An ex vivo Study of Foam Cell Formation and Cholesterol Clearance in Macrophages Isolated from Patients with Hypertriglyceridemia and Low High-Density Lipoprotein Cholesterol Treated with High-Dose Simvastatin

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Abstract
High-density lipoproteins (HDL) are thought to be cardio-protective due to their role in decreasing cholesterol accumulation and in enhancing cholesterol removal from foam cells. Our study will examine foam cell formation and HDL-mediated cholesterol removal from macrophages isolated from patients with hypertriglyceridemia and low HDL. Peripheral blood monocytes and lipoproteins will be isolated from donors before and after 6–12 weeks on high-dose simvastatin (80 mg). The isolated lipoproteins from each donor will be incubated with their own macrophages for the study of foam cell formation and HDL-mediated cholesterol efflux. We expect to show that simvastatin increases HDL cholesterol, decreases triglyceride levels, and is associated with less foam cell formation and greater HDL-mediated cholesterol clearance.

Introduction
The protective role of high-density lipoproteins (HDL) in clinical disease has become more apparent with the findings from recent clinical trials. The results from the Copenhagen Male Study showed that hypertriglyceridemia and low HDL cholesterol levels were as powerful predictors of ischemic heart disease in men as were elevated low-density lipoprotein (LDL) cholesterol levels [1]. As well, results from the Air Force/Texas Coronary Atherosclerosis Prevention Study showed the benefit of treating clinically asymptomatic patients with average cholesterol and low HDL cholesterol levels with lovastatin [2].

In a study of American veterans, patients with low HDL cholesterol on treatment with gemfibrozil showed significant risk reductions for major cardiovascular events compared with control patients [3].

The mechanisms behind the cardioprotective effect of HDL is thought to be its participation in reverse cholesterol transport and its ability to limit LDL oxidation [4]. Reverse cholesterol transport is a process by which HDL removes cholesterol from foam cells and transports it back to the liver for disposal [4]. HDL cholesterol can be directly transferred to the liver via uptake by receptors.
(SR-BI) or through intermediate steps, such as the transfer of cholesteryl esters (CE) from HDL to very-low-density lipoprotein (VLDL) particles by CE transfer protein [4].

Much of our current understanding of the effects of HDL on cholesterol removal from foam cells is derived from studies using cultured cells, most commonly macrophages. CE-enriched macrophages (foam cells) are a predominant cell type within atherosclerotic lesions. Cultured human macrophages can become CE enriched by receptor and nonreceptor uptake of aggregated LDL or modified LDL [5]. Excess unesterified cholesterol (UC) is converted into CE by the action of an integral membrane protein located in the endoplasmic reticulum, acyl-CoA:cholesterol acyltransferase (ACAT). Human macrophages can release UC to medium that does and does not contain added cholesterol acceptors (i.e. HDL, apolipoprotein A1 particles). This process of release of UC to the medium is the earliest step in reverse cholesterol transport.

Our study proposes to examine foam cell formation and cholesterol efflux in macrophages isolated from patients with hypertriglyceridemia and low HDL cholesterol and on treatment with high-dose simvastatin. This ex vivo approach will help to clarify the effect of simvastatin on HDL composition and on the ability of HDL to reduce foam cell formation and enhance cholesterol removal from macrophages.

**Study Design**

**Inclusion/Exclusion Criteria**

Patients older than 18 years of age with fasting hypertriglyceridemia (200–400 mg/dl) and hypoalphalipoproteinemia (below 40 mg/dl) will be invited to participate in the study. Study patients will have normal renal, liver and thyroid function tests. Patients will not have diabetes mellitus, and will consume no more than two alcoholic beverages per week. Women of child-bearing age must be using an acceptable method of birth control. The expected enrollment will be 20 patients.

**Investigations**

**Baseline.** Each patient will donate approximately 150 ml of blood for lipid analysis [total cholesterol, triglyceride, LDL cholesterol, HDL cholesterol, apoB-100, apoA1, and lipoprotein (a)], comprehensive metabolic profile, thyroid function tests (free T4 and TSH), fasting insulin levels, and lipoprotein fraction and monocyte isolation. Subfraction analysis of VLDL, LDL and HDL will be performed by nuclear magnetic resonance spectroscopy (Lipomed Inc., Raleigh, N.C., USA).

**Lipoprotein Isolation and Characterization.** The lipoprotein fractions (LDL density 1.019–1.063 g/ml, and HDL density 1.063–1.210 g/ml) will be isolated by sequential ultracentrifugation [6]. The following components of HDL will be characterized: apolipoprotein content (denaturing gradient gel electrophoresis), triglyceride content (commercially available kit), phospholipid content (measurement of inorganic phosphate as described by Sokoloff and Rothblat [7]) and cholesterol content (unesterified and esterified mass measured by gas liquid chromatography).

**Cultured Macrophages.** Theuffy coat will be harvested from the separated blood. The white cells will be suspended in an equal volume of cold phosphate-buffered saline, and then underlayered with Ficoll Paque. Each individual donor’s macrophages will be cultured for 10 days and then induced to foam cell formation by incubation with their own aggregated LDL-C or modified LDL-C (acetylated LDL) [8]. Cholesterol efflux will be determined by incubating macrophage foam cells with their respective donor HDL-C and monitoring the decrease in intracellular cholesterol mass by gas liquid chromatography and the appearance of radiolabeled free cholesterol in the medium.

**Treatment Phase**

Donors will be treated with maximum doses of simvastatin (40 mg titrated to 80 mg daily after 6 weeks) for a total treatment period of 3 months. At both 6 and 12 weeks, lipoproteins and monocytes will be isolated and processed as described above. The plasma will be analyzed by nuclear magnetic resonance to document the shift and reduction of particles in the lipoprotein subfractions. Foam cell formation and HDL-C-mediated cholesterol efflux will be measured using each donor’s own macrophages, LDL-C and HDL-C.

**Posttreatment Phase**

At the end of 12 weeks, drug therapy will be withdrawn and 6 weeks later, patients will be asked to donate blood for lipoprotein analysis and monocyte isolation.

**Data Analysis**

Each experiment will be performed in at least triplicate wells. Statistical significance will be determined using Student’s t test with probability values <0.05.
Discussion

The study design is a novel one, in that we will be able to isolate monocytes and lipoproteins from individual donors with type IV dyslipidemia, with each person serving as their own control. We will be able to fully characterize the effects of high-dose simvastatin on lipoprotein particle number and size, as well as its composition. The results will show whether alterations in lipoprotein composition by simvastatin affect foam cell formation and/or HDL-mediated cholesterol efflux.

The effect of simvastatin on macrophage function will also be elucidated, as we will be examining the effect of the HMG-CoA reductase inhibitor on foam cell formation and cholesterol efflux. Other potential beneficial effects of HMG-CoA reductase inhibitors have been noted in the atherosclerotic process. The reductase inhibitors simvastatin and lovastatin were found to have inhibitory effects on rabbit intestinal ACAT [9]. One aspect of this study will be the measurement of ACAT activity in macrophages before and after simvastatin treatment. The results should determine whether simvastatin decreased ACAT activity and reduced foam cell formation.

It has been shown that high-dose simvastatin significantly increased HDL cholesterol levels and apolipoprotein A1 (the major protein moiety of HDL) as compared with atorvastatin [10]. During the course of our study, we will be able to assess the effect of high-dose simvastatin on changes in HDL composition, particle size, and on its ability to mediate cholesterol removal from foam cells. We expect these results will further clarify the beneficial effect of simvastatin on HDL metabolism and its role in reverse cholesterol transport.

References

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