Association of Angiotensin-Converting Enzyme With Low-Density Lipoprotein in Aortic Valvular Lesions and in Human Plasma

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Background—Recent studies have demonstrated that lesions of aortic sclerosis and stenosis share several similarities with lesions of atherosclerosis. In atherosclerosis, angiotensin-converting enzyme (ACE) is expressed by a subset of macrophages. This study was undertaken to determine whether ACE might be present in aortic sclerosis or stenosis lesions.

Methods and Results—Immunohistochemistry was performed on 26 paraffin-embedded human aortic valves. Monospecific antibodies were used to identify ACE, macrophages, angiotensin II type I receptor (AT-1 receptor), angiotensin II, and apolipoprotein B. Human low-density lipoprotein (LDL) and high-density lipoprotein (HDL) were isolated from plasma of normal volunteers by sequential density-gradient ultracentrifugation. ACE was not present in normal valves but was present in all valves with aortic sclerosis or stenosis lesions. ACE was detected on a subset of lesion macrophages but was present primarily in an extracellular distribution, where it colocalized with apolipoprotein B. ACE was detected by Western blotting on plasma LDL but not on HDL isolated from normal volunteers. Angiotensin II, the enzymatic product of ACE, was colocalized with ACE in valve lesions. ACE also was colocalized with apolipoprotein B in an adjacent coronary atherosclerotic plaque.

Conclusions—ACE is present in aortic sclerosis and stenosis lesions, where it may participate in lesion development, as is evidenced by the presence of its enzymatic product, angiotensin II. The observation of an association of ACE with LDL in both lesions and plasma suggests that LDL may deliver ACE to lesions and has implications for the role of ACE-containing LDL in other diseases, such as atherosclerosis. (Circulation. 2002;106:2224-2230.)

Key Words: angiotensin ■ apolipoproteins ■ immunohistochemistry ■ lipoproteins ■ valves

Historically, calcific aortic stenosis had been viewed as a “degenerative” and therefore unmodifiable disease. Recent studies have demonstrated, however, that aortic sclerosis and stenosis lesions share several similarities with atherosclerosis. These include the presence of a chronic inflammatory infiltrate of macrophages and T lymphocytes,1–3 deposition of “atherogenic” lipoproteins such as low-density lipoprotein (LDL) and lipoprotein(a) [Lp(a)],4,5 and active mediators of calcification, such as osteopontin.6,7 Epidemiological studies have confirmed that aortic stenosis and atherosclerosis share several common risk factors, including older age, male sex, diabetes, smoking, hypertension, and elevated levels of LDL and Lp(a). These observations have led to the novel proposal that pharmacological strategies effective in atherosclerosis, such as hepatic hydroxymethylglutaryl coenzyme A reductase-inhibitor (statin) therapy, might slow the progression of AS.4,9 Recent, retrospective, nonrandomized trials using electron beam tomography (EBT)10,11 or echocardiography12,13 have shown that statin use may be associated with a decreased rate of AS progression.

Another class of therapies shown to be effective in decreasing clinical events in patients with atherosclerosis is the angiotensin-converting enzyme inhibitors.14 Angiotensin-converting enzyme (ACE) has been detected in human atherosclerotic lesions, where it is associated with a subset of lesion macrophages.15–17

Though in the present study, we sought to determine whether ACE might also be present in aortic sclerosis and stenosis lesions. ACE was present in both aortic sclerosis and stenosis lesions and the enzymatic product of ACE, angiotensin II, also was present in lesions. More surprisingly, although a subset of lesion macrophages contained ACE, the vast majority of...
lesion ACE was extracellular and associated with retained apolipoprotein B (apoB). This observation suggested that ACE might be associated with LDL particles. This possibility was confirmed by Western blotting, which demonstrated that ACE was present on LDL that had been isolated by ultracentrifugation from the plasma of normal volunteers.

Methods

Human Aortic Valves
Twenty-one human aortic valves were included in the primary analyses. Of these, 17 were obtained at autopsy and were classified as either normal or as having aortic sclerosis lesions based on the absence or presence of histologically detectable calcium, respectively. The remaining 4 valves were excised during valve replacement surgery for clinical aortic stenosis and so were classified as having aortic stenosis. Tissue from these patients was used in previous studies evaluating the cellular components, osteopontin content, and presence of apolipoproteins in aortic valve lesions. Sections of the valve leaflets were fixed in methanol-Carnoy’s solution (60% methanol/30% chloroform/10% glacial acetic acid) and paraffin-embedded. Five additional valves were obtained from native hearts of patients undergoing cardiac transplantation for ischemic cardiomyopathy and were fixed in 10% neutral-buffered formalin within 2 hours of organ removal. For histological and immunohistochemical studies, 6 μm-thick tissue sections were used.

Antibodies and Antisera
Immunohistochemical studies were performed using the following commercially-available monoclonal antibodies at the concentrations and incubation periods indicated: anti-CD68 (titer 1:1000 for 60 minutes; DAKO Corporation, Carpinteria, Calif) for macrophages and anti-α-actin (titer 1:1000 for 60 minutes; Boehringer-Mannheim Biochemica, Indianapolis, Ind) for smooth muscle cells or myofibroblasts. ApoB was identified using a rabbit polyclonal antisera (titer 1:1000 for 60 minutes; kind gift of Dr Thomas Innerarity, Gladstone Institute, San Francisco, Calif).

To evaluate specific components of the renin-angiotensin system, the following commercial available polyclonal antisera were used at the concentrations and incubation periods indicated: anti-ACE (titer 1:750 overnight, Chemicon International Inc, Temecula, Calif); anti-angiotensin II (Ang II) (titer 1:500, overnight, Cortex Biochem, San Leandro, Calif); and anti-angiotensin type 1 receptor (AT-1 receptor) (titer 1:200 overnight at 4°C).

Single-Label Immunohistochemistry
Single-label immunohistochemistry was performed as described previously. Briefly, tissue sections were de-paraffinized using xylene and were rehydrated with graded alcohols. The slides were blocked with 3% H2O2 for 5 minutes, washed with PBS, incubated with the primary antibody for 60 minutes or overnight, and then washed again with PBS. A biotinylated secondary antibody (anti-mouse for CD68 and α-actin; anti-rabbit for apoB or angiotensin II; anti-sheep for ACE) was then applied for 30 minutes, followed by an avidin-biotin-peroxidase conjugate (ABC Elite, Vector Laboratories) for 30 minutes. Standard peroxidase enzyme substrate, 3,3′-diaminobenzidine with nickel chloride, then was added to yield a brown reaction product identifying ACE; 3,3′-diaminobenzidine [Sigma] to yield a blue reaction product identifying apoB.

Double-Label Immunohistochemistry
For double label immunohistochemistry, tissues were deparaffinized with xylene and rehydrated with graded alcohols. Slides then were incubated overnight at 4°C with the first primary antibody (either anti-ACE or anti-apoB). Slides were washed in PBS and a biotinylated secondary antibody (anti-sheep for ACE or anti-rabbit for apoB) then was applied for 30 minutes. Slides were again washed with PBS, and either avidin-biotin-alkaline phosphatase (ABC-alkphos, Vector; for ACE staining) or an avidin-biotin peroxidase conjugate (ABC Elite, Vector; for apoB staining) then was applied. Chromogens then were applied (New Fuchsin, DAKO, to yield a red product identifying ACE; 3,3′-diaminobenzidine [Sigma] to yield a brown product identifying apoB).

Western Blotting
Western blotting was performed to determine the presence or absence of ACE in LDL or HDL isolated from normal human plasma. LDL and HDL samples were electrophoresed on 4% to 12% polyacrylamide gels (Figure 3) under reducing conditions and transferred to nitrocellulose. Nonspecific binding sites on nitrocellulose strips were blocked with PBS containing 1% (wt/vol) fatty acid free bovine serum albumin and 1% (wt/vol) nonfat dry milk, followed by incubation with either a mouse monoclonal antibody against human ACE (#MAB 3502, Chemicon International, Inc; titer 1:500) or a sheep polyclonal antibody against ACE (#AB 1273, Chemicon; titer 1:500) for 2.5 to 4 hours at room temperature. After five 10-minute washes with PBS containing 5% (wt/vol) dry milk, the blots were incubated with the appropriate immunoglobulin G-peroxidase-linked secondary antibody (Boehringer-Mannheim; titer 1:8000), and again washed 5 times with 5% (wt/vol) dry milk in PBS. The peroxidase reaction was visualized using a chemiluminescence detection system (Supersignal, Pierce) and Hyperfilm MP (Amersham Pharmacia Biotech).

Statistical Analyses
Statistical analyses were performed using GraphPad Prism, Version 3.02 software (GraphPad Prism). Correlation coefficients were determined to test for relationships between continuous variables and Fisher’s exact test was used to compare categorical variables.

Results

Detection of ACE in Aortic Valve Lesions
ACE was not detected in normal aortic valves (data not shown). ACE was present in all valves with lesions, however. Previous studies of human atherosclerosis have detected ACE in association with a subset of atherosclerotic lesion macrophages. In aortic valve lesions, ACE was detected in a subset of macrophages, but the majority of aortic valve macrophages did not contain detectable ACE (Figure 1A). In contrast, ACE was detected primarily in an extracellular distribution in aortic valve lesions (Figure 1A).

Colocalization of ACE With Apolipoprotein B in Aortic Valve Lesions
We recognized that the extracellular location of ACE in aortic valve lesions was similar to that of apoB, which is the primary

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protein found on LDL cholesterol particles. Thus, adjacent aortic valve sections were stained for apoB (Figure 1B). Extracellular ACE did, in fact, colocalize to regions of apoB deposition (Figure 1A and 1B). Although extracellular ACE staining was restricted to regions containing apoB, the extracellular ACE staining was not as extensive as that for apoB.

To confirm the relationship between extracellular ACE and apoB, computer-assisted morphometry (ImagePro Plus, Version 4.0 software, MediaCybernetics, Inc) was used to determine the immunostained areas for ACE and apoB for all 17 methacarn-fixed valves in which the leaflet was intact. Immunostained areas were expressed as a percentage of total leaflet cross-sectional area. Therefore, this quantitation was not performed on surgically excised valves, which typically were heavily fragmented as a result of surgical removal. The mean (±SD) percent of extracellular ACE-stained area was 37±35% of extracellular apoB area. In addition, there was a strong correlation ($R^2=0.90$, $P<0.0001$) between ACE and apoB areas, expressed as percents of total leaflet cross-sectional areas (Figure 2).

Figure 1. ACE in a human aortic valve lesion. A, Double immunostaining for macrophages (blue stain) and ACE (red stain) demonstrate that the majority of macrophages are blue, indicating the absence of ACE protein. A minority of macrophages contain ACE protein, identified by their purple stain. In contrast, the vast majority of red ACE staining is extracellular. B, Double immunostaining for macrophages (blue stain) and apoB, the primary protein of LDL cholesterol particles (brown stain), demonstrate the presence of extensive extracellular apoB staining, which colocalizes with extracellular ACE. Original magnification ×400.

Figure 2. Correlation of ACE and extracellular apoB in human aortic valves. For each of the 17 intact, methacarn-fixed leaflets, apoB immunostained area, expressed as a percentage of total leaflet area, is plotted on the X axis, and ACE immunostained area, also expressed as a percentage of lesion area, is plotted on the Y axis. The correlation between apoB and ACE immunostained areas is high ($R^2=0.90$, $P<0.01$).

Figure 3. Identification of ACE on normal human plasma LDL by Western blotting. With either a mouse monoclonal anti-ACE antibody (A) or the sheep polyclonal anti-ACE antiserum used in immunohistochemical experiments (B), a single band of the predicted molecular size of the ACE protein was detected on LDL. In contrast, ACE was not detected on either apoE-containing HDL (HDL+E) or apoE-free HDL (HDL-E) (B).
The strong correlation of ACE with apoB in lesions suggested that ACE was associated with retained lipoprotein particles. This raised the question of whether ACE might be associated with LDL in plasma. To investigate this possibility, Western blotting was performed on LDL that had been isolated from normal human volunteers by sequential density gradient ultracentrifugation (Figure 3). A single band of the predicted molecular size of ACE was detected on LDL using either a mouse monoclonal antibody to ACE (Figure 3A) or the

Detection of ACE on Plasma LDL

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Figure 4. Colocalization of apoB, ACE, and angiotensin II (AngII) in a human aortic valve lesion. Single-label immunostaining (black stains) was performed on adjacent sections using antibodies to apoB (A), ACE (B), and AngII (C). ApoB and ACE are colocalized. AngII, the enzymatic product of ACE, also colocalizes in the regions that contain ACE. Original magnification ×40, methyl green counterstain.

Figure 5. ACE colocalizes with extracellular apoB in a human atherosclerotic lesion. Photomicrographs show immunostaining (black immunoreaction product) for apoB (A), ACE (B), and macrophages (C) performed on neighboring sections of an atherosclerotic plaque. Arrowheads indicate regions that contain extracellular apoB (A) and ACE (B), but no macrophages. Original magnification ×100, methyl green counterstain.
polyclonal antiserum used in immunohistochemistry (Figure 3B). In contrast, ACE was not detected on HDL fractions (Figure 3B).

Detection of Angiotensin II, the Enzymatic Product of ACE, in Human Aortic Valve Lesions

To determine whether the ACE detected in aortic valve lesions might be enzymatically active, immunohistochemistry also was performed for angiotensin II on formalin-fixed valves obtained from 5 explanted hearts removed at the time of cardiac transplantation. Formalin-fixed valves were used because the anti-angiotensin II antibody did not give positive immunostaining on methacarn-fixed specimens. Immunostaining of serial sections for apoB, ACE, and angiotensin II confirmed both the observation from methacarn-fixed valves that ACE colocalized with apoB and also that angiotensin II was present in areas with extracellular ACE immunostaining (Figure 4). Thus, detection of angiotensin II in association with ACE in valve lesions is consistent with the hypothesis that the extracellular aortic valve lesion ACE is enzymatically active.

Figure 6. AT-1 receptor is not detected in normal aortic valves. Photomicrographs demonstrate immunostaining (brown stain) of adjacent sections of a normal aortic valve for \( \alpha \)-actin (A) and for the AT-1 receptor (B). Neither \( \alpha \)-actin (A) nor AT-1 receptors (B) are detected in normal valve fibroblasts. As positive controls, regions of aorta from the same sections are shown, in which SMC stain positively for both \( \alpha \)-actin (C) and AT-1 receptor (D). Original magnification \( \times 100 \), hematoxylin counterstain.

Figure 7. AT-1 receptor is present in myofibroblasts of stenotic aortic valve. Photomicrographs demonstrate adjacent sections of a surgically excised stenotic aortic valve immunostained (brown stain) for \( \alpha \)-actin (A and C) and for the AT-1 receptor (B and D). Several \( \alpha \)-actin positive cells are present (A), including \( \alpha \)-actin-expressing vascular pericytes (arrowheads) and myofibroblasts (arrows). Many of these \( \alpha \)-actin positive cell types also contain AT-1 receptor protein (B). Shown at higher power is a region of \( \alpha \)-actin-positive myofibroblasts (C) that also contain AT-1 receptor (D). Original magnification 100 (A and B) or \( \times 200 \) (C and D); hematoxylin counterstain.
Extracellular ACE and apoB in an Atherosclerotic Lesion

One of the valve specimens studied also contained a portion of a proximal coronary artery with an atherosclerotic lesion. Interestingly, extracellular ACE also was detected in association with apoB in this human atherosclerotic lesion (Figure 5). Thus, the retention of ACE-containing LDL particles on extracellular matrix also may be characteristic of human atherosclerotic lesions, as well as of aortic valvular lesions.

AT-1 Receptor Is Rarely Detected in Normal Valves but Is Present in Stenotic Valves

AT-1 receptor expression was determined in the 21 methacarn-fixed valve specimens. In normal or nonstenotic aortic valve leaflets, AT-1 receptor protein was generally not detected (Figure 6). In stenotic valves, AT-1 receptor protein typically was detected on a subset of -actin—expressing cells. These included vascular pericytes, which are a smooth muscle cell phenotype, and myofibroblasts (Figure 7). With respect to valve fibroblasts, this set of observations suggests that AT-1 receptor expression is not typical of fibroblasts, but that factors that modulate phenotypic conversion of fibroblasts into myofibroblasts (which express contractile proteins, such as -actin) is associated with up-regulation of AT-1 receptor expression. Overall, AT-1 receptor expression was detected in only 18% (3/17) of nonstenotic valves but in 75% (3/4) of stenotic valves (P=0.053 by Fisher’s exact test).

Discussion

This study is the first to provide evidence of a potential role for the renin-angiotensin system in the pathogenesis of calcific aortic valve disease, by demonstrating the presence of ACE and angiotensin II in aortic valve lesions and by demonstrating the presence of the AT-1 receptor, a major receptor for angiotensin II, in advanced aortic valve lesions. An additional surprising finding is not only that ACE and extracellular apoB colocalize in lesions but also that ACE associates with plasma LDL. This suggests that ACE may be concentrated in aortic valve lesions (and also in atherosclerotic lesions) through the retention of plasma lipoproteins.

There are a number of possible explanations for the role of angiotensin II in the pathogenesis of aortic valve disease. Angiotensin II has been detected in stable, unstable, and ruptured human coronary plaques,9 in coronary lesions of primate models of atherosclerosis,16 and in human atherectomy specimens.16 In atherosclerosis, the source of angiotensin II could be either the circulation or local production by plaque macrophages. Angiotensin II has a number of proinflammatory effects that potentially contribute to the atherosclerosis process. These include serving as a chemotactic factor for monocytes,20,21 thus stimulating the accumulation of macrophages within lesions; promoting smooth muscle cell adhesion22; enhancing uptake of modified LDL23; reducing macrophage cholesterol efflux24; and reducing the efficiency of the intrinsic fibrinolytic system via production of plasminogen activator inhibitor-1.25 With aortic valve lesions, each of these mechanisms also may play important pathogenic roles.

In addition, this study suggests that circulating lipoproteins, specifically LDL cholesterol, may play a role in the disease process by delivering ACE to aortic valve lesions. This is particularly intriguing in light of recent, nonrandomized, retrospective studies suggesting that treatment with lipid lowering drugs is associated with lower rates of aortic valve lesion progression, as measured by electron beam tomography-assessed rates of aortic valve calcium accumulation10,11 or by echocardiographically-measured rates of aortic valve area decrease.12,13

It remains unclear whether the bulk of aortic valve lesion ACE is produced locally by lesion macrophages or whether it is carried into lesions on LDL cholesterol particles. The observation that ACE is present on plasma LDL and the strong correlation between lesion apoB content and lesion ACE content are most consistent with the hypothesis that at least a substantial portion of ACE is carried into lesions on LDL particles. In addition, the observation that angiotensin II is present in regions with LDL-associated ACE suggests that this LDL-associated extracellular lesion ACE is enzymatically active.

This study has some interesting therapeutic implications for aortic valve lesion development. Specifically, is raises the possibility that ACE inhibitors or angiotensin receptor antagonists might inhibit aortic valve lesion development. In addition, it raises the possibility that, because LDL may carry ACE into lesions, pharmacological therapies that lower plasma LDL levels might decrease lesion formation at least in part by decreasing the amount of plasma LDL available to carry ACE into aortic valve lesions.

Study Limitations

This study has several limitations. First, it was performed on a relatively small number of human aortic valve specimens. The observation that ACE was consistently present in lesions, however, suggests a general role for ACE in lesion development. Second, the evidence that ACE is enzymatically active in lesions is circumstantial, in that angiotensin II was detected in lesions, but we provide no direct evidence of a cellular effect of angiotensin II in lesion cells. Third, the study provides strong evidence that ACE is carried on plasma LDL, but leaves several interesting questions unanswered, such as what proportion of plasma LDL particles contain ACE, whether modifications in LDL, such as LDL particle size, affect the affinity of ACE for LDL particles, and whether known polymorphisms in the ACE protein, such as the insertion deletion polymorphism, affect the affinity of ACE for LDL particles. These intriguing questions should form the basis for additional studies designed to characterize the ACE/LDL interaction demonstrated in the present study.

Conclusion

This study has applied immunohistochemical techniques to a series of aortic valves, ranging from normal to stenotic, to evaluate a potential role for the renin-angiotensin system in aortic valve disease pathogenesis. The study documents the presence of ACE and angiotensin II in sclerotic and stenotic aortic valve lesions, thereby suggesting a role for the renin-angiotensin system in the pathogenesis of calcific aortic
valve disease. These findings also raise the possibility that renin-angiotensin system inhibition with ACE inhibitors or angiotensin receptor antagonists may favorably affect the disease process. Given the consistent benefit of ACE inhibition in a similar disease, namely atherosclerosis, further study of this therapy in calcific aortic valvular disease seems warranted.

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