental adipose tissue of obese patients with type 2 diabetes compared with lean and obese normoglycemic subjects, and although less pronounced, the levels are also reduced in subcutaneous adipose tissue of type 2 diabetic patients (8). Plasma adiponectin concentrations in patients with acute coronary syndrome, both acute myocardial infarction and unstable angina pectoris, are significantly lower than those in patients with stable angina pectoris and in controls, and a low adiponectin concentration is correlated independently with the development of an acute coronary disease (9). Plasma adiponectin levels are an inverse predictor of the cardiovascular outcome in patients with end-stage renal disease (10). Tietge et al. (11) reported that plasma adiponectin levels in cirrhosis are increased significantly, because the liver is a major source of adiponectin extraction and the adiponectin levels in cirrhosis do not correlate with parameters of body composition or metabolism but exclusively with reduced liver function and altered hepatic hemodynamics.

As for physiological function, adiponectin was reported to inhibit processes that could be related to atherosclerotic plaque formation and suggested to act as an antiatherogenic factor (12–15). In 2002, using adiponectin knockout mice, adiponectin was directly demonstrated to have antiatherogenic functions and to prevent neointimal thickening and increased proliferation of vascular smooth muscle

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Abbreviations: CRP, C-reactive protein; GBP28, gelatin binding protein of 28 kDa; HMW, high-molecular-weight; POD-IH7, horseradish peroxidase-conjugated IH7 Fab′.

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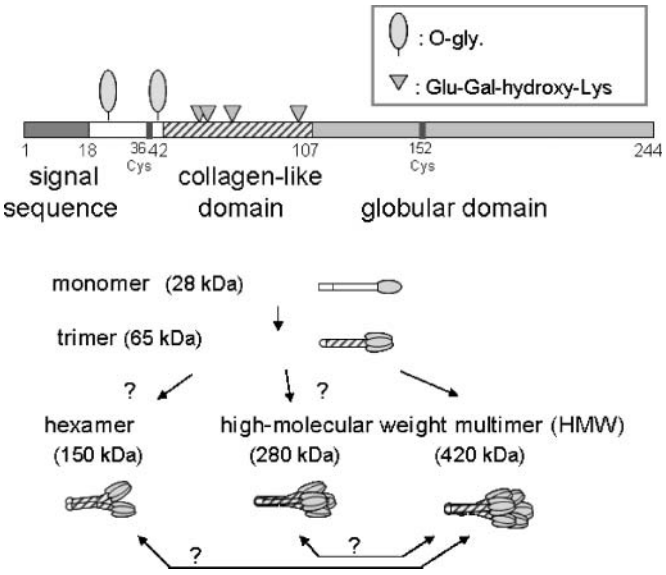

Fig. 1. Structure of human adiponectin. The protein consists of an N-terminal signal sequence of 18 amino acids followed by a sequence of 24 amino acids in which two *O*-glycoside chains are attached to threonine 22 and threonine 37. This region is followed by a stretch of 22 collagen repeats, and the C-terminal 137 amino acids form a globular domain. By means of the C-terminal globular domain and collagen-like domain, three adiponectin monomers form one stable trimer, and these trimers further multimerize to form bouquet forms. HMW, high-molecular-weight.

cells in mechanically injured arteries (16, 17). In addition, adiponectin was recently shown to inhibit the binding of LDL to biglycan (18). On the other hand, after Fruebis et al. (19) reported that adiponectin increased fatty acid oxidation in muscle and caused weight loss in mice in 2001, adiponectin was reported to have a number of antidiabetic activities (16, 20–22). Since 2002, glycosylation-dependent and oligomerization state-dependent activities of adiponectin have been reported (23–26), and in 2004, the amount of HMW adiponectin, not the absolute amount of adiponectin, was reported to be important in antidiabetic activities and vascular protective activities (27, 28).

With the ELISA systems that are commercially available now, total adiponectin can be measured, and because of this, the adiponectin levels reported to date have been those of total adiponectin. Here, we describe the development and evaluation of a sandwich ELISA method for HMW adiponectin involving monoclonal antibodies raised against HMW adiponectin, GBP28. Our sandwich ELISA system is available as a kit from Fujirebio Co. (Tokyo, Japan).

MATERIALS AND METHODS

Materials

A HiLoad 16/60 Superdex 200 prep-grade column, a Superdex 200 HR 10/30 column, protein molecular weight markers for size-exclusion chromatography, and ECL Plus Western blotting detection reagent were purchased from Amersham Biosciences (Uppsala, Sweden). Gelatin-Cellulofine was from Seikagaku Kogyo (Tokyo, Japan). LipofectAMINE reagent was from Invitrogen. A commercial ELISA kit for human adiponectin was purchased from Otsuka Pharmaceuticals (Tokyo, Japan). A monoclonal anti-human adiponectin/Acrp30 antibody and a biotinylated anti-human adiponectin/Acrp30 antibody were obtained from R&D Systems. Alkaline phosphatase- and peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories. Horseradish peroxidase streptavidin (streptavidin-conjugated horseradish peroxidase) was from Vector Laboratories. Tetramethylbenzidine solution was purchased from Dako Japan Co. (Kyoto, Japan), and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were from Sigma Chemical. The bicinchoninic acid protein assay kit was from Pierce. Human plasma was kindly provided by the Japan Red Cross.

HMW adiponectin purification and preparation of antibodies

From human plasma, HMW adiponectin was purified by affinity to gelatin-Cellulofine as described previously (4). Briefly, pooled human plasma was applied to the gelatin-Cellulofine column, and after thorough washing with 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl (TBS), HMW adiponectin was eluted with 10 mM Tris-HCl, pH 7.4, containing 1 M NaCl. After dialysis against TBS, the eluate was applied to a sulfate-Cellulofine column. The flow-through fractions were pooled, concentrated, and applied to the HiLoad 16/60 Superdex 200 prep-grade column repeatedly.

Mouse monoclonal antibodies against HMW adiponectin were produced by the ordinary method. Balb/c mice were immunized with human HMW adiponectin using Freund's complete adjuvant, and spleen cells were fused with mouse myeloma cell line P3U1 using polyethylene glycol 4000. For screening, an HMW adiponectin coat plate was used, and the specificity of antibodies was verified by Western blotting. Rabbit anti-sera against HMW adiponectin (anti-HMW), the C-terminal 20 amino acids of adiponectin (anti-C), and the N-terminal 20 amino acids of adiponectin (anti-N) were described previously (4, 29). One of the obtained monoclonal antibodies against HMW adiponectin, IH7 (IgG_{2a}), was digested with pepsin, reduced with 2-mercaptoethanol, and then reacted with maleimidoyl horseradish peroxidase to prepare horseradish peroxidase-conjugated IH7 Fab′ (POD-IH7).

Sandwich ELISA for HMW adiponectin

A sandwich ELISA for adiponectin was developed using IH7 as the capture antibody and POD-IH7 as the detection antibody. A 96-well microtiter plate was coated with 100 μl/well IH7 (1 μg/ml 10 mM Tris-HCl, pH 7.4), at 4°C overnight and then blocked with TBS containing 1% BSA at 4°C overnight. After washing of the plate with TBS containing 0.05% Tween 20 (TTBS), 100 μl aliquots of HMW adiponectin standards (2–50 ng/ml; five point calibration curve) or adequately diluted serum samples (400- to 1,000-fold) with TBS containing 1% BSA were added and the plate was incubated at room temperature for 1 h. After the plate had been washed three times with TTBS, 100 μl of POD-IH7 (50 ng/ml TBS containing 1% BSA) was added to each well, followed by incubation at room temperature for 30 min. After the plate had been washed three times with TTBS, 100 μl of the tetramethylbenzidine solution was added, followed by incubation at room temperature for 30 min. After the color had developed, 50 μl of 0.36 M H_2SO_4 was added to stop the reaction, and then the plate was read at 450 nm with a Bio-Rad model 680 microplate reader.

Subjects

For HMW adiponectin analysis, serum samples from 246 subjects (age range, 25–83 years) were randomly chosen as a sub-

sample for the Health Examination Survey study carried out in Japan. The serum samples were stored at −70°C before HMW adiponectin analysis.

Size-exclusion chromatography of human serum

Human serum was diluted with the same volume of phosphate-buffered saline (10 mM sodium phosphate and 150 mM NaCl, pH 7.4). To separate 0.5 ml of serum, the HiLoad 16/60 Superdex 200 prep-grade column equilibrated with the same buffer was used, and for 75 μl of serum, the Superdex 200 HR 10/30 column was used.

Expression of recombinant adiponectins in CHO-K1 cells

The full-length adiponectin cDNA was inserted into expression vector pZeoSV2(+). cDNAs of the N-terminal cysteine mutant, Cys36Ser, and the C-terminal cysteine mutant, Cys152Ser, were also prepared and inserted into pZeoSV2(+). These constructs were transfected into CHO-K1 cells by the LipofectAMINE method according to the manufacturer's instructions, and stably transfected cell lines for the wild-type adiponectin and two mutants were established.

General procedures

SDS-PAGE was performed by the method of Laemmli, followed by Coomassie staining or Western blotting. After treatment with each antibody and alkaline phosphatase- or peroxidase-conjugated secondary antibodies of a nitrocellulose membrane, bands were detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate or ECL Plus Western blotting detection reagent according to the manufacturer's instructions and quantified with a GS-800 Calibrated Densitometer (Bio-Rad). For protein size analysis, a prestained SDS-PAGE standard, Precision Plus Protein Standards Dual Color (Bio-Rad), was used.

Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) version 12.0J (SPSS, Chicago, IL). The values are expressed as means ± SD. Correlation analysis was performed with the Pearson correlation test using unadjusted values, and statistical significance was tested using unpaired or paired *t*-tests. $P < 0.05$ was considered significant.

Ethics

This study was approved by the Ethical Committees of Showa University and Keio University Ise Keio Hospital. The investigations were conducted according to the principles outlined in the Declaration of Helsinki, and all patients gave informed consent.

RESULTS

Antibodies against HMW adiponectin

Mice were immunized with human HMW adiponectin, GBP28, and three mouse monoclonal antibodies, IH5, IH6, and IH7, were obtained (for IH5, IH6, and IH7, contact the corresponding author by e-mail). As described previously (4), because adiponectin possesses a collagen-like domain, the protein shows a very special character on SDS-PAGE (**Fig. 2**). As shown in **Fig. 3A**, lanes 4 and 2, when HMW adiponectin was boiled in Laemmli's loading buffer, it gave a single band corresponding to 28 kDa under reducing conditions, which represents the adiponectin monomer,

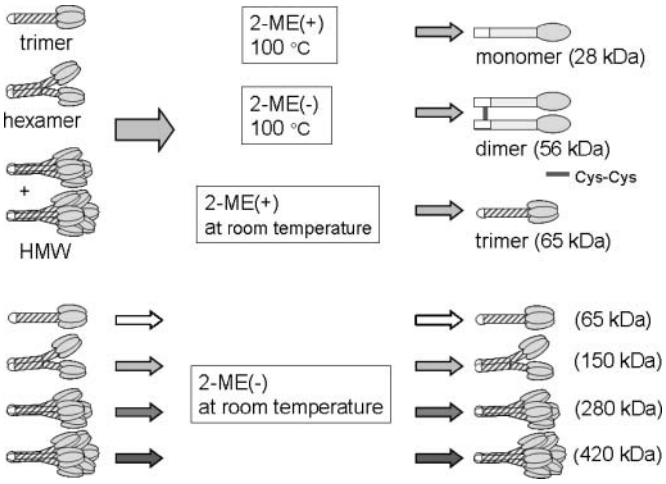

Fig. 2. Schematic presentation of HMW adiponectin characteristics on SDS-PAGE. When the trimer, hexamer, and HMW adiponectin are boiled in Laemmli's loading buffer under reducing conditions, they all give a single band of 28 kDa, which represents the adiponectin monomer, and when boiled under nonreducing conditions, they give a single band corresponding to 56 kDa, which represents the disulfide-bonded adiponectin dimer. On the other hand, without boiling under reducing conditions, they give a band corresponding to 65 kDa, which represents the adiponectin trimer, and under nonreducing conditions, the trimer, hexamer, and HMW adiponectin give bands of 65 kDa, which represents the adiponectin trimer, 150 kDa, which represents the hexamer, and 280 and 420 kDa, which represent HMW multimers, respectively. 2-ME, 2-mercaptoethanol.

and a single band corresponding to 56 kDa under nonreducing conditions, which represents the disulfide-bonded adiponectin dimer. On the other hand, without boiling, under reducing conditions, HMW adiponectin gave a band

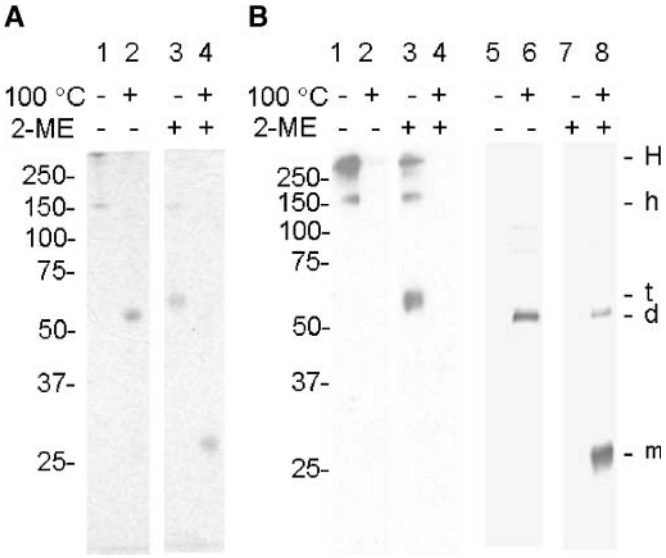

Fig. 3. Immunoblot analysis and 12.5% Laemmli's SDS-PAGE of HMW adiponectin. A: Coomassie staining. B: Immunoblotting with IH7 (lanes 1–4) and anti-C (lanes 5–8). Lanes 1, 3, 5, and 7 were not heat-denatured, and lanes 2, 4, 6, and 8 were heat-denatured at 100°C for 3 min under nonreducing or reducing conditions. H, HMW; h, hexamer; t, trimer; d, dimer; m, monomer; 2-ME, 2-mercaptoethanol.

corresponding to 65 kDa, which represents the adiponectin trimer, and under nonreducing conditions, it gave a band corresponding to 150 kDa and a major band at almost the top of the gel, which represent the adiponectin hexamer and the HMW multimer, respectively (Fig. 3A, lanes 3, 1). When rabbit anti-HMW serum is used for immunoblotting, it can only detect bands of adiponectin without heat denaturation (i.e., that of the trimer and multimer bands of 65 kDa, 150 kDa, and higher molecular mass). On the other hand, rabbit anti-C and anti-N sera can only detect bands of heat-denatured adiponectin (i.e., the monomer and dimer bands of 28 and 56 kDa) (Fig. 3B, lanes 6, 8). When the reactivity of the three monoclonal antibodies, IH5, IH6, and IH7, was analyzed, they showed the same characteristics as anti-HMW (i.e., they only reacted with the non-heat-denatured bands: the trimer, hexamer, and higher multimer bands) (Fig. 3B, lanes 1, 3).

Reactivity of antibodies

To elucidate the recognition sites for IH5, IH6, and IH7, reactivity with the wild-type recombinant and two cysteine mutants, Cys36Ser and Cys152Ser, was analyzed. As shown in **Fig. 4A**, lane 1, they recognized the three bands of the wild-type recombinant (i.e., the HMW, hexamer, and trimer). When the elution profile was analyzed by gel-filtration chromatography, the wild-type recombinant was eluted as the HMW, hexamer, and trimer molecules, the ratio being almost 1:1:1, and they were all detected by IH5, IH6, and IH7 (Fig. 4B, lanes 1–3). The N-terminal cysteine, cysteine 36, was reported to be important for the formation of the HMW and hexamer, although the C-terminal cysteine, cysteine 152, after mutation to alanine, did not have any effect on multimer formation (26). The Cys36Ser and Cys152Ser mutants were secreted into the medium far less than the wild-type recombinant, and their amounts were ~1/30th and 1/10th that of the wild-type recombinant, respectively, on densitometry analysis. When their elution profiles were analyzed by gel-filtration chromatography, these two cysteine mutants were mostly eluted as the trimer (Fig. 4C, D, lanes 7–9). In contrast to the cysteine-to-alanine mutation, a cysteine-to-serine mutation might induce some conformational change, and the Cys36Ser and Cys152Ser mutants did not form stable multimers. Unexpectedly, IH5, IH6, and IH7 scarcely reacted with the HMW, hexamer, and trimer forms of the Cys152Ser mutant (Fig. 4C, lanes 1–3). As described previously (4), heat denaturation or acidic pH treatment of HMW adiponectin induces a conformational change of the collagen-like domain, and after these treatments, HMW adiponectin could be detected with anti-C. After acetate treatment of the same nitrocellulose membrane, the HMW, hexamer, and trimer bands of Cys152Ser could be detected with anti-C (Fig. 4C, lanes 4–6). These results suggested that, although the Cys152Ser mutant could form the trimer and higher multimers, their conformations are different from the native forms, at least with respect to the IH5, IH6, and IH7 recognition sites. The Cys36Ser mutant was secreted far less efficiently than the wild type and the Cys152Ser mutant, and as shown in Fig. 4D, lanes 7–9, almost all of the secreted molecules were proteolytically cleaved into an ~26 kDa form similar to the Cys36Ala mutant reported by Pajvani et al. (25). Instead of its instability, the Cys36Ser mutant could form low amounts of HMW and hexamer, which could react with IH5, IH6, and IH7 (Fig. 4D, lanes 1–3). After acetate treatment, in addition to the HMW and hexamer bands, anti-C detected monomer and dimer bands of ~26 and ~52 kDa, which might be formed through cross-linking of collagen-like domains. This means that the Cys36Ser mutant could not form a trimer that is stable in SDS-containing loading buffer like the native adiponectin, suggesting the importance of cysteine 36 for the formation of a stable trimer. From these results, IH5, IH6, and IH7 were suggested to react with the C-terminal globular domain of one trimer.

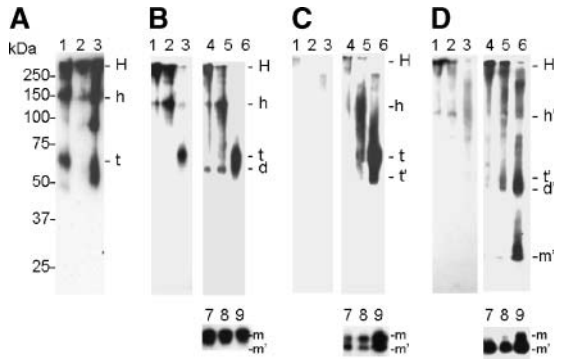

Fig. 4. Reactivity analysis of IH7. A: The wild-type recombinant (lane 1), human serum HMW adiponectin (lane 2), and recombinant adiponectin purchased from R&D (lane 3) were detected with IH7. B–D: The wild-type recombinant (B), Cys152Ser mutant (C), and Cys36Ser mutant (D) were fractionated by gel chromatography, and HMW adiponectin (lanes 1, 4, and 7), the hexamer (lanes 2, 5, and 8), and the trimer (lanes 3, 6, and 9) were detected with IH7 (lanes 1–3) or anti-C (lanes 4–9). The samples in lanes 1–6 were neither heat-denatured nor reduced, and those in lanes 7–9 were heat-denatured under reducing conditions. Lanes 4–6 show data for the same nitrocellulose membrane as lanes 1–3, and after acetate treatment, it was detected by anti-C. H, h, t, d, and m are as in Fig. 3. h′, t′, d′, and m′ are assumed to be proteolytically cleaved bands of the hexamer, trimer, dimer, and monomer, respectively.

Adiponectin contains at least two *O*-glycoside chains, which are attached to threonine 22 and threonine 37 in the N-terminal nonglobular domain (Fig. 1). The wild-type recombinant contains two more *O*-glycoside chains that are attached to threonine 20 and threonine 21, and these additional *O*-glycoside chains do not influence the reactivity of IH5, IH6, and IH7. Also, *O*-glycoside digestion of these adiponectins with neuraminidase and *O*-glycanase did not have any effect on the reactivity with IH5, IH6, and IH7 (data not shown). These data might further support the notion that the recognition epitopes of IH5, IH6, and IH7 are in the C-terminal globular domain of one trimer.

Using an HMW adiponectin coat plate, the reactivity of three monoclonal antibodies was also analyzed. After treatment with one monoclonal antibody, the plate was treated with each of the other monoclonal antibodies, which were biotinylated, and the bound biotinylated antibody was detected with streptavidin-conjugated horseradish peroxidase. In these experiments, it was shown that IH5, IH6,

and IH7 bind to same site or nearby sites to block binding of the other two antibodies.

Development of an ELISA for adiponectin

Using IH5, IH6, and IH7, we tried to develop sandwich ELISA systems and found that with any combination of two of the three antibodies and even with the same antibody, a similar sandwich ELISA system could be constructed. Finally, using IH7 as the capture antibody and POD-IH7 as the detecting antibody, a sandwich ELISA was developed. As the adiponectin standard, HMW adiponectin, GBP28, purified from human serum was used. The concentration of standard HMW adiponectin was decided based on the absorbance at 280 nm and the results of quantitative amino acid composition analysis and the bicinchoninic acid protein assay with BSA as a standard. These three analyses gave almost the same concentration for the HMW adiponectin standard, and the absorbance of 1.0 at 280 nm was about 1.0 mg/ml. From these data, the absorbance at 280 nm was chosen as the concentration of the HMW adiponectin standard.

When human serum is separated by gel chromatography on a HiLoad 16/60 Superdex 200 prep-grade column, adiponectin is eluted as three peaks corresponding to apparent molecular masses of 420, 240, and 180 kDa, which represent HMW and the hexamer and trimer. Although adiponectin molecules give three peaks, which could be detected on immunoblotting with anti-C, IH5, IH6, or IH7, with the developed ELISA, the HMW molecule could only be measured effectively when the result was compared with quantitative data obtained on densitometry (**Fig. 5A, B**). Additionally, as reported previously (4), only HMW adiponectin showed affinity to gelatin and could be affinity-precipitated with gelatin-Cellulofine. When serum with a low concentration of adiponectin was analyzed, even though more hexamer and trimer existed than HMW, similar results were obtained (i.e., only HMW adiponectin could be measured effectively) (Fig. 5C, D).

Next, to develop an ELISA for total adiponectin, using an HMW adiponectin coat plate, a competition ELISA system was tested. Briefly, each fraction was preincubated with POD-IH7 and then added to an HMW adiponectin coat plate. Again, only HMW adiponectin could be measured. No combination of IH5, IH6, and IH7 could detect molecules except HMW molecules. Competition ELISA with rabbit anti-HMW polyclonal antibodies was also performed, and again only HMW adiponectin was measured. We could not establish an ELISA with any antibodies against HMW adiponectin to measure the hexamer or trimer effectively. Finally, with the obtained monoclonal antibodies against HMW adiponectin, only a novel ELISA system specific for HMW adiponectin could be developed.

With our ELISA system with IH7 and POD-IH7, appropriate calibration curves were obtained with human plasma-derived HMW adiponectin and concentrations ranging from 0.4 to 50 ng/ml. The intra-assay coefficient of variation for replicate measurement of four samples ranged from 2.4% to 3.0%, and the interassay coefficient of variation ranged from 4.2% to 5.1%. With food intake, the HMW adiponectin concentration was decreased slightly after 1–2 h with a carbohydrate diet and after 4–5 h with an ordinary diet containing lipid (data not shown). After surgical operation, the HMW adiponectin concentration was observed to

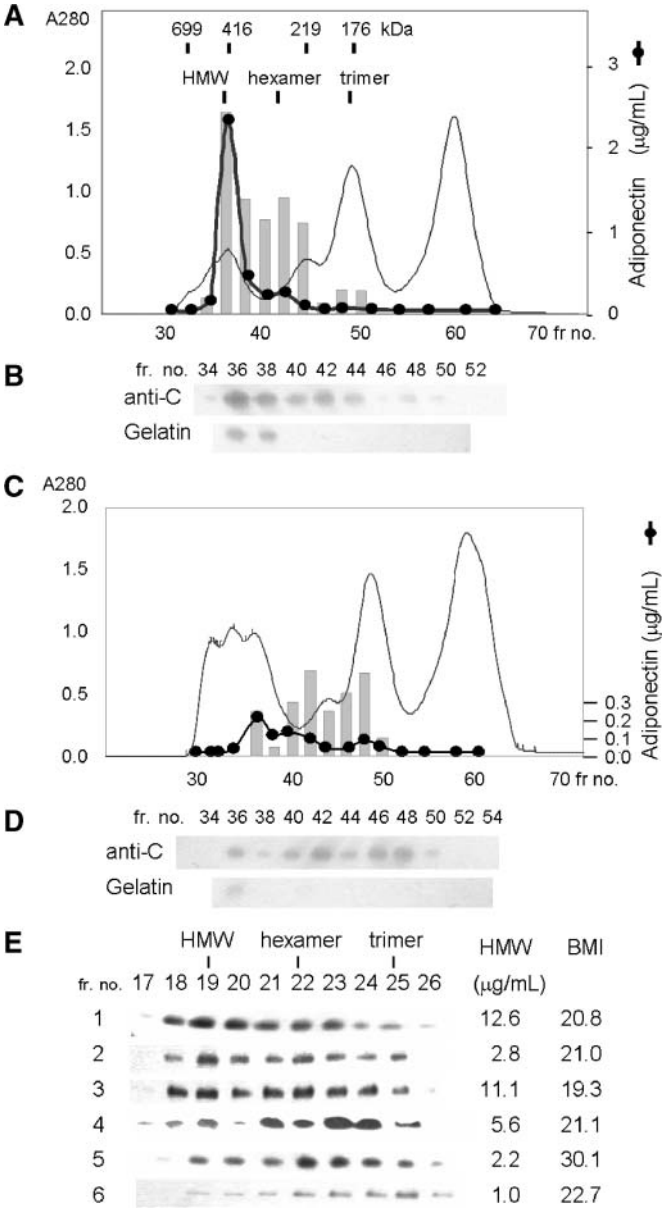

Fig. 5. Specificity of a novel ELISA with IH7. A: Gel filtration analysis of a lean individual's serum using the HiLoad 16/60 Superdex 200 prep-grade column. The elution profile was monitored for absorbance at 280 nm (A280; thin line). The closed circles show adiponectin concentrations in individual fractions obtained with the novel ELISA with IH7, and the gray bars show immunoblotting data for anti-C quantified by densitometry (see B). B: Each fraction (fr.) in A was heat-denatured under reducing conditions, analyzed by immunoblotting with anti-C, affinity-precipitated with gelatin-Cellulofine (Gelatin), and analyzed by immunoblotting with anti-C. C, D: Data for an obese individual. E: Sera with various HMW adiponectin concentrations were analyzed by gel chromatography on a Superdex 200 HR 10/30 column and immunoblotting. Rows 1 and 2 show data for women, and rows 3–6 show data for men. BMI, body mass index.

decrease, and after C-reactive protein (CRP) normalized, it increased to the original level (data not shown). With a very few plasma samples, we experienced a marked decrease in the HMW adiponectin concentration after several months of storage at −70°C. Thus, we recommend serum as a sample, which should be obtained after overnight fasting from an individual without obvious inflammation and stored at −70°C for long-term storage.

Ratio of HMW, hexamer, and trimer in each serum

When serum samples with various HMW adiponectin concentrations were analyzed by gel chromatography and immunoblotting, variable ratios of HMW, hexamer, and trimer were observed. In sera from women, independent of the HMW adiponectin concentration, HMW was most abundant, the hexamer was next, and the trimer was least (Fig. 5E, rows 1, 2). In contrast to those from women, sera from men showed variable ratios. Although sera with high concentrations of HMW adiponectin showed a similar pattern to sera from women, as shown in Fig. 5E, row 3, sera with lower concentrations showed variable patterns. In some, the trimer was the most abundant molecule, and in some, the hexamer was most abundant, as shown in Fig. 5E, rows 4–6. These results suggest that the HMW adiponectin concentration and the total adiponectin concentration do not correlate with each other, so each adiponectin molecule concentration has a different meaning.

Comparison with commercial ELISA kits

There are many commercial ELISA kits for human adiponectin, so next we checked which molecules were measured with these kits. We purchased a kit from Otsuka Pharmaceuticals. We also purchased an anti-human adiponectin/Acrp30 antibody (antiglobular), a biotinylated antiglobular, and a recombinant human adiponectin as a standard from R&D Systems and constructed an ELISA system according to the manufacturer's instructions. For the analysis of many samples, a Superdex 200 HR 10/30 column was used to separate human serum instead of the HiLoad 16/60 Superdex 200 prep-grade column. As shown in **Fig. 6A**, from the Superdex 200 HR 10/30 column, human adiponectin elutes faster than from the HiLoad 16/60 Superdex 200, and the hexamer and trimer were eluted at positions corresponding to apparent molecular masses of ~320 and 220 kDa. When each fraction was assayed with these ELISA systems, both of them detected three peaks (i.e., all molecules), with a different efficiency for each molecule. Somehow, the reactivities of the ELISA systems from both Otsuka Pharmaceuticals and R&D Systems with HMW, and the ELISA system from R&D Systems with the trimer, were low compared with quantitative densitometry (Fig. 6B, C). From these results, each ELISA was shown to have a different specificity. Again, only our ELISA was shown to be HMW adiponectin-specific and to measure it effectively.

Comparison of IH5, IH6, and IH7 with the antiglobular

The antiglobular from R&D Systems was raised against recombinant adiponectin with a 6× histidine tag at the

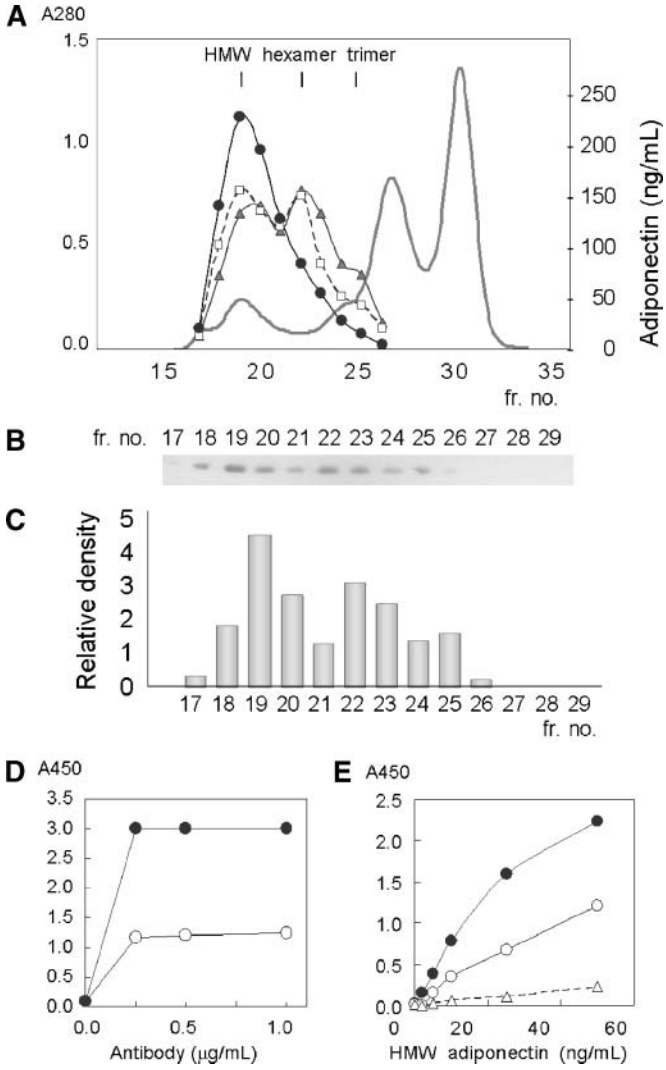

Fig. 6. Comparison of ELISAs and antibodies. A: Human serum was fractioned on a Superdex 200 HR 10/30 column, and each fraction was assayed with our novel ELISA with IH7 (closed circles), an ELISA kit from Otsuka Pharmaceuticals (closed triangles), and an ELISA with the antiglobular (R&D Systems) (open squares). The gray line shows the absorbance at 280 nm (A280). B: Each fraction (fr.) was heat-denatured under reducing conditions and then analyzed by immunoblotting with anti-C. C: Densitometry data for B. D: The antiglobular (open circles) and IH7 (closed circles) were added to an HMW adiponectin coat plate, and after 1 h of incubation at room temperature, bound antibodies were detected with peroxidase-conjugated anti-mouse IgG. E: After HMW adiponectin had been captured on an IH7 coat plate, the plate was incubated with 1 μg/ml antiglobular (open circles) or IH7 (open triangles) or with no antibody (closed circles) for 1 h at room temperature, and after thorough washing, horseradish peroxidase-conjugated IH7 Fab′ was added.

C terminus and is reported to detect an epitope in the globular domain (amino acids 104–244), according to the manufacturer. Also, the purchased recombinant human adiponectin contained almost equal amounts of HMW and the hexamer and trimer, with an extra multimer of ~100 kDa, which might be one trimer with an extra disulfide-linked monomer, as judged on analysis by im-

munoblotting with IH7 (Fig. 4A, lane 3). The antiglobular could not react with heat-denatured bands but could react with non-heat-denatured bands (i.e., HMW and the hexamer and trimer, like IH5, IH6, and IH7) when it was used for immunoblotting (data not shown). From this, the antiglobular was suggested to recognize a tertiary structure in the globular domain of one trimer. On the other hand, the antibodies in the Otsuka ELISA kit can only react with heat- and SDS-denatured adiponectin. Therefore, next we studied whether the antiglobular could compete with IH5, IH6, and IH7 using an HMW adiponectin coat plate. As shown in Fig. 6D, the binding of the antiglobular to HMW adiponectin was much lower than that of IH7, and this result suggested that the number of epitopes in HMW adiponectin recognized by the antiglobular was fewer than by IH7. After HMW adiponectin had been captured on an IH7 coat plate, the plate was incubated with the antiglobular for 1 h at room temperature, and then, after thorough washing, POD-IH7 was added. With the antiglobular treatment, the reactivities of IH5, IH6, and IH7 were reduced to approximately one-third (Fig. 6E). From these results, IH5, IH6, and IH7 are again suggested to react with the C-terminal globular domain in one trimer and to recognize nearby, but different, sites from the epitope recognized by the antiglobular.

HMW adiponectin concentrations in serum samples from healthy subjects

Using our novel ELISA method, HMW adiponectin concentrations were determined in serum samples from 246 subjects (age range, 25–83 years) in a fasting state and randomly chosen as a subsample for the Health Examination Survey study. Among the 246 subjects, 159 were women aged 60.7 ± 11.4 years with body mass index of 23.5 ± 2.8 kg/m^2 and fat percentage of 30.3 ± 5.9%, and 87 were men aged 61.9 ± 12.2 years with body mass index of 23.9 ± 3.2 kg/m^2 and fat percentage of 22.5 ± 5.5%. Only fat percentage was significantly different ($P < 0.001$). The mean concentration of HMW adiponectin in the 159 women was 7.8 ± 4.9 μg/ml (mean ± SD; range, 0.5–22.2 μg/ml). The mean concentration in the 87 men was 4.8 ± 3.7 μg/ml (range, 0.4–17.0 μg/ml), significantly lower than that in women ($P < 0.001$). The total adiponectin concentration determined with the commercial ELISA kit (Otsuka) was also examined. The mean concentration of total adiponectin in the 159 women was 9.7 ± 5.1 μg/ml (range, 1.9–26.0 μg/ml) and that in the 87 men was 6.9 ± 4.1 μg/ml (range, 1.7–23.0 μg/ml), significantly lower than that in women ($P < 0.001$). Total adiponectin concentration was significantly higher than HMW adiponectin concentration ($P < 0.001$), and the differences were 2.2 ± 1.0 μg/ml (range, −1.3–6.7 μg/ml) and 1.8 ± 1.1 μg/ml (range, 0.0–6.0 μg/ml) in men and women ($P = 0.024$), respectively.

The correlation between the HMW adiponectin concentration and the total adiponectin concentration was also analyzed. As shown in **Fig. 7A, B**, the HMW adiponectin concentration and total adiponectin concentration showed good correlation ($r = 0.976$, $P < 0.001$), but the total adiponectin concentration was much higher than the HMW adiponectin concentration, especially below an HMW adiponectin concentration of 3 μg/ml. Although the ratio of the HMW adiponectin concentration to the total adiponectin concentration (HMW/total) could not be the real ratio of HMW adiponectin molecules to total molecules in a serum sample, because of the different standards, HMW/total was calculated and plotted against the HMW adiponectin concentration. The mean HMW/total in the 159 women was 0.76 ± 0.15 (range, 0.27–1.07), and that in the 87 men was 0.62 ± 0.18 (range, 0.23–1.00), significantly lower than that in women ($P < 0.001$). As shown in Fig. 7C, sera containing 3 μg/ml or less HMW adiponectin concentration exhibited extremely lower HMW/total values. A similar tendency, but an obviously lower correlation, was observed for the total adiponectin concentration and HMW/total (Fig. 7E). Judging from these results, with lower HMW adiponectin concentrations, HMW adiponectin is a minor molecule and the hexamer and/or trimer should be major.

To determine the real ratio of HMW adiponectin molecules to total molecules (i.e., HMW%), serum samples were applied to a Superdex 200 HR 10/30 column and

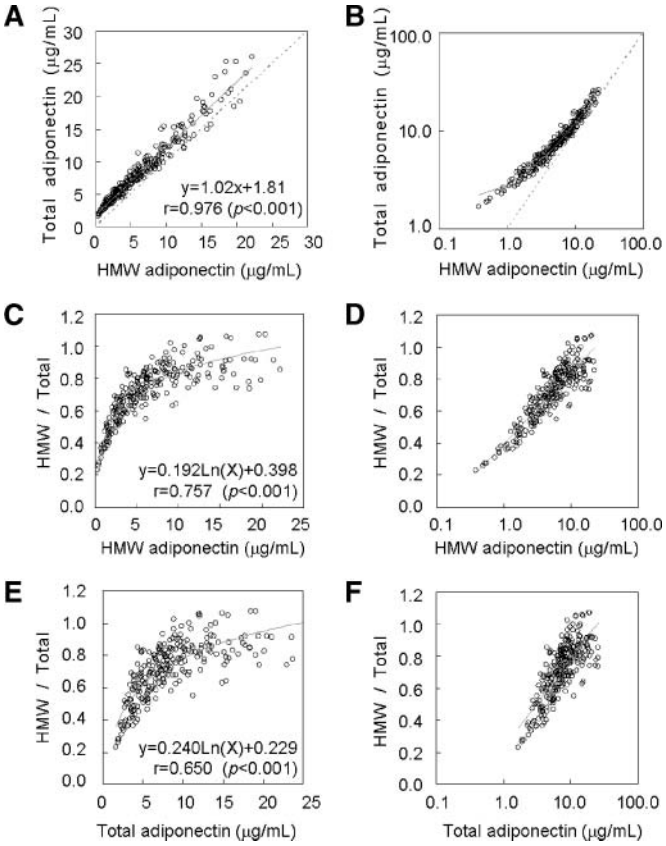

Fig. 7. Correlations between HMW adiponectin and total adiponectin with an ELISA kit from Otsuka Pharmaceuticals (A, B), HMW/total (C, D), and HMW/total and total adiponectin (E, F). HMW/total is the ratio of the HMW adiponectin concentration to the total adiponectin concentration of each subject. In B, D, and F, the HMW and total adiponectin concentrations are shown on a log scale.

fractions were analyzed by immunoblotting and densitometry, as shown in Fig. 6. The HMW% was calculated using relative density data. For example, the sum of the densities of fractions 18–20 was divided by the total density of fractions 18–26 in Fig. 6C. As described and shown in Fig. 5E, independent of the HMW adiponectin concentration, the ratio of HMW and the hexamer and trimer in women's sera did not differ greatly, and in **Fig. 8**, only data for three women whose HMW adiponectin concentrations were 2.9, 6.6, and 13.9 μg/ml are shown. For men, the ratio varied, and as shown in Fig. 8A, the HMW% in 15 men showed significant correlation with the HMW adiponectin concentrations ($r = 0.731$, $P = 0.002$), and the sera with lower HMW concentrations exhibited lower HMW%. Although the HMW/total values were higher than the HMW%, there was significant correlation ($r = 0.553$, $P = 0.03$). For convenience, it might be possible to use HMW/total instead of HMW%. HMW/total in women and men plotted against the HMW adiponectin concentration is shown in Fig. 8C, D, respectively. For the 159 women, HMW/total was >0.55 with the exception of only 11 sera in which the HMW adiponectin concentrations were 1.8 μg/ml or less. HMW/total in men was low, and 35 of the 87 men (i.e., 40%) had HMW/total of <0.55 and HMW adiponectin concentrations of <3.0 μg/ml.

Among the 246 subjects, even though they were clinically healthy, 116 had hypertension defined as systolic blood pressure levels of ⩾140 mm Hg and/or diastolic blood pressure levels of ⩾90 mm Hg. Forty-nine subjects had blood glucose levels of ⩾110 mg/dl and/or glycated hemoglobin A1c levels of ⩾5.8%, the averages being 139.7 mg/dl and 6.1%, respectively. Eight subjects had HDL levels of <40 mg/dl, 106 had LDL levels of ⩾140 mg/dl, and 29 had triglyceride levels of ⩾150 mg/dl. Also, three subjects had CRP levels of ⩾0.1 mg/dl. Finally, only 58 subjects were healthy: 43 women and 15 men. The mean concentrations of HMW adiponectin in healthy women and healthy men were 8.4 ± 5.5 and 6.2 ± 3.6 μg/ml ($P = 0.174$), the mean concentrations of total adiponectin were 10.4 ± 6.4 and 8.6 ± 4.1 μg/ml ($P = 0.303$), and HMW/total values were 0.76 ± 0.14 and 0.69 ± 0.12 ($P = 0.092$), respectively. There were no significant differences between healthy women and healthy men, or between healthy subjects and others, as shown in **Fig. 9** and **Table 1**, except HMW/total between healthy men and other men. Between healthy women or other women and other men, there were significant differences ($P < 0.001$).

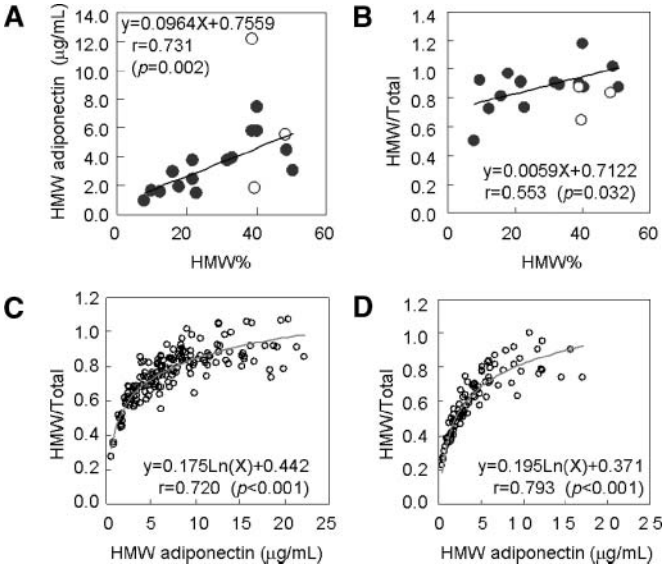

Fig. 8. Correlation between HMW adiponectin and the HMW% (A), HMW/total and the HMW% (B), and HMW/total and HMW adiponectin in women (C) and men (D). To determine the HMW%, each serum was fractioned by gel chromatography, and fractions were analyzed by immunoblotting and densitometry as in Fig. 6. Then, HMW% was calculated by dividing the sum of the densities of HMW adiponectin fractions 18–20 by that of adiponectin fractions 18–26. In A, B, data for three women (open circles) are plotted for reference.

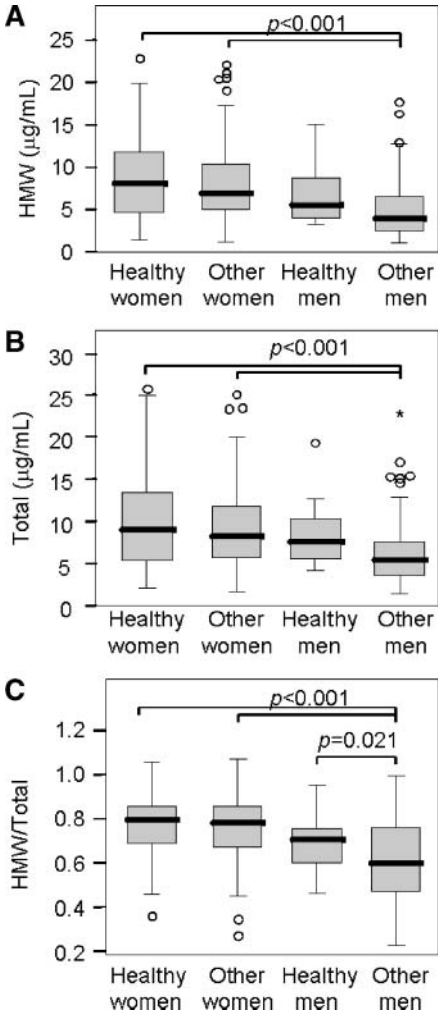

Fig. 9. Comparison of the HMW adiponectin concentration (A), total adiponectin concentration (B), and HMW/total (C) among healthy women (43 subjects), other women with abnormal biomarkers (116 subjects), healthy men (15 subjects), and other men with abnormal biomarkers (72 subjects).

DISCUSSION

In human plasma, adiponectin exists as a trimer, a hexamer, and HMW multimers. Only HMW adiponectin can bind to gelatin-Cellulofine and be eluted with 1 M NaCl. Using this gelatin binding HMW adiponectin, GBP28, as an antigen, monoclonal antibodies IH5, IH6, and IH7 were obtained. To date, all antibodies except our monoclonal antibodies have been raised against recombinant adiponectin, and only IH5, IH6, and IH7 are antibodies raised against native adiponectin purified from human serum. When they are used for immunoblotting, they recognize all native molecules (i.e., the trimer, hexamer, and HMW) but cannot recognize the monomer or dimer that arises when adiponectin is heat-denatured under reducing or nonreducing conditions. From these results, IH5, IH6, and IH7 are suggested to recognize a tertiary structure formed by three monomers and existing within one intact trimer. Because IH5, IH6, and IH7 could not recognize the Cys152Ser mutant, which forms mainly the trimer, they were suggested to recognize the tertiary structure of a C-terminal globular part in one adiponectin trimer.

Using IH7, we developed a novel sandwich ELISA system. When human serum fractions separated by gel chromatography were analyzed, HMW adiponectin could be measured effectively and specifically. On the other hand, a commercially available ELISA kit from Otsuka Pharmaceuticals was found to measure all adiponectin molecules. For this ELISA method, samples should be heat-denatured in SDS-containing buffer, and all adiponectin molecules should be denatured to be detected. With this ELISA, HMW adiponectin was measured with less efficiency than with our ELISA and gave lower values. In addition, we developed another sandwich ELISA with an antiglobular according to the manufacturer's instructions. According to the manufacturer's information, the antiglobular was raised against recombinant full-size human adiponectin with a 6× histidine tag at its C terminus and detects an epitope in the C-terminal globular domain. The antiglobular and IH5, IH6, and IH7 showed some competition in reaction with HMW adiponectin, and this further supports the notion that IH5, IH6, and IH7 recognize an epitope or epitopes in the C-terminal globular domain. For ELISA with the antiglobular, the sample does not need to be denatured, and after dilution, as intact molecules, adiponectin can be measured. Again with this ELISA, all adiponectin molecules could be measured with less efficiency for the HMW and trimer forms. It is very interesting that the measured adiponectin molecules depend on the antibodies used and that each antibody shows a different preference.

Next, using our ELISA, HMW adiponectin concentrations were determined in serum samples of a subsample for the Health Examination Survey study. As expected, the adiponectin concentrations determined with our ELISA and the ELISA kit from Otsuka Pharmaceuticals were different, and the HMW adiponectin concentrations determined with our ELISA were lower than the total adiponectin concentrations determined with the ELISA kit from Otsuka Pharmaceuticals. Surprisingly, the HMW adiponectin concentration and total adiponectin concentration showed very good correlation ($r = 0.976$, $P < 0.001$). Although they showed very good correlation as a whole, especially at <3 μg/ml HMW adiponectin, the concentrations in individual sera differed greatly, and the HMW adiponectin concentrations were much lower than the total adiponectin concentrations ($P < 0.001$).

The serum total adiponectin concentration in men was reported to be significantly lower than that in women, because androgens decrease plasma adiponectin by reducing adiponectin secretion from adipocytes (30). Pajvani et al. (27) reported that the majority of adiponectin in male mouse sera is the hexamer, whereas female mice have similar levels of the hexamer and HMW, and surprisingly, both male and female mice have similar and higher propor-

TABLE 1. Clinical characteristics of the subjects

Characteristic	Healthy Women	Other Women	*P*	Healthy Men	Other Men	*P*
Number	43	116		15	72	
HMW adiponectin (μg/ml)	8.4 ± 5.5	7.6 ± 4.7	0.418	6.2 ± 3.6	4.5 ± 3.7	0.095
Total adiponectin (μg/ml)	10.4 ± 6.1	9.4 ± 4.7	0.358	8.6 ± 4.1	6.6 ± 4.1	0.086
HMW/total	0.76 ± 0.14	0.76 ± 0.15	0.987	0.69 ± 0.12	0.60 ± 0.18	0.021
Age (years)	57.5 ± 15.3	61.9 ± 9.3	0.085	54.9 ± 14.4	63.3 ± 11.3	0.014
Body mass index (kg/m^2)	22.4 ± 2.6	23.8 ± 2.8	0.014	21.6 ± 1.9	24.3 ± 3.2	0.002
Fat (%)	28.4 ± 5.7	31.1 ± 5.8	0.005	18.3 ± 3.7	23.4 ± 5.5	0.001
Systolic blood pressure (mm Hg)	119.1 ± 14.8	133.5 ± 18.5	<0.001	118.6 ± 9.9	138.8 ± 20.6	<0.001
Diastolic blood pressure (mm Hg)	69.6 ± 10.9	78.0 ± 11.5	<0.001	73.0 ± 8.7	84.1 ± 12.1	0.001
Aspartate aminotransferase (U/l)	20.9 ± 5.5	24.2 ± 7.9	0.015	22.6 ± 5.7	25.9 ± 8.6	0.161
Alanine aminotransferase (U/l)	15.9 ± 4.6	23.2 ± 13.9	<0.001	18.6 ± 5.6	27.5 ± 18.1	0.001
γ-Glutamyl transpeptidase (U/l)	16.5 ± 5.5	30.1 ± 25.2	<0.001	23.2 ± 5.9	50.3 ± 47.7	<0.001
Creatinine (mg/dl)	0.62 ± 0.09	0.62 ± 0.12	0.845	0.84 ± 0.13	0.82 ± 0.14	0.563
Glycated hemoglobin A1c (%)	4.8 ± 0.4	5.2 ± 0.8	0.01	4.7 ± 0.2	5.4 ± 1.0	<0.001
Fasting plasma glucose (mg/dl)	88.3 ± 6.2	99.2 ± 26.5	<0.001	90.9 ± 7.8	112.5 ± 36.4	<0.001
Atherogenic index	1.75 ± 0.47	2.81 ± 1.12	<0.001	2.07 ± 0.71	2.94 ± 1.02	0.002
HDL-cholesterol (mg/dl)	71.6 ± 14.0	64.0 ± 16.7	0.009	64.7 ± 18.1	57.3 ± 14.9	0.095
LDL-cholesterol (mg/dl)	109.7 ± 18.4	144.5 ± 31.7	<0.001	112.0 ± 19.5	136.1 ± 34.3	0.001
Tryglyceride (mg/dl)	72.6 ± 24.4	124.7 ± 65.8	<0.001	80.7 ± 22.0	133.7 ± 61.0	<0.001

HMW, high-molecular-weight; Other, subjects with abnormal biomarker(s); All values except number are presented as means ± SD. *P* values were determined by means of unpaired *t*-tests. Atherogenic index = (total cholesterol − HDL-cholesterol)/HDL-cholesterol.

tions of HMW adiponectin within their adipose tissue than in sera. Pajvani et al. (27) also reported similar results for human serum and subcutaneous abdominal adipose adiponectin. In 2005, Xu et al. (31) showed that testosterone selectively inhibits HMW adiponectin secretion from rat adipocytes. In our study, the serum HMW adiponectin concentration and HMW/total were significantly lower in men than in women, and these findings further confirmed androgen-induced hypoadiponectinemia in men as a result of reduced HMW adiponectin secretion. The resulting low HMW adiponectin concentration might be related to the high risks of insulin resistance and atherosclerosis in men.

Peroxisome proliferator-activated receptor γ agonist (thiazolidinedione) treatment and weight reduction upregulate plasma total adiponectin (32, 33), and in 2004, increased total adiponectin was shown to be a result of an increase in HMW (27). With thiazolidinedione treatment, serum HMW adiponectin increased greatly and the improvement of the HMW/(HMW + hexamer) ratio was strongly correlated with the improvement of insulin sensitivity. From this finding, Pajvani et al. (27) concluded that the HMW adiponectin amount, not the absolute amount of adiponectin, was important for antidiabetic activities. With weight reduction, serum HMW adiponectin also increased greatly, with little changes in the hexamer and trimer (28). In these studies, gel filtration or velocity sedimentation was used to separate HMW adiponectin from other molecules, and each fraction was analyzed by ELISA or immunoblotting. By simply examining sera with our novel ELISA, we observed that HMW adiponectin significantly increased on thiazolidinedione treatment or on weight reduction before a significant increase in total adiponectin (data not shown). We also monitored the change in the HMW adiponectin concentration after a meal, the results being similar to those for mice obtained by Pajvani et al. (25) using velocity sedimentation. These findings suggest that our novel ELISA is an easy and useful method for monitoring HMW adiponectin concentrations.

In conclusion, the developed ELISA is specific for HMW adiponectin, and by simply examining sera with our novel ELISA, one can measure the HMW adiponectin concentration and monitor its changes, which we hope will be useful for the prevention and treatment of metabolic syndrome.

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