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

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**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

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**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.
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% CO2 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% CO2 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 interleukin-1-β. 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-β (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, triglycerides, 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
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 = 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).
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 kinase (27,28). 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 (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 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.

**REFERENCES**