Valvular Heart Disease

Multimodality Molecular Imaging Identifies Proteolytic and Osteogenic Activities in Early Aortic Valve Disease

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Background—Visualizing early changes in valvular cell functions in vivo may predict the future risk and identify therapeutic targets for prevention of aortic valve stenosis.

Methods and Results—To test the hypotheses that (1) aortic stenosis shares a similar pathogenesis to atherosclerosis and (2) molecular imaging can detect early changes in aortic valve disease, we used in vivo a panel of near-infrared fluorescence imaging agents to map endothelial cells, macrophages, proteolysis, and osteogenesis in aortic valves of hypercholesterolemic apolipoprotein E–deficient mice (30 weeks old, n = 30). Apolipoprotein E–deficient mice with no probe injection (n = 10) and wild-type mice (n = 10) served as controls. Valves of apolipoprotein E–deficient mice contained macrophages, were thicker than wild-type mice (P < 0.001), and showed early dysfunction detected by MRI in vivo. Fluorescence imaging detected uptake of macrophage-targeted magnetofluorescent nanoparticles (24 hours after injection) in apolipoprotein E–deficient valves, which was negligible in controls (P < 0.01). Valvular macrophages showed proteolytic activity visualized by protease-activatable near-infrared fluorescence probes. Ex vivo magnetic resonance imaging enhanced with vascular cell adhesion molecule-1–targeted nanoparticles detected endothelial activation in valve commissures, the regions of highest mechanical stress. Osteogenic near-infrared fluorescence signals colocalized with alkaline phosphatase activity and expression of osteopontin, osteocalcin, Runx2/Cbfa1, Osterix, and Notch1 despite no evidence of calcium deposits, which suggests ongoing active processes of osteogenesis in inflamed valves. Notably, the aortic wall contained advanced calcification. Quantitative image analysis correlated near-infrared fluorescence signals with immuneactive vascular cell adhesion molecule-1, macrophages, and cathepsin-B (P < 0.001).

Conclusions—Molecular imaging can detect in vivo the key cellular events in early aortic valve disease, including endothelial and macrophage activation, proteolytic activity, and osteogenesis. (Circulation. 2007;115:377-386.)

Key Words: valves ▪ stenosis ▪ inflammation ▪ atherosclerosis ▪ hypercholesterolemia ▪ imaging

Aortic valve disease is a progressive disorder that ranges from mild valve thickening to severe calcification with impaired leaflet motion or aortic valve stenosis.1 Aortic valve stenosis is the most common valvular heart disease; no effective therapy is currently available, however, other than surgical valve replacement, performed in ≈85 000 patients in the United States and 275 000 worldwide annually.2,3 Clinical evidence suggests that coronary atherosclerosis and aortic valve stenosis share similar epidemiological risk factors, such as age, sex, hypercholesterolemia, and hypertension.4–6 Clinicopathological studies of human stenotic aortic valves identified lesions similar to those in atherosclerotic plaques that contained inflammatory cells and calcific deposits.7,8 Preclinical studies further demonstrated atherosclerosis-like lesions in the aortic valve leaflets in rabbit and mouse models of atherosclerosis.9–11 The cellular and molecular factors involved in the development of aortic valve stenosis remain largely obscure, however. Understanding the mechanisms of aortic valve inflammation and calcification will provide mechanistic insights into the pathogenesis of aortic valve disease. Furthermore, detection of early molecular and functional abnormalities in aortic valves in vivo may predict the future risk of subclinical valvular lesions and identify targets for effective therapeutic strategies to prevent aortic valve stenosis.

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We previously demonstrated a common paradigm of interstitial and endothelial cell activation during valve development, disease, surgical transplantation, and tissue engineering.12–17 Activated valvular cells overexpress matrix
metalloproteinases (eg, MMP-1, MMP-2, MMP-9, and MMP-13),12,13,16,17 cysteine proteases (cathepsin S and cathepsin K),12 adhesion molecules (vascular cell adhesion molecule-1 [VCAM-1] and intercellular adhesion molecule-1),17 embryonic myosin,12,13,17 and interleukin-1β.12 These molecules play various roles in inflammation and tissue remodeling and may represent surrogate endpoints for valve development, remodeling, and disease progression. Newer imaging technologies currently available may detect in vivo expression and activity of molecules responsible for valve pathology and monitor maturation/remodeling of native and tissue-engineered valves in individual patients. We recently used such imaging approaches to demonstrate endothelial cell and macrophage activation in aortas of hypercholesterolemic apolipoprotein E-deficient (apoE−/−) mice, an established model of atherosclerosis.18–20 We therefore hypothesized that multimodality molecular imaging can detect in vivo early changes in valvular cell functions and identify novel molecular targets for prevention of aortic valve stenosis. The present study aimed to map key cellular and molecular functions, such as (1) endothelial cell activation, (2) macrophage activation, (3) proteolytic enzyme activity, and (4) osteoblastic activity in the aortic valves of hypercholesterolemic apoE−/− mice using a recently developed comprehensive set of near-infrared fluorescent (NIRF) imaging probes that specifically target biological processes associated with inflammation.

**Methods**

**Animal Protocol**

We studied aortic valves of 30 apoE−/− mice at 30 weeks of age that had received an atherogenic diet (Teklad TD 88137; 42% milk fat, 0.2% total cholesterol, Harlan, Indianapolis, Ind) for 20 weeks. Age-matched wild-type C57/B6 mice (n=10, Jackson Laboratory, Bar Harbor, Me) and apoE−/− mice with no probe injection (n=10) served as controls. Serum cholesterol levels were determined at the time of euthanasia with a colorimetric assay according to the manufacturer’s instructions (Raichem, San Diego, Calif). The Subcommittee on Research Animal Care at Massachusetts General Hospital approved all procedures.

**Macrosopic Fluorescence Reflectance Imaging**

Mice received imaging agents or saline (control) via intravenous injection 24 or 48 hours before imaging. After mice had been euthanized, aortas were perfused with saline, dissected, and imaged to map the macrosopic NIRF signals elaborated from each imaging agent at the aortic root with a custom-built fluorescence reflectance imaging system equipped with multichannel filter sets, including green (green fluorescent protein/fluorescein isothiocyanate; excitation, 406 to 450 nm; emission, 495 to 525 nm), far red (VT680; excitation, 615 to 645 nm; emission, 680 to 720 nm), and near-infrared (indocyanine green; excitation, 716 to 756 nm; emission, 780 to 820 nm; Omega Optical, Brattleboro, Vt). Fluorescence images were obtained with an exposure time of 1 to 60 seconds. Subsequently, aortic roots dissected through the area of high NIRF signal were processed for histological analysis.

**Microscopic Laser Scanning Fluorescence Imaging**

After the aortic root was isolated, multichannel fluorescence imaging was performed with a laser scanning fluorescence microscope (IV100, Olympus Corp, Tokyo, Japan) specifically developed for imaging small experimental animals. Three laser lines at 450, 680, and 750 nm were used. Images of 512×512 pixels with a pixel size of ~2.75×2.8 μm/pixel were collected with the Fluoview 300 software program (Olympus) and stored as multilayer, 16-bit image file format (TIFF) files. Subsequently, aortic roots with leaflets were processed for histological analyses.

**Magnetic Resonance Imaging**

For high-resolution ex vivo magnetic resonance imaging (MRI), the aortic root was immersed in liquid agar to minimize air–tissue interface susceptibility artifacts and movement during imaging. The aorta was then placed in a 14.0-T vertical-bore system with microimaging capabilities (Bruker BioSpin MRI, Inc, Billerica, Mass). Gradient-recalled echocardiographic images were obtained with the following parameters: in-plane resolution, 70×70 μm; slice thickness, 0.5 mm; repetition time, 200 ms; and echo time, 7.0 ms. Images were analyzed with a shareware software package (OsiriX version 1.7.1). After imaging, the aorta was removed from the agar and embedded for histological analyses.

In vivo MRI was performed under inhalation anesthesia (isoflurane 1% to 2% vol/vol plus 4 L of O2) on a 9.4-T horizontal-bore scanner (Bruker Biospec, Billerica, Mass). We obtained bright-blood cine images with ECG and respiratory gating (SA Instruments, Stony Brook, NY) using a gradient-echo FLASH sequence and a dedicated mouse cardiac surface coil. Imaging parameters were as follows: in-plane resolution, 125×125 μm; slice thickness, 1 mm; 16 frames per RR interval (repetition time, 7.0 to 8.0 ms); echo time, 2.7 ms; and number of excitations, 8.

**Correlative Histopathological Assessment**

**Morphological Characterization**

Tissue samples were frozen in OCT compound (Sakura Finetech, Torrance, Calif), and 5-μm serial sections were cut through the aortic valves. All 3 cusps were stained with hematoxylin and eosin for general morphology. Activity of alkaline phosphatase (crucial for initiating mineralization) was detected on cryosections that were directly incubated with conjugated antibody (red alkaline phosphatase substrate kit; Vector Labs, Burlingame, Calif). Von Kossa silver stain was used to visualize inorganic phosphate calcium salts and Alizarin red to detect calcium deposits on adjacent sections. Aortic arches with prominent calcification were used as positive controls.

**Multichannel Fluorescence Microscopy**

We performed multichannel fluorescence microscopy to examine activation of valvular cells and proteolytic and osteogenic activity. Sections were imaged with an upright epifluorescence microscope (Eclipse 80i, Nikon Instruments, Melville, NY) with a cooled CCD camera (Cascade, Photometrics, Tucson, Ariz). Fluorescence images were obtained at a wavelength of green (filter, 480±20 nm; excitation, 535±25 nm; emission, Q505LP bandpass), far red (filter, 650±22.5 nm; excitation, 710±25 nm; emission, Q680LP bandpass), or near-infrared (filter, 775±25 nm; excitation, 845±27.5 nm; emission, Q810LP bandpass), depending on the probe. The same exposure time, which ranged from 500 to 2000 ms, was used for each probe.

**Immunohistochemistry**

For validation of NIRF signals, we used immunohistochemistry for macrophages (rat monoclonal antibody against mouse Mac3, BD Biosciences, San Jose, Calif), myofibroblasts (α-smooth muscle actin, 1A4, Dako, Carpinteria, Calif), activated endothelial cells (anti-mouse VCAM-1/CD106, BD Pharmingen, San Diego, Calif), gelatinases (polyclonal rabbit anti-mouse MMP-2 and MMP-9, Chemicon International, Temecula, Calif), cathepsin B and K (goat polyclonal antibodies, Santa Cruz Biotechnology, Santa Cruz, Calif), osteoblast differentiation markers (goat polyclonal antibodies against mouse osteocalcin and osteopontin, Abcam, Cambridge, Mass), osteogenic transcription factors and signaling (goat polyclonal anti-human Cbfal/Runx2 antibody, RD Systems, Minneapolis, Minn; rabbit polyclonal antibody to Sp7/Osterix, Abcam, Cambridge, Mass; and rabbit polyclonal antibody to cleaved Notch1, Cell Signaling Technology, Inc, Danvers, Mass). The avidin-biotin peroxidase method was used for immunohistochemistry. The reaction
was visualized with a 3-amino-9-ethyl-carbazol substrate (AEC, Sigma Chemical, St Louis, Mo), which yielded red reaction products. Adjacent sections treated with nonimmune IgG provided controls for antibody specificity. Images were captured with a digital camera (Nikon DXM 1200-F, Nikon Inc, Melville, NY).

Targeted and Activatable Molecular Imaging Agents

Macrophage-Targeted Magnetofluorescent Nanoparticles

Magnetofluorescent nanoparticles (MFNPs) are internalized by macrophages after systemic administration and therefore serve as imaging agents for in vivo detection of these proinflammatory phagocytes. The nanoparticle was covered with a layer of dextran, contained a superparamagnetic iron oxide core detectable by MRI, and was labeled with far-red fluorochromes for fluorescence detection (VT680; excitation/emission, 673/694 nm; Amersham Biosciences, Amersham, United Kingdom).

VCAM-1–Targeted Imaging Agent

Imaging of VCAM-1 expression in vivo used a peptide-derivated nanoparticle (VCAM-1 internalizing nanoparticle-28) that triggers cellular internalization and “trapping” in VCAM-1–expressing cells, which leads to a biological signal amplification recently developed by in vivo phage display in apoE<sup>−/−</sup> mice. This linear peptide is homologous to the integrin very late antigen-4, a known ligand of VCAM-1. VCAM-1–targeted peptides were then conjugated with MFNPs with an average of 2 fluorochromes per nanoparticle (fluorescence in NIRF window; excitation/emission 673/694 nm).

Protease-Activatable NIRF Agents

Activatable NIRF agents, well validated in a mouse model of atherosclerosis, visualized activity of gelatinases (MMP-2/9; Gelsense680, VisEn Medical, Inc, Woburn, Mass) and cysteine proteases (predominantly cathepsin B; Prosense680, VisEn Medical, Inc) in inflamed aortic valves. These quenched substrate probes produce negligible fluorescence at baseline because of closely spaced fluorochromes. However, on protease-mediated cleavage and fluorochrome release, the NIRF signal increases by approximately 200-fold. Both agents fluoresce in the NIRF window of excitation/emission 673/694 nm wavelength.

Calcification

Recent studies demonstrated that bisphosphonate-conjugated imaging agents can detect the functions of osteoblasts and calcific deposition in bone and coronary arteries. OsteoSense750 (VisEn Medical, Inc) binds to sites of calcification in vivo, particularly to hydroxyapatite, and serves as an imaging agent for detection of osteoblastic activity. This agent elaborates fluorescence detectable through the NIRF window (excitation/emission 750/780 nm).

Quantitative Assessment and Statistical Analysis

Overall thickness of the leaflets averaged over 5 equally distributed length measurements throughout the valve for both wild-type and apoE<sup>−/−</sup> mice was quantified with imaging software (IPLab version 3.9.3; Scanalytic, Inc, Rockville, Md). Digitized NIRF and immunohistochemistry images were analyzed with imaging software (IPLab version 3.9.3). NIRF images were segmented by thresholding grayscale intensity at a fixed level (20 000 arbitrary fluorescence units), and 5 regions of interest were randomly selected along each leaflet. The same sections were then processed for immunohistochemistry (Mac3, VCAM-1, and cathepsin B), digitized, and segmented by thresholding red intensity at a fixed level (100 arbitrary units). The percent-positive area per leaflet was calculated and correlated with average NIRF signal intensity of the same region of interest. The valve target-to-background ratio was calculated as valve signal/adjacent aorta background signal.

Data are presented as mean±SEM. The Student (unpaired) t test was performed with GraphPad Prism (version 4.00, GraphPad Software, San Diego, Calif). Probability values less than 0.05 were considered significant.

Results

Aortic Valves of Hypercholesterolemic Mice Develop Lesions Similar to Early Atherosclerotic Plaques

Aortic valve leaflets of wild-type mice were thin and composed predominantly of quiescent fibroblast-like cells with no macrophage infiltration (Figure 1A; serum cholesterol, 108±26 mg/dL). In contrast, cholesterol-fed apoE<sup>−/−</sup> mice of 30 weeks of age (serum cholesterol, 588±47 mg/dL; P<0.001 versus wild-type controls) had thickened leaflets with macrophage-rich subendothelial lesions on the aortic site of the valve, located mostly in the base of the leaflet (Figure 1A). Quantitative analysis revealed significantly thicker leaflets in apoE<sup>−/−</sup> mouse valves than in wild-type mice.
Endothelial Cell Activation Occurs in the Commissures of Diseased Aortic Valves

We examined endothelial cell activation gauged by VCAM-1 expression in aortic valves of apoE−/− mice that received the VCAM-1–targeted agent (VCAM-1 internalizing nanoparticle-28, 30 mg/kg iron, n=5) or 0.9% saline (n=3) via intravenous injection 48 hours before imaging. High-resolution 14.0-T MRI demonstrated signal enhancement ex vivo. As visualized on short-axis slices through the aortic root, VCAM-1 internalizing nanoparticle-28 predominantly targeted the commissures of the aortic valve, providing a robust landmark for histopathological assessment (Figure 2A). Fluorescence NIRF microscopy (680 nm) of VCAM-1 internalizing nanoparticle-28 distribution visualized ex vivo by MRI correlated with VCAM-1 expression detected by immunohistochemistry ($R^2=0.784$, $P<0.001$; Figure 2B and 2C).

Macrophages Accumulate in Early Aortic Valve Lesions

A subset of apoE−/− mice received macrophage-targeted MFNPs (15 mg/kg iron, n=5) or 0.9% saline (n=3) 24 hours before imaging. Fluorescence reflectance imaging through the 680-nm channel detected NIRF signals in the areas corresponding to the aortic root of mice that received MFNPs (Figure 3A). On cross sections through the aortic valve, immunoreactive macrophages colocalized with NIRF signals. In contrast, we found no significant NIRF signal enhancement in saline-injected controls either ex vivo or in situ (Figure 3B). The target-to-background ratio was 4-fold higher in apoE−/− mice than in controls (6.0 versus 1.6; $P<0.01$). Quantitative histopathological analysis correlated NIRF signals (detected by fluorescence microscopy through the 680-nm channel) with macrophages (detected by Mac3 staining) in valvular lesions ($R^2=0.879$, $P<0.001$; Figure 3C).

Macrophages and Activated Valvular Myofibroblasts Elaborate Proteolytic Activity in Early Aortic Valve Disease

We further assessed the activity of matrix-degrading enzymes in aortic valves of cholesterol-fed apoE−/− mice injected with NIRF protease-activatable probes: cysteine proteinases (cathepsin B, n=5) or gelatinases (MMP-2 and MMP-9, n=5). Wild-type control mice produced undetectable levels of NIRF signals determined by macroscopic fluorescence reflectance imaging (data not shown) and multichannel fluorescence imaging.

Figure 2. Endothelial cell activation occurs in the commissures of diseased aortic valves. A, Ex vivo MRI. Left, Long-axis view demonstrated the aortic arch and root. Dotted line demonstrates slice position of short-axis view. Middle, Short-axis view shows negative signal enhancement (darkening) caused by uptake of VCAM-1–targeted nanoparticles. Right, Color-coded signal intensities (red) show focused uptake of VCAM-1 in commissures (arrows). B, Immunoreactive VCAM-1 colocalizes with NIRF signal (excitation/emission 673/694 nm, exposure time 500 ms) in aortic valve commissure. Magnification ×400. C, Quantitative image analysis correlates immunoreactive VCAM-1 with NIRF signals.

(89.3±7.5 μm, n=20 versus 31.3±2.1 μm, n=10; $P<0.0001$; Figure 1B).
microscopy (Figure 4A). Notably, normal valves in wild-type mice did not contain immunoreactive cathepsin B. Fluorescence reflectance imaging in apoE<sup>−/−</sup> mice injected with the cathepsin-activatable NIRF probe yielded strong signals at the level of aortic valves (Figure 4B). Correlative histopathological analysis colocalized NIRF signals with immunoreactive cathepsin B in the macrophage-rich lesions (Mac3) of aortic valves (Figure 4C). Quantitative analysis further demonstrated a close positive correlation between NIRF-positive areas and cathepsin B–immunopositive areas per leaflet \( (R^2=0.858, P<0.001; \text{Figure } 4D) \). Sites of atherosclerotic valves of apoE<sup>−/−</sup> mice that exhibited fluorescence signals elaborated from an activatable NIRF probe for gelatinases colocalized with activated myofibroblast-like cells (α-smooth muscle actin–positive cells) and macrophages (Mac3) bearing immunoreactive MMP-2 and MMP-9 (Figure 5).

Valvular Myofibroblasts Exhibit Osteoblastic Activity in the Early Stage of Aortic Valve Stenosis

To detect osteoblastic activity in inflamed aortic valves, we administered OsteoSense750 in apoE<sup>−/−</sup> mice 24 hours before imaging \( (n=10) \). Mice were coinjected with spectrally distinct NIRF MFNPs to visualize macrophages. Ex vivo imaging with multichannel, high-resolution, laser scanning fluorescence microscopy on opened aortic root detected signals for osteogenic activity elaborated from OsteoSense750 \( (750 \text{ nm}, \text{target-to-background ratio}=29.7\pm1.6, P<0.01; \text{Figure } 6A) \). A macrophage-
targeted NIRF MFNP (680 nm, target-to-background ratio = 5.1 ± 0.6, P < 0.01) visualized inflammation (Figure 6A). Fluorescence microscopy on cross sections of the same aortic valve further colocalized NIRF signals for osteoblastic activity (750 nm) in myofibroblasts with alkaline phosphatase activity, immunoreactive osteopontin and osteocalcin (differentiated osteoblasts), Runx2/Cbfa1 and Osterix (osteogenic transcription factors), and cleaved Notch1 (indicative of activated Notch signaling), whereas Alizarin red stain and von Kossa stain detected no evidence of mineralization (Figure 6B), which suggests the active ongoing processes of osteogenesis in inflamed valves (detected by Mac3; data not shown). In contrast, cross sections through aortic arches of the same animals demonstrated prominent calcification detected by both Alizarin red and von Kossa methods. Runx2/Cbfa1, Osterix, and cleaved Notch1 were undetectable in aortic valves of wild-type mice (data not shown).

Figure 4. Macrophages contribute to elastolytic activity in early aortic valve disease. A, Cross sections of wild-type (WT) control valves elaborating negligible NIRF signal enhancement were not immunoreactive to cathepsin B. B, Macrosscopic fluorescence imaging exhibited NIRF signals in the aortic root of apoE−/− mice that received cathepsin-activatable agent (VT680; excitation, 615 to 645 nm; emission, 680 to 720 nm). C, Correlative histopathological analysis with multichannel NIRF microscopy (excitation/emission, 673/694 nm; exposure time, 1000 ms) and immunohistochemistry colocalized NIRF signals with macrophages (Mac3) expressing cathepsin B. Bottom panels demonstrate high-power views. Magnification ×100 (top) and ×400 (bottom). D, Quantitative analysis correlated NIRF signals and immunoreactive cathepsin B.
myofibroblasts (colocalizes with MMP-2 and MMP-9 expressed by activated valve stenosis and the feasibility of imaging early changes in present study has further explored the pathogenesis of aortic valve abnormalities in low-density lipoprotein rece-

work by Drolet and colleagues demonstrated significant valve sclerosis that resembles human aortic stenosis. The mice (95 weeks old) on regular chow developed degenerative features (eg, macrophage accumulation and calcification).7–9 A recent study by Tanaka et al10 reported that aged apoE mice injected with gelatinase MMP-2/9 agent colocalizes with MMP-2 and MMP-9 expressed by activated myofibroblasts (α-smooth muscle actin–positive cells) and mac-

rophages (Mac3) in the valve leaflets. Magnification ×400.

MRI Detected In Vivo Early Valvular Dysfunction in Aortic Valve Disease

High-resolution cine magnetic resonance images examined in vivo the functional integrity of the aortic valve. Three consecutive early diastolic frames from one such cine demonstrated that a jetlike area of flow-related spin dephasing, which produces a loss of signal, propagates across the leaflets of the aortic valve into the left ventricle (Figure 7). These findings are consistent with aortic valve regurgitation, previously described in apoE−/− mice by echocardiography.10,11

Discussion

Accumulating preclinical and clinical evidence suggests that aortic valve stenosis and atherosclerosis share similar mechanisms, genetic mutations, risk factors, and histopathological features (eg, macrophage accumulation and calcification).7–9 A recent study by Tanaka et al10 reported that aged apoE−/− mice (95 weeks old) on regular chow developed degenerative valve sclerosis that resembles human aortic stenosis. The work by Drolet and colleagues11 demonstrated significant aortic valve abnormalities in low-density lipoprotein receptor–deficient mice fed a high-fat/high-carbohydrate diet. The present study has further explored the pathogenesis of aortic valve stenosis and the feasibility of imaging early changes in aortic valve disease. The present study demonstrated that a high-cholesterol diet induced valve thickening in 30-week-old apoE−/− mice, which was associated with the presence of the macrophage-rich lesions that are highly prone to calcification (Figures 1 through 6); therefore, these mice likely have an accelerated rate of disease progression and can serve as a much-needed animal model to study the pathogenesis of aortic valve stenosis. The present study also visualized key cellular and molecular events (eg, endothelial cell activation, macrophage activation, proteolytic activity, and osteoblastic activity/premieralization) in early lesions that may be involved in the development of valvular disease and cause structural changes resulting in valve insufficiency (Figure 7). Moreover, it unraveled dynamic biological processes in vivo in valve disease progression rather than merely analyzing the static and degenerative conditions at the time of death. In addition, the present study linked hypercholesterolemia and valvular inflammation, which suggests that atherosgen factors also contribute to the pathogenesis of aortic valve sclerosis.

Aortic valves open and close ≈100 000 times a day and therefore bear repetitive changes in shape and dimension throughout the cardiac cycle. The flexion area of the aortic leaflets near the attachment of the aortic root (commissure) encounters the highest mechanical forces,26 which might induce endothelial cell activation/injury and expression of adhesion molecules. Indeed, several studies demonstrated increased VCAM-1, intercellular adhesion molecule-1, and E-selectin expression in surgically removed diseased heart valves.27,28 Strict temporal and spatial regulation makes these adhesion molecules ideal targets for molecular imaging. The present study using MRI and NIRF microscopy clearly demonstrates ex vivo that distribution of a VCAM-1–targeted agent mostly occurs in valve commissures and correlates well with immunoreactive VCAM-1 (Figure 2). These results further suggest that endothelial cell activation/damage occurs at the regions of high flexure and increased mechanical forces and that inflammatory cells likely enter the leaflets via circulation in response to endothelial cell activation or injury.

In addition to mechanical stresses, various atherosgenic components such as elevated plasma lipids may induce valve endothelial cell activation that results in an amplification cascade of events such as monocyte recruitment, visualized in the present study by macrophage-targeted MFNPs. In addition, we and others have shown that macrophages and valvular interstitial cells (activated myofibroblast-like cells) elaborate excessive levels of proteolytic enzymes (eg, collagenase-1/MMP-1, collagenase-3/MMP-13, gelatinase-A/MMP-2, and gelatinase-B/MMP-9) and cysteine endoproteases (cathepsins) and contribute critically to collagen and elastin degradation that leads to vascular and valvular remodeling and subsequent structural changes.12,13,15,16,29–31 Furthermore, elastolytic enzymes (eg, gelatinases, stromelysin, and cathepsins) may initiate elastin degradation, which provides a nidus for hydroxyapatite crystal formation,32 and therefore, these enzymes may play a considerable role in aortic calcification. Several lines of evidence also suggest osteopontin-induced MMP-9 activity in aortic mesenchymal cells, which may contribute to the vascular inflammation and calcification.33 In the present study, we demonstrated os-
teopontin expression and MMP-9 activity in inflamed aortic valves. In addition to extracellular matrix, MMPs cleave various substrates (e.g., interleukin-1β precursor and tissue factor pathway inhibitor) and therefore may enhance valvular inflammation.\textsuperscript{34,35} The multimodality imaging approach used in the present study visualized proteolytic activity elaborated from valvular macrophages and activated myofibroblasts (Figures 3 through 5) and thus may provide a biological readout of inflammation and matrix degradation and predict the risk of subclinical aortic valve stenosis.

The high morbidity and mortality rates in patients with aortic sclerosis and the significant portion of those who subsequently develop aortic stenosis suggest the need for close follow-up and serial evaluation once lesions are identi-
Conventional structural imaging modalities can identify pronounced late-stage calcification; no current imaging methods can detect in vivo early mineralization and osteogenesis in cardiac valves, however. The present study detected an amplified osteoblastic NIRF signal that resulted from binding of the imaging agent to nanomolar concentrations of calcium/hydroxyapatite complexes elaborated by α-smooth muscle actin-positive cells. These valvular myofibroblast-like cells exhibited alkaline phosphatase activity, osteopontin, osteocalcin, and osteogenic transcription factors such as Runx2/Cbfa1 and Osterix, which indicates highly regulated active processes of ongoing mineralization before development of macroscopic and microscopic calcification (Figure 6). We also found that large populations of myofibroblast-like cells contained a cleaved form of Notch1, which may direct osteoblast differentiation. In addition, a spectrally distinct NIRF signal in the same valves colocalized with macrophages that expressed osteoclastic cathepsin K (expressed during bone resorption; data not shown), which suggests that the early development of aortic valve stenosis involves osteoblastic and osteoclastic activities in parallel. What causes the imbalance of these activities toward mineralization in early aortic valve disease, however, requires further investigation. Nevertheless, the results of the present study documented here show an association of valvular lesions with features of typical atherosclerotic plaques, including endothelial activation, inflammation, proteolytic activity, and osteogenesis. Therefore, modification of atherogenic factors and pharmacological therapies that target proinflammatory pathways may retard the progression of aortic valve calcification when introduced early. Molecular imaging of the earliest stages of calcification may identify high-risk valves while disease is silent and may enable the monitoring of valvular osteogenic activity during therapeutic interventions such as lipid lowering.

Collectively, our present findings visualizing early changes of cellular functions in vivo support a concept of the development of calcific aortic valve disease (see review by O’Brien). Mechanical forces and oxidized lipids may activate valvular endothelium and initiate recruitment of inflammatory monocytes/macrophages. Activated macrophages produce a variety of cytokines, growth factors, and matrix-degrading enzymes. Proteolytic enzyme action, in turn, may cause extracellular matrix remodeling and thickening/stiffening of the leaflets, resulting in valvular dysfunction. The resultant altered mechanical stresses and disturbed flow patterns may further induce inflammation and differentiation of valvular fibroblasts into activated myofibroblasts and, subsequently, into osteoblast-like cells through upregulation of the Runx2/Cbfa1 pathway, leading to the deposition of calcium primarily in the valvular commissures and to immobilization of the aortic leaflets. Therefore, a better understanding of the molecular mechanisms of calcification that causes dysfunction of aortic valves is required to improve therapies for aortic valve stenosis. Ongoing efforts that combine rigorous assessment of valve pathology and further development of imaging technologies will provide novel insights into an optimum therapeutic strategy for patients with aortic valve stenosis and other valvular diseases.


**CLINICAL PERSPECTIVE**

Aortic valves with severe stenosis and calcification that may cause clinical complications often are surgically replaced. Although current valve substitutes behave similarly in most recipients, responses of engineered tissue implantation may have considerable patient-to-patient variability. Therefore, it is important not only to understand how individual factors and responses to injury affect the structure and function of tissue-engineered valves, but also to monitor, noninvasively, tissue healing and remodeling in vivo, which requires emerging technologies such as molecular imaging, which is able to assess tissue composition, microstructure, and biological processes. The agents used in the present study can potentially be tested in a clinical setting and detected by high-resolution magnetic resonance imaging or optical approaches. Catheter-based fluorescence sensors and confocal microscopy are expected to allow high-resolution, multichannel, in vivo imaging of the different biological processes such as inflammation and osteogenesis. In addition, imaging of inflammatory and calcific foci in the earliest aortic valve lesions will permit testing of pathophysiological hypotheses in vivo and could serve as an intermediate end point based on biological function in addition to anatomy in the evaluation of novel therapies that target inflammation and calcification and, hence, permit intervention before aortic stenosis develops. Our findings suggest that molecular imaging approaches will (1) improve our understanding of the molecular bases of development of aortic stenosis and other valve diseases, (2) identify subclinical valvular lesions, (3) predict the future risk of such lesions, (4) help to establish individualized therapeutic strategies, (5) evaluate effects of novel therapies, and (6) monitor remodeling of implanted tissue-engineered heart valves.
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