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Potential pathological roles for oxidized low-density lipoprotein and scavenger receptors SR-AI, CD36, and LOX-1 in aortic valve stenosis

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ABSTRACT

Objective: To clarify the potential mechanisms by which oxidized low-density lipoprotein (oxLDL) could contribute to the progression of aortic valve stenosis (AVS).
Methods: We investigated a total of 46 stenotic and 20 control human aortic valves. The mRNA expression levels of scavenger receptor class A type 1 (SR-A1), CD36, Lectin-like oxidized LDL receptor-1 (LOX-1), and scavenger receptor class B type 1 (SR-B1) were studied using qPCR. Their cellular distribution in the valves was assessed by immunohistochemistry, and the potential effects of oxLDL were studied in cultured myofibroblasts isolated from the aortic valves.
Results: In AVS, the proinflammatory SR-A1 and the angiogenic LOX-1 were upregulated ($p = 0.003$ and $p = 0.002$), whereas the antiangiogenic CD36 was downregulated ($p = 0.02$). The expression of the atheroprotective SR-B1 remained unchanged. Immunohistochemistry revealed that SR-A1 was expressed by macrophages, whereas the expression of CD36 and LOX-1 was confined to myofibroblasts and endothelial cells in the diseased valves. In cultured valvular myofibroblasts, mast cell-derived components and TNF-α induced LOX-1 expression ($p = 0.05$ and $p < 0.001$), whereas oxLDL promoted the expression of proinflammatory cytokines. Furthermore, the expression of osteoprotegerin, an inhibitor of valvular calcification, decreased in response to oxLDL. Finally, myofibroblasts derived from stenotic valves accumulated more DiI-labeled oxLDL than myofibroblasts derived from macroscopically healthy valves ($p = 0.035$), so revealing enhanced foam cell-forming potential of myofibroblasts in the diseased valves.
Conclusion: This study unveils the presence of SR-A1, CD36, and LOX-1 in aortic valves and suggests potential mechanisms by which they may contribute to the pathological angiogenesis, inflammation, calcification, and lipid accumulation in AVS.

1. Introduction

Aortic valve stenosis (AVS) is an active atheroinflammatory disease with many similarities to atherosclerosis [1]. Risk factors common to AVS and atherosclerosis include smoking, hypertension and hypercholesterolemia [2]. Moreover, oxidatively modified low-density lipoprotein (LDL), aggregated lipid particles, and foam cells typically found in atherosclerotic lesions also accumulate in stenotic valve leaflets [3–5]. Inflammation, fibrosis, angiogenesis, and active calcification are prominent features of advanced AVS [5–8]. Inflammatory infiltrates in the stenotic valves may even associate with clinical stenosis [9,10].

Despite the similarities between the two diseases, attempts to apply pharmacotherapies commonly used for prevention and treatment of coronary artery disease have failed when explored in AVS [1]. Regardless of the apparent association between AVS and hypercholesterolemia in the epidemiological studies, the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) were ineffective in the prevention of this disease [11].

Abbreviations: AVS, aortic valve stenosis; IL(-6/8), interleukin(-6/8); LOX-1, lectin-like oxidized LDL receptor-1; MCP-1, monocyte chemoattractant protein-1; M-CSF, macrophage colony-stimulating factor; OxLDL, oxidized low-density lipoprotein; qPCR, quantitative polymerase chain reaction; SR-A1, scavenger receptor class A type 1; SR-B1, scavenger receptor class B type 1; Statins, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors; TNF-α, tumor necrosis factor-alpha.

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Therefore, instead of mere elevation of plasma LDL-cholesterol levels, the valvular modification of LDL and its subsequent uptake by the valvular cells could be crucial in the initiation and progression of AVS. Indeed, oxidized LDL (oxLDL) present in stenotic aortic valves colocalizes with inflammatory cells and calcium deposits [3]. OxLDL also induces the formation of calcific nodules by transformed osteoblastic valvular myofibroblasts, thus accelerating valvular calcification [12].

The importance of LDL modification in AVS progression is supported by the findings that plasma oxLDL levels associate with more advanced histopathological changes in stenotic valves, and that cigarette smoking, a risk factor of AVS, is related to elevated circulating oxLDL levels [13]. Furthermore, the plasma proportions of small dense LDL particles, potential sources of oxLDL, associate with AVS progression [14].

Scavenger receptors are a diverse group of membrane proteins and soluble proteins that are able to bind modified forms of LDL [15], e.g. oxLDL [16]. Indeed, several members of the scavenger receptor family have been implicated in the pathogenesis of atherosclerosis. Scavenger receptor class A member 1 (SR-AI) and CD36 are involved in macrophage foam cell formation, and can also be expressed by endothelial cells [17]. Moreover, CD36 can interact with thrombospondins resulting in the inhibition of angiogenesis [18], thrombospondin-2 being upregulated in AVS [19]. Lectin-type oxidized LDL receptor 1 (LOX-1), in turn, was first discovered in endothelial cells and it mediates the uptake of oxLDL by these cells. In addition, LOX-1 has pro-angiogenic potential by being involved in the signaling cascade which mediates capillary tube formation by oxLDL [20]. In contrast to the above, scavenger receptor class B member 1 (SR-BI) participates in reverse cholesterol transport by binding high density lipoprotein (HDL), and is thus considered atheroprotective [17]. The expression of these receptors in the healthy and diseased aortic valves is currently unknown.

In the present work, our aim was to study the expression of oxLDL-binding receptors in the aortic valve, and to learn whether their expression would differ in healthy and stenotic valves. In addition, we investigated the effects of oxLDL on cultured human aortic valvular interstitial cells (myofibroblasts). Furthermore, we explored the ability of cultured valvular myofibroblasts to transform into foam cells when exposed to oxLDL, and whether this potentially disease-accelerating event is more pronounced in AVS.

2. Materials and methods

2.1. Samples and study population

We obtained stenotic aortic valves from a total of 46 patients undergoing valve replacement surgery due to symptomatic, moderate to severe AVS. All the patients had isolated AVS, the exclusion criteria being echocardiographically moderate or severe aortic regurgitation or mitral valve disease. We also excluded patients with a history of myocardial infarction, proximal coronary artery stenosis exceeding 50%, complicated diabetes, renal insufficiency, or endocarditis. The samples used in immunohistochemical analyses ($n = 24$) were snap-frozen in liquid nitrogen and embedded in OCT compound, and subsequent samples were either snap-frozen for qPCR analyses ($n = 10$) or used freshly for cell culture ($n = 12$). Control valves without visible valvular disease ($n = 16$) came from patients undergoing cardiac transplantation due to dilated or ischemic cardiomyopathy ($n = 4$, used in immunohistochemistry), or from deceased organ donors whose hearts were unsuitable for use as grafts because of advanced age, resuscitation, or suspected ischemia ($n = 12$; 9 for qPCR analyses, 1 for immunohistochemistry, and 2 for both qPCR and immunohistochemistry). The most common cause of death in the organ donor group was traumatic brain injury ($n = 8$). All the frozen samples are further characterized in Supplemental Table I. Additional control valves for cell culture studies came freshly from patients undergoing valve replacement surgery due to aortic regurgitation ($n = 4$). The study complies with the Declaration of Helsinki. The protocol was approved by the Ethics Committee of Helsinki University Central Hospital (approval ref. nr. 141/13/03/01/10). All the patients signed an informed consent. The use of organ donor tissue was approved by The National Authority for Medicolegal Affairs of Finland (approval ref. nr. 4444/32/300/05).

2.2. Quantitative real-time PCR analyses (qPCR)

Total cellular RNA was isolated from frozen aortic valve leaflets (10 stenotic and 11 controls) and from cultured valvular myofibroblasts (4 stenotic and 4 controls), using RNeasy columns (Qiagen). Tissue material was homogenized in Trizol reagent (Invitrogen/Life Technologies) prior to RNA isolation. RNA was converted to cDNA using MMLV reverse transcriptase (SuperScript III, Invitrogen) and random hexamers (Promega). The cDNAs were amplified on ABI Prism 7500 Sequence detector system (Applied Biosystems) using Power SYBR-Green PCR Master Mix (Applied Biosystems) or TaqMan Universal PCR Master Mix (Applied Biosystems) and FAM-labeled fluorogenic probes with gene-specific primers, and run in parallel with a standard TaqMan PCR program. All the primers and probes, when applicable, are presented in Supplemental Table II. TaqMan primers were purchased from Applied Biosystems, and SYBR-Green primers from biomers.net. The data were normalized to the same endogenous control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and relative units were calculated with a comparative Ct method [21].

2.3. Immunohistochemical and immunofluorescence stainings

Cryostat sections of aortic valves (24 stenotic and 7 controls) were immunostained with EnVision System-HRP (DAB) kits (DAKO) according to instructions provided by the manufacturer. Alexa fluor secondary antibodies (Invitrogen) were used in double immunofluorescence stainings as described [22]. All the antibodies used in this study are listed in Supplemental Table III. Non-immune mouse and rabbit isotype-specific immunoglobulins (mouse IgG2b, Biosite; others, Serotec) were used as negative controls. The sections were observed by light and epifluorescent microscopy, and the immunopositivity for scavenger receptors SR-A1, CD36, and LOX-1 was semi-quantitatively graded from none (−) to abundant (+++) staining.

2.4. LDL isolation, oxidation, and labeling

Human LDL ($d = 1.019–1.050$ g/ml) was isolated from the plasma of healthy volunteers (Finnish Red Cross) by sequential ultracentrifugation as described [23]. The protein concentration in the preparations was measured by the Lowry method using bovine serum albumin as a standard [24]. The amounts of the lipoproteins in this study are expressed in terms of their protein concentration. Cigarette smoke was selected as an oxidative agent in addition to the standard $CuSO_4$ method based on literature [25]. Cigarette-smoked PBS was prepared by connecting a burning cigarette with plastic tubing to a 20 ml syringe containing 1 ml sterile PBS. Three cigarettes were smoked into 1 ml of PBS by drawing the smoke into the syringe and mixing vigorously. For oxidation, lipoproteins were first dialyzed overnight at +4 °C against PBS and then incubated in the presence of 10 μM $CuSO_4$ or 50% cigarette smoke-treated PBS at +37 °C for at least 18 h. The oxidation was terminated by adding EDTA to the final concentration of 100 μM. The degree of oxidation