# Role of Simvastatin as an Immunomodulator in Type 2 Diabetes

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**OBJECTIVE** — To test the hypothesis that simvastatin reduces the levels of circulating immune complexes (ICs) containing modified lipoproteins (mLDLs; mLDL-ICs), which may represent an additional mechanism for the reduced incidence of cardiovascular events in patients treated with simvastatin.

**RESEARCH DESIGN AND METHODS** — A total of 26 patients with type 2 diabetes and triglyceride levels <400 mg/dl who were not receiving lipid-lowering medications or CYP 3A4 inhibitors were enrolled in the study. After 2 weeks on a lipid-lowering diet and exercise, the patients were started on simvastatin 20 mg/day. The dose of simvastatin was adjusted until the levels of LDL cholesterol were ≤100 mg/dl. Blood was collected at baseline, 3 and 6 months after LDL cholesterol levels reached target, and 3 months after stopping simvastatin to measure advanced glycation end product LDL and oxidized LDL antibodies, mLDL-IC, intracellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, metalloproteinase-1 (MMP-1), lipid profile, liver function tests, creatinine kinase, glucose, and $HbA_{1c}$.

**RESULTS** — Twenty-one patients completed the study. Their $HbA_{1c}$ remained within 1% of baseline levels. There was a highly significant decrease in mLDL-IC levels after 3 and 6 months of treatment with simvastatin, with a return to near baseline levels after discontinuation.

**CONCLUSIONS** — Simvastatin significantly reduced the concentration of mLDL-IC, probably as a consequence of both a decrease in the formation of mLDL and to a reduction in the titers of mLDL antibodies. This effect is likely to have a beneficial impact in the inflammatory reaction associated with atherosclerosis.

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Diabetes is associated with increased incidence of macrovascular complications including coronary heart disease (CHD) as well as cerebrovascular and peripheral vascular disease (1,2). The mechanisms behind the accelerated development of atherosclerosis in diabetes and decreased survival after an acute cardiovascular event are poorly understood. Increased levels of modified lipoproteins (mLDLs) have been proposed as significant factors contributing to the accelerated development of macrovascular complications in diabetes (3). The mLDLs emerge, at least in part, as a consequence of chronic hyperglycemia that leads to protein glycation throughout the body. Glycated proteins, including LDL, are more susceptible to oxidation (3,4). The synergy of glycation and oxidation results in formation of advanced glycation end products (AGEs) or glycoxidation products (5), which are able to induce a humoral autoimmune response and, as a consequence, lead to the formation of immune complexes detectable in both serum (6) and atheromatous lesions (7,8). Immune complexes (ICs) isolated from the serum of patients with diabetes contain malondialdehyde-modified and AGE-modified LDL (9), as well as the corresponding antibodies (10), which have been shown to be predominantly of the proinflammatory isotypes IgG1 and IgG3 (9,11).

Significantly increased levels of ICs containing mLDL (mLDL-ICs) have been demonstrated in patients with both type 1 and type 2 diabetes and established CHD. In a small group of patients with type 2 diabetes and CHD, the levels of mLDL-IC were significantly elevated when compared with those determined in control subjects and nondiabetic patients with CHD (10). In a prospective case-control study performed in the Pittsburgh Epidemiology of Diabetes Complications Study cohort (type 1 diabetes), we found that the levels of mLDL-IC at entry into the study were significantly higher in the group of patients in whom macrovascular disease developed within a 7-year follow-up period than in the matched control group without macrovascular disease (12,13).

Several clinical trials, among them the Scandinavian Simvastatin Survival Study (4S) (14) and Cholesterol and Recurrent Events (CARE) trial (15), have shown that statins significantly reduce the incidence of cardiovascular events in diabetes. Because statins have immunomodulatory (16) and anti-inflammatory properties (17) in addition to their well-known lipid-lowering properties, we decided to determine whether treating type 2 diabetic patients with simvastatin would lead to a reduction in autoantibodies against AGE-LDL and oxidized LDL as well as to a reduction in the levels of circulating mLDL-IC. Furthermore, we investigated whether a reduction in the levels of

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**Abbreviations:** AGE, advanced glycation end product; apoB, apolipoprotein B; CHD, coronary heart disease; IC, immune complex; ICAM-1, intracellular adhesion molecule-1; MHC-II, major histocompatibility complex II; mLDL, modified lipoprotein; MMP-1, metalloproteinase-1; VCAM-1, vascular adhesion molecule-1.

mLDL-IC would affect markers of endothelial dysfunction such as cell-adhesion molecules and metalloproteinase-1 (MMP-1).

## RESEARCH DESIGN AND METHODS

— A total of 26 patients with type 2 diabetes were enrolled into the study. The requirements for entrance into the study were that patients should not have been treated with lipid-lowering medications in the last 6 months and should have none of a list of excluding conditions or laboratory abnormalities. The criteria for exclusion included triglyceride levels >400 mg/dl at entry, active liver disease or liver dysfunction (transaminase levels >1.5-fold of the upper limit of normal), and creatinine kinase levels threefold greater than the upper limit of normal. Patients receiving CYP 3A4 inhibitors (cyclosporine, itraconazole, ketoconazole, erythromycin, clarithromycin, and nefazodone) were also excluded. Women who were pregnant and/or breast-feeding were excluded. Patients with class III or IV angina refractory to medication, class III or IV congestive heart failure, transient ischemic attacks, or documented cerebrovascular accident or myocardial infarction within the last 6 months were excluded. Also excluded were patients with malignancies, life-threatening diseases, end-stage renal disease, nephrotic syndrome, or other endocrinopathies, except corrected hypothyroidism. Macrovascular disease was diagnosed based on complete history and physical examination, 12-lead electrocardiography, and Doppler examinations (ankle-to-arm ratio <0.9 was considered positive). Records of coronary angiographic studies were reviewed when available. Only two patients had microvascular or macrovascular complications. One patient had peripheral neuropathy and history of CHD and peripheral vascular disease. Another patient had macroalbuminuria, peripheral neuropathy, and history of CHD. Four patients were being treated with diet alone, one was taking insulin and metformin, three patients were taking metformin alone, and four patients were taking sulfonylureas alone. The remaining patients were being treated with a combination of sulfonylureas and metformin. The therapeutic regimen was not changed in any of the patients during the course of the study, but it was adjusted to maintain proper glycemic control. Of the 26 patients recruited, 18 had hypertension. Three of these 18 patients were taking ACE inhibitors, 10 were taking ACE inhibitors and hydrochlorothiazide, and the remaining 5 patients were taking calcium channel blockers (2 patients) or a combination of triamterene with either hydrochlorothiazide (1 patient), furosemide (1 patient), or atenolol (1 patient). One patient was taking levothyroxine and was euthyroid, one patient was on allopurinol, one patient was taking fluoxetine, six patients were taking terazosin, and four patients were taking cimetidine. All patients were taking aspirin during the trial.

After they had been screened, the patients were placed on a lipid-lowering diet (<300 mg cholesterol, 25–30% total fat, <7% saturated fat, 15% protein, and the remainder carbohydrates). The patients were also encouraged to exercise at least three to four times weekly. Approximately 2 weeks after the initial screening, the patients were enrolled in the study if they still fulfilled the entrance criteria. They were all placed on simvastatin 20 mg/day, and a lipid profile was obtained. A second lipid profile was obtained 4–6 weeks after initiation of therapy. The dose of simvastatin was adjusted if LDL cholesterol was still >100 mg/dl, and another lipid profile was obtained 4–6 weeks later. Six patients required 40 mg/day simvastatin, and one patient required 80 mg/day to reach goal. Most patients required 20 mg simvastatin daily to reach goal. At baseline, 3 and 6 months after the goal for LDL was reached, and 3 and 6 months after cessation of simvastatin, blood samples were collected to measure AGE-LDL and oxidized LDL antibodies, mLDL-IC, soluble markers of endothelial dysfunction (intracellular adhesion molecule-1 [ICAM-1], vascular adhesion molecule-1 [VCAM-1], and E-selectin), and metalloproteinase-1 (MMP-1) and to perform routine tests (lipid profile, liver function tests, as well as creatinine kinase, glucose, and $HbA_{1c}$ levels).

Two patients failed to return after the first two visits, one due to problems with transportation and the other due to myalgia with normal creatinine kinase levels. After 6 months of treatment, the drug was discontinued in the remaining 24 patients and the follow-up continued for another 6 months (washout period). Three patients did not return after discontinuation of the drug. Blood samples were collected in the remaining 21 patients after 3 months of drug discontinuation; blood samples were collected in 9 of these patients after 6 months.

### Determination of the levels of circulating antibodies to mLDL

The levels of circulating ("free") oxidized LDL and AGE-LDL antibodies were determined by the competitive enzymoimmunoassay originally described for oxidized LDL antibodies (18), modified for the assay of antibodies to other forms of mLDL. In the case of the oxidized LDL antibody assay, the results were expressed as an absolute value (19). The values for AGE-LDL antibodies were expressed as the optical density difference between unabsorbed and absorbed aliquots of the same serum (9). The interassay variations for these assays were 14% for AGE-LDL antibodies and 14% for oxidized LDL antibodies at the low level of the measurements and 3.9% for values in the middle to the high levels.

### Isolation and characterization of soluble ICs

Soluble ICs were precipitated from serum samples with polyethylene glycol 6000 at a final concentration of 3.5% (12,20). To measure specifically those ICs that contained LDL or other apolipoprotein B (apoB) and cholesterol-rich lipoproteins, we determined the cholesterol content of ICs by gas chromatography and the concentration of apoB in ICs by quantitative immunoelectrophoresis (10,20). The interassay variation for these assays was 11% for both total cholesterol and apoB content in ICs.

### Measurement of soluble cell adhesion molecules and matrix MMP-1

Soluble adhesion molecules were measured using commercially available immunoassays from Research and Diagnostic Systems (Minneapolis, MN). These immunoassays have a coefficient of variation of <5%. MMP-1 was assayed by enzymoimmunoassay as previously described (21). The intra-assay and interassay coefficients of variation range from 3.3 to 5.6% (intra-assay) and from 5.6 to 10.2% (interassay).

### Statistical analysis

Paired Student's *t* tests and the McNemar test for matched pairs were used in uni-

**Table 1—*Baseline clinical characteristics of the patients who completed the study***

| Parameter | Mean | SD | Range |
|---|---|---|---|
| Age (years) | 60.4 | 8.2 | 45–77 |
| Race | 7 black/1 Hispanic/13 white | | |
| Sex (men/women) | 17/4 | | |
| BMI (kg/m$^2$) | 33.6 | 10.5 | 24–62 |
| Duration of diabetes (years) | 5.62 | 5.84 | 1–20 |
| Systolic blood pressure (mmHg) | 137 | 16 | 110–165 |
| Diastolic blood pressure (mmHg) | 80 | 7.5 | 70–95 |
| Smoking habits | None at present (12 ex-smokers) | | |
| Drinking habits | 11 social drinkers<br>1 regular drinker | 9 non-drinkers | |
| Exercise level | 10 moderately active | 7 active | 4 sedentary |

variate analysis to assess treatment differences. Correlations were performed using the Pearson test after log transformation when appropriate.

**RESULTS** — The characteristics of the patients, including age, sex, race, BMI, smoking and drinking habits, hypertension, and degree of exercise are summarized in Table 1. The mean age of the patients who completed the trial was 60 years, and most of the patients were overweight or obese. None of the patients smoked at entry or during the course of the study. The results of the lipid profile, glucose, and $HbA_{1c}$ during the trial are shown in Table 2. Liver function tests and creatinine kinase were within normal limits during the course of the study in all patients. The 21 patients who completed the study showed no change in glucose homeostasis. $HbA_{1c}$ was maintained within 1% of the patient's initial levels. The patients were kept in good to fair control (Table 2). As expected, marked decreases in total and LDL cholesterol levels were observed during the treatment period; return to near baseline levels was noted after discontinuation of the drug. No significant changes in HDL cholesterol or triglycerides were observed (Table 2).

There were no significant differences in the levels of soluble ICAM-1, VCAM-1, E-selectin, and MMP-1 during the observation period. The levels of mLDL-IC and antibodies against oxidized LDL and AGE-LDL during the trial are summarized in Table 3. There was a highly significant decrease in levels of mLDL-IC, as determined by the content of apoB and cholesterol in the IC, after 3 and 6 months of treatment with simvastatin. There were no significant differences in oxidized LDL and AGE-LDL antibody levels with treatment, but there was a clear trend to lower levels at the end of the washout period. Unfortunately, data were collected after the 6-month washout period in only nine patients, but in these patients, the levels were frankly lower (0.085 ± 0.056 [OD] for AGE-LDL antibodies and 33.95 ± 19.47 [mg/l] for oxidized LDL antibodies).

Correlations between oxidized LDL and AGE-LDL antibody levels and the cholesterol and apoB content of IC with the several components of the lipoprotein profile are shown in Table 4. A significant correlation between the apoB and cholesterol content of IC was found, as expected, because they both reflect the levels of mLDL-IC. A strong correlation between serum LDL cholesterol and the cholesterol content of IC was also observed, but the correlation between apoB in the IC and serum LDL cholesterol was less consistent.

**CONCLUSIONS** — In our group of 21 patients with type 2 diabetes, we observed a marked decrease in mLDL-ICs during treatment with simvastatin, followed by a return to baseline levels after a 3- to 6-month washout period. All other parameters measured—oxidized LDL and AGE-LDL antibodies, adhesion molecules (ICAM-1, VCAM-1, and E-selectin), and MMP-1—did not show significant changes during the study.

Some very interesting questions are raised by the quantitative data concerning mLDL-IC and mLDL antibodies. The significant decrease in the concentration of circulating mLDL-IC associated with simvastatin therapy is most likely secondary to a decrease in the formation of mLDL and/or to a reduction in the formation of mLDL antibodies. The reduction of mLDL levels most likely results from the decrease in the formation of modified LDL, as a consequence of both a reduction in the levels of native LDL, limiting the availability of substrate to generate immunogenic LDL modifications, and of the antioxidant effect of statins (21). The reduction of native LDL levels by itself could not account for the reduction on mLDL-IC levels because the antibodies



**Table 2—*Effect of simvastatin on lipid/lipoprotein levels, glucose, and $HbA_{1c}$ in 21 patients with type 2 diabetes***

| Parameter | Baseline | Treatment | | Washout 3–6 months |
|---|---|---|---|---|
| | | 3 months | 6 months | |
| Total cholesterol (mg/dl) | 205 ± 27 (156–269) | 161 ± 34 (112–254) | 167 ± 38 (124–281) | 201 ± 34 (151–269) |
| LDL cholesterol (mg/dl) | 124 ± 28 (53–168) | 85 ± 31 (50–166) | 82 ± 23 (39–130) | 122 ± 27 (86–166) |
| HDL cholesterol (mg/dl) | 42 ± 10.8 (28–68) | 41 ± 10.9 (25–65) | 45 ± 11.5 (25–62) | 42 ± 12 (28–77) |
| Triglycerides (mg/dl) | 185 ± 87 (52–340) | 166 ± 69 (67–283) | 184 ± 116 (58–483) | 193 ± 86 (83–378) |
| Glucose (mg/dl) | 152 ± 36 (87–228) | 147 ± 40 (91–216) | 169 ± 72 (90–415) | 150 ± 36 (91–229) |
| $HbA_{1c}$ (%) | 6.8 ± 1 (5.5–9.0) | 6.8 ± 1 (5.5–8.3) | 7.1 ± 1.37 (5.6–9.8) | 7.25 ± 0.89 (5.7–8.9) |

Data are means ± SD (range).

**Table 3—*Effect of simvastatin on AGE-LDL and oxidized LDL antibodies and mLDL-IC in 21 patients with type 2 diabetes***

| Parameter | Baseline | Treatment | | Washout 3–6 months |
|---|---|---|---|---|
| | | 3 months | 6 months | |
| AGE-LDL Ab (OD) | 0.183 ± 0.4 (0–1.82) | 0.186 ± 0.34 (0.09–1.41) | 0.196 ± 0.39 (0.07–1.62) | 0.155 ± 0.23 (0.08–0.89) |
| Oxidized LDL-Ab (mg/l) | 91 ± 143 (13.9–655) | 107 ± 178 (20.4–720) | 99.4 ± 154 (16.4–558) | 81 ± 111 (12.5–414) |
| mLDL-IC (cholesterol) (mg/l) | 644 ± 188 (285–983) | 397 ± 117 (189–653) | 435 ± 133 (201–756) | 613 ± 189 (303–948) |
| mLDL-IC (apoB) (mg/l) | 118 ± 43 (32.9–179.8) | 83 ± 30 (29–133.9) | 87.2 ± 30 (30–143.2) | 114 ± 39 (44.7–177.8) |

Data are means ± SD (range). mLDL-IC (cholesterol) baseline versus 3 and 6 months of treatment, $P < 0.0001$; baseline versus washout, NS; 3 vs. 6 months, NS; 3 months versus washout, $P < 0.0007$; 6 months versus washout; $P < 0.0002$. mLDL-IC (apoB) baseline versus 3 and 6 months of treatment, $P < 0.0001$; baseline versus washout, NS; 3 vs. 6 months, NS; 3 months versus washout, $P < 0.0001$; 6 months versus washout, $P < 0.0002$. Ab, antibody.

isolated from mLDL-IC do not recognize native LDL and native LDL is not precipitated by polyethylene glycol (20)

Because of the lack of reliable assays for modified types of LDL in serum samples, we could not document a reduction in the levels of modified LDL. On the other hand, if the effect of simvastatin was limited to reducing the formation of mLDL, we should have detected an increase in free antibody levels as a consequence of reduced formation of IC. The fact that such an increase was not observed can be considered indirect evidence for a reduction in total mLDL antibody levels. An alternative explanation would be that the rate of antibody synthesis decreased as a consequence of the reduction in the formation of mLDL. This hypothesis is unlikely because the response to mLDL in these patients has been ongoing for an extended period of time and simvastatin therapy reduces but not completely suppresses the formation of mLDL. Considering that immune responses to protein antigens may persist for 10 years or longer without boosting, it is unlikely that a decrease in the rate of formation of antibodies takes place after such a short period of time, particularly when the inducing antigen is likely to remain present. The slight decrease in free mLDL antibody levels measured after washout, in turn, could reflect the capture of mLDL antibodies from the circulating pool by a rebound in the formation of mLDL. Therefore, the documented decrease in mLDL-ICs may be the consequence of a dual effect of simvastatin: a decreased generation of oxidized LDL and AGE-LDL and a reduced synthesis of antibodies to modified lipoproteins.

A reduced synthesis of mLDL antibodies could be explained by the immunomodulatory effects of statins (16). Atorvastatin and, to a lesser extent, lovastatin and pravastatin inhibit the upregulation of major histocompatibility complex II (MHC-II) molecules by antigen-presenting cells stimulated with interferon-γ. This effect is due to a transcriptional block of MHC-II expression but does not affect the constitutive expression of MHC-II. Because the activation of $T_H2$ helper cells assisting the humoral immune response involves interaction with MHC-II–associated peptides in activated antigen-presenting cells (22), a downregulation of MHC-II expression should have an indirect negative effect on B-cell activation, explaining the trend for a decrease in total antibody concentrations seen in pa-

**Table 4—*Correlations between mLDL-IC, lipoprotein levels, and mLDL antibody levels***

| | mLDL-IC (cholesterol) | mLDL-IC (apoB) | LDL cholesterol | Cholesterol | AGE-LDL antibodies | *P* |
|---|---|---|---|---|---|---|
| mLDL-IC (apoB) | | | | | | |
| Baseline | 0.9547 | | | | | <0.00001 |
| 6 months treatment | 0.9403 | | | | | <0.00001 |
| Washout | 0.8943 | | | | | <0.00001 |
| LDL cholesterol | | | | | | <0.0088* |
| Baseline | 0.5689* | 0.4777† | | | | <0.0332† |
| 6 months treatment | 0.7899 | 0.7506 | | | | <0.0002 |
| Washout | 0.5341 | 0.2695 | | | | <0.0185 |
| Total cholesterol | | | | | | <0.0314* |
| Baseline | 0.4704* | 0.4052 | 0.6496† | | | <0.0011† |
| 6 months treatment | 0.4185 | 0.2969 | 0.7934 | | | <0.0001 |
| Washout | 0.4234 | 0.1485 | 0.8754 | | | <0.00001 |
| AGE-LDL antibodies | | | | | | |
| Baseline | 0.1680 | 0.1951 | −0.1175 | −0.0914 | | |
| 6 months treatment | 0.2038 | 0.2780 | −0.1788 | −0.0109 | | |
| Washout | 0.0132 | 0.0622 | −0.1654 | −0.2785 | | |
| Oxidized LDL antibodies | | | | | | |
| Baseline | 0.1484 | 0.1170 | −0.1983 | −0.2132 | 0.3059 | |
| 6 months treatment | 0.3541 | 0.3443 | −0.1267 | −0.0021 | 0.5960 | <0.0148 |
| Washout | 0.2034 | 0.3006 | −0.2255 | −0.1535 | 0.5283 | <0.02 |

tients taking simvastatin. Although simvastatin was not one of the statins included in the study by Kwak et al. (16), there is no reason to believe that this statin would be different from all others.

It must be stressed that this possible effect of statins on the synthesis of mLDL antibodies would not have been apparent from the results of circulating antibody assays. The literature on this subject, recently reviewed by Lopes-Virella and Virella (6), is almost evenly distributed between reports suggesting that autoantibodies to mLDL are associated with or can predict progression of CHD and reports that fail to show such an effect or even show an inverse correlation between mLDL antibodies and CHD or other forms of atherosclerosis. We have previously demonstrated significantly increased levels of mLDL-IC in patients with diabetes and established CHD when compared with control subjects and nondiabetic patients with CHD (10). Of special note is the fact that the levels of free antibodies against oxidized LDL and glycated LDL were not significantly different in patients and control subjects. In another study performed with a type 1 diabetes cohort from the Pittsburgh Epidemiology of Diabetes Complications Study, we demonstrated that the levels of mLDL-IC measured in serum specimens obtained upon entrance into the study (when the patients were free of macrovascular disease) were significantly higher and the levels of free oxidized LDL antibodies were significantly lower in the group of patients who had CHD at the end of the study (12,13).

The mechanisms by which mLDL-IC may induce or accelerate the progression of arteriosclerosis have been the subject of detailed studies. Human autoantibodies to oxidized LDL and AGE-LDL are predominantly of the IgG1 and IgG3 subclasses (9), known to interact with higher affinity with Fc$\gamma$ receptors and to activate complement (23). Model ICs prepared with human LDL and rabbit antibodies promote cholesterol ester accumulation in macrophages and activate the release of proinflammatory cytokines (interleukin-1$\beta$ and tumor necrosis factor) and matrix metalloproteinases (21,24), well-known players in the inflammatory process of arteriosclerosis (25).

In conclusion, our results strongly suggest that the marked reduction in cardiovascular events induced by simvastatin may be secondary, at least in patients with diabetes, both to a decrease in LDL cholesterol and to a decrease in LDL oxidation/glycoxidation and autoantibody formation, both changes resulting in an inhibition of the formation of proinflammatory ICs, thus having a downregulating effect on the inflammatory component of atherosclerosis.

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