Elevated Titer of Autoantibodies Against oxidized LDL Forms in Patients with Coronary Artery Disease

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Introduction: OxLDL plays an important role in the pathogenesis of atherosclerosis and coronary artery disease (CAD). The oxidative modification of LDL induces immunogenic epitopes located on the LDL particle. These epitopes are primarily formed by the interaction of oxidized phospholipids with the apoB-100 moiety. Human plasma PAF-acetylhydrolase (PAF-AH) is an anti-inflammatory and an anti-atherogenic enzyme and is distributed primarily on lipoproteins, 70% of which is associated with LDL. PAF-AH hydrolyzes the oxidized phospholipids formed during the oxidative modification of LDL. The presence of autoantibodies to oxidized LDL seems to correlate with the progression of atherosclerosis and has been reported in many pathologic conditions. In this study, we evaluated the levels of oxLDL autoantibodies in relation to the phase of LDL oxidation and to PAF-AH activity in patients with coronary artery disease (CAD).

Methods: Sixty-five patients with angiographically verified CAD and thirty-two healthy controls participated in the study. LDL was isolated from whole plasma by three sequential ultracentrifugations. LDL oxidation was performed either in the presence of active PAF-AH or after the enzyme inactivation. These LDL preparations were oxidized in the presence of 5 μM CuSO4 for 3h in 37˚C. Three different forms of oxLDL were received, oxLDLL (at the end of lag phase), oxLDLP (at the end of propagation phase) and oxLDLD (at the end of decomposition phase). The detection of autoantibodies was performed using an ELISA method.

Results: The Lag time of LDL oxidation was significantly higher in both patients and healthy controls in the presence of active PAH-AH, compared to that observed when the enzyme was inactivate. The PAF-AH activity was no different in either study groups. Titers of autoantibodies against all forms of oxLDL were present in both patients and healthy controls. Patients with CAD had significantly higher levels of oxLDL autoantibodies than the control group when oxLDLP (1.467± 0.727 vs 0.994±0.214, p=0.0002) and oxLDLD (1.217±0.385 vs 1.029±0.254, p=0.01) was used as antigen. Furthermore, a positive correlation between the titers of autoantibodies against oxLDLP and oxLDLL and PAF-AH activity was observed. There was no observed difference between patients and controls when oxLDLL and MDA-LDL were used as antigens. In competition experiments the titers of autoantibodies against oxLDL were significantly reduced by 80% when the samples were incubated with Lyso-PC, whereas no significant reduction was observed when the forms of oxLDL(–) were used as antigens.

Conclusions: The titers of autoantibodies against all forms of oxLDL in both study groups were higher when PAF-AH was inactivated, before the LDL oxidation. However, these titers cannot discriminate patients with CAD from healthy individuals. Nevertheless, patients with CAD have elevated titers of autoantibodies against the oxidized forms of oxLDLP and oxLDLD compared to healthy controls. Consequently, the epitope that could discriminate the CAD patients from healthy controls is lyso-PC, which is the product of PAF-AH activity.

Cardiovascular diseases constitute the primary cause of mortality in the Western world and are due to an extensive atheromatosis of the coronary vessels. The pathogenesis of atherosclerosis includes complex interactions among the arterial wall cells (endothelial cells, macrophages, smooth muscle cells) and plasma lipoproteins, mainly low density lipoprotein (LDL) and high density lipoprotein (HDL)[1]. According to the predominant opinion, the formation of atheromatous plaque commences with the adhesion of blood monocytes to the vessel endothelium, which is induced by oxidised LDL (oxLDL), and their penetration in the subendothelium space. In the subendothelium space, the monocytes differentiate in macrophages, take up large quantities of cholesterol, and are transformed into foam cells. Macrophages are known to have receptors that differ from standard LDL-receptors. These receptors recognize and bind modified forms of LDL and mainly oxLDL, but not natural LDL. oxLDL induces the accumulation of cholesterol in macrophages and plays a significant role in the pathogenesis of atheromatous disease[2].

LDL oxidation is a complex procedure that brings various changes to the LDL particle. During this procedure, both the lipid and protein content of LDL are subject to chemical modifications that depend on the locally produced free radicals[3-7]. One of the most important changes noted during LDL oxidation is the peroxidation of polyunsaturated fatty acids that are esterified at the *sn-2* phospholipid location. This peroxidation results in the formation of oxidized phospholipids (oxPL), that have small chains of oxidized fatty acids at the *sn-2* location. These lipids exhibit an elevated antigenicity, either separately or as complexes with LDL proteins (mainly with apolipoprotein B-100), and play a significant role in the formation of autoantibodies against oxLDL in vivo[8-11]. In addition, oxPL participate in many of the biological actions of oxLDL[8].

The bioactive oxPL formed in oxLDL are sensitive to the action of the enzyme acetylhydrolase of the platelet activation factor (PAF-acetylhydrolase, PAF-AH), that is bound with lipoproteins and mainly with LDL in the plasma. The hydrolysis of oxPL formed during LDL oxidation, by the action of PAF-AH, results both in the reduction of the biological activity of oxLDL and the prevention of the modification of apolipoprotein B-100[12-13]. To date, it is not known whether oxPL hydrolysis induced by PAF-AH reduces the antigenicity of oxLDL, as such hydrolysis leads to the formation of lysophosphatidylocholine (lyso-PC), a lipid that also exhibits antigenicity, since it is responsible for many of the biological actions of oxLDL on the arterial wall[3], and leads to the formation of autoantibodies[14-16]. Several recent studies have shown the presence of autoantibodies to oxLDL and/or LDL modified with malonic dialdehyde (MDA-LDL) in patients with coronary artery disease[9,17-19]. In these studies, patients have shown higher autoantibody titers to oxLDL as compared to normal subjects, a finding that indicates that these titers may have a diagnostic and/or prognostic value [20-31]. However, there are studies according to which no differences are observed in the titers of autoantibodies between patients and healthy controls. The causes of inconsistency in the results of various clinical studies are not known.

All studies carried out until now have studied titers of autoantibodies against an extensively oxidized LDL (oxidation for 24h). It is known, however, that such LDL forms cannot be formed under normal conditions, while the oxidized LDL forms that are possibly formed in vivo are partially oxidized ones, i.e. LDL in the early stages of oxidative conversion. In addition, to date no study has taken into account the intrinsic PAF-AH activity of LDL which, as mentioned above, plays a significant role in the oxPL degredation and the formation of lyso-PC. This paper studies the presence of autoantibodies against oxidatively modified LDL forms with or without inactivation of intrinsic PAF-AH in three oxidation stages, as well as the presence of autoantibodies to LDL modified with malonic dialdehyde (MDA-LDL) in the serum of patients with coronary artery disease and in healthy controls. Possible association between the titer of autoantibodies and PAF-AH activity was also investigated.

Material-Method

Study population

Sixty five (65) sequential patients participated in the study (58 men - 89.2% and 7 women – 10.8%, with an average age of 62 ± 9.5 and 67.8 ± 7.8 years, respectively) that were subjected to coronary arteriography in the hemodynamic laboratory of our hospital from January through April 2000. Thirty eight (38) patients (58.5%) were subjected to coronary arteriography because they suffered from stable angina and 27 patients

(41.5%) in the context of an investigation of unstable coronary syndrome (unstable angina, acute transmural or non-Q myocardial infarction) as an initial manifestation of coronary artery disease in a period less than a month from the coronary arteriography, and while they were free of symptoms for at least 7 days. All patients had a positive exercise test and were treated with β-blockers, statins and aspirin. Twenty nine (29) patients suffered from one-vessel disease (lumen stenosis of at least one epicardial artery >50%), 13 patients suffered from 2-vessel disease, and 23 patients suffered from 3-vessel disease. Patients with a history of hepatic or renal disease, rheumatic etiology diseases, or neoplasias, and patients that had been subjected to coronary arteriography during the acute phase of an unstable coronary syndrome or in the context of cardiomyopathy and valvulopathy investigation, were excluded from the study.

Blood sampling was performed on the morning prior to coronary arteriography. Standard risk factors were recorded for all patients (smoking, history of diabetes mellitus and arterial hypertension, hereditary history of early cardiovascular disease, body mass index), and serum triglycerides, total cholesterol, LDL- and HDL-cholesterol levels were measured. Thirty two (32) healthy volunteers (subjects that did not suffer of chronic or acute disease, and had no history of cardiovascular disease) were used as a control group. The clinical particulars of the study subjects are shown in table 1.

Isolation of LDL using sequential ultracentrifugations

Venous blood sampling was performed in tubes containing angicoagulative EDTA. Plasma was subjected to three sequential ultracentrifugations lasting 10 hours each, at 40,000 rpm at 14°C. Upon completion of the third ultracentrifugation, LDL was collected at the top of the tube[32]. Then, the collected LDL was subjected to a 24-hour dialysis. The quantification of LDL was performed using the protein assay per BCA method.

Table 1. Clinical characteristics and lipid profile of patients and healthy controls.

	Control group (n=32)	Patients with CAD (n=65)
Gender (men/women)	18/14	57/8 ++
Age, years	52,4±17,7	62,5±9,5 ++
Body mass index (kg/m^2)	26,1±6,5	26,3±4,35
Arterial hypertension, n	5	42 ++
Diabetes mellitus, n	2	12
Smokers (active/former)	8/3	28/18
Total cholesterol, mg/dl	232,9±48,7	239,3±60,6
HDL-cholesterol, mg/dl	43,3±9,2	37,8±11,4 +
LDL-cholesterol, mg/dl	163,0±69,3	173,9±54,6
Triglycerides, mg/dl	126,1±79,4	172,5±107

+ $p<0.05$, ++ $p<0.001$ as compared to the control group

Inactivation of intrinsic PAF-AH

The irreversible inactivation of PAF-AH of LDL was performed with the addition of a 100mM Pefabloc solution. Upon completion of incubation, LDL with its intrinsic PAF-AH inactivated was extensively dialysed. Then, the protein was assayed.

LDL oxidation

LDL with its intrinsic PAF-AH inactivated or non-activated was diluted at 100μg protein/ml. Oxidation was performed with the addition of a 0.5mM $CuSO_4$, 10μl/ml LDL solution (final Cu^{2+} concentration: 5μM). The oxidation was monitored at 234 nm by recording the sigmoid curve of the conjugate dienes production. Three forms of oxLDL with active intrinsic PAF-AH were prepared, i.e. $oxLDL_L$ (at the end of the lag phase), $oxLDL_P$ (at the end of the propagation phase), and $oxLDL_D$ (at the end of the decomposition phase). Three forms of oxLDL were also prepared following PAF-AH inactivation, i.e. $oxLDL(-)_L$ (at the end of the lag phase), $oxLDL(-)_P$ (at the end of the propagation phase), and $oxLDL(-)_D$ (at the end of the decomposition phase)[32].

Chemical modification of LDL with malonic dialdehyde

The chemical modification of LDL was performed by its incubation, under appropriate conditions, with MDA. The modified LDL (MDA-LDL) was extensively dialysed. Then, the protein was assayed.

Autoantibodies assay with sandwich-type ELISA

The ELISA plate was coated with the antigen at a concentration of 10μg/ml PBS/BHT/EDTA/well, and was stored at 4°C for 24h. Then, it was washed-out using the washout reagent, and the supernatant was removed in such a way as to remove the quantity of the antigen that had not been bound, hence to ensure a higher precision for the method. Next, 100μl of a gelatin 1% solution were added to bind the

empty sites. Incubation was performed for 1h at room temperature. Then, it was washed-out using the washout reagent, and the supernatant was removed in such a way as to remove the largest liquid quantity possible. Next, 50μl of serum diluted at 1:50 were added in the 1% gelatin solution. Incubation was performed for 1h at room temperature. Then, it was washed-out using the washout reagent and the supernatant was removed. Next, 50μl of the secondary antibody were added, that was conjugated with horseradish peroxidase diluted to a 1:1000 volume in 1% gelatin solution. The incubation was performed for 1h at room temperature. It was then washed-out using the washout reagent, and the supernatant was removed. 50μl of the color developer reagent were added in each well. Incubation followed for 3-4 min at room temperature. 50 μl of the reaction terminator reagent (2N HCl) were added in each well. The absorption was measured at 492 nm in a microelisa photometer.

In the inhibition experiments, serums were preincubated for 1h with 10 μg/ml lyso-PC prior to their addition to the ELISA plate. The titer of autoantibodies was assessed as the ratio of absorption when the oxLDL was used as antigen to the absorption when natural LDL was used as antigen.

Activity of serum PAF-AH

The activity of serum PAF-AH was assayed using the precipitation method with trichloroacetic acid (TCA), as previously described[33]. In order to measure serum PAF-AH activity, 50μl of diluted serum (dilution to 1/50 v/v using HEPES buffer solution) were used. The samples were mixed with HEPES to a final volume of 90μl. The reaction commenced with the addition of 10μl of the 1mM [^{3}H]-PAF solution in BSA/saline (final concentration 100μM in the reaction mixture). The enzymatic reaction lasted 10 minutes, and was performed via incubation at 37°C. The PAF-AH activity was expressed as the quantity of PAF (in nmol) hydrolyzed per minute per ml of serum.

Statistical analysis

The continuous variables in each group of subjects were expressed as mean values ± standard deviation (SD), while the distinct variables were expressed as an absolute figure. The titers of autoantibodies were expressed as mean values ± standard deviation. The groups were compared using the Student's t-test for the continuous variables, and the x^2 test for the distinct variables. The Pearson test was performed to demonstrate a possible correlation between the PAF-AH activity and the titer of autoantibodies against all the modified LDL forms used.

Results

Clinical and laboratory characteristics of the study population

As shown in Table 1, the healthy volunteers compared to the patients were younger and developed hypertension (15.6% vs. 64.6%, p=0.0001), and diabetes mellitus (6.25% vs. 18.5%, p=0.179) at a lower rate. No statistically significant differences were noted in the triglycerides levels, and the serum total- and LDL-cholesterol levels, between the 2 groups of the study. However, the healthy volunteers exhibited relatively higher levels of HDL cholesterol (p=0.05).

Autoantibodies against oxidised LDL

The titer of autoantibodies in the serum of patients and normal volunteers was studied in relation to the extent of LDL oxidation. As shown in figures 1A, 1B, LDL oxidation follows a sigmoid curve, regardless whether the intrinsic PAF-AH has been inactivated prior to the oxidation. It should be noted that the latent time of LDL oxidation with active PAF-AH (Figure 1A) is higher than the respective time observed when the intrinsic PAF-AH is inactivated prior to the oxidation (120±10 minutes vs. 75±15 minutes respectively, p<0.05) (Figure 1B). This finding confirms the protective role of PAF-AH as regards the sensitivity of LDL in oxidation. Titers of antibodies were observed to all oxLDL forms, both in patients with coronary artery disease and in healthy volunteers. As regards oxLDL with active PAF-AH, the titer of autoantibodies was higher in both groups of the study when $oxLDL_P$ was used as antigen, as compared to the titers observed when $oxLDL_L$ and $oxLDL_D$ were used as antigens (Figure 1C). In contrast, when oxLDL forms were used as antigens following the inactivation of intrinsic PAF-AH prior to oxidation, the titer of autoantibodies was elevated both against $oxLDL(-)_P$ and $oxLDL(-)_D$ as compared to the titers observed when $oxLDL(-)_L$ was used as antigen (Figure 1D). As shown in figure 2, the titer of autoantibodies in both groups of the

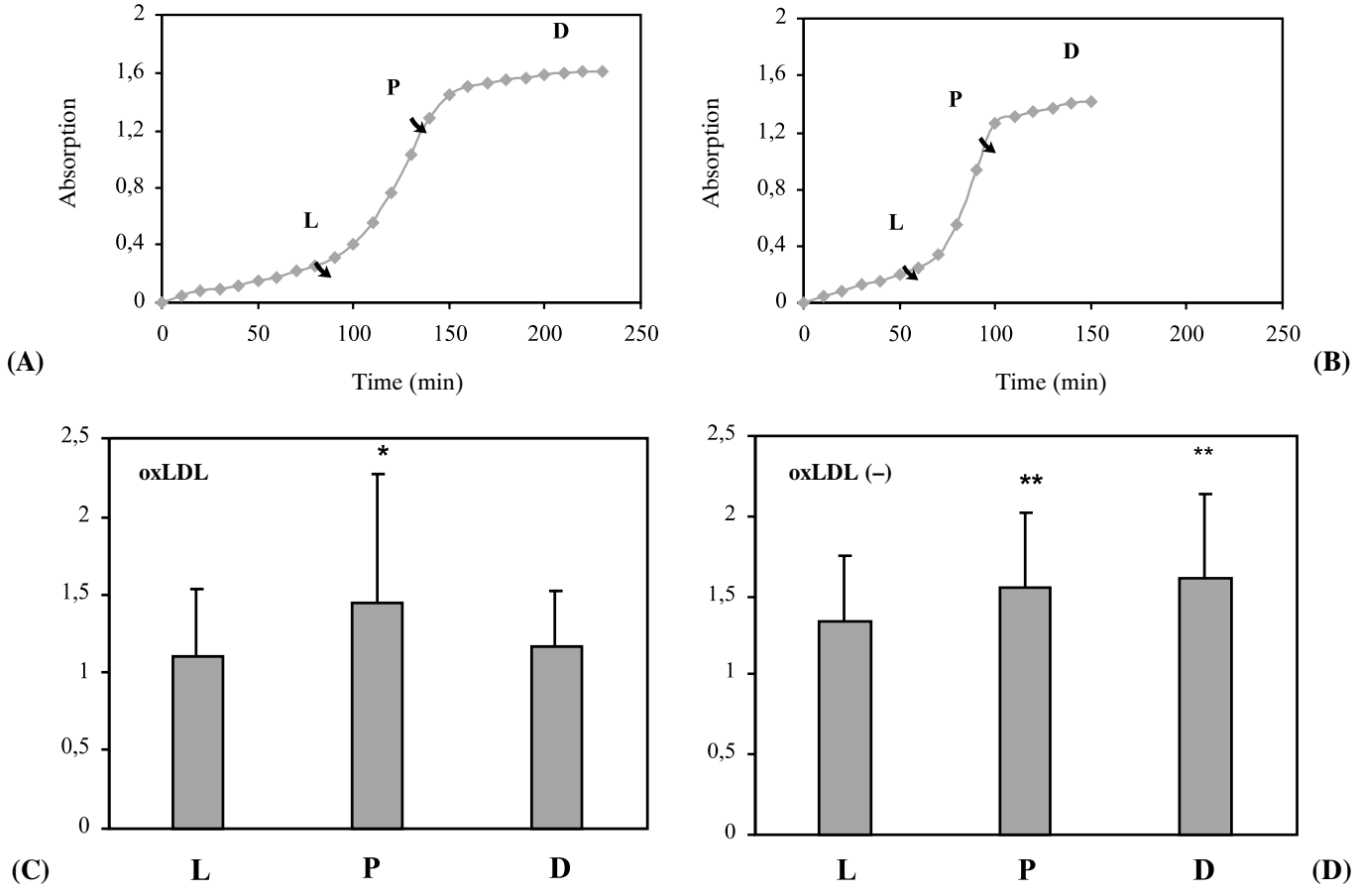

Figure 1. The three phases of LDL oxidation are shown with the activated (A) and inactivated form of PAF-AH (B). Autoantibody titres of the entire study population to oxLDL in three phases of oxidation (L=oxLDL$_L$, P=oxLDL$_P$ και D= oxLDL$_D$) with the activated (*p<0.001) (C) and inactivated PAF-AH (p<0.0001) (D).

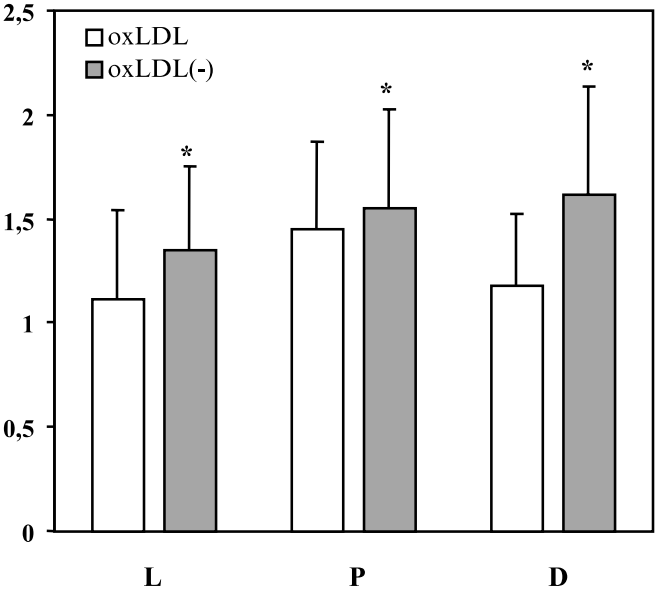

Figure 2. Autoantibody titers to oxLDL with the activated form of PAF-AH expressed in the entire study population were compared to the autoantibody titers to oxLDL with the inactivated form of PAF-AH during the same oxidation state (p<0.001).

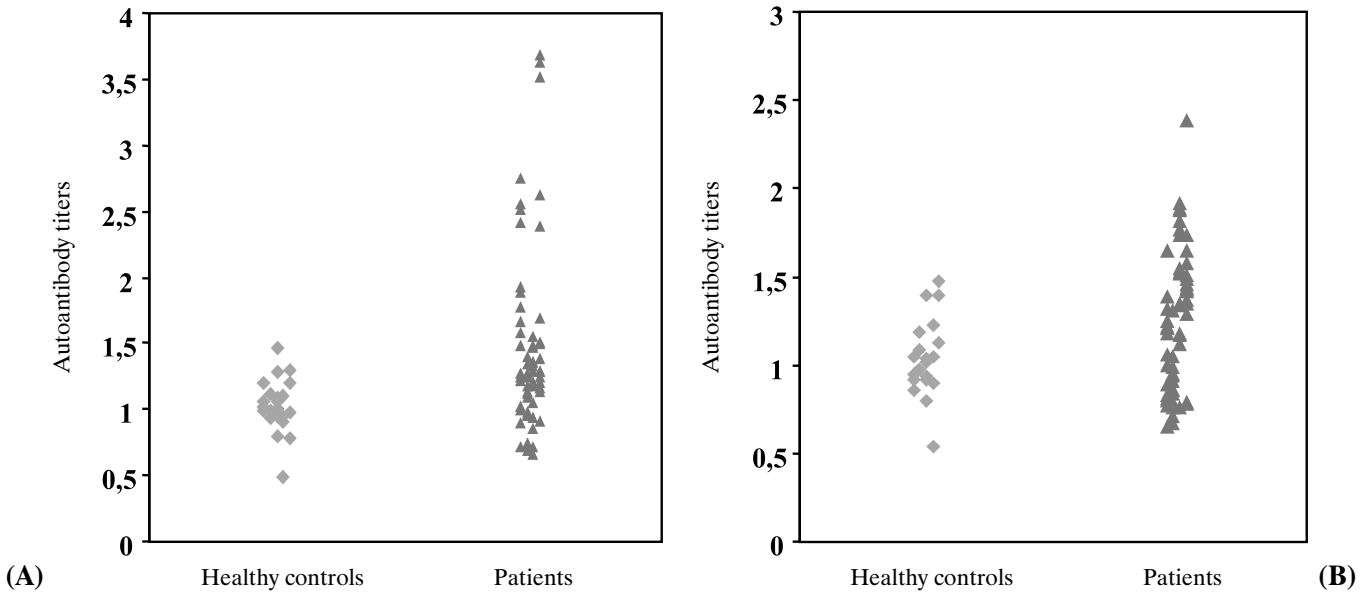

Figure 3. Scatter plots of autoantibody titers to $oxLDL_P$ (A) and $oxLDL_D$ (B) in healthy controls and patients with coronary heart disease.

study was higher for the oxLDL(–) forms than the oxLDL forms, for each antigen used. The titer of autoantibodies in patients was significantly higher as compared to the titers observed in healthy controls when $oxLDL_P$ (1.467±0.727 vs. 0.994±0.214, p=0.0002) (Figure 3A) and $oxLDL_D$ (1.217±0.385 vs. 1.029±0.253, p=0.01) (Figure 3B) were used as antigens. No differences were observed between the 2 groups when $oxLDL_L$ was used as antigen (1.131±0.430 vs. 1.078 ±0.251). The titers of autoantibodies against $oxLDL_L$ and $oxLDL_P$ showed a positive correlation with serum PAF-AH activity (Figure 4A, 4B). The serum PAF-AH activity was not different between healthy controls and patients (50.987±22.354 vs. 57.254± 15.274, respectively). Also, in inhibition experiments, the titer of autoantibodies to oxLDL was reduced by 83±6.5% when the serums were pre-incubated with lyso-PC, while no significant reduction was observed when oxLDL(–) was used as antigen (Figure 5). Also, no differences were observed regarding the titer of autoantibodies between patients suffering of 1-, 2-, or 3-vessel disease. It is worth mentioning that no differences were observed regarding the titer of autoantibodies between patients and healthy subjects when $oxLDL(–)_L$ (1.401±0.446 vs. 1.231±0.408), $oxLDL(–)_P$ (1.628±0.530 vs. 1.340 ±0.417) and $oxLDL(–)_D$ (1.644±0.621 vs. 1.504 ±0.430) were used as antigens. Finally, no differences were observed between the two groups as regards the titer of autoantibodies against MDA-LDL (1.438±0.900 vs. 1.224±0.257).

Discussion

This is the first time that the correlation of the titer of autoantibodies in the serum of patients with coronary artery disease to the LDL oxidation extent (oxidation phase), as well as to the activity of both PAF-AH that is bound to LDL and the total enzymatic activity of the serum, is investigated. The results of our study showed that the LDL-related PAF-AH activity plays a significant role both to the serum autoantibodies levels and their antigenic specificity.

Indeed, when oxLDL(–) forms (i.e. oxLDL with PAF-AH inactivated) were used as antigens, the titers of serum autoantibodies in both patients and the control group were higher as compared to those observed when oxLDL was used as antigen. This effect proves that the titer of autoantibodies totally depends on the oxPL levels that are formed during LDL oxidation. As already mentioned, the oxPL are hydrolyzed and degraded by the intrinsic PAF-AH of LDL upon their formation. Consequently, when PAF-AH is inactivated, the quantity of oxPL increases continuously during LDL oxidation, resulting to a higher titer of autoantibodies, when the oxLDL(–) forms are used as antigens, as compared to the titers

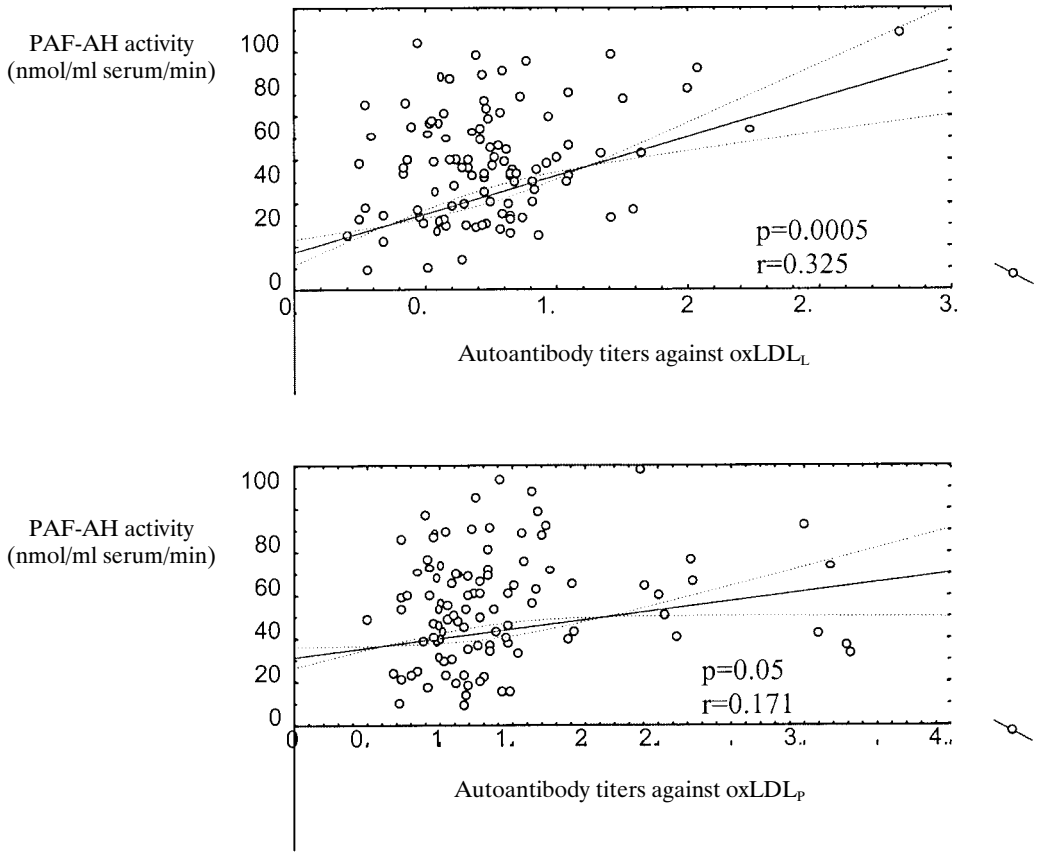

Figure 4. Correlation diagrams of autoantibody titers to oxLDL with PAF-AH activity.

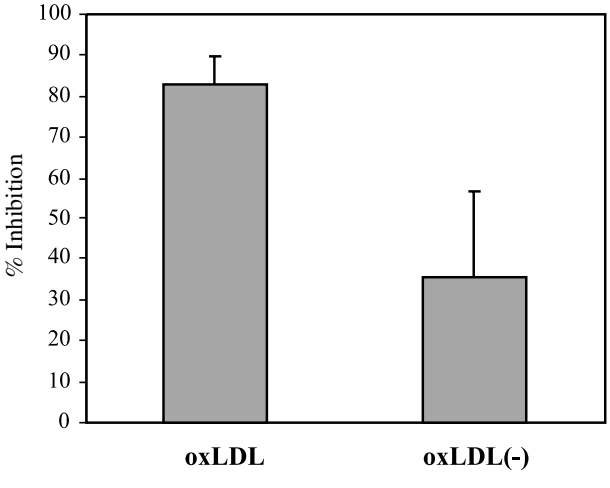

Figure 5. % inhibition by 10μg/ml lyso-PC of serum binding to oxLDL and oxLDL(-)-coated plates (83±6.5 vs 35.3±21.5, $p<0.02$).

observed when the oxLDL forms are used as antigens. However, the titers of these antibodies against oxLDL(–) cannot differentiate the patients with coronary artery disease from healthy subjects.

In contrast, significantly higher titers of autoantibodies were observed in patients with coronary artery disease as compared to the normal subjects, when oxidized LDL forms were used as antigens, prepared without prior inactivation of the intrinsic PAF-AH. As we have shown in a prior study, these oxidized LDL forms are enriched with lyso-PC, a phospholipid formed by oxPL by the action of PAF-AH. Consequently, these oxLDL forms differ from the oxLDL(–), since they are rich in lyso-PC, while they contain a small quantity of oxPL[33]. As already reported by other researchers, apart from oxPL, lyso-PC located in atheromatous plaques shows a

potent antigenicity[11]. Therefore, based upon the results of our study, we may assume that the elevated titer of autoantibodies to oxLDL in the presence of active PAF-AH observed in patients, as compared to the control group, is mainly due to lyso-PC. This hypothesis is further supported by the results of the inhibition experiments in the presence of extrinsic lyso-PC, that have shown that the titer of autoantibodies to oxLDL was reduced by 80% in both groups of the study, when the serums were preincubated with lyso-PC prior to the assay (Figure 5). The positive correlation observed between the serum PAF-AH activity and the titer of autoantibodies against oxLDL, but not against oxLDL(–) (Figure 4), conforms with the above hypothesis. This correlation may be interpreted only if the titers of autoantibodies refer to lyso-PC, since lyso-PC is the product resulting from the action of PAF-AH.

In conclusion, this is the first time that autoantibodies against oxPL and lyso-PC are observed both in the serum of patients with coronary artery disease and of healthy subjects. However, lyso-PC is the epitope that may discriminate the patients from the healthy subjects, i.e. a lipid formed by the action of PAF-AH. These findings indicate the significant role of PAF-AH as regards the type of epitopes formed in oxLDL, and consequently as regards the type of autoantibodies formed against this atherogenic lipoprotein.

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