Original Article

Activation of Peripheral Blood CD14$^+$ Monocytes Occurs in Diabetes

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Blood levels of inflammatory markers associated with endothelial dysfunction and atherosclerosis are increased in diabetic patients; the highest levels occur in poorly controlled diabetes. We investigated the activation state of peripheral blood monocytes in diabetes with respect to scavenger receptor (CD36) expression and monocyte chemoattractant protein-1, intracellular adhesion molecule-1, vascular cell adhesion molecule-1, and peroxisome proliferator–activated receptors mRNA expression. CD14$^+$ monocytes were isolated from peripheral blood of type 1 and type 2 diabetic patients with good (HbA_{1c} <7.0%) or poor (>9.4%) glycemic control and a group of nondiabetic subjects. Monocytes from diabetic subjects displayed increased CD36 cell surface expression ($P < 0.0005$) and increased uptake of oxidized LDL ($P < 0.05$). Monocyte chemoattractant protein-1 gene expression was increased in monocytes from both groups of diabetic subjects ($P < 0.05$). Both CD68 and peroxisome proliferator–activated receptor-γ gene expression were increased in the poorly controlled diabetic group ($P < 0.05$ for each), whose monocytes also displayed increased attachment to endothelial monolayers ($P < 0.0005$ vs. nondiabetic control subjects). In poorly controlled diabetes, CD14$^+$ monocytes are functionally activated and show some of the differentiation markers associated with macrophages. These monocytes also demonstrate an increased ability for attachment to normal endothelial cells, one of the early stages in atherogenesis. *Diabetes* 54:2779–2786, 2005

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15d-PGJ2, 15-deoxy-δ 12, 14 prostaglandin J2; AGE, advanced glycation end product; CMFDA, 5-chloromethylfluorescein diacetate; CRP, C-reactive protein; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate; HAoEC, human aortic endothelial cell; ICAM-1, intracellular adhesion molecule-1; IL-6, interleukin-6; MCP-1, monocyte chemoattractant protein-1; oxLDL, oxidized LDL; VCAM-1, vascular cell adhesion molecule-1.

It has become clear that inflammatory processes play a major role in atherogenesis and that the endothelium is intimately involved at all stages of atheroma formation. With this has come a major change in our understanding of the function of the endothelium as a diffuse organ, involved in prevention of thrombosis and in modulation of both relaxation and contraction of the blood vessels (1,2). Raised levels of the inflammatory marker C-reactive protein (CRP) are associated with altered endothelial function and arterial stiffness in healthy individuals (3,4) and are predictive of future coronary heart disease in healthy middle-aged men (5). Views on the importance of inflammatory markers have progressed to the extent that it has been suggested by some that CRP is a stronger predictor of first cardiovascular events than LDL cholesterol (6); however, others suggest a more cautious interpretation of available data (7). In vitro studies have shown that CRP induces endothelial cells to produce the inflammatory cytokine interleukin-6 (IL-6), which, at least in part, is responsible for upregulation of vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) (8). CRP also induces production of monocyte chemoattractant protein-1 (MCP-1) by both endothelial (8) and vascular smooth muscle (9) cells; MCP-1 enhances the movement of mononuclear cells into the subendothelial space.

Although a great deal has been published on vessel wall stiffness and the prevalence of inflammatory markers in association with endothelial dysfunction, very little attention has been paid to the state of the peripheral blood monocyte in diseases associated with atherosclerosis. Usually the monocyte develops into the mature macrophage within the subendothelial space, and it is here that upregulation of scavenger receptors occurs, equipping the macrophage to bind and take up oxidized LDL (oxLDL) (10). However, it is possible that maturation of monocytes could take place in any location where the conditions were permissive.

In diabetes, there is a substantially increased risk of vascular disease (11). Inflammatory markers have been found to be predictive of coronary heart disease (12) and even of type 2 diabetes itself (12,13). Many of the inflammatory markers now associated with atherosclerosis are found in diabetes at higher levels than in the nondiabetic population. There are raised levels of CRP and IL-6 in type 1 (14) and type 2 (15) diabetes; this is also the case for

TABLE 1
Characteristics of the subject groups

Characteristic	NDC	WCD	PCD
n	13	25	27
Age (years)	51.8 ± 7.9	58.2 ± 16.8*	50.6 ± 16.1†
Sex (male/female)	7/5	16/9	12/15
Ratio of type 1 to type 2 diabetic patients	–	7:16	14:13‡
Duration of diabetes (years)	–	12.4 ± 12.7	16.0 ± 11.9
A1C (%)	ND	6.01 ± 0.60	10.11 ± 0.76
BMI (kg/m^2)	25.0 ± 3.4	28.7 ± 4.8§	29.5 ± 8.9
Systolic blood pressure (mmHg)	125 ± 5	136 ± 15†	134 ± 22¶
Diastolic blood pressure (mmHg)	75 ± 6	77 ± 9	79 ± 8‖
Total cholesterol (mmol/l)	5.17 ± 0.65	4.81 ± 1.15	5.14 ± 1.10
LDL cholesterol (mmol/l)	3.15 ± 0.61	2.59 ± 1.07	2.89 ± 0.90
HDL cholesterol (mmol/l)	1.54 ± 0.31	1.44 ± 0.47	1.30 ± 0.36
Family history of diabetes (*n*)	4	12	12
Current smoker (*n*)	0	4	2
Vascular complications			
Microvascular (*n*)	0	5	11
Macrovascular (*n*)	0	1	2

Data are means ± SD unless otherwise indicated. NDC, nondiabetic subjects; WCD, well-controlled diabetic patients; PCD, poorly controlled diabetic patients. *P = 0.0143 vs. NDC. †P = 0.0279 vs. WCD. ‡One type 2 diabetic subject in the poorly controlled group was GAD antibody positive. §P = 0.0249 vs. NDC. ¶P = 0.0078 vs. NDC. ‖P = 0.0007 vs. NDC.

MCP-1 levels in both type 1 (16) and type 2 (17) diabetes. Not surprisingly, endothelial dysfunction occurs in diabetes (2). However, in addition to this, many of the conditions usually thought to induce activation/maturation of the monocyte/macrophage and uptake of oxLDL in the subendothelial space are present within the peripheral circulation in diabetes. First, many of the molecules associated with upregulation of the scavenger receptor, CD36, are raised in the blood in diabetes; these include oxLDL (18), advanced glycation end products (AGEs) (19), IL-6 (20), and MCP-1 (21) [rev. in 22]. Second, oxLDL is found in the circulation in diabetes (23,24) and thus may be available for uptake if scavenger receptors are present.

The purpose of this project was *1*) to investigate whether upregulation of the CD36 scavenger receptor occurs in peripheral blood CD14$^+$ monocytes in diabetes and whether this is influenced by the state of glycemic control and *2*) to determine whether glycemic control affects the function of these monocytes with respect to uptake of oxLDL and endothelial cell attachment.

RESEARCH DESIGN AND METHODS

After local ethical committee approval and written informed consent, patients with type 1 and type 2 diabetes and nondiabetic subjects were recruited. Patients with diabetes were selected according to their level of HbA_{1c} (A1C) from the routine diabetic outpatient clinic; those with A1C values of 5.4–7.0% were considered to be well controlled, whereas those with A1C values of 9.4–11.5% were classified as poorly controlled. Patients taking vitamin supplements, antioxidants, thiazolidinediones, hydroxymethylglutaryl-CoA reductase inhibitors, or fibrate drugs were excluded. Patient characteristics are described in Table 1. Healthy control subjects were taking none of the drug groups listed above.

Monocyte isolation. Human venous blood was collected in a lithium heparin tube and processed within 1–2 h. CD14$^+$ monocytes were isolated directly from total blood with a magnetic activated cell sorting system (AutoMACS; Miltenyi Biotech) using microbeads coated with CD14 antibody (MACS CD14 MicroBeads; Miltenyi Biotech). Briefly, 5 ml of blood was incubated with CD14 microbeads (250 μl) at 4°C for 15 min. Then the blood sample was diluted with 5 ml of FACSflow buffer and centrifuged (10 min, 2,000 rpm) at room temperature. The supernatant was carefully removed, and the cell pellet was diluted with FACSflow buffer. The cell suspension was then run on the AutoMACS system, and purified CD14$^+$ monocytes were isolated by positive selection. The positive cells were at least 90% CD14$^+$, as measured by flow cytometry. Percentage of monocyte recovery was 85.6 ± 4.7%, 95.5 ± 1.4%, and 95.1 ± 1.5% in the nondiabetic control, well-controlled diabetic patient, and poorly controlled diabetic patient groups, respectively; of these, 93.8 ± 1.5%, 91.0 ± 1.7%, and 91.9 ± 1.2% were CD14$^+$, respectively; no significant difference was observed among the three groups.

Quantification of CD36 expression on monocytes. Fluorescein isothiocyanate–conjugated CD14 antibody was used to identify monocytes, and the anti-CD36 antibody was conjugated to phycoerythrin. Fluorescein isothiocyanate–conjugated mouse IgG1 and phycoerythrin-conjugated mouse IgM were used as isotype-matched negative controls. Staining and flow cytometric analysis of freshly obtained monocytes were carried out by standard procedures, using a Coulter Epics Elite flow cytometer equipped with an argon ion laser.

Briefly, 200 μl of monocyte suspension was incubated for 15 min with 5.0 μl of appropriately diluted monoclonal antibody at 4°C, in the dark. Cells were then washed once with 1 ml PBS containing 2% human serum and centrifuged (5 min, 1,000 rpm). Stained cells were fixed with 2% paraformaldehyde and stored at 4–8°C until data acquisition by flow cytometry on a Beckman Coulter Epics Elite flow cytometer.

Lipoprotein labeling. LDL (Calbiochem) was labeled with the fluorescent probe 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (DiI; Molecular Probes). Briefly, 300 μg of the fluorescent probe DiI was added to 1 mg of LDL protein. The mixture was then incubated at 37°C for 18 h, protected from light. The DiI-labeled LDL was reisolated by ultracentrifugation (100,000 rpm for 1 h) at d = 1.060 g/ml and extensively dialyzed against 5 mmol/l Tris, 154 mmol/l NaCl, and 0.1 g/l EDTA, pH 7.4, at 4°C. DiI-labeled LDL (1 mg) was oxidized in a solution of $CuCl_2$ (10 μmol/l) for 24 h at 37°C. All lipoprotein preparations were stored at 4°C in sterile containers after filtration sterilization (25,26).

DiI-OxLDL uptake. After isolation on the AutoMacs system, monocytes were seeded into a 24-well plate and incubated in RPMI-1640 (Sigma) containing 5% lipoprotein-deficient human serum, L-glutamine (2 mmol/l), streptomycin (100 μg/ml), penicillin (100 IU/ml), nonessential amino acids (2 mmol/l), D-glucose (5 mmol/l), and DiI-OxLDL (50 μg/ml) to quantify the scavenger receptor activity. Blocking of CD36 receptor activity was achieved by preincubating with anti-CD36 monoclonal antibody SMO (20 μg/ml, Sigma) for 10 min. Monocytes were incubated at 37 or 4°C for 4 h. At the end of the incubation period, the cells were transferred into polypropylene centrifuge tubes and washed twice with PBS containing 0.4% BSA and 0.25 g/l EDTA and twice with PBS/EDTA alone. After each wash, cells were centrifuged at 690 × *g* for 5 min and resuspended in 5 ml of the buffer. The centrifuge tubes were changed before the last wash to reduce contamination with free DiI-OxLDL adhering to the plastic. After the last centrifugation (2,750 × *g*; 5 min), the buffer was removed, and 1 ml of cell lysis reagent (1 g/l SDS and 0.1 mol/l NaOH) was added to the cell pellet. Cells were then incubated under gentle shaking for 1 h at room temperature. Fluorescence was measured in duplicates of 200 μl of the lysate on black plates with excitation and emission wavelengths set at 520 and 580 nm, respectively (25,26). Cellular protein

content was determined using a bicinchoninic acid protein assay reagent kit (Pertio Science); BSA was used as a standard. The internalized DiI-OxLDL was calculated as the difference between the cell-associated DiI-OxLDL fraction at 37°C and the fraction associated with the cells at 4°C. Uptake was measured against a DiI-OxLDL standard curve.

Real-time RT-PCR analysis of gene expression. Total RNA was extracted using a RNeasy Mini kit (Qiagen). Real-time RT-PCR was performed using the ABI Prism 7700 sequence detection system (Applied Biosystems). Sequence-specific PCR primers for CD36 (GenBank accession no. NM000072), CD68 (GenBank accession no. NM001251), LDL receptor (GenBank accession no. NM002776), MCP-1 (GenBank accession no. X14768), peroxisome proliferator–activated receptor (PPAR)-α (GenBank accession no. NM005036), PPARδ (GenBank accession no. AF187850), PPARγ (GenBank accession no. AB0055215), ICAM-1 (GenBank accession no. NM000201), VCAM-1 (GenBank accession no. X53051), and glyceraldehyde-3-phosphate dehydrogenase (GenBank accession no. M33197) were designed using the Primer Express software version 1.5 (Applied Biosystems) and synthesized by Invitrogen Life Technologies.

Real-time PCR assays were performed in a sealed 96-well microtiter plate (Applied Biosystems) on a spectrofluorometric thermal cycler (Applied Biosystems 7700 Prism). Amplification of each sample was performed in triplicate as follows: 95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescent signal was measured and plotted during each 60°C annealing and extension step for all samples. Each reaction was characterized by a value, the cycle threshold, defined as the fractional number of cycles at which the reporter fluorescent emission reached a fixed threshold level in the exponential region of the amplification plot (27).

Monocyte adhesion assays

Fluorescent labeling of monocytes. After isolation on the AutoMacs system, peripheral blood monocytes were fluorescently labeled with 5-chloromethylfluorescein diacetate (CMFDA; Molecular Probes) for quantitative adhesion assay. The fluorescence labeling of circulating monocytes was done by incubating cells (1×10^6 cells/2 ml) with 5 μmol/l CMFDA in RPMI-1640 (45 min, 37°C, and 5% CO_2). At the end of the incubation period, excess dye was removed by washing the cells once with complete medium. To allow the cleavage by the intracellular esterase into a highly charged fluorescent marker that is retained by viable cells, cells were then incubated in complete RPMI for 30 min; cells were then washed with complete RPMI and resuspended at a density of 5×10^5 cells/ml (28).

Monocyte adhesion to human aortic endothelial cells. Human aortic endothelial cells (HAoECs) (PromoCell) were cultured to confluence on 13-mm-diameter cover-glass placed in a 24-well plate at 37°C and 5% CO_2. CMFDA-labeled $CD14^+$ monocytes (5×10^5cells/well) were coincubated with HAoEC for 45 min at 37°C. After incubation, nonadherent cells were removed by washing each well three times with PBS. After fixation of the attached cells with paraformaldehyde (4%), cells were washed with PBS. Five randomly chosen 1-mm^2 regions in each cover-glass were evaluated; the number of attached cells was counted using an inverted microscope.

GAD antibodies. GAD antibodies were measured in type 2 diabetic subjects using a World Health Organization–standardized radioimmunoassay.

Statistical analysis. Comparisons among the three groups of subjects were performed using a two-way ANOVA test. Differences with $P < 0.05$ were considered as significant.

RESULTS

CD36 cell surface expression on circulating monocytes. To investigate whether CD36 cell surface expression was upregulated by the degree of glycemic control in peripheral blood monocytes from diabetic patients, circulating monocytes were isolated using anti-CD14–coated magnetic beads; this method has been shown previously to induce less cell activation than Ficoll centrifugation (29). CD36 scavenger receptors were expressed in each group of subjects studied. However, only 4% of the circulating monocytes from the nondiabetic control group were positive for CD36 compared with 37 and 64% for the circulating monocytes from the well-controlled and poorly controlled diabetic patient groups, respectively (Fig. 1). (One patient with type 2 diabetes was GAD antibody positive; this patient was in the poorly controlled group.)

When all diabetic subjects were analyzed as a single group, there was a highly significant correlation between

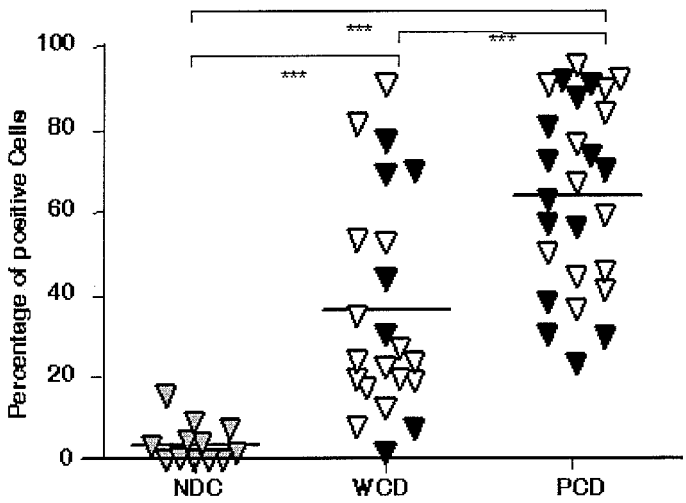

FIG. 1. Cell surface expression of scavenger receptor CD36. Flow cytometric analysis of expression of CD36 in $CD14^+$ circulating monocytes from nondiabetic control subjects (NDC), well-controlled diabetic patients (WCD), and poorly controlled diabetic patients (PCD). After isolation by positive selection on the AutoMACS cell sorting system, $CD14^+$ monocytes were labeled with an anti–CD36-phycoerythrin antibody. △, patients with type 2 diabetes; ▲, patients with type 1 diabetes; ◬, control subjects. Data are expressed as percentage of positive cells for CD36; $n = 13$ (NDC), $n = 22$ (WCD), $n = 27$, (PCD); ***$P < 0.0005$.

A1C and CD36 cell surface expression ($P < 0.0001$). The ratio of type 1 and type 2 diabetic subjects is different in the well-controlled and poorly controlled groups (Table 1); however, this did not influence the outcome with respect to CD36 surface expression (Fig. 1). When type 1 and type 2 diabetic subjects were analyzed separately with respect to CD36 cell surface expression, there was no difference among the groups (ANOVA, $P = 0.259$). Similarly, although there was a difference in the sex ratios between the two groups of diabetic subjects, no sex difference in CD36 cell surface expression was observed within each group (not shown).

As a consequence, all the above patients were included in this study. However, because of restrictions on the amount of blood that could be taken from each subject, all subjects did not have all tests carried out; in that case, care was taken to ensure that there was a large representative subgroup for each test.

CD36, CD68, and LDL receptor gene expression on circulating monocytes

CD36 mRNA. CD36 gene expression showed a significant, progressive increase from nondiabetic subjects to well-controlled diabetic patients and from well-controlled to poorly controlled patients (Fig. 2*A*); this gave ~50% increase in well-controlled diabetic patients and 130% increase in poorly controlled diabetic patients above the nondiabetic control group. There was no difference between type 1 and type 2 diabetic subjects ($P = 0.645$).

CD68 mRNA. Macrosialin/CD68 is a heavily glycosylated trans-membrane protein that is highly and specifically expressed by tissue macrophages. To determine the degree of monocyte differentiation into macrophages, CD68 mRNA expression was quantified. CD68 gene expression was constitutively expressed in monocytes from control subjects (Fig. 2*B*). There was a small increase in CD68 mRNA expression in both diabetic groups. This increase

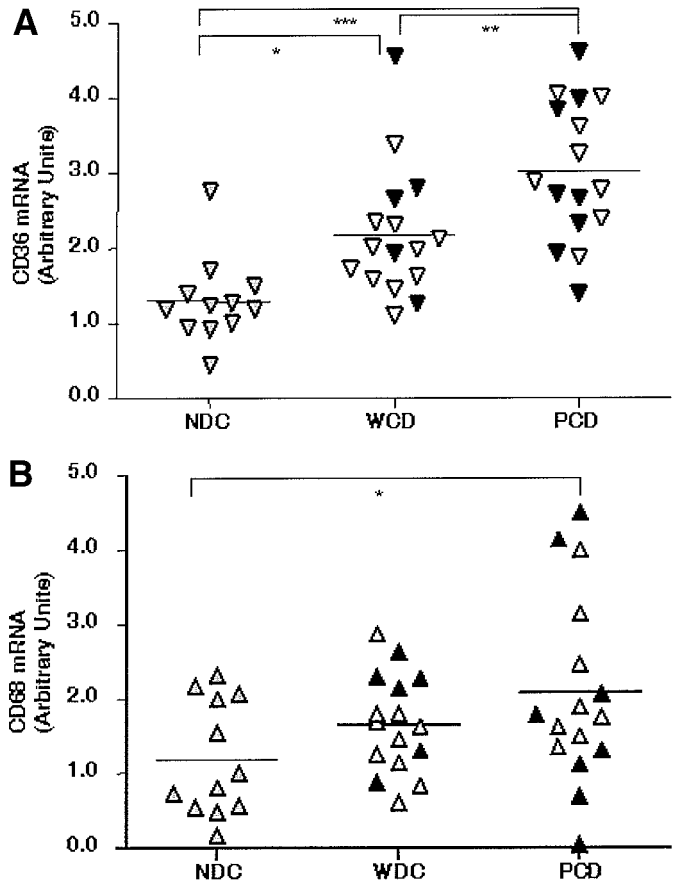

FIG. 2. CD36 and CD68 mRNA expression. Expression of CD36 mRNA (*A*) and expression of CD68 mRNA (*B*) in $CD14^+$ monocytes from control subjects (NDC), well-controlled diabetic patients (WCD), and poorly controlled diabetic patients (PCD). △, patients with type 2 diabetes; ▲, patients with type 1 diabetes; and △, control subjects. The mRNA level was quantified using real-time RT-PCR; $n = 12$ (NDC), $n = 16$ (WCD), $n = 16$ (PCD). *$P < 0.05$; ***$P < 0.005$; ***$P < 0.0005$.

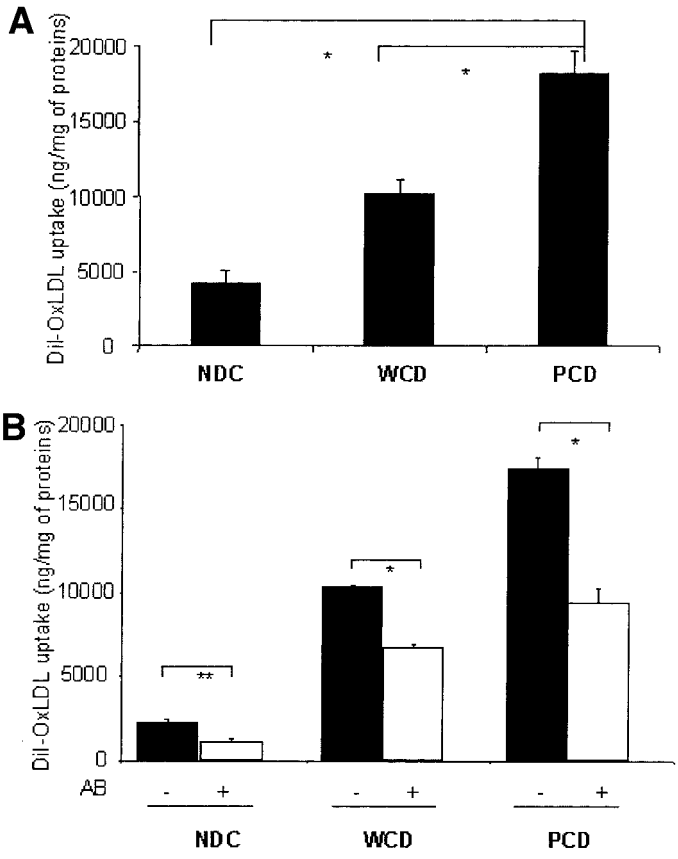

FIG. 3. DiI-OxLDL uptake by $CD14^+$ circulating monocytes. $CD14^+$ monocytes from control subjects (NDC), well-controlled diabetic patients (WCD), and poorly controlled diabetic patients (PCD) were incubated in presence of DiI-OxLDL (50 μg/ml) alone (*A*) or with an anti-CD36 monoclonal antibody (20 μg/ml) (*B*) for 4 h at 4°C or 37°C. After washes and lysis of the cells, DiI-OxLDL uptake was assessed by spectrofluorimetry (excitation, 520 nm; emission, 580 nm). Data are expressed as means ± SE. For the DiI-OxLDL uptake experiments, $n = 7$ (NDC), $n = 7$ (WCD), $n = 9$ (PCD); *$P < 0.05$, **$P < 0.005$. For the DiI-OxLDL uptake in presence of anti-CD36 monoclonal antibody, three samples per group were analyzed.

was significant in poorly controlled patients compared with nondiabetic control subjects, 2.09 vs. 1.20 arbitrary units (AU) ($P = 0.0257$) (Fig. 2*B*), although there was variability within the group; CD68 was not significantly increased in well-controlled patients compared with nondiabetic control subjects, 1.66 vs. 1.20 AU ($P = 0.4785$). There was no difference between type 1 and type 2 diabetic subjects ($P = 0.908$).

LDL receptor mRNA. Because no significant difference was found among the LDL cholesterol levels of the three groups, it was not surprising that no significant difference was found in LDL receptor mRNA expression among the groups studied. LDL receptor mRNA expression was 1.10 ± 0.28, 0.70 ± 0.25, and 0.96 ± 0.37 AU in nondiabetic control subjects, well-controlled diabetic patients, and poorly controlled diabetic patients, respectively.

DiI-OxLDL uptake by freshly isolated circulating monocytes. To determine whether the scavenger receptor CD36 expressed at the surface of the freshly isolated circulating monocytes was fully functional, we assessed the uptake or internalization of fluorescently labeled oxLDL, DiI-OxLDL. Freshly isolated monocytes were able to internalize DiI-OxLDL (Fig. 3*A*). DiI-OxLDL uptake was increased by 243% ($P = 0.0023$) and 433% ($P = 0.0002$) in circulating monocytes from well-controlled diabetic patients and from poorly controlled diabetic patients, respectively, compared with nondiabetic subjects. However, this effect was only significant in poorly controlled patients (Fig. 3*A*). Addition of a specific antibody blocking the ligand-binding domain of CD36 reduced the uptake of DiI-OxLDL by 49% ($P = 0.0079$), 34% ($P = 0.0262$), and 48% ($P = 0.0252$) in monocytes from nondiabetic control subjects, well-controlled diabetic patients, and poorly controlled diabetic patients, respectively (Fig. 3*B*).

Expression of atherosclerotic markers (MCP-1, ICAM-1, and VCAM-1) mRNA in circulating monocytes. To understand the mechanisms and genes involved in the upregulation of CD36 expression in monocytes from poorly controlled diabetic patients, we assessed the gene expression of different proinflammatory mediators such as MCP-1 and adhesion molecules (ICAM-1 and VCAM-1).

MCP-1 gene expression was significantly increased in monocytes from subjects with diabetes. MCP-1 expression was increased by 237% (3.59 vs. 1.51 AU; $P = 0.0488$) and by 278% (4.20 vs. 1.51 AU; $P = 0.0143$) in monocytes from well-controlled diabetic patients and monocytes from poorly controlled diabetic patients, respectively, compared with the nondiabetic control group (Fig. 4*A*); there

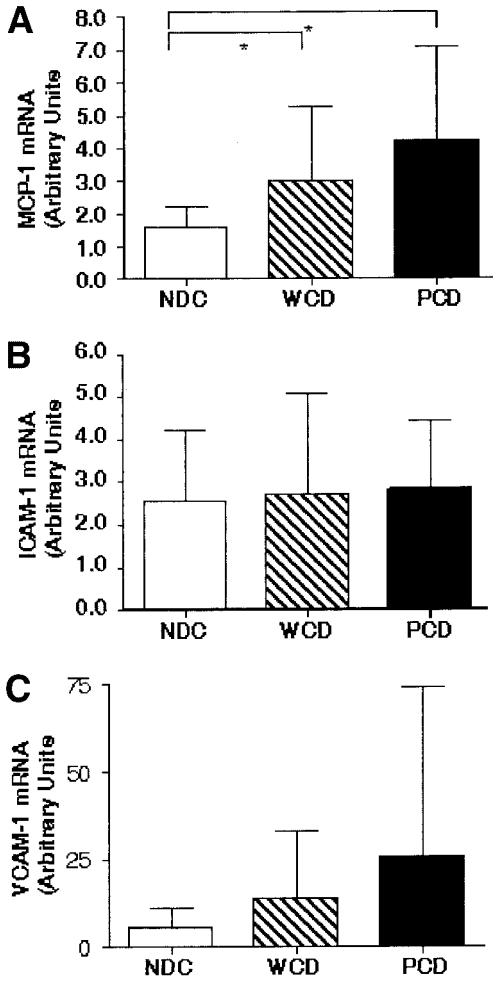

FIG. 4. Expression of MCP-1 (*A*), ICAM-1 (*B*), and VCAM-1 (*C*) mRNA in CD14$^+$ monocytes from control subjects (NDC), well-controlled diabetic patients (WCD), and poorly controlled diabetic patients (PCD). Expression of gene mRNA level was determined by real-time RT-PCR; n = 12 (NDC), n = 16 (WCD), n = 16 (PCD). Data are expressed as means ± SD,*P < 0.05.

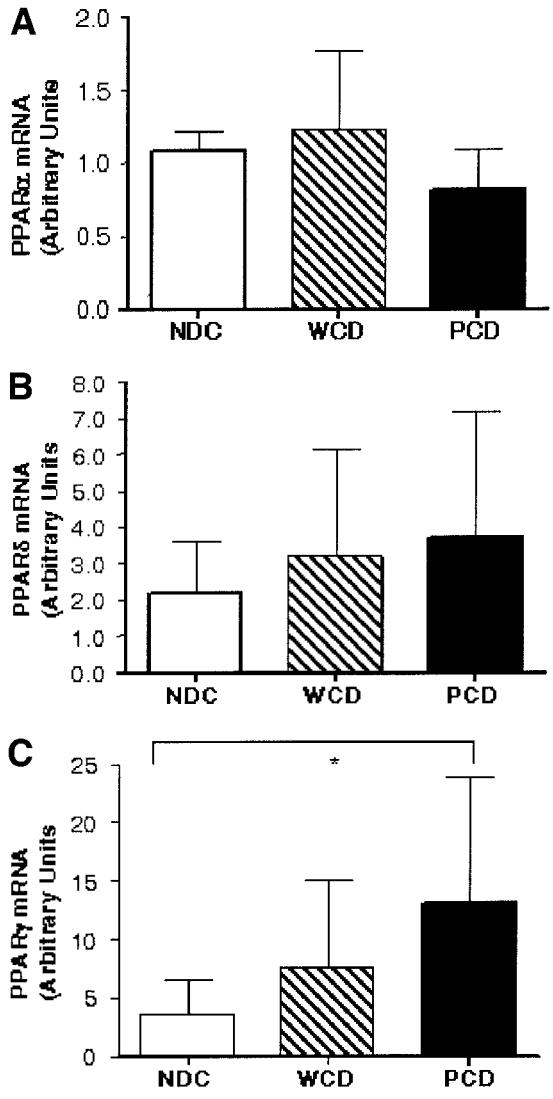

FIG. 5. Expression of PPAR α (*A*), PPAR δ (*B*), and PPARγ (*C*) mRNA in CD14$^+$ monocytes from control subjects (NDC), well-controlled diabetic patients (WCD), and poorly controlled diabetic patients (PCD). The mRNA level was quantified using real-time RT-PCR; 12 (NDC), n = 16 (WCD), n = 16 (PCD). Data are expressed as means ± SD,*P < 0.05.

was no significant difference between the two diabetic patient groups. Although a relationship of BMI with MCP-1 has been shown in severe obesity, in this study, when all subjects were analyzed as a single group, there was no correlation between MCP-1 and BMI (P = 0.781). With respect to type of diabetes, there was no difference in MCP-1 gene expression (type 1 vs. type 2 diabetes, P = 0.818).

No significant difference in ICAM-1 and VCAM-1 gene expression was observed among the three groups of subjects studied (Fig. 4*B* and *C*). In the case of VCAM-1, there was a tendency for increased levels in the two groups of diabetic subjects that was more marked in the poorly controlled group (Fig. 4*C*); however, this was not statistically significant because of wide variation within the group. With respect to type of diabetes, there was no difference between type 1 and type 2 subjects for ICAM-1 (P = 0.234) or VCAM-1 (P = 0.983).

Expression of PPARs mRNA in circulating monocytes. No significant differences were observed in PPAR α and PPARδ mRNA expression among the three groups (Fig. 5*A* and *B*). However, PPARγ expression was significantly increased in monocytes from poorly controlled diabetic patients compared with the monocytes from nondiabetic control subjects (Fig. 5*C*). There was no difference between type 1 and type 2 diabetic subjects (P = 0.401).

Monocyte adhesion to HAoEC. To determine whether the reactivity of circulating monocytes from diabetic patients was different from nondiabetic control subjects, we performed a test of adhesion on a monolayer of human aortic endothelial cells that had not been treated in any way to enhance adhesiveness. Fluorescently labeled, freshly isolated peripheral blood CD14$^+$ monocytes attached to HAoEC. There was a modest increase in the well-controlled diabetic group compared with nondiabetic subjects; in poorly controlled diabetic subjects, there was a greater than twofold, significant increase in monocyte attachment to the endothelial monolayers compared with nondiabetic control subjects and a significant, ~60% increased attachment compared with monocytes from well-controlled diabetic subjects (Fig. 6).

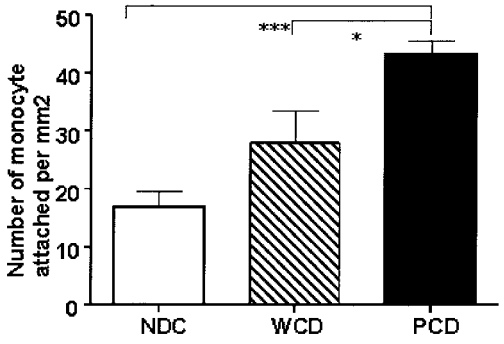

FIG. 6. Circulating monocyte adhesion to HAoEC. After being labeled with CMFDA, CD14$^+$ monocytes from control subjects (NDC), well-controlled diabetic patients (WCD), and poorly controlled diabetic patients (PCD) were incubated in presence of HAoEC at 37°C for 45 min. After PBS washes, the numbers of attached monocytes per square millimeter were quantified on five randomly chosen fields, using an inverted microscope. Data are expressed as means ± SE. For monocyte adhesion experiments, n = 10 (NDC), n = 8 (WCD), n = 6 (PCD), ***P < 0.0005.

DISCUSSION

The results clearly show an increase in functional CD36 scavenger receptors in peripheral blood CD14$^+$ monocytes of subjects suffering from diabetes. Furthermore, monocytes from those with high levels of A1C demonstrated a significantly increased ability to attach to endothelial cells, one of the early steps leading to atheroma formation. Gene expression of CD68, another scavenger receptor, was also significantly increased in monocytes from poorly controlled diabetic subjects. Increase in CD36 and CD68 expression has been previously associated with the maturation of monocytes toward macrophages (30). CD36 is a type B scavenger receptor, which is normally thought to be responsible for ~50% of oxLDL uptake. Scavenger receptors can recognize oxidized lipids or oxidized apoproteins (22). Initial work had shown that CD36 recognized lipid moieties of oxLDL (31); however, recent work has shown that the binding of oxLDL to CD36 is mediated by oxidized phospholipids associated with both the lipid and protein moieties of the lipoprotein (32). In the present study, the increase in CD36 surface expression was paralleled by an increase in uptake of oxLDL, which could be blocked by 40–50% with antibodies to the ligand binding domain of CD36. The present study also revealed a significant increase in MCP-1 mRNA in subjects with poor glycemic control, another sign of monocyte activation. All CD14$^+$ cells were included in the measurements, because monocytes were isolated solely on the basis of CD14 positivity and not on any criterion that would give selective advantage to activated cells, such as prior attachment to a substratum (30).

It has been reported that severe atherosclerosis is associated with a reduction in the expression of CD36 on peripheral blood monocytes (33). In the present study, therefore, it is unlikely that the degree of atherosclerosis, per se, is the cause of the increase in CD36 found in the poorly controlled diabetic group. However, PPARγ (32), oxLDL (19), AGEs (19), and MCP-1 (21) have all been associated with upregulation of the CD36 receptor.

PPARγ mRNA was significantly increased in monocytes from poorly controlled patients. The regulation of PPARγ mRNA in monocytes is not yet fully understood, but it can be induced by oxLDL (18,34); additionally, oxidized metabolites of linoleic acid (34,35) and arachidonic acid (15-deoxy-δ 12, 14 prostaglandin J2 [15d-PGJ2]) (35) can induce PPARγ activation, and it is known that glucose causes oxidative stress (36). This is of possible importance because upregulation of CD36 receptors by oxLDL is PPARγ dependent (37). However, it is difficult to implicate oxLDL or oxidized metabolites of linoleic and arachidonic acid as the cause of differences between the two groups of diabetic patients with respect to PPARγ gene expression and CD36 upregulation because the absolute concentrations of oxLDL in the plasma in well-controlled and poorly controlled diabetes are similar (24). Furthermore, in the case of the arachidonic acid metabolite, 15d-PGJ2, there appears to be no difference in the concentrations between nondiabetic and diabetic subjects (38), and the levels measured with the more recent, sensitive assays for 15d-PGJ2 are believed to be below those needed to activate PPARγ (38).

AGEs upregulate CD36 gene expression in a dose-dependent fashion, and they also upregulate PPARγ gene expression (18). The increased levels of AGEs present in poorly controlled diabetic subjects (39) could account, in large part, for the difference in CD36 gene expression between poorly controlled and well-controlled patients in the present investigation. In contrast to AGEs, transient increases in plasma glucose have no additional effect on CD36 expression in monocytes from well-controlled type 2 diabetic subjects (40).

The results of the present investigation indicate that peripheral blood monocytes are activated in poorly controlled diabetes; this also occurs in hypertension (41). In the present study, several patients from both diabetic groups had a history of hypertension, but all had good blood pressure control at the time of study. When the individual results for CD36 were subjected to subanalysis, those patients with a history of hypertension did not have higher monocyte CD36 expression than those without a history of hypertension within their own A1C-defined groups (individual data not shown). It has also been shown that (total) peripheral monocytes, from glucose-tolerant, grossly obese subjects (BMI in the range 37–60 kg/m^2) are in a proinflammatory state (42,43). In contrast, in the mildly obese, diabetic subjects of the present study, there is an important association between the level of A1C and the degree of proinflammatory activation of CD14$^+$ monocytes.

Activation of peripheral blood monocytes is an important additional facet of the inflammatory profile of diabetic vascular disease to be added to endothelial dysfunction. The CD36 scavenger receptors were functional with respect to uptake of oxLDL, and there was also a significant increase of CD68 gene expression in patients with poor glycemic control. These changes are usually associated with maturation toward fully differentiated macrophages. However, it cannot be deduced from the results of this study that these activated monocytes will easily become foam cells in the peripheral blood because activators of PPARγ that induce gene expression of CD36 may also induce the scavenger receptor CLA-1/SR-B1, which binds HDL with high affinity and promotes cholesterol efflux from macrophages as the first step in the reverse choles-

terol transport pathway (44); on the other hand, HDL cholesterol tends to be lower in diabetes, as in the present study, and the reverse transport pathway may be compromised. Whether PPARγ activation in monocytes will result in a net increase or decrease in lipid accumulation and whether PPARγ activation, per se, induces or inhibits atherogenesis is unclear. Although increased PPARγ expression may be induced by cytokines and its activation may have proinflammatory effects (45), it has also been shown to inhibit production of monocyte inflammatory cytokines (46) and be a negative regulator of monocyte/macrophage activation (47).

However, the results of this study leave little doubt that peripheral blood $CD14^+$ monocytes in subjects with poorly controlled diabetes are functionally activated and show some of the differentiation markers associated with macrophages. Of major importance is the fact that the monocytes demonstrate an increased ability for endothelial cell attachment, one of the early stages in atheroma formation.

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