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ORIGINAL ARTICLE

Circulating Levels of Lectin-Like Oxidized Low-Density Lipoprotein Receptor-1 are Associated with Inflammatory Markers

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Abstract Lectin-like oxidized-low-density lipoprotein receptor-1 (LOX-1) is increasingly linked to atherosclerotic plaque formation and the soluble form of this receptor may reflect activities of disease. We investigated the associations among levels of sLOX-1, oxidized-low-density lipoprotein (ox-LDL), cytokines and the extension of atherosclerosis in patients with coronary artery disease (CAD). Lipid, TNF-α, IL-6, C reactive protein (CRP), ox-LDL, peroxy radical and sLOX-1 levels were measured in 29 controls and 60 patients with CAD, 30 of which with one or two vessels involved (group 1), and 30 patients with three or four vessels involved (group 2). The serum levels of sLOX-1 were significantly and progressively higher in group 1 [611 (346–1,313) pg/ml, median (interquartile range)] and in group 2 [2,143 (824–3,201) pg/ml] than in control subjects [268 (111–767) pg/ml]. LOX-1 levels positively correlated with IL-6 ($r = 0.38$, $P = 0.0042$), TNF-α ($r = 0.38$, $P = 0.0037$), CRP levels ($r = 0.32$, $P = 0.027$) and age ($r = 0.25$, $P = 0.048$). In the multivariate analysis TNF-α resulted the only independent determinant of LOX-1 serum levels (β-value = 0.304, $P = 0.017$). These findings suggest that sLOX-1 levels are up-regulated during CAD progression and are associated with inflammatory markers. The measurement of the circulating soluble form of this receptor may be potentially useful in predicting CAD progression in humans.

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Introduction

Lectin-like oxidized-low-density lipoprotein receptor-1 (LOX-1) was initially identified as the major receptor for ox-LDL (oxidized low-density lipoprotein) in endothelial cells and was later found also to have an inducible expression in macrophages and smooth muscle cell [1, 2]. Besides ox-LDL, LOX-1 can recognize apoptotic/aged cells, activated platelets, and bacteria, implying versatile physiological functions as a "scavenger" receptor. LOX-1 activation by ox-LDL binding stimulates intracellular signalling, gene expression and production of superoxide radicals [3, 4]. In vivo, its expression is enhanced in pro-atherogenic settings including, hypertension, hyperlipidemia, diabetes, and atherosclerosis. Transgenic mouse models for LOX-1 overexpression or gene knockout suggests that LOX-1 contributes to atherosclerotic plaque formation and progression [5, 6]. Besides being involved in the activation of endothelial cells, LOX-1 is also involved in the transformation of smooth muscle cells, and accumulation of lipids in macrophages, resulting in cell injury that facilitates the development of atherosclerosis.

Like many cell-surface receptors with a single transmembrane domain, LOX-1 can be cleaved at the juxtamembrane region, most likely by serine proteases, and secreted in a soluble form [7]. Since elevated levels of soluble receptors in plasma may reflect increased expression of membrane-bound receptors and disease activities, circulating soluble LOX-1 (sLOX-1) is increasingly viewed as a vascular disease biomarker and

a potential therapeutic target in heart attack and stroke prevention [8].

Serum sLOX-1 have been shown to be increased early in patients with acute coronary syndrome, suggesting that it may reflect vulnerable unstable atherosclerotic plaques with enhanced proinflammatory and protease activities [9]. Recently, it has been shown that sLOX-1 correlated with oxidative stress markers in stable coronary artery disease [10]. However, the association of circulating sLOX-1 with the extension of CAD and inflammatory markers has not been properly addressed. We therefore attempted to clarify the link between sLOX-1 with plasma ox-LDL, cytokines and the extension of coronary atherosclerosis in patients affected by CAD.

Methods

The study included 60 patients admitted to our Institute because of documented or suspected ischemic heart disease and who, after undergoing coronary angiography, were diagnosed as being affected by coronary artery disease (CAD). Our controls were 29 healthy subjects who had not undergone coronary angiography. Physical examination and routine laboratory tests excluded the presence of other major disease (e.g. diabetes, hypertension, renal or liver diseases, infectious, chronic inflammatory or immunologic diseases, or malignancies). Exclusion criteria were any acute cardiovascular events during the last 3 months before hospitalization, and assumption of statins, other hypolipemic therapy, vitamins or antioxidant dietary supplements. Prior to drawing blood, patients were taken off antianginal medication, while sublingual nitrates were allowed only in the case chest pain were to persist for more than 3 minutes. Of the initial patients, 4 required sublingual nitrates and were therefore excluded from the study. All other medication, except antiplatelets, was suspended 7 days before taking the blood sample.

All subjects gave written informed consent for their participation in the study which was approved by the local ethics committee and conforms to the principles outlined in the Declaration of Helsinki.

Of the 60 patients enrolled, 17 had a reduced lumen diameter in one coronary artery, 14 patients had two arteries involved, 15 patients had three arteries involved and 14 patients had four arteries involved; in each case the mean reduction lumen was at least about 50%.

Analytical Methods

All blood samples were collected at 4 °C the day before the cardiac catheterization and were immediately centrifuged. Plasma or serum samples were stably stored at −80 °C for no more than 15 days. Plasma concentrations of TC, HDL and Triglycerides (Tg) were determined by standard laboratory methods. The concentration of LDL was calculated using the Friedewald equation.

To measure the generation of peroxy radicals—the initial products of the reaction between free radicals and oxygen—a colorimetric method (D-Roms test, Diacron international, Italy) was used as previously described [11]. This test is based on the ability of transition metals to catalyze in the presence of peroxides with the generation of free radicals that are trapped by an alchilamine whose reaction yields a colored radical detectable at 505 nm. The results are expressed as Carratelli Units (UC) (1 UC = 0.08 mg H_2O_2/dl).

High sensitivity (hs)-IL-6 and hs-TNFα were quantified using sandwich ELISA kits (Biosource International, CA) according to the manufactures' instructions. Inter-assay coefficients of variation was 7.8%.

High sensitivity C reactive protein (hs-CRP) was measured by the Immulite System (Diagnostic Products Corporation, CA).

Ox-LDL was assessed by a sandwich ELISA kit (Mercordia AB, Sweden) according to the manufactures' instructions. The inter-assay coefficient of variation was 4.7%.

Serum LOX-1 levels were measured by an in-house double-sandwich ELISA kits. Briefly, 96-well microplates (Nunc-Immuno Plates Maxisorp, Nunk, Roskilde, Denmark) were coated with monoclonal anti-human LOX-1 antibody (R & D systems, Minneapolis, MN) (0.25 μg/ml) in coating buffer (10 mmol/l PBS, pH 7.2) and incubated overnight at room temperature (RT). The serum specimens were initially inactivated at 56 °C for 30 min to block non-specific protein binding sites. Four rinses with washing buffer (PBS containing 0.05% Tween 20, PBST) followed each incubation step. After blocking with reagent buffer (1% BSA in PBS) at RT for 1 h, 100 μl of heat-inactivated sample (diluted 1:2 in reagent diluent) was added, and incubated at RT for 2 h. Recombinant human LOX-1 (R & D systems) was used as the standard in a concentration range of 100–10,000 pg/ml. Then, 100 μl of polyclonal goat anti-human LOX-1 antibody (R & D systems) (0.25 μg/ml) was added and incubated at RT for 2 h. Next, 100 μl of rabbit antigoat-HRP antibody (Santa Cruz Biotechnology, Inc., CA) (diluted 1:400) was added and incubated for 1 h at RT. Finally, 100 μl of tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO) was added. After 5–15 min, 50 μl of 2 mol/l sulphuric acid was added to stop the reaction, and optical density at 450 nm determined by an ELISA plate reader. The inter-assay coefficient of variation value was 7.2%. The lower limit of detection for LOX-1 was 30 pg/ml.

Statistical Analysis

Data are given as the mean ± SD. Variables with a skewed distribution are expressed as median and interquartile range. Appropriate variable transformations were made to reduce the skewness of the data, such as natural logarithmic transformation for sLOX-1, TNF-α, IL-6 etc. Differences among different groups of patients and controls were evaluated by one-way ANOVA followed by Bonferroni post hoc test for continuous variables and by X^2 test for noncontinuous variables. Relationships between serum sLOX-1 and other factors were assessed by Pearson correlation analysis. In order to evaluate the factors affecting sLOX-1 levels, all variables significantly associated with sLOX-1 were included in the multiple regression analysis, plus gender and BMI as confounding factors. A P value lower than 0.05 was considered statistically significant.

Results

To determine whether the circulating parameters varied with the severity of the disease, we divided the patients into two groups. Patients with one or two vessels involved were clustered in group 1, while patients with three or four vessels involved were clustered in group 2. Table 1 summarizes age, gender and lipid profiles in each group of patients and controls. Controls and patients were comparable for sex, age, BMI, pressure, glycemia and triglycerides, while HDL values were more elevated in controls than in patients. Instead, total cholesterol and LDL values resulted significantly lower in the group 2 compared to the controls (Table 1).

Circulating sLOX-1, as well as peroxy radical, CRP, TNF-α, IL-6 levels resulted significantly higher in patients than controls and tended to increase with the severity of CAD (values of group 2 were higher than values of group 1) (Table 2). Instead, ox-LDL values were significantly higher in group 1, while those of the group 2 resulted comparable to control values (Table 2).

As shown in the Fig. 1a–d, LOX-1 serum levels positively correlated with TNF-α ($r = 0.38$, $P = 0.0037$), IL-6 ($r = 0.38$, $P = 0.0042$), CRP levels ($r = 0.32$, $P = 0.027$) and age ($r = 0.25$, $P = 0.048$), and did not correlate with peroxy radicals, ox-LDL, and with all the other parameters reported in Table 1. The multivariate analysis revealed that, after correction for confounding factors, TNF-α was the only determinant of LOX-1 levels (β-value = 0.304, $P = 0.017$).

Discussion

Atherosclerosis is associated with oxidative stress, inflammation, and upregulation of LOX-1. We found that sLOX-1 is significantly more elevated in patients affected by CAD than in controls and that this increase correlated with the number of affected vessels.

This increase raises the question of the cell source and the triggers for this soluble receptor. Although we do not know whether the measured sLOX-1 levels in serum are related to tissue LOX-1 values, some studies have highlighted that circulating concentrations of this receptor are indicative of endothelial-surface bound receptor levels [9, 12]. In vitro, the cell-surface expression of LOX-1 is induced by many inflammatory cytokines, oxidative stress,

Table 1 Biochemical and clinical features of study subjects

	Controls	Patients		*P*
		Group 1	Group 2	
Age (years)	62.0 ± 13.6	65.8 ± 13.0	67.9 ± 12.5	n.s.
Gender (female, male)	8/21	7/24	4/25	n.s.
BMI (kg/m^2)	26.3 ± 0.9	26.8 ± 4.2	25.0 ± 5.7	n.s.
Systolic pressure	130.0 ± 19.8	127.7 ± 23.9	121.6 ± 21.5	n.s.
Diastolic pressure	85.0 ± 9.5	68.7 ± 10.6	70.9 ± 8.5	n.s.
Glycemia (mg/dl)	98.96 ± 30.9	86.6 ± 11.7	107.4 ± 34.4	n.s.
Triglycerides (mg/dl)	90.8 ± 41.9	117.6 ± 43.2	111.3 ± 62.8	n.s.
Total cholesterol (mg/dl)	194.0 ± 24.6	186.6 ± 33.2	155.1 ± 45.8*,§	0.0007
HDL (mg/dl)	50.7 ± 15.9	38.3 ± 8.9§	35.4 ± 11.7**	0.0001
LDL (mg/dl)	124.1 ± 18.2	124.5 ± 28.7	97.4 ± 36.7#, †	0.0017

Group 1 patients with one- or two-vessel coronary artery disease; *Group 2* patients with three- or four-vessel coronary artery disease; *BMI* body mass index; *HDL* high density lipoprotein; *LDL* low density lipoprotein

* $P < 0.01$ versus Group 1; § $P < 0.001$ versus controls; ** $P < 0.0001$ versus controls; # $P < 0.05$ versus controls; † $P < 0.01$ versus Group 1

Table 2 Oxidative and inflammatory parameters of study subjects

	Controls	Patients		*P*
		Group 1	Group 2	
LOX-1 (pg/ml)	268 (111–767)	611 (346–1,313)*	2,143 (824–3,201)§,**	0.0015
Peroxy radicals (UC)	315 ± 92	345 ± 90	410 ± 110$^{\S,\#}$	0.0018
CRP (mg/dl)	0.09 (0.06–0.29)	0.25 (0.12–0.53)*	0.43 (0.18–1.57)§,**	0.0038
TNF-α (pg/ml)	1.07 (0.09–1.8)	1.74 (0.62–3.52)*	2.00 (0.91–3.97)§	0.032
IL-6 (pg/ml)	0.18 (0.04–0.61)	0.79 (0.23–2.17)†	1.2 (0.4–3.0)§	0.0059
ox-LDL (U/l)	72.5 (58.6–154.1)	143 (79.7–272.3)*	77.7 (32.8–157.2)$^{\#}$	0.0073

Group 1 patients with one or two diseased vessels; *Group 2* patients with three or four diseased vessels; *LOX-1* lectin-like oxidised lipoprotein receptor-1; *CRP* C reactive protein; *TNF-α* tumor necrosis factor; *IL-6* Interleukin-6; *ox-LDL* oxidized low density lipoprotein

* $P < 0.05$ versus controls; § $P < 0.001$ versus controls; ** $P < 0.05$ versus Group 1; $^{\#}$ $P < 0.01$ versus Group 1; † $P < 0.01$ versus controls

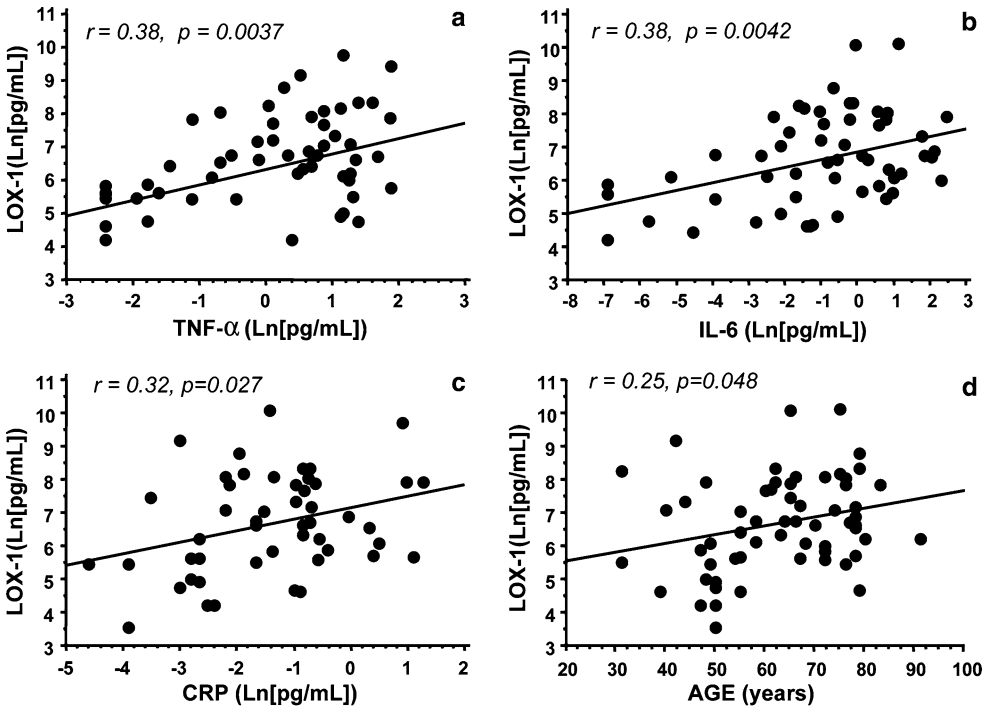

Fig. 1 Correlations between serum sLOX-1 concentrations and TNF-α (**a**), IL-6 (**b**), CRP (**c**) and age (**d**) in all subjects of the study

hemodynamic stimuli [13, 14]. Expression of LOX-1 can be induced after macrophage-like differentiation in vitro in human peripheral blood monocytes [15]. In endothelial cells activated by TNF-α, the cell-surface expression of LOX-1 precedes soluble LOX-1 production [7], suggesting that the regulation of LOX-1 cleavage is correlated to LOX-1 cell expression. Our present study clearly shows a positive correlation between circulating sLOX-1 and TNF-α plasma concentrations, indicating this latter as independent determinant and possible trigger of the s-LOX-1 increase. Likewise, CRP and IL-6 enhance, in a dose- and time-dependent manner, endothelial LOX-1 mRNA and protein expression [14]. In line with this finding, we found a significant relationship between circulating sLOX-1 and CRP or IL-6. However, our results do not agree with those of other authors [9, 10] who have reported no correlation between sLOX-1 and CRP or IL-6. This discrepancy with our results probably stems from a difference in the selection of patients. Contrary to them, we excluded patients with diabetes, hypertension, and/or under the respective therapy that could influence the results. Another explanation for this discrepancy could be the higher sensitivity of our method (10-fold more sensible) that is able to reveal correlations with the other parameters which could have remained hidden with the less sensitive methodology used in the above-mentioned studies.

Similarly to other studies [16, 17], our data confirm the link between CAD and elevated plasma concentrations of peroxy radicals, cytokines, and CRP, and between their levels and the severity of the disease.

However in our study, contrary to expectations, ox-LDL plasma levels were higher in less severe patients (group 1) and lower in more severe patients (group 2).

We can assume that, the reduced levels of ox-LDL in more severely affected patients, could be due to increased uptake of ox-LDL by sLOX-1 which are present in high levels in this group. In support to this hypothesis, some studies have shown that soluble membrane receptors can modulate disease activity by binding to its ligand and preventing ligand uptake by the receptor at the cell-surface [18–20]. For instance, the soluble TNF receptors, whose shedding is induced by TNF itself as well as other cytokines [18], have extracellular regulatory functions affecting local or systemic TNF bioavailability. Circulating sLOX-1 could hence be an important regulator of plasma ox-LDL levels. To explore whether the antibodies used in our ELISA could bind the complex ox-LDL ↔ sLOX-1 in serum as well, we performed a sLOX-1 assay in the presence of exogenously added high levels of ox-LDL; no interference of this ligand was observed in sLOX-1 detection (data not shown). Alternatively, we performed an ox-LDL assay in the presence of high levels of sLOX-1. Also in this case, the adding of sLOX-1 did not interfere with the assay (data not shown). Thus, we can speculate that, cytokines, ox-LDL and the soluble form of its receptor constitute a complex network, which interactively influence the development of the atherosclerotic vascular lesion. However, unlike the inflammatory cytokines and ox-LDL that have a clear pathogenetic role in the atherosclerotic process, little is known about the regulation of sLOX-1 shedding, the biological mechanisms underlying the kinetics of sLOX-1 production and removal and the complex interaction between the soluble receptor and its ligands in health and disease states.

Conclusion

The measurement of sLOX-1 in serum may be potentially useful in predicting atherosclerotic disease progression in humans. Nevertheless, in order to have a clear picture of the pathophysiological roles of sLOX-1 and establish the diagnostic value of sLOX-1 in atherogenesis and vascular diseases, further studies are required.

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