Inflammatory Biomarkers and Progression of Diabetic Retinopathy in African Americans With Type 1 Diabetes

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PURPOSE. We examined whether baseline plasma levels of markers of inflammation and endothelial dysfunction are associated with the incidence of diabetic retinopathy (DR) in African Americans with type 1 insulin-dependent diabetes mellitus (T1DM).

METHODS. At baseline and follow-up examinations, detailed ocular examination, structured clinical interview, venous blood specimens, and masked grading of seven standard field retinal photographs were obtained. Baseline plasma levels of 28 inflammatory biomarkers, measured using multiplex bead analysis system, were measured in the participants.

RESULTS. After adjusting for age, glycemic control, and other potential confounders, baseline plasma levels of E-selectin were associated significantly with progression of DR, E-selectin and tumor necrosis factor- α (TNF- α) levels with incidence of proliferative DR (PDR), and soluble intercellular adhesion molecule-1 (sICAM-1) and TNF- α levels with incidence of macular edema (ME).

CONCLUSIONS. In African Americans with T1DM, inflammation and endothelial dysfunction precede the development of DR, thus supporting the notion that inflammation may influence progression/incidence of disease.

Keywords: inflammation, diabetic retinopathy, African Americans, incidence, biomarkers

Occlusion of retinal capillaries and leakage from the retinal vasculature are early clinical features of diabetic retinopathy (DR).^{1,2} While the pathogenesis of such changes remains unclear, there is experimental and clinical evidence to suggest that inflammation may be one of the pathways involved in the development of DR.^{3,4} In animal models of diabetes mellitus (DM), entrapment of leukocytes with loss of adjacent endothelial cells, extravascular macrophage accumulation, and capillary occlusion occur early on following induction of DM, and are associated with retinal VEGF-induced retinal expression of the inflammatory intercellular adhesion molecule-1 (ICAM-1).^{5–9} In diabetic animals, treatment with ICAM-1 antibodies, deletion of the ICAM gene or its leukocyte ligand, CD18, or intravitreal corticosteroids, all significantly decrease leukostasis and retinal vascular leakage.^{7,8,10,11} In addition, systemic administration of nonsteroidal anti-inflammatory drugs that target TNF- α also reduces leukostasis and retinal vascular leakage in diabetic animals, suggesting that TNF- α participates in these processes.¹²

Clinical studies also support the role of inflammation in relation to DR. Compared to nondiabetic persons, patients with either proliferative diabetic retinopathy (PDR) or macular edema (ME) have higher vitreous levels of cytokines, for example IL-6; IL-1 β ; TNF- α ; growth factors, for example VEGF; platelet derived growth factor (PDGF); chemokines, for example stromal derived factor-1 (SDF-1), monocyte chemo-attractant protein-1 (MCP-1), interferon-inducible protein (IP10), and IL-8; and adhesion molecules, for example E-

selectin, soluble ICAM-1 (sICAM-1), and soluble vascular adhesion molecule-1 (sVCAM-1).^{13–26} Similarly, serum levels of inflammatory markers, for example sVCAM-1, sICAM-1, IL-6, TNF- α , E-selectin, Regulated on Activation Normal T Cell Expressed and Secreted (RANTES), SDF-1 α , MCP-1, and/or C-reactive protein (CRP) have been shown, albeit inconsistently, to be higher in DR than in non-DR patients or nondiabetic persons.^{21,26–39} Significantly, intravitreal injection of either steroids or VEGF neutralizing antibodies has now become part of the treatment of diabetic patients with ME.^{40,41} Because of the complex interactions between inflammatory biomarkers, VEGF may not be the only one involved. Interpretation of the clinical data also has been limited by small sample sizes, mixed populations of types 1 and 2 DM, measurement of only either one or a few biomarkers, and/or the use of cross-sectional designs. Two longitudinal studies failed to support this hypothesis.^{35,42} In one multicenter longitudinal study in T1DM, none of the four serum biomarkers examined predicted all of the DR outcomes in a consistent manner.⁴³

Thus, it still is unclear whether inflammation either precedes and participates in the development of DR, and if so, which biomarker(s) may be involved, or accompanies already established DR. Our study investigates the hypothesis that specific plasma markers of inflammation and endothelial dysfunction are associated with the progression of DR, incidence of PDR, and/or incidence of ME using a secondary analysis of longitudinal data gleaned from a large cohort of African Americans with T1DM.

METHODS

Study Population

The original cohort consisted of 725 African Americans with T1DM who participated in the New Jersey 725 study between 1993 and 1998.^{44,45} Patients were identified from among 68,455 African Americans listed in the New Jersey Department of Health computerized Hospital Discharge Data as having a diagnosis of DM. Of those, a review of 13,615 randomly chosen patient charts was conducted in 31 participating hospitals. Patients with a discharge diagnosis of T1DM, acute onset of DM, treated with insulin before 30 years of age, and currently on insulin were included. The current use of insulin was confirmed at the time of first contact with the patient. Excluded were patients diagnosed with an acute onset of DM before age 30 but not currently on insulin, those diagnosed with DM after age 30, and patients with a discharge diagnosis of maturity-onset diabetes of youth.⁴⁶ Of the 875 eligible patients, 725 (82.9%) participated.

Of the 725 patients, 508 (70.1%) underwent a 6-year follow-up examination, 44 (6.1%) could not be located, 34 (4.7%) refused examination, and 139 (19.2%) had died.⁴⁷ At the 6-year follow-up, 25 (4.9%) participants who were no longer receiving insulin were excluded, leaving 483 eligible for analysis. All 483 patients were confirmed to have used insulin continuously since their first visit. This report concerns the 412 (85.3%) of the 483 patients who had baseline plasma samples available for measurement and had at least one follow-up examination (Table 1). The mean ($\pm$ SD) follow-up time was 6.0 ($\pm$ 0.3) years for the 370 patients examined after a 6-year interval, and 8.4 ($\pm$ 1.8) years for the 42 patients who had a second visit 7 to 11 years after baseline. Outcome measures reflect the data from the follow-up visit showing the worst retinopathy status.

Procedures

Patients were examined in the Eye Clinic of University Hospital in Newark, New Jersey. The same procedures were followed at baseline and follow-up visits. Upon arrival, informed written consent was obtained from each patient. Patients underwent a complete eye examination, including dilated retinal examination (done by MSR) and seven standard stereoscopic Diabetic Retinopathy Study (DRS) retinal photographs. Also obtained were height and weight. Using a random zero sphygmomanometer, blood pressure was measured twice in the sitting and standing positions.⁴⁸ The average of the two measurements was used. Each patient underwent a structured clinical interview that included medical (infections; use of statin medications; and past history of coronary disease, stroke, or lower extremity arterial disease [LEAD]) and ophthalmologic histories, and sociodemographic factors and lifestyle variables (i.e., self-reported measures of cigarette smoking, alcohol consumption, and illicit drug use).

Venous blood was drawn for measurement of total glycosylated hemoglobin using high-pressure liquid chromatography (Bio-Rad, Labcorp Laboratory, Hercules, CA). High- and low-density lipoprotein cholesterol (HDL-C and LDL-C), and total cholesterol blood levels were measured using an enzymatic assay and separation spectrophotometry (Genzyme Diagnostics, Cambridge, MA). A 4-hour timed urine collection was obtained for measurement of the albumin excretion rate (AER) and creatinuria, using spectrophotometry (SmithKline Beecham Clinical Laboratory, Philadelphia, PA). A 2 mL venous blood sample, collected by venipuncture in an EDTA-coated Vacutainer tube (BD Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ), was mixed thoroughly, and

plasma was separated by centrifugation and stored frozen at -70°C for future assay.

The research followed the tenets of the Declaration of Helsinki, and The Institutional Review Board of the University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey, approved the study.

Diabetic Retinopathy Grading

Color fundus photographs obtained at both visits were graded for DR severity in a masked fashion by the Wisconsin Fundus Photograph Reading Center in Madison, Wisconsin. The modified Early Treatment Diabetic Retinopathy Study (ETDRS) Airlie House classification of DR was used.^{49,50} DR severity level for a patient was determined using the severity level in the worse eye. ME was evaluated clinically by the PI (MSR) and was considered present only if there was thickening of the retina with or without partial loss of retinal transparency within 1 disc diameter from the center of the macula, and/or focal laser photocoagulation scars in the macular area, and/or a documented history of ME. The presence of ME was confirmed by the Reading Center.⁴⁹

For eyes that could not be graded, because of either opacities of the media, phthisis, or enucleation, review of previous medical records was done by MSR. When a history of either photocoagulation or pars plana vitrectomy for PDR was documented by chart review, the DR level was scored as 85. Eyes that had an ETDRS grading < 61 at the time of examination and had previously had laser photocoagulation or vitrectomy for PDR, documented by chart review, also were classified as grade 85.

Measurements of the Inflammatory Biomarkers

The investigator (JC) who did the measurements of the inflammatory markers was masked to the clinical data. Baseline plasma samples stored at -70°C were analyzed for 28 inflammatory biomarkers selected either after review of the literature or because of their potential role in the pathogenesis of DR. Included were 10 cytokines and soluble receptors (IL-1 α , IL-2 receptor [sIL-2R], IL-6, IL-8, IL-10, IL-12 p40, IL-12p70, soluble CD40 ligand [sCD40], TNF- α , and IFN- γ), three growth factors (granulocyte macrophage colony stimulating factor [GM-CSF], PDGF, and VEGF), 11 chemokines (eotaxin, fractalkine, growth-related oncogene- α [GRO- α], MCP-1, MCP-3, RANTES, macrophage inflammatory protein-1 α [MIP-1 α], MIP-1 β , IP-10, SDF-1, and neutrophil-activating peptide [ENA-78]), and 4 soluble adhesion molecules (E-selectin, sICAM-1, sVCAM-1, and CRP).

The biomarker concentrations were measured using a multiplex bead analysis system (Milliplex X-MAP; Millipore Corp., Billerica, MA). Intra-assay and interassay variations were below 15% and 18%, respectively. Inflammatory marker measurements were done using 25 μL samples. After overnight incubation in 96-well plates, the specific fluorescence corresponding to each set of biomarkers was measured on the Luminex 100 instrument (Luminex, Austin, TX). Quantification was done against 4-parameter logistic regression-generated standard curves using the reference cytokine standards supplied by the kit manufacturer. For statistical analysis, the biomarkers concentrations below the lowest concentration point in the standard curves were given a value of zero.

Definitions

The ETDRS grading in each eye was used to generate an 8-step scale as follows: ETDRS grading < 20 = step 1, 20 = step 2, 35 = step 3, 43 = step 4, 47 = step 5, 53 = step 6, 61 = step 7, and

TABLE 1. Baseline Characteristics of the 412 African Americans With Type 1 Diabetes by Retinopathy Status

Baseline Characteristics	EDTRS Retinopathy Severity Group				ME	
	Mean (SD)				No, N = 360	Yes, N = 36
	0–19, N = 169	20–37, N = 134	43–53, N = 49	≥61 or Laser, N = 46		
Age, y	21.4 (8.9)	28.6 (9.3)	32.3 (8.9)	38.2 (9.9)	26.2 (10.3)	35.4 (10.0)
BMI, kg/m ²	26.3 (8.7)	28.2 (7.6)	26.7 (7.1)	30.6 (11.9)	26.9 (8.0)	33.1 (12.7)
Age at diagnosis of diabetes, y	16.3 (8.0)	17.1 (7.2)	17.3 (7.4)	16.2 (7.0)	16.6 (7.6)	17.4 (7.1)
Duration of diabetes, y	4.9 (5.0)	10.9 (7.3)	14.5 (5.6)	21.4 (8.5)	9.2 (7.9)	17.3 (7.6)
Glycosylated hemoglobin, %	13.5 (4.6)	14.6 (3.9)	14.1 (3.9)	12.2 (4.1)	13.9 (4.3)	12.8 (3.9)
Mean arterial BP, mm Hg	85.6 (12.3)	90.0 (11.3)	96.6 (12.4)	101.4 (14.3)	88.8 (12.4)	102.3 (14.8)
Cholesterol, mg/dL						
Total	197.4 (49.1)	196.9 (51.6)	218.7 (62.5)	200.7 (41.7)	200.6 (52.5)	197.4 (39.0)
HDL	59.8 (21.7)	58.3 (18.4)	60.5 (22.1)	54.6 (19.1)	59.2 (20.5)	55.0 (18.8)
LDL	115.3 (38.2)	114.3 (33.9)	123.4 (47.4)	110.4 (31.0)	116.1 (37.9)	109.3 (32.1)
CRAE	170.2 (15.4)	170.3 (15.3)	168.8 (15.2)	156.1 (18.5)	169.1 (15.8)	165.5 (18.8)
CRVE	251.0 (22.4)	256.6 (23.5)	266.5 (29.7)	255.0 (38.2)	254.2 (23.8)	265.5 (40.8)
%						
Sex, female	53.3	61.9	46.9	69.6	56.6	60.0
SES, low	53.8	53.7	51.0	50.0	52.8	51.4
Smoking						
Never	54.7	56.7	60.4	60.9	58.1	48.6
Past	8.4	13.3	6.2	13.0	10.7	11.4
Current	36.8	30.0	33.3	26.1	31.2	40.0
Alcohol abuse	7.5	11.8	16.7	11.1	10.6	7.7
Statin medication use, yes	0.6	2.2	4.1	6.5	1.6	8.6
ASA use, yes	1.8	1.5	8.2	4.3	2.1	8.6
Heart disease, yes	2.4	4.5	14.3	17.4	4.8	22.9
Stroke, yes	1.2	2.2	4.1	8.7	2.1	5.7
LEAD, yes	1.8	5.2	10.2	10.9	3.7	20.0
Proteinuria*						
None	80.7	62.4	27.7	4.3	64.7	11.8
Micro	13.3	25.6	25.5	34.8	19.4	35.3
Overt	6.0	12.0	46.8	60.9	15.9	52.9

BP, blood pressure; SES, socio-economic status; ASA, aspirin.

* Albumin excretion rate: none, <20 µL/min; microproteinuria, 20 to 200 µL/min; overt proteinuria, >200 µL/min, dialysis, or kidney transplant.

≥61 or laser = step 8. Progression of DR was defined as the difference in steps between baseline and follow-up in the worse eye, and was evaluated for the 398 patients with ETDRS grading < 61 at baseline, had not had laser or vitrectomy for PDR, and who had progression of one or more steps at follow-up.

Incidence of PDR was evaluated for patients who had no PDR in either eye at baseline (ETDRS levels < 61 and no laser photocoagulation or vitrectomy for PDR, *N* = 374) and who had progression in at least one eye at follow-up to ≥61, or had received laser photocoagulation or undergone vitrectomy for PDR (*N* = 79).

Incidence of ME was calculated for patients who had no ME or had not received focal laser photocoagulation for ME in either eye at baseline (*N* = 375), and who either had ME in at least one eye or had received focal laser photocoagulation for ME in either eye since baseline (*N* = 60).

Patient's age was defined as the age at baseline. Mean arterial blood pressure (MAP) was calculated as follows: diastolic blood pressure + 1/3(systolic blood pressure – diastolic blood pressure). Microproteinuria was defined as present if baseline AER was 20 to 200 µg/min, and overt proteinuria if baseline AER was >200 µg/min, or the patient was on dialysis or had received a kidney transplant. Cardiovascular disease (CVD) was considered present if, at baseline,

the patient reported LEAD if the patient had undergone foot or leg amputation for a circulatory problem, or heart disease if the patient had coronary disease or had had a myocardial infarction, or stroke.⁵¹ Baseline CVD was confirmed using standardized criteria by review of hospital records. Smoking was defined as “never,” “past,” or “current.” Alcohol consumption was defined as heavy if the patient reported ever consuming 4 or more alcoholic beverages a day for at least one year. The average diameter of retinal arterioles (central retinal arteriolar equivalent [CRAE]) and venules (central retinal venular equivalent [CRVE]) was measured by the Ocular Epidemiology Reading Center in Madison using computer-assisted grading from digitized images of field 1 of baseline retinal photographs.⁵² An index of recent systemic infection at baseline was computed based on medical history and recent hospital admissions.

Statistical Analysis

Statistical analyses were performed using IBM SPSS (v.20; IBM, Armonk, NY). Because preliminary inspection showed that the distribution of all baseline inflammatory biomarkers was skewed positively, those data were first rank transformed. Pearson correlations were used to describe the association between (ranked) inflammatory markers with either preva-

TABLE 2. Baseline Levels (pg/mL) of Inflammatory Markers in 412 African Americans With Type 1 Diabetes Who Had Baseline and Follow-up Examinations

	Median (Interquartile Range)
Cytokines	
IL-1 α	3.20 (32.10)
sIL-2R	3.20 (23.26)
IL-6	0.62 (3.20)
IL-8	3.00 (4.56)
IL-10	0.01 (3.2)
IL-12p40	3.20 (14.18)
IL-12p70	0.97 (3.07)
sCD40	106.57 (186.53)
TNF- α	4.789 (3.64)
INF- γ	1.75 (3.04)
Growth factors	
GM-CSF	34.17 (30.67)
PDGF	12,960 (12,341.95)
VEGF	48.76 (76.87)
Chemokines	
Eotaxin	38.11 (37.01)
Fractalkine	59.81 (63.30)
GRO α	708.14 (498.78)
MCP-1	193.69 (133.01)
MCP-3	7.73 (17.74)
MIP-1 α	5.76 (8.19)
MIP-1 β	25.64 (24.18)
IP-10	239.68 (194.84)
SDF-1	1,638.32 (1,630.84)
ENA-78	1,275.45 (1,420.51)
RANTES	75,315.97 (63,941.70)
Soluble adhesion molecules	
E-selectin	36.12 (37.62)
sICAM-1	156.83 (89.49)
sVCAM-1	900.41 (374.81)
Acute phase marker CRP	3,848.24 (9,686.96)

lence or incidence of DR. To balance conflicting concerns about multiple comparisons and the conservation of statistical power, only P values < 0.01 were considered significant. Pearson correlations also were used to examine the association between markers, and the infection index and the use of statin medications.

Multiple regression analyses (linear for progression of DR, and logistic for incidence of either PDR or ME) were run forcing the target inflammatory biomarker in the first model, and then adding sex, age, body mass index (BMI), and glycosylated hemoglobin levels in step 2. In steps 3 and greater, the software was allowed to select significant ($P < 0.05$) additional contributions made to progression of DR, incidence of PDR, or incidence of ME from the following baseline characteristics: AER, MAP, LEAD, CVD, CRAE, CRVE, total cholesterol, alcohol consumption, and smoking.

RESULTS

Baseline plasma levels of the 28 inflammatory biomarkers in the 412 patients are shown in Table 2. Associations between baseline plasma biomarkers and known predictors of incident DR are presented in Table 3. There was no significant association between baseline biomarker levels and either the index of infection or use of statins (data not shown).

Progression of DR

Univariate associations between baseline plasma levels of the inflammatory markers and progression of DR are presented in Table 4. Three markers, ENA-78, E-selectin, and sICAM-1, were associated significantly with progression of DR. None of these was associated significantly with the prevalence of DR at baseline, indicating that different inflammatory markers are associated with incidence and prevalence of DR.

When all three markers were entered in a multiple linear regression, only E-selectin and ENA-78 showed independent associations with progression of DR ($P < 0.001$ and $P < 0.001$, respectively). E-selectin levels were associated strongly with levels of sICAM-1 (and, to a lesser extent, with levels of VEGF and MIP-1 β). Levels of ENA-78 were associated significantly with plasma levels of GRO- α (data not shown).

In further regression models, progression of DR was examined separately for E-selectin and ENA-78 (Table 5). After controlling for age, sex, and BMI, baseline E-selectin levels remained associated significantly with progression of DR ($P = 0.05$). As shown in Table 5A (model 2), glycosylated hemoglobin levels also predicted progression of DR, and reduced, but did not eliminate, the association between E-selectin and progression of DR. Squaring the beta weights indicated that 4.6% (0.242) of the variance in progression of DR was attributable to either E-selectin or glycosylated hemoglobin levels, and 1.2% (0.112) was uniquely attributable to E-selectin. In model 3, where proteinuria was added to the model, E-selectin remained significantly associated with progression of DR, despite the fact that E-selectin levels also are associated strongly with proteinuria at baseline ($r = 0.22$, $P < 0.001$). There was no further change in the association between E-selectin and progression of DR when adjusting for MAP, CRVE, or CVD (data not shown). These data suggested that E-selectin predicts progression of DR through a mechanism that is independent of either glycosylated hemoglobin or other known risk factors for DR progression.

The crude and adjusted association between ENA-78 and progression of DR is shown in Table 5B. Adding glycemic control in model 2 eliminated the significant association between ENA-78 levels and progression of DR; there was no further change when adjusting for proteinuria, MAP, or CVD (data not shown). These data suggested that ENA-78, predicts progression of DR through a mechanism that is linked to glycosylated hemoglobin.

Incidence of PDR

Univariate analyses showed that four markers were associated significantly with the incidence of PDR, IL-8, TNF- α , E-selectin, and sICAM-1 (Table 4). TNF- α also was associated significantly with the prevalence of PDR at baseline.

When all four markers were entered in a multiple logistic regression, only baseline E-selectin and TNF- α had an independent association with the incidence of PDR ($P < 0.001$ and $P < 0.001$, respectively). After controlling for baseline age, glycosylated hemoglobin, MAP, and AER, E-selectin and TNF- α analyzed separately remained significant predictors of the incidence of PDR (Tables 5C, 5D). These data suggested that E-selectin, a marker of endothelial dysfunction, and TNF- α , a proinflammatory marker, predict incidence of PDR through a mechanism that is independent of glycosylated hemoglobin, age, proteinuria, or CVD.

Incidence of ME

Univariate analysis showed that TNF- α , E-selectin, and sICAM-1 were associated significantly with the incidence of ME (Table 4).

TABLE 3. Relationship Between Baseline Biomarker Levels and Known Predictors of DR, PDR, and ME in African Americans With Type 1 Diabetes Univariate Analyses

	Age		BMI		HbA1c		MAP		Total-C		HDL-C		LDL-C		Serum Creatinine	
	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value
Cytokines																
IL-1 α	-0.23	<0.001	-0.15	0.003	NS		NS		NS		NS		NS			
sIL-2R	NS		NS		-0.14	0.005	NS		0.12	0.02	NS		NS		NS	
IL-8	NS		-0.10	0.04	NS		NS		NS		NS		NS		0.14	0.005
sCD40	NS		NS		NS		NS		NS		NS		NS		NS	
TNF- α	NS		NS		NS		0.14	0.004	NS		-0.13	0.01	NS		0.14	0.005
INF- γ	NS		NS		NS		NS		NS		NS		NS		NS	
Growth factors																
VEGF	NS		-0.10	0.04	0.11	0.03	NS		NS		NS		NS		NS	
Chemokines																
Eotaxin	0.13	0.01	-0.20	0.001	0.11	0.04	NS		NS		NS		NS		NS	
GRO- α	NS		NS		NS		NS		NS		NS		NS		NS	
MCP-1	0.13	0.007	NS		NS		0.12	0.01	NS		NS		-0.12	0.02	NS	
MIP-1 β	NS		NS		0.13	0.007	NS		NS		NS		NS		NS	
IP-10	0.16	0.002	0.14	0.006	-0.16	0.001	NS		-0.10	0.05	-0.15	0.003	NS		NS	
SDF-1	NS		NS		0.10	0.04	NS		NS		NS		-0.11	0.03	NS	
ENA-78	NS		NS		0.26	<0.001	NS		NS		NS		NS		NS	
Soluble adhesion molecules																
E-selectin	NS		NS		0.28	<0.001	NS		NS		-0.12	0.02	NS		0.14	0.006
sICAM-1	NS		NS		0.17	0.001	NS		NS		-0.14	0.005	NS		NS	
sVCAM-1	NS		-0.23	0.001	0.17	0.001	0.10	0.04	NS		NS		NS		NS	
CRP	0.25	<0.001	0.46	<0.001	NS		0.15	0.003	0.10	0.04	NS		0.13	0.01	NS	

r, correlation coefficient; NS, nonsignificant *P* values < 0.05. Only biomarkers significantly associated with predictors of DR are shown.

When these three markers were entered in a multiple logistic regression, only sICAM-1 ($P = 0.002$) and TNF- α ($P = 0.02$) were shown to be associated independently with incidence of ME; both remained independent predictors of the incidence of ME after adjusting for age, BMI, glycosylated hemoglobin, MAP, proteinuria, or CVD (Tables 5E, 5F). These data suggested that E-selectin and TNF- α predict incidence of ME through a mechanism that is independent of glycosylated hemoglobin, age, BMI, proteinuria, blood pressure, or CVD.

The risk of incident DR for each significant biomarker expressed as quartiles is shown in Table 6: For baseline E-selectin levels > 40 pg/mL, the risk of incident PDR almost doubles and that of DR progression is on average 1 step greater than for lower levels of E-selectin. For baseline TNF- α > 3.4 pg/mL there is a 3- to 4-fold increase in risk of incident PDR and a 2- to 3-fold increase in the risk of incident ME. For baseline sICAM-1 > 165 pg/mL the risk of incident ME triples.

TABLE 4. Relationship Between Baseline Biomarker Levels (pg/mL) and Prevalence or Progression/Incidence of DR, PDR, and ME in African Americans With Type 1 Diabetes: Univariate Analyses

	Diabetic Retinopathy				PDR				Macular Edema			
	Prevalence		Progression		Prevalence		Incidence		Prevalence		Incidence	
	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value	<i>r</i>	<i>P</i> Value
Cytokines												
sIL-2R	0.160	0.002	NS		0.18	<0.001	NS		NS		NS	
IL-8	0.160	0.002	NS		NS		0.14	0.007	NS		NS	
TNF- α	0.23	<0.001	NS		0.17	<0.001	0.23	<0.001	NS		0.18	<0.001
Chemokines												
MCP-1	0.2	<0.001	NS		0.19	<0.001	NS		NS		NS	
ENA-78	NS		0.17	<0.001	NS		NS		NS		NS	
Soluble adhesion molecules												
E-selectin	NS		0.23	<0.001	NS		0.22	<0.001	NS		0.16	0.001
sICAM-1	NS		0.18	<0.001	NS		0.21	<0.001	NS		0.21	<0.001
CRP	NS		NS		0.15	0.003	NS		NS		NS	

Only inflammatory biomarkers significantly ($P < 0.01$) associated with either prevalence or incidence of DR are shown.

Table 5. Relationship Between Baseline Inflammatory Markers and Progression of DR in African Americans With Type 1 Diabetes: Multivariate Analysis

	Model 1*			Model 2†			Model 3‡			Model 4§		
	Beta	(95% CI)	P Value	Beta	(95% CI)	P Value	Beta	(95% CI)	P Value	Beta	(95% CI)	P Value
A. Progression of DR from E-selectin												
E-selectin, per 10 ranks	0.25	(0.16, 0.34)	<0.001	0.14	(0.04, 0.23)	0.05	0.17	(0.07, 0.26)	0.01			
GlycoHb, %				0.41	(0.33, 0.49)	<0.001	0.40	(0.32, 0.48)	<0.001			
Microproteinuria 0 vs. 2							−0.13	(−0.23, −0.03)	0.05			
B. Progression of DR from ENA-78												
ENA-78, per 10 pg/mL	0.17	(0.06, 0.27)	<0.001	0.05	(−0.06, 0.16)	0.14						
GlycoHb, %				0.45	(0.36, 0.53)	<0.001						
OR	OR	(95% CI)	P	OR	(95% CI)	P	OR	(95% CI)	P	OR	(95% CI)	P
C. Incidence of Proliferative DR from E-selectin												
E-selectin, per 10 ranks	1.02	(1.01, 1.03)	<0.001	1.02	(1.01, 1.03)	0.001	1.02	(1.01, 1.03)	0.008	1.02	(1.01, 1.03)	<0.001
Age, y				1.10	(1.06, 1.13)	<0.001	1.09	(1.05, 1.12)	<0.001	1.09	(1.05, 1.13)	<0.001
GlycoHb, %				1.18	(1.10, 1.28)	<0.001	1.20	(1.11, 1.30)	<0.001	1.17	(1.08, 1.26)	<0.001
Microproteinuria 0 vs. 2							2.69	(1.16, 6.25)	0.02			
MAP, per 10 mm										1.38	(1.06, 1.81)	0.02
D. Incidence of Proliferative DR from TNF-α												
TNF-α, per 10 ranks	1.03	(1.02, 1.04)	<0.001	1.03	(1.02, 1.04)	<0.001	1.02	(1.01, 1.04)	<0.001	1.03	(1.01, 1.04)	<0.001
Age, y				1.09	(1.06, 1.13)	<0.001	1.09	(1.05, 1.12)	<0.001	1.09	(1.05, 1.12)	<0.001
GlycoHb, %				1.21	(1.12, 1.30)	<0.001	1.23	(1.14, 1.33)	<0.001	1.20	(1.11, 1.30)	<0.001
Microproteinuria 0 vs. 2							2.61	(1.12, 6.06)	0.03			
MAP, per 10 mm										1.36	(1.04, 1.79)	0.03
E. Incidence of macular edema from sICAM-1												
sICAM-1, per 10 ranks	1.02	(1.01, 1.03)	<0.001	1.02	(1.01, 1.03)	0.002	1.01	(1.00, 1.03)	0.02	1.02	(1.00, 1.03)	0.008
Age, y				1.05	(1.01, 1.08)	0.004	1.02	(0.99, 1.06)	0.21	1.03	(1.00, 1.06)	0.09
BMI, per kg/m ²				1.03	(1.00, 1.06)	0.07	1.02	(0.99, 1.05)	0.25	1.02	(0.99, 1.05)	0.35
GlycoHb, %				1.11	(1.03, 1.20)	0.008	1.13	(1.04, 1.23)	0.003	1.12	(1.03, 1.21)	0.005
Microproteinuria 0 vs. 1¶							2.82	(1.26, 6.31)	0.01			
Microproteinuria 0 vs. 2							7.57	(3.30, 17.34)	<0.001			
MAP, per 10 mm												
LEAD, yes versus no										1.65	(1.26, 2.15)	<0.001
										3.41	(0.94, 12.40)	0.06
F. Incidence of macular edema including TNF-α												
TNF-α, per 10 ranks	1.02	(1.01, 1.04)	<0.001	1.02	(1.01, 1.04)	<0.001	1.02	(1.01, 1.03)	0.01	1.02	(1.01, 1.03)	0.002
Age, y				1.05	(1.02, 1.08)	0.003	1.02	(0.99, 1.05)	0.23	1.03	(1.00, 1.06)	0.09
BMI, per kg/m ²				1.03	(1.00, 1.06)	0.11	1.02	(0.99, 1.05)	0.34	1.02	(0.98, 1.05)	0.36
GlycoHb, %				1.13	(1.05, 1.22)	0.002	1.15	(1.06, 1.24)	0.001	1.14	(1.05, 1.23)	0.001
Microproteinuria 0 vs. 1¶							3.30	(1.47, 7.39)	0.004			
Microproteinuria 0 vs. 2							7.15	(3.07, 16.67)	<0.001			
MAP, per 10 mm										1.54	(1.18, 2.01)	0.001
LEAD, yes versus no										4.66	(1.31, 16.60)	<0.001

Overall *P* values are shown. CI, confidence interval; Beta, standardized regression coefficient; GlycoHb, glycosylated hemoglobin; OR, odds ratio.

* No adjustment.

† Adjusted for age, sex, body mass index, and glycosylated hemoglobin.

‡ Adjusted in addition to what is in model 2 for baseline presence of microproteinuria.

§ Replacing microproteinuria in model 2 with heart disease/stroke, lower extremity amputation, and MAP.

|| Comparing patients with AER > 200 μL/min to patients with AER < 20 μL/min.

¶ Comparing patients with AER = 20 to 200 μL/min to patients with AER < 20 μL/min.

TABLE 6. Relationship Between Unadjusted Baseline Plasma Inflammatory Biomarkers Expressed in Quartiles and Progression of DR in African Americans With Type 1 Diabetes

Inflammatory Biomarkers	Outcome					
	Progression of DR		Incidence of PDR		Incidence of ME	
	N*	M (SD)†	N*	%	N*	%
E-selectin, pg/ml						
First quartile ≤ 25.2	96	3.5 (2.3)	96	11.1		
Second quartile 25.3–39.0	97	3.9 (2.2)	75	15.7		
Third quartile 39.1–65.1	107	4.5 (2.3)	71	29.0		
Fourth quartile ≥ 65.2	85	4.8 (2.2)	53	31.2		
TNF- α , pg/ml						
First quartile ≤ 3.4			121	7.4	118	8.5
Second quartile 3.5–5.3			108	24.1	108	13.9
Third quartile 5.4–8.7			96	31.2	97	22.7
Fourth quartile ≥ 8.8			49	28.6	52	23.1
sICAM-1, pg/ml						
First quartile ≤ 122.9					99	11.1
Second quartile 123–164.3					114	7.0
Third quartile 164.4–232.2					89	21.3
Fourth quartile ≥ 232.3					73	28.8

* Number at risk.

† Mean number of steps (SD).

Multiple regression analyses for all three outcomes also were run excluding 21 patients with <1 year of DM since elevated inflammatory markers might be related to autoimmune destruction of the pancreatic beta cells. Results were unchanged except that when E-selectin was examined as a predictor of progression of DR overt proteinuria no longer was a covariate (data not shown).

DISCUSSION

In our study, baseline plasma levels of specific inflammatory markers were found to be significant predictors of incident DR in T1DM African Americans. Specifically, E-selectin was associated with progression of DR and incidence of PDR, TNF- α with incidence of PDR and ME, and sICAM-1 with incidence of ME. The data also showed that this role appears to be independent of glycemic control or other known risk factors for incident DR, for example hypertension, CVD, or renal disease.

Interestingly, results of cross-sectional studies indicate that serum levels of E-selectin and ICAM-1 are increased in DR patients compared to either non-DR patients or nondiabetic persons.^{27,28,30} A strength of our study is its prospective design, since cross-sectional studies do not distinguish between increased levels of inflammatory molecules resulting from activation of endothelial cells from those resulting from damaged endothelial cells, and thus cannot infer causal relationship(s).⁵³ Moreover, plasma levels of E-selectin and other inflammatory biomarkers are affected by systemic factors, for example glycemic control, hypertension, CVD, obesity, and blood lipoproteins.^{27,53–55} For instance, in the longitudinal study of Spijkerman et al., plasma levels of E-selectin no longer were associated with progression of DR after controlling for glycemic control.³⁵ In our longitudinal study, the association between E-selectin with either progression of DR or incidence of PDR was not eliminated when adjusting for glycemic control. This suggested a causal relationship between the two via pathway(s), at least partial, other than hyperglycemia. Thus, baseline levels of E-selectin may provide unique information regarding incident DR, information that differs significantly from that of other predictors for example, age,

glycemic control, proteinuria, retinal venular diameter, or hypertension.^{47,52}

In our study, increased baseline levels of sICAM-1 were predictive of the development of ME. These data were consistent with reports of intense ICAM retinal immunostaining in one patient with ME, and elevated vitreous levels of sICAM-1 in diabetic patients with ME.^{22,31} In the Diabetes Control and Complications Trial, there was a trend for increasing serum levels of ICAM-1 to be associated with a higher risk of incident retinal hard exudates.⁴³ It also is noteworthy that intravitreal injections of anti-VEGF medications are successful in reducing ME and improving visual acuity in some diabetic patients.^{40,41} Our study suggested that in diabetic patients biomarkers other than VEGF also may be involved in ME.

The possible role of adhesion molecules in the early stages of the development of DR is supported by the findings that in induced DM entrapment of leukocytes and leukocyte-endothelial adhesion occur early on, and are associated with areas of retinal capillary occlusion, increased expression of retinal ICAM, and breakdown of the blood-retinal barrier.^{5,7,8}

E-selectin and sICAM-1 are adhesion molecules responsible for the rolling and adhesion of the leukocytes to endothelial cells.^{56,57} By upregulating the expression of E-selectin and ICAM on endothelial cells, VEGF participates in this process.⁵⁸ Whether the local accumulation of any or all of these inflammatory molecules results in the retinal capillary degeneration seen in early DR still is unclear.^{1,2}

Another result of our study was that increased baseline TNF- α was associated independently with the incidence of PDR and ME. Interestingly, elevated TNF- α also was associated with baseline prevalence of PDR and ME (Table 4), suggesting that levels of this marker remain elevated once either PDR or ME is present. In previous reports, vitreous and serum levels of TNF- α were higher in patients with PDR compared to either nondiabetic persons or non-DR patients.^{14,18,20,24,32,36,37,39} Infliximab, a chimeric monoclonal antibody specific for human TNF- α inhibitors, also appears to improve visual acuity in some diabetic patients with ME.⁵⁹ Moreover, in diabetic animals devoid of TNF- α , leukostasis, retinal vascular leakage, and vascular and neuronal apoptosis associated with DR were prevented.⁶⁰

TNF- α is produced by a variety of cells in reaction to a wide range of stimuli, for example cytokines and ischemia/hypoxia, and in turn influences a complex network of cells and mediators of inflammation.⁶¹ Thus, our African American patient data might be consistent with the actions of TNF- α in upregulating expression of adhesion molecules (e.g., ICAM-1) resulting in ME, and angiogenesis in the setting of hyperglycemia or ischemia leading to PDR.^{61,62}

In our study baseline levels of E-selectin, s-ICAM-1, and TNF- α all were associated significantly with incident DR independent of either glycemic control, blood lipids, or presence of micro- or macroangiopathy. This raises the question of what other pathway(s) (i.e., accumulation of advanced glycation end-products, ischemia-reperfusion injury, oxidative stress, or some other process) may initiate the upregulation of these inflammatory biomarkers.^{63–65}

Limitations of the study include the fact that inflammatory markers were measured only in plasma. Plasma samples were not obtained concurrently in normal nondiabetic persons. Because the samples were stored for a period ranging from 14 to 19 years, there is the possibility of protein degradation despite storage at -70°C .⁶⁶ Repeated documentation of changes in DR status, glycosylated hemoglobin values, and inflammatory marker levels over time was not available. Elevated levels of biomarkers at baseline may have been associated with the presence of subclinical retinal disease. Finally, because this is a group of patients with poor glycemic control and high mortality rates, we cannot exclude the possibility of survival bias for this group of patients despite the fact that the follow-up rate was good. Thus, our data may be specific only to this population and should be confirmed in other T1DM persons.

In summary, data from our exploratory study indicated that baseline plasma levels of certain markers of inflammation and endothelial dysfunction are independent predictors of the incidence of DR in T1DM African Americans, suggesting that those markers either may participate in or be an indicator of DR development or progression.

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