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First quantitative assay of alpha-Gal in soft tissues: Presence and distribution of the epitope before and after cell removal from xenogeneic heart valves

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ABSTRACT

Decellularized xenograft heart valves might be the ideal scaffolds for tissue engineered heart valves as the alternative to the currently used biological and mechanical prostheses. However, removal of the alpha-Gal epitope is a prerequisite to avoid hyperacute rejection of untreated xenograft material. The aim of this study was to develop an ELISA soft-tissue assay for alpha-Gal quantification in xenograft heart valves before and after a detergent-based (TriCol) or equivalent cell removal procedure. Leaflets from porcine valves were enzymatically digested to expose the epitope and reacted with the alpha-Gal monoclonal antibody M86 for its recognition. Rabbit erythrocytes were used as a reference for the quantification of alpha-Gal. Native aortic and pulmonary leaflets exhibited different epitope concentration: 4.33×10^{11} vs. 7.12×10^{11}/10 mg wet tissue ($p < 0.0001$). Sampling of selected zones in native valves revealed a different alpha-Gal distribution within and among different leaflets. The pattern was consistent with immunofluorescence analysis and was unrelated to microvessel density distribution. After TriCol treatment alpha-Gal was no longer detectable in both pulmonary and aortic decellularized valves, confirming the ability of this method to remove both cells and alpha-Gal antigen. These results hold promise for a reliable quantitative evaluation of alpha-Gal in decellularized valves obtained from xenograft material for tissues engineering purposes. Additionally, this method is applicable to further evaluate currently used xenograft bioprostheses.

1. Introduction

Valve replacement is the most common surgical intervention for patients affected by end-stage valvular disease. Different types of prosthetic valves are currently used, either mechanical or biological. Mechanical valves are more durable, but require long-term anticoagulation therapy. Unlike mechanical valves, biological valves do not require life-long anticoagulation but have limited durability due to structural degeneration [1]. Moreover, glutaraldehyde fixation suppresses tissue viability, and seems to be one of the initiators of the calcification process [2]. Conversely, homograft valves are thought to retain the fibroblast viability, but are immunogenic [3–5] and their supply is limited.

The shortage of organ donors has led surgeons to explore the field of xenotransplantation through the use of heart valves from animals. The pig is now considered the most likely source of human replacement organs due to dimensional matching compatibility, similar physiology and growth to human size in a short period of time [6]. In clinical medicine, pigs are a reliable source of insulin, vascular grafts, dura mater, urinary bladder, and orthopaedic and dermal graft applications [7]. However, when transplanted into a primate, native as well as incompletely decellularized porcine heart valves trigger a violent hyperacute rejection, similar to that occurring in ABO-incompatible transplants [6,8,9].

The rejection of porcine tissue is due to the presence of the alpha-Gal epitope (galactose-alpha1–3-galactose) remaining on the surface of the endothelial cells [6,8,10,11]. This sugar moiety is expressed in most mammalian tissues, excluding humans and higher primates [12]. In humans, the continuous antigenic stimulation by gastrointestinal flora results in the production of anti-alpha-Gal antibodies, accounting for 1% of the circulating immunoglobulins, both IgM and IgA [13,14]. Once a xenogenic tissue is recognized, the complement cascade is activated and it triggers endothelial cell dysfunction, platelet aggregation and vascular thrombosis [15–17]. Several studies have been carried out in order to clarify which cell type expresses the epitope as well as to identify its distribution within different organ districts [12]. Moreover, many procedures to remove the antigen from tissues have been investigated, although only a few have resulted in a practical clinical use [13,14,16]. So far, the safest, and most expensive, way to circumvent the presence of the alpha-Gal epitope is the production of knockout animals for the 1,3-galactosiltransferase gene [8,18].

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The purpose of this study is the development of a quantitative enzyme-linked immunosorbent assay (ELISA) alpha-Gal soft-tissue test to assess the amount of the epitope in xenogenic aortic and pulmonary leaflets before and after cell removal by a detergent-based (TriCol) or equivalent procedure [19]. The TriCol procedure is characterized by the combined use of two different non-denaturing detergents: Triton X-100 (a non-ionic surfactant) and sodium cholate (a water-soluble anionic bile acid detergent), allowing the solubilization of both plasmatic and intracellular organelle membranes. TriCol has been reported to be suitable for the preparation of acellular and biocompatible heart valve scaffolds [20].

An anti-alpha-Gal primary monoclonal antibody (M86) was used to devise a novel, indirect ELISA assay. The number of epitopes in the tissue was determined with reference to an alpha-Gal standard source (rabbit erythrocytes) [21].

2. Materials and methods

2.1. Collection and sampling

Six hearts were harvested from Dutch Landrace × Large White pigs at a local abattoir. Animals were adults (10–12 months old), with body mass ranging from 160 to 180 kg. Hearts were stored and delivered to the laboratory in cold isotonic saline. The aortic and pulmonary valves were isolated within 2 h from death, and rinsed with cold saline for blood removal. The ELISA alpha-Gal assay was carried out on aortic and pulmonary leaflets grouped as follows:

Group A: 18 native leaflets (9 aortic and 9 pulmonary) were processed immediately and the results categorized as native samples (Nt);

Group B: 18 leaflets (9 aortic and 9 pulmonary) were treated with TriCol before performing the assay, and the results categorized as decellularized samples (Dc).

The following nomenclature was applied in order to differentiate the aortic leaflets (Fig. 1A): LCC (left coronary cusp), RCC (right coronary cusp), NCC (non-coronary cusp). The pulmonary leaflets were identified as follows: PC (posterior cusp) the cusp positioned near and opposite to the aortic LCC and, anticlockwise from PC, RAC (right anterior cusp) and LAC (left anterior cusp).

According to the paper of Weind et al. [22], we also investigated whether the distribution of endothelial cells related to the valve microvasculature might contribute to the density distribution of alpha-Gal epitopes. Thus, each Nt and Dc leaflet was divided into four regions, named BASE, DX, CE and SX, as shown in Fig. 1B. In order to allow for different weights, thicknesses and leaflet surface areas from different sectors, the amount of alpha-Gal antigen was expressed as number of epitopes/10 mg wet weight, total number/whole leaflet (absolute amount) and number/mm^2 surface area, when appropriate (Table 1). Surface area was determined on digitalized images by "Image J ver. 1.42I" (Wayne Rasband, National Institutes of Health, USA) software.

2.2. Decellularization procedure

Decellularization was carried out as previously described [19]. Briefly, samples were extracted for 8 h with 1% (w/v) Triton X-100 (Sigma, St. Louis, MO) after treatment with protease inhibitors at 4 °C in hypotonic conditions. After a further 8 h extraction samples were treated for two 8 h periods in phosphate-buffered saline (PBS) containing 0.5 M NaCl at 4 °C. Then Triton X-100 was replaced by 10 mM sodium cholate in hypotonic PBS and extraction was resumed for two 8 h periods at room temperature. All samples were washed with saline and 10% (v/v) isopropanol (Sigma), and equilibrated in isotonic PBS. All extraction procedures were carried out in the presence of 0.04% NaN_3 (Sigma) in fully degassed, sterile solutions (0.22 μm filter; Millipore) with 10 mM sodium ascorbate under a nitrogen atmosphere [19].

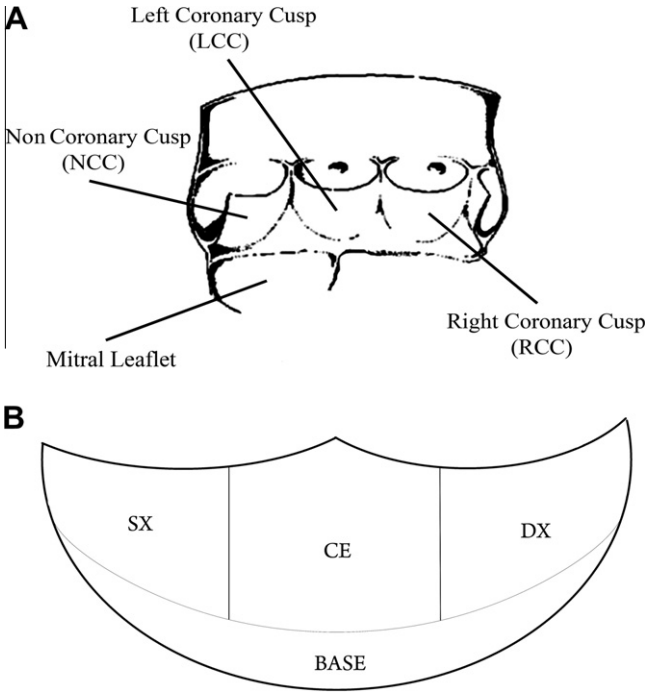

Fig. 1. (A) Image of the internal aspect of the aortic root, showing the nomenclature assigned to the leaflets. The pulmonary leaflets (not shown in the figure) were identified as follows: PC (posterior cusp) positioned near and opposite to the aortic LCC and, in an anticlockwise direction from PC, RAC (right anterior cusp) and LAC (left anterior cusp). (B) Four assigned regions of the leaflet, called BASE, DX, CE and SX. The upward surface of the leaflet is the fibrosa layer.

2.3. Alpha-Gal ELISA test sample processing

Each leaflet was gently blotted with filter paper (Whatman filter paper No. 3), divided into different zones (Fig. 1B) and the weight determined. In order to make the epitopes available, each sample was minced, digested (30 mg wet weight/2 ml) at 60 °C for 15 min with papain (2.8 mg/ml) (Sigma) in PBS and the enzyme inactivated by subsequent heating for 5 min at 100 °C. The sample was then incubated with the primary antibody M86 [1:50] (Axxora, Notthingham, UK) diluted in PBS, for 2 h at 37 °C (determined after testing different incubation times, to optimize conditions without reaching the saturation level), with gentle stirring and finally centrifuged in a Minispin Plus centrifuge (Eppendorf) at 14,340 g for 30 min at 4 °C.

2.4. Alpha-Gal ELISA test

A solution of alpha-Gal/bovine serum albumin (BSA) in PBS at 5 μg/ml (7.6 sugar residue per each BSA molecule; Dextra Laboratories, UK) was used to coat a Polisorp 96 well plate (Nunc, Rochester, NY) with 50 μl per well, incubating for 2 h at 37 °C. After three washes with PBS (250 μl/well per wash, 5 min for the first and 3 min for the other two, taking care to avoid air bubble formation) the blocking procedure was performed using 250 μl per well of 1% human serum albumin (Sigma) in PBS for 2 h at room temperature in darkness; then wells were washed three times as above.

Erythrocytes from whole blood (Giant White rabbit) were suspended in cold isotonic saline, counted in a Burker chamber, aliquoted and stored at -20 °C. A calibration curve was produced