Circulating Concentrations of the Adipocyte Protein Adiponectin Are Decreased in Parallel With Reduced Insulin Sensitivity During the Progression to Type 2 Diabetes in Rhesus Monkeys

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Adiponectin is a novel adipose-specific collagen-like molecule that belongs to the collectin family (12-14). Adiponectin bound to collagens I, III, and V (major components of the vascular intima) in a solid-phase binding assay and accumulated in the vascular wall when the endothelial barrier was damaged (15). The concentration of adiponectin in plasma ranged from 5 to 10 μg/ml in healthy humans (14). Obese patients, type 2 diabetic patients, and patients with coronary artery disease showed significantly lower levels of plasma adiponectin (14,16,17). We found that administration of adiponectin decreased the attachment of monocytic cell line THP-1 cells to human aortic endothelial cells (16,18), which is an early event in atherosclerotic vascular damage. Adiponectin decreases the expression of multiple adhesion molecules, including in endothelial cells via the modulation of NFκB signaling (16,18). Adiponectin also dramatically suppressed the secretion of TNF-α from human monocyte-macrophages (19). These clinical and experimental observations suggest that adiponectin plays some protective role against the atherosclerotic vascular change and that the decreased plasma adiponectin in type 2 diabetic patients may contribute to the development of atherosclerotic complications. The mechanism of decreased plasma adiponectin in type 2 diabetes, however, has not yet been clarified.

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Insulin resistance is one of the important risk factors associated with atherosclerosis and diabetes. Insulin resistance often accompanies visceral fat accumulation (1). Recent studies have provided evidence that adipose tissue may play a crucial role in the development of insulin resistance, type 2 diabetes, and their complications through the secretion of a variety of biologically active molecules (adipocytokines) (1). Hotamisligil et al. (2) reported that tumor necrosis factor-α (TNF-α) over-produced in adipose tissue of obesity contributes to the development of insulin resistance. Leptin is an adipose-specific hormone contributing to the regulation of energy expenditure and food intake (3). Leptin also affects insulin sensitivity and may participate in the development of hypertension (4-8). Plasminogen activator inhibitor-1 (PAI-1) increases in obesity and diabetes and may play a part in thrombosis and the development of vascular disease (9-11). These adipocytokines may cause the atherosclerotic vascular disease in type 2 diabetes directly or through the development of insulin resistance.

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followed in this model. A decrease in insulin sensitivity and a progressive increase of fasting plasma insulin (followed by a decline in β-cell function) precedes the manifestation of diabetes in rhesus monkeys (20–22). In the current study, we investigated the plasma concentrations and adipose mRNA expression of adiponectin during the development of obesity, insulin resistance, and type 2 diabetes in rhesus monkeys.

RESEARCH DESIGN AND METHODS
Male rhesus monkeys (Macaca mulatta) were individually housed and maintained in accordance with the National Academy of Sciences guidelines for the care and use of laboratory animals. Either the nutritionally complete liquid diet Ensure (Ross Laboratories, Columbus, OH) or Monkey Chow (Purina Mills, St. Louis, MO) and fresh water were provided to monkeys ad libitum (8 h/day). For measurement of adiponectin and leptin, the monkeys to be studied were sorted into the following three groups: lean (body fat <22%), obese (body fat >22%), or type 2 diabetic. Diabetes was diagnosed according to American Diabetes Association criteria (24) (fasting plasma glucose >7 mmol/l).

Procedures. Plasma samples were obtained under light anesthesia (ketamine hydrochloride, as described above, or immediately after anesthetization by intravenous sodium pentobarbital). Tissue samples were stored instantly in liquid nitrogen and stored at −80°C.

After a 16-h fast, the monkeys were placed under light anesthesia (ketamine hydrochloride 10 mg/kg body wt) after an 16-h fast. Plasma samples were obtained under ketamine hydrochloride, as described above, or immediately after anesthetization by intravenous sodium pentobarbital. Tissue samples were stored instantly in liquid nitrogen and stored at −80°C.

Enzyme-linked immunosorbent assay of plasma adiponectin and leptin. We measured plasma adiponectin levels in monkeys using the enzyme-linked immunosorbent assay (ELISA) system developed for the measurement of human plasma adiponectin concentrations, as described previously (14). Human recombinant adiponectin was used as a standard. The affinity of the monoclonal anti-adiponectin antibody (used as the first antibody of ELISA) to the monkey adiponectin was as strong as that to human adiponectin; however, the affinity of the polyclonal antibody (which was used for the second antibody) was weaker. Thus, the values were indicated as an arbitrary unit.

First, we evaluated the plasma concentrations of adiponectin and leptin in the lean monkeys (n = 14, age 10 ± 2 years, 10.5 ± 0.7 kg body wt, 16 ± 1% body fat); the obese monkeys (n = 23, age 16 ± 1 years, 16.7 ± 0.7 kg body wt, 34 ± 1% body fat); and the diabetic monkeys (n = 10, age 23 ± 1 years, 13.2 ± 0.6 kg body wt, 31 ± 2% body fat). The lean group showed normal fasting glucose (3.3 ± 0.1 mmol/l) and insulin (351 ± 31 pmol/l) levels. The obese group showed normal fasting glucose levels (4.0 ± 0.2 mmol/l), but they had significantly higher plasma insulin levels (1,009 ± 278 pmol/l, P < 0.05) compared with normal monkeys. In the diabetic group, fasting plasma glucose levels were elevated (11.2 ± 0.9 mmol/l) and fasting plasma insulin levels had declined from previously elevated levels to the normal range (251 ± 56 pmol/l), as described previously (20–23). The obese monkeys showed significantly higher plasma leptin concentrations than did lean monkeys (20.4 ± 3.0 vs. 4.3 ± 1.0 ng/ml, P < 0.001) as reported previously (Fig. 1) (26,27). In contrast, the plasma adiponectin concentrations in obese monkeys were significantly lower than in lean monkeys (1.4 ± 0.2 vs. 2.7 ± 0.5, P < 0.01) (Fig. 1). When the monkeys developed diabetes, the fat weight decreased. Accordingly, plasma leptin levels in the diabetic monkeys returned to near lean levels (Fig. 1). However, the plasma adiponectin levels in diabetic monkeys remained lower than those in lean monkeys (Fig. 1). These results were similar to our previous data in humans (17).

Longitudinal changes of plasma adiponectin during the development of obesity and diabetes. Longitudinal changes of plasma adiponectin were investigated in the individual monkeys during the development of obesity and type 2 diabetes. The time course during the development of type 2 diabetes has been divided into eight phases (20). At phase 1, monkeys are young (age <10 years), lean

FIG. 1. Fat weight (A) and the plasma levels of leptin (B) and adiponectin (C) in lean, hyperinsulinemic obese, and type 2 diabetic rhesus monkeys. The plasma levels of adiponectin and leptin from lean (n = 14), obese (n = 23), and type 2 diabetic (n = 10) monkeys were measured by ELISA. Values represent means ± SE. *P < 0.05; **P < 0.01; ***P < 0.001.
(body fat < 22%), and have normal fasting plasma insulin and glucose levels (Fig. 2). At phase 2, the monkeys are lean and normal, although they are middle-aged (>10 years). High plasma levels of adiponectin were observed at these phases (Fig. 2). During phases 3 through 5, monkeys became obese, and progressive increases in fasting plasma insulin and leptin were observed. Plasma levels of adiponectin were greatly decreased in this phase. Obesity and
hyperinsulinemia were prominent at phases 5 through 7. The deterioration of $K_G$ values (the index of glucose tolerance) became evident. The plasma levels of leptin further increased during these phases, reflecting the progression of obesity. The plasma levels of adiponectin reached quite low levels (Fig. 2). At phase 8, fasting plasma insulin levels dropped and the fasting plasma glucose increased. The plasma leptin levels were decreased in association with the decrease in body and fat weight. In contrast, the plasma adiponectin levels remained at low levels (Fig. 2).

**Relationship between plasma adiponectin concentration and insulin sensitivity.** Obese monkeys are often insulin resistant and hyperinsulinemic. The degree of insulin resistance varies in the individual obese monkey (21,23). In rhesus monkeys, the plasma levels of adiponectin decreased in phases 3–5. Insulin sensitivity decreased in phases 3–5 preceding the development of diabetes (21). The change in the plasma levels of adiponectin was similar to the change in insulin sensitivity (Fig. 2). Thus, the reduction of plasma adiponectin concentration might be related to the development of insulin resistance. We evaluated $M$ rates using the euglycemic-hyperinsulinemic technique in 24 nondiabetic monkeys. Plasma adiponectin concentrations were closely correlated with $M$ rates ($r = 0.66, P < 0.001$, Fig. 3) as well as with body weight ($r =$...
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-0.46, \( P < 0.05 \), body fat weight (\( r = -0.42, P < 0.05 \)), and plasma insulin (\( r = -0.41, P < 0.05 \)). Plasma adiponectin levels were not correlated with age, \( K_B \), or AIR (\( r = -0.12, 0.15, \) and \( -0.21 \), respectively). Multiple regression analysis showed that decreased \( M \) rate (\( P < 0.05 \)) and increased fat weight (\( P < 0.05 \)) were significantly and independently associated with decreased levels of adiponectin. Fasting plasma insulin was not significantly associated with the plasma levels of adiponectin, because fasting plasma insulin levels progressed through an inverted U-shaped curve. We divided nondiabetic monkeys into the following three groups: lean (body fat <22%, \( n = 7 \)), obese with high plasma levels of adiponectin (body fat >22%, adiponectin >1.4, \( n = 9 \)), and obese with low plasma levels of adiponectin (body fat >22%, adiponectin <1.4, \( n = 8 \)). The body weight, body fat, and plasma glucose levels were not different in the obese monkeys with higher and lower levels of adiponectin (Fig. 4). In the obese monkeys with lower levels of plasma adiponectin, the \( M \) rate was significantly lower than it was in those with higher levels of plasma adiponectin (\( t \) test, \( P < 0.05 \)) (Fig. 4). Thus, hypoadiponectinemia could be related to insulin resistance.

Longitudinally, the \( M \) rate began to decrease at the earliest phase of obesity then reached and sustained low levels in subsequent phases. The plasma levels of adiponectin decreased as the monkeys developed insulin resistance (Fig. 2). The close relationship was indicated in this prospective longitudinal study.

**Plasma adiponectin concentrations and mRNA levels in adipose tissue.** Plasma adiponectin concentrations decreased when the monkeys became obese. To clarify whether the plasma adiponectin levels are determined by the mRNA expression levels in the adipose tissue, we measured the adiponectin mRNA levels in subcutaneous adipose tissue. We first cloned the monkey adiponectin cDNA. Sequence analysis revealed that the predicted monkey adiponectin protein (excluding the signal peptide) was identical in length (224 amino acids) and showed a 96% identity with human adiponectin (12,13) (Fig. 5A). Northern blot analysis showed that monkey adiponectin mRNA was expressed exclusively in adipose tissue, as observed in humans in earlier studies (Fig. 5B) (12,13).

Leptin mRNA levels were significantly correlated to fat weight (\( r = 0.62, P < 0.01 \)) as previously reported (26). On the other hand, adiponectin mRNA levels were not correlated to fat weight (\( r = -0.11 \)) (Fig. 6) or \( M \) rate (\( r = 0.36 \)). No clear correlation between the plasma and mRNA levels of adiponectin was observed (\( r = -0.02 \)). Plasma leptin levels were significantly correlated with leptin mRNA levels in adipose tissue (\( r = 0.60, P < 0.01 \)) (Fig. 6). Adjustment of each mRNA level for HSP83 produced similar results, as shown in Fig. 6.

**DISCUSSION**

Adiponectin is an adipose-specific plasma protein that inhibits the expression of adhesion molecules in endothelial cells and the secretion of TNF-\( \alpha \) from monocyte-macrophages (16,18,19). Therefore, it possesses possible antiatherogenic and anti-inflammatoryary properties. The plasma levels of adiponectin were decreased in obese subjects and in patients with type 2 diabetes (14,17). The mechanism of reduced adiponectin in these metabolic disorders has not been clear. Rhesus monkeys provide excellent models of human obesity and type 2 diabetes (20–23), and the natural histories of obesity and of diabetes have been clearly demonstrated in this model. Plasma adiponectin concentrations were decreased in rhesus monkeys with obesity and in those with type 2 diabetes. The current data were compatible with our previous data observed in humans (14,17). One of the important observations in this study was the demonstration of longitudinal changes in plasma adiponectin levels during the development of obesity and type 2 diabetes. The plasma levels of adiponectin began to decrease in the earliest stage of obesity, when the insulin resistance and hyperinsulinemia were progressing. This pattern was very similar to the change in insulin sensitivity as measured by the \( M \) rates, suggesting the close relationship between the \( M \) rate and the plasma adiponectin levels. Indeed, the plasma levels of adiponectin were strongly correlated to the plasma adiponectin levels during the development of obesity and type 2 diabetes. The plasma levels of adiponectin decreased as the monkeys developed insulin resistance (Fig. 2). The close relationship was indicated in this prospective longitudinal study.

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**FIG. 5.** Amino acid sequence and expression of rhesus monkey adiponectin. A: Comparison of the amino acid sequences from monkey and human adiponectin. Amino acids that are identical to the monkey sequence are represented by dashed lines. The predicted signal sequence is underlined. The glycine in a region encoding Gly-X-Y triplets (Glys-X-Y repeats) is indicated in bold type. The noncollagenous region, which is homologous to VIII collagen, X collagen, and the C chain of C1q, is double-underlined. B: Tissue distribution of monkey adiponectin mRNA. Total RNA (10 \( \mu \)g) isolated from various tissues was applied to each lane. Northern blot analysis was carried out as described in research design and methods.
sults suggested that hypoadiponectinemia associates with insulin resistance.

Insulin binding to adipocytes was reduced in obese and diabetic monkeys (29). Basal and insulin-stimulated glucose utilization dropped markedly as hyperinsulinemia progressed into diabetes (29). A defect in the covalent activation of glycogen synthase by in vivo insulin has been identified in insulin resistance and type 2 diabetes in subcutaneous adipose tissue (30). Thus, adipose tissue as well as skeletal muscle plays an important role in insulin resistance in rhesus monkeys (30,31). Insulin regulates secretion of various molecules from adipocytes (32,33). Scherer et al. (34) demonstrated that the secretion of the mouse adiponectin homologue Acrp30 was stimulated by insulin in 3T3-11 cells. Secretion of adiponectin from adipocytes may be disturbed in the insulin-resistant state.

One of the possible mechanisms of insulin resistance in obesity is overproduction of TNF-α from adipose tissue. TNF-α suppresses the release of leptin and PAI-1 from adipocytes (35–40). Thus, the increased TNF-α may also reduce the secretion of adiponectin from adipose tissue in insulin-resistant obesity.

Recent studies have determined that the cellularity of adipose tissue is heterogeneous and that the size of adipocytes is also importantly related to the insulin resistance. Okuno et al. (41) reported that the adipocytes in Zucker obese rats with insulin resistance were larger than those in lean littermates without insulin resistance. Treatment with the insulin-sensitizing thiazolidinedione troglitazone increased the number of small adipocytes and improved insulin sensitivity in Zucker obese rats without changing the total adiposity. Therefore, small adipocytes

FIG. 6. A: The correlation between mRNA levels of adiponectin and fat weight ($r = -0.11$, NS) and between mRNA levels of $ob$ (leptin) and fat weight ($r = 0.62$, $P = 0.01$). A total of 5 μg of total RNA was electrophoresed. Hybridization was performed as described in RESEARCH DESIGN AND METHODS. B: Correlation between the plasma and mRNA levels of adiponectin ($r = -0.02$, NS) and between the plasma leptin and $ob$ mRNA levels of ($r = 0.60$, $P = 0.01$). The mRNA levels of adiponectin and $ob$ were determined by Northern blot hybridization. The plasma levels of adiponectin and leptin were analyzed by ELISA.
may be more insulin-sensitive than larger ones. Insulin-sensitive small adipocytes may secrete more adiponectin, and obese monkeys with more of the small adipocytes (i.e., hyperplastic obesity) may have higher plasma adiponectin levels than obese monkeys with hypertrophic obesity.

We have reported that adiponectin suppressed the TNF-α signaling in endothelial cells (16). Although we have not examined this specifically, adiponectin may attenuate the TNF-α signaling in adipocytes and protect them from the development of insulin resistance. TNF-α increases in obesity (2) and may suppress the secretion of adiponectin from adipocytes. The decreased adiponectin in obesity may further deteriorate insulin resistance caused by TNF-α. In insulin resistance, secretion of adiponectin would decrease further. Such a vicious cycle may be involved in the insulin-resistant state.

It is not clear whether the plasma level of adiponectin is determined at mRNA levels in humans. In this study, we assayed the mRNA levels of adiponectin using monkey adipose tissue. The mRNA levels of adiponectin did not correlate with either adiposity or insulin sensitivity. Plasma adiponectin levels did not correlate with mRNA levels in adipose tissue in the current study. Therefore, the plasma adiponectin concentration in rhesus monkeys may be regulated at a postranscriptional level, including the translation and/or secretion level.

In summary, the antiatherogenic plasma protein adiponectin decreased before the onset of diabetes, in parallel with the decrease of insulin sensitivity. Hypoadiponectinemia is closely related to insulin resistance. Our present data suggests that the decreased plasma levels of adiponectin may be related to the development of insulin resistance and possibly of atherosclerosis.

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