Atherosclerosis and Lipoproteins

Presence of Oxidized Low Density Lipoprotein in Nonrheumatic Stenotic Aortic Valves

Margareta Olsson, Johan Thyberg, Jan Nilsson

Abstract—The aim of the present study was to analyze if LDL particles trapped in stenotic aortic valve tissue undergo oxidative modification. Degenerative aortic stenosis affects >3% of the population >75 years of age in the Western world. Recent studies have revealed the presence of a chronic inflammatory process similar to what has been described in other degenerative diseases such as atherosclerosis. However, the underlying disease mechanisms of degenerative aortic stenosis still remain largely unknown. Six tricuspid stenotic valves, obtained at valve replacement, were compared with 3 control valves collected from hearts taken out during transplantation. The stenotic valves and the control valves were examined by immunohistochemistry, using antibodies against apoB, 4-hydroxynonenal-modified LDL, leukocytes, and HLA-DR. All valves were also stained with oil red O for neutral lipids. Extracellular neutral lipids were found in all stenotic valves, extending from the bases along the fibrosa layer. This lipid colocalized with apoB- and 4-hydroxynonenal–modified LDL immunoreactivity. 4-Hydroxynonenal–modified LDLs were present around calcium deposits, subendothelially, and in the deeper layer of the fibrosa. There was also a colocalization with macrophages, T lymphocytes, and HLA-DR expression. Control valves had a thin area of neutral lipid accumulation, a small amount of apoB, but no signs of inflammation. A distinct colocalization between oxidized LDLs, T-lymphocyte accumulation, and calcium deposits suggests that oxidized lipids may play a role in the disease process. (Arterioscler Thromb Vasc Biol. 1999;19:1218-1222.)

Key Words: degenerative aortic stenosis ■ low density lipoprotein ■ lipid oxidation ■ T-lymphocyte accumulation

With the decline in the incidence of rheumatic fever, degenerative valvular aortic stenosis has become the most common valvular disorder in the Western world.1 It affects >3% of the population >75 years of age and is a major cause of disability and death in this age group.2 The cause as well as the underlying disease mechanisms remain largely unknown. Epidemiological studies have revealed no major risk factors for the disease, with the exception of the presence of bicuspid valves.3,4 The latter observation has been taken as an indication of an involvement of mechanical stress in the disease process. This notion is also supported by the finding that valvular fibrosis and calcification initially occurs at the base of the cusps.5–7 By using immunohistochemical techniques, we and others have recently characterized the cellular composition of degenerative aortic stenotic valves.8,9 The increase in valve thickness is caused by the combined effect of increased cellularity, increased extracellular matrix deposition, and focal accumulation of calcium deposits. Most cells in aortic stenotic valves are fibroblast-like mesenchymal cells expressing HLA-DR antigen as well as the smooth muscle cell markers, such as α-actin and desmin, in a manner closely resembling that observed in other chronic fibrotic disorders.10 Aortic stenotic valves also contain numerous macrophages and activated T lymphocytes, suggesting involvement of local immune reactions.8

Lipids are known to accumulate in aortic valves with increasing age.11,12 More recently, O’Brien et al13 demonstrated the presence of a large amount of apoB in aortic stenotic valves, indicating that lipid accumulation occurs as a result of retention of LDLs in valve tissue. The aim of the present study was to analyze if LDL particles trapped in aortic stenotic valve tissue undergo oxidative modification. Previous studies have shown that oxidized LDLs are cytotoxic, stimulate inflammatory activity, and promote connective tissue cell proliferation.14,15 If present in aortic valve tissue, oxidized LDLs may thus play a role in the changes leading to the development of valvular aortic stenosis.

Methods

Six tricuspid stenotic aortic valves, obtained at valve replacement, were examined. The ages of the patients ranged from 72 to 79 years. The valves were fixed in 4% formaldehyde:0.02 mol/L butylated hydroxytoluene immediately after removal to avoid oxidation. Three control valves were collected from hearts obtained at transplantation and fixed in 4% formaldehyde. All 3 cusps from each control valve were examined. The ages of the control patients ranged from 25 to 58 years.

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1218
Primary Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Source</th>
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<tr>
<td>ApoB</td>
<td>1:400</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>NA59 (4HNE-modified LDL)</td>
<td>1:400</td>
<td>Kindly provided by Dr J. Witztum</td>
</tr>
<tr>
<td>MDA2 (malondialdehyde-modified LDL)</td>
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<td>Kindly provided by Dr J. Witztum</td>
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<tr>
<td>CD6 (pan T cells)</td>
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<td>DAKO</td>
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<td>CD68 (macrophages)</td>
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<tr>
<td>HLA-DR</td>
<td>1:400</td>
<td>DAKO</td>
</tr>
<tr>
<td>FVIII (endothelium)</td>
<td>1:400</td>
<td>DAKO</td>
</tr>
</tbody>
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4HNE indicates 4-hydroxynonenal; FVIII, von Willebrand factor.

The study was approved by the Ethics Committee of the Karolinska Hospital and the patients were included after informed consent.

**Histological Analysis**

All valves were stained for neutral lipids with oil red O (Sigma).

**Immunohistochemical Analysis**

To optimize antigen preservation, the specimens were not decalcified before sectioning. Suitable tissue samples, not including the commissures, were cryosectioned in 10-μm-thick sections. Indirect immunohistochemistry was performed by using the Avidin-Biotin-Complex method with peroxidase. Endogenous peroxidase was blocked by incubation with 0.3% hydrogen peroxide in methanol for 30 minutes. This step was preceded by incubation with 0.2% Triton X-100 in PBS when staining for apoB was performed. The slides were then rinsed in PBS, incubated with 5% horse serum for 30 minutes, and incubated with primary antibodies for 60 minutes (Table). As evidenced by using this protocol, there might be an oxidation of lipids during the procedure. However, incubation with antibodies against oxidized LDLs before pretreatment with hydrogen peroxide showed the same results. Negative controls were incubated with irrelevant mouse sera. To demonstrate the specificity of the staining against 4-hydroxynonenal–modified LDLs, a blocking procedure with NA59 antibodies and oxidized LDLs was performed (100 μg/mL of oxidized LDLs in F10 medium + 1:400 anti-NA59).

After rinsing in PBS the specimens were incubated with a biotin-labeled horse anti-mouse antibody for 30 minutes followed by an avidin-biotin complex conjugated with peroxidase for 30 minutes. The sections were rinsed in PBS and exposed to diaminobenzidine solution for 5 minutes followed by rinsing in PBS. The slides were counterstained with Gill’s hematoxylin, dehydrated in ethanol and xylene, and mounted in Eukitt (O. Kindler Gimbtt Co).

Double staining was performed to test for possible colocalization between apoB and oxidized LDLs. The specimens were first incubated with a sheep antibody against human apoB (Boehringer-Mannheim) in 1% BSA/PBS overnight, rinsed, and incubated with a biotin-labeled rabbit anti-sheep IgG (Vector) for 30 minutes followed by an alkaline phosphatase–conjugated avidin-biotin complex for another 30 minutes and finally stained by using fast red substrate (Vector). After blocking in 5% horse serum for 30 minutes, the sections were then incubated with the NA59 antibody against oxidized LDLs for 30 minutes, rinsed, and exposed to hydrogen peroxide for 30 minutes. After rinsing they were subsequently incubated with a biotin-labeled horse anti-mouse IgG antibody for 30 minutes followed by an avidin-biotin complex conjugated with peroxidase for 30 minutes. The sections were rinsed in PBS and exposed to diaminobenzidine solution for 5 minutes followed by rinsing in PBS.

**Results**

**Macroscopic Evaluation**

All aortic valves examined were tricuspid. One of the control valves showed signs of tissue hypertrophy at the base of 1 cusp but was without visible calcification, whereas the remaining 2 control valves were thin and transparent. All valves obtained from patients undergoing valve replacement were markedly hypertrophic and contained multiple calcium nodules. There was no commissural fusion present in any of the stenotic valves.

**Oil Red O and Immunohistochemical Staining**

In the control valves, oil red O staining demonstrated presence of lipids in all cusps. In the youngest control valve, lipids were present only as a thin border between the fibrosa and the spongiosa (Figure 1). The staining was most clearly seen close to the base of the cusps, whereas essentially no staining could be observed in the tip region. The valvular lipid content appeared to increase with age, and in the oldest control valve 1 cusp showed an early lesion, with lipids present in the entire fibrosa part of the valve. Immunohistochemical analyses demonstrated presence of apoB, small amounts in the thin and transparent cusps and more markedly in the cusp with the early lesion. This immunoreactivity appeared in the same areas that were stained by oil red O. 4-Hydroxynonenal–modified LDL immunoreactivity was present and colocalized with apoB and oil red O staining in the cusp with the early lesion.

All stenotic valves were intensively stained by oil red O. Staining was most prominent in the endothelial region of the fibrosa, but it was also present in the deeper layers of the valve and, in particular, close to the lamina elastica. Staining was also strong in the vicinity of calcified areas and no calcium deposits could be detected in areas devoid of lipids (Figure 2a). Stenotic valves contained markedly more apoB immunoreactivity than control valves (Figure 2b). As in the control valves, there was a distinct colocalization between apoB immunoreactivity and oil red O staining.

![Figure 1](http://atvb.ahajournals.org/Downloadedfrom Olsson et al May 1999 1219)
Antibodies raised against 4-hydroxynonenal–modified LDLs and malondialdehyde-modified LDL were used to detect presence of oxidized LDLs in the valves. These antibodies have previously been used to identify oxidized LDLs in human atherosclerotic plaques.\textsuperscript{21} 4-Hydroxynonenal–modified LDL immunoreactivity was present in all of the stenotic valves. The 4-hydroxynonenal–modified LDL immunoreactivity was present predominantly in the endothelial region of the fibrosa and around calcium deposits (Figure 2c), thus demonstrating a clear colocalization with apoB immunoreactivity and oil red \(\text{O}\) staining. The colocalization between apoB and 4-hydroxynonenal–modified LDL could also be demonstrated by using double staining (Figure 3). Preincubation of the 4-hydroxynonenal–modified LDL antibodies with 100 \(\mu\)g/mL of copper-oxidized LDL resulted in a complete removal of the immunostaining, further supporting the specificity of the immune reaction (Figure 4).

Relation Between Markers of Inflammatory Activity and Oxidized LDLs

In the control valves unaffected by tissue hypertrophy, only occasional T lymphocytes and macrophages could be detected. These were uniformly distributed in the valve tissue and did not colocalize with lipid deposition. In the control valve with 1 cusp demonstrating an early lesion, T lymphocytes and macrophages were found at the base of the valve and in the endothelial region of the fibrosa. A few foam cells were present in these areas. Expression of HLA-DR was also observed on some cells colocalized with lipid depositions.

All stenotic valves contained T-cell and macrophage immunoreactivity in the subendothelial layer of the fibrosa, in the vicinity of calcium deposits, and along the lamina elastica. Thus, these cells were clearly localized to regions also staining for neutral and oxidized lipids (Figure 2d). No T lymphocytes were present in lipid-poor areas, whereas some
macrophages could be detected in these regions. HLA-DR–expressing cells were most prominent in regions with lipid accumulation but could also be found in lipid-poor areas of the valves.

Discussion

Lipids have been shown to accumulate in aortic valves with increasing age. The present findings demonstrate that aortic valves affected by degenerative stenosis contain markedly higher amounts of lipids than healthy valves. Our findings also confirm previous studies by O’Brien et al. showing a colocalization between lipid staining and apoB immunoreactivity in stenotic valves. This colocalization is a strong indication that the lipid deposits encountered in stenotic valves are derived from LDLs, the major carriers of apoB in the circulation. In accordance with observations of O’Brien et al., we found that most lipids and apoB immunoreactivity were present in the subendothelial area of the fibrosa. This part of the valve faces the aortic lumen and is exposed to much lower shear stress than the opposite, ventricular side of the valve. It is noteworthy that areas exposed to low shear stress also show an enhanced uptake of LDLs in blood vessels and are predisposed to develop atherosclerosis in response to hyperlipidemia. The mechanism responsible for the increased accumulation of LDLs at low shear stress areas remains to be fully clarified, but it may involve an enhanced endothelial permeability and/or retention of LDLs by extracellular matrix.

The present findings also demonstrate that LDLs that have become trapped in stenotic valves undergo oxidative modification. The role of LDL oxidation in atherosclerosis has attracted considerable interest, and the chemical characteristics and pathophysiological properties of oxidized LDLs have therefore been studied intensely. The oxidative modification of LDLs in extracellular tissues is initiated by reactive oxygen intermediates or radicals generated by cellular enzymes. Oxidized LDL particles are taken up by macrophage scavenger receptors, resulting in the formation of foam cells. In the core region of advanced atherosclerotic plaques, lipid deposition is associated with massive accumulation of macrophage foam cells and necrosis. It is clearly in contrast to the situation in stenotic valves, where lipid deposition is more pronounced in the subendothelial than in the deeper layers. Macrophages are uniformly present in lipid-rich areas but are markedly fewer than the surrounding connective tissue cells. Moreover, stenotic valves do not contain areas with extensive lipid-associated necrosis. This difference in response to lipid accumulation and oxidation between the atherosclerotic plaque and the stenotic valve may be of considerable pathophysiological relevance. The clinical manifestations of atherosclerosis are closely linked to the necrotic degeneration and rupture of the plaque. In contrast, the clinical manifestations of aortic stenosis are due to tissue hypertrophy and calcium deposition. One may speculate that differences in the rate of accumulation and/or clearance of oxidized LDLs between atherosclerotic plaques and stenotic valves may account for some of these differences in the tissue response.

Oxidized LDLs are highly cytotoxic for most cells. In atherosclerotic plaques, accumulation of toxic concentrations of oxidized lipids is believed to result in extensive cell death eventually causing plaque rupture. A similar process clearly does not take place in stenotic valves. However, it is reasonable to believe that oxidized LDLs, to some extent, are also toxic for valve fibroblasts. An interesting possibility is that the combination of extracellular lipid deposits and matrix vesicles released from valve fibroblasts exposed to oxidized toxic components of these lipid deposits may serve as nuclei for calcium deposition and nodules formation. The ability of extracellular phospholipid deposits to polymerize calcium crystals has been shown in atherosclerotic plaques. Mineralization of bone is activated by small, membrane-surrounded matrix vesicles released from osteoblasts. Vesicles of similar structure are also found as cells undergo necrotic degeneration. The possible role of oxidized lipids in valve fibroblast cytotoxicity and calcium deposition clearly requires further study and may even represent a possible mechanism for therapeutic intervention and/or prevention of the development of vulvular aortic stenosis.

Components of oxidized LDLs have also shown proinflammatory and growth-stimulatory properties. It is thus possi-

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**Figure 4.** Specificity of the oxidized LDL immunoreactivity. Parallel sections demonstrating part of a stenotic valve stained with antibodies against oxidized LDLs (NA59) preincubated with 100 μg/mL of copper-oxidized LDLs (a) and NA59 antibodies alone (b). Preincubation with oxidized LDLs removes all immunoreactivity, demonstrating the specificity of the immune reaction. Original magnification ×400.
able that products generated by lipid oxidation are involved in the inflammatory process present in the stenotic valves. Are, then, atherosclerosis and degenerative aortic stenosis essentially the same disease? This is probably not the case, because aortic stenosis and coronary artery disease show little covariation and because the major risk factors for coronary artery disease do not appear to predispose for development of aortic stenosis. However, both disease processes are likely to involve a combination of toxic and mechanical injuries resulting in inflammation and fibrosis, subsequently leading to tissue necrosis in 1 disease and massive calcium deposition in the other.

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References
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