Extracellular Matrix Remodeling and Organization in Developing and Diseased Aortic Valves

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Abstract—Heart valve disease is an important cause of morbidity and mortality worldwide. Little is known about valve disease pathogenesis, but increasing evidence implicates a genetic basis for valve disease, suggesting a developmental origin. Although the cellular and molecular processes involved in early valvulogenesis have been well described, less is known about the regulation of valve extracellular matrix (ECM) organization and valvular interstitial cell (VIC) distribution that characterize the mature valve structure. Histochemistry, immunohistochemistry, and electron microscopy were used to examine ECM organization, VIC distribution, and cell proliferation during late valvulogenesis in chicken and mouse. In mature valves, ECM organization is conserved across species, and developmental studies demonstrate that ECM stratification begins during late embryonic cusp remodeling and continues into postnatal life. Cell proliferation decreases concomitant with ECM stratification and VIC compartmentalization. Explanted, stenotic bicuspid aortic valves (BAVs) from pediatric patients were also examined. The diseased valves exhibited disruption of the highly organized ECM and VIC distribution seen in normal valves. Cusps from diseased valves were thickened with increased and disorganized collagens and proteoglycans, decreased and fragmented elastic fibers, and cellular disarray without calcification or cell proliferation. Taken together, these studies show that normal valve development is characterized by spatiotemporal coordination of ECM organization and VIC compartmentalization and that these developmental processes are disrupted in pediatric patients with diseased BAVs. (Circ Res. 2006;98:1431-1438.)

Key Words: valve disease extracellular matrix valvular interstitial cells cardiac development

Valve replacement, usually for aortic valve disease, is the second most common cardiac operation performed in the US, and the need for reintervention is common.1,2 Little is known about valve disease pathogenesis, but increasing evidence implicates a genetic basis for valve malformation, suggesting a developmental origin.3–7 Valve development is initiated with endothelial-mesenchymal transformation of the endocardium in the outflow tract and atrioventricular (AV) canal to form endocardial cushions, and this process has been extensively studied.8,9 Endocardial cushions subsequently elongate as a result of cell proliferation and undergo expansion and remodeling of the extracellular matrix (ECM) to form the mature semilunar valve cusps and AV valve leaflets. The morphogenetic events that characterize valvulogenesis during late embryonic and postnatal development are largely unknown.10

The mature valve structure is composed of ECM, valvular interstitial cells (VICs), and overlying endothelial cells. The ECM is composed of 3 highly organized overlapping layers with distinct mechanical properties arranged in orientation to blood flow in the semilunar and AV valves.11,12 The primary components of these layers are collagens, proteoglycans, and elastin. In aortic valves, the fibrosa, or arterial aspect of the cusp, is composed primarily of collagen fibers; the spongiosa, or central aspect, consists predominantly of loosely arranged proteoglycans; and the ventricularis, or ventricular aspect, contains elastin fibers.13–15 Studies of diseased valves in adult patients have shown cusp and leaflet thickening, collagen fiber disorganization, increased VIC density, and calcification.16,17 However, the interpretation of these histopathological studies has been limited by common valve disease comorbidities such as aging, hypertension, and hypercholesterolemia.

In this study, we examined the processes of ECM stratification and VIC distribution during valve development in chicken and mouse. These findings were applied to histopathological assessment of explanted, stenotic bicuspid aortic valves (BAV) from pediatric patients. We found that late valvulogenesis is characterized by spatiotemporal coordination of ECM organization and VIC compartmentalization, and the developmental programs underlying cusp remodeling contribute to ongoing valve growth and maintenance in postnatal life. These developmental processes...
are disrupted in pediatric patients with a diseased aortic valve resulting from a viable, congenital malformation (BAV).

Materials and Methods

Chicken and Mouse Embryo Collection
White leghorn chicken eggs (Spafas Inc, Roanoke, Ill) were incubated at 38°C in high humidity, and hearts were collected at Hamburger–Hamilton stages 29, 36, and 42, corresponding to embryonic day 6 (E6), E10, and E16, respectively. Juvenile chicken hearts (Tewes Poultry Farm, Erlanger, Ky) were collected at 8 weeks. Mouse embryos were obtained from timed pregnancies with observation of a copulation plug designated as E0.5. Mouse hearts were collected at E12.5, E14.5, E18.5, 2 weeks, and 4 months. Valve tissue was also obtained from rabbit (3 months) and sheep (20 months). All animal procedures were performed in accordance with institutional guidelines.

Pediatric Aortic Valve Tissue Collection
Human specimens were obtained from pediatric patients with aortic valve disease undergoing aortic valve replacement (affected) and at the time of autopsy from age-matched pediatric patients who died of noncardiac causes (control). Medical records were reviewed, and patients with a history of a genetic syndrome, infective endocarditis, or rheumatic heart disease were excluded. These studies were approved by the Institutional Review Board at Cincinnati Children’s Hospital Medical Center.

Histochemistry
Hearts or valve tissue were dissected, fixed, and processed as previously described. Movat’s modified pentachrome stain was used to examine ECM organization and VIC distribution on 6- to 8-μm sections of developing, mature, and diseased valves. Von Kossa staining was used to evaluate the presence of calcification in control and diseased valves. Images were obtained using an Olympus BX60 microscope and captured using Advanced SPOT image software.

Immunohistochemistry
Mouse monoclonal antibodies directed against aggrecan (Chemicon; 1:200), collagen type III (Iowa 3B2; 1:200), elastin (Sigma; 1:1000), and tenascin (Iowa M1-B4; 1:200) were used to evaluate ECM organization as previously described. The tenascin and collagen type III antisera were purchased from the Developmental Studies Hybridoma Bank developed with the National Institute of Child Health and Human Development and maintained by the University of Iowa. Antigen retrieval was used for the elastin antibody using antigen unmasking solution (Vector Laboratories). Pretreatment was used for the aggrecan and collagen type III antibodies, as previously described with modification. A universal streptavidin/biotin and diaminobenzidine detection system (Pierce) was used for colorimetric detection.

Electron Microscopy
Transmission electron microscopy (Hitachi 7600) of mouse valve tissue was performed as previously described. For visualization of collagens and elastins, sections were stained with 5% tannic acid aqueous solution, followed by 1% uranyl acetate, and counterstained with lead citrate.

Analysis of Cell Proliferation, Distribution, and Morphology
Cell proliferation was assessed on 8-μm mouse sections using the rabbit polyclonal antibody anti–phospho-Histone H3 (P-Histone H3, 1:100; Upstate). The proliferation index (proliferating cells per total cells) was calculated at serial time points (E12.5, E14.5, E18.5, 2 weeks, 4 months). In chicken, cell number, cusp thickness, and cusp area were measured in sectioned valve structures. Cell density (cell number per area) was calculated in whole valve structures (E6, E10, E16, and 8 weeks) and in individual cusp layers (cE16, 8 weeks) using ImageJ software (NIH). Cusp thickness in developing, mature, and diseased valves was measured in the proximal half of the valve. For each data point, measurements were made in triplicate from 3 independent samples.

Statistical Analysis
Student t test or 1-way ANOVA was used to compare groups. Findings are reported as mean with SEM. A probability value of <0.05 was considered significant.

Results

ECM Organization of the Mature Aortic Valve Is Conserved Across Species
Organization of the mature aortic valve in animals with 4 chambered hearts commonly used in cardiovascular research was examined histologically (Figure 1). Aortic valve cusps from human, sheep, chicken, rabbit, and mouse demonstrated similar ECM protein composition and stratification, as well as qualitatively increased cell density in the ventricularis or ventricular aspect of the cusp. In all species, there was collagen (yellow) in the fibrosa or arterial aspect of the cusp, elastic fibers (black) in the ventricularis, and proteoglycans (blue) in the spongiosa, as indicated by Movat’s pentachrome stain. Elastic fibers were concentrated at the proximal portion of the cusp and extended to the leading or closing edge (arrowheads, Figure 1A, 1C, 1E, 1G, and 1I). Although the overall pattern of ECM organization in the mature valve was conserved, differences were observed among species. For example, larger mammals (human and sheep) have more abundant collagen and elastin in the fibrosa and ventricularis, respectively, and more distinctly defined ECM organization compared with chicken, rabbit, and mouse (Figure 1A through 1F). Further, the chicken has a disproportionately thicker cusp with a well-defined ECM, similar to human and sheep, but relatively increased proteoglycan compared with collagen and elastin. In contrast, the rabbit and mouse have less distinct ECM layering and qualitatively increased VIC density throughout the valve cusp (Figure 1G through 1I). In rabbit, there is overlap of the fibrosa and spongiosa layers of the cusp, as evidenced by the teal appearance caused by blue and yellow staining (Figure 1G and 1H). The mature aortic valve structure in mouse has relatively less collagen in the fibrosa and less elastin in the ventricularis compared with larger animals (Figure 1I and 1J). These differences in ECM organization may be attributable, in part, to variation in size and hemodynamic burden of the different species; however, overall ECM protein composition and basic trilaminar organization in the mature valve structure is conserved.

Valve Remodeling Is Characterized by Stratified ECM and Decreased Cell Density
To determine the timing of ECM organization in the developing chicken (c) and mouse (m) semilunar and AV valves, serial time points corresponding to 3 major morphogenetic processes during valvulogenesis, namely endocardial cushion formation, cushion elongation, and cusp and leaflet remodeling, were examined using Movat’s pentachrome stain. Similar results were observed in semilunar and AV valves in both chicken and mouse (Figure 2). During cushion formation...
proteoglycan is diffusely expressed throughout developing valves, and ECM stratification is not observed. Distinct layers (fibrosa and spongiosa), reminiscent of the mature valve structure, were first appreciated during cusp and leaflet remodeling (cE16, mE18.5), marking the beginning of ECM stratification (arrows, Figure 2C, 2F, 2I, and 2L). During cusp and leaflet remodeling, collagen is expressed in the fibrosa of both semilunar (arterial aspect) and AV (ventricular aspect) valves. Proteoglycan is the primary component of the spongiosa in both semilunar and AV valves. Elastin is present in the aorta at all time points but appears relatively late in the ventricularis. ECM stratification is a central feature of chicken and mouse valvulogenesis and begins during late embryonic cusp and leaflet remodeling; however, the mature valve structure is not appreciated until postnatal life (compare with Figure 1).

To investigate VIC distribution relative to valve morphogenesis in the embryonic (E6, E10, and E16) and juvenile (8 weeks) chicken aortic valve, cell number, cusp thickness, and cusp area were quantified (Table). Cusp area increased approximately 50-fold from the endocardial cushion stage to the postnatal juvenile stage (0.065 versus 3.4 mm²), corresponding with a 7-fold decrease in cell density (11289 versus 1589 cells/mm²). In the early stages of valve development (cE6, mE12.5) and cushion elongation (cE10, mE14.5), proteoglycan is diffusely expressed throughout developing valves, and ECM stratification is not observed. Distinct layers (fibrosa and spongiosa), reminiscent of the mature valve structure, were first appreciated during cusp and leaflet remodeling (cE16, mE18.5), marking the beginning of ECM stratification (arrows, Figure 2C, 2F, 2I, and 2L). During cusp and leaflet remodeling, collagen is expressed in the fibrosa of both semilunar (arterial aspect) and AV (ventricular aspect) valves. Proteoglycan is the primary component of the spongiosa in both semilunar and AV valves. Elastin is present in the aorta at all time points but appears relatively late in the ventricularis. ECM stratification is a central feature of chicken and mouse valvulogenesis and begins during late embryonic cusp and leaflet remodeling; however, the mature valve structure is not appreciated until postnatal life (compare with Figure 1).
(cushion formation, cushion elongation), there was uniform cell density in the valve primordia. During later stages (cusp remodeling, juvenile), variation in cell distribution was observed in the emerging stratified ECM layers, as evidenced by different cell densities in the layers. During both cusp remodeling and postnatal juvenile stages of development, cell density was significantly higher in the fibrosa and ventricularis layers of the aortic valve cusps when compared with the spongiosa (Table). Importantly, the findings demonstrating VIC compartmentalization in juvenile chicken correlate with previous reports in juvenile human valves. In contrast to VIC density, endothelial cell density was constant from the cushion stage to the juvenile stage. These findings demonstrate that VIC compartmentalization follows a similar time course as ECM stratification during cusp remodeling.

### Aortic Valve ECM Stratification Begins During Cusp Remodeling and Continues Into Postnatal Life

Expression patterns of specific ECM proteins were examined during cushion elongation (E10), cusp remodeling (E16), and the juvenile cusp (8 weeks) in chicken to assess ECM organization (Figure 3). Aggrecan, a chondroitin sulfate proteoglycan and a constituent of cartilage, is expressed during cushion elongation and is present in the fibrosa and spongiosa during the cusp remodeling and juvenile stages (Figure 3A through 3C). Aggrecan is strongly expressed in the annulus during cusp remodeling but is absent from the annulus during the juvenile stage. Collagen type 3, an abundant collagen in valve tissue and cartilage, is present in the fibrosa, annulus, and aorta during cusp remodeling, and juvenile stages (Figure 3D through 3F). Elastin, a vascular matrix protein, is present in the proximal ventricularis during cusp remodeling and expression increases during the juvenile stage (Figure 3G through 3I). Tenascin, a large structural matrix protein also expressed in tendon, is localized to the annulus during cusp elongation, and extends into the fibrosa and the ventricular endothelium during cusp remodeling (Figure 3J through 3L). Interestingly, aggrecan and tenascin (markers of cartilage and tendon respectively) are expressed in the fibrosa. Both collagen type 3 and elastin are expressed in the aorta throughout embryonic development, but only collagen type 3 is present in the annulus. These studies demonstrate that specific ECM proteins organize into stratified layers during cusp remodeling. This ECM remodeling continues into postnatal life, suggesting that mechanisms of prenatal valve development may also be active in postnatal valve growth and maintenance.
Reduction in Cell Proliferation Is a Feature of Valve Remodeling

To investigate cell proliferation, developing and mature semilunar and AV valves in mice were examined during cushion formation (E12.5), cusp remodeling (E18.5), juvenile (2 weeks), and adult (4 months) stages using the mitotic cell marker P-Histone H3 (Figure 4). In the semilunar valves, the cell proliferation index decreased from 30% in the outflow tract endocardial cushions (Figure 4A) to 5% during cusp remodeling (Figure 4B) and 1% during the juvenile stage. In the AV valves, the cell proliferation index decreased from 36% in the AV endocardial cushions (Figure 4C) to 6% during leaflet remodeling (Figure 4D) and 2% during the juvenile stage. Cell proliferation, as indicated by Histone H3 phosphorylation, was not detected in adult semilunar cusps or AV leaflets (Figure 4E). As previously reported, myocardial cell proliferation was evident at all embryonic time points examined (data not shown).25 These findings demonstrate that cell proliferation in developing valves is dramatically reduced during the cusp remodeling and juvenile stages, suggesting that the increased valve size noted during these stages is attributable, in large part, to increased ECM production but not cell hyperplasia.

Ultrastructural Analysis of the Mouse Aortic Valve Demonstrates That ECM Organization Begins During Cusp Remodeling and Continues After Birth

Transmission electron microscopy was used to assess ECM organization in developing and mature aortic valves in mouse (Figure 5). During cushion elongation (E14.5; Figure 5A and 5B), unassembled collagens, proteoglycans, and elastin precursors are distributed throughout the primordial valve structure (Figure 5B). During cusp remodeling (E18.5), rudimentary collagen and elastin fiber fragments are in close proximity to proteoglycans. Short collagen fibers are present predominantly in the fibrosa, whereas elastin fiber fragments are present in the ventricularis (data not shown). During the juvenile stage (2 weeks; Figure 5C and 5F), extensive networks of collagen fibers are present in the fibrosa (Figure 5C and 5E), and organized elastin fibers are present in the ventricularis (Figure 5C and 5F). Whereas elastin precursors and fiber fragments are still observed at the juvenile stage, the majority of elastin fibers are highly organized, consistent with ongoing remodeling during postnatal life. These findings in mouse aortic valves demonstrate that ECM stratification begins during late embryonic development, and the ECM becomes more highly organized during postnatal life.

VIC distribution and morphology were also evaluated in the developing aortic valves of mouse (Figure 5). VICs were distinguished from endothelial cells by position and the lack of gap junctions. During cushion elongation, VICs were mesenchymal in appearance and loosely associated with ECM. However, during the juvenile stage, VICs become differentially distributed in ECM layers. In the fibrosa and ventricularis, VICs are embedded in collagens and elastins, but in the spongiosa, mesenchymal VICs are loosely associated with proteoglycans. VICs with a synthetic phenotype, as indicated by abundant mitochondria, rough endoplasmic reticulum, hyperactivated nuclei, and exocytosis of synthetic vesicles, were present in the cusp remodeling and juvenile stages26(Figure 5D). These findings demonstrate VIC compartmentalization corresponds temporally with ECM synthesis and stratification.

ECM Organization and VIC Compartmentalization Are Disrupted in Pediatric Aortic Valve Disease

ECM organization and VIC compartmentalization were examined in pediatric aortic valve disease, in which primary effects on these processes can be determined without the confounding factors of aging, hypertension, and hypercholes-
terolemia present in adults with valve disease. Explanted valve tissue was obtained from pediatric patients with BAV undergoing aortic valve replacement (median age 11 months [range, 1 to 184], n=6) and compared with age-matched controls (median age 6 months [range, 1 to 168], n=6). In each case, aortic stenosis was the indication for surgery. Three cases had previous balloon angioplasty, and 1 case had prior surgical valvotomy. Histopathology with Movat’s pentachrome stain demonstrated that ECM organization was similarly disrupted in all explanted diseased valves when compared with controls (Figure 6). Specifically, ECM trilaminar stratification was lost, as evidenced by the presence of disorganized collagens and proteoglycans in all layers (Figure 6B and 6C). Elastin fiber content appeared reduced, but collagen and proteoglycan content were increased substantially, as evidenced by the significant increase in cusp thickness (diseased valves=1.68±0.14 mm, control valves=0.32±0.04 mm, P<0.0001; Figure 6D). In addition, collagen and elastin fibers are disorganized, as evidenced by abnormal fiber orientation and fragmentation. VIC compartmentalization was also disrupted, as evidenced by cellular disarray in the form of cell clusters and large relatively cell-free areas in all aspects of the interstitium. However, there was no increase in cell proliferation, as determined by P-Histone H3 immunohistochemistry, and there was no evidence of calcification by von Kossa staining (data not shown). These findings demonstrate that pediatric aortic valve disease with underlying BAV is characterized by excessive ECM production, ECM disorganization, and VIC disarray.

Discussion

ECM organization and VIC compartmentalization, hallmarks of the mature valve structure, begin during late embryonic cusp remodeling and continue into postnatal life (Figure 7). The substantial cell proliferation appreciated during early valve development is markedly reduced during cusp remodeling, suggesting that late embryonic and postnatal valve growth is a function of increased ECM production. The coordination of ECM organization and VIC compartmentalization during valvulogenesis suggests that developmental mechanisms regulate both processes. In diseased aortic valves from pediatric patients with BAV, excessive ECM production, ECM disorganization, and VIC disarray without calcification are observed (Figure 7D). The pathogenesis of aortic valve disease in pediatric patients with BAV may be the result of dysregulation of the molecular hierarchies that control late valve development, ultimately leading to increased cusp thickness and ECM disorganization.

**ECM Organization and VIC Compartmentalization Begin During Late Embryonic Remodeling and Continue Into Postnatal Life**

The highly organized trilaminar stratification of ECM proteins in aortic valves was conserved across mammalian and avian species. During late embryonic cusp remodeling, we observed that cell proliferation decreased and ECM organization and VIC compartmentalization were spatially and temporally coordinated. Similar findings were recently demonstrated in fetal and postnatal human valves. Considerable valve growth occurs during postnatal life after formation of the organized trilaminar ECM stratification that characterizes the mature valve structure. The marked increase in cusp area compared with cell number indicates that the major increase in valve size is caused by increased ECM protein synthesis that far outpaces cell proliferation. ECM stratification is initiated with the diversification of VICs that express specific matrix proteins and are less proliferative. The differential distribution of VICs in the mature valve structure may reflect functional diversity within these cell populations.

**ECM Protein Gene Mutations Cause Human Valve Disease**

Mutations in several ECM genes result in valve disease in humans and clearly indicate the importance of strict ECM
regulation for normal valve function. For example, mutations in the fibrillin 1 gene cause BAV and mitral valve prolapse, whereas mutations in the elastin gene cause disease of the aortic, mitral, and pulmonary valves. In addition, mitral valve prolapse and pulmonary valve stenosis have been linked to mutations in the collagen type 3 and tenascin X genes. Therefore, a structural or functional defect in a single ECM protein is sufficient to cause valve disease, but the mechanisms by which ECM protein insufficiency or dysfunction lead to valve pathogenesis is not known. The disorganization of ECM structure in congenitally malformed valves may lead to abnormal signaling cascades, which in turn result in VIC dysregulation of ECM synthesis, consistent with the idea that valve disease has developmental origins.

Increased ECM Production, ECM Disorganization, and VIC Disarray Are Cardinal Features of the Stenotic BAV in Pediatric Patients

In every age group, but particularly in the pediatric patient, BAV underlies the majority of cases of aortic valve disease. BAV is the most common congenital cardiovascular malformation, with an incidence of approximately 1% in the general population. Recent studies have found that BAV is heritable, or caused by major genetic effects. A benefit of studying aortic valve disease in the pediatric patient is that it avoids confounding valve disease comorbidities, such as aging, hypertension, and hypercholesterolemia, that complicate the interpretation of histopathological studies of aortic valve disease in adults. A major finding of this study is that stenotic BAVs in pediatric patients exhibit increased ECM production, ECM disorganization, and VIC disarray without calcification, providing further support for the idea that faulty developmental programs result in abnormal and increased ECM and consequently valve disease. Taken together, these findings suggest that aortic valve disease has its origins in valve development.

Normal valve development is characterized by the spatiotemporal coordination of ECM organization and VIC compartmentalization. Signaling pathways involved in valvulogenesis are increasingly becoming known; however, the molecular regulation of normal valve development and the role of these pathways in valve disease are unclear. In diseased aortic valves from pediatric patients with BAV, these developmental processes are disrupted, resulting in cusp thickening, ECM disorganization, and VIC disarray without calcification. Further studies are required to determine the extent to which these features can be extrapolated to other types of valve disease. However, based on the findings reported here, we speculate that a viable congenital malformation (BAV) can lead to progressive dysregulation of ECM organization and VIC compartmentalization characteristic of valve disease.

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