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Pulmonary autograft valve explants show typical degeneration

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Objectives: We sought to evaluate the microscopic characteristics of pulmonary autograft valve explants.

Methods: Cell density and thickness of the autograft valve ventricularis were determined and compared with those of normal aortic and pulmonary valves (n = 11). Cellular phenotype and extracellular matrix involvement were assessed with immunohistochemistry. Collagen 3-dimensional architecture was studied by means of confocal microscopy.

Results: The autograft valve exhibited characteristic thickening of the ventricularis compared with the normal aortic and pulmonary valves (137 vs 77 [$P = .058$] vs 37 μm [$P = .002$], respectively). Its cell number was increased compared with those of the normal aortic and pulmonary valves (396 vs 230 [$P = .02$] vs 303 [$P = .083$], respectively). Myofibroblasts and stressed endothelial cells, both of which were present in pulmonary autografts, were absent in control valves. The exclusive presence of matrix metalloproteinase 1 was an additional sign of extracellular matrix turnover. Apoptosis, elastinolysis, cell proliferation, and senescence were not expressed. Dense fibrosis of the autograft ventricularis with relatively well-aligned collagen fibers was observed with confocal microscopy.

Conclusions: Fibrous hyperplasia of the ventricularis and cellular and extracellular matrix characteristics of active remodeling were a consistent finding in pulmonary autograft valve explants. The observations suggest a primary valve-related cause to be involved in pulmonary autograft valve failure. (J Thorac Cardiovasc Surg 2010;139:1416-9)

Pulmonary autograft valve regurgitation and root dilatation have emerged as important complications of the Ross procedure at short-term and midterm follow-up.[1-3] Valve regurgitation has generally been assumed to be secondary to root dilatation, although a primary valve-related cause has never been excluded. In our previous study fibrotic thickening of the autograft ventricularis was identified as a common feature.[4] Here we systematically studied 11 explanted autografts and 11 control valves to assess and quantify the histopathologic characteristics to support any clinicopathologic correlates.

MATERIALS AND METHODS

Pulmonary autografts (n = 11) were obtained from adults undergoing reoperations for pulmonary autograft failure between 2003 and 2007 in the University Medical Centres of Rotterdam and Leiden, The Netherlands (Table 1). In 10 patients reoperation was indicated because of progressive dilatation of the neoaortic root combined with significant neoaortic valve regurgitation. One patient underwent reoperation because of progressive dilatation of the neoaortic root alone. Age-matched heart valves (n = 11; aortic, n = 5; pulmonary, n = 6) obtained from the Heart Valve Bank of the Erasmus MC, Rotterdam, The Netherlands, served as controls.

Sample Staining

Radial valve sections (4 μm) were cut from paraffin-embedded leaflets and mounted on slides. Von Gieson staining was used to visualize elastin and collagen fibers, and Alcian blue staining was used to visualize glycosaminoglycans and nuclei. Sections were photographed with a Zeiss microscope (Zeiss, Oberkochen, Germany) with a Sony DXC 950p full-color 3CCD camera (Sony Corp, Tokyo, Japan) and captured with KS-400 image analysis software, version 3.0 (Zeiss). Von Gieson–stained sections were analyzed with ImageJ version 1.37V to determine the thickness of the ventricularis and total valve thickness. The mean number of nuclei was calculated in Alcian blue–stained sections by counting 4 different sample fields.

Immunohistochemical analysis with 13 different markers was performed to compare autograft valves (n = 11) with control valves (n = 11, Table 2). In general, sections were deparaffinized, followed by antigen retrieval by boiling in 0.1 mol/L citrate buffer, pH 6.0; blocked for endogenous peroxidases; and incubated for 1 hour with the primary antibody. Next, sections were washed and incubated with an Envision horseradish peroxidase–conjugated secondary antibody (DAKO, Glostrup, Denmark). Subsequently, all slides were incubated with diaminobenzidine and analyzed with KS-400 image analysis software to quantify the staining percentage of the ventricularis.

Confocal Microscopy

Three-dimensional images were made from 20-μm-thick, paraffin-embedded, Sirius red–stained leaflets with an LSM510 confocal microscope with a C-Apochromat 40x/1.2W Corr water immersion objective (Zeiss).

Statistical Methods

Continuous data, such as those obtained from histochemical analyses, were compared with the unpaired *t* test. Discrete data, such as those obtained

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TABLE 1. Clinical data of patients undergoing the Ross procedure and control subjects

	Autograft (n = 11)	Control (n = 11)
Age at operation (y)	24.4 (10.5–39.2)	NA
Age at explantation (y)	35.8 (23.6–47.8)	31.9 (19.7–49.7)
Interval (y)	11.2 (7.3–15.4)	NA
Male	9	7
Female	2	3
Bicuspid native valves	7	0

NA, Not applicable.

from the immunohistochemical analysis of desmin and α–smooth muscle actin, were analyzed with Fisher's exact test. Analysis of the correlation between the variables studied and the time to explantation was performed with Pearson's correlation coefficient. All comparative analyses were performed with SPSS 12.0.1 for Windows (SPSS, Inc, Chicago, Ill).

RESULTS

Histochemical Analysis

Autograft valves did not differ significantly from normal aortic and pulmonary valves (Figure 1) in their mean total thickness (372 vs 474 [normal aortic valve] and 292 μm [normal pulmonary valve]; $P > .2$ and $P = .067$, respectively). Thickness of the ventricularis of the autograft valve (137 μm) was significantly greater than that of the normal aortic (77 μm) or pulmonary (37 μm) valves ($P = .058$ and $P = .002$, respectively). It comprised 36% of total autograft thickness compared with normal aortic (17%) and pulmonary (12%) valves ($P = .02$ and $P < .001$, respectively). The number of cells per square millimeter in the ventricularis was 396 in autografts compared with numbers seen in normal aortic (230) and pulmonary (303) valves ($P = .02$ and $P = .083$, respectively). Matrix cell morphology was spindle shaped, and cells lining the ventricularis surface appeared large and cubical.

Immunohistochemical Analysis

The autograft ventricularis was lined by endothelial cells, as identified by means of CD31 staining. Autograft valves showed α–smooth muscle actin–positive cells and desmin-positive cells in 91% and 36% of samples, respectively, compared with 36% and 0%, respectively, in control samples ($P = .024$ and $P = .035$, respectively). The expression of these markers indicates a myofibroblast-like phenotype of the cells present in the extracellular matrix of the autograft ventricularis. In contrast to controls, the autograft ventricularis stained positive for interleukin 6, matrix metalloproteinase 1, and transforming growth factor β. A very low number of inflammatory cells were present in the ventricularis of both autograft and control samples, as identified by means of CD45 staining. Apoptosis, matrix degradation, cell proliferation, and senescence markers were not expressed. Interestingly, no correlation was present between the variables studied and the time to explantation (data not shown).

Confocal Microscopy

Collagen fiber density was more pronounced in the ventricularis of autograft valves compared with that seen in normal aortic and pulmonary valves (Figure 2). Moreover, these fibers showed a high degree of geometric organization. Fiber alignment was typically in the radial direction (ie, from wall to rim).

DISCUSSION

Pulmonary autograft valve explants consistently showed a thickened ventricularis. Its presence along the entire cusp, as well as focal expression of the fibroblast activators transforming growth factor β and interleukin 6, suggest this thickening is the result of a local adaptive response rather than fibrous overgrowth (pannus), as suggested by Rabkin-Aikawa and colleagues,[5] who observed similar thickening

TABLE 2. Antibodies used in immunohistochemical analysis

Markers	Cellular aspects	Antigen retrieval	Primary antibody	Secondary antibody	Company
α-SMA	Myofibroblasts	−	1:400	Mouse	Sigma
Active caspase 3*	Apoptosis	+	1:200	Rabbit	Cell Signaling
Cathepsin K	Elastinolysis	+	1:1000	Rabbit	TNO
Cathepsin S	Elastinolysis	+	1:1000	Rabbit	TNO
CD31*	Endothelium	+	1:400	Mouse	DAKO
CD45	Inflammatory infiltrate	+	1:100	Mouse	DAKO
Desmin	Myofibroblasts	−	1:20	Mouse	LUMC
IL-6	Differentiation	+	1:500	Goat ‡	Biogenesis
Ki67	Proliferation	+	1:200	Mouse	DAKO
MMP1	Collagen breakdown	−	1:1000	Mouse	Chemicon
MMP9	Collagen breakdown	+	1:250	Rabbit	TNO
$p^{16INK4a}$†	Senescence	+	1:800	Mouse	Neomarkers
TGF-β	Differentiation	+	1:500	Rabbit	LUMC

α-SMA, α-Smooth muscle actin; *IL-6*, interleukin 6; *MMP*, matrix metalloproteinase; *TGF-β*, transforming growth factor β. *Diaminobenzidine enhancement by means of incubation with Cu_2SO_4 for 5 minutes. †Antigen retrieval with Tris/ethylenediamine tetraacetic acid. ‡Rabbit α goat + streptavidin-biotin-peroxidase complex.