Adipocyte-Derived Plasma Protein, Adiponectin, Suppresses Lipid Accumulation and Class A Scavenger Receptor Expression in Human Monocyte-Derived Macrophages

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Background—Excessive lipid accumulation in macrophages plays an important role in the development of atherosclerosis. Recently, we discovered an adipocyte-specific plasma protein, adiponectin, that is decreased in patients with coronary artery disease. We previously demonstrated that adiponectin acts as a modulator for proinflammatory stimuli and inhibits monocyte adhesion to endothelial cells. The present study investigated the effects of adiponectin on lipid accumulation in human monocyte-derived macrophages.

Methods and Results—Human monocytes were differentiated into macrophages by incubation in human type AB serum for 7 days, and the effects of adiponectin were investigated at different time intervals. Treatment with physiological concentrations of adiponectin reduced intracellular cholesteryl ester content, as determined using the enzymatic, fluorometric method. The adiponectin-treated macrophages contained fewer lipid droplets stained by oil red O. Adiponectin suppressed the expression of the class A macrophage scavenger receptor (MSR) at both mRNA and protein levels by Northern and immunoblot analyses, respectively, without affecting the expression of CD36, which was quantified by flow cytometry. Adiponectin reduced the class A MSR promoter activity, as measured by luciferase reporter assay. Adiponectin treatment dose-dependently decreased class A MSR ligand binding and uptake activities. The mRNA level of lipoprotein lipase as a marker of macrophage differentiation was decreased by adiponectin treatment, but that of apolipoprotein E was not altered. Adiponectin was detected around macrophages in the human injured aorta by immunohistochemistry.

Conclusions—The adipocyte-derived plasma protein adiponectin suppressed macrophage-to-foam cell transformation, suggesting that adiponectin may act as a modulator for macrophage-to-foam cell transformation. (Circulation. 2001;103:1057-1063.)

Key Words: atherosclerosis ■ macrophages ■ receptors ■ adiponectin

Obesity is the most common risk factor for cardiovascular morbidity and mortality.1–3 Adipose tissue is not simply an energy storage organ, but also a secretory organ, producing a variety of bioactive substances, including leptin, tumor necrosis factor-α, plasminogen activator inhibitor type 1, and adiponectin, that may directly contribute to the development of vascular diseases.4–9 Adiponectin is an adipocyte-specific plasma protein homologous to collagen VIII, collagen X, and complement factor C1q,10 and it is abundantly present in human plasma, accounting for 0.01% of the total plasma protein.11 Plasma adiponectin levels are reduced in patients with coronary artery disease, including myocardial infarction.9 We recently demonstrated that plasma adiponectin accumulated in the subendothelial space in the rat injured carotid artery model and that adiponectin inhibited tumor necrosis factor-α–induced expression of adhesion molecules in vascular endothelial cells.9,12 Therefore, the decrease in the plasma adiponectin may directly correlate with the development of vascular diseases as an adipocyte-derived endocrine modulator for proinflammatory stimuli when the endothelial barrier is injured.

In the early stages of atherosclerosis, the circulating monocytes attach to injured endothelial cells and infiltrate the subendothelial space, leading to differentiation into macrophages.13–16 Subsequently, the macrophages take up modified LDL and transform into foam cells by accumulating intracel-
The accumulation of lipid-laden foam cells and the ongoing macrophage-related inflammation are key features in early atherosclerotic lesions. The scavenger receptor family proteins, such as class A macrophage scavenger receptor (MSR) and class B MSR (CD36), play a major role in lipid accumulation and the foam cell formation of macrophages by taking up modified LDL. However, the endogenous regulator of MSR has not been fully elucidated. In this study, we investigated the effects of the adipocyte-derived plasma protein adiponectin on lipid accumulation and class A MSR expression in human monocyte-derived macrophages.

Methods

Cell Culture

Mononuclear cells were isolated from peripheral blood by density gradient centrifugation, as previously described. The cells were suspended in RPMI-1640 (Gibco) supplemented with 10% human type AB serum and incubated for 1 hour at 37°C in cell culture dishes. Nonadherent cells were removed by washing twice with PBS. The remaining adherent cells were cultured in the same medium. After 7 days of incubation, the cells were used as human monocyte-derived macrophages. The medium was replaced every 2 or 3 days. Human monocyte-derived macrophages were cultured in the medium for 7 days and then incubated with the indicated amount of adiponectin in the same medium for the times indicated. Human recombinant adiponectin was prepared as previously described.

Analysis of Cellular Cholesteryl Ester Contents and Lipid Accumulation

Human monocyte-derived macrophages were treated with or without 30 μg/mL adiponectin for 3 days. The cellular cholesterol content was determined by the enzymatic, fluorometric method, as previously described. Briefly, the cellular lipids were extracted with hexane/isopropanol (3/2, v/v), dried under a nitrogen flush, and then dissolved in isopropanol as previously described. For the determination of free cholesterol, the supernatant was added to enzyme mixtures containing cholesterol oxidase (0.16 IU/mL) and cholesterol esterase (60 U/mL). The reaction mixtures for measuring free cholesterol and those for measuring total cholesterol were incubated at 37°C for 1 hour and 2 hours, respectively, followed by the addition of sodium hydroxide to terminate the reaction. Fluorescent intensity was measured with excitation at 310 nm and emission at 407 nm. The mass of cholesteryl ester was calculated by subtracting free cholesterol from total cholesterol. After lipid extraction, the cellular protein was dissolved in sodium hydroxide, and the protein concentration was determined by the method of Lowry et al. For detecting lipids accumulated in macrophages, oil red O staining was performed as previously described.

Immunoblot Analysis

Human monocyte-derived macrophages were treated with or without 30 μg/mL adiponectin for 2 days. Whole cell lysates were resolved on 7.5% SDS-polyacrylamide gels, followed by electrophoretic transfer to nitrocellulose membranes (Amersham). The membranes were exposed to mouse monoclonal anti-human class A MSR antibody (ANOC 9108) were used as previously reported.

Figure 1. Effects of adiponectin on cholesteryl ester contents in human monocyte-derived macrophages. Human monocyte-derived macrophages were differentiated and treated with or without 30 μg/mL of adiponectin, as described in Methods. Cellular cholesteryl ester content was determined by an enzymatic, fluorometric method, as described in Methods. Columns and vertical bars denote mean and SD of 3 experiments. *P<0.05 vs untreated control. Control cholesteryl ester content was 126±11 nmol/mg of cell protein. Representative results from 3 experiments are shown.

Figure 2. Effects of adiponectin on lipid droplets in human monocyte-derived macrophages. Human monocyte-derived macrophages were treated with (B) or without (A) 30 μg/mL adiponectin for 2 days. Cells were stained with oil red O (red color) and with anti-adiponectin monoclonal antibody ANOC 9108 (green color), as described in Methods. Bar indicates 20 μm.
FACScan flow cytometer (Becton Dickinson). Data were analyzed with the Cell Quest program. Specific fluorescent intensity was calculated by subtracting autofluorescent intensity from the mean fluorescent intensity of DiI-labeled cells.

Figure 3. Inhibitory effect of adiponectin on class A MSR protein level in human monocyte-derived macrophages. A, Human monocyte-derived macrophages were treated with or without 30 µg/mL adiponectin for 2 days. B, Effect of ANOC 9104 on inhibition of class A MSR protein expression by adiponectin. Human monocyte-derived macrophages were treated with adiponectin (30 µg/mL) or vehicle in absence of ANOC 9104 (30 µg/mL) or control nonimmune IgG (60 µg/mL). Expression of class A MSR protein was examined by immunoblot analysis. Representative results from 3 experiments are shown.

Immunofluorescent Flow Cytometry
To detect the cell-surface expression of CD36, immunofluorescence flow cytometric analysis was performed using FITC-conjugated mouse monoclonal antibodies against human CD36 (OKM-5, Ortho Diagnostic System). Human monocyte-derived macrophages were treated with adiponectin (30 µg/mL) or vehicle in presence of ANOC 9104 (30 µg/mL) or control nonimmune IgG (60 µg/mL). Expression of class A MSR protein was examined by immunofluorescence flow cytometric analysis. Representative results from 3 experiments are shown.

Northern Blot Analysis
Human monocyte-derived macrophages were treated with the indicated concentrations of adiponectin for 2 days. Total cellular RNA was prepared by RNA-TRIZOL extraction (Gibco). Total RNA (10 µg per lane) was electrophoresed and transferred to a nylon membrane. Human class A MSR cDNA (1350 bp, nucleotides 1 to 1350 in the human class A MSR cDNA sequence) was kindly provided by Dr Tatsuhiko Kodama (Tokyo University). Human full-length lipoprotein lipase (LPL) or apolipoprotein E (apoE) cDNA was amplified by reverse transcriptase polymerase chain reaction, subcloned into pBlueScript KS (TOYOBO), and used as a cDNA probe. The membranes were hybridized with human class A MSR, LPL, or apoE cDNA probes labeled with [α-32P]dCTP by means of a random primer labeling system (Amersham). Hybridized membranes were exposed to Kodak XAR-5 film between 2 intensifying screens.

Cell Transfection and Measurement of Luciferase Activity
Class A MSR promoter fragment (from −630 to +50 bp) was kindly provided by Dr Akio Matsumoto (National Institute of Health and Nutrition, Tokyo, Japan). The promoter fragment was subcloned into the luciferase reporter vector with pGL3-Basic (Promega). For the transfection study, a human monocytic cell line (THP-1 cells; Japanese Cancer Research Resources Bank) was used. THP-1 cells were transfected using the DEAE-dextran sulfate method, as follows: 2×10⁵ cells were cotransfected with 20 µg of class A MSR pGL3 plasmid and 1 µg of SV40 control plasmid (Promega) in 250 µg of DEAE-dextran for 60 minutes and shocked with 10% DMSO for 2 minutes. After transfection, the cells were incubated with RPMI-1640 supplemented with 10% fetal calf serum for 24 hours and then treated with phorbol 12-myristate 13-acetate at a final concentration of 50 ng/mL for 24 hours to differentiate monocytes from macrophage-like cells. THP-1 macrophages were treated with or without 30 µg/mL adiponectin for 24 hours. Luciferase activity was measured using a dual luciferase assay kit and a luminometer.

AcdLDL Binding and Uptake
Macrophage binding and uptake of acetylated LDL (AcdLDL) were analyzed by flow cytometry using lipoproteins labeled with the fluorescent probe 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI). DiI-AcdLDL and unlabeled AcdLDL were purchased from Biomedical Technologies. After treatment with the indicated concentrations of adiponectin for 2 days, the cells were incubated with varying concentrations of DiI-AcdLDL dissolved in RPMI-1640 containing 2% lipoprotein-deficient human serum. Cells were incubated at 4°C for 30 minutes for binding assays and at 37°C for 3 hours for uptake experiments. For competition assays, unlabeled AcdLDL in excess amounts (50-fold) were added with DiI-AcdLDL. The cells were resuspended in PBS and analyzed using a FACSscan flow cytometer (Becton Dickinson). Data were analyzed by the Cell Quest program. Specific fluorescent intensity was calculated by subtracting autofluorescent intensity from the mean fluorescent intensity of DiI-labeled cells.

Figure 4. Effect of adiponectin on surface expression of CD36 protein on human monocyte-derived macrophages. Human monocyte-derived macrophages were treated in presence (right) or absence (left) of 30 µg/mL adiponectin for 2 days. Surface expression of CD36 was examined by flow cytometric analysis. Cells were incubated with FITC-labeled mouse anti-human CD36 antibodies (OKMS; peak A) or FITC-conjugated control mouse IgG (peak B). Representative results from 3 experiments are shown.

Figure 5. Dose-response effect of adiponectin on class A MSR mRNA levels in human monocyte-derived macrophages. Human monocyte-derived macrophages were treated with indicated amounts of adiponectin for 2 days. Levels of class A MSR mRNA were determined by Northern blot analysis. Representative results from 5 experiments are shown.
Immunohistochemical Staining
Human abdominal aorta was obtained from a human subject who underwent an operation for an abdominal aortic aneurysm, and the aorta was then embedded in paraffin. Adiponectin immunohistochemical staining was performed using anti-adiponectin monoclonal antibodies (ANOC 9108), as previously described.12 Macrophages were detected using anti-CD68 antibody (DAKO), as previously described.25 This subject gave written informed consent. The Ethics Committee of Osaka University approved the study.

Statistic Analysis
Data are presented as mean±SD. Differences were analyzed by Student’s unpaired t test or 1-way ANOVA. P<0.05 was considered statistically significant.

Results
Adiponectin Reduces Cholesteryl Ester Contents and Lipid Droplets in Human Monocyte-Derived Macrophages
We first investigated the effects of adiponectin on cholesteryl ester accumulation in human monocyte-derived macrophages. Treatment of macrophages with physiological concentrations of adiponectin (30 μg/mL) for 3 days significantly reduced cholesteryl ester contents by ~50% compared with untreated controls (Figure 1). Double staining of the macrophages with anti-adiponectin antibody and oil red O was performed. The adiponectin-treated macrophages contained fewer lipid droplets and appeared more elongated than the untreated macrophages (Figure 2). Adiponectin was detected only on the adiponectin-treated macrophages and not on the untreated macrophages. These results indicated that adiponectin suppressed the macrophage-to-foam cell transformation in vitro.

Adiponectin Suppresses the Expression of Class A MSR Without Affecting that of CD36
The scavenger receptor family proteins play an important role in lipid accumulation and the foam cell formation of macrophages. Therefore, we next investigated whether adiponectin could modulate the expression of 2 major scavenger receptors, class A MSR and CD36, in human monocyte-derived macrophages. The immunoblot analysis revealed that the major isoform of class A MSR was type I protein in human monocyte-derived macrophages. Treatment of human macrophages with adiponectin (30 μg/mL) for 2 days markedly suppressed the expression of class A MSR proteins (Figure 3A). Cotreatment of the anti-adiponectin monoclonal antibody ANOC 9104 with adiponectin reversed the adiponectin-induced suppression of class A MSR protein expression (Figure 3B). In contrast to class A MSR, adiponectin treatment (30 μg/mL) had no effect on CD36 protein levels by flow cytometric analysis (Figure 4). The effects of adiponectin on class A MSR steady-state mRNA levels and promoter activity were further examined by Northern blot and luciferase assay, respectively. Human monocyte-derived macrophages expressed type I and II class A MSR mRNAs (Figure 5). Adiponectin treatment suppressed both type I and II class A MSR mRNA levels in a dose-dependent manner (Figure 5). The promoter activity of class A MSR standardized by SV40 promoter was significantly reduced by adiponectin treatment in the transfected human monocytes cell line of THP-1.
derived macrophages (Figure 6). These results indicated that adiponectin decreased the class A MSR expression at the transcriptional level.

Adiponectin Reduces Binding and Uptake of DiI-AcLDL

The function of class A MSR was examined by flow cytometric analysis using DiI-AcLDL. After treatment with or without 30 µg/mL adiponectin for 2 days, cells were incubated with varying concentrations of DiI-AcLDL. Adiponectin treatment reduced the specific and saturable binding of DiI-AcLDL to human monocyte-derived macrophages observed in a dose-dependent manner (Figure 7B). Adiponectin treatment did not affect nonspecific binding in the presence of excess unlabeled AcLDL (Figure 7A). Additionally, the binding of Dil-AcLDL to macrophages was not suppressed when adiponectin was added at the same time as Dil-AcLDL (data not shown), suggesting that adiponectin does not occupy the binding sites of Dil-AcLDL to macrophages.

Uptake of DiI-AcLDL by human macrophages was further analyzed by flow cytometry. Cells treated with the indicated concentrations of adiponectin for 2 days were incubated with 5 µg/mL Dil-AcLDL for 3 hours at 37°C. Adiponectin treatment dose-dependently reduced Dil-AcLDL uptake in human macrophages (Figures 8A and 8B). Treatment with 30 µg/mL adiponectin resulted in a 40% reduction of Dil-AcLDL uptake (Figure 8B). The inhibitory effect of adiponectin on Dil-AcLDL uptake was partially but significantly reversed by cotreatment with the anti-adiponectin monoclonal antibody ANOC 9104 (Figure 8C). These findings indicated that adiponectin suppressed class A MSR function in human monocyte-derived macrophages.

Effect of Adiponectin on LPL, ApoE, and Class A MSR mRNA Levels

We next investigated whether adiponectin could modulate macrophage differentiation. Because LPL and apoE are well-characterized markers of macrophage differentiation, the effects of adiponectin on LPL, apoE, and class A MSR mRNA levels were analyzed by Northern blotting. Adiponectin treatment reduced the steady-state mRNA levels of LPL and class A MSR without affecting those of apoE (Figure 9).

Expression of Adiponectin in Human Injured Vascular Wall

Finally, we examined the localization of adiponectin in the lesions of the human injured aorta by immunohistochemical analysis. Double immunostaining of adiponectin and macrophages revealed that adiponectin was abundant in the endo-
helium and in the subendothelial space, which also contains macrophages; the endothelial injury was confirmed by thrombus attachment (Figure 10). However, adiponectin was not detected in the subendothelial space of atherosclerotic lesions with an intact endothelium (data not shown).

**Discussion**

In the present study, we demonstrated that physiological concentrations of adiponectin (3 to 30 μg/mL) had significant inhibitory effects on lipid accumulation, class A MSR expression, and class A MSR ligand binding and uptake activities in human monocyte-derived macrophages in vitro.

The generation of lipid-laden foam cells is considered a key step in the pathogenesis of atherosclerosis.\textsuperscript{13–16} Class A MSRs play a pivotal role in foam cell formation from macrophages by mediating the uptake of modified LDL.\textsuperscript{17,18} We and others have demonstrated by immunohistochemical analyses that class A MSR protein is detected in the macrophages from human atherosclerotic lesions.\textsuperscript{18,25,27} Recent studies showed that certain double-knockout mice (class A MSR/apoE) had smaller atherosclerotic lesions than single-knockout mice (apoE).\textsuperscript{28} These findings indicate that class A MSRs contribute to the generation of atherosclerotic lesions. Therefore, our present observations suggest that adiponectin may act as a negative endocrine modulator for foam cell formation through the inhibition of class A MSR expression.

The regulation of the scavenger receptor family proteins is poorly understood, although it plays an important role in the development of atherosclerosis. The expression of class A MSR and CD36 increased during monocyte-to-macrophage differentiation.\textsuperscript{21,29} However, a recent study showed that the expression of these 2 proteins was differentially regulated by the nuclear receptor pathways.\textsuperscript{30} Although CD36 is positively regulated by the nuclear receptor peroxisome proliferator-activated receptor γ,\textsuperscript{30} the nuclear receptor related to the regulation of class A MSR has not been well characterized. In the current study, adiponectin specifically decreased the protein levels of class A MSR without altering those of

![Figure 10. Expression of adiponectin in human injured vascular wall. Immunohistochemical double staining of adiponectin and macrophages was performed as described in Methods. Positive immunoreactivity to adiponectin (green color) and macrophages (red color) was observed. Bar indicates 100 μm.](image-url)
CD36. In addition, adiponectin decreased the steady-state mRNA levels of class A MSR and LPL mRNA without altering those of apoE. Because class A MSR, LPL, and apoE reportedly increase during monocyte-to-macrophage differentiation,\textsuperscript{29,31,32} adiponectin may partially modulate macrophage differentiation and affect lipid accumulation in the macrophages. The promoter assay of class A MSR revealed that adiponectin suppressed the class A MSR expression at the transcriptional level, although further studies are needed to clarify the precise transcriptional regulatory mechanism by which adiponectin decreases the class A MSR expression.

Recently, we detected adiponectin in the rat catheter-injured vascular walls but not in the intact vascular walls.\textsuperscript{12} In addition, adiponectin accumulated in the subendothelial space of the vascular wall from the plasma at an early phase of catheter injury. In the present study, adiponectin was detected around macrophages in the human injured aorta where a thrombus was attached. These observations suggest that adiponectin may rapidly accumulate in the vascular wall when the endothelial barrier is injured and modulate the macrophage-to-foam cell transformation in vivo.

In summary, we demonstrated that adiponectin reduced lipid accumulation in human monocyte-derived macrophages through an inhibition of class A MSR expression. Our findings suggest that the adipocyte-specific plasma protein adiponectin is not only a negative regulator of the endothelial adhesion molecule expression,\textsuperscript{9} but also a modulator for macrophage foam cell formation, thus providing a fundamental mechanism for the link between overnutrition and atherosclerosis.

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