Low-Density Lipoprotein Postsecretory Modification, Monocyte Function, and Circulating Adhesion Molecules in Type 2 Diabetic Patients With and Without Macrovascular Complications

The Effect of \(\alpha\)-Tocopherol Supplementation

S. Devaraj, PhD; I. Jialal, MD, PhD

**Background**—Although diabetes confers an increased propensity toward accelerated atherogenesis, data are lacking on monocyte activity in type 2 diabetic patients with (DM2-MV) and without (DM2) macrovascular disease compared with control subjects. Thus, we tested whether (1) postsecretory modifications of LDL (glycation and oxidation), monocyte proatherogenic activity, and circulating levels of soluble cell adhesion molecules (sCAMs) are more pronounced in DM2-MV than in DM2 and control subjects and (2) RRR-\(\alpha\)-tocopherol (AT) therapy, 1200 IU/d for 3 months, has a similar effect in the 3 groups (n=25 per group).

**Methods and Results**—Although LDL glycation was increased in both diabetic groups compared with control subjects, AT therapy had no significant effect on glycation. AT therapy significantly decreased LDL oxidizability in all 3 groups. Diabetic monocytes released significantly more superoxide anion (\(O_2^-\)) and interleukin-1\(\beta\) (IL-1\(\beta\)) and exhibited greater adhesion to endothelium than control subjects. AT therapy significantly decreased the release of \(O_2^-\), IL-1\(\beta\), tumor necrosis factor-\(\alpha\), and monocyte-endothelium adhesion in all 3 groups. There was no significant difference between the 2 diabetic groups for any of the above parameters. sICAM levels were significantly elevated in both diabetic groups compared with controls. AT therapy resulted in a significant decrease in sCAMs.

**Conclusions**—This is the first demonstration of increased IL-1\(\beta\) secretion and increased adhesion of monocytes to endothelium from normotriglyceridemic diabetic subjects and of decreased monocyte activity and sCAMs with AT therapy in diabetic subjects with and without macrovasculopathy. ([Circulation. 2000;102:191-196.])

**Key Words:** vitamins ■ diabetes ■ cells ■ cell adhesion molecules ■ proteins

The diabetic state per se confers an increased propensity to accelerated atherogenesis. However, the precise mechanisms by which this occurs remain to be elucidated. Data are relatively scarce on postsecretory modifications of lipoproteins and their role in atherogenesis in type 2 diabetic patients with (DM2-MV) and without (DM2) macrovascular disease. The best-studied modification of lipoproteins in diabetes is nonenzymatic glycation. The effect of \(\alpha\)-tocopherol (AT) supplementation on LDL and protein glycation is controversial. Several lines of evidence support a proatherogenic role for oxidized LDL. Factors that promote LDL oxidation in diabetic patients include antioxidant deficiencies, increased production of reactive oxygen species, and protein glycation. Numerous antioxidant deficiencies have been reported in diabetic patients, and several investigators have reported evidence for increased oxidative stress and LDL oxidation in diabetes. Although several investigators have shown the beneficial effects of antioxidant supplementation, especially with AT in nondiabetic patients, data are lacking on the effect of AT supplementation on LDL oxidation in DM2-MV.

The monocyte-macrophage is a crucial and the most readily accessible cell that is involved in atherogenesis. Diabetic monocytes have been shown to produce increased levels of reactive oxygen species on activation. In addition, hyperglycemia promotes monocyte binding to endothelial cells, and diabetic monocytes display increased binding to endothelium. However, no studies to date have examined the functional alterations in monocyte activity in DM2 and DM2-MV compared with control subjects. We have recently shown, in nondiabetic patients, that AT supplementation decreases production of reactive oxygen species from human monocytes; decreases lipid oxidation; inhibits the release of proatherogenic, proinflammatory cytokines, such as interleukin (IL)-1\(\beta\), from human monocytes; and decreases adhesion...
of monocytes to endothelium, the latter by inhibition of adhesion molecules CD11b and VLA-4 and decreased nuclear factor-kB activity.\textsuperscript{16,17} Also, we have shown that the inhibition of IL-1B release from activated human monocytes is via inhibition of 5-lipoxygenase.\textsuperscript{18}

Increasing evidence supports the role of soluble cell adhesion molecules (sCAMs), such as intercellular adhesion molecule (sICAM)-1, vascular cell adhesion molecule (sVCAM)-1, and E-selectin, as molecular markers of atherosclerosis.\textsuperscript{19} Recently, it was shown that supplementation with N-acetyl-L-cysteine resulted in an increase in erythrocyte glutathione and a concomitant reduction in plasma VCAM-1 levels.\textsuperscript{20} However, data on the effect of AT supplementation on sCAM levels are lacking.

Thus, the aims of this study were (1) to determine whether postsecretory modifications of LDL (glycation and oxidation) as well as monocyte proatherogenic activity and sCAMs are more pronounced in DM2 and DM2-MV than in control subjects and (2) to test whether AT therapy, 1200 IU/d for 3 months, has a similar effect on control subjects, DM2, and DM2-MV on LDL oxidation and glycation, monocyte function, and sCAMs.

Methods
A total of 75 subjects were recruited. They were divided into 3 groups (DM2, n=25, type 2 diabetic patients without vascular complications, DM2-MV, n=25), and age- and sex-matched healthy control subjects (n=25). This protocol was approved by the Institutional Review Board, and all subjects gave informed consent. The following inclusion criteria were adopted for the study: nonsmokers; not on antioxidant vitamin supplements, vitamins, lipid-lowering drugs, b-blockers, or nonsteroidal anti-inflammatory drugs; no renal or liver dysfunction; normal complete blood count; alcohol consumption <1 oz/d; and no gastrointestinal disorders, such as malabsorption. There were 3 dropouts in the study (1 DM2 and 2 DM2-MV) because of unsuccessful phlebotomy or because of lipid-lowering medication being initiated during the study. Thirteen subjects in the DM2 group and 6 subjects in DM2-MV group were on oral hypoglycemic agents (OHAs) alone, 7 patients in DM2 and 15 patients in DM2-MV were on insulin alone, and the remaining 5 patients in the DM2 and 4 in the DM2-MV groups had been given both OHAs and insulin. The OHAs used included metformin and glyburide. Estrogen therapy was prescribed in 3, 4, and 5 subjects in the control, DM2, and DM2-MV groups, respectively. Antihypertensive drugs, including diuretics, enalapril, verapamil, benazepril, quinapril, and hydrochlorothiazide, were prescribed to 9 of the DM2 and 18 of the DM2-MV patients. Criteria for macrovascular disease in diabetic patients included evidence of cardiovascular disease (clinical presentation and ECG evidence of myocardial infarction or positive stress tests or coronary angiography), cerebrovascular disease (stroke, transient ischemic attacks, or MRI evidence), or peripheral vascular disease (amputation, intermittent claudication, evidence of vascular disease with color flow Doppler by B-mode ultrasound, or ankle-brachial index <0.8 and toe pressures <45 mm Hg). Before entry into the study, the subjects had a complete laboratory evaluation, including complete blood count, routine chemistry, and spot urine for microalbumin and creatinine. Twenty-four-hour urine samples were collected if a spot microalbumin/creatinine ratio was >30 mg/g creatinine.

Fasting blood was obtained from all the subjects at baseline and after 3 months of supplementation with all-rac-AT (1200 IU/d) and after 2-month washout phase. Blood was drawn from 1 subject from each of the 3 groups (ie, age- and sex-matched control, DM2, and DM2-MV) on the same day for LDL isolation and isolation of monocytes. Subjects were asked to adhere to their regular diet and activity levels and not change any medications during the entire period of the study. Compliance was monitored by pill count and by analysis of plasma fatty acid content and plasma, LDL, and mononuclear cell AT levels.

Fasting blood (180 mL) was obtained from all subjects at each phase: 120 mL of heparinized blood for studies on monocyte function and 60 mL of blood anticoagulated with EDTA for studies of LDL modification and for plasma levels of fatty acids and AT. LDL was isolated by preparative ultracentrifugation from plasma as previously described.\textsuperscript{10} LDL (100 mg protein/mL) was oxidized with 5 mM/L copper at 37°C for 8 hours as reported previously.\textsuperscript{10} The amounts of conjugated dienes, lipid peroxides, and apolipoprotein B fluorescence were determined, and lag phase was computed as described previously.\textsuperscript{6,10}

LDL glycation was measured by aminophenyl borate affinity chromatography as described previously.\textsuperscript{21} Also, glycated hemoglobin and plasma protein levels were measured in all subjects by automated affinity chromatography and as reported previously.\textsuperscript{10}

Plasma and LDL fatty acid composition was measured by gas chromatography after extraction and transmethylation as described previously.\textsuperscript{10} Plasma and LDL AT levels were measured by reverse-phase high-performance liquid chromatography, after ethanol precipitation and hexane extraction.\textsuperscript{2}

Mononuclear cells were isolated from fasting heparinized blood (120 mL) by Ficoll-Hypaque centrifugation as described previously.\textsuperscript{16} All the assays of monocyte function were undertaken on the day of isolation. All reagents used to assay monocyte function were tested for endotoxin contamination by the Limulus endotoxin assay.\textsuperscript{16} Lipopolysaccharide (LPS), a known activator of monocytes, was used to activate monocytes.\textsuperscript{16}

Superoxide anion release from monocytes was measured after activation with LPS (10 mg/mL) by the superoxide dismutase-inhibitable reduction of ferricytochrome c as described previously.\textsuperscript{16} Results are expressed as nmol superoxide - min -1 - mg cell protein -1.

The release of IL-1B and tumor necrosis factor-a (TNF-a) was measured in LPS-activated monocytes with highly sensitive human immunoasay reagents from Amersham (Biotrak) as reported previously.\textsuperscript{16} IL-1B and TNF-a secretions from monocytes are expressed as pg/mg cell protein.

Monocyte–endothelial cell adhesion was assayed by phase-contrast microscopic evaluation as reported previously.\textsuperscript{16} The number of attached monocytes in 5 high-power microscopic fields was counted for LPS-activated endothelial cells with a phase-contrast microscope.

Circulating adhesion molecules, sVCAM, sICAM, and sE-selectin, in frozen plasma samples were also layered in a single batch with an ELISA methodology with reagents and controls from Amersham. The mean intra-assay coefficient of variation for these assays in 7 samples run in quintuplicate and in the ranges reported in this study was <5%.

Statistics
Nonparametric tests were implemented because of skewed distribution of data in some of the variables. Kruskal-Wallis ANOVA was performed to assess differences in responses after supplementation in the different parameters between the 3 groups, and the level of significance was set at 0.05. On all significant parameters, Mann-Whitney U tests were performed for pairwise comparisons, and the level of significance was set at P<0.02 to adjust for multiple testing. Spearman’s rank correlation was performed to examine associations between parameters tested.

Results
There were no significant differences in age (55±7, 57±2, and 60±7 years), male/female ratio, or body mass index (27.5±5, 32.6±6, and 31.7±8 kg/m²) among the control, DM2, and DM2-MV groups, respectively. Although none of the DM2 group had evidence of diabetic retinopathy, 7 of the DM2 patients had evidence of incipient nephropathy, as evidenced by microalbuminuria. There were no significant
differences in the lipid and lipoprotein levels or plasma fatty acids among the 3 groups at baseline and after supplementation (data not shown).

Levels of hemoglobin A\textsubscript{1c}, glycated plasma proteins, and glycated LDL were significantly increased in both diabetic groups compared with control. However, AT supplementation did not have any significant effect on measures of glycation in any of the 3 groups (data not shown). At baseline, levels of plasma, LDL, and mononuclear cell AT were similar in all 3 groups. AT supplementation resulted in a significant 2.5-fold increase in plasma lipid–standardized, LDL, and mononuclear cell AT compared with baseline in all 3 groups (data not shown). There was a trend toward increased LDL oxidative susceptibility in both diabetic groups compared with control for conjugated dienes ($P<0.037$ for DM2 and $P<0.026$ for DM2-MV compared with control, respectively); however, there was no significant difference between DM2 and DM2-MV. AT supplementation resulted in significant and similar increases in lag phase of oxidation as measured by conjugated dienes in all 3 groups (Figure 1). Similar observations were obtained for lipid peroxides and apo B fluorescence (data not shown).

LPS-activated diabetic monocytes secreted similar and significantly increased levels of $O_2^-$ compared with matched controls. AT supplementation resulted in a significant reduction in $O_2^-$ release in all 3 groups (Fig 2A). Levels of IL-1\beta were significantly increased from LPS-activated monocytes from the DM2 and DM2-MV compared with the control groups. Although levels of TNF were increased in both diabetic groups compared with control, the increases were not statistically significant. AT supplementation decreased the secretion of both IL-1\beta and TNF-\alpha in all 3 groups (Figure 3A and 3B). The percent inhibition of IL-1\beta release was greater than that of TNF-\alpha for all 3 groups (control, 71\% versus 54\%, $P=0.0012$; DM2, 81.6\% versus 43.6\%, $P<0.001$; and DM2-MV, 82.1\% versus 58.4\%, $P<0.001$). Diabetic monocytes exhibited significantly greater adhesion to human endothelium than in control groups. AT supplementation resulted in a similar and significant decrease in adhesion of monocytes to human umbilical vein endothelial cells in all 3 groups (Figure 2B).

There was a similar and significant increase in sICAM-1 levels in both the DM2 and DM2-MV groups compared with control. AT supplementation resulted in a similar and significant decrease in sCAMs in all 3 groups compared with baseline (Table).

In the DM2 group, 7 patients had microalbuminuria. After exclusion of these 7 individuals in the DM2 group, comparing the remaining 17 patients with the DM2-MV and control groups did not change any of the findings reported above.

For all the parameters studied, there were no significant differences between baseline and washout phases in any of the 3 groups.

**Discussion**

Preliminary findings in large lipid-lowering and antihypertensive trials suggest that lowering LDL cholesterol and blood pressure reduces cardiovascular events in diabetic patients.\textsuperscript{22–25} However, in some trials, although there was a significant benefit in the entire group, the benefit in the diabetic subgroup was borderline or nonsignificant.\textsuperscript{26–28} This emphasizes that observations in the nondiabetic cannot be readily extrapolated to the diabetic individual. Although some data exist on the effects of other modalities, such as antioxidant supplementation, on the prevention of cardiovascular events in nondiabetic patients,\textsuperscript{29–31} data in diabetic patients are lacking. It is important to determine whether the diabetic state attenuates the effects of AT supplementation seen in normal volunteers.
In this study, we have shown increased glycation of plasma proteins and LDL in DM2 and DM2-MV compared with the control group. There were no significant differences between the 2 diabetic subgroups. This is supported by observations by Yegin and Ozben, who reported increased levels of all glycated lipoproteins in diabetic patients with and without vascular complications, with no significant differences between diabetic patients with and without complications except for glycated VLDL. However, Kobayashi et al demonstrated increased glycated LDL values in diabetic patients with complications compared with those without. With regard to the effect of AT on protein glycation, the data appear to be confusing. Whereas some investigators have clearly shown that AT decreases glycation of proteins such as hemoglobin, others have failed to show this. Jain et al reported that AT supplementation (100 IU/d) can significantly lower glycohemoglobin levels in type I diabetic patients. In this study, we investigated the effect of high-dose AT supplementation on glycated hemoglobin, glycated plasma proteins, and glycation of LDL in 47 diabetic patients. As in our previous studies, we do not demonstrate any beneficial effects of AT on LDL glycation in either diabetic subgroup. It appears that the favorable effect of AT on protein glycation is seen mainly in type 1 diabetic patients.

Although several investigators have shown increased susceptibility of LDL to oxidation in diabetic patients compared with controls, data are lacking on LDL oxidizability in these patients in the presence of vascular complications. We demonstrate that in vitro LDL oxidizability was not significantly different between the control, DM2, and DM2-MV groups. This is supported by recent observations by Leinonen et al, in which lag phase was not significantly different between control subjects and diabetic patients with or without coronary artery disease. Also, in this article, we show that AT supplementation resulted in a decrease in LDL oxidative susceptibility. This finding confirms the reports of previous investigators. However, this is the first study to document that AT supplementation has an equivalent effect in DM2-MV. The previous reports have largely focused on DM2.

Many data support a major role for inflammation in atherogenesis. Thus, it is clearly important to study monocyte proatherogenic activity in diabetes. In this study, we report, for the first time, increased proatherogenic activity of diabetic monocytes compared with control as assessed by superoxide anion, IL-1β release, and adhesion to endothelium. Although this increase was more pronounced in DM2-MV, none of the parameters were significantly elevated in DM2-MV compared with DM2. It is possible that with a larger sample size, this trend would become significant. Furthermore, because all patients in the DM2 group were not specifically tested for complications, it is possible that they had clinically silent vascular complications. Future studies using noninvasive measures will delineate differences between DM2 and DM2-MV groups.

Previous studies have reported increased O$_2^-$ release in diabetic monocytes compared with matched controls. However, none of these studies have examined O$_2^-$ release in DM2 and DM2-MV. Whereas Hill et al and Kitahara et al reported increased O$_2^-$ levels in monocytes of uncomplicated diabetic patients compared with control, Hiramatsu et al reported increased O$_2^-$ levels only in mononuclear cells from diabetic patients with hypertriglyceridemia without complications. Thus, our study is the first to report increased O$_2^-$ levels in LPS-activated monocytes in DM2 and DM2-MV compared with the control group.
Several lines of evidence suggest that the proinflammatory cytokines IL-1β and TNF-α are proatherogenic. There was a significant increase in IL-1β and a nonsignificant increase in TNF release from activated monocytes in both diabetic groups. In contrast to our study, Desfaits et al observed increased levels of LPS-stimulated TNF-α release from monocytes in poorly controlled diabetics. The present study is the first report of increased levels of the proinflammatory, proatherogenic cytokine IL-1β from activated monocytes of diabetic subjects.

The most novel aspect of this study was to investigate the effect of AT on monocyte function in diabetic subjects. We clearly show in this study that in both DM2 groups, AT supplementation decreased O2 release. In addition, we showed beneficial effects on IL-1β and TNF-α. The effect of AT on IL-1β release was significantly greater than its effect on TNF-α. However, because TNF-α production has been linked to insulin resistance, modulation of TNF expression in type 2 diabetic patients with AT could be potentially beneficial. In a recent report, 600 IU/d of AT for 4 weeks in diabetic patients (n=11) had no effect on IL-1β and TNF in whole blood. Monocyte cytokine release was not studied. Also, the dose of AT was much lower (600 IU/d). A dose-response study of AT supplementation in diabetic subjects is urgently needed to determine the threshold dose that is anti-inflammatory.

Previous groups have reported increased adhesion of monocytes to endothelial cells in DM2 compared with control. However, Hoogerbrugge et al demonstrated significantly increased binding of monocytes to endothelium only in type 2 diabetic patients with hypertriglyceridemia, and in Carantoni’s study, the patients were also hypertriglyceridemic, and mononuclear cell adherence and non monocyte adhesion was studied. In neither study were the patients with vascular disease separated from those without complications. This is therefore the first study to report enhanced adhesion of monocytes to endothelium in both DM2 and DM2-MV, who were normotriglyceridemic, compared with matched control subjects. Furthermore, this study goes further in demonstrating that AT enrichment of diabetic monocytes decreased their adhesion to endothelium.

In this study, although there was no significant difference in levels of sVCAM-1 and sE-selectin in diabetic patients compared with control, sICAM-1 levels were significantly increased in both DM2 and DM2-MV compared with control. Several investigators have reported increased levels of different sCAMs in type 1 and type 2 diabetic patients. Data comparing sCAMs in DM2 and DM2-MV are lacking. In diabetic patients, there appear to be limited data on therapies to modulate sCAMs, except for supplementation with N-acetyl-L-cysteine, which decreased sVCAM-1. Also, therapy with troglitazone, which has a structure similar to that of AT but is hepatotoxic, resulted in a decrease in plasma E-selectin levels in DM2. In the present study, we show a significant reduction in all 3 sCAMs in both diabetic groups. Thus, AT, in addition to improving monocyte function, may also improve endothelial function.

Thus, it appears that many data support the evidence that AT supplementation in diabetic patients is beneficial. Also, AT supplementation in type 1 diabetic patients has been shown to ameliorate diabetic microvascular complications. AT, in addition to decreasing LDL oxidative susceptibility and platelet aggregation, appears to have significant effects on monocyte and endothelial function.

In conclusion, we clearly demonstrate increased inflammation in diabetic subjects that can be modulated with AT therapy. Thus, it is conceivable that AT supplementation in diabetic patients could also result in a reduction in macrovascular disease.

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