ORIGINAL PAPERS

Adv Clin Exp Med 2011, **20**, 2, 157–164
ISSN 1230-025X

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Participation of Tenascin C in Native and Homograft Aortic Valve Degeneration*

Udział tenascyny C w degeneracji natywnych i homogennych aortalnych zastawek serca

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Abstract

Background. Tenascin C (TnC) is a connective tissue matrix glycoprotein influencing cell proliferaton, migration and apoptosis. TnC participates in the development of pathological processes in the circulatory system including, as recently demonstrated, valvular degeneration leading to aortic valve stenosis. Homograft durability is limited by its degeneration, similar to changes appearing in cases of a typical aortic stenosis, which currently are regarded as an active remodeling process with expression of inflammatory modulators, as well as participation of cells and extracellular factors (including TnC), which under physiological conditions are involved in bone formation.
Objectives. The aim of the study was a comparison of TnC expression in native stenotic aortic valves and degenerating aortic valve homografts.
Material and Methods. The material included two groups of aortic valves removed during routine surgery: native stenotic valves (n = 10) and dysfunctional aortic valve homografts (n = 10). Frozen valve sections were used for immunohistochemical detection of TnC, and also for identification of cells differentiated towards myofibroblasts (SMA), macrophages (CD68) and endothelial cells (CD34, vWF).
Results. TnC immunoreactivity was observed in both valve types, being significantly higher in native stenotic valves than in homografts ($p = 0.007$). The proportion of myofibroblasts in the total valvular cell population was insignificantly higher in native valves than in homografts. In homografts showing significantly lower total celullarity than native valves ($p = 0.003$), macrophages were more numerous ($p = 0.005$), and located mostly on the valve leaflet surfaces, while in native valves they were almost absent in this location.
Conclusions. The presence of TnC in the stroma of native stenotic aortic valves and in homografts indicates a participation of active remodeling mechanisms in the pathogenesis of degeneration in both types of valves. However, a significantly lower level of TnC and lower cellularity in homografts suggests that changes leading to their dysfunction, to some extent, include passive degeneration (**Adv Clin Exp Med 2011, 20, 2, 157–164**).

Key words: tenascin, aortic valve, homograft, extracellular matrix proteins, immunofluorescence.

Streszczenie

Wprowadzenie. Tenascyna C (TnC) jest glikoproteiną macierzy tkanki łącznej biorącą udział w modyfikacji aktywności proliferacyjnej, migracyjnej oraz różnicowaniu i apoptozie komórek. TnC uczestniczy w rozwoju procesów patologicznych w układzie naczyniowym, a ostatnio stwierdzono jej udział również w powstawaniu zastawkowych zmian degeneracyjnych prowadzących do stenozy aortalnej. Czas funkcjonowania homograftów aortalnych jest ograniczony ich degeneracją, przypominającą zmiany zachodzące w przypadku typowej stenozy aortalnej, które obecnie są uważane za proces aktywnej przebudowy (ekspresja modulatorów procesu zapalnego, udział komórek i czynników pozakomórkowych – w tym tenascyny C, fizjologicznie warunkujących osteogenezę).
Cel pracy. Porównanie ekspresji TnC w natywnych stenotycznych aortalnych zastawkach i degenerujących homograftach aortalnych.

*This study was supported by a statutory grant (K/ZDS/000987 to G.J.L.) from the Jagiellonian University Medical College.

Materiał i metody. Badanie obejmowało dwie grupy zastawek usuniętych w czasie rutynowych zabiegów wymiany zastawki: natywne stenotyczne zastawki aortalne (n = 10) i homografty (n = 10). Na skrawkach mrożeniowych płatków zastawek oznaczano immunofluorescencyjnie TnC, a także identyfikowano komórki zróżnicowane w kierunku miofibroblastów (SMA) oraz makrofagi (CD68) i komórki śródbłonka (vWF, CD34).
Wyniki. W natywnych zastawkach stenotycznych stwierdzono istotnie wyższą ekspresję TnC niż w zastawkach homogennych (p = 0,007). Proporcja miofibroblastów w populacji komórek w natywnych zastawkach stenotycznych była wyższa niż homograftach. W homograftach przy istotnie mniejszej całkowitej gęstości komórek, w porównaniu z zastawkami natywnymi (p = 0,003), stwierdzono większy udział makrofagów (p = 0,005), zwłaszcza umiejscowionych na powierzchni płatka, podczas gdy w zastawkach natywnych były one prawie nieobecne w tym miejscu.
Wnioski. Obecność TnC w natywnych zastawkach stenotycznych i homograftach wskazuje na udział mechanizmów aktywnej przebudowy w patogenezie degeneracji obu typów zastawek. Istotnie niższy poziom ekspresji TnC w homograftach oraz mniejsza liczba komórek zrębu sugeruje, że zmiany prowadzące do ich dysfunkcji w pewnym stopniu mają charakter biernej degeneracji (**Adv Clin Exp Med 2011, 20, 2, 157–164**).

Słowa kluczowe: tenascyna, zastawka aortalna, homograft, białka macierzy pozakomórkowej, immunofluorescencja.

Aortic valve degeneration is an age-related process leading to valvular sclerosis and stenosis affecting up to 5% of elderly patients [1]. In the course of the disease, dystrophic calcification is a common phenomenon. Over the years it was regarded as a passive, purely destructive, non-modifiable process, leading to calcium salt accumulation in defective valves. However, recent years have brought strong evidence supporting the concept of active mechanisms participating in the pathophysiology of valvular as well as vascular calcifications. The players involved include endothelial, inflammatory and activated interstitial cells, cytokines produced by these cells (TNF-alpha, TGF-beta1), extracellular lipids (apolipoproteins), various matrix proteins (metalloproteinases, bone morphogenetic proteins, bone matrix glycoproteins) and renin-angiotensin systems [2].

Participation of the above mentioned active mechanisms in homograft pathology is still poorly understood. In calcified areas of explanted aortic valve homografts, Shetty et al. [3] found interstitial cells which expressed markers of osteoblastic differentiation and core binding factor α1 (RUNX2/Cbfa1) as well as osteopontin, osteonectin, RANK and RANKL, indicating the osteogenic potential of the homograft valve tissue.

It is postulated that chondroblastic and/or osteoblastic transdifferentiation of cells localized in the valvular stroma can lead to calcification and heterotopic bone formation [4, 5].

Dystrophic calcification is also observed in atherosclerosis, however, comparing to aortic valve stenosis (AVS), it seems to be a relatively late event. Studies have shown that coronary artery disease risk factors (hyperlipidemia, hypertension, diabetes) are also valid in cases of AVS. Many similarities between atherosclerosis and AVS exist, including at pathophysiological and molecular levels: endothelial cell damage, lipid deposition, oxidation of lipoproteins and inflammatory infiltration [6].

Tenascin C (TnC) is a member of a highly conserved family of large connective tissue matrix glycoproteins [7]. During embryogenesis, TnC participates in the modification of cellular activity (proliferation, migration, differentiation and apoptosis). It plays also an important role in the development of vascular pathology, and the involvement of TnC in aortic valve degeneration leading to stenosis has been demonstrated recently [8]. However, its role in homograft pathology is not known.

The aim of this study was to examine if TnC is expressed in degenerating homograft aortic valves and to compare its expression level and distribution with those in native stenotic aortic valves. Additional morphological parameters characterizing valvular degeneration such as abundance and distribution of myofibroblastic cells and macrophages, interstitial cell density and continuity of endothelial lining were also investigated.

Material and Methods

The valves were collected from patients undergoing aortic valve replacement surgery. The study protocol was approved by the Jagiellonian University Medical College Bioethical Committee. The material included 10 native aortic valves excised due to stenosis (6 men and 4 women, mean age 59 ± 12.3 years, max./min.: 73/24), 10 aortic valve homografts (7 men and 3 women, mean age 50.7 ± 12.3 years, max./min.: 67/27) originating from nonbeating-heart donors, implanted as "fresh", antibiotic-preserved and removed due to dysfunction during repeat surgery carried out within 6 to 16 years (mean 13 years) of grafting. Differences in the mean age of patients between both groups as well as age differences between sexes within each group were statistically insignificant.

After initial macroscopic evaluation, the valves were fixed for 24 hours in 4% buffered formalin.

For routine histology, paraffin-embedded cross sections (7 μm thick) of central segments of the cusps (from the base to the free edge) were prepared and stained with hematoxylin and eosin. For immunohistochemistry, 12 μm thick cryostat sections were prepared.

Immunohistochemistry

After preincubation with 5% normal goat serum for 40 min, sections were incubated overnight with primary antibodies (Table 1) in a humid chamber at room temperature. Next, sections were washed extensively in PBS and incubated for 90 min with goat anti-mouse Cy-3-conjugated and/or goat anti-rabbit Cy-2-conjugated antibodies (Table 1). Cell nuclei were counterstained with DAPI (Sigma, Saint Louis, MO, USA). Sections were washed three times in PBS and mounted in a glycerol/PBS solution (pH = 8.6). Negative controls were performed by omitting the primary antibodies during the first incubation.

Morphometry and Statistics

Sections were examined under an Olympus BX50 light/fluorescence microscope. Images were recorded using a DP-71 digital CCD camera (Olympus, Japan) coupled to an IBM PC-class computer equipped with AnalySIS-FIVE® (Soft Imaging System GmbH, Münster, Germany) image analysis system.

TnC expression was estimated semiquantitatively, using a score range from 0 to 4 (0 = none, 1 = trace, 2 = < ¼ leaflet section area (l.s.a.), 3 = ≤ ½ l.s.a., 4 = > ½ l.s.a.).

For the estimation of abundance and distribution of myofibroblastic cells (alpha-SMA positive) the F_{SMA} coefficient was calculated, defined as: $F_{SMA} = R \times G \times P$, where R denotes an area occupied by myofibroblasts: 0 = none, 1 = a few scattered cells, 2 = <¼ l.s.a., 3 = < ½ l.s.a., 4 = < ¾ l.s.a.. 5 = ≥ ¾ l.s.a.; G denotes myofibroblast density in clusters: 1 = < 250 cells/mm^2, 2 = < 500 cells/mm^2, 3 = ≥ 500 cells/mm^2; P denotes the proportion of myofibroblasts to other cells: 1 = < 1%, 2 = ≤ 10%, 3 – > 10%

Total interstitial cell density was estimated by using a score range from 0 to 3 (0 = ≤ 10 cells/mm^2, 1 = ≤ 100 cells/mm^2, 2 = ≤ 300 cells/mm^2, 3 = > 300 cells/mm^2).

The statistical analysis employed (in compliance with the specific category of collected data) a t-Student test for unpaired data, a Mann-Whitney test, and the Fisher's exact test. The value of $p < 0.05$ was considered statistically significant.

Results

The authors found the presence of TnC in both groups of valves with significantly higher immunoreactivity in native aortic valves than in homograft valves ($p = 0.007$) (Fig. 1). In the cases of native valves (Fig. 2a) as well as homografts (Fig. 3), tissue distribution of TnC was similar: strongly TnC positive areas were seen within focal calcifications (Fig. 2b), positive immunostaining was also visible in the walls of blood vessels located in tissue adjacent to the calcifying areas (Fig. 2c). In most cases (except for one homograft and two native valves), segmental TnC immunoreactivity was also observed along both leaflet surfaces, in a basal lamina-associated zone (Fig. 2a, 3).

The native aortic valves revealed significantly higher density of interstitial cells than the homografts ($p = 0.003$).

The proportion of myofibroblasts/activated interstitial cells (alpha-SMA positive) in the total interstitial cell population was more than twice as high in native valves as compared with homograft valves (F_{SMA} = 7.5 and 3, respectively), however

Table 1. Primary and secondary antibodies used in immunofluorescentic examination. MC = monoclonal; PC = polyclonal

Tabela 1. Przeciwciała pierwotne i wtórne użyte w badaniach immunofluorescencyjnych. MC = monoklinalne; PC = poliklonalne

Antibody (Przeciwciało)	Type (Typ)	Dilution (Rozcieńczenie)	Manufacturer (Producent)	Code (Kod)
Primary (Pierwotne)				
mouse anti-human tenascin	MC	1:200	Chemicon, Temecula, CA	MAB19101
mouse anti-actin, smooth muscle	MC	1:200	Chemicon, Temecula, CA	MAB1522
mouse anti-human CD68	MC	1:1	Chemicon, Temecula, CA	IHCR2113-6
mouse anti CD34	MC	1:50	Novocastra, Newcastle, UK	NCL-END
rabbit anti von Willebrand Factor	PC	1:200	Novocastra, Newcastle, UK	NCL-vWFp
rabbit anti-laminin	PC	1:50	Sigma, Saint Louis, MO	L9393
Secondary (Wtórne)				
Cy3-conjugated goat anti-mouse		1:400	Jackson IR, West Grave, PA	115-165-146
Cy2-conjugated goat anti-rabbit		1:400	Jackson IR, West Grave, PA	111-225-144

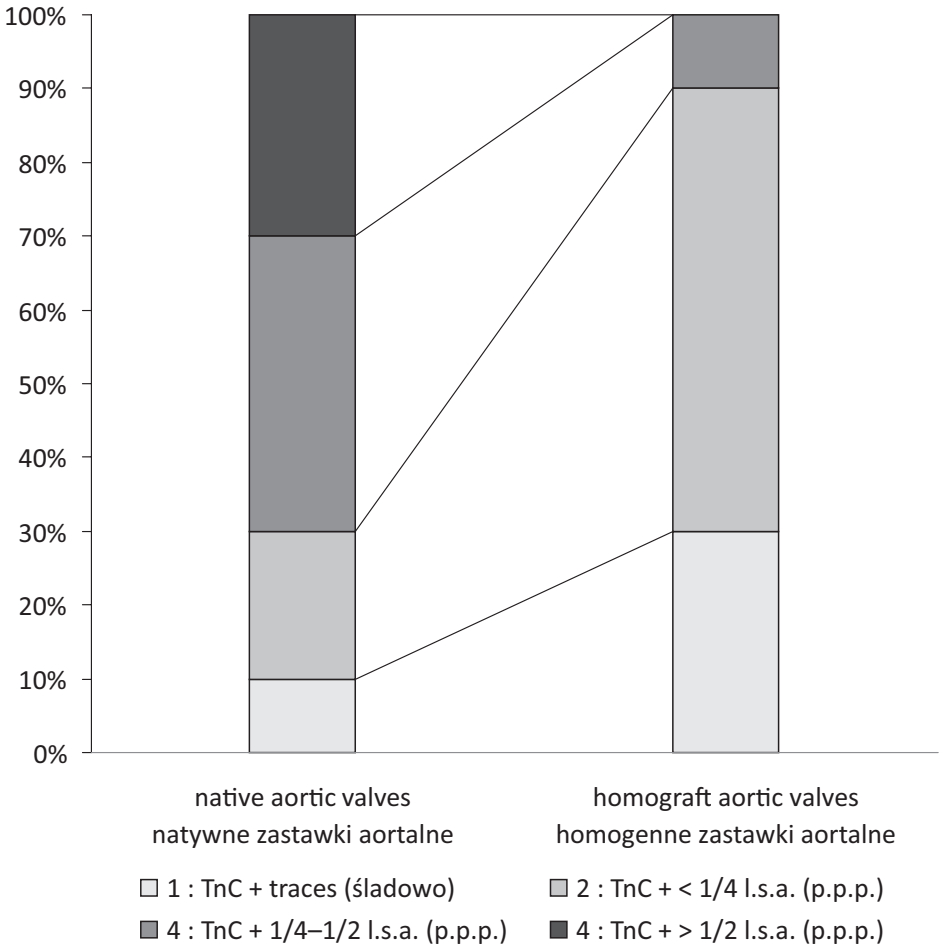

Fig. 1. TnC in the examined groups of valves. Graph shows the percentage of cases with TnC immunoreactivity at the particular score values (1–4). l.s.a. – leaflet section area

Ryc. 1. TnC w badanych grupach zastawek. Wykres przedstawia odsetek przypadków wykazujących dodatnią reakcję w danym przedziale (1–4). p.p.p. – powierzchnia przekroju płatka

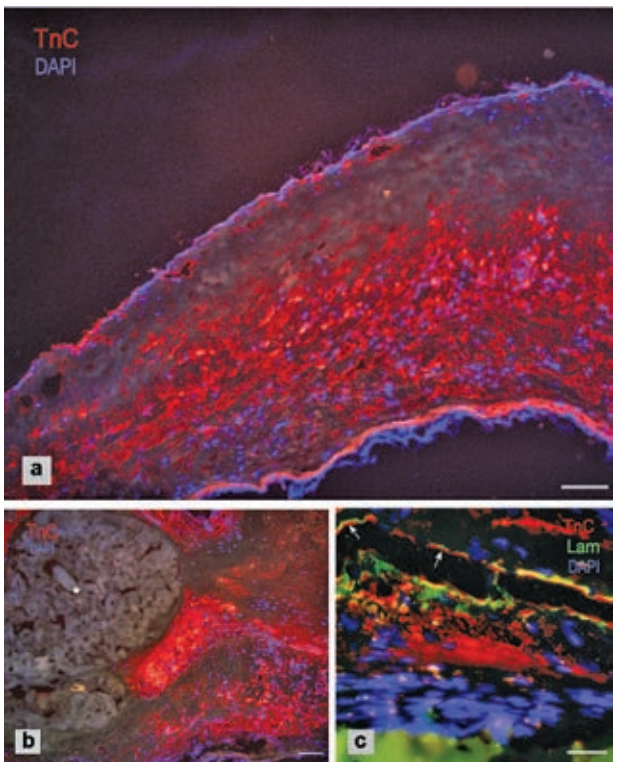

Fig. 2. TnC (red) in native stenotic aortic valve leaflet. **a**: immunoreactivity in the leaflet stroma and segmentally along the surface in a basal lamina-associated zone; **b:** high immunoreactivity in the vicinity of focal calcifications (asterisk); **c:** immunoreactivity in the wall of blood vessel located in tissue adjacent to the calcifying areas (→). Green fluorescence: laminin in basal lamina. **a,b,c:** cell nuclei stained with DAPI (blue). Scale bar: a, b = 200μm, c = 50 μm

Ryc. 2. TnC (czerwony) w płatku natywnej stenotycznej zastawki aortalnej; **a:** immunoreaktywność w zrębie płatka i odcinkowo wzdłuż powierzchni w rejonie blaszki podstawnej; **b:** wysoki poziom immunoreaktywności w sąsiedztwie wapniejących złogów (gwiazdka); **c:** immunoreaktywność w ścianie naczyń krwionośnych umiejscowionych w tkance sąsiadującej z ogniskami zwapnień (→). Zielona fluorescencja – laminina w blaszce podstawnej. a,b,c: jądra komórkowe barwione DAPI (niebieskie). Znacznik skali: a,b = 200 μm, c = 50 μm

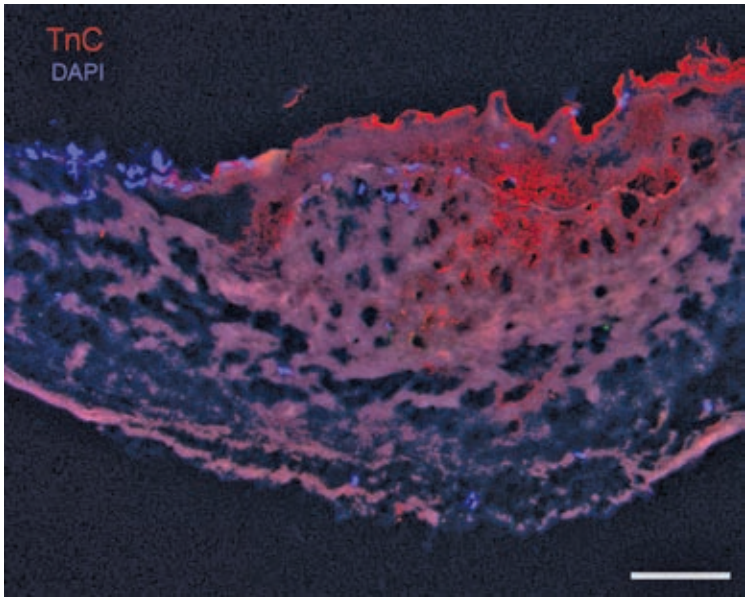

Fig. 3. TnC (red) in homograft aortic valve. Segmental localization in a basal lamina-associated zone and weak expression in leaflet stroma. Note almost acellular leaflet stroma. Cell nuclei stained with DAPI (blue). Scale bar = 200 μm

Ryc. 3. TnC (czerwony) w homogennej aortalnej zastawce. Odcinkowa lokalizacja w rejonie blaszki podstawnej oraz słaba ekspresja w zrębie płatka. Widoczny prawie bezkomórkowy zrąb płatka. Jądra komórkowe barwione DAPI (niebieskie). Znacznik skali = 200 μm

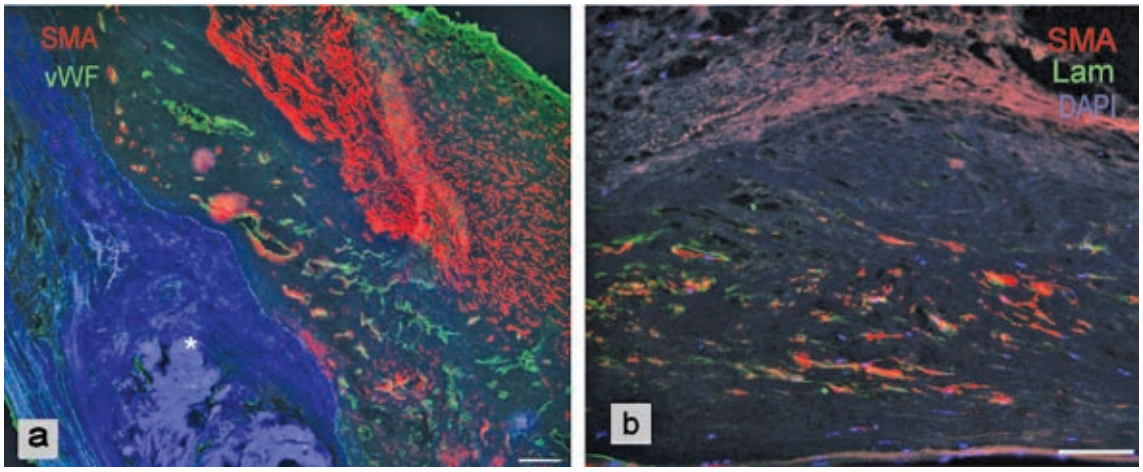

Fig. 4. Myofibroblastic/activated valvular interstitial cells (red). **a**: in native stenotic aortic valve leaflet. Note blood vessels with SMA positive cells in their walls in deeper areas of valve stroma, close to focal calcifications (*). Endothelial cells (green). **b:** in homograft valve stroma. Cell nuclei stained with DAPI (blue). Scale bar = 200 μm

Ryc. 4. Komórki miofibroblastyczne/aktywowane komórki śródmiąższowe (czerwone). **a:** w płatku natywnej zastawki stenotycznej. W głębszych warstwach w sąsiedztwie wapniejących złogów (*) widoczne naczynia z komórkami SMA-pozytywnymi w ścianie. Komórki śródbłonkowe (zielony). **b:** w zrębie homograftu. Jądra komórkowe barwione DAPI (niebieskie). Znacznik skali = 200 μm

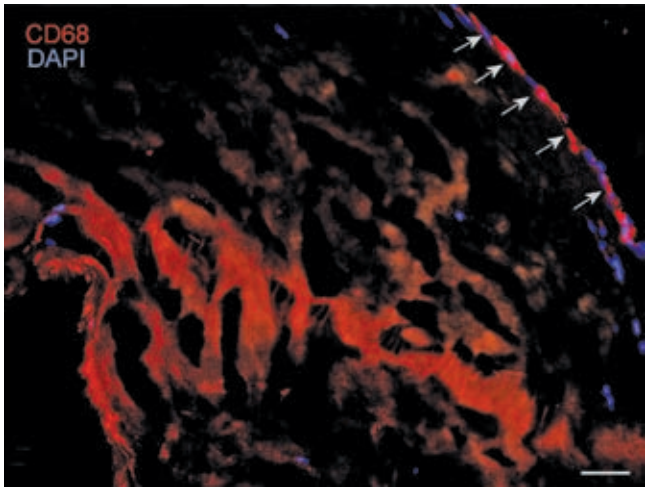

Fig. 5. Macrophages (red) arranged in epithelial-like fashion on the surface of homograft valve. Note virtually acellular leaflet stroma. Cell nuclei stained with DAPI (blue). Scale bar = 50 μm

Ryc. 5. Makrofagi (czerwone) ułożone nabłonkowato na powierzchni homograftu. Zrąb płatka praktycznie zupełnie bezkomórkowy. Jądra komórkowe barwione DAPI (niebieskie). Znacznik skali = 50 μm

this difference did not reach statistical significance ($p = 0.07$). In five native valves and two homografts, alpha-SMA positive cells were also found in the walls of small blood vessels (Fig. 4).

In all native and homograft valves, macrophages were located in the areas of focal calcifications. They were also present in other stromal areas in all homografts and in six native valves. In eight homografts and in only one native valve, we found macrophages distributed along the leaflet surface in an epithelial-like manner (Fig. 5).

In almost all (nine) native valves, newly formed blood vessels were located in areas of focal calcifications (in three cases also beneath the leaflet surface in the form of relatively wide vascular channels with variable diameter, partly lined with endothelial cells). In these areas, inflammatory infiltrations were also observed, predominantly composed of mononuclear cells. Blood vessels were found in only four homograft valves.

In the majority of homografts (six), the layered structure of the valves was almost completely obscured, which was only partly observed in heavily stenotic native valves. The valves showed a substantial loss of endothelial cells, more prominent in homografts. In these groups of valves, interstitial cells were unevenly distributed, and in most cases acellular areas were prominent (Fig. 3). Small mononuclear (in one case mixed) inflammatory infiltrations were seen in half of the homograft valves.

Discussion

TnC is associated with the morphogenesis of vascular, skeletal and nervous systems. In normal adult tissue, TnC expression remains at a very low level only in some areas of the connective tissue. In the course of inflammation, in malignant tumors or during intense tissue regeneration its expression is significantly elevated [7].

Healthy valve stroma, as evidenced by immunohistochemistry and in-situ hybridization methods in non-stenotic valves, shows only minimal TnC expression in basement-membrane-associated zones [8]. Present study has demonstrated the presence of TnC in both native stenotic and in insufficient homograft aortic valves. In cases of native stenotic valves, this finding is consistent with the observations of Satta et al. [8] who reported increased expression of TnC correlating with the degree of valve pathology. In authors' material, particularly strong TnC immunoreactivity appeared in leaflet areas involved in intensified remodeling processes leading to massive fibrosis, nodular calcification and blood vessel formation.

These results and previous observations of other authors show that TnC expressed in pathologically altered aortic valves is produced by valvular interstitial cells (including alpha-SMA positive myofibroblasts, which are regarded as a subpopulation of activated valvular interstitial cells involved in matrix remodeling and osteoblastic valvular interstitial cells responsible for the production of matrix components characteristic for bone tissue), as well as by endothelial cells and macrophages [8,9]. TnC inducing factors include cytokines (e.g. interleukin 1, TGF-beta 1) released by inflammatory cells, as well as mechanical/hemodynamic stress [10].

The impact of mechanical stress on the initiation of tissue mineralization is widely recognized [11, 12]. It seems to be a more important player in the pathology of the heart valves than of arteries, contributing to the earlier and more prominent calcification observed in the former ones, particularly in places of maximal leaflet flexion (near the base), which are exposed to the largest loads [13].

A notably accelerated degenerative process with prominent calcification has been demonstrated in homograft aortic valves [14–16]. Although explanted homografts can show similar pathological changes to those observed in native stenotic valves (including interstitial cell transdifferentiation and production of ground substance elements typical for bone tissue) [3], they also reveal some specific pathological features. Important factors modifying the process of homograft degeneration include a variable (but always substantial) level of endothelial and interstitial cell damage, dependent on the applied graft preimplantation preservation method (as "fresh", antibiotic-preserved or cryopreserved), the source of the valve (multiorgan donor, cardiac transplantation or nonbeating heart donor), as well as host and donor immune responses [17–19].

Present study has demonstrated for the first time the participation of TnC in homograft valve pathology. In view of the fact that homograft durability is limited by the degenerative processes analogous to those observed in native stenotic valves, the TnC immunoreactivity found by us in homografts as well as its similar location in leaflet tissue, provide further evidence for a common pathobiology of both homograft and native aortic valve degenerative processes. However, the substantially lower level of TnC immunoreactivity observed in the homografts suggests some differences between both processes.

Due to almost absent or heavily damaged endothelial lining, the grafted valves seem to be much more exposed to factors inducing their degeneration (e.g. lipoproteins and/or inflammatory

cells), in particular those originating directly from the bloodstream, than the native valves [20, 21]. Localization of macrophages on the surface of the homograft leaflets, very often observed in authors' material, indicate that they originate from blood monocytes adhering to the areas of the valve devoid of endothelial lining.

In the aortic homografts the authors examined in this and the previous study [22], they found completely acellular areas in valve stroma. The total number of interstitial cells was also significantly lower. Studies on the viability of the homograft cells have shown that many of them are lost (partly due to apoptosis) in the months immediately following the grafting, which significantly reduces valve regenerative abilities [19]. Thus, it seems probable that the chances of successful prevention of valve degeneration (e.g. by controlling the activity of the cells involved) are limited in cases of homografts. Also the lower number of homograft valves revealing formation of new blood vessels and limited myofibroblastic transdifferentiation of interstitial cells in these valves, as compared with native stenotic valves, seem to support this opinion.

The relatively small number of valves examined is a limitation of the present study, therefore our results are preliminary and require confirmation on a larger group of valves.

The authors conclude that the appearance of TnC in native stenotic aortic valves and in aortic valve homografts indicates the involvement of active remodeling mechanisms in the pathogenesis of the degeneration in both types of valves. In homograft aortic valves, however, significantly lower TnC immunoreactivity and limited total as well as SMA positive interstitial cell number suggests that mechanisms leading to their dysfunction are also – to some extent – passive, which should be taken into account in determining preventive strategies.

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Conflict of interest: None declared

Received: 21.12.2010
Revised: 8.02.2011
Accepted: 24.03.2011