Coronary Artery Disease

Simvastatin Inhibits the Monocyte Expression of Proinflammatory Cytokines in Patients With Hypercholesterolemia

Domenico Ferro, MD,* Sandro Parrotto, MD,* Stefania Basili, MD,† Cesare Alessandri, MD,‡ Francesco Violi, MD*

Rome, Italy

OBJECTIVE	The purpose of this study was to assess if simvastatin has an anti-inflammatory activity in patients with hypercholesterolemia.
BACKGROUND	Simvastatin, an inhibitor of 3-hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) reductase, reduced cardiovascular events in patients with myocardial infarction and hypercholesterolemia.
METHODS	Sixteen patients with polygenic hypercholesterolemia were randomly allocated to diet (n = 8) or diet plus 20 mg/day simvastatin (n = 8) for eight weeks. Before and at the end of treatment period, lipid profile and monocyte expression of tumor necrosis factor-α (TNF) and interleukin-1β (IL-1β) were measured.
RESULTS	At baseline no difference in lipid profile and monocyte expression of TNF and IL-1β were observed between the two groups. In patients allocated to diet alone, no change in lipid profile and monocyte expression of TNF and IL-1β was seen. In patients with diet plus simvastatin, significant decreases of total cholesterol (-27%, $p < 0.02$), low density lipoprotein-cholesterol (-33%, $p < 0.02$), and monocyte expression of TNF (-49%, $p < 0.02$) and IL-1β (-35%, $p < 0.02$) were observed. At the end of treatment period, patients treated with simvastatin had lower cholesterol and monocyte TNF and IL-1β than did patients assigned to diet alone.
CONCLUSION	This study suggests that simvastatin possesses anti-inflammatory activity via the inhibition of pro-inflammatory cytokines TNF and IL-1β expressed by monocytes. (J Am Coll Cardiol 2000;36:427–31) © 2000 by the American College of Cardiology

High serum levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDL-C) have been associated with atherosclerosis and increased incidence of thrombotic complications, especially in patients with coronary heart disease (1–3). Interventional studies showed that cholesterol-lowering therapy reduces the risk of vascular events in primary and secondary prevention clinical trials (4,5).

Clinical trials with inhibitors of 3-hydroxy-methyl-glutaryl coenzyme A (HMG-CoA) reductase (statins) reinforced the concept that cholesterol is an important risk factor for cardiovascular complications. In fact, recent studies demonstrated that, in patients with elevated as well as average LDL-C levels, statins reduce the risk of cardiovascular events (6–9).

The mechanisms accounting for this effect include the regression and/or the inhibition of progression of atherosclerotic plaque, which, in turn, would prevent thrombotic events (10). However, changes of atherosclerotic plaques are usually observed after four years from statin administration (11), whereas only two years of such therapy are necessary to reduce cardiovascular events (6–9). Hence, alternative mechanisms have been considered for explaining the effects

of statins on atherothrombosis. Among these, the anti-inflammatory activity is particularly attractive because inflammation is considered an early step in the formation and progression of atherosclerotic plaque (12). Experimental models showed that cholesterol reduction by pravastatin is associated with diminished amount of inflammatory cells in the plaque (13); furthermore, hypercholesterolemic patients treated with HMG-CoA reductase inhibitors have diminished monocyte adhesiveness to endothelium (14).

Herein we report the first evidence that simvastatin reduces the monocyte expression of pro-inflammatory cytokines in patients with polygenic hypercholesterolemia.

MATERIALS AND METHODS

Subjects. We selected 16 patients affected by polygenic hypercholesterolemia (7 males and 9 females; age 52 ± 9 [35 to 70] years). Five patients were current smokers and four had therapeutically controlled hypertension. All subjects gave written informed consent to participate in the study. Exclusion criteria were diabetes mellitus, history of alcohol or drug abuse, body mass index >25, peripheral-, cardio-, and cerebro-vascular atherosclerotic diseases, concomitant inflammatory diseases, drugs modifying lipid metabolism, or blood coagulation.

Abbreviations and Acronyms

HDL-C	= high density lipoprotein cholesterol
HMG-CoA	= 3-hydroxy-methyl-glutaryl coenzyme A
IL-1β	= interleukin 1-beta
LDL-C	= low density lipoprotein cholesterol
TC	= total cholesterol
TNF	= tumor necrosis factor-alpha

Design of study. Patients attended our department after an overnight fast between 8:00 and 9:00 AM. After a rest period of at least 20 min, two blood samples were withdrawn from each patient, without stasis, from the antecubital vein using a 20-G needle and centrifuged at 1500 g for 10 min. Serum aliquots were immediately used to measure lipid parameters.

Hypercholesterolemic patients were randomly allocated to receive diet (total fat intake <30% of total calories, cholesterol <300 mg/day, polyunsaturated/saturated fatty acids ratio = 1.0) plus simvastatin (20 mg/day (n = 8; 3 men and 5 women; 49 ± 10 years) or diet alone (n = 8; 4 men and 4 women; 54 ± 9 years) for eight weeks. Safety biochemical parameters (serum aspartate aminotransferase, serum alanine aminotransferase, and creatine kinase), lipid profile, and cytokines expressed by monocytes were measured before and after the treatment period. The measurement of each variable was performed blind.

METHODS

Total cholesterol (TC) (Cholesterol-ES TM reagent, Beckman Instruments Inc., Galway, Ireland), triglycerides (Triglycerides-INT reagent, Beckman), high density lipoprotein cholesterol (HDL-C) (HDL-C reagent, Beckman) were assayed on all samples. The LDL-C was calculated according to Friedewald's formula (15).

Isolation and incubation of blood mononuclear cells. Peripheral blood mononuclear cells were isolated from heparinized venous blood using aseptic technique. Platelets were removed by using two-step centrifugation, once at 140 g and twice at 100 g in PBS at room temperature for 10 min. Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on lymphoprep (Nyegaard, Oslo Norway) at 1200 g for 20 min at 20°C (16). Monocytes, identified by May-Grünwald Giemsa staining comprised 16% to 22% (mean: 19%).

Monocytes (adherent cells) were obtained by incubation of the PBMCs for 90 min at 37°C in humidified atmosphere of 5% CO_2 in air in Petri dishes containing RPMI-1640, supplemented with 2 mmol/liter glutamine; lymphocytes (nonadherent cells) were removed by aspiration with a Pasteur pipette and washing of the dishes with warm media (17). The purified monocyte preparation contained 85% to 95% monocytes. After isolation, cells were washed twice in PBS and incubated at 2 × 10^5 cells/ml in RPMI-1640 at 37°C 5% CO_2 for 6 h. Cultures were performed in the presence of LPS (*Escherichia coli* OB11: B4, Sigma, St.

Louis, Missouri) at a final concentration of 0.4 ng/ml. At the end of the incubation period, the cells and media were separated by centrifugation (2,000 g for 15 min).

The cells were washed with Tris-NaCl buffer (0.1 mmol/ liter NaCl, 0.1% bovine serum albumin, pH 7.4) and then lysed in the same buffer by adding 15 mmol/liter *n*-octyl-β-D-glycopyranoside at 37°C for 30 min (18). Cells count and trypan blue exclusion were performed on cell suspensions after washing.

Tumor necrosis factor-alpha and inteleukin-1-beta. Monocyte tumor necrosis factor-alpha (TNF) was assayed in duplicate by and enzyme immunoassay (EIA) (Biokine TNF test kit, T-Cell Diagnostics, Cambridge, Massachusetts) (19). The detection limit was calculated to be 10 pg/ml. Intra- and interassay coefficients of variation were 8% and 9%, respectively. Interleukin-1-beta (IL-1β) levels (Genzyme Diagnostic, Cambridge, Massachusetts) were measured by enzyme-immunometric assay according to the manufacturer's instructions. Intra- and interassay coefficients of variation were 4% and 11%, respectively. The low detection limit of the assay was 6 pg/ml. All samples were tested in duplicate.

Statistical analysis. Previous study showed that sample size would have to consist of at least six patients in each group (alpha = 0.05 and 1-beta = 0.90) (20,21), assuming that simvastatin reduced monocyte tissue factor by 40%. We postulated that a same sample size was necessary to observe at least 40% reduction in monocyte TNF and IL-1β.

Statistical analysis was performed by chi-square statistic and by appropriate *t*-test. The linear regression test was used to study the different correlation. When necessary, log transformation was used to normalize the data. The effect of treatment was analyzed by two-way repeated measures analysis of variance (ANOVA). Data were presented as mean ± SD, median, and 95% confidence limits. Only two-tailed probabilities were used for testing statistical significance. A p value <0.05 was regarded as statistically significant (22). All calculations were made using personal computer software (Stat View II, Abacus Concepts, Berkeley, California).

RESULTS

At baseline no difference in TC, trygliceries, HDL-C, LDL-C, and monocyte expression of TNF and IL-1β was observed between the groups assigned to diet or diet plus simvastatin (Table 1). A significant direct correlation between monocyte expression of TNF and IL-1β was detected (rho = 0.84, p < 0.0001). After eight weeks of treatment, patients allocated to diet alone did not show significant changes in lipid profile and monocyte expression of TNF and IL-1β (Table 1). Conversely, in patients treated with diet plus simvastatin, a significant decrease of TC (−27%) and LDL-C (−33%) was observed, whereas triglycerides and HDL-C did not significantly change; furthermore, a

Table 1. Laboratory Indexes in Hypercholesterolemic Patients Treated for Eight Weeks With Diet or Diet Plus Simvastatin

| Assay | Diet | | | Diet Plus Simvastatin | | | Difference Between Groups after Treatment |
	Baseline (N = 8)	p Value*	8th Week (N = 8)	Baseline (N = 8)	p Value*	8th Week (N = 8)	p Value†
Total cholesterol (mg/dl)	268.6 ± 41.8 264 (200–338)	> 0.05	265.1 ± 42.4 263 (200–341)	289 ± 20.8 288 (265–331)	0.012	217.6 ± 31.5 211 (161–255)	0.045
Triglycerides (mg/dl)	107.7 ± 51 113.5 (48–206)	> 0.05	125.8 ± 41.2 123.5 (70–206)	148.2 ± 77.9 152.0 (60–305)	> 0.05	131.7 ± 60.8 134.0 (69–248)	> 0.05
HDL-cholesterol (mg/dl)	59 ± 9.8 57 (47–77)	> 0.05	57.4 ± 4.8 57 (52–64)	49.1 ± 11.9 49 (35–70)	> 0.05	52.5 ± 9.1 54 (38–62)	> 0.05
LDL-cholesterol (mg/dl)	188 ± 41.8 186 (127–261)	> 0.05	182.6 ± 49 170 (121–270)	210 ± 31.5 212 (162–259)	0.012	138.7 ± 32.8 137 (71–179)	> 0.05
TNF (pg/2×10^5 monocytes)	136.3 ± 95.1 118.8 (45.4–342.8)	> 0.05	147.6 ± 98.8 108.7 (38.0–350.2)	174.7 ± 106.3 134.7 (61.6–342.5)	0.017	72.4 ± 19.8 71.5 (42.2–110.6)	0.027
IL-1β (pg/2×10^5 monocytes)	74.6 ± 14.3 77.4 (47.9–92.6)	> 0.05	82.1 ± 13.9 78.6 (65.1–105.8)	88.9 ± 21.1 89.3 (60–118.4)	0.017	58.8 ± 9.6 57.7 (44.6–74.7)	0.0023

Data are: mean ± SD and median (range).

*p Value of Wilcoxon signed rank test; †p value of Mann-Whitney test used to compare the percentage change from baseline.

significant decrease of monocyte expression of TNF and IL-1β was detected (Table 1).

Figures 1 and 2 report on individual changes of monocyte expression of TNF and IL-1β in patients allocated to diet alone or diet plus simvastatin. In patients treated with diet plus simvastatin, median decrease of monocyte TNF was 49%: the reduction of monocyte TNF was observed in all but one patient. A similar finding was observed for IL-1β, whose median value decreased by 35%: also, for this variable the reduction was observed in all but one patient. Conversely, in patients treated with diet alone, both monocyte TNF and IL-1β remained somewhat stable over the

follow-up period. The data were also analyzed by two-way repeated measures ANOVA, which demonstrated a significant decrease of TNF ($p < 0.02$) and IL-1β ($p < 0.002$) after diet plus simvastatin treatment; the two-way interaction between TNF and IL-1β decreases were statistically significant (Rao R [2.27] = 6.64; $p < 0.004$).

DISCUSSION

Inflammation and atherosclerosis. There is a growing body of evidence suggesting that inflammation has a crucial role in the onset and progression of atherosclerosis (12).
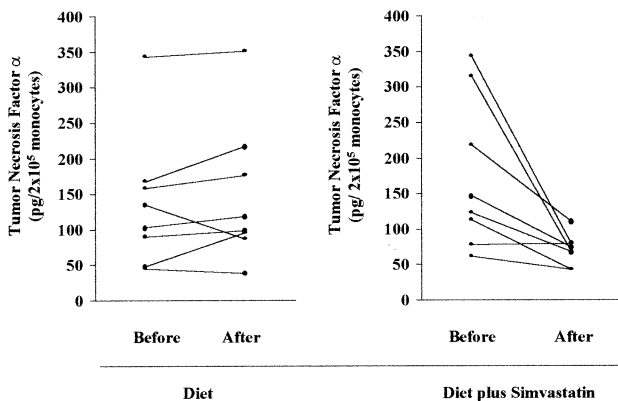

Figure 1. Tumor necrosis factor-α expressed by monocytes of hypercholesterolemic patients at the baseline and after diet **(left panel)** or diet plus sinvastatin **(right panel)** treatment.
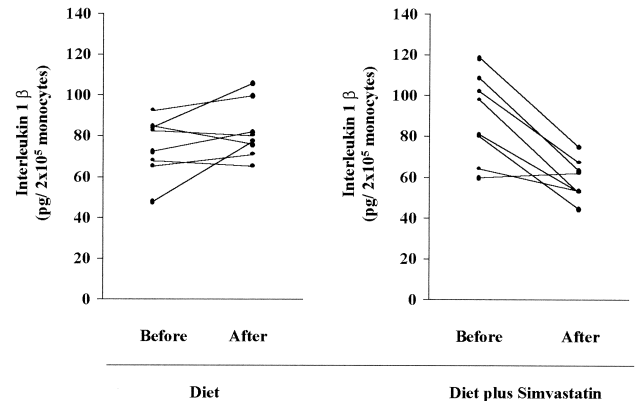

Figure 2. Interleukin-1β expressed by monocytes of hypercholesterolemic patients at the baseline and after diet **(left panel)** or diet plus sinvastatin **(right panel)** treatment.

The LDL-C oxidation by cells resident in the vessel wall represents the *primum movens* of a sequence of events leading to monocyte adhesion to endothelial cell with ensuing infiltration in the intima (23,24). Resident macrophages, in turn, would contribute to atherosclerotic progression by virtue of secretion of pro-inflammatory cytokines that may induce important changes in endothelial function and favor activation of clotting system. Accordingly, macrophages are present in the site of plaque rupture and seem to be responsible for acute coronary syndrome by activating clotting system through over-expression of tissue factor (25,26). The mechanism by which macrophages contribute to plaque rupture is still unknown; however, release of pro-inflammatory cytokines may induce plaque instability and in turn favor its rupture. Hence, knowledge of the mechanisms leading to pro-inflammatory cytokines release by macrophages may be potentially relevant to modulate plaque instability.

Recent studies suggested that cholesterol biosynthesis is associated with pro-inflammatory cytokine formation via mevalonate metabolites. In particular, protein farnesylation has been shown to induce posttranslation modification of G-proteins, including RAS, and to elicit activation of MAP kinase (27,28). Interestingly, incubation of macrophages with sodium phenylacetate, an inhibitor of mevalonate phyrophospahte decarboxylase, inhibited formation of pro-inflammatory cytokines such as TNF and IL-1β (29). A similar effect was observed by incubating macrophages with lovastatin, an inhibitor of HMG-CoA reductase; thus, lovastatin inhibited the monocyte expression of TNF and IL-1β, thus corroborating the importance of mevalonate pathway in the intracellular formation of pro-inflammatory cytokines (29). As this effect has never been demonstrated after supplementation with an inhibitor of HMG-CoA reductase we undertook this study to assess if inhibition of mevalonate pathway is associated with reduced inflammatory cytokines.

Simvastatin treatment and cytokines. We observed that, after eight weeks of supplementation with simvastatin, patients with polygenic hypercholesterolemia showed lower monocyte expression of TNF and IL-1β in comparison to baseline values, while no significant changes were observed in patients assigned to diet alone. The effect of simvastatin on monocyte activity was also reinforced by the fact that at the end of the treatment period, patients assigned to simvastatin had lower monocyte expression of TNF and IL-1β than did patients assigned to diet alone. Reduction of pro-inflammatory cytokines expression might have potential clinical implication because of the important role played by these cytokines in the inflammation of atherosclerotic plaque (12). In particular, the decreased expression of TNF may be relevant as TNF promotes formation of oxygen free radicals, thus contributing to the oxidative stress occurring within the vessel wall (30). Also, TNF represents an important link between inflammation and thrombosis inas-

much as it elicits macrophage expression of tissue factor, a protein of extrinsic clotting pathway that converts factor X to Xa (31). The decreased expression of TNF by macrophages after simvastatin treatment may contribute to explain our previous data (20) showing that simvastatin reduces in vivo thrombin generation by inhibiting the monocyte expression of tissue factor.

Conclusions. This study shows that simvastatin has anti-inflammatory property by inhibiting the monocyte expression of TNF and IL-1β. An intriguing corollary of this study is that cholesterol biosynthesis is a potential flogogen pathway that contributes to the formation of pro-inflammatory cytokines. Inhibition of cholesterol biosynthesis is therefore important not only for reducing intracellular cholesterol accumulation but also for inhibiting the formation of cytokines, which play an important role in favoring the atherosclerotic plaque instability and potentially contribute to oxidize LDL-C. Hence, we hypothesize that the reduction of inflammatory components of atherosclerotic plaque with eventual plaque stabilization may represent another mechanism accounting for the reduction of cardiovascular events observed after simvastatin treatment.

Reprint requests and correspondence to: Prof. Francesco Violi, MD, I Clinica Medica, Policlinico Umberto I, Viale del Policlinico, 00185 Rome, Italy. E-mail: violi@uniroma1.it.

REFERENCES

1. Gotto AM Jr., La Rosa SC, Hunninghake D, et al. The cholesterol facts. A summary of the evidence relating dietary fats, serum cholesterol, and coronary heart disease. Circulation 1990;81:1721–33.
2. Pekkanen J, Linn S, Heiss G, et al. Ten-year mortality from cardiovascular disease in relation to cholesterol level among men with and without preexisting cardiovascular disease. N Engl J Med 1990;322:1700–7.
3. Kannel VB. Range of serum cholesterol values in the population developing coronary artery disease. Am J Cardiol 1995;76:69–77.
4. Carlson LA, Danielson D, Ekberg I, Klintemar B, Rosenhamer G. Reduction of myocardial reinfarction by the combined treatment with clofibrate and nicotinic acid. Atherosclerosis 1977;27:81–6.
5. Frick MH, Elo O, Happa K, et al. Helsinki Heart Study: primary-prevention with gemfibrozil in middle-aged men with dyslipemia. N Engl J Med 1987;377:1237–45.
6. Scandinavian Simvastatin Survival Study Group. Randomised trial of cholesterol lowering in 4,444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S). Lancet 1994;344:1383–9.
7. Shepherd J, Cobbe SM, Ford I, et al. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. N Engl J Med 1995;333:1301–7.
8. Sacks FM, Pfeffer MA, Moye LA, et al. The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators. N Engl J Med 1996;335:1001–9.
9. Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group. N Engl J Med 1998;339:1349–57.
10. Vaughan CJ, Murphy MB, Buckley BM. Statins do more than just lower cholesterol. Lancet 1996;348:1079–82.

11. MAAS investigators. Effect of simvastatin on coronary atheroma: the Multicentre Anti-Atheroma Study (MAAS). Lancet 1994;344:633–8.
12. Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med 1999;340:115–26.
13. Williams JK, Sukhova GK, Herrington DM, Libby P. Pravastatin has cholesterol-lowering independent effects on the artery wall of atherosclerotic monkeys. J Am Coll Cardiol 1998;31:684–91.
14. Weber C, Erl W, Weber KS, Weber PC. HMG-CoA reductase inhibitors decrease CD11b expression and CD11b-dependent adhesion of monocytes to endothelium and reduce increased adhesiveness of monocytes isolated from patients with hypercholesterolemia. J Am Coll Cardiol 1997;30:1212–7.
15. Friedewald WT, Lewy RI, Fredrickson DS. Estimation of the concentration of low density lipoprotein cholesterol in plasma without use of the preparative ultracentrifuge. Clin Chem 1972;18:499–502.
16. Calmus Y, Robert A. Increased procoagulant activity in peripheral blood mononuclear cells from patients with liver cirrhosis. Thromb Res 1992;68:103–8.
17. Kornberg A, Blank M, Kaufman S, Shoenfeld Y. Induction of tissue factor-like activity in monocytes by anti-cardiolipin antibodies. J Immunol 1994;153:1328–32.
18. Meszaros K, Aberle S, Dedrick R, et al. Monocyte tissue factor induction by lipopolysaccharide (lps): dependence on LPS-binding protein and cd14, and inhibition by a recombinant fragment of bactericidal/permeability-increasing protein. Blood 1994;83:2516–25.
19. Scuderi P, Sterling K, Lam K, et al. Raised serum levels of tumor necrosis factor in parasitic infections. Lancet 1986;2:1364–5.
20. Ferro D, Basili S, Alessandri C, Mantovani B, Cordova C, Violi F. Simvastatin reduces enhanced monocyte tissue factor expression in patients with type IIa hypercholesterolemia. Lancet 1997;350:1222.
21. Pocock SJ. Clinical trials. A pratical approach. New York: Wiley, 1988.
22. Armitage P, Berry G. Statistical Methods in Medical Research. 2nd ed. Blackwell Scientific, 1990.
23. Steinberg D. Lipoproteins and the pathogenesis of atherosclerosis. Circulation 1987;76:508–14.
24. Diaz MN, Frei B, Vita JA, Keaney JF Jr. Antioxidants and atherosclerotic heart disease. N Engl J Med 1997;337:408–16.
25. Moreno PR, Bernardi VH, López-Cuéllar J, et al. Macrophages, smooth muscle cells, and tissue factor in unstable angina. Circulation 1996;94:3090–7.
26. Ardissino D, Merlini PA, Ariëns P, Coppola R, Bramucci E, Mannucci PM. Tissue-factor antigen and activity in human coronary atherosclerotic plaque. Lancet 1997;349:769–71.
27. Casey PJ, Solski PA, Der CJ, Buss JE. p21ras is modified by a farnesyl isoprenoid. Proc Natl Acad Sci U S A 1989;86:8323–7.
28. Kikuchi A, Williams LT. The post-translational modification of ras p21 is important for Raf-1 activation. J Biol Chem 1994;269:20054–9.
29. Pahan K, Sheikh FG, Namboodiri AM, Singh I. Lovastatin and phenylacetate inhibit the induction of nitric oxide synthase and cytokines in rat primary astrocytes, microglia, and macrophages. J Clin Invest 1997;100:2671–9.
30. Kizaki M, Sakashita A, Karmaker A, Lin CW, Koeffler HP. Regulation of manganese superoxide dismutase and other antioxidant genes in normal and leukemic hematopoietic cells and the relationship to cytotoxicity by tumor necrosis factor. Blood 1993;82:1142–50.
31. Nemerson Y. Tissue factor and hemostasis. Blood 1988;71:1–8.