Mechanisms Underlying Aortic Dilatation in Congenital Aortic Valve Malformation

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Background—The high incidence of aortic disease in subjects with congenital aortic valve malformations suggests a causative relationship between these 2 conditions. The histological observation in aortic dilatation/aneurysm/dissection is Erdheim cystic medial necrosis (CMN), a noninflammatory loss of smooth muscle cells (SMCs), fragmentation of elastic fibers, and mucoid degeneration.

Methods and Results—To examine whether apoptosis is 1 of the mechanisms underlying CMN and aortic medial layer SMC loss, ascending aortic wall specimens from 32 patients were collected at cardiothoracic surgery and examined by histochemical staining and terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling. From echocardiography results, 4 groups of patients were identified: bicuspid valve carriers with (bi/dil) or without (bi/0) aortic dilatation and tricuspid valve carriers with (tri/dil) or without (tri/0) aortic dilatation. Massive focal apoptosis was observed in the medial layers of bi/dil (mean apoptotic index [mAI], 8.1 ± 6.0) and tri/dil (mAI, 8.1 ± 8.3) compared with tri/0 (mAI, 0.9 ± 1.2; P = 0.0079 and P = 0.037). In bi/0 (mAI, 9.1 ± 5.7) compared with tri/0 (mAI, 0.9 ± 1.2), rates of medial SMC apoptosis were increased (P = 0.0025). Bi/dil (mean age, 40.6 ± 15.7 years) were significantly younger than tri/dil (mean age, 56.4 ± 12.8 years) undergoing the same operation (P = 0.0123).

Conclusions—Premature medial layer SMC apoptosis could be part of a genetic program underlying aortic disease in patients with aortic valve malformations. (Circulation. 1999;99:2138-2143.)

Key Words: apoptosis ■ aneurysm ■ valves ■ aorta

Bicuspid aortic valve is among the most common congenital heart malformations, with a prevalence of 1% to 2% in the general population and a strong male predilection.1 Its association with clinically serious abnormalities of the ascending aorta, including aortic dilatation, aneurysm, dissection,2,3 and coarctation of the aorta,4,5 has suggested a common underlying developmental defect involving the aortic valve and the wall of the ascending aorta. In fact, the common neuroectodermal origin of both structures was suggested.6,7 In addition, the malformative effects of experimental neural crest ablation on cardiac outflow tract formation have been demonstrated.8

The histological abnormality underlying ascending aortic dilatation, aneurysm, and dissection is Erdheim’s cystic medial necrosis (CMN),9,10 which is characterized by a triad of noninflammatory smooth muscle cell (SMC) loss, fragmentation of elastic fibers, and accumulation of basophilic ground substance within cell-depleted areas of the medial layer of the vessel wall. Infection, atherosclerosis, or severe shear stress11–13 can lead to the same histological picture of CMN of the aortic wall as hereditary connective tissue disorders14 such as Marfan syndrome, Ehlers-Danlos syndrome, or a spectrum of mutations in the fibrillin or type II procollagen genes.15

Apoptosis, which is a form of programmed cell death,16 has been recognized as a central feature of fundamental biological processes, including embryonic morphogenesis,17 remodeling of mature tissues,18,19 and cell replacement in certain adult tissues, eg, the thymus.20 In contrast to necrosis, apoptosis occurs in isolated cells without any accompanying cellular reaction.21 Because of the noninflammatory nature of Erdheim’s CMN, we investigated whether apoptosis could be 1 of the mechanisms underlying this histological pattern. In a next step, the rates of medial SMC apoptosis were compared between dilated and nondilated ascending aortic wall specimens from bicuspid and tricuspid valve carriers. Furthermore, experiments were designed to support the hypothesis that the cells undergoing apoptosis are of neuroectodermal origin.

Methods

Patients and Tissues

Asking aortic wall specimens from 32 patients undergoing cardiothoracic surgery in the Vienna General Hospital were analyzed. Preoperatively, ascending aortic diameters were measured by

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transthoracic echocardiography at the level of the aortic ring as the most apical site of attachment of the aortic valve cusps, at the level of the sinotubular junction, and at the ascending aorta at its widest diameter accessible from the parasternal window at end diastole. Aortic diameters at the sinotubular junction were found to be representative of ascending aortic diameters. Mean aortic valve gradients were assessed during cardiac catheterization or echocardiography. Aortic regurgitation was graded by cardiac catheterization and by review of the echocardiographic width of the regurgitant jet, retrograde flow in the ascending aorta, and slope of the continuous-wave Doppler spectrum. History of hypertension was assessed by a review of medical history, blood pressure measurements, and history of antihypertensive drug treatment (the Table). Patients with atherosclerosis of the ascending aorta, aortitis, infective endocarditis, and primary connective tissue disorders, eg, Marfan disease, were not included in the study.

Four groups of patients were identified on the basis of ascending aortic diameters and the number of aortic valve cusps (the Table). The patient groups were bicuspid valve carriers without or with aortic dilatation and tricuspid valve carriers without or with aortic dilatation, which was defined as aortic width >40 mm, well above the normal range of 20 to 37 mm.

Samples were harvested by the surgeon during replacement of the ascending aorta, aortic valve surgery, and/or aortocoronary bypass surgery or correction of aortic coarctation from the site of the aortic cannulation after inspection of valve anatomy and any signs of atherosclerosis in the ascending aorta. In addition, 2 specimens from normal abdominal aorta were harvested from transplant donors as control tissues for the DNA laddering experiments (Figure 2, lanes 4 and 5). The protocol was approved by the Medical Ethics Committee of the University of Vienna, Vienna, Austria (EKZ 96/294).

One of us was present in the operating room in each case for immediate sample collection into both 7.5% buffered formalin and liquid nitrogen.

**Histological Examination**

A modified trichrome stain was used to examine the degree of Erdheim’s CMN, defined as pooling of mucoid material, elastin fragmentation with disruption of elastin lamellae, fibrosis with increase in collagen at the expense of SMCs, and medionecrosis with areas of apparent loss of nuclei. CMN was classified into 4 grades. Because several different degrees of CMN were observed within a single specimen, the most severe changes were chosen for grading. Investigators performing the histological evaluation were blinded to the clinical data.

Samples displaying histological signs of atherosclerosis or inflammation of any other cause were excluded from further analysis because of the potential confounding effect on the assessment of apoptosis. Minimal atherosclerotic changes were encountered focally in all vessel specimens from patients and control tissue but were unrelated to apoptotic foci.

**Immunohistochemistry**

Immunohistochemistry was performed as described. Rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP: 1:400, DAKOPATTS) and mouse monoclonal anti-human α-actin antibody (10 μg/mL, DAKOPATTS) were used. Aminoethyl carbazole (AEC) served as chromogenic substrate.

**In Situ Detection of Apoptotic Cells**

Terminal transferase–mediated dUTP nick end labeling (TUNEL; in situ apoptosis detection kit ApoTag, Oncor Inc) was carried out according to manufacturer’s instructions. For quantification of TUNEL–positive cells, 4 fields per section within the SMC layer displaying the highest respective degree of CMN were examined at 200-fold magnification. The apoptotic index was calculated with the following formula: 100 × (number of TUNEL–positive cell nuclei per field/total number of cell nuclei per field).

**DNA Laddering Experiments**

Genomic DNA was isolated from 200 mg arterial tissue according to a standard procedure. The DNA pellet was subjected to ligase-mediated polymerase chain reaction (PCR; ApoAlert LM-PCR Assay Kit, Clontech). Then, 20 μL of each reaction was electrophoresed on an ethidium bromide containing 1.5% agarose gel. DNA was visualized under UV (302 nm) light.

**Double-Staining Immunohistochemistry**

After identification of aortic medial SMCs by α-actin stains (Figure 1D), the TUNEL protocol was combined with anti-GFAP immunohistochemistry for further characterization of apoptotic cells. TUNEL was performed by use of alkaline phosphatase generating a blue immunoreactivity with nitro blue tetrazolium chloride/5-bromo-4–3-indoxyl-phosphate toluidine-salt. AEC was used as substrate for GFAP detection.

**Statistical Analysis**

Statistical differences between groups were evaluated by use of the unpaired t test and ANOVA. A value of P < 0.05 was considered significant.

**Results**

**Cell Death Analysis in Erdheim’s CMN Lesions**

To set the stage for patient group analysis, initial experiments were designed to examine the possibility of SMC apoptosis as a mechanism of medial SMC loss. In a series of TUNEL stains of specimens with CMN grades 3 and 4 (the Table, n = 20), strong evidence for medial SMC apoptosis was obtained in 17 samples (Figure 1). Typically, apoptotic cell nuclei were identified at the edge of the oval CMN lesions, with nuclear remnants visible in the center (Figure 1). A biochemical criterion distinguishing apoptosis from necrosis is the production of mononucleosomal or oligonucleosomal DNA fragments at multiples of 180 to 200 bp resulting from DNA cleavage by endonucleases (Figure 2, arrows). Because of the focal nature of apoptosis in CMN, an adapter-based PCR technique relying on 5'-phosphorylated blunt ends in mammalian DNA fragments was used for DNA laddering experiments. DNA analysis of dilated ascending aortic wall specimens (Figure 2, lane 2) revealed oligonucleosomal DNA fragments resembling those obtained from a parallel experiment with involuting thymus cells (Figure 2, lane 1). In contrast, genomic DNA isolated from nondilated nonatherosclerotic ascending and descending aortic wall tissue did not show fragmentation (Figure 2, lanes 3 through 5).

**Characterization of Patient Groups**

No statistical differences were identified between patient groups with regard to history of hypertension or hemodynamic parameters of the aortic valve. In no case was subvalvular left ventricular outflow tract obstruction noted. A correlation between hemodynamic parameters and aortic width was lacking, which is in accordance with published data. Bicuspid aortic valve carriers with aortic dilatation (mean age, 40.6 ± 15.7 years) were significantly younger than tricuspid valve carriers undergoing the same procedures (mean age, 56.4 ± 12.8 years; P = 0.0123), which is in contrast to the contention that CMN shows a striking correlation with age and represents a normal histological aging process for the aorta.
### Patient Characteristics, Degree of CMN, and Apoptotic Index of Ascending Aortic Medial SMCs in 4 Patient Groups Defined by Number of Aortic Valve Cusps and Aortic Root Diameter

<table>
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<tr>
<th>Sample</th>
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<th>Sex, F/M</th>
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40.6±15.7† n=4/5 n=1/8 30.8±33.3† II‡ 54.8±12.9† 3.2±0.7† 8.1±6.0†

### Bicuspid Valve Carriers Without Ascending Aortic Dilatation

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43.9±19.9† n=0/7 n=2/5 36.0±42.0† II‡ 28.9±6.7† 2.6±1.4† 9.1±5.7†

### Tricuspid Valve Carriers With Ascending Aortic Dilatation

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56.4±12.8† n=3/6 n=3/6 19.9±24.8† II‡ 61.6±13.0† 3.4±0.6† 8.1±8.3†

### Tricuspid Valve Carriers Without Ascending Aortic Dilatation

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32     | 53     | M Yes    | 44                            | II/III                          | 29                            | Aortic stenosis/regurgitation | 1             | 0.0            |

65.1±11.1† n=0/7 n=2/5 31.4±7.2† II‡ 30.6±3.0† 1.6±0.8† 0.9±1.2†

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*Aortic root diameters at the sinotubular junction are reported.
†Mean±SD.
‡Median value.
§Specimens used for counting cells double-positive for anti-GFAP and TUNEL.
Comparative Quantitative Analysis of Medial Layer SMC Apoptotic Rates Within Patient Groups

In a second series of experiments, specimens from all patients were examined by TUNEL. This technique permits in situ detection of apoptotic cell nuclei and is an adequate tool for their quantitative analysis. Because of apoptotic foci in the medial layer of the vessel wall, counting of apoptotic nuclei was performed in these areas and used to calculate apoptotic indexes. Quantification of medial layer SMC apoptosis in specimens from bicuspid valve carriers with and without aortic dilatation revealed markedly increased apoptotic indexes (with: range, 2.5 to 18.8; mean, 8.1±6.0; without: range, 1.7 to 18.8; mean, 9.1±5.7) compared with control specimens from tricuspid valve carriers without aortic dilatation (range, 0.0 to 3.0; mean, 0.9±1.2; P=0.0079 and P=0.037). However, severe SMC depletion and CMN grades 3 and 4 were found in 8 of 9 bicuspid valve carriers with and in 3 of 7 patients without aortic dilatation (the Table). In the 4 remaining specimens from bicuspid valve carriers without aortic dilatation, apoptotic indexes ranged from 3.0 to 11.0, exceeding those considered to be due to normal tissue turnover,30 and were statistically increased as a group (P=0.0025). Apoptotic cells were distributed in foci within the medial layer of the vessel wall. No significant differences in apoptotic indexes of medial SMCs were found in specimens from bicuspid and tricuspid valve carriers with aortic dilatation (range, 0.1 to 28.0; mean, 8.1±8.3; the Table).

Immunohistochemical Analysis of Ascending Aortic Wall Specimens Using an Antibody Directed Against GFAP

To substantiate the hypothesis that neural crest–derived SMCs are undergoing apoptosis, immunohistochemistry with anti-GFAP, an antibody directed against intermediate filaments of astrocytes and other neural crest–derived cells, and double-staining experiments using both GFAP immunohistochemistry and TUNEL were performed. Anti-GFAP immunoreactive cells were found clustered within the central parts of the medial layer of ascending aortas (Figure 3B). Close-up examination of apoptotic areas (in the specimens marked with § in the Table) revealed that 40.3±23.0% of TUNEL-positive cell nuclei were found in anti-GFAP immunoreactive cells (Figure 3C, arrows).

Discussion

The data demonstrate that apoptosis is a key mechanism underlying SMC loss in the ascending aortas of bicuspid aortic valve carriers undergoing cardiothoracic surgery in the present study. We speculate that the same may be found in individuals who do not require aortic valve or ascending aortic surgery, as suggested by the example of patient 12 (the
Table 1. It has become widely accepted that apoptosis serves as a major mechanism for the precise control of cell numbers in developing and mature tissues under physiological and pathological conditions. Apart from intrinsic signals, death signals may originate outside a cell. In the vascular wall, these include, for example, mechanical forces, oxidized lipoproteins within atherosclerotic lesions, and inflammatory cytokines. A recent study has investigated apoptotic SMC loss in descending aorta with medial degradation and formation of abdominal aneurysm in the presence of atherosclerosis and chronic inflammation. In the cases under discussion, atherosclerotic changes in ascending aortas of bicuspid valve carriers were minimal and did not explain the degree of aortic dilatation. Apoptosis as a mechanism of aortic aneurysm formation in the absence of inflammation and atherosclerosis is a novel observation.

Increased apoptosis rates in aortas of bicuspid valve carriers without aortic dilatation strongly support the concept of an independent aortic remodeling process that may be preceding overt aortic dilatation. This hypothesis is currently being tested by follow-up examinations.

Furthermore, a number of observations allow room for interesting speculations. Descriptions of familial bicuspid valve associated with aortic root enlargement and evidence of a 9- to 18-fold-higher incidence of aortic aneurysms in individuals with bicuspid aortic valves favor the hypothesis of a developmental fault involving the aortic valves and ascending aortic wall. During embryogenesis, neural crest cells derived from the cranial neural fold migrate into the cardiac outflow tract. Late in development, these ectomesenchymal cells are located between the proximal aorta and pulmonary trunk and are thought to participate in outflow tract septation. A few ectomesenchymal cells are scattered in the cusps of the arterial valves. From these data, a hypothesis is that the valve-sculpting neural crest cells are involved in valvular pathogenesis, whereas the neuroectodermal immigrants into the ascending aorta are prematurely eliminated later in life. Therefore, a search for neural crest markers in ascending aortic specimens was undertaken.

Anti-GFAP-immunoreactive cells were observed in a focal distribution pattern in areas of CMN (Figure 1C) within the medial layer. GFAP is a major protein constituent of glial intermediate filaments in differentiated astrocytes. Because a group of proteins with similar molecular weights and isoelectric points as GFAP and immunoreactivity with anti-GFAP has been found in neural crest–derived cells, it is possible that GFAP-positive SMCs are of neuroectodermal origin. The low number of double-staining cells in our study may be explained by the observation of a loss of GFAP gene expression in cells undergoing apoptosis.

Tricuspid valve carriers with aortic dilatation showed similar apoptotic indexes and degree of CMN as bicuspid valve carriers. Although these patients were significantly older and CMN could result from aging and shear stress, the mechanism outlined earlier may play a role in these patients. Support for a quantitative genetic influence is rendered from a study who showed valvulo(patho)genesis in a laboratory-inbred family of Syrian hamsters with a high incidence of bicuspid aortic valves. They documented a continuous phenotypic valve spectrum, ranging from the bicuspid to a tricuspid condition, with intermediate stages represented by the tricuspid aortic valve with different degrees of leaflet fusion.

In conclusion, the present data suggest that medial SMC apoptosis is associated with ascending aortic aneurysms in bicuspid aortic valve carriers. A search for a genetic background of premature programmed cell death in ascending aortic disease and its relation to the expression of connective tissue-associated genes needs to be undertaken.

References


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Diana Bonderman, Elisabeth Gharehbaghi-Schnell, Gregor Wollenek, Gerald Maurer, Helmut Baumgartner and Irene M. Lang

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