

# Evidence of Increased Inflammation and Microcirculatory Abnormalities in Patients With Type 1 Diabetes and Their Role in Microvascular Complications

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**OBJECTIVE**—Type 1 diabetes is associated with increased microvascular complications and inflammation. The monocyte-macrophage is a pivotal cell in atherogenesis. There are scanty data on noninvasive measures of microvascular abnormalities and inflammation in type 1 diabetic subjects with microvascular complications. Thus, we examined systemic and cellular biomarkers of inflammation in type 1 diabetic patients with microvascular complications (T1DM-MV patients) and type 1 diabetic patients without microvascular complications (T1DM patients) compared with matched control subjects and determined the microcirculatory abnormalities in the T1DM and T1DM-MV patients using computer-assisted intravital microscopy (CAIM).

**RESEARCH DESIGN AND METHODS**—Fasting blood, 24-h urine, and CAIM measurements were obtained from the T1DM and T1DM-MV patients and matched control subjects. C-reactive protein, E-selectin, nitrotyrosine, monocyte superoxide, and cytokines were elevated in the T1DM and T1DM-MV patients compared with control subjects ( $P < 0.01$ ).

**RESULTS**—Severity index, as assessed by CAIM, was significantly increased in the T1DM and T1DM-MV patients compared with the control subjects ( $P < 0.001$ ). There was a significant increase in C-reactive protein, nitrotyrosine, vascular cell adhesion molecule and monocyte superoxide anion release, and interleukin-1 release in T1DM-MV compared with T1DM patients ( $P < 0.05$ ). T1DM-MV patients had significantly increased CAIM severity index and microalbumin-to-creatinine ratio compared with T1DM patients ( $P < 0.05$ ). Furthermore, pp38MAPK, pp65, and pERK activity were significantly increased in monocytes from the T1DM and T1DM-MV patients compared with those from the controls subjects, and pp38MAPK and pp65 activity were significantly increased in the T1DM-MV compared with the T1DM patients ( $P < 0.01$ ).

**CONCLUSIONS**—T1DM-MV patients have increased inflammation compared with T1DM patients. CAIM provides an effective biomarker of microvascular complications, since it is signifi-

cantly elevated in T1DM-MV compared with T1DM patients and can be monitored following therapies targeted at improving inflammation and/or microvascular complications of type 1 diabetes. *Diabetes* 56:2790–2796, 2007

**C**oronary 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 and/or retinopathy have a significantly 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 remain to be elucidated. Inflammation is pivotal in atherosclerosis (2). The monocyte-macrophage, a crucial cell in atherogenesis, is readily accessible for study. We and others have previously shown that monocytes from type 2 diabetic patients with and without complications exhibit increased proatherogenic activity compared with matched control subjects (3–5). Recently, we demonstrated that type 1 diabetic subjects exhibit increased inflammation as evidenced by increased plasma C-reactive protein (CRP) levels and increased monocyte pro-atherogenic activity (6). However, there are scanty data on biomarkers of monocyte function and inflammation in type 1 diabetic patients with microvascular complications (T1DM-MV patients) and on noninvasive measures of microvascular abnormalities. Thus, the main objective was to assess monocyte function and associated biomarkers of inflammation in type 1 diabetic patients without and with microvascular complications compared with matched control subjects and to examine microvascular abnormalities using the technique of computer-assisted intravital microscopy (CAIM) (7–9).

## RESEARCH DESIGN AND METHODS

Type 1 diabetic patients (onset <20 years and on insulin therapy since diagnosis) presenting at age  $\geq 15$  years with duration of diabetes  $\geq 1$  year (to avoid the autoimmune component of the disease) were recruited without restriction to sex, race, or socioeconomic status by the endocrinologists S. Griffen, T. Aoki, and N. Glaser at UC Davis Medical Center through fliers and advertisements in the local newspaper. None of the patients were on glucophage and/or the thiazolidinediones. Female subjects were studied in the follicular phase of the menstrual cycle. Postmenopausal women on estrogen replacement therapy were excluded, since estrogen decreases LDL oxidation, preserves endothelial function, reduces levels of soluble cell adhesion molecules, and raises CRP (10,11). Exclusion criteria were as follows: mean A1C over the last year >10%; inflammatory disorders (e.g., rheumatoid arthritis); macrovascular complications such as strokes, myocardial infarction, etc.;

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CAIM, computer-assisted intravital microscopy; CRP, C-reactive protein; hsCRP, high sensitive CRP; IL, interleukin; LPS, lipopolysaccharide; NF, nuclear factor; TNF, tumor necrosis factor; sVCAM, soluble vascular cell adhesion molecule.

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abnormal liver, renal, or thyroid function; malabsorption; steroid therapy; anti-inflammatory drugs except aspirin (81 mg/day), as recommended by the American Diabetes Association, since this dose is not anti-inflammatory (12); use of antioxidant supplements in the past 3–6 months; pregnancy; smoking; abnormal complete blood count and alcohol consumption >1 oz/day; consumption of n-3 polyunsaturated fatty acid capsules (>1 g/day), since n-3 polyunsaturated fatty acids have a significant anti-inflammatory effect on cytokines and adhesion molecules (13); and chronic high-intensity exercisers, since intense exercise can stimulate cytokine release (14). None of the subjects were on lipid-lowering drugs.

Microvascular complications were defined as retinopathy, nephropathy, and neuropathy and determined in type 1 diabetic patients. Presence of retinopathy was diagnosed by Ophthalmology by fundal photography and grading as defined by the Early Treatment Diabetic Retinopathy Study (ETDRS), performed by trained technicians who were blinded; also, fluorescein angiography was performed in some patients with proliferative retinopathy. Average Early Treatment Diabetic Retinopathy Study score in type 1 diabetic patients without macrovascular complications (T1DM patients) was 12 and in T1DM-MV patients was 42. Nephropathy status was determined on consistent results from at least two timed urine specimens and confirmed using 24-h urine. Microalbuminuria was defined as microalbumin-to-creatinine ratio of >30–300  $\mu\text{g}/\text{mg}$  creatinine, and overt nephropathy was defined as albumin excretion rate of >300  $\mu\text{g}/\text{mg}$  creatinine. For assessment of neuropathy, participants were questioned on sensory, motor, and autonomic symptoms such as numbness, hypersensitivity to touch, burning, aching, or stabbing pain in the hands or feet, and the standard neurological examination included evaluation of reflex activity, sensation to light touch (cotton wool), pain (pinprick), vibration (tuning fork), and proprioception. Neuropathy was defined as the presence of two or more of the above symptoms, sensory and/or motor signs, and absent tendon reflexes.

After history and physical examination, baseline electrocardiograms and ankle-brachial indexes by Doppler studies were undertaken on the T1DM patients to rule out macrovascular disease. Informed consent was obtained from the participants.

**Study design.** T1DM patients ( $n = 54$ ), T1DM-MV patients ( $n = 48$ ) matched for age (within 10 years), and ethnicity- and sex-matched control subjects were studied ( $n = 54$ ). Fasting blood (90 ml) was obtained to assess monocyte function and other biomarkers of inflammation.

A complete blood cell count, plasma lipid and lipoprotein profile, creatinine, liver function tests, blood glucose, glycated hemoglobin, thyroid-stimulating hormone, and urinary microalbumin were assayed in the Clinical Pathology Laboratory using standard laboratory techniques.

**Computer-assisted intravitreal microscopy.** Each experimental subject was seated and requested to relax for at least 10 min. Subjects were cautioned not to rub their eyes. If there was any eye irritation, two drops of nonmedicated ophthalmic saline was applied, and excessive saline was blotted by a tissue at the corner of the eye. Subjects again relaxed before videotaping, with the subject's forehead and chin resting on a head chin rest and elbows resting steadily on the bench. The microcirculation of the bulbar conjunctiva was videotaped using CAIM in each experimental subject using a charge-coupled device (CCD) video camera (COHU Model CCD-64153-000) as described previously (7–9). A fiber-optics light source (Fiber-Lite Model 3100) with a Kodak #58 Wratten (anti-red) filter was focused on the peri-limbal vessels of the bulbar conjunctiva for epi-illumination. The perilimbal region of the bulbar conjunctiva was videotaped. Each subject was seated and asked to relax for at least 5 min and then asked to rest his or her head on a chin-forehead restraint securely mounted to a desk. The height of the CAIM system was adjusted to align horizontally with the peri-limbal region of the eye. Once in focus, the conjunctival vessels appear as sharp black lines and tubes on-screen. A 15-min videotape sequence was made of each experimental subject in at least five different fields. The videotapes on the conjunctival microcirculation in all experimental subjects were coded and blindly analyzed to maintain objectivity during data analysis.

All coded video sequences were studied in their entirety to identify morphometric microvascular abnormalities in the conjunctival microcirculation, with the identity of the patients and their medical records blinded to the investigators. Normally, five or more video sequences (with at least one video sequence from each of the five different fields videotaped) from each patient and control subject were selected. A well-resolved video frame from each video sequence was captured for detailed analysis. A few vessels of interest were selected for detailed analysis using in-house developed imaging software VASCAN and VASVEL and public-domain software SCION (7–9). A severity index was then computed from the 15 different microvascular abnormalities found, some of which include percent presence of abnormal wide vessel diameter, vessel distribution, vessel morphology, vessel (flow) sludging, boxcar flow pattern (same as trickled flow), microaneurysms, blood flow velocity, and whole blood viscosity. The advantages of this technique are that

it is noninvasive and rapid. In addition, it has been previously validated (7–9) and has an intra-assay coefficient of variation of <5% and an interassay coefficient of variation of 12%.

Circulating biomarkers of inflammation that were assessed include high sensitive CRP (hsCRP), plasma soluble cell adhesion molecules (soluble vascular cell adhesion molecule [sVCAM], soluble intercellular adhesion molecule [sICAM], and soluble E-selectin), and nitrotyrosine. Parameters of monocyte function that were assessed include superoxide anion, interleukin (IL)-1 $\beta$ , IL-6, IL-8, IL-10, and tumor necrosis factor (TNF)- $\alpha$  release and adhesion to human aortic endothelium.

**Soluble cell adhesion molecules.** Plasma soluble intercellular adhesion molecule-1, VCAM-1, and E-selectin were measured by enzyme-linked immunosorbent assay using reagents from R&D Biosystems as reported previously (3).

**CRP.** Plasma hsCRP levels were measured by an ultra-sensitive assay (4).

**Nitrotyrosine.** Plasma nitrotyrosine levels were measured by enzyme-linked immunosorbent assay using reagents from Oxis. The inter- and intra-assay coefficient of variation of all these enzyme-linked immunosorbent 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 CD14 positive by flow cytometry. Isolated monocytes were activated using lipopolysaccharide (LPS) (10  $\mu\text{g}/\text{ml}$  for  $\text{O}_2^-$  measurements and 1  $\mu\text{g}/\text{ml}$  for cytokine and chemokine release, as obtained from our preliminary studies) and the following functions were studied:  $\text{O}_2^-$  release, release of cytokines, and adhesion to human aortic endothelial cells.

**Superoxide anion.**  $\text{O}_2^-$  generation in resting and LPS-activated monocytes were measured as the superoxide dismutase-inhibitable reduction of acetylated ferricytochrome C as described previously (3).

**Cytokines and chemokines.** The release of the cytokines IL-1 $\beta$ , IL-6, IL-10, and TNF- $\alpha$  and the chemokine IL-8 were measured in the supernatants of resting and LPS-activated monocytes after a 24-h incubation at 37°C using a Becton Dickinson Fluorescence-Activated Cell Scan Array (3).

**Monocyte adhesion.** Adhesion of human monocytes to confluent monolayers of human aortic endothelial cells (obtained from Clonetics) were carried out by a fluorescence method as described previously (3).

**Cell signaling studies.** Monocyte lysates and nuclear extracts were prepared as described previously (15), and phosphorylated and total p38MAPK, ERK, and JNK activity in the lysates and nuclear factor (NF)- $\kappa\text{B}$  p65 activity in the nuclear extracts were assessed using reagents from Bio-Rad using the Bioplex multiplex phosphoprotein detection assays following the manufacturer's instructions. The intra-assay coefficients of variations of the assays were <14%. Results were confirmed by Western blotting using specific antibodies to the targeted phosphoproteins.

**Statistical analysis.** After one-way ANOVA, parametric data were analyzed using paired  $t$  tests, and nonparametric tests (Wilcoxon's signed rank) 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/Pearson correlation was performed to examine associations between parameters tested.

## RESULTS

Baseline subject characteristics are provided in Table 1. While T1DM-MV patients were significantly older than T1DM patients and control subjects, there were no significant differences in BMI, systolic/diastolic pressure, duration of diabetes, lipid profile, fasting glucose, and A1C among the T1DM and T1DM-MV patients (Table 1). Furthermore, 30% of T1DM-MV patients had evidence of diabetic retinopathy, 66% had evidence of incipient nephropathy (as evidenced by microalbuminuria), and 21% had diabetic neuropathy. As expected, urine microalbumin-to-creatinine ratio was significantly elevated in T1DM-MV compared with T1DM patients and control subjects ( $P < 0.001$ ). Furthermore, the severity index as assessed by CAIM was elevated in both T1DM and T1DM-MV patients compared with control subjects, with the increase being significantly higher in T1DM-MV compared with T1DM patients alone ( $P < 0.01$ ) (Fig. 1). In addition, among the microvascular abnormalities, there was significantly greater vessel sludging and abnormal

TABLE 1  
Baseline subject characteristics

	Control subjects	T1DM patients	T1DM-MV patients
<i>n</i>	54	54	48
Age (years)	28.9 ± 12.3	26.2 ± 11.3	36.4 ± 15.1*
BMI (kg/m <sup>2</sup> )	26.4 ± 5.9	24.8 ± 3.6	26.9 ± 5.5
Systolic blood pressure (mmHg)	111.6 ± 10.9	110.9 ± 13.8	115.1 ± 11.8
Diastolic blood pressure (mmHg)	70.1 ± 12	70 ± 16.2	72.7 ± 9.4
Duration of diabetes (years)	—	14 (6–20)	20 (14–30)
Glucose (mg/dl)	73.5 ± 16.7	172.9 ± 101.6*	161.1 ± 81.3*
Total cholesterol (mg/dl)	181.1 ± 37.3	176.4 ± 30.9	187.2 ± 39.1
HDL cholesterol (mg/dl)	47.5 ± 13.4	52.3 ± 13.2	54.4 ± 20.5
LDL cholesterol (mg/dl)	113.9 ± 32.1	109.8 ± 21.7	112.8 ± 35.4
Triglyceride (mg/dl)	79 (60–108)	69 (60–101)	66 (61–89)
A1C (%)	5.4 ± 1.39	8.6 ± 1.8*	8.8 ± 1.9*
Urine microalbumin-to-creatinine ratio	5.6 ± 5.19	5.9 ± 3.7	80.9 ± 147.2*†
CAIM severity index	2.3 ± 1.35	5.6 ± 1.1*	6.6 ± 1.8*†
Retinopathy %	—	—	30
Nephropathy %	—	—	66
Neuropathy %	—	—	12

Data are means ± SD or median (interquartile range) unless otherwise indicated. \**P* < 0.05 compared with control subjects, and †*P* < 0.05 compared with T1DM patients by one-way ANOVA followed by Bonferroni's post-test.

blood flow pattern (box-car phenomenon), and blood flow velocity was increased in T1DM-MV compared with T1DM patients, indicative of a more pronounced microvascular abnormality in these patients.

Among the circulating biomarkers of inflammation, age-adjusted hsCRP levels were significantly increased in plasma of T1DM and T1DM-MV patients compared with control subjects and also significantly increased in T1DM-MV compared with T1DM patients (35% increase, *P* < 0.01) (Table 2). Also E-selectin levels were significantly increased in both T1DM and T1DM-MV patients compared with control subjects, with no significant differences between the two type 1 diabetic groups (Table 2). sVCAM levels were significantly higher in T1DM-MV compared with T1DM patients and control subjects, and plasma nitrotyrosine levels were significantly increased in both T1DM and T1DM-MV patients compared with control subjects, and the increase in T1DM-MV patients was significantly higher than in T1DM patients (Fig. 2).

With regard to cellular biomarkers of inflammation, i.e., monocyte pro-atherogenic activity, in the resting state,

T1DM and T1DM-MV monocytes secreted significantly increased O<sub>2</sub><sup>-</sup> compared with those of control subjects (data not shown). After LPS activation, T1DM-MV monocytes secreted significantly increased levels of O<sub>2</sub><sup>-</sup> compared with those of T1DM patients and control subjects (Table 3, Fig. 3A). Monocytic release of IL-1, IL-6, and TNF-α were significantly increased in T1DM and T1DM-MV patients compared with control subjects in both the resting (data not shown) and LPS-activated state (Table 3). In addition, monocyte release of IL-1β was significantly increased in T1DM-MV compared with T1DM patients and control subjects (Fig. 3B). Monocyte adhesion to endothelium was also significantly increased in both T1DM and T1DM-MV patients compared with control subjects, with no significant differences between the two diabetic groups (Table 3).

To gain mechanistic insights into differences observed between biomarkers of inflammation in T1DM patients, we examined phosphorylation of the MAPK, p38MAPK, pERK, and pJNK activities in the lysates of monocytes and NF-κB activity in nuclear extracts of monocytes from control subjects, T1DM patients, and T1DM-MV patients. pERK/ERK ratio, pp38MAPK/p38MAPK, and pp65/total p65 ratio were significantly increased in both T1DM and T1DM-MV patients compared with control subjects, with no significant differences in JNK activity. Also, pp65 and pp38MAPK activity were significantly increased in T1DM-MV compared with T1DM patients (Fig. 4).

**Correlation.** There was a significant positive association between severity index as assessed by CAIM and IL-1β levels (*r* = 0.35, *P* = 0.03). Furthermore, increased pp38MAPK and pp65 activity in T1DM-MV patients was significantly associated with increased IL-1β levels (*r* = 0.36, *P* = 0.04, and *r* = 0.43, *P* = 0.03, respectively).

DISCUSSION

Inflammation and oxidative stress are pivotal in atherosclerosis (16). Type 1 diabetes is associated with increased cardiovascular morbidity and mortality (1). We have previously reported increased hsCRP and monocyte pro-atherogenic activity in T1DM-MV patients compared with control subjects. Whereas macrovascular complications

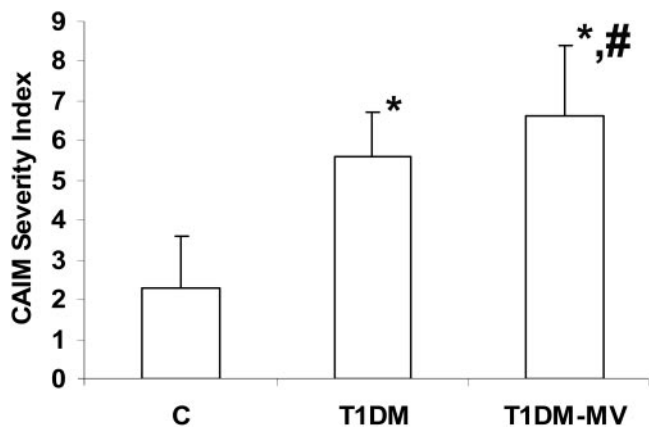


FIG. 1. Increased microvascular abnormalities in T1DM-MV compared with T1DM patients and control subjects (C). Severity index was computed from CAIM measurements of the bulbar conjunctiva from control subjects and T1DM and T1DM-MV patients, as described in RESEARCH DESIGN AND METHODS. \**P* < 0.001 by one-way ANOVA compared with control subjects, and #*P* < 0.05 compared with T1DM patients.

TABLE 2  
Plasma biomarkers of oxidative stress and inflammation

	Control subjects	T1DM patients	T1DM-MV patients
<i>n</i>	54	54	48
hsCRP (mg/l)	0.95	1.7*	2.6*†
sVCAM (ng/ml)	106.6 ± 16.8	122 ± 24.7	128 ± 34.6*†
sICAM (ng/ml)	25 ± 10.1	25.3 ± 7.3	29.3 ± 9.6*
sCD40L (ng/ml)	0.46 (0.13–0.63)	0.51 (0.31–0.78)	0.57 (0.26–0.54)
E-selectin (ng/ml)	10.2 ± 3.8	13.5 ± 5.1*	14.5 ± 3.5*
Nitrotyrosine (ng/ml)	119 ± 78	291.8 ± 125.8*	505.1 ± 232.6*†

Data are means ± SD or median (interquartile range) unless otherwise indicated. \**P* < 0.05 compared with control subjects, and †*P* < 0.05 compared with T1DM patients by one-way ANOVA followed by Bonferroni's post-test.

are the major cause of morbidity and mortality in type 2 diabetes, in type 1 diabetes, the risk associated with microvascular complications is enormous (1). However, there is a paucity of data examining both biomarkers of oxidative stress, inflammation, and monocyte function in patients with type 1 diabetes with and without microvascular complications compared with age-, sex-, BMI-, and race-matched control subjects and testing a noninvasive marker, CAIM. In the present study, we provide novel evidence that T1DM-MV patients have significantly higher nitrotyrosine levels, CRP, and increased monocyte superoxide anion and IL-1β compared with T1DM patients and control subjects. Furthermore, we document for the first time that CAIM is an effective noninvasive biomarker of complications in type 1 diabetes.

Schalkwijk et al. (17) reported elevated CRP levels in T1DM patients compared with control subjects. Furthermore, in accordance with the present study, they showed that CRP was higher in type 1 diabetic patients with microalbuminuria (18). In the EURODIAB study, levels of CRP, plasma IL-6, TNF, and VCAM and E-selectin were significantly higher in type 1 diabetic subjects with versus without macrovascular complications (19). Also, Targher et al. (20) reported increased CRP levels in T1DM patients, but failed to study T1DM-MV patients. The Finnish Diabetic Nephropathy study also revealed increased CRP levels in type 1 diabetic patients with nephropathy (21). In the present study, we report increased levels of CRP in North American T1DM-MV patients compared with both T1DM patients and control subjects.

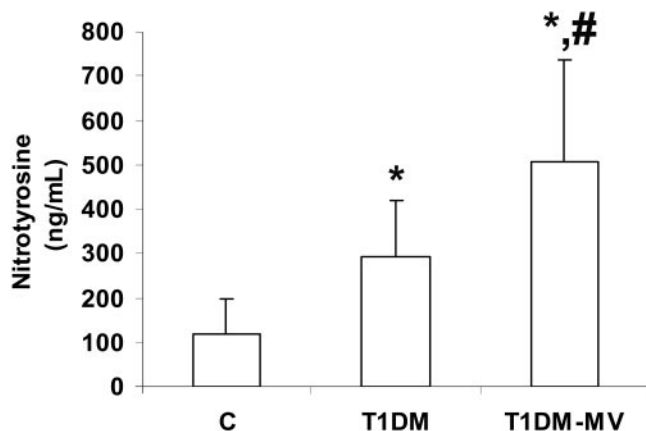


FIG. 2. Nitrotyrosine levels in control subjects (C) and T1DM and T1DM-MV patients. Levels of plasma nitrotyrosine were assessed in T1DM-MV and T1DM patients and matched control subjects as described in RESEARCH DESIGN AND METHODS. \**P* < 0.001 by one-way ANOVA compared with control subjects, and #*P* < 0.05 compared with T1DM patients.

With regard to monocyte cytokines, studies in type 1 diabetic patients with duration of diabetes >1 year have not yielded consistent results. Kulseng et al. (22) have reported increased mononuclear cell TNF secretion in T1DM patients. Jain et al. (23) showed that hyperketone-mic 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 T1DM patients, the sample size has been relatively small (*n* ≤ 20). We also previously reported that monocyte IL-1β levels following activation of monocytes were significantly increased in T1DM patients compared with control subjects (6). In this study, we go further to demonstrate that there is accentuation of the pro-inflammatory state in T1DM-MV patients, as evidenced by a significant increase in IL-1β levels compared with T1DM patients and matched control subjects. This is especially important since increased IL-1β

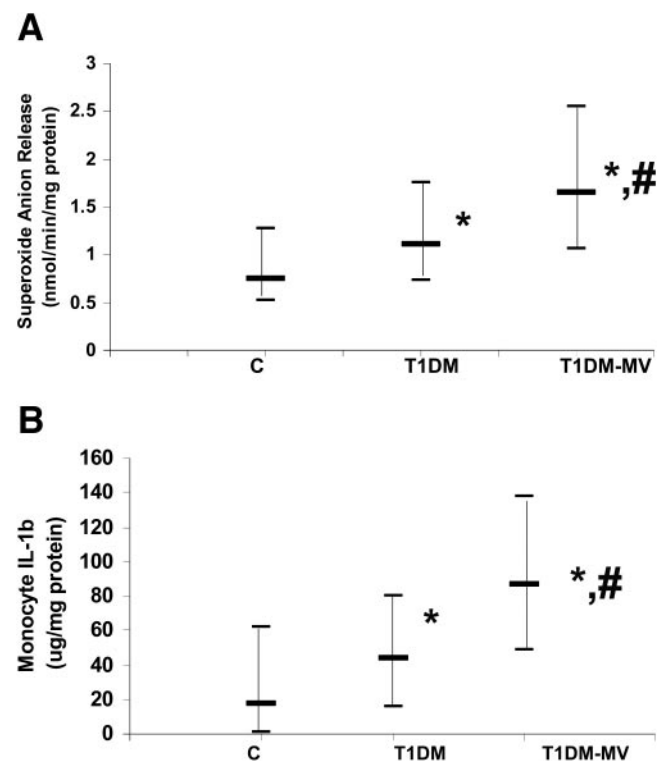


FIG. 3. Monocyte proatherogenic activity in T1DM-MV compared with T1DM patients and control subjects (C). Monocytes were isolated from fasting heparinized blood and superoxide anion release (A). IL-1β release (B) was measured in LPS-activated monocytes from control subjects and T1DM and T1DM-MV patients, as described in RESEARCH DESIGN AND METHODS. \**P* < 0.01 by one-way ANOVA compared with control subjects, and #*P* < 0.05 compared with T1DM patients.

TABLE 3  
Monocyte proatherogenic activity

	Control subjects	T1DM patients	T1DM-MV patients
<i>n</i>	54	54	48
Superoxide anion release (nmol · min <sup>-1</sup> · mg <sup>-1</sup> protein)	0.75 (0.52–1.27)	1.11 (0.74–1.76)*	1.65 (1.1–2.6)*†
Monocyte-endothelial cell adhesion (%)	28 ± 13	41 ± 15*	41 ± 22*
IL-8 (μg/mg protein)	102 (10–271)	183.4 (23–287)*	182.3 (122–227)*
IL-1β (μg/mg protein)	17.7 (1.1–62)	55.3 (15.3–80.2)*	86.4 (49–138)*†
IL-6 (μg/mg protein)	16.8 (1–92)	46.4 (20–84)*	64.3 (53–102)*
IL-10 (μg/mg protein)	503 (27–2,586)	346 (103–1,719)	514 (145–2,775)
TNF-α (μg/mg protein)	1.8 (0.2–4.9)	4.5 (2–13)*	6.2 (3–15)*

Data are means ± SD or median (interquartile range) unless otherwise indicated. \**P* < 0.01 compared with control subjects, and †*P* < 0.05 compared with T1DM patients by one-way ANOVA followed by Bonferroni's post-test.

levels have previously been shown to be involved in the rat model to be associated with increased diabetic retinopathy (24).

Increasing evidence supports the role of plasma levels of cell adhesion molecules, soluble intercellular adhesion molecule-1, sVCAM-1, and E-selectin as emerging biomarkers of atherosclerosis (25). In type 1 diabetic patients, different laboratories demonstrate different results (26–29). We showed previously that soluble intercellular adhesion molecule and soluble E-selectin levels are significantly increased in T1DM patients. In the present study, we further demonstrate pronounced increases in sVCAM in T1DM-MV compared with T1DM patients and control subjects.

T1DM patients are prone to increased oxidant stress. A decrease in total free radical trapping capacity of serum has been reported in T1DM patients as well as increased thiobarbituric acid reactive substances and increased serum superoxide levels (30–32). Additionally, marked increase in DNA and protein oxidation has been observed in T1DM patients (33,34). Also, nitrotyrosine, a marker of protein oxidation, has previously been shown to be increased in T1DM patients compared with control subjects (35). We have previously reported increased plasma nitrotyrosine levels in T1DM patients and also that monocytes from T1DM patients exhibit increased superoxide anion levels compared with those of matched control subjects (6). There is another small report of increased nitroty-

rosine levels in T1DM patients (36); however, those without and with microvascular complications were not studied. In the present study, we provide evidence that T1DM-MV patients have accentuated oxidative stress compared with T1DM patients and control subjects, as evidenced by significantly increased nitrotyrosine levels and increased monocytic superoxide anion release. In addition, we report a significant association between CAIM severity index and nitrotyrosine levels. It is possible that the increased oxidative stress in T1DM-MV patients is mediated via the mitochondrial electron transport chain (uncoupling protein 1), activation of phagocyte NADPH oxidase, or glycooxidation via AGE receptor interactions, and these mechanisms will be explored in future studies.

In an attempt to tease out molecular pathways that could explain the increased oxidative stress and inflammation in T1DM-MV compared with T1DM patients and control subjects, we explored activation of NF-κB as well as MAPK. Only p38MAPK and ERK activity were increased in T1DM patients. While JNK activation appears to be crucial in pancreatic β-cell dysfunction, it does not appear to be activated in monocytes of T1DM patients. Previously, Hofmann et al. (37) showed increased NF-κB mononuclear activity in T1DM patients; however, individuals with complications were not assessed. In addition, p38MAPK activity is increased in diabetic nephropathy. We have previously shown that high glucose activates monocyte IL-1β via upregulation of p38MAPK and NF-κB activity *in vitro* (38). In addition, Aljada et al. (39) have shown that glucose activates mononuclear NF-κB activity. In the present study, increased IL-1β levels in T1DM-MV patients was associated with increased *in vivo* activity of both p38 MAPK and NF-κB. IL-1β has previously been demonstrated to accelerate apoptosis of retinal capillary cells via activation of NF-κB, and the process is exacerbated in high glucose conditions (40). Furthermore, in macrophages isolated from *db/db* mice, Li et al. (41) have shown increased activation of pro-inflammatory cytokines and NF-κB transcriptional activation. Our data lend support to the role of increased NF-κB activation in T1DM-MV patients, resulting in increased IL-1β levels, which in turn could contribute to increased microvascular complications.

Schram et al. (19) reported their cross-sectional analyses of the EURODIAB study and showed that a combined inflammatory Z score (CRP, TNF, and IL-6) was associated with retinopathy, albuminuria, and cardiovascular disease in their type 1 diabetic population. Here we provide novel data that CRP, IL-1β, nitrotyrosine, and monocyte superoxide are significantly increased in T1DM-MV patients

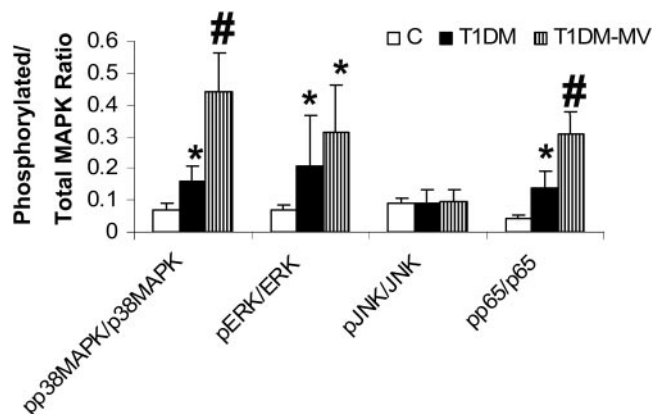


FIG. 4. Activation of MAPK in monocytes of T1DM patients. Monocyte lysates were prepared, and activity of phosphor-to-total p38MAPK, ERK, and JNK were measured in control subjects (C) and T1DM and T1DM-MV patients, as described in RESEARCH DESIGN AND METHODS. Also, nuclear extracts of monocytes from these three groups were prepared, and pp65 activity to total p65 was assessed as described in RESEARCH DESIGN AND METHODS. \**P* < 0.01 by one-way ANOVA compared with control subjects, and #*P* < 0.05 compared with T1DM patients.

compared with control subjects. A noninvasive biomarker of complications, the severity score as assessed by CAIM, is significantly increased in T1DM-MV compared with T1DM patients and control subjects, indicative of significantly increased microvascular abnormalities in this group. Microvascular abnormalities are associated with and causative of the development of end-stage organ complications in diabetes. The easy noninvasive accessibility of the conjunctival vessels and the ability to identify and locate the same vessels repeatedly for longitudinal evaluations emphasize the usefulness of this real-time technology in the evaluation of microvascular abnormalities in T1DM patients. The increased CAIM severity index in T1DM-MV patients demonstrates the utility of this rapid noninvasive marker in assessing microvascular abnormalities. Furthermore, this is the first study to report a strong correlation of CAIM with a biomarker of inflammation, IL-1 $\beta$ , which is significantly elevated in T1DM-MV compared with T1DM patients. In addition, we report a strong association of urinary microalbumin with nitrotyrosine. It is possible that increased systemic and cellular inflammation contribute to increased microvascular complications of type 2 diabetes as well, and this will be examined in future studies. Future studies will also examine the effect of pharmacological intervention (such as with low-dose statins) on oxidative stress and inflammation in T1DM-MV patients and examine the defined biomarkers of IL-1 $\beta$ , nitrotyrosine, CRP, monocyte superoxide anion in conjunction with microalbumin-to-creatinine ratio, and CAIM severity index, in an attempt to reverse/retard progression of complications in type 1 diabetes.

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