Cellular Cholesterol Efflux Mediated by HDL Isolated from Subjects with Low HDL Levels and Coronary Artery Disease

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Objective - *The aim of this study was to verify whether HDL particles isolated from patients with coronary artery disease (CAD) and low HDL-C had diminished ability to promote cholesterol efflux from cultured cells compared with HDL isolated from subjects without CAD and with normal HDL-C.*

Methods - *Smooth muscle cells isolated from human aortas cultured and radiolabeled with ^{3}H-cholesterol were loaded with cholesterol and incubated with increasing concentrations of HDL isolated from 13 CAD patients with low HDL-C (CAD group) or from 5 controls without CAD (C group). Efflux of cellular cholesterol was measured by cellular depletion of radiolabeled cholesterol and by the appearance of ^{3}H-cholesterol into experimental medium expressed as a percentage of total labeled cholesterol.*

Results - *Cholesterol efflux increased with the amount of HDL present in the medium, and no difference was found between groups at various HDL protein concentrations: efflux was 28 ± 6.3% (C) and 25.5 ± 8.9% (CAD) with 25 µg/mL; 34 ± 4.3% (C) and 31.9 ± 6.6% (CD) with 50 µg/mL and 39.5 ± 3.5% (C) and 37.1 ± 4.4% (CAD) with 100 µg/mL, HDL.*

Conclusion - *Because the HDL fraction of CAD patients with low HDL-C have normal ability to extract cholesterol from cells of the vessel wall, it is suggested that low HDL-C atherogenicity should be ascribed to diminished concentrations of HDL particles rather than to the qualitative properties of the HDL fraction.*

Key words: coronary artery disease, cholesterol efflux, high density lipoprotein

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The HDL particle is constituted of phospholipids, cholesteryl esters, free cholesterol, triglycerides, and apolipoproteins (apo). Although apo E and Cs may also be present, apo A-I and A-II synthesized in hepatocytes and enterocytes are the main proteins in the HDL particles. While circulating, an HDL particle receives free cholesterol and phospholipids from the cells. This is the first step of reverse cholesterol transport, in which cholesterol is transported from peripheral tissues to the liver for excretion in the bile. Free cholesterol taken up by HDL particles is then esterified by the action of lecithin cholesterol acyl transferase (LCAT), using apo AI as a co-factor. In a process mediated by cholesteryl ester, transfer protein (CETP) HDL cholesteryl ester is transferred to VLDL and LDL, whereby it is captured and excreted by the liver [1].

Low HDL concentration (HDL-C) estimated both as low HDL cholesterol and apo AI is considered one of the major risk factors for CAD. Low HDL is mostly related to increased removal of the lipoprotein from the plasma rather then diminished lipoprotein synthesis. The increased removal from the plasma has been mostly ascribed to the composition of the HDL particles. Among the most common factors affecting HDL composition, CETP mutations are considered one of the major determinants of HDL concentration in a given population [2]. Genetic alterations in LCAT, apo AI, and other proteins involved in HDL metabolism can also result in diminished HDL levels [3]. Environmental factors, such as smoking, physical activity, diet composition, and caloric intake, are also important contributors to HDL composition and concentration in the plasma [4].

In many studies [5,6], the focus has been on the ability of peripheral cells from subjects with low HDL plasma levels to release cholesterol to the HDL fraction.

The opposing aspect, ie, the ability of HDL from subjects with low HDL levels to extract cholesterol from peripheral cells, has been scarcely explored.

The current study aimed to verify whether the HDL particles of CAD patients with low HDL-C had a diminished

ability to promote cholesterol efflux from a standard preparation of human aorta smooth muscle cells. Cholesterol efflux from the cells was determined in incubates with HDL fractions isolated from CAD patients with low HDL-C and from a control group of subjects with normal HDL-C concentration and without CAD.

Methods

Patients and controls were recruited from the ambulatory clinic of the Atherosclerosis and Coronary Unit of the Heart Institute (InCor) of the University of São Paulo Medical School, Brazil. Subjects gave written consent to participate in the study, which was approved by the Medical Ethics Committee of the Heart Institute.

Fasting morning blood samples were collected by venipuncture and centrifuged at 2500 g for 15 minutes at 4°C for plasma separation and were utilized for HDL-C isolation with the standard preparation technique by ultracentrifugation. HDL was isolated in the density interval of 1.125 to 1.21 g/mL and was subjected to heparin agarose chromatography to remove apolipoprotein E or B. HDL-C isolated from each patient was sterilized by filtration through 0.22 µm cellulose acetate membrane, and protein was quantified by the method of Lowry et al [7]. HDL particles were then added to experimental medium for efflux studies.

Human aortic smooth muscle cells were isolated from primary cultures of aorta fragments. Cells were plated at 15,000 cells per 16 mm culture dishes and maintained in Dulbecco´s modified Eagle´s medium (DMEM) containing 10% fetal bovine serum (FBS) 4mM glutamine 100U/mL penicillin, and 100 µg/mL streptomycin in a 37°C humidified incubator with 5% CO_2 as previously described [8]. To label cellular cholesterol, subconfluent cells were maintained in DMEM containing 10 % FBS and 0.5 µCi/mL ^{3}H-cholesterol until confluence. Labeled cells were subsequently loaded with 30 µg/mL nonlipoprotein cholesterol for 24 hours and then incubated with DMEM containing 1 mg/mL bovine serum albumin (BSA) to allow equilibration of cell cholesterol pools for 24 hours. Cells were then incubated with DMEM containing 1mg/mL BSA alone or with DMEM containing 1mg/mL BSA and increasing concentrations of HDL protein isolated from 13 subjects with low HDL cholesterol (HDL-C <35 mg/dL) and CAD (CAD group) or from 5 controls (HDL-C >40 mg/dL) and no CAD (C group) for 24 hours. Cell medium was collected, centrifuged for 10 minutes at 1500 x g, and aliquots taken for scintillation counting.

Cell layers were washed with PBS/BSA and cellular lipids were extracted with hexane:isopropanol (3:2, v:v) and separated by thin liquid chromatography (TLC). and developed in heptane:ether:methanol:acetic acid (80:30:3:2;v:v:v:v). Free and esterified cholesterol radioactivity were quantitated by scintillation counting. The efflux of labeled cholesterol from cells was measured by the appearance of ^{3}H-cholesterol into the culture medium in the presence or absence of HDL. Cellular cholesterol content (free and esters) were expressed as a percentage of total labeled cholesterol (medium + cellular content).

Each experiment was made in triplicate cultured dishes and results were expressed as mean + SD. Comparison between groups were analyzed by the paired Student *t* test. When not indicated, statistical significance were assumed for P value less than 0.05.

Results

Patient population is summarized in Table I. It shows the age, sex, and plasma lipids of both subject groups. The low HDL patients were older and their plasma triglyceride concentration was greater than that of the controls. Total and LDL cholesterol were not different .

Figure 1A shows the data of the labeled free cholesterol that was transferred to the culture medium in the presence of increasing amounts of HDL. On the other hand, Figure 1B and 1C show the data of labeled free and esterified cholesterol that remained in the cells while the amounts of HDL were being increased.

In Figure 1A, it is clear that the greater the amounts of HDL in the medium the greater the cholesterol efflux. It is also clear that the cholesterol efflux was unchanged regardless of whether the HDL in the medium was that from low or normal HDL cholesterol subjects. Accordingly, as seen in Figure 1B and C, both the free and esterified cholesterol that remained in the cells diminished proportionally to the amount of HDL present in the culture medium, and the depletion of the intracellular cholesterol was unrelated to the HDL being from normal- or from low-HDL cholesterol subjects.

Discussion

This study shows that HDL from low-HDL-C subjects with CAD is equally efficient as that of normal-HDL-C subjects without CAD in promoting the cholesterol efflux.

The transfer of cholesterol from the intracellular pools to the plasma membrane depends on the interaction between apo AI on the HDL surface and the ABCA-1 membrane protein [9,10]. The transfer of cholesterol by diffusion from the cell membrane to the medium is minimal. The bulk of the cholesterol is transferred to the HDL particles, by exchange with HDL phospholipids. Apparently, apo AI has no major

Table I - Patient Population and Lipid Profile

	Group C (n = 5)	Group HA (n = 13)
Age	48 ± 5 years	67 ± 7 years*
Sex	Male (n=5)	Male (n = 10) Female (n = 3)
Total Cholesterol	170 ± 30	194 ± 42
LDL	115 ± 25	114 ± 33
HDL	45 ± 3	30 ± 4*
Triglyceride	150 ± 20	231 ± 68*

* P<0.01 Student *t* test. Values are listed as means ± SD; total cholesterol, LDL: low density lipoprotein HDL: high density lipoprotein and triglyceride are expressed in mg/dL.

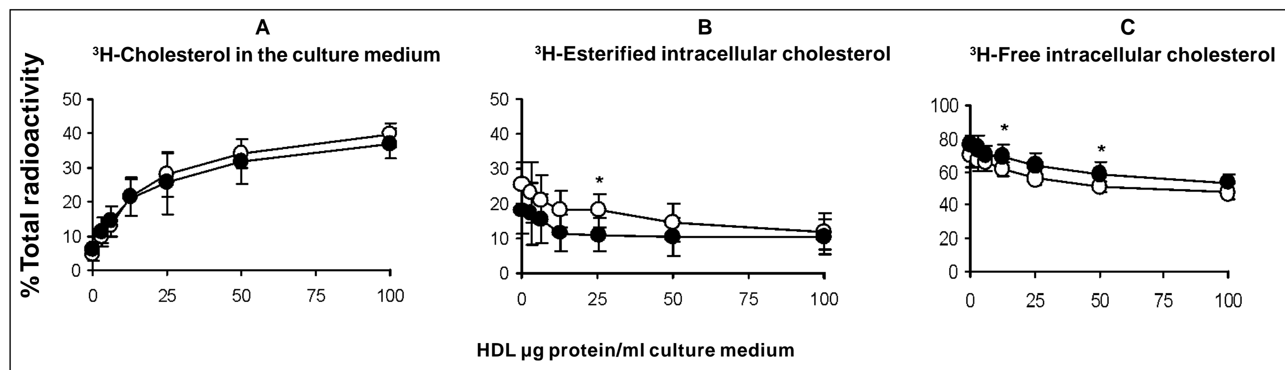

Fig. 1 - HDL-mediated Cholesterol Efflux and Intracellular Cholesterol - Preconfluent human aortic smooth muscle cells were incubated with DMEM containing 10% FBS and 0.4µCi/mL ^{3}H-cholesterol until reached confluence. Cells were cholesterol-enriched by incubation with 30 µg/mL nonlipoprotein cholesterol in DMEM containing 2 mg/mL BSA for 24 hours followed by incubation for 24 hours in DMEM containing 1 mg/mL BSA alone to allow equilibration of cholesterol pools. All cells were subsequently incubated with DMEM containing 1 mg/mL BSA alone or the same medium with increasing HDL protein concentrations ranging from 3.1 up to 100 µg/mL HDL protein isolated from Control patients with normal HDL plasma concentration (**C,○**) or low HDL-C and CAD (**CAD, ●**) for 24 hours. After 24 hours, medium and cellular content of free and esterified ^{3}H-cholesterol were measured as previously described [9]. ^{3}H-Cholesterol efflux (fig. 1A) and cellular ^{3}H-cholesterol esters (fig. 1B) and ^{3}H-free cholesterol (fig. 1C) are the mean ± SD of 3 dishes expressed as a percentage of total (medium + cell esterified and free cholesterol) radioactivity. * indicates $P < 0.04$ by Student t test compared with controls.

role in the extraction of cholesterol from the membrane to the lipoprotein [8].

It is noteworthy that the group of low HDL-C subjects differed from that of normal HDL-C subjects not only in respect to HDL-C levels but also in age and presence of CAD in the former group. Although HDL composition was not assessed in this study, it is presumed that it could differ between the 2 groups as long as those variables were present. Nonetheless, cholesterol efflux rates were not affected by the quality of the HDL preparations, as established in this protocol. This outcome, however, does not exclude the possibility that HDL from low-HDL-C subjects with apo AI mutations would have less ability to extract cholesterol from the cells. It is unlikely that our patients bear those mutations. Another important issue refers to the fact that, in our study, transfer proteins and CETP, which intervene in the reverse transport processes, were not at play in the experimental setup.

Our results suggest that the HDL particles of CAD patients with low HDL-C do not have a diminished ability to extract cholesterol from cells of the vessel wall. Therefore, the increased incidence of CAD in subjects with low HDL-C should be consequent not to altered composition of the HDL particles but indeed to the diminished concentration of those particles in the plasma.

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