Age-Associated Aortic Stenosis in Apolipoprotein E-Deficient Mice

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The present study was designed to assess aortic valve morphology and function in mice of advanced age. We also evaluated the potential contribution of bone-marrow-derived cells to the pathogenesis of aortic stenosis.

**OBJECTIVES**

- Age-associated valvular degeneration is characterized by lipid accumulation, collagen deposition, and calcification containing smooth muscle-like cells and osteoblast-like cells. Cellular and molecular factors that mediate these changes remain unknown.

**METHODS**

We extensively examined the aortic valves of senile wild-type and apolipoprotein E (ApoE)−/− mice with echocardiography. The aortic valves were analyzed by immunohistochemistry and electron microscopy. The bone marrow of wild-type and ApoE−/− mice was reconstituted with that of green fluorescent protein (GFP) or beta-galactosidase (LacZ) mice, which expressed GFP or LacZ ubiquitously.

**RESULTS**

Transaortic flow velocity was correlated with age in wild-type and ApoE−/− mice. The aortic valves of old ApoE−/− mice showed sclerosis that resembled the pathology of human aortic stenosis. A significant number of GFP-positive cells (10.7 ± 4.1%) in the sclerotic valves of ApoE−/− mice expressed alpha-smooth muscle actin, whereas most of the GFP-positive cells were identified as endothelial cells or macrophages in wild-type mice. There were bone-marrow-derived cells that were positive for osteoblast-related proteins near the sites of ectopic calcification. The sclerotic valves displayed frequent apoptotic cell death and chemokine expression. Smooth muscle-like cells observed in degenerative valves might derive, at least in part, from bone marrow. ([J Am Coll Cardiol 2005;46:134–41] © 2005 by the American College of Cardiology Foundation)

**CONCLUSIONS**

Senile ApoE-deficient mice display aortic valve sclerosis that is similar to that observed in humans. The sclerotic valves displayed frequent apoptotic cell death and chemokine expression. Smooth muscle-like cells observed in degenerative valves might derive, at least in part, from bone marrow.

**METHODS**

- **Animals and bone marrow transplantation (BMT).** Green fluorescent protein (GFP) mice (C57BL/6 background), beta-galactosidase (LacZ) mice (C57BL/6J background), which expressed GFP or LacZ ubiquitously, and ApoE−/− mice were already described (7,8). Wild-type C57BL/6 mice were purchased from SLC (Shizuoka, Japan). Bone marrow transplantation was performed after lethal X-irradiation with a total dose of 8.7 Gy (MBR-1520RB, Hitachi, Tokyo) as described previously (7). A total of 80% to 90% of peripheral leukocytes had been reconstituted as determined by flow cytometry or fluorescence in situ hybridization for Y-chromosome. All mice were fed regular chow. All procedures involving experimental animals were performed in accordance with protocols approved by the institutional committee for animal research.
- **Echocardiography.** Mice were anesthetized by intraperitoneal injection of nembutal (50 mg/kg). Color Doppler imaging was obtained through a parasternal approach with a 12-MHz linear probe and an ultrasound imaging system (LOGIQ 7, GE Medical Systems, Tokyo, Japan). Transaortic flow velocity was evaluated by pulse and continuous waves recorded through a near apical approach with
a 12-MHz sector probe and an echocardiography imaging apparatus (EnVisor M2540A, PHILLIPS, Tokyo, Japan) (9). The sample volume cursor was placed at the aortic root with angle correction (37° to 60°). The heart rates during examination were about 300 to 550 beats/min.

**Immunohistochemistry.** At death, aortic valves of C57BL/6 and ApoE−/− mice were observed using a cooled CCD camera (VB-6010, Keyence, Osaka, Japan). The hearts were excised and snap-frozen in optimal cutting temperature (OCT) compound (TissueTek, Tokyo, Japan). Immunohistochemistry was performed as described (7,8), using first antibodies against α-SMA (clone 1A4, Sigma, St. Louis, Missouri), cluster of differentiation 31 (CD31, clone MECl3, BD Biosciences, San Jose, California), osteocalcin (Biogenesis, Kingston, New Hampshire), mouse macrophage/monocytes-2 (MOMA-2, Dai-Nippon, Tokyo, Japan), CD3ε (Santa Cruz Biotechnology, Santa Cruz, California), monocyte chemotactic protein-1 (MCP-1, R&D Systems, Minneapolis, Minnesota), PDGF-B (sc-7878, Santa Cruz Biotechnology), vascular endothelial growth factor (VEGF, AF-293-NA, R&D Systems), stromal cell-derived factor-1 (SDF-1, R&D Systems), and LacZ (ICN, Aurora, Ohio) followed by the avidin-biotin complex technique and Vector red substrate (Vector, Burlingame, California). Isotype-matched normal immunoglobulins were used as negative controls in immunohistochemistry and immunofluorescence study. Calcification was detected by von Kossa staining followed by counterstaining with hematoxylin and eosin. LacZ was detected as described (7,8). LacZ-positive cells were counted and expressed as a proportion of the total number of nuclei in the valve.

**Plastic embedding, immunofluorescence double-staining, and transmission electron microscope.** Plastic embedding to detect GFP signal was performed as described (8). Thin sections (4 μm) were stained with the Cy3-conjugated anti-α-SMA (Sigma) antibody or antibodies against CD31, mouse pan endothelial cell antigen 32, osteopontin (sc-10593, Santa Cruz Biotechnology), or osteocalcin followed by incubation with Cy3 or rhodamine-conjugated secondary antibodies. Nuclei were counterstained with Hoechst 33258 (Sigma). GFP-positive cells were counted and expressed as a proportion of the total number of nuclei. The TdT-mediated dUTP nick end labeling (TUNEL) staining was performed on frozen sections as described elsewhere (8). The sections were observed under a confocal microscope (FLUOVIRONMENTV300, Olympus, Tokyo, Japan). Transmission electron microscopic observation was performed as described (10).

**RESULTS**

**Age-associated aortic stenosis in mice.** We extensively examined the aortic valves of wild-type and ApoE−/− mice of various ages with echocardiography. Transaortic flow velocity could be measured in wild-type and ApoE−/− mice (9). There was a significant correlation between age and transaortic flow velocity in both groups, indicating that murine aortic sclerosis progresses with age like valvular degeneration observed in humans (Figs. 1A and 1B) (12). Gender did not influence the relationship between age and transaortic flow velocity. We did not detect any pathological increase in transaortic flow in wild-type mice (Fig. 1C). Although the regression line of ApoE−/− mice was not significantly steeper than that of the wild-type mice, aortic flow velocity was faster than 150 cm/s in more than half of the ApoE−/− mice older than 43 weeks of age. In a 103-week-old female ApoE−/− mouse, aortic flow velocity was increased to as high as 427 cm/s (Fig. 1D). Color Doppler imaging did not detect any aortic regurgitation in the wild-type mice (Video 1; see the July 5 issue of JACC at www.onlinejacc.org). Mild aortic regurgitation could be observed in 9 of 30 ApoE-deficient mice older than 68 weeks of age (Fig. 1E; Videos 2 and 3; see the July 5 issue of JACC at www.onlinejacc.org). The aortic velocity in the ApoE−/− mice with aortic regurgitation was significantly faster (n = 9, 205 ± 33 cm/s) than that in the mice without aortic regurgitation (n = 21, 143 ± 10 cm/s, p = 0.023). These results suggest that age-associated aortic sclerosis causes pathological stenosis and regurgitation in ApoE−/− mice of advanced age.

**Histological examination of the sclerotic aortic valves.** Next, the sclerotic aortic valves were examined histologically. An en face observation revealed that the aortic valve leaflets of 95-week-old ApoE−/− mice showed increased opacity, while those of 96-week-old C57BL/6 mice were transparent (Fig. 2A). Von Kossa staining demonstrated ectopic calcification in the aortic valves of the 88- to 97-week-old ApoE-deficient mice (Fig. 2B), while calcification was not detected in the aortic valves of the 94- to 98-week-old wild-type mice. Osteocalcin, an ossification-related protein, was expressed in the sclerotic valves of ApoE-deficient mice. Valve leaflets of age-matched C57BL/6 mice were covered with an endothelial cell layer as determined by immunostaining for an endothelial marker, CD31 (Fig. 2C). The valves of 74- to 97-week-old ApoE−/− mice showed sporadic sites of endothelial denudation. The sclerotic valves of the ApoE−/− mice contained a number of α-SMA positive cells, which were

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**Abbreviations and Acronyms**

ApoE = apolipoprotein E  
BMT = bone marrow transplantation  
GFP = green fluorescent protein  
LacZ = beta-galactosidase  
SMA = smooth muscle actin  

Statistical analysis. All values are presented as means ± SEM. Means were compared by unpaired Student t test. Correlation between age and transaortic blood flow velocity was performed with linear regression analysis. Comparison of regression lines was performed with standard methods (11). A p value <0.05 was considered statistically significant.
seldom detected in the valves of the wild-type mice. Many macrophages and T cells (Fig. 2C) infiltrated in the degenerated valves of 74- to 97-week-old ApoE−/− mice, whereas a few macrophages could be detected in the aortic valves of 94- to 98-week-old ApoE−/−/− mice. The cell types observed in the degenerated valves were confirmed by ultrastructural analysis under an electron microscope (Figs. 3A and 3B). Besides macrophages (Fig. 3C), osteoblast-like cells with well-developed Golgi apparatus and rough endoplasmic reticulum could be identified (Fig. 3E) near the site of calcium deposition (Fig. 3F). There were smooth muscle-like cells that had a basement membrane, muscle fibers, and mitochondria (Figs. 3B and 3D).

**Contribution of bone marrow-derived cells to valvular remodeling.** To investigate the origin of the cells that could be found in sclerotic valves, bone marrow cells from GFP-mice were transplanted to 59-week-old ApoE−/− mice (BMTGFP−/−ApoE mice, n = 3). After 34 weeks, there was a significant number of GFP-positive cells (16.0 ± 3.5%) in the aortic valves of BMTGFP−/−ApoE mice (Fig. 4A). Immunofluorescence study detected GFP-positive cells that expressed α-SMA (Fig. 4B). GFP-positive cells on the valvular surface were integrated to the endothelium and expressed endothelial cell markers including MECA32 and CD31 (Fig. 4C). Near the sites of ectopic calcification, there were GFP-positive cells that expressed osteoblast-related proteins including osteopontin and osteocalcin (Fig. 4D). Bone marrow transplantation was performed from LacZ mice to 31-week-old ApoE−/−/− mice (BMTLacZ−/−ApoE mice); 49 weeks after BMT, the aortic valves of the BMTLacZ−/−ApoE mice were thickened and calcified. Anti-LacZ immunostaining revealed that 23.3 ± 2.1% cells were LacZ-positive in the aortic valves of BMTLacZ−/−ApoE mice (Fig. 4E). Immunofluorescence study identified LacZ-positive cells that were positive for α-SMA (data not shown) or CD31 (Fig. 4F). These results suggest

**Figure 1.** Age-associated increase in transaortic flow velocity in wild-type mice and in apolipoprotein E (ApoE)−/− mice. (A and B) Mice were anesthetized by intraperitoneal injection of nembutal. Transaortic flow signals were evaluated by continuous waves recorded through a near apical approach with a 12-MHz sector probe and an echocardiography imaging apparatus (EnVisor M2540A, PHILLIPS, Tokyo, Japan) in wild-type (A, 8- to 120-week-old, male n = 18, female n = 2) and ApoE−/− mice (B, 9- to 115-week-old, male n = 23, female n = 22). As the mice grew older, the velocity increased in both groups. There was a significant correlation between age and transaortic valve flow velocity ([AV] flow velocity) in both groups. (C and D) Transaortic flow patterns of a 98-week-old male C57BL/6 mouse (C) and a 103-week-old female ApoE−/− mouse (D). The maximum aortic flow velocity was 427 cm/s in the ApoE−/− mouse. (E) B-mode (upper panels) and color Doppler (lower panels) images were obtained through a parasternal approach with a 12-MHz linear probe and an ultrasound imaging system (LOGIQ 7, GE Medical Systems, Tokyo, Japan). Functional aortic regurgitation could be detected in senile ApoE−/− mice (arrowheads). Ao = aorta; AR = aortic regurgitation signal; LA = left atrium; LV = left ventricle; RA = right atrium; RV = right ventricle.
that bone-marrow-derived cells were recruited and differenti-ated into α-SMA-positive cells, endothelial-like cells, or osteoblast-like cells that might contribute to valvular sclerosis. Bone marrow transplantation was also performed from GFP mice to C57BL/6 mice (BMTGFP−/− mice, n = 3). GFP-positive cells could be detected in the aortic valves of the BMTGFP−/− mice at 44 weeks after BMT (Fig. 4G). There was no significant difference in the relative amount of GFP-positive cells in the aortic valves between BMTGFP−/− and BMTGFP−/−ApoE mice (18.5 ± 2.1% vs. 16.0 ± 3.5%, p = NS). Most of the GFP-positive cells were identified as endothelial cells (Fig. 4H) or macrophages (Fig. 4I). Few GFP-positive cells (0.3 ± 0.3%) expressed α-SMA in BMTGFP−/− mice, whereas α-SMA expression was detected in 10.7 ± 4.1% of the bone-marrow-derived cells in the aortic valves of BMTGFP−/−ApoE mice (Fig. 4G).

Cell death and cytokine expression in sclerotic aortic valves. To understand the mechanisms by which bone-marrow-derived cells were recruited to the site of aortic valvular degeneration, we investigated cell death and cytokine expression in the sclerotic valves. The sclerotic valves of 97-week-old female ApoE−/− mice expressed high amounts of MCP-1, VEGF, PDGF-BB, and SDF-1, which were absent in the aortic valves of 8-week-old wild-type mice (Fig. 5A). The expression of chemokines and cytokines was associated with frequent apoptosis of endothelial cells and interstitial cells as determined by TUNEL staining (Fig. 5B). Double immunofluorescence imaging revealed that the chemokine and cytokines were expressed particularly around the apoptotic cells (Fig. 5C).

DISCUSSION
Epidemiologic studies revealed that age-associated aortic stenosis is associated with clinical risk factors similar to those for atherosclerosis, such as age, male-gender, history of smoking, hypercholesterolemia, hypertension, and diabetes, suggesting that age-associated aortic stenosis process might be caused by the mechanisms similar to those of atherosclerosis (13). In this study, we found that aortic valve flow velocity increases with aging in wild-type mice as well as in ApoE−/− mice. However, marked increase in aortic valve flow velocity was detected only in ApoE−/− mice. It
is likely that both altered lipid metabolism and aging are essential for the development of murine aortic sclerosis, which potentially causes functional stenosis and regurgitation.

Taking advantage of the mouse model of valvular sclerosis, we investigated the potential contribution of bone marrow cells to the pathogenesis of aortic sclerosis. Our findings suggest that some of the smooth muscle-like and osteoblast-like cells in degenerative valves might derive from bone marrow. Bone marrow-derived cells were also integrated to the endothelium of the aortic valve. We and others (7,14–16) reported that bone-marrow-derived cells can contribute to the pathogenesis of vascular diseases and that neo-intima formation might be similar to the healing process in response to mechanical and humoral stimuli (17). It was observed that severe damage in the vessel wall is essential for bone marrow progenitor cells to participate in arterial remodeling (8). In this regard, aortic valves are continuously subjected to a high mechanical stress at the flexion area of the aortic cusps near the attachment of the aortic root and the line of coaptation (18,19). In addition to mechanical stress, aortic valves are always exposed to various atherogenic substances, such as oxidized low-density lipoprotein, homocysteine, angiotensin II, and lipopolysaccharides, which induce apoptosis in endothelial cells on the surface of the valves (20). Presumably, bone-marrow-derived cells home at the injured surface to replace the apoptotic endothelial cells. However, in the presence of hypercholesterolemia, this reparative process may turn to be degenerative as bone-marrow-derived cells also differentiate into SMC-like cell or osteoblast-like cells that may potentially contribute to cellular accumulation or calcification, as suggested in the pathogenesis of atherosclerosis (7,21). Thus, mechanical and humoral injuries to the endothelial lining seem to constitute the earliest phase of the valvular degeneration (2).
The molecular mechanism by which bone marrow cells are mobilized and recruited to the site of valvular degeneration remains to be elucidated. Recent evidence suggests that apoptotic cells express chemokines and cytokines, thus potentially provoking inflammatory responses (22,23). In aortic valves of senile ApoE−/− mice, there were many apoptotic cells that were associated with the expression of high amounts of MCP-1, PDGF-BB, VEGF, and SDF-1. It was reported that these cytokines and chemokines are essential for recruitment of bone-marrow-derived cells to vascular lesions (24–27). It is likely that those factors may play a role, at least in part, in the recruitment and homing of bone-marrow-derived cells to the site of valvular remodeling.

Our study also might have an implication for regenerative medicine. There is increasing enthusiasm for the use of somatic stem cells for “cell transplantation therapy” and “tissue engineering.” However, given the pluripotency and heterogeneity of bone marrow cells, they may potentially turn into disease-aggravating cells (28) and accelerate pathological processes, such as atherosclerosis and valvular sclerosis (29,30).
Apolipoprotein E-/- mice develop severe hypercholesterolemia and atherosclerotic lesions that may resemble human lesions (31,32). They were widely used to investigate the specific molecules or cells that play a crucial role in the pathogenesis of atherosclerosis (7,31–34). In most studies, atherosclerotic lesions in the aorta, coronary arteries, and pulmonary arteries were analyzed in the ApoE-/- mice up to 23 weeks of age (31,35,36). Although a few reports have described pathological changes in aortic valves of hypercholesterolemic mice (37,38), no physiological examination was performed to detect functional abnormalities in ApoE-/- mice. In this study, echocardiogram and histological examination successfully detected sclerotic changes with functional abnormality in the aortic valves of ApoE-/- mice of advanced age.

Mouse genetics have been extensively characterized, and the mice full genome sequence is available. Furthermore, recent advances in gene-manipulating techniques have enabled us to produce various genetically modified mice to determine the role of specific molecules in a variety of biological phenomena including vascular remodeling. Our mouse model of aortic sclerosis might be useful to understand the pathogenesis of human aortic stenosis and to develop therapeutic strategies (39).

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REFERENCES


APPENDIX

For accompanying videos, please see the July 5, 2005, issue of JACC at www.onlinejacc.org.