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Paradoxical Decrease of an Adipose-Specific Protein, Adiponectin, in Obesity

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We isolated the human adipose-specific and most abundant gene transcript, apM1 (Maeda, K., et al., *Biochem. Biophys. Res. Commun.* 221, 286–289, 1996). The apM1 gene product was a kind of soluble matrix protein, which we named adiponectin. To quantitate the plasma adiponectin concentration, we have produced monoclonal and polyclonal antibodies for human adiponectin and developed an enzyme-linked immunosorbent assay (ELISA) system. Adiponectin was abundantly present in the plasma of healthy volunteers in the range from 1.9 to 17.0 mg/ml. Plasma concentrations of adiponectin in obese subjects were significantly lower than those in non-obese subjects, although adiponectin is secreted only from adipose tissue. The ELISA system developed in this study will be useful for elucidating the physiological and pathophysiological role of adiponectin in humans.
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Obesity, defined as an accumulation of excess body fat frequently accompanies insulin resistance, hypertension, dyslipoproteinemia and vascular diseases and is a major health problem in the industrialized countries. The molecular basis of the pathogenesis of obesity-linked disorders has not been fully elucidated. We performed a systematic analysis of expressed genes in human adipose tissue (2). Unexpectedly, human adipose tissue expressed a variety of genes for secretory proteins. One of the examples is plasminogen activator inhibitor type 1 (PAI-1), which is a potent inhibitor of the fibronolytic system. Expression of PAI-1 mRNA was augmented in adipose tissue, especially intra-abdominal fat during the development of obesity in a rodent model (3). Plasma PAI-1 levels were closely correlated with the intra-abdominal fat depot in humans (3), suggesting its significance in the development of vascular disease. Substantial research has indicated that adipose tissue is not simply an energy storage organ, but is a secretory organ which produces a variety of proteins that influence the metabolism of the body. Leptin (4) transmits a satiety signal through the bloodstream to the brain. Tumor necrosis factor α (TNFα) interferes with insulin receptor signaling and is a possible cause of the development of insulin resistance in obesity (5). Those 'adipocytokines' may be relevant to obesity-linked disorders.

Through the analysis of human adipose tissue cDNA library, we have isolated a novel cDNA which is specifically and abundantly expressed in adipose tissue (1). The cDNA encoded a 244-amino-acid protein highly homologous to collagen VIII, X and complement factor C1q with a putative signal sequence. The gene product was predicted to be a kind of matrix protein synthesized by adipose tissue and we named it adiponectin. The physiological role of adiponectin in humans has not yet been elucidated. In the current study, we have developed an enzyme-linked immunosorbent assay (ELISA) system for the measurement of plasma adiponectin and determined its plasma levels in non-obese and obese subjects.

MATERIALS AND METHODS

Expression of recombinant adiponectin and preparation of antibodies. A 693-bp adiponectin cDNA (nt 69-761) encoding a protein eliminating a leader peptide was subcloned into the pET3c expression vector (Novagen Inc, USA.) and was used to transform host E. coli, BL21(DE3)pLysS. Synthesis of recombinant adiponectin protein was induced by isopropylthio-β-D-galactoside (IPTG) at the final concentration at 0.4 mM. Bacterial cells were precipitated and suspended in 50 mM Tris-HCl (pH 8.0) for one hour and added Triton X-100 at the final

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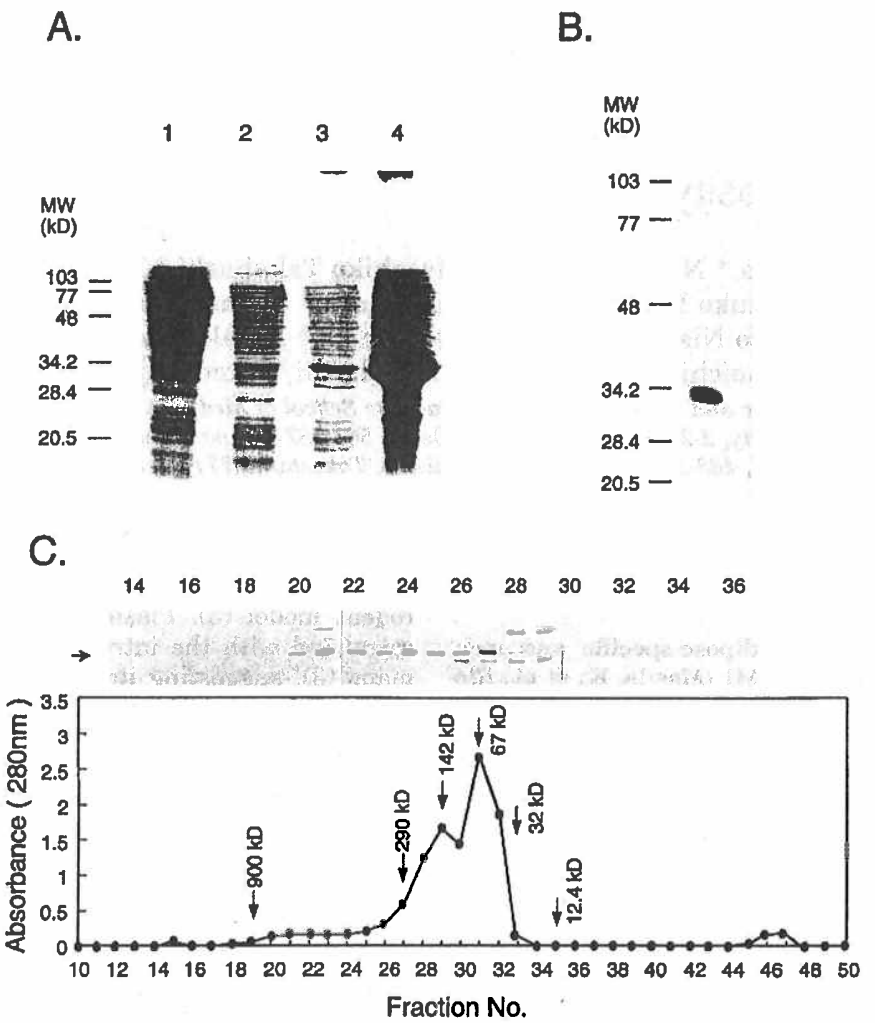

FIG. 1. Expression of adiponectin in E. coli and detection of adiponectin in plasma. Adiponectin cDNA was subcloned into the pET3c vector and recombinant protein was expressed in E. coli, BL21(DE3)pLysS. The lysates of E. coli cells which did not expressed adiponectin (lane 1), cells before IPTG induction (lane 2), cells after IPTG induction (lane 3) and the inclusion body (lane 4) were subjected to SDS/PAGE and stained with Coomassie Brilliant Blue (A). Human plasma sample was subjected to SDS/PAGE. Adiponectin protein was detected by monoclonal antibody, ANOC9108 (B). Human plasma sample was fractionated by gel chromatography and an aliquot of each fraction was subjected to immunoblotting (C). Arrow shows the position of adiponectin. Dual bands in fraction 28 and 29 correspond to IgG.

concentration at 0.2% and sonicated. The suspended buffer was centrifuged and the pellet was washed by the same treatment. The pellet, inclusion body was precipitated and solubilized by 100 mM Tris-HCl (pH 8.0) containing 7 M guanidine HCl, 1% β-mercapto-ethanol. The solubilized protein was refolded in the presence of 200 volumes of 2 M urea, 20 mM Tris-HCl (pH 8.0) for 3 days at 4°C. The refolded protein was concentrated by centrifugal filter, dialyzed with 20 mM Tris-HCl (pH 8.0), and purified by DEAE-5PW ion-exchange high performance liquid chromatography (Toso, Japan) equilibrated in 20 mM Tris-HCl (pH 7.2) using a linear gradient of NaCl (0-1M). Mouse monoclonal antibodies (ANOC 9101-9111) and rabbit polyclonal antibodies (OCT 9101-9105) were raised against recombinant adiponectin. Specificity of monoclonal antibodies was verifyed by Western blotting. Native molecular mass of adiponectin in human plasma was analyzed by gel chromatography. One hundred μl of human plasma sample and molecular weight standards; human IgM (900 kilodalton, kD), glutamate dehydrogenase (290kD), lactate dehydrogenase (142kD), enolase (67kD), adenylate kinase (32kD), cytochrome C (12.4kD) were subjected onto Superose 6 (Pharmacia) and eluted with a buffer containing 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl. An aliquot of each fraction was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blotting using monoclonal antibody, ANOC 9108.

Enzyme-linked immunosorbent assay (ELISA). A 96-well plate was incubated with 5 mg/ml of monoclonal antibody, ANOC 9108 at 4°C overnight, and blocked with phosphate buffer saline containing 0.1% bovine serum albumin (BSA) and 0.05% sodium azide. Human plasma (10 μl each) was mixed with 90 μl of the sample buffer, 31.25mM Tris-HCl (pH 6.8), containing 2.3% SDS and boiled for 5 min. Sequentially the samples were diluted with the sample buffer and 50 μl of sample at a final 1:5000 dilution was applied to each well of the antibody-coated plate and incubated overnight at room temperature. The recombinant adiponectin was used as the standard. The wells were washed three times with 5 mM Tris-HCl (pH 8.0) containing 15 mM NaCl and 0.05% Tween 20, then 100 μl of a final 1:10,000 dilution of rabbit polyclonal antibody, OCT 9104 was added and incubated for three hours at room temperature. Each well was washed three times with wash buffer and the binding of OCT 9104

was determined by the O-phenylene-diamine dihydrochloride (OPD)-horse raddish peroxidase (HRP) method.

Plasma concentrations of adiponectin were measured in 87 non-obese subjects (51 men and 36 women; mean [± SD] age, 50.4 ± 12.6 years) and 57 obese subjects (32 men and 25 women; mean [± SD] age, 42.3 ± 12.9 years). Obese subjects, defined as those with a body mass index (BMI) greater than 26.4. The BMI was 22.8 ± 2.2 (16.1–26.2) for normal weight and 31.9 ± 5.5 (26.4-46.1) for obese subjects. Following a 12 hr fast, venous blood samples were drawn and the plasma samples were frozen at −80°C until analysis. The results in the normal weight and obese subjects were compared by means of Mann-Whitney U-test. Spearman's correlation coefficient was used to estimate linear relationships between variables. A regression model was fitted to determine the relation between plasma adiponectin levels and BMI. Regression analyses were performed to evaluate the relation of other variables to the plasma adiponectin levels. All analyses were conducted with JMP (SAS Institute Inc).

RESULTS

Detection of Adiponectin in Human Plasma

Adiponectin cDNA encoding a protein eliminating a putative signal peptide was subcloned into the pET3c vector and was transformed into E. coli. Synthesis of recombinant protein was induced by IPTG. A 30 kD protein corresponding to the position of the monomer of adiponectin was observed by SDS- PAGE (Fig. 1A). The inclusion body was solubilized and refolded. The resultant protein was purified by HPLC. The amino acid sequence of the purified protein was analyzed and verified to be identical to the predicted amino acid sequence of adiponectin. Rabbits and mice were immunized by the recombinant protein. Polyclonal antibodies (OCT 9101-9105) and monoclonal antibodies (ANOC 9101-9111) were raised. Western blotting analysis revealed that each of antibodies recognized recombinant adiponectin. Human plasma samples were also subjected to Western blotting. Of these antibodies, ANOC 9108 specifically detected adiponectin as a single band of 30 kD corresponding to a monomer form on SDS-PAGE (Fig. 1B). Thus, ANOC 9108 was used for further analysis.

To evaluate the native form of adiponectin in plasma, plasma sample was fractionated by gel chromatography and an aliquot of each fraction was subjected to immunoblotting. Proteins reacting with ANOC 9108 were widely distributed in the fractions from 20 to 27, corresponding to a molecular mass greater than 290 kD (Fig. 1C). The result suggests that adiponectin exists as a large multimeric molecule or forms a complex with other protein(s) in plasma.

Preliminarily, we evaluated plasma concentration of adiponectin by semi-quantative Western blotting using recombinant adiponectin as standards. Adiponectin was relatively abundant in plasma with a range of 2-10 μg/ml.

Development of ELISA System for Determination of Plasma Adiponectin

We tried to establish a sandwich ELISA system in which adiponectin specific antiboby, ANOC 9108 was used as a first antibody. When the plasma sample was directly subjected to ELISA system, the absorbance obtained was lower than expected by Western blotting. The result might be accounted for multimeric formation of adiponectin in plasma. Therefore, plasma samples were boiled with a buffer containing SDS to convert adiponectin to a monomeric form. Figure 2A shows the determined plasma adiponectin level with a different boiling time after treatment with SDS sample buffer. After boiling the plasma in SDS buffer for 5 min, adiponectin could be detected as the expected value judged by Western blotting. A linear curve was obtained with the range of recombinant adiponectin between 3.5 pg/well and 1000 pg/well and the dilution curve of human plasma was parallel to the standard curve (Fig. 2B). The intra-

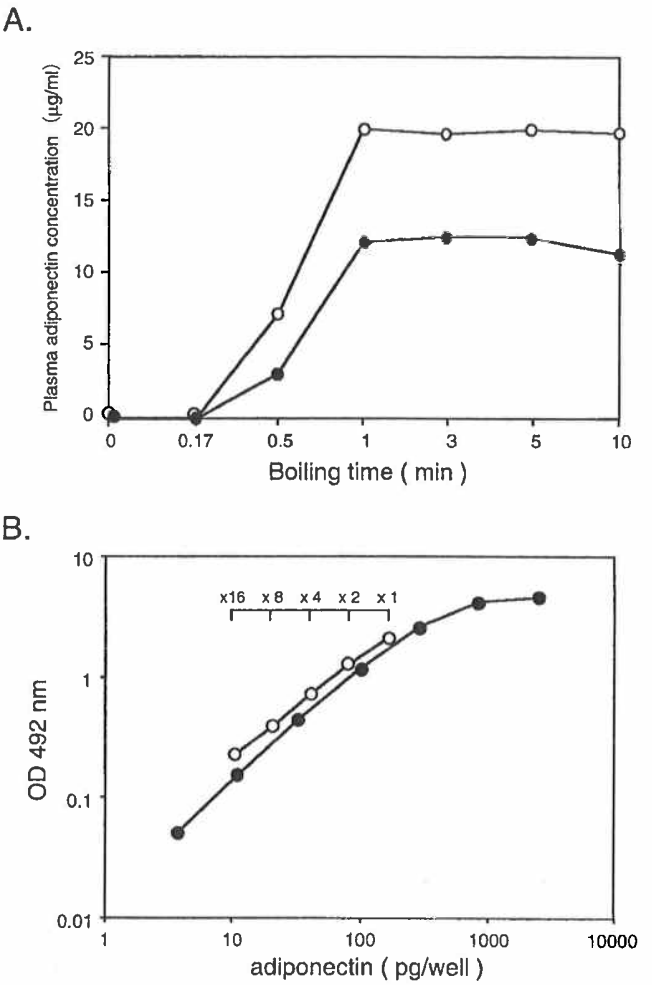

FIG. 2. Determination of plasma adiponectin concentration by ELISA system. Plasma samples from two volunteers (open circle and closed circle) were boiled with sample buffer containing SDS for the indicated time and were subjected to the ELISA system (A). Absorbance curve at 492 nm with the range of recombinant adiponectin between 3.5 pg/well and 1000 pg/well (closed circle) and dilution curve of human plasma (open circle).

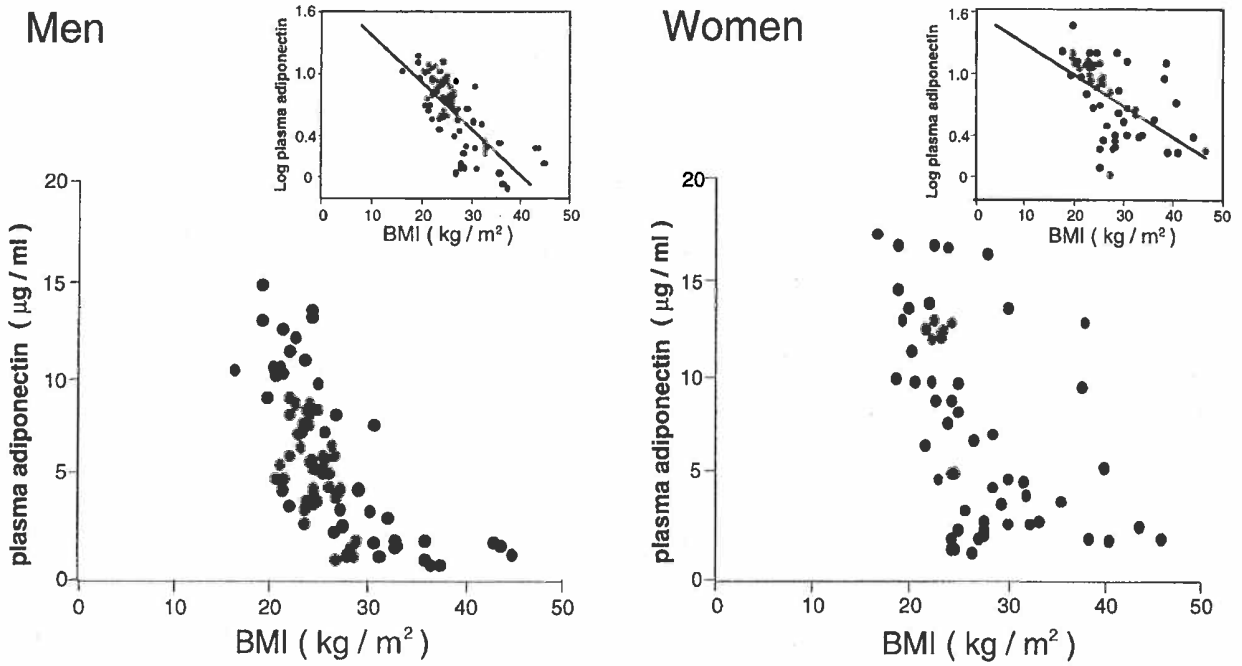

FIG. 3. Plasma adiponectin concentration and BMI in clinically normal subjects. The inset shows the natural log of the plasma adiponectin concentration plotted against BMI.

and inter-assay coefficiencies of variations were 3.3% and 7.4%, respectively.

Plasma Adiponectin Concentrations in Non-Obese and Obese Subjects

Plasma concentrations of adiponectin were determined in 87 non-obese subjects and 57 obese subjects. Adiponectin was abundantly present in the plasma of non-obese subjects. Mean (±S.D.) plasma concentration of adiponectin in non-obese subjects was 8.9 ± 5.4 mg/ml (ranging from 1.9 to 17.0 mg/ml). Unexpectedly, the plasma levels of adiponectin in the obese subjects (3.7 ± 3.2 mg/ml, mean ± S.D.) were lower than those in the non-obese subjects ($p < 0.0001$). A strong negative correlation was observed between plasma adiponectin levels and body mass indices (BMIs) in both men ($r = -0.66$, $p < 0.0001$) and women ($r = -0.48$, $p < 0.0001$) (Fig. 3). The correlation between BMI and logarithmically transformed plasma adiponection concentration more closely fitted a simple linear regression model both in men ($r = -0.71$, $p < 0.0001$) and women ($r = -0.51$, $p < 0.0001$) (inset of Fig. 3). Plasma adiponectin levels in men were significantly lower in women among non-obese subjects (men; 7.7 ± 3.1 μg/ml, women; 10.6 ± 7.3 μg/ml, $p < 0.0001$) and among obese subjects (men; 2.8 ± 2.1 ml/ml, women; 4.8 ± 4.0 ml/ml, $p < 0.0001$). Plasma adiponectin concentrations were not correlated with age when adjusted for BMI.

DISCUSSION

Adipose tissue secretes a variety of proteins into circulating blood. Recently a novel adipose-specific secretory protein was identified independently by different approaches. We have isolated apM1/adiponectin cDNA by large-scale random sequencing of a 3'-directed human adipose tissue cDNA library (1). Scherer et al. and Hu et al. cloned cDNA for Acrp30 (6) and AdipoQ (7) by the differential display before and after the differentiation of mouse 3T3-L1 and 3T3-F442 cells, respectively. Adiponectin cDNA has 85% homology to Acrp30 and AdipoQ, hence they must be a mouse counterpart. Nakano et al. purified GBP28 (8) from human plasma with a gelatin-affinity column. Amino acid sequence of the peptides of GBP28 was identical to the predicted amino acid sequence of adiponectin. The physiological role of the protein, however, has not been elucidated. In the current study, we developed an ELISA system for quantitative measurement of adiponectin in human plasma and found that adiponectin was abundantly present in human plasma.

Plasma concentrations of most proteins produced by adipose tissue are increased in obesity because of an increase in the total body fat mass. For example, the plasma concentration of leptin is increased when BMI is increased (9). When the expression level per unit RNA is upregulated in obesity as is the cases of leptin and TNFa, the plasma concentrations will be augmented. Unexpectedly, the plasma levels of adiponectin in obesity were

lower than those in non-obese subjects. The mechanism by which plasma adiponectin is decreased in obesity has not been clarified. Although the adipoQ/Acrp30 mRNA increased according to the differentiation of preadipocytes, the marked reduction of adipoQ mRNA in obese human and mice adipose tissues was reported. Therefore, the decreased plasma adiponectin levels in obesity might at least partly account for the decrease in mRNA levels in adipose tissue. The possibility that secretion of adiponectin from adipocytes is disturbed in obesity remains obscure. The ELISA system developed in this study will serve as a useful tool to investigate the physiological and the pathophysiological significance of adiponectin.

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