Cardiovascular Pathology 24 (2015) 102–109

Contents lists available at ScienceDirect

Cardiovascular Pathology

Original Article

Morphologic studies of cell endogenous repopulation in decellularized aortic and pulmonary homografts implanted in sheep

Mila Della Barbera, Marialuisa Valente, Cristina Basso, Gaetano Thiene *

Department of Cardiac, Thoracic and Vascular Sciences, University of Padua Medical School, Via A. Gabelli 61, 35121 Padova, Italy

ARTICLE INFO

Article history:
Received 11 June 2014
Received in revised form 7 October 2014
Accepted 9 October 2014

Keywords:
Experimental
Homograft
Morphology
Valves
Vascular

ABSTRACT

Purposes: The rationale of this study was to assess morphologically the effects of implantation of decellularized aortic and pulmonary homografts into growing sheep, with the objective to establish type and extent of cell repopulation and propensity to dystrophic calcification over a prolonged period of time.
Methods: Pulmonary and aortic homografts were obtained from healthy euthanized juvenile sheep (35–45 kg). Complete decellularization was accomplished in 0.5% sodium deoxycholate and 0.5% sodium dodecylsulfate for 24 h. Twelve homografts from 11 animals were studied as follows:

- 2 native (1 pulmonary and 1 aortic) from the same animal;
- 2 decellularized unimplanted (1 aortic and 1 pulmonary), from two different animals;
- 8 decellularized (2 aortic and 6 pulmonary), implanted in 8 female animals for 14–21 months.

Gross, X-ray, histology, immunohistochemistry, morphometry, transmission electron microscopy and calcium content spectroscopy investigations were carried out.
Results: Decellularization appeared complete in unimplanted homografts. The extracellular matrix was intact. Explanted homografts showed soft, pliable cusps without gross calcium deposits and tears; calcium content showed slight difference between aortic and pulmonary cusps (5.505±2.04 vs. 2.77±1.06 mg/g dry weight, $P=.04$). Microscopic calcifications were observed in two aortic homografts on smooth muscle cells of repopulated homograft wall and on valvular interstitial cells, respectively. Inflammatory infiltrates were never seen.
Cell repopulation occurred in homograft wall with actin smooth muscle and vimentin positive cells in media lamellar units (cell density per millimeter squared, 885.4±424.38 in native vs. 172.64±160.33 in implanted homograft, $P<.01$) as well as in cusps (cell density per millimeter squared, 495.96±63.92 in native vs. 184.66±140.74 in implanted homograft, $P<.01$). The percentage area of recellularization was 71.27±3.03 in the homograft wall and 22.16±3.06 in the cusps.
Thickness of pulmonary explanted homograft wall and cusps was 900.68±321.52 μm vs. 994.36±135.92 μm and 204.75±66.64 μm vs. 231.04±105.94, respectively ($P=$ NS), whereas in aortic homograft wall and cusps it was 1358.604±423.79 μm vs. 2065.32±431.46 μm, $P=.016$, and 248.01±93.95 μm vs. 390.30±104.81 μm, $P=.03$, respectively. The endothelial lining was restored.
Conclusion: Endogenous cell repopulation in decellularized homografts occurs and persists following implantation, at both wall and cusp level, without evidence of immune reaction.
Even in the long term, the cusps exhibit no structural deterioration and negligible calcification.

1. Introduction

The ideal graft for aortic and pulmonary valve replacement has not been accomplished so far. Tissue engineering in the heart valve field represents a promising alternative to native homografts or glutaraldehyde-fixed xenografts for valve substitution, aiming to create nonimmunogenic, biologically viable grafts [1]. None of the currently employed heart valve substitutes shows growth potential or regeneration capability for use in children or in adults. Many attempts have been done so far to realize an ideal graft that has the possibility to grow, repair, and remodel while the living recipient grows up, eliminating the need of redo surgery: (a) in vitro cell seeding on biodegradable synthetic scaffolds, (b) in vitro cell seeding of decellularized natural scaffolds, and (c) in vivo repopulation of decellularized natural scaffolds by circulating endogenous cells [2]. The decellularization of natural scaffolds consists of the elimination of immunogenic allograft cells from the valvular matrix, by using various decellularization experimental protocols

* Corresponding author. Department of Cardiac, Thoracic and Vascular Sciences, University of Padua Medical School, Via A. Gabelli 61, 35121 Padova, Italy. Tel.: +39-049-8272283; fax: +39-049-8272285.
E-mail addresses: mila.dellabarbera@unipd.it (M. Della Barbera), marialuisa.valente@unipd.it (M. Valente), cristina.basso@unipd.it (C. Basso), gaetano.thiene@unipd.it (G. Thiene).

http://dx.doi.org/10.1016/j.carpath.2014.10.001
1054-8807/

[3–8], in place of glutaraldehyde fixation that triggers mineralization. Several groups have described methods to obtain decellularized tissues comprising (a) ionic detergents, i.e., sodium deoxycholate (SDC) and sodium dodecylsulfate (SDS) [5,7]; (b) combination of ionic and nonionic detergents, i.e., *tert*-octylphenyl-polyoxyethylene (Triton X-100) and SDC [3]; (c) enzymatic extraction methods and chelating agents, i.e., trypsin with ethylenediaminetetraacetic acid (EDTA) [3,7]; (d) combination of ionic or nonionic detergents with enzymatic extraction, i.e., *N*-lauroylsarcosinate and recombinant endonuclease [4], SDC and SDS as well as DNase digestion [6], Triton X-100, and SDC followed by ribonuclease and deoxyribonuclease digestion [3]; and combination of ionic and nonionic detergents and chelating agents, i.e., glycerin, SDC, SDS, Triton X-100, and EDTA [8].

In previous studies, it was observed that, in vivo, cell repopulation occurs in decellularized tissues after implant with various success depending on the physical, chemical, enzymatic, or combined methods used [7,9–12]. A protocol that considers the use of detergents [12] without significant toxicity to cell seeding revealed to be effective for endogenous cell repopulation of homograft implants within 6 months [13]. However, effects in the long term were not assessed so far.

The hypothesis of this investigation was that the decellularized homografts may serve as a scaffold for endogenous self repopulation, transforming the homograft into an autograft, thus preventing immune reaction as well as dystrophic calcification that occurs in glutaraldehyde-fixed bioprostheses.

2. Materials and methods

2.1. Animals

Twelve homografts from 11 growing sheep were studied. Four homografts served as controls: (a) 1 native aortic and 1 native pulmonary from the same animal, (b) 1 decellularized unimplanted aortic, and 1 decellularized unimplanted pulmonary from two different animals. Eight female animals were operated with implantation of decellularized homografts (age at implant: 6.6±2.3 months), 2 aortic and 6 pulmonary in aortic and pulmonary position, respectively. Among pulmonary homografts, 3 were downsized bicuspid to match the size of recipients for limited availability of small-sized homografts [14] and 3 were standard tricuspid implanted following Ross operation. Overall, 21 semilunar implanted cusps (6 aortic and 15 pulmonary) were examined, in Hannover, Germany.

All animal experiments and surgical procedures were performed in compliance with the Guide for the Care and Use of Laboratory Animals as published by the US National Institutes of Health (Publication 85-23, revised 1996) and were approved by the local animal care committees.

2.2. Homograft decellularization preparation

Aortic and pulmonary homografts, including both sinusal (with semilunar cusps) and tubular portions, were retrieved from healthy euthanized sheep and decellularized in Hannover, Germany, as previously reported [6]. In brief, decellularization was performed in 0.5% SDC (Sigma, St. Louis, MO, USA) and 0.5% SDS (Carl Roth, Karlsruche, Germany) for 24 h followed by nine wash cycles (8 h each) with phosphate-buffered saline (PBS) supplemented with penicillin and streptomycin (100 μg/ml, P/S; Biochrom, Cambridge, UK) to remove of remaining detergents and cell debris for an overall of 96 h.

All decellularization steps were performed at room temperature and under continuous shaking. The decellularized homografts were stored in PBS at 4°C 1 day prior to implantation [13].

2.3. Homograft implantation

Surgery and follow-up were carried out as previously reported [6,13]. After a follow-up of 14 and 18.5 months for the 2 aortic homografts and of 20–21 months for the pulmonary homografts, the animals were euthanized by intravenous pentobarbital (1 ml/kg body weight; WDT) following heparinization. The explants were fixed in 4% buffered formalin [15] for 48–72 h and then underwent to pathological investigation.

2.4. Morphological evaluation

After gross examination, the explanted homografts were submitted to X-ray. Thereafter, tissue sampling was performed in the homograft wall and cusps with transverse and/or longitudinal orientation.

2.4.1. Histology

The samples, dehydrated in crescent ethanol series, were paraffin embedded and 4- to 5-μm-thick sections were stained with hematoxylin–eosin to detect cells (nucleus and cytoplasm) as sign of recellularization and inflammation, Weigert-Van Gieson to evidence collagen and elastin, Heidenhein modified Azan Mallory for both cells and fibrillar extracellular matrix, Alcian PAS for ground substance extracellular matrix, and Von Kossa for calcium deposits.

2.4.2. Immunohistochemistry

For characterization of cell phenotype, other serial paraffin sections were incubated with the following antibodies: mouse antihuman smooth muscle actin (SMA), rabbit antihuman von Willebrand factor (vWF), mouse antihuman vimentin, mouse antihuman CD57 (HNK-1), mouse antihuman CD31, mouse antihuman vascular endothelial growth factor (VEGF), and rabbit antihuman CD117/c-kit (Dakocytomation, Glostrup, Germany); rabbit antihuman VEGF receptor 1 (R1) (Novus Biologicals, Littleton, USA), mouse antihuman VEGF receptor 2 (R2) (Santa Cruz Biotechnology, Santa Cruz, USA), and mouse antisheep CD45 (Serotec, Kidlington, UK). Primary antibodies incubation was followed by Envision HRP Kit Complex (Dakocytomation).

Endogenous peroxidase was quenched for 20 min in 0.3% hydrogen peroxide (H_2O_2) in PBS. Sections were incubated for 1 h at room temperature with the primary antibodies: mouse antihuman SMA (1:200), rabbit antihuman vWF (1:10.000), mouse antihuman vimentin (1:200), mouse antihuman CD57 (HNK-1) (1:100), mouse antihuman CD31 (1: 100), mouse antihuman VEGF (1:3.000), rabbit antihuman CD117/c-kit (1:200), rabbit antihuman VEGF R1 (1:100), mouse antihuman VEGF R2 (1:250), and mouse antisheep CD45 (1:100).

Prior to incubation with all primary antibodies, sections were submitted to antigen retrieval by heating in microwave oven (90°C) for 10 min in 0.01 citrate buffer, pH 6.0 (anti-SMA, anti-vWF, anti-CD117/c-kit, anti-VEGF R1, and anti-VEGF R2), and in 1 mM EDTA, pH 8 (anti-VEGF, anti-CD57, and anti-CD45).

Nuclear staining was performed with Mayer's hematoxylin solution. Antibodies incubation was also performed on native and decellularized homograft samples, as positive and negative controls, respectively.

Double staining with mouse antihuman SMA and one of the following antibodies (rabbit antihuman CD117/c-kit, mouse antihuman CD57, mouse antihuman VEGF, mouse antihuman VEGF R1, and rabbit antihuman VEGF R2) was performed on other slides to detect precursor cells. Antibodies were visualized with antimouse and antirabbit IgG AlexaFluor488-conjugated (Life Technologies, Molecular Probes, Carlsbad, USA) and with antimouse and antirabbit IgG Texas-Red-conjugated (Santa Cruz Biotechnology, Dallas, USA). Micrographs were taken using a laser scanner confocal microscope TCS-SL (Leica, Heidelberg, Germany).

2.4.3. Assessment of homograft cell repopulation: morphometrical analysis

Mean cell number per millimeter squared (cell density) was calculated in order to quantify the recellularization process.

Hematoxylin–eosin-stained slides were examined by light microscope (Zeiss Axioplan 2, Carl Zeiss, Oberkochen, Germany), equipped with digital camera (AxioVision, Carl Zeiss, Oberkochen, Germany)