Original Article

Increased Monocytic Activity and Biomarkers of Inflammation in Patients With Type 1 Diabetes

Sridevi Devaraj,[1] Nicole Glaser,[2] Steve Griffen,[2] Janice Wang-Polagruto,[1] Eric Miguelino,[1] and Ishwarlal Jialal[1,2]

Type 1 diabetes is associated with increased vascular complications, and monocytes are pivotal cells in atherogenesis. However, there are few data on monocyte function and inflammation in type 1 diabetes. The aim of this study was to compare monocyte function and biomarkers of inflammation in type 1 diabetic subjects without macrovascular disease with that in matched control subjects ($n = 52$ per group). Fasting blood was obtained for biomarkers of inflammation (C-reactive protein [CRP], plasma-soluble cell adhesion molecules [CAMs], monocyte chemoattractant protein 1, nitrotyrosine, CD40 ligand [CD40L], and monocyte function). High-sensitive CRP, soluble intracellular adhesion molecule (sICAM), sCD40L, and nitrotyrosine levels were significantly elevated in type 1 diabetic subjects compared with in control subjects ($P < 0.05$). Monocyte superoxide anion release was significantly increased in the resting (37%; $P < 0.05$) and activated state (26%; $P < 0.005$) in type 1 diabetic compared with in control subjects. Monocyte interleukin (IL)-6 levels were significantly elevated in type 1 diabetic subjects compared with in control subjects in the resting state (51%; $P < 0.05$) and after lipopolysaccharide activation (31%; $P < 0.01$). Monocyte IL-1β levels were increased in the activated monocytes in type 1 diabetic compared with in control subjects. There were no significant differences in monocyte tumor necrosis factor levels or adhesion between the two groups. Thus type 1 diabetes is a proinflammatory state, as evidenced by increased levels of monocyte IL-6, superoxide anion, and plasma CRP, sICAM, sCD40L, and nitrotyrosine levels. These results have a major implication on our understanding of the role of inflammation in vasculopathies in type 1 diabetes. *Diabetes* 55:774–779, 2006

Coronary artery disease is the main cause of death in type 1 diabetes. Type 1 diabetes is associated with an increased risk of vascular complications, and type 1 diabetic patients with proteinuria have a 15- to 37-fold increased risk of fatal coronary artery disease (1). Most studies have indicated that this excess risk for macrovascular complications cannot be explained solely by conventional risk factors such as dyslipidemia, hypertension, and smoking. Therefore, the diabetic state, per se, confers an increased propensity to accelerated atherogenesis; however, the precise mechanisms by which this occurs remain to be elucidated. Several lines of evidence point to the role of increased inflammation in the pathogenesis of these vasculopathies. Inflammation is pivotal in atherosclerosis. C-reactive protein (CRP), the prototypic marker of inflammation, is a cardiovascular risk marker; in addition, increased CRP levels predispose patients to the development of diabetes, and CRP may be proatherogenic (2). The monocyte macrophage, a pivotal cell in atherogenesis, is readily accessible for study. We and others have previously shown that monocytes from type 2 diabetic subjects exhibit increased proatherogenic activity compared with those from matched control subjects (3–5). However, there are few data on monocyte function and inflammation in type 1 diabetes, especially in type 1 diabetic patients without macrovascular disease. The main objective of this study was to assess monocyte function and associated biomarkers of inflammation in type 1 diabetes without clinical macrovascular disease in comparison with matched control subjects.

RESEARCH DESIGN AND METHODS

For this study, type 1 diabetic patients (with a disease onset <20 years and having been treated with insulin therapy since diagnosis) who had no clinical macrovascular complications, were age ≥15 years, and had a diabetes duration ≥1 year (to avoid the autoimmune component of the disease) were recruited without restriction to sex, ethnicity, or socioeconomic status by the endocrinologists S.G. and N.G. at the University of California, Davis Medical Center through fliers and advertisements in the local newspaper. None of the patients were on glucophage or a thiazolidinedione. Female subjects were studied in the follicular phase of the menstrual cycle. Postmenopausal women on estrogen replacement therapy were excluded from the study because estrogen decreases LDL oxidation, preserves endothelial function, reduces levels of soluble cell adhesion molecules (CAMs), and raises CRP levels (6,7). Exclusion criteria included mean HbA_{1c} (A1C) >10% over the last year; the presence of an inflammatory disorder (e.g., rheumatoid arthritis); an abnormal liver, renal, or thyroid function; malabsorption; current steroid therapy; a current regimen with anti-inflammatory drugs (except aspirin [81 mg/day] as recommended by the American Diabetes Association because this dose is not anti-inflammatory) (8); the use of antioxidant supplements in the previous 3–6 months; current pregnancy; current smoking; an abnormal complete blood

From the [1]Department of Pathology, Laboratory for Atherosclerosis and Metabolic Research, General Clinical Research Center, University of California at Davis Medical Center, Sacramento, California; and the [2]Department of Internal Medicine, Laboratory for Atherosclerosis and Metabolic Research, University of California at Davis Medical Center, Sacramento, California.

Address correspondence and reprint requests to Ishwarlal Jialal, MD, PhD, Director, Laboratory for Atherosclerosis and Metabolic Research, Departments of Pathology and Internal Medicine, UC Davis Medical Center, Sacramento, CA 95817. E-mail: ijialal@ucdavis.edu.

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CAM, cell adhesion molecule; CRP, C-reactive protein; ELISA, enzyme-linked immunosorbent assay; HAEC, human aortic endothelial cell; hsCRP, high-sensitivity CRP; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein 1; sCD40L, soluble CD40 ligand; sICAM, soluble intracellular adhesion molecule; sVCAM, soluble vascular cell adhesion molecule; TNF-α, tumor necrosis factor-α.

count; alcohol consumption >1 oz/day; consumption of N-3 PUFA capsules (N-3 PUFA has a significant anti-inflammatory effect on cytokines and adhesion molecules) (9); and being a chronic high-intensity exerciser (intense exercise can stimulate cytokine release) (10). None of the subjects were on lipid-lowering drugs.

After a history was taken and physical examination was performed in 55 type 1 diabetic patients, patients' baseline and exercise electrocardiograms, ankle-brachial indexes (by Doppler studies), and sonograms of the carotids for stenoses were obtained to rule out macrovascular disease. Any type 1 diabetic patient with macrovascular complications was excluded from the study ($n = 2$). Also, subjects underwent two spot urine tests for the evaluation of microalbuminuria. If the results were in the microalbuminuric range (30–300 mg/g creatinine), a 24-h urine was collected. Subjects with macroalbuminuria (>300 mg/g creatinine) were excluded ($n = 1$). Subjects with background/proliferative retinopathy and pregnant women were not entered into the study. In all, 52 type 1 diabetic patients entered the study. Informed consent was obtained from all participants.

Type 1 diabetic patients without macrovascular disease ($n = 52$) and age- and sex-matched control subjects ($n = 52$/group) were studied. Fasting blood (90 ml) was obtained to assess subjects' monocyte function and other biomarkers of inflammation. A complete blood cell count, plasma lipid and lipoprotein profiles, creatinine levels, liver function tests, and blood glucose, A1C, thyroid-stimulating hormone, and urinary microalbumin levels were assayed at baseline in the Clinical Pathology Laboratory using standard laboratory techniques. Parameters of monocyte function that were assessed included superoxide anion, interleukin (IL)-1β and -6, and tumor necrosis factor-α (TNF-α) release and adhesion to human aortic endothelium. Other biomarkers of inflammation that were assessed include high-sensitivity CRP (hsCRP), plasma soluble cell adhesion molecules (CAMs: soluble vascular CAM [sVCAM], soluble intracellular adhesion molecule [sICAM,] soluble E-selectin [sE-selectin], and soluble P-selectin [sP-selectin]), monocyte chemoattractant protein 1 (MCP-1), soluble CD40 ligand (sCD40L), and nitrotyrosine.

sICAM-1, sVCAM-1, sE-selectin, and sP-selectin were measured by enzyme-linked immunosorbent assay (ELISA) using reagents from R&D Biosystems, as previously reported (3). Plasma hsCRP levels were measured by an ultrasensitive assay (4). sCD40L levels were measured in plasma by a sandwich ELISA. Plasma nitrotyrosine levels were measured by ELISA using reagents from Oxis. The inter- and intra-assay coefficient of variation of all these ELISA assays was <10%.

Monocyte isolation. Mononuclear cells were isolated from fasting heparinized blood (90 ml) by Ficoll-Hypaque gradient (3). Monocytes were isolated by magnetic cell sorting using the depletion technique (Miltenyi Biotech). Using this technique in our laboratory, we have shown that at least 88% of cells are positive for CD14 by flow cytometry. Isolated monocytes were activated using lipopolysaccharide (LPS; 10 μg/ml for O_2^- measurements and 1 μg/ml for cytokine and chemokine release, as obtained from our preliminary studies), and the following functions were studied: release of O_2^-, IL-1β and -6, and TNF-α and adhesion to human aortic endothelial cells (HAECs). Study subjects were assessed in pairs (i.e., one type 1 diabetic subject and the respective control subject).

Other monocyte assays. O_2^- generation in resting and LPS-activated monocytes were measured as the superoxide dismutase–inhibitable reduction of acetylated ferricytochrome C, as previously described (3). The release of the cytokines IL-1β and -6 and TNF-α and the chemokine MCP-1 were measured in the supernatants of resting and LPS-activated monocytes after a 24-h incubation at 37°C using a highly sensitive immunoassay, as previously described (3). The adhesion of human monocytes to confluent monolayers of HAECs (obtained from Clonetics) was carried out by a fluorescence method, as previously described (3).

Statistical analyses. Parametric data were analyzed using paired *t* tests, and nonparametric tests (Wilcoxon's signed-rank test) were implemented because of skewed distribution of data in some of the variables. The level of significance was set at $P < 0.05$. Spearman's rank correlation test was performed to examine associations between parameters tested.

RESULTS

There were no significant differences in age, male-to-female ratio, BMI, or systolic/diastolic blood pressure between the type 1 diabetic and control groups (Table 1). Although none of the type 1 diabetic subjects had evidence of diabetic retinopathy, five did have evidence of incipient nephropathy, as evidenced by microalbuminuria. Levels of glucose and A1C were significantly increased in the type 1 diabetic compared with in the control group (Table 1).

TABLE 1
Subject characteristics at baseline

	Control subjects	Type 1 diabetic subjects
n	52	52
Age (years)	25 ± 10	24 ± 11
Male/female	25/27	25/27
BMI (kg/m^2)	25 ± 5	24 ± 4
Systolic blood pressure (mmHg)	107 ± 10	116 ± 10
Diastolic blood pressure (mmHg)	67 ± 9	70 ± 11
Total cholesterol (mg/dl)	183 ± 39	171 ± 34
LDL cholesterol (mg/dl)	119 ± 34	106 ± 28
HDL cholesterol (mg/dl)	48 ± 10	52 ± 13
Total triglycerides (mg/dl)	80 ± 49	61 ± 37
A1C (%)	5.2 ± 0.2	7.7 ± 1.2*
Glucose (mg/dl)	85 ± 9	169 ± 74*

Data are means ± SD. *$P < 0.001$ vs. control subjects.

There were no significant differences in the lipid profile between the two groups (Table 1).

Among biomarkers of inflammation, which were measured in blood from fasted subjects, hsCRP levels were significantly increased in plasma of type 1 diabetic subjects compared with that of control subjects (42% increase; $P < 0.001$) (Fig. 1*A*). sCD40L levels were significantly increased in type 1 diabetic compared with in control subjects (Fig. 1*B*). There were no significant differences in MCP-1 levels in serum between type 1 diabetic and control subjects (62 ± 39 vs. 55 ± 43 ng/ml). sICAM, sE-selectin, and sP-selectin levels were significantly increased in type 1 diabetic compared with in control subjects, whereas sVCAM levels were not significantly different between the two groups (Fig. 2*A* and *B*).

Plasma nitrotyrosine levels were significantly increased in type 1 diabetic compared with in control subjects (Fig. 3*A*). Furthermore, monocytes from type 1 diabetic subjects secreted significantly increased levels of O_2^- compared with those from matched control subjects, both in the resting state and after activation with LPS (37 and 26% increase, respectively, vs. control subjects; $P < 0.05$) (Fig. 3*B*).

Monocytic release of IL-6 was significantly increased in type 1 diabetic compared with in control subjects in both the resting and LPS-activated state ($P < 0.05$) (Fig. 3*C*). Levels of IL-1β were significantly increased only from LPS-activated monocytes from type 1 diabetic compared with control subjects (Table 2). Although levels of TNF-α were increased in monocytes from type 1 diabetic subjects compared with in those from control subjects, the increase was not statistically significant (Table 2). However, there were no differences in the adhesion of monocytes from type 1 diabetic subjects to HAECs when compared with monocytes from control subjects (Table 2). Monocyte CD11b levels were evaluated in a subgroup of patients and were not significantly different in type 1 diabetic compared with in control subjects (138 ± 49 vs. 133 ± 43 mfi; $n = 14$; $P = 0.56$). In the type 1 diabetic group, five patients had microalbuminuria; after we excluded these five individuals from the analysis, comparing the remaining 47 type 1 diabetic subjects with the control subjects did not change any of the findings reported above.

There were no significant correlations among levels of CRP, CAMs, or parameters of monocyte function with BMI

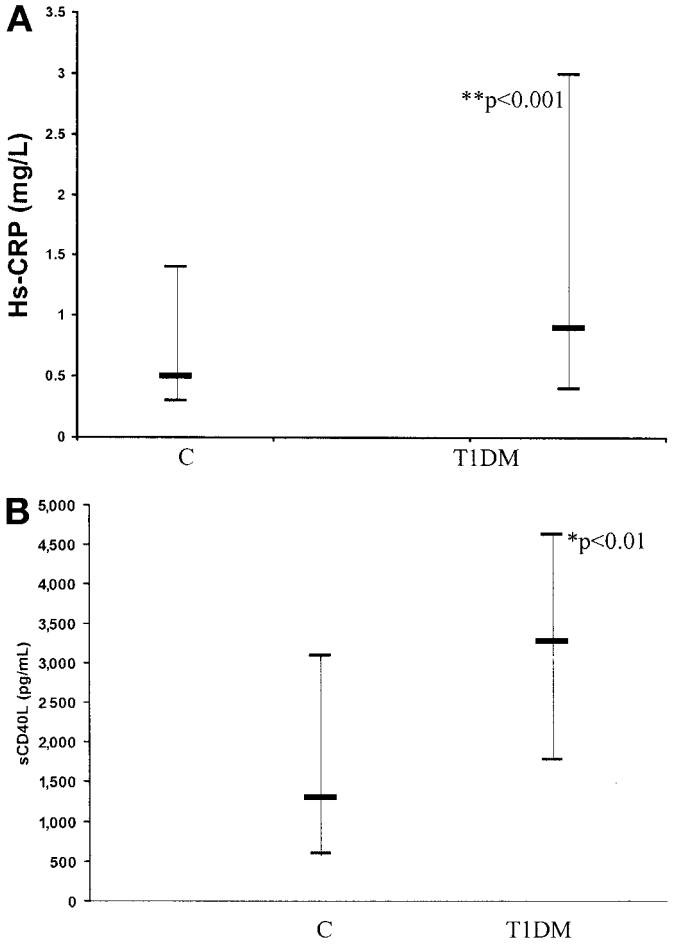

FIG. 1. hsCRP and sCD40L levels in control (C), and type 1 diabetic (T1DM) patients. Levels of plasma hsCRP (*A*) and sCD40L (*B*) were assayed in type 1 diabetic patients (n = 52) and matched control subjects (n = 52) as described in RESEARCH DESIGN AND METHODS. Data are medians and interquartile ranges. **P < 0.001 and *P < 0.01 vs. control subjects.

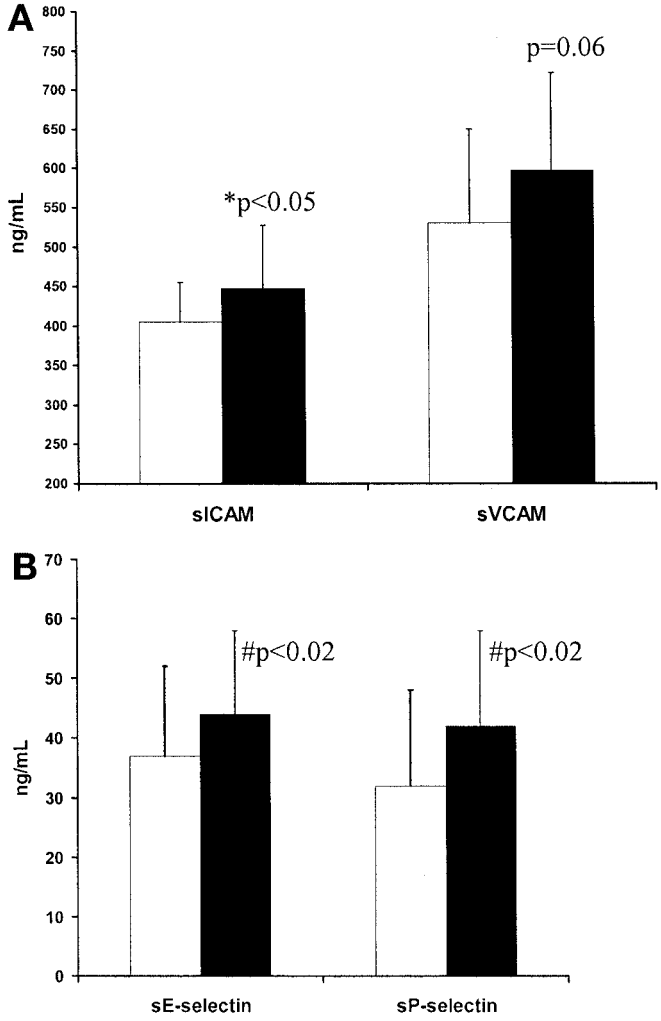

FIG. 2. Levels of sICAM, sVCAM, sE-selectin, and sP-selectin in control (□) and type 1 diabetic (■) subjects. Levels of plasma sICAM and sVCAM (*A*) and sE-selectin and sP-selectin (*B*) were assayed in type 1 diabetic patients (n = 52) and matched control subjects (n = 52) as described in RESEARCH DESIGN AND METHODS. Data are means ± SD. #P < 0.02 and *P < 0.05 vs. control subjects.

or the level of glycemic control (A1C or plasma glucose) in this study.

DISCUSSION

Inflammation and oxidative stress are pivotal in atherosclerosis (11), and type 1 diabetes is associated with increased cardiovascular morbidity and mortality (1). However, there are few data examining biomarkers of oxidative stress, inflammation, and monocyte function in patients with type 1 diabetes who do not have clinical macrovascular disease compared with age-, sex-, BMI-, and ethnicity-matched control subjects. The present study was a comprehensive report in type 1 diabetic subjects of increased oxidative stress, as evidenced by increased nitrotyrosine levels and monocyte superoxide anion, and increased inflammation, as evidenced by increased CRP, CAM, sCD40L, and monocyte IL-6 levels, compared with that seen in control subjects. This was the first study to report increased monocyte proatherogenic activity in type 1 diabetic patients without macrovascular complications compared with control subjects.

Schalkwijk et al. (12) reported elevated CRP levels in type 1 diabetic patients without macrovascular disease compared with control subjects. However, no evidence for macrovascular disease was assessed clinically. In a later study, Schwalkwijk's group showed that CRP was higher in type 1 diabetic patients with microalbuminuria (13). Although Ciarla et al. (14) and Gomes et al. (15) confirmed that CRP levels are increased in type 1 diabetes, Myrup et

TABLE 2
Monocyte IL-1β and TNF-α levels and monocyte endothelial cell adhesion in type 1 diabetic and healthy control subjects

	Control subjects	Type 1 diabetic subjects
n	52	52
Monocyte IL-1β (μg/mg protein)		
Resting	2.3 ± 0.6	2.8 ± 0.7
Activated	4.9 ± 1.6	8.3 ± 1.7*
Monocyte TNF-α (μg/mg protein)		
Resting	0.81 ± 0.24	0.85 ± 0.53
Activated	1.27 ± 0.86	1.89 ± 1.00
Monocyte endothelial cell adhesion (% bound)		
Resting	20.2 ± 14.8	20.6 ± 10.9
Activated	24.6 ± 16.1	25.4 ± 10.1

Data are means ± SD. *P < 0.05 vs. control subjects.

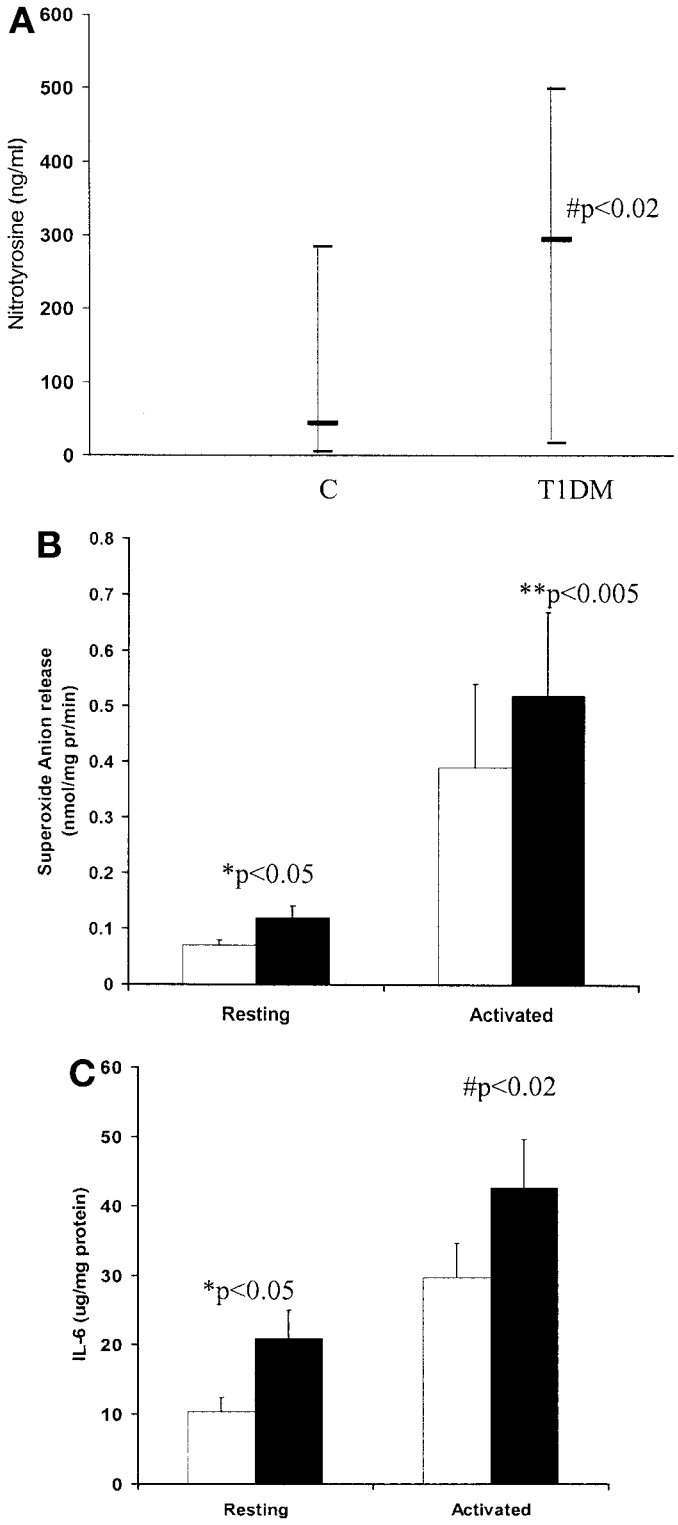

FIG. 3. Levels of plasma nitrotyrosine, monocyte superoxide anion, and monocyte IL-6 release in control (C) and type 1 diabetic (T1DM) subjects. Levels of plasma nitrotyrosine (*A*), monocyte superoxide anion (3*B*), and monocyte IL-6 (*C*) were assayed in type 1 diabetic patients (■; $n = 52$) and matched control subjects (□; $n = 52$) as described in RESEARCH DESIGN AND METHODS. Data are medians and interquartile ranges for nitrotyrosine and means ± SD for monocyte superoxide and IL-6 release.$P < 0.005$, #$P < 0.02$, and *$P < 0.05$ vs. control subjects.**

al. (16) failed to show that CRP levels were increased in type 1 diabetes. In the EURODIAB study, levels of CRP, plasma IL-6, TNF-α, VCAM, and E-selectin were significantly higher in type 1 diabetic subjects with macrovascular complications versus those without (17). Hayaishi-Okano et al. (18) showed that CRP levels were higher in type 1 diabetic subjects compared with in control subjects and correlated this with intima-media thickness. Also, Targher et al. (19) recently reported increased CRP levels in patients with type 1 diabetes with no complications. We have shown previously that type 2 diabetic patients with and without macrovascular complications have increased levels of hsCRP compared with control subjects (4). In the present study, we reported increased levels of CRP in North American type 1 diabetic patients without macrovascular disease, as assessed by carotid sonogram and ankle brachial indexes.

Studies have recently supported the role of CD40-to-CD40L interactions in atherosclerosis (20). Furthermore, sCD40L levels, a marker of increased plaque burden, appear to be significantly increased in type 1 diabetic compared with in control subjects (21,22). Yngen et al. (23) have demonstrated that sCD40L levels are increased in type 1 diabetic patients with microangiopathy. In the present study, we confirmed that type 1 diabetic patients without macrovascular disease have increased levels of sCD40L compared with matched control subjects.

Diabetic patients are prone to increased oxidant stress. A decrease in the total free radical–trapping capacity of serum has been reported in type 1 diabetes as well as the increased presence of thiobarbituric acid reactive substances and increased serum superoxide levels (24–26). In addition, a marked increase in DNA and protein oxidation has been observed in type 1 diabetes (27,28). Also, nitrotyrosine, a marker of protein oxidation, has been observed to be increased in type 2 diabetic compared with in control subjects (29). In this study, we reported increased plasma nitrotyrosine levels in type 1 diabetes and also showed that monocytes from type 1 diabetic patients exhibit increased superoxide anion levels compared with those from matched control subjects, thus confirming earlier observations of increased oxidative stress in type 1 diabetes. Furthermore, we previously observed increased monocyte superoxide in type 2 diabetic subjects with and without macrovascular complications compared with control subjects and demonstrated in vitro that hyperglycemia results in increased activation of protein kinase C-α and p47phox, leading to increased superoxide anion generation from monocytes (30). It is possible that the increased oxidative stress in type 1 diabetes is mediated via the mitochondrial electron transport chain (uncoupling protein 1), activation of phagocyte NADPH oxidase, or glycoxidation via interactions with advanced glycation end product receptors; these mechanisms will be explored in future studies.

With regard to monocyte cytokines, studies in type 1 diabetic patients with a diabetes duration >1 year have not yielded consistent results. The most consistent data to date involve increased levels of serum IL-6 in type 1 diabetes; however, monocyte IL-6 levels had not been studied (31–35). Here we demonstrated that monocytes from type 1 diabetic patients secrete increased levels of IL-6, the predominant driver of CRP release in the liver. We previously showed that CRP production in human endothelial cells is augmented by macrophage-conditioned media via IL-1 and -6 (34). Ohno et al. (31) reported

decreased secretion of 1L-1 and -6 but not TNF-α in peripheral blood monocytes from 16 type 1 diabetic Japanese patients. Kulseng et al. (32) reported increased mononuclear cell TNF-α secretion in type 1 diabetes. Also, Hussain et al. (33) reported increased serum TNF-α levels in patients with long-standing type 1 diabetes but not serum IL-1β levels; however they failed to use a sensitive assay for these cytokines (they did not detect any IL-1β in any samples) and did not study monocyte cytokine release. Jain et al. (35) recently showed that hyperketonemic type 1 diabetic subjects had increased levels of plasma IL-6. It should be emphasized that in all these previous studies examining cytokine release from type 1 diabetes, the sample size has been relatively small ($n \leq 20$). There are few data on the release of these proatherogenic cytokines from monocytes in type 1 diabetic patients without macrovascular complications. In our study, we also observed that monocyte IL-1β levels after activation of monocytes were significantly increased in type 1 diabetic compared with in control subjects.

An early event in atherogenesis is the binding of monocytes to the endothelium and their entry into the intima (36). There have been at least two reports of increased monocyte adhesion in type 1 diabetic patients. Setiadi et al. (37) reported increased adhesion of monocytes to fibronectin in type 1 diabetic patients with vascular complications compared with in control subjects. All patients had retinopathy and some had nephropathy and macrovascular disease. Kunt et al. (38) demonstrated increased monocyte-endothelial adhesion in normolipidemic type 1 diabetic patients compared with in control subjects with a concomitant elevation in CD11b expression. No mention of vascular complications was made in that study. However, another study did not report increased monocyte-endothelial adhesion in normolipidemic type 1 diabetic patients (39); some of the patients in that study did have vascular complications. Thus, although two of the three studies to date have reported increased monocyte adhesion in type 1 diabetes, it needs to be emphasized that all three studies were small ($n < 20$). In this study, we failed to observed increased adhesion of monocytes to endothelium from type 1 diabetic subjects without macrovascular complications, possibly due to no increase in monocyte CD11b.

Increasing evidence supports the role of plasma levels of the CAMs (sICAM-1, sVCAM-1, and E-selectin) as emerging biomarkers of atherosclerosis (40). In type 1 diabetic patients, different laboratories have demonstrated different results (41–44). Fasching et al. (41) reported increased levels of sICAM-1 and sVCAM-1 but not sE-selectin in type 1 diabetic patients and noted a significant association between levels of sVCAM-1 and microvascular complications. Cominacini et al. (42) report elevated levels of only sE-selectin and no change in sVCAM and sICAM in type 1 diabetic patients. The results of studies by Roep et al. (43) and Mysliwiec et al. (44) suggest that sICAM-1 levels are significantly elevated in type 1 diabetic patients and, in fact, are increased to a greater extent in preclinical diabetes compared with in overt diabetes. In our study, we also showed that sICAM levels are significantly increased in type 1 diabetes without macrovascular complications. In addition, we reported increased levels of the selectins (sE-selectin and sP-selectin) in type 1 diabetes, which may be markers of endothelial or endothelial/platelet activation. Thus it appears that disturbances of monocytes as well as endothelium in diabetes could lead to increased adhesion. Although we report increased activation of the endothelium, as evidenced by increased sICAM and sE-selectin levels, the in vitro monocyte adhesion assay failed to reveal any significant differences on monocyte-endothelial cell adhesion; this could have been due to the lack of an increase in monocyte counterreceptors (CD11b) in type 1 diabetic patients compared with in matched control subjects.

It appears that there are several similarities in the inflammatory status in type 1 and type 2 diabetic patients, as evidenced by these patients' increased CRP and CAM levels and increased monocyte superoxide anion and IL-6 release. However, type 2 diabetic patients also exhibit increased monocyte IL-1β release, increased VCAM and IL-8 levels (45), and increased monocyte–endothelial cell adhesion and lower levels of adiponectin compared with control subjects. Although prospective studies are needed to establish which of these biomarkers are the best predictors of vascular complications, Schram et al. (17), in reporting the results of their cross-sectional analyses of the EURODIAB study, showed that a combined inflammatory Z-score (CRP, TNF, and IL-6) was associated with retinopathy, albuminuria, and cardiovascular disease.

In conclusion, type 1 diabetes is associated with increased oxidative stress (nitrotyrosine and monocyte superoxide) as well as a proinflammatory state as evidenced by increased levels of hsCRP, monocyte IL-6, sICAM, sE-selectin, sP-selectin, and sCD40L. Therapies to modulate inflammation in type 1 diabetes may be beneficial in forestalling diabetic vasculopathies.

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