ISSN 2304-2354
Number 2, Volume 44, 2013

INTERPLAY HDL PARTICLES AND INFLAMMATION MARKERS IN CORONARY STENOSIS

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Abstract

Introduction: Inflammation provides important links between risk factors and the mechanisms of atherosclerosis. The clinical value and the interrelationship of HDL was followed with acute phase proteins hsCRP, fibrinogen and SAA, with apo-lipoproteins, A-I and B and serum levels of cytokines in 198 patients with cardiovascular disease.

Methods: On exclusion criteria (MI, heart failure, CHD >2 years, anticoagulant therapy, 198 patients were recruited and were subdivided with stenosis <50% and >50% in accordance with CASS. Lipids were measured on OLYMPUS AU640. LDL-ox was determined by immunosorbent assay and SAA by immunonephelometry. Plasma MDA was assayed using a high performance liquid chromatography method based on the classic thiobarbituric acid (TBA) reaction. Serum levels of cytokines and hsCRP were analyzed by solid-phase chemiluminescent immunometric assay on DPC Immulite 1.000.

Results: Highest ox-LDL was associated with highest percent of stenosis and HDL is highly inversely related to the degree of stenosis. The HDL data were confirmed with a similar significant change of apo A(I) concentration from 134mg% with normal vessels and 123,0 mg with >50% stenosis. HDL-C and apo A(I) are directly inversely related to the degree of stenosis and directly to the acute phase proteins. TNFα ($p<0.1$) and IL6 are related to the degree of stenosis.

Conclusions: HDL-c is highly inversely related to the degree of stenosis, directly related to the APP and inversely to pro-inflammatory cytokines. SAA is responsible for the reassembly and dysfunction of HDL. Cytokines are mainly related to the dysfunction of HDL.

Keywords: cytokines, acute phase proteins, cardiovascular events, high density lipoproteins, and apoproteins.

Introduction

Atherosclerosis is an inflammatory process that can be initiated by infectious or endothelium damaging agents and aggravated by accepted and recognized risk factors such as LDL-C and ox-LDL (1,2). Many of the mechanisms involved in the process of atherogenesis are very similar to those seen in other chronic inflammatory diseases. Plasma markers of inflammation are increased in patients with atherosclerosis and varied with the site and extent of the disease, but do not provide diagnostic power above established risk factors (3). HDL is a truly independent predictor of risk and evidence now shows that increasing the precursor, apo A(I), is nearly always protective (4). There are many plausible and proven mechanisms by which HDL can inhibit atherosclerosis, including removal of cholesterol, anti-oxidation, anti-inflammation and very importantly, anti-monocyte adherence actions. Thus, an understanding of HDL metabolism and the structural changes as the remodeling are critical to explaining why increased HDL is protective (5).

Chronic infection creates a proinflammatory state which is characterized by long term elevation of cytokines and acute phase proteins and the conditions which are associated with the clinical outcome and manifestations of atherosclerotic disease (6). The most prominent cells that invade in evolving lesions are monocyte-derived macrophages and T-lymphocytes producing a wide array of soluble inflammatory mediators important in the initiation and perpetuation of the disease (7). The production of pro-inflammatory cytokines in chronic inflammation is the result of the T cell monocytes interactions and that mechanism seems to be blocked by the negative acute phase protein apolipoprotein A-I. Prospective epidemiologic studies have consistently demonstrated that markers of inflammation are independent predictors of cardiovascular events (8,9). The gap in preventive strategy prompted the search for new biomarkers in risk stratification of cardiovascular

ISSN 2304-2354
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events. Acute phase proteins, inflammatory responses, coagulation, platelet aggregation and genetic makers are major candidates (10,11). Chronic low grade elevations of CRP and serum amyloid A (SAA) are associated with increased cardiovascular risk. The major function of APP SAA is expressed through the remodeling of HDL (12,13) creating dysfunction of HDL. More detailed analysis of the utility of CRP and SAA in predicting CHD are needed (14,15) and studies addressing the pathogenic mechanisms are a help to clarify the associations.

The interplay of HDL in the inflammation process and in the expression of the acute phase proteins are the targets for new ways of treatment of the oxidative stress. In the present study, patients with angiographically documented stenosis were examined and studied in function of the extent of the disease. The oxidative stress biomarkers are evaluated in function of the degree of stenosis and related to the acute phase proteins. The clinical value is followed by the interplay and the interrelationship of HDL with the cytokines expression and the APP response are followed. The LDL and HDL particle size changes are analyzed and followed by apo A-I and apo B analysis. The study is very helpful in understanding the role of acute phase proteins in the reassembly of HDL particles and to understand the dysfunction of HDL during the acute phase of inflammation. A mechanistic model of the HDL interplay can be presented.

Materials and methods

Patients from the Department of Cardiology in the University Hospital Center "Mother Teresa", were selected based on exclusion criteria (MI, heart failure, CHD >2 years, anticoagulant therapy and antibiotic therapy since two months before). The patients underwent coronarography examination and were screened and subdivided in percentage of stenosis in accordance with CASS guidelines classifications.

On the basis of exclusion criteria we recruited 198 patients, males and females, between 45 and 75 years with CVD from the coronarography unit and 43 were screened with metabolic syndrome (MS). We subdivided the positive screened patients in two groups, group 1 non significant stenosis <50%, and group 2 significant stenosis >50%. Venous blood was sampled in serum tubes and heparinized-tubes after overnight fasting. Serum and heparinized-plasma samples were obtained by centrifugation (1.000xg, 10 min) and kept frozen at -80°C until assayed. On each sample, analytical procedures and measurements of the different lipid biomarkers were always performed within the same day to avoid repetitive freezing and thawing of the sample. All subjects gave their consent to storing blood for this study, which was approved by the Ethical Committee of Tirana hospital.

Methods

Serum ox-LDL concentration.

Serum ox-LDL concentration was measured by an enzyme-linked immunosorbent assay (ELISA) based on a murine monoclonal antibody, mAb-4E6 (16), specific for a neo-epitope in the aldehyde-substituted lysine residues of the apolipoprotein B-100 moiety of ox-LDL (Mercodia Uppsala, Sweden). The bound ox-LDL was detected with a peroxidase-conjugated anti-apolipoprotein B antibody and a colorimetric reaction with 3,3',5,5'-tetramethylbenzidine reading at 450 nm.

Plasma MDA concentration

Plasma MDA (malon-dialdehyde) was assayed using a high performance liquid chromatography (HPLC) method based on the classic thiobarbituric acid (TBA) reaction (17). An aliquot of 200 µl plasma was diluted with 750 µl H_3PO_4. 0.44 moL/L and mixed with 350 µL TBA, 42 mmol/L. After heating at 100°C for one hour, an aliquot of 20 µL was injected into the HPLC system (Merck LaChrom, Darmstadt, Germany). The TBA-MDA adduct was separated on a reversed-phase column (NOVA-pak C18 3.9 x 150mm. Waters, Milford, MA) and monitored by fluorescence detection (£ex = 515 nm,£m = 543 nm). The column was isocratically eluted at 1 ml/min with CH3oh/0.6% KH2PO4 pH 6.0 (30/70 v/v) The method was calibrated using 1,1,3,3-tetraethoxypropane as standard (18).

Other Biochemical parameters

Serum concentrations of total Cholesterol, HDL-cholesterol and Triglycerides were determined by commercially available colorimetric-enzymatic methods on Olympus AU640 analyser. Serum LDL cholesterol concentrations were calculated using the Friedewald formula. Apolipoprotein A-I (apoA-I) and apolipoprotein B (apo B) concentrations were measured by immunonephelometry on a BN Prospec nephelometer (Siemens Healthcare Diagnostics, Marburg, Germany).

Serum Amyloid A (SAA) was determined in human serum by means of particle immunonephelometry (Dade Behring).

We analyzed the serum levels of following cytokines, TNF, IL 6, IL 10, IL 8, IL 1beta, IL 2R and hs-CRP by solid-phase chemiluminescent immunometric assay on DPC Immulite 1.000.

Statistics

Data are presented as mean ±SD when normally described and median when not normally distributed. Statistical significance was performed using SPSS for Windows, 14.0 and MEDCALC version, 9.3. Statistical significance was considered at the level of $P<0.05$.

Results

The baseline characteristics of the study population group are described under patient identification in table 1. A significant higher number of patients were screened in group two, n=72 in group one against n=126. The average body weight was similar in both groups. There are very important data, the ratio of Males/Females increased significantly p>0.01 in group II with >50% stenosis and confirm the gender relationship to disease development. It is well known that males have higher risk for CV-events and females are more protected. We observed a significant low percentage of diabetes in the group with >50% stenosis, although diabetes is a primary risk factor for arterial damage (19). As expected hypertension is significantly (p<0.001) higher in the patients group with >50% stenosis and confirm earlier inclusions in the guidelines regarding blood pressure. Also in the lines of prospective studies a higher percentage of smokers were screened in the group with the highest percentage of stenosis.

Serum total cholesterol and LDL-c were not significantly different between the two groups of stenosis and are rather near the normal zones (Table nr.2). LDL-ox was 77±28 in group I and 78±30 in group II and is linear related to LDL-C, p<0.001. Apo B is higher in group II however not significantly, there is however a more direct relation to LDL-ox with a p<0.001. The increase of apo B suggests a higher number of LDL particles with a smaller size increasing the small LDL particles. An exceptional change in HDLs was observed, the lowest values (39mg%) were measured in patients with > 50% stenosis and the highest values (44 mg%) in the group with <50% stenosis. The HDL data were

Table nr.1 Patient identification; baseline characteristics of the study population, group I < 50% stenosis, group II > 50 stenosis

Patients	group I	group II	p value
Sample size	72	126	
Male/Females	54% (males)	79%	p>0.01
Age	54	59	
Weight	78,09	78,8	NS
Diabetes	59%	26%	p<0.001
Hypertension	65%	26%	p<0.001
Smoking	35%	68%	p<0.001

Table nr.2 Lipid risk factors analysis in function of % stenosis (<50% group I against >50% group II)

CVD Risk factors	Group I	Group II	p value
TC (mg/dl)	193±56	186±49	NS
TG (mg/dl)	138±66	163±118	p<0.1
LDL-c (mg/dl)	118±45	114±37	NS
LDL-ox (U/L)	77±28	78±30	NS
MDA (mM)	2.4±0.9	2.2±1.4	NS
HDL (mg/dl)	44±9	39±8	p<0.001
Apo A(I) (mg/dl)	134±25	123±20	p<0.001
Apo B (mg/dl)	95±26	100 ±31	NS

Table nr.3 Acute phase proteins in function of the degree of arterial stenosis, group I <50% against group II >50%

Acute Phase protein	Group I	Group II	p value
Fibrinogen (mg/dl)	304±81	330±88	p< 0.04
SAA (mg/dl)	1.13±0.2	3.79±1.2	p < 0,10
hsCRP (mg/dl)	0.58±0.8	0.85± 0.6	p < 0.05

ISSN 2304-2354
Number 2, Volume 44, 2013

confirmed with a similar significant ($p > 0.001$) change of Apo A(I) concentration from 134 mg% in group I and 123 mg% in the arteries with more than 50% stenosis.
We followed the acute phase response during inflammation and there was a linear relationship to hsCRP, fibrinogen and degree of stenosis, both acute phase proteins increased significantly (table nr.3). Fibrinogen and hsCRP were more significant than SAA which correspond with literature data (20). hsCRP increased from 0.58mg/dl to 0.85 mg/dl. Both APP, hsCRP and fibrinogen, showed a significant inverse relationship to HDL and especially to apo A(I) peptide ($p<0.001$). We investigated also the relationship between CV-risk factors and the APP, two important significant relations were observed between BMI and SAA, $p < 0.001$ and hypertension was significantly related to hsCRP $p<0.05$.
In table (4) we summarize the most important significant data between the lipid risk factors, the oxidative stress and the acute phase proteins. HDL and especially Apo A1 and MDA and not apo B are highly related to hs-CRP and SAA is only significantly related to apo A1, which is very important for further clarification of the role of HDL response on acute phase inflammation and to understand the dysfunction of HDL by remodeling. In the remodeling process of HDL smaller particles are processed with a great loss of anti-atherogenic properties of HDL.

Table nr.4 The most important significant relationships between the CV lipid risk factors and protein inflammation markers

CV- lipid risk factors	hsCRP *P value*	SAA *P value*
HDL	<0.004	0.276
Apo (A-I)	<0.0001	<0.009
Apo B	0.395	0.351
MDA	<0.008	0.226
LDL-ox	0.367	0.533

To understand the role of cytokines in the degree of stenosis the cytokine concentrations in both groups were determined and we calculated an increase of IL6 and of TNFα in function of percentage stenosis (Table nr.5). To understand the role of HDL in the inflammation process we analyzed the relationship of HDL to the individual cytokines (Table 6). All investigated cytokines except for IL10 are negatively correlated to HDL and they are significant for IL2R, IL6 and IL 8. The cytokines were also studied in function of the APP and we calculated the relationship. There was a linear relation between IL 6 (<0.0001), IL 8 ($p<0.01$), IL 10 ($p<0.02$) and TNFα ($p<0.01$) and hsCRP. Also between SAA and Il 6 we observed a highly linear relationship and they were less significant between SAA and IL 10 ($p<0.03$) and TNFα ($p<0.05$).

Table nr.5 The cytokine concentration in function of percent stenosis

Cytokines	Group I	Group II	P value
IL 10 pg/ml	4.19±1.7	4.79±4.5	NS
IL-2R (U/ml)	724±333	778±346	NS
IL 6 pg/m	5.92±3.8	6.50± 4,1	p< 0.1
IL-8 pg/ml	11.03±8.2	11.37±7.8	NS
TNFα pg/ml	16.90±11.02	14.78±9.4	p< 0.1

Table nr.6 Correlation of HDL to different cytokines

CV-risk Factor	Cytokines	P value
HDL	IL 10	p< 0.2
HDL (-)	IL 2R	p< 0.002
HDL (-)	IL 6	p< 0.001
HDL (-)	IL 8	p< 0.01
HDL (-)	TNF	p< 0.9

Discussion

Atherosclerosis, the major risk for coronary heart disease events is no longer considered as a lipid disorder. It is a process of dynamic interactions, where increased cytokines, cell adhesion molecules and acute phase proteins (hsCRP and SAA) are key players in the CV-events (20).
Baseline characteristics of the study population, group I < 50% stenosis, and group II > 50 stenosis, demonstrated important significant differences. There are more females in group I as we know from gender differences (21). The percentage diabetes are higher in group I (59%) than in group II (26%), which is in opposite regarding the risk factors guidelines for cv-events. However the percentage hypertensions 65% in group I against 75% in group II are significant higher in the patient group with > 50% stenosis, those results are in accordance with literature data. We analyzed also the influence of smoking on the degree of stenosis and we observed a higher percentage of smokers in group two. Low-density lipoproteins (LDLs) are susceptible to structural modifications by oxidation, particularly the small dense LDL particles (22). Oxidized LDL (LDL-ox) formation in the subendothelial space of the arterial wall is a key initiating step in atherosclerosis because it contributes to foam cell generation, endothelium dysfunction, and inflammatory processes. The Asklepios study (23), investigating 2524 healthy middle-aged subjects showed that circulating LDL-ox is affected

by many biological and lifestyle factors, as well as subclinical atherosclerosis. Baseline characteristics of the Asklepios study population showed for LDL-ox concentration an average of 91.5±38.4 (U/L) for males and 100.7±38.8 (U/L) for females. The included patient data are lower (77±28 U/L) and there was no difference inside the % stenosis, however the LDL-ox is significantly related to TC, and LDL-C ($p < 0.001$). We measured a significant increase of ferritine in function of % stenosis ($p < 0.05$), group I of the patients has an average of 138±83 mg/dl against 194±83 mg/dl. Oxidation of PUFA by redox-active metals as iron (Fe^{2+}) is considered the initiating step in LDL oxidation (18) generating the formation of malondialdehyde (MDA). MDA is related to ischemic syndromes and more related to inflammation and may be a marker of plaque instability. In our study we observed a linear association between Ox-LDL, LDL-c and MDA, this suggest that plasma MDA is direct related to the LDL-c concentration. MDA was on the same concentration level in function of the % of stenosis. Apo B results confirm the observations of the obtained data on low density lipoproteins. The increase of apo B/LDL-c in function of percentage stenosis confirms the changes of the particle size distribution resulting in a higher percentage of small LDL particles (22,24).

One of our main data demonstrated however a significant decrease of HDL in function of the degree of stenosis, which is confirmed by the apoprotein AI analysis. One of the main results in our study is the relationship of HDL to the degree of stenosis. How lower the HDL concentrations as higher the arterial stenosis. Several prospective epidemiological studies provided overwhelming evidence that a low plasma HDL-c is a major, independent risk factor for the development of an acute coronary event. Studies in patients with rare disorders of HDL metabolism and in genetically modified animal models support a causal relationship between low HDL and development of atherosclerotic vascular disease (25). HDL has the unique ability to remove cholesterol from tissues and transport it to the liver (26). HDL has a number of antiatherogenic properties such as protection of the endothelium, prevention of LDL oxidation and there is mounting evidence that anti-inflammatory properties may protect against atherosclerosis (20). Therefore we studied the APP regarding the process of stenosis. Only hsCRP and Fibrinogen are significantly related to the degree of stenosis. HsCRP related very well and significant to HDL, apo A(I) and MDA. SAA related only significantly to Apo (A-I). Our data confirmed the function of SAA in the reassembly of HDL and the role of the APP in the dysfunction of HDL (27). SAA interact with HDL during the acute phase of inflammation and apo A-I is removed and replaced by the SAA particles. The remodeling of HDL is responsible for the dysfunction of HDL changing to a pro-atherogenic particle. The cytokines were inversely related to HDL and especially to Apo (A-I), only IL 10 was directly related and only IL 6 was significantly related to the degree of stenosis. The acute phase response can impair the anti-inflammatory functions of HDL. These results suggest and confirm that cytokines decrease apo A(I) expression via inhibiting of PPARα activation and increase acute phase proteins (SAA), via the nuclear factor NF-κB (27). Cytokines decrease also the expression of apo A-1, through a replacement of apo A-1 in HDL by SAA. Inflammation decreases HDL and converts HDL to a more pro-atherogenic form. The mechanistic model of the function of HDL is show in figure nr.1 and explains the interplay and the interaction of HDL and the inflammation markers.

Our data confirm that LDL-C and Ox-LDL are key players in cardiovasular events and are essential in

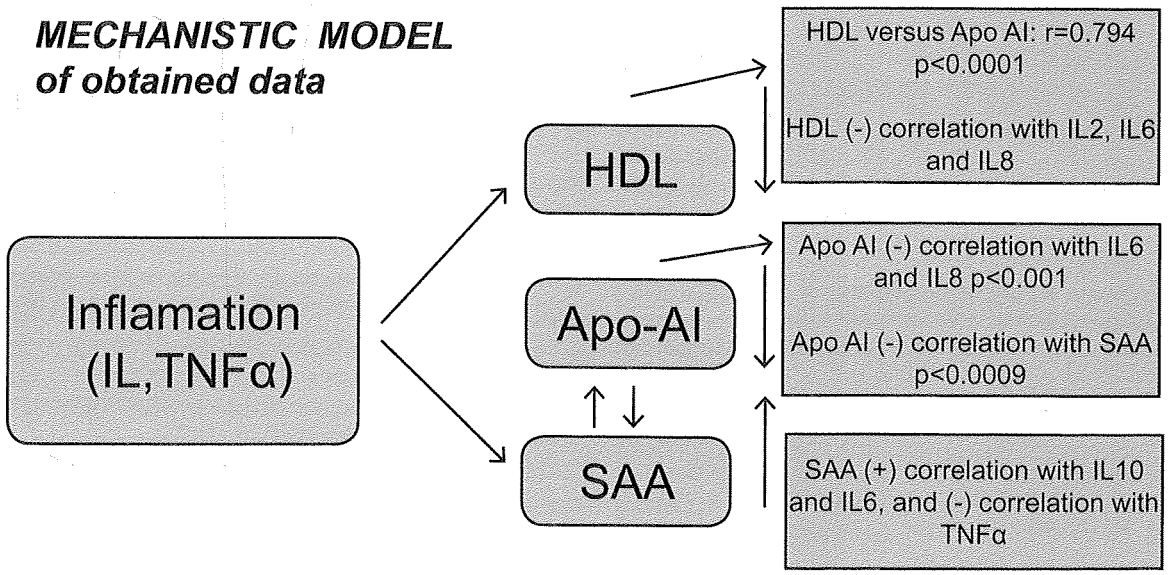

Figure nr.1 The mechanistic model of HDL remodeling during the acute phase of inflammation

diagnosis and treatment. HDL and inflammation factors, inversely related to HDL, are the new targets for treatment. Finally cytokines are related to HDL decrease and to the dysfunction of HDL converting into a more pro-atherogenic form by an exchange of SAA molecules with apoprotein A(I).

Ox-LDL plays a more significant role in the extension of the stenosis and seems less important at the higher degree of stenosis. HDL on the other hand plays an important role over the whole process of arterial damages and there is an inverse linearity between the percent increase of stenosis and the decrease of the HDL concentration which is confirmed by the Apo-A1 peptide concentration.

Conclusion

HDL-c is highly inversely related to the degree of stenosis, directly related to the APP and inversely to pro-inflammatory cytokines. SAA is responsible for the reassembly and dysfunction of HDL. HDL and cytokines are key players in the reverse cholesterol transport. Our major conclusions are LDL-c; ox-LDL is accepted essential in the cardiovascular events and they are key players in diagnosis and treatment. HDL and inflammation factors are the targets for new ways of treatment of the oxidative stress. Cytokines are mainly related to the dysfunctions of HDL.

Acknowledgment: We thank the financial grant support of the BFOS –Brugge.

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