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In vivo xenogeneic scaffold fate is determined by residual antigenicity and extracellular matrix preservation

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ABSTRACT

The immunological potential of animal-derived tissues and organs is the critical hurdle to increasing their clinical implementation. Glutaraldehyde-fixation cross-links proteins in xenogeneic tissues (e.g., bovine pericardium) to delay immune rejection, but also compromises the regenerative potential of the resultant biomaterial. Unfixed xenogeneic biomaterials in which xenoantigenicity has been ameliorated and native extracellular matrix (ECM) architecture has been maintained have the potential to overcome limitations of current clinically utilized glutaraldehyde-fixed biomaterials. The objective of this work was to determine how residual antigenicity and ECM architecture preservation modulate recipient immune and regenerative responses towards unfixed bovine pericardium (BP) ECM scaffolds. Disruption of ECM architecture during scaffold generation, with either SDS-decellularization or glutaraldehyde-fixation, stimulated recipient foreign body response and resultant fibrotic encapsulation following leporine subpannicular implantation. Conversely, BP scaffolds subjected to stepwise removal of hydrophilic and lipophilic antigens using amidosulfobetaine-14 (ASB-14) maintained native ECM architecture and thereby avoided fibrotic encapsulation. Removal of hydrophilic and lipophilic antigens significantly decreased local and systemic graft-specific, adaptive immune responses and subsequent calcification of BP scaffolds compared to scaffolds undergoing hydrophile removal only. Critically, removal of antigenic components and preservation of ECM architecture with ASB-14 promoted full-thickness recipient non-immune cellular repopulation of the BP scaffold. Further, unlike clinically utilized fixed BP, ASB-14-treated scaffolds fostered rapid intimal and medial vessel wall regeneration in a porcine carotid patch angioplasty model. This work highlights the importance of residual antigenicity and ECM architecture preservation in modulating recipient immune and regenerative responses towards xenogeneic biomaterial generation.

1. Introduction

Xenogeneic tissues have found clinical utility as surgical patches in various sites of the body. For instance, towards vascular repair, glutaraldehyde-fixed bovine pericardium (BP) patches are advantageous due to ready availability, decreased suture line bleeding, durability, and strength [1,2]. Further, ability for immediate insonation and reduced restenosis risk motivates use of BP over synthetic patches [2]. Unfortunately, current glutaraldehyde-fixation processes employed to increase BP immune-compatibility for clinical applications limit recipient cellular ingrowth and subsequent extracellular matrix (ECM) remodeling in response to the dynamic physiological environment [3–5]. With limited ability to adaptively remodel [5], these non-viable biomaterials are associated with risk for either aneurysmal dilation or stenosis [1]. By avoiding fixation, ECM-based biomaterials derived from xenogeneic tissues may retain capacity for cellular repopulation and regenerative processes.

Xenoantigenicity represents the major barrier to immune-compatibility (i.e., avoidance of destructive, recipient, graft-specific immune responses) and expanded use of unfixed xenogeneic biomaterials in clinical practice. Galactose-α(1,3)-galactose (α-gal) and major histocompatibility complex class I (MHC I) are two xenoantigens known to elicit immune rejection upon implantation [6,7]. The carbohydrate moiety α-gal is ubiquitous in non-primate mammals and New World Monkeys; absence of α-gal in humans

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and Old World Monkeys results in constitutive production of the corresponding xenoreactive anti-Gal antibody [8,9]. Consequently, α-gal is the primary mediator of hyperacute rejection in discordant xenotransplantation [7]. Present on the surface of all nucleated cells, MHC I molecules elicit both innate and adaptive xenogeneic immune responses [6,7]. Recognition and presentation of non-self, major or minor histocompatibility antigens to T-cells activates the adaptive immune response [10]. Resultant cytokine secretion promotes recruitment and activation of additional innate and adaptive immune cells for graft-specific, cell-mediated immune rejection [7]. Concurrently, recognition and presentation of non-self antigens to helper T-cells by B-cells initiates B-cell maturation, isotype switching, and antibody secretion [10]. Local lymphoid follicle formation creates a site for new graft-specific lymphocyte development. These immunological challenges motivate efforts to achieve xenoantigen removal from tissues and organs, to yield intact ECM to serve as a scaffold for tissue engineering and regenerative medicine applications. Non-fixation approaches to reduce ECM scaffold xenoantigenicity include decellularization [11,12], targeted removal of known antigens [13–16], and physiochemical property-based removal of unknown antigens [17,18]. Stepwise, solubilization-based removal of xenoantigens from BP, using dithiothreitol and KCl for hydrophiles, followed by amidosulfobetaine-14 (ASB-14) for lipophiles, significantly reduces residual hydrophilic [17] and lipophilic [18] antigens, including detectable α-gal and MHC I in the resultant scaffold. However, the extent to which persistent hydrophilic and lipophilic antigens in xenogeneic scaffolds modulate recipient adaptive immune responses *in vivo* is currently unknown.

Maintenance of native ECM architecture to promote recipient recognition of the scaffolds as self in origin represents a second critical hurdle to generation of regenerative xenogeneic biomaterials. Fibrous encapsulation of synthetic implants is attributed to innate cells being unable to recognize the implant as self [19,20]. Cells of the innate immune system perpetually survey tissues for foreign substances and have been linked to cell-mediated xenograft rejection [7]. Persistence of foreign (i.e., non-self) material stimulates a foreign body response, characterized by giant cell formation and graft fibrous encapsulation [19]. Following isolation from the rest of the body by formation of a fibrous capsule [21], the graft is rendered non-viable [22] and subsequently unable to undergo regenerative processes. Through preservation of native ECM architecture (e.g., morphology, collagen alignment, and thermal stability), it is possible that innate cells probing the xenogeneic scaffold may identify the implant as self. In turn, foreign body response and subsequent fibrous encapsulation of the graft may be avoided. However, the degree to which alterations in ECM architecture of xenogeneic scaffolds affect *in vivo* innate and regenerative responses has not been previously been investigated.

In this study, we determined the ability of various levels of residual antigenicity and ECM preservation to modulate beneficial and deleterious *in vivo* adaptive, innate, and regenerative responses towards BP-based biomaterials.

2. Materials and methods

Detailed Materials and Methods can be found as Supplementary Information. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

2.1. Tissue harvest

Frozen bovine pericardium from adult cattle (Spear Products) was thawed and placed into PBS (pH 7.4) with 0.1% (w/v) EDTA and 1% (v/v) Antibiotic Antimycotic Solution (AAS). Pericardial fat and loose connective tissue was removed. Circumferentially trimmed strips were stored in Dulbecco's Modified Eagles Medium (DMEM) containing 15% (v/v) dimethyl sulfoxide (DMSO) at −80 °C.

2.2. Sample preparation

BP was subjected to antigen removal 4 °C and 125 rpm unless otherwise stated, as previously described [17,18]. For *in vitro* characterization and rabbit implantation, sets of four anatomically-adjacent BP pieces (1.0 cm × 1.5 cm) were generated: one piece for native control and one for each of three treatments (Table 1): (i) hydrophilic antigen removal using optimized solubilizing antigen removal buffer (opt SARB; 10 mM Tris-HCl, pH 8.0 with 100 mM dithiothreitol, 100 mM KCl, 2 mM $MgCl_2$–$6H_2O$, 0.5 mM Pefabloc SC (Roche), 1% (v/v) AAS) only for 96 h; (ii) hydrophilic antigen removal using opt SARB for 48 h, followed by lipophilic antigen removal using 1% (w/v) ASB-14 in opt SARB at room temperature for 48 h; or (iii) decellularization using 0.1% (w/v) SDS (Bio-Rad) in basic antigen removal buffer (BARB; 10 mM Tris-HCl, pH 8.0 with 0.5 mM Pefabloc SC, 1% (v/v) AAS) for 48 h, followed by 1% (w/v) SDS in BARB at room temperature for 48 h. Antigen removed or decellularized BP then underwent nucleic acid digestion (10 mM Tris-HCl, pH 7.6 with 2.5 Kunitz units/mL DNase I, 7.5 Kunitz units/mL RNase A, 0.15 M NaCl, 2 mM $MgCl_2$–$6H_2O$, and 1% (v/v) AAS in) for 24 h, and washout (20 mM Tris-buffered saline, pH 7.5 with 0.5 mM Pefabloc SC and 1% (v/v) AAS) for 48 h. For pig implantation, BP pieces (2.0 cm × 1.5 cm) were subjected to antigen removal using either opt SARB only or opt SARB, followed by ASB-14.

2.3. Western blot

One-dimensional electrophoresis and Western blot was performed using equal volumes of residual hydrophilic or lipophilic BP protein extract ($n = 6$ per group), as previously described [17,18]. To assess persistence of known xenoantigens, blots were probed with either anti-Galα1-3Galβ1-(3)4GlcNAc-R) (M86; Enzo Life Sciences) or anti-bovine MHC I (IL-A88; Bio-Rad AbD Serotec).

2.4. Scanning electron microscopy

Two 5 mm diameter discs from each scaffold ($n = 3$ per group) were mounted with opposite sides facing up and analyzed on a scanning electron microscope (Philips XL30 TMP; FEI Company).

2.5. Differential scanning calorimetry

ECM thermal stability was determined using an adaptation of a method previously described [23]. Briefly, 5 mm diameter discs (punched from an initial 0.2 g piece, $n = 6$ per group) were lyophilized (0.8–5.7 mg in dry weight), crimped in aluminum pans, and heated (30–280 °C at 20 °C/min and 10 min hold at 120 °C) using a differential scanning calorimeter (DSC 6000; Perkin Elmer, Waltham, MA). Denaturation temperature was determined with Pyris software (version 11.1.1.0497, Perkin Elmer).

Table 1
Unfixed BP scaffold treatments.

Sample	Hydrophile solubilization	Lipophile solubilization
Native BP	–	–
opt SARB	opt SARB	–
ASB-14	opt SARB	1% (w/v) ASB-14 in opt SARB
SDS	0.1% (w/v) SDS in BARB	1% (w/v) SDS in BARB

Scaffolds were generated using hydrophile solubilization only (opt SARB), stepwise hydrophile and lipophile solubilization (ASB-14), or SDS-decellularization (SDS).