Accumulation of oxidized LDL in human semilunar valves correlates with coronary atherosclerosis

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Abstract

Objective: Recent data indicate that oxidized low-density lipoprotein (ox-LDL) has several proatherogenic effects, e.g. induction of macrophage chemoattractants, adhesion molecules, cytokines, type-I plasminogen activator inhibitor and platelet-derived growth factor A-chain by smooth muscle cells. Therefore, ox-LDL has been utilized as a marker of oxidative modification of proteins in atherosclerosis. Because heart valves consist of smooth muscle cells, fibroblasts and endothelial cells, and because valvular disease and coronary atherosclerosis could result from similar biological processes, we investigated ox-LDL accumulation in isolated aortic and pulmonary valves and coronary arteries from patients with angiographically proven coronary heart disease (CHD, \(n = 19\)), patients with idiopathic congestive heart failure (IDCM = idiopathic dilated cardiomyopathy, \(n = 20\)), and transplant donors. Methods: Masson–Goldner staining and immunohistochemistry utilizing anti ox-LDL and CD68 were performed on paraffin sections of freshly isolated semilunar valves. Data were analyzed by digital image planimetry and by visual scoring of staining intensity. Results: Ox-LDL immunoreactivity was identified in the vascular aspect of the attachment line, in the deep valve stroma, and in the ventricular and vascular endothelium of the semilunar valves, colocalizing with macrophages. Valvular ox-LDL area was significantly increased in CHD-patients (\(P < 0.03\)) and IDCM-patients (\(P < 0.04\)) compared with controls. More ox-LDL was accumulating in the pulmonary valves than in the aortic valves (\(P = 0.04\)) as assessed by area and staining intensity. Valvular ox-LDL area in pulmonary valve and aortic valve was significantly correlated with ox-LDL accumulation in the intimal layer (\(P < 0.001\)) and medial layer (\(P < 0.001\)) of coronary arteries from the same patients. Conclusion: The data suggest that the biological process leading to ox-LDL accumulation in coronary atherosclerosis also involves heart valves. Therefore, accumulation of the oxidative stress marker ox-LDL in heart valves illustrates atherosclerosis as an additional mechanism accelerating valvular degeneration in these patients. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

Elevated plasma low-density lipoprotein (LDL)-cholesterol is a key risk factor for the development of coronary atherosclerosis [1,2]. LDL is a complex of a large-molecular-mass protein, apolipoprotein B, neutral and polar lipids, and lipophilic antioxidants, mainly vitamin E and beta-carotene. Intracellular cholesterol levels down-regulate LDL-receptor expression, but not expression of scavenger receptors. Oxidative modification of LDL by free oxygen radicals [3–5] leads to fragmentation of apoB. As a result, the particle loses its affinity for the LDL receptor and binds avidly to scavenger receptor, resulting in foam cell formation [6,7]. Ox-LDL has been shown to amplify the atherogenicity of LDL by increasing the expression of P-selectin, leukocyte adhesion mediated through various adhesion molecules [8,9] and by reducing the anti-aggregatory properties of the endothelial cells by interfering

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with NO- and PGI₂-synthesis [10]. Furthermore, during the lipid peroxidation process, a wide range of biologically active products, such as peroxides, malondialdehyde, 4-hydroxynonenal, lyssolecithin, platelet activating factor (PAF) and PAF-like substances and oxysterols are formed [11–14]. Consequently, ox-LDL or by-products of lipid peroxidation affect vasomotor properties and promote thrombosis, as well as lesion initiation and progression.

Although more than 85% of LDL entering the vascular wall will leave, a variable fraction undergoes oxidation in microdomains sequestered from antioxidants within the particle itself, plasma and tissue. It is assumed that the oxidative modification of LDL occurs primarily in the arterial intima. Arterial vessels throughout the body, as well as cardiac valves are exposed to circulating lipoproteins. Previous studies have demonstrated an association of LDL, with collagen as a key step in lipid aggregation in the intima of heart valves in hyperlipidemic rabbits [15]. Lesions in the animals were similar to those found in native human heart valves [16–18]. In contrast to coronary atherosclerosis, lesions appeared in areas exposed to low shear stress and high hydrostatic pressure [19]. Several groups have investigated the onset of the atherosclerotic process in cardiac valves of various animal models [20, 21]. The results revealed that hypercholesterolemia produces atherosclerotic lesions in the cardiac valves, which can deform the leaflets thus altering their normal function.

Because coronary and valvular atherosclerosis are similar processes and may occur coincidentally [22], we performed a semiquantitative comparative evaluation of immunohistochemical staining intensity of the oxidative stress marker ox-LDL in freshly explanted cardiac valves and coronary arteries of patients undergoing heart transplantation for chronic heart failure (CHF).

2. Materials and methods

2.1. Patients characteristics

Patient characteristics are depicted in Table 1. All patients underwent right and left heart catheterization including contrast ventriculograms before heart transplantation including a complete hemodynamic evaluation (Table 1). Furthermore, aortic stenosis was excluded by echocardiography. CHF was defined as left ventricular ejection fraction <30% and NYHA class IV. Based on a clinical history of myocardial infarction and coronary angiograms, the etiology of CHF was determined as either due to coronary artery disease (CHD) or due to other causes (IDCM, idiopathic dilated cardiomyopathy). While 15 patients suffered from mitral regurgitation (8 patients in the CHD and 7 patients in the IDCM group), there was thickening of the aortic valve with mild regurgitation, but no gradient in four CHD- and two IDCM-patients, and a functionally normal aortic valve in the remaining patients. Pulmonary valves were considered normal by echocardiogram.

Table 1

<table>
<thead>
<tr>
<th>Parameters</th>
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<tr>
<td></td>
<td>CHD (n=19)</td>
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<td></td>
<td>IDCM (n=20)</td>
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<td>Controls (n=6)</td>
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<td>PAMP (mmHg)</td>
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<td>PCWP (mmHg)</td>
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<tr>
<td>RVEDP (mmHg)</td>
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</tr>
<tr>
<td>PVR (dynes.cm.sec⁻¹)</td>
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<tr>
<td>Coronary Stary grade</td>
<td>3.9±2.6</td>
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</table>

* Hemodynamic data were measured during right and left heart catheterization. Values are presented as means±S.D. No statistical differences were observed for any value within the groups.

* Patients who had a supine systolic or diastolic blood pressure above 140/90 mmHg or had been on antihypertensive medication were regarded as hypertensives.

* LVEF = left ventricular ejection fraction, CO = cardiac output, PAMP = pulmonary arterial mean pressure, PCWP = pulmonary capillary wedge pressure, RVEDP = right ventricular enddiastolic pressure, PVR = pulmonary vascular resistance, AV = aortic valve.
2.2. Tissue isolation

Semilunar valves and coronary arteries were isolated from patients with dilative ($n=20$, IDCM) or ischemic ($n=19$, CHD) cardiomyopathy. In 18 patients, tissues were available from both coronary arteries and semilunar valves. Nine of these patients had been classified as IDCM patients, and nine as CHD patients.

Control coronary arteries and semilunar valves were from heart donors ($n=6$), who had been primarily intended for transplantation, but were eventually deferred because of circumstances making the organs not suitable for transplantation, e.g., left bundle branch block or subepicardial bleeding.

2.3. Sample processing

Explanted hearts were immediately immersed into cardioplegic solution containing 1 mmol/1 EDTA and 50 µmol/1 butylated hydroxytoluene at 4°C to prevent ex vivo formation of ox-LDL. Tissue harvest was performed 3–6 h later. Because all tissues were harvested following the same protocol, the potential of ex vivo ox-LDL formation was not likely to interfere with the data.

During isolation, the valve leaflets were kept attached to the valve ring, which mainly consists of connective tissue. Samples were fixed immediately after removal in 7.5% formaldehyde (in phosphate buffered saline, pH 7.2).

2.4. Immunoperoxidase staining

Tissue embedded in paraffin wax was cut in 3–5 µm thick sections, dried at 55°C for 2 h and then deparaffinized in xylene for 20 min followed by dehydration through graded alcohols. The endogenous peroxidase activity was blocked with 3% H$_2$O$_2$ in methanol. Tissue proteolysis was performed by treatment with 0.1% protease (protease XIV, EC 3.4.24.31, Sigma, Vienna, Austria) in 0.05 M Tris–HCl, pH 7.6. After a wash in Tris-buffered saline (0.15 M sodium chloride, 0.05 M Tris–HCl, pH 7.6), sections were incubated with anti-ox-LDL [23] 1:100, or antibody CD68 (10 µg/ml), followed by the addition of biotinylated goat–anti-rabbit antibody (Dako, Glostrup, Denmark; 15 min, RT). After repeated washes with Tris–HCl buffer (pH 7.4), sections were incubated with streptABCComplex/HRP (Dakopatts, Glostrup, Denmark, 15 min, RT). The red reaction product was developed using aminoethylcarbazole (Dako). Counterstaining was performed with hematoxylin. Sections were coverslipped in Aquatex (Merck, Darmstadt, Germany). The specificity of the reaction was assessed by competitive binding assays using ox-LDL. In these experiments the binding of the antibody to tissue ox-LDL was inhibited in a dose-depen-
dent manner. Cross reactivity of anti-ox-LDL with native LDL was minimized by preincubation of the antibody with native LDL and removal of complexes by centrifugation [23].

2.5. Computer-assisted quantitative histological evaluation of the atherectomy specimens

All specimens were digitized in their full size utilizing a slide scanner (Nikon 6.0 35 mm film scanner, LS-20, Nikon Corporation, Tokyo, Japan). Images were processed and the color contrast enhanced using the Adobe Photoshop 3.0 software package (Adobe Systems Inc., San Jose, CA, USA). Planimetric analysis of the specimen area staining with anti-ox-LDL in percent (%) of total specimen area (immunoreactivity area or percentage (% enrichment) were performed using computer-based planimetry (NIH Image 1.61/pic, Bethesda, MD, Table 1). In addition, staining intensity was analyzed using a score, ranging from 0 to 4 estimated units (eU) as follows: (0=no immunoreactivity, 1=minimal staining, 2=light staining, 3=intense staining and 4=very intense staining). At least three sections from the aortic and pulmonary valves and from the coronary arteries were analyzed from each patient. The mean values of these determinations were used for statistical calculations. There was 100% reproducibility for the assessment of staining intensity, and a percentage difference between two observers (interobserver variability) of 1 estimated unit (eU) or 20%.

Masson–Goldner stains were utilized to assess general specimen histology. In this technique erythrocytes stain yellow, fibrin is staining red, collagen stains green and elastic fibers stain dark-blue. The internal elastic lamina was utilized as the landmark for identifying the intimal–medial interface in coronary artery specimens.

2.6. Statistical analysis

Values are presented as mean±S.D.. Analysis of variance (ANOVA) with the Scheffé procedure as a post-hoc test or the Student’s t-test was used. A P-value of <0.05 was considered to be significant. Linear regression analysis was used to assess the correlation between enrichment of ox-LDL and hemodynamic parameters, or the correlation between ox-LDL accumulation in the semilunar valves compared with ox-LDL accumulation in the intima and media of coronary arteries.

3. Results

3.1. Analysis of anti-ox-LDL immunoreactivity

Ox-LDL immunoreactivity was identified in semilunar valves of all individuals. In the normal valves and coronary arteries of normal individuals ox-LDL immunoreactivity
was confined to endothelial cells (Figs. 1 and 2, panels B). In contrast, intra- and extracellular deposits of ox-LDL immunoreactivity were identified in patient tissues (compare Figs. 1 and 2, panels B and E, respectively). Ox-LDL immunoreactivity was identified in the vascular aspect of the attachment line, in the deep valve stroma, and in the ventricular and vascular endothelium of the semilunar valves, colocalizing with macrophages. Relative ox-LDL area in the aortic valves was increased in valves from CHD-patients (14.96 ± 3.65%) and IDCM-patients (13.58 ± 4.55%), compared with donor valves (9.97 ± 1.46%). The differences between ox-LDL immunoreactivity area and staining intensity between CHD aortic valves and control valves was significant (P = 0.033 and P = 0.044, respectively, Fig. 3). Ox-LDL immunoreactivity was noted in macrophages (Figs. 1 and 2, panels B and E, note the colocalization of immunoreactivity with CD68 staining in panels C and F) and in the valve matrix, possibly in association with collagen (compare panels D and E).

Fig. 1. Immunohistochemical detection of human ox-LDL in an aortic valve of a representative control subject (panels A–C) and of a representative patient with coronary heart disease (panels D–F). Red color deposits resulting from the oxidation of AEC as described in Methods represent positive immunoreactivity. Panels A and D represent parallel sections stained with a modified trichrome stain where green represents collagen. Panels B and E are ox-LDL stains, while panels C and F are parallel sections stained with CD68 for the visualization of monocytes and macrophages. Note the colocalization of ox-LDL staining and macrophages. All magnifications are 200-fold.
Relative ox-LDL area in the pulmonary valves was increased in valves from CHD-patients (17.25±5.01%) and IDCM-patients (15.18±4.48%), compared with donor valves (9.55±1.67%) (immunoreactivity area in CHD patients: \( P=0.0029 \) vs. control; immunoreactivity area in CHD patients: \( P=0.035 \) vs. control; staining intensity in CHD patients: \( P=0.029 \) vs. control). The differences between ox-LDL immunoreactivity area and staining intensity between all patient and control pulmonary valves was significant (\( P=0.033 \), Fig. 4).

Pulmonary valves of CHD-patients demonstrated more ox-LDL immunoreactivity than aortic valves of CHD-patients (%area: \( P=0.04 \)). In control valves no differences in ox-LDL immunoreactivity between pulmonary and aortic valves was observed (%area: \( P=0.703 \) and intensity: \( P=1 \)).
3.2. Analysis of risk factors

No statistical relationship between patient age, history of hypertension, smoking status, diabetes and ox-LDL accumulation was observed. Furthermore, neither serum triglyceride and nor total cholesterol values correlated with ox-LDL accumulation in the semilunar valves in both patient groups (Table 1).

3.3. Correlation between ox-LDL accumulation in semilunar valves and coronary arteries

Because ox-LDL immunoreactivity analysis had indicated increased ox-LDL accumulation in semilunar valves of CHD-patients, coronary artery ox-LDL accumulation was correlated with valvular accumulation of ox-LDL. Coronary artery segments of all patients were collected and analyzed in parallel with valve tissue in 18 patients (Fig. 5), including all twelve patients in whom histological analysis of coronary artery specimens had disclosed significant atherosclerotic coronary artery disease despite negative coronary angiograms. Ox-LDL immunoreactivity was identified both in the intimal layer of the coronary arteries in association with macrophages (Fig. 2, panels E and F), but also in the medial layer (Fig. 2, panels D and E) colocalizing with collagen bundles (note collagen staining blue in panel D of Fig. 2). Interestingly, ox-LDL immunoreactivity was identified in coronary atherosclerotic lesions in 12 out of 20 IDCM-patients who had been classified as normal by routine coronary angiograms. Relative ox-LDL area and intensity in the semilunar valves correlated significantly with relative ox-LDL area and intensity in the intima and media of the coronary arteries from the same patient (P<0.001, data for ox-LDL area are shown in Fig. 5).

Although the number of patients is small, there was no significant correlation between LDL- and HDL-cholesterol and ox-LDL accumulation in both coronary arteries and semilunar valves. It was noted though that serum LDL was weakly related to ox-LDL accumulation in both semilunar valves (P=0.06) and that serum HDL cholesterol levels were inversely related with ox-LDL area in the pulmonary valves (P=0.06). However, none of these observations reached statistical significance.
aging [24], ox-LDL accumulation in cardiac valves could be a marker for valvular degeneration. In agreement with these data, recent reports indicate that aortic sclerosis, defined as valve thickening without restriction of leaflet motion on echocardiography, is associated with an increased risk of death from cardiovascular causes [25]. Aortic valve disease and coronary disease share many risk factors [26–28].

Heart valves are composed chiefly of extracellular matrix surrounded by an endothelial cell monolayer and few scattered cells, that are known to have characteristics intermediary between fibroblasts and smooth muscle cells. For example, similar to smooth muscle cells, they are coupled by communicating junctions, have intracellular bundles of actin filaments, express cyclic GMP-dependent protein kinase activity and are capable of contraction [29]. In diet-induced hyperlipidemia they become loaded with large lipid droplets [15,16,21] as smooth muscle cells of the arterial wall, and are therefore an excellent model of vascular intima. While ox-LDL area was strikingly high in controls, ox-LDL intensity of staining was clearly less. Based on this observation the authors had initially chosen to include “intensity” as an important discriminating parameter for the study. In addition, the authors do recognize the difficulty of identifying true normal controls as a general confounding factor in the study of human tissues. As indicated in the manuscript, heart donors were chosen as controls. Given the current observation, one may speculate that ox-LDL accumulation in these subjects may impact on late vascular complications in cardiac transplant recipients.

3.4. Influence of intracardiac pressures on ox-LDL accumulation

The hemodynamic parameters of heart transplant recipients are summarized in Table 1. Linear regression analysis showed that relative ox-LDL area correlated with pulmonary capillary wedge pressure (PCWP, \( P = 0.02 \) for aortic valve, \( P = 0.03 \) for pulmonary valve). In addition, a correlation between ox-LDL area and RVEDP (\( P = 0.02 \) for both semilunar valves), and ox-LDL area and PVR (\( P = 0.048 \) for aortic valve and \( P = 0.04 \) for pulmonary valve) could be observed.

4. Discussion

The present study demonstrates that ox-LDL, a marker of oxidative modification of proteins, accumulates in cardiac valves of patients with coronary atherosclerosis, within macrophage-rich lesions that resemble atherosclerotic plaques. Because increasing protein oxidation is a general biochemical feature of both atherosclerosis [7] and

Fig. 5. Linear regression analysis between medial (panel A) and intimal (panel B) ox-LDL area of coronary arteries versus pulmonary and aortic ox-LDL area of the same patients (n=18).

Enrichment of ox-LDL in the aortic valves was highest in patients with severe coronary artery disease. Grouping of patients was performed based on the cardiac catheterization data, allowing the oversight of non-obstructive coronary atherosclerosis. In fact, ox-LDL immunohistochemical analysis disclosed 12 patients in the IDCM group who demonstrated non-occlusive coronary atherosclerosis that was not diagnosed by angiography. In controls only trace amounts of ox-LDL accumulation were evident (Fig. 1B). These findings explain the relatively high ox-LDL accumulation in the valves of IDCM-patients when depicted as a group in Figs. 3 and 4. We were not able to show any significant correlation of valvular ox-LDL accumulation and serum risk factors of atherosclerosis, in particular total serum cholesterol and triglycerides. These values were determined years before the patients underwent transplantation, and are not reflecting values of severely sick patients.

Valvular ox-LDL accumulation correlated significantly with hemodynamic parameters of left ventricular failure, i.e. elevated PCWP and RVEDP. Whether the trend of increased ox-LDL accumulation in the pulmonary valves reflects right ventricular systolic pressure elevation in the presence of a left ventricular systolic pressure decrease in chronic heart failure, is not known. Increased adhesion of
blood cells, e.g., monocyte/macrophages on the valve surface as a consequence of lower shear forces, locally accumulating cytokines and decreased release of NO and prostaglandins from valvular endothelium under these conditions, could serve as mechanisms explaining differential gene expression in the pulmonary vascular bed [30]. Although plasma cytokines have been correlated with the in situ overproduction of cytokines, e.g., in ARDS, sepsis or chronic heart failure [31–33], they exert their effects in an autocrine/paracrine fashion and regulate gene expression in situ over short distances, without detectable changes of plasma levels. Therefore, significant effects may occur without the protein mediator appearing in plasma. Atherosclerosis is a generalized process involving all intravascular structures that are subject to intramural stress beyond a certain threshold [17,19]. The data suggest that a combination of extended mechanical stress due to elevated intracardiac pressures and slow flow may lead to increased valvular accumulation of serum lipids and consequently to an increase in oxidation injury. These mechanisms may independently enhance valvular degeneration.

Immunohistochemical analyzes revealed that positive ox-LDL staining was most intense in areas where Masson–Goldner stains suggested the presence of collagen. This observation is in good agreement with animal data, where LDL accumulation after a high cholesterol–high lipid diet was observed in association with extracellular collagen fibers [15]. Our data expand this observation by suggesting that aggregating LDL particles may undergo oxidation while being immobilized in collagen of the extracellular matrix. In addition, ox-LDL accumulation was observed around small valvular calcifications (data not shown). LDL can induce apoptosis in vascular smooth muscle cells, particularly ox-LDL [34,35], which may colocalize with apoptotic smooth muscle cells in human atherosclerotic plaques [36]. The effect of ox-LDL appears to occur via ketocholesterol by downregulation of Bcl-2 [35]. The possible contribution of valvular smooth muscle cell apoptosis to the process of valvular degeneration deserves further studies.

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