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ORIGINAL ARTICLE

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Differences in xenoreactive immune response and patterns of calcification of porcine and bovine tissues in α-Gal knock-out and wild-type mouse implantation models

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Abstract

OBJECTIVES: Although bioprostheses are widely used in cardiovascular surgery, their durability is limited due to degeneration. Degeneration of bioprostheses limiting its clinical use results from multiple factors, and immune reaction has been considered to be one of the most important factors. The study objectives were to compare the mechanical characteristic differences of porcine and bovine prostheses, assess the differences in immune reaction among different species and tissues as well as elucidate bioprosthetic failure patterns in α-Gal knock-out (KO) and wild-type mouse implantation models.

METHODS: Six groups of different xenogeneic tissues (porcine pericardium, aortic valve, aortic wall; bovine pericardium, aortic valve and aortic wall) were implanted into the subcutaneous tissue of the wild-type mouse ($n = 4$) and the KO mouse ($n = 4$) (four xenogeneic tissue segments per each mouse). Mechanical and chemical tests, including tensile strength measurement and thermal stability test for pericardial tissues and pronase test for different xenogeneic tissues, were performed before implantation. Anti-α-Gal antibody titres (IgM and IgG antibodies) were measured using serum enzyme-linked immunosorbent assay analyses before implantation and 30, 60 and 90 days after implantation. Implanted tissues were harvested after 90 days and studied for histopathology and quantification of calcification.

RESULTS: There were no significant differences in tensile strength and shrinkage temperature between the porcine and bovine pericardia, although the bovine pericardia showed a greater elasticity than the porcine pericardia (elongation at tensile strength, 74.8 ± 4.5% vs 50.0 ± 8.7%, $P < 0.001$). Resistance towards pronase degradation was not different among the groups of tissues (Groups 1–6, 89.1 ± 7.6, 95.1 ± 1.8, 90.3 ± 5.3, 93.7 ± 3.3, 89.1 ± 2.4 and 89.1 ± 3.0%, respectively; $P = 0.061$). The IgM titres of the α-Gal KO mice were significantly higher at 30 days after implantation (0.71 ± 0.27 vs 1.07 ± 0.48, $P = 0.004$), whereas the IgG titres of the α-Gal KO mice remained higher until 60 days after implantation (at 30 days, 0.81 ± 0.07 vs 1.28 ± 0.79, $P = 0.017$; at 60 days, 0.54 ± 0.16 vs 1.43 ± 1.10, $P = 0.045$) than those of the wild-type mice. Calcium levels of tissues implanted into the α-Gal KO mice were significantly higher than those implanted into the wild-type mice regardless of tissue type (from Groups 1–6, 4.72 ± 1.75 vs 27.76 ± 22.73 μg/mg; 3.05 ± 1.04 vs 15.90 ± 6.98 μg/mg; 2.13 ± 1.48 vs 29.76 ± 30.71 μg/mg; 1.02 ± 0.53 vs 5.97 ± 1.40 μg/mg; 3.18 ± 3.41 vs 30.55 ± 66.69 μg/mg; 6.21 ± 5.56 vs 21.65 ± 17.77 μg/mg, all $P \leq 0.002$).

CONCLUSIONS: Chronic immune response to the α-Gal antigen may cause more severe tissue calcification in α-Gal KO mice. Removal of α-Gal antigenicity is strongly advised in xenogeneic bioprosthetic tissue implantation.

Keywords: Bioprosthesis • Xenotransplantation • Immune response

INTRODUCTION

Bioprostheses made of bovine and porcine tissues are widely used during cardiovascular surgery. However, the durability of bioprostheses is limited due to tissue degeneration resulting from multiple factors including immunological, chemical, mechanical and surgical factors [1]. The exact mechanisms of bioprosthetic degeneration are not fully understood yet. Even the same biological heart valve may have different structural deteriorations according to the implanted position. The mitral bioprostheses have a tendency to degenerate earlier than the aortic bioprostheses because the mitral valve has to withstand the systolic pressure of left ventricle. Immune reaction has also been considered to be an important factor, and the xenoreactive Galα1,3-Galβ1–4GlcNAc-R (α-Gal) epitope has been regarded as one of the major antigens [2]. The α-Gal epitope exists as a cell surface molecule in most

species, except humans and old world monkeys [3]. Immune reactions between the epitope on bioprosthesis and antibodies against the epitope in humans could play a major role in immune-mediated tissue degeneration and dystrophic calcification. Various bioprostheses including bovine or porcine pericardium and porcine heart valves have individual composition, function and morphological characteristics [4, 5]. However, differences in immune reactions among different species and tissues have not been studied previously.

The aims of this study were (i) to compare the mechanical characteristic differences of porcine and bovine prostheses, (ii) to assess the differences in immune reaction among different species and tissues and (iii) to elucidate bioprosthetic failure patterns in α-Gal knock-out (KO) and wild-type mouse implantation models.

MATERIALS AND METHODS

Tissue preparation

Fresh bovine and porcine tissues (pericardium, aortic valve and aortic wall) were obtained from the local slaughterhouse, placed in cold phosphate-buffered saline (PBS, 0.1 M, pH 7.40) and immediately transported to the laboratory. On arrival, the tissues were rinsed with normal saline and freed from adherent fat and areolar tissue. The tissues were fixed in PBS-buffered 0.5% glutaraldehyde (GA) solution for 3 days, and then fixed in 0.25% GA solution for 7 additional days. All the treatments were done at room temperature. After the fixation process, the tissues were stored in 4% propylene oxide solution before use. The tissue preparation methods were similar to those used commercially, which included GA and ethanol treatment. In addition, this study elucidated the role of immune reaction by implanting the tissues into the α-Gal KO mice.

Experimental design

Xenogeneic tissues were divided into six groups according to the species and types of tissue: Groups 1 (porcine pericardium), 2 (porcine aortic valve), 3 (porcine aortic wall), 4 (bovine pericardium), 5 (bovine aortic valve) and 6 (bovine aortic wall). Four segments of 6 × 6 mm circular tissues from each group were implanted into subcutaneous pouches created in the dorsal area of the wild-type ($n = 4$) and α-Gal KO ($n = 4$) mice, and the wound was closed with 4-0 Nylon sutures. Therefore, each group included 32 segments of implanted tissues, and a total of 48 mice (C57BL/6, 3 weeks old, weighing approximately 20 g; 24 α-Gal KO mice and 24 wild-type mice) were used. The implanted tissues were harvested 90 days after implantation, and differences in immune reaction and calcification patterns were examined. Blood samples for immune response analysis were obtained from each mouse before implantation and 30, 60 and 90 days after implantation.

All animals were treated under the Guide for the Care and Use of Laboratory Animals (National Academy of Science, Washington, DC, USA). This study was approved by the Institutional Animal Care and Use Committee of Clinical Research Institute, Seoul National University Hospital (IACUC No. 12-0185). This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Mechanical and chemical tests for the glutaraldehyde-treated porcine and bovine tissues before implantation

Mechanical test. Uniaxial testing was done to compare the mechanical properties of the tissues. Tissue strips (5 × 50 mm, 12 porcine and 12 bovine pericardial strips) were prepared. Thickness of the tissue was measured at the centre of the tissue strip using a thickness gauge (SM-112, TECLOCK, Japan). Tensile properties were examined using a tensile testing machine (K-ML-1000N, M-TEC, Republic of Korea), which is equipped with a digital force gauge (DS2–200N, IMADA, Japan). The digital force gauge had an extension rate of 100 mm/min, and a preload of 0.0 N. Ultimate strength and strain at fracture were calculated from the recorded stress–strain curves.

Thermal stability test. Shrinkage temperatures, which reflect the degree of fixation (cross-linking) of the tissues, were measured by using a custom-built extensometer. Tissue strips (8 × 30 mm; five porcine and five bovine pericardial strips) were loaded to 95 g weight, which was held along the long axis, and placed in a water bath. The temperature of the water was increased by ∼5°C/min, and at the same time, the width of the tissue strip was measured with a microscope. By plotting the width against the temperature curve, we identified the sharp deflection point at which shrinkage occurred (shrinkage temperature).

Mechanical and thermal stability tests were only performed in porcine and bovine pericardial tissues because the tests required rather long tissue segments (5 × 50 mm for the mechanical test, and 8 × 30 mm for the thermal stability test) for accurate measurement.

Pronase test. Tissue stability was evaluated by measuring the resistance to enzymatic degradation using protease (Pronase, Roche, Germany). Tissue samples (10 × 10 mm; five tissue samples for each group) were weighed and initially soaked in a HEPES solution (10 mM, pH 7.4) containing glycine (0.1 M) for 24 h at 50°C. Samples were then transferred to a solution of the same buffer containing the enzyme at a concentration of 0.5 mg/ml. To assure optimal enzymatic activity of the solution, the enzyme was added along with $CaCl_2$ (0.01 M). Samples were incubated for 24 h at 50°C, washed and freeze-dried. After renewed determination of the dry weight, the actual weight loss due to enzymatic degradation was calculated.

Histological study

Tissue samples harvested after implantation were examined with light microscopy. Harvested tissue samples were fixed in 10% formalin and were embedded in paraffin wax. Those were sliced into 6 μm thick sections and then stained with haematoxylin–eosin (H&E) and von Kossa stains. The von Kossa stain was additionally performed, because it is a useful analytic method to observe calcifications. The structure of collagen fibres, degree of inflammatory cell infiltrations and calcification were examined.

Immunohistochemisty

Harvested tissue samples were stained for mouse macrophages and T-cells. The primary antibodies used were anti-mouse F4/80

antigen (eBioscience, San Diego, CA, USA; a marker for mouse macrophages) at 1 : 300 dilution or anti-mouse CD4 (eBioscience; a marker for mouse T-cells) at 1 : 300 dilution. The horseradish peroxidase-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Grove, PA, USA) was used as a secondary antibody with 1 : 500 dilution. Diaminobenzidine was used as a chromogen and haematoxylin was used for counterstaining.

Enzyme-linked immunosorbent assay

Anti-α-Gal serum IgM and IgG antibodies in α-Gal KO and wild-type mice were measured before implantation and 30, 60 and 90 days after implantation by enzyme-linked immunosorbent assay (ELISA). Blood samples of 0.2–0.4 ml per mouse (four mice per group) were collected from the infraorbital venous plexus. Synthetic α-Gal epitopes linked to bovine serum albumin (α-Gal BSA; Dextra, Reading, UK) were used as solid-phase antigens. Diluted serum samples (1 : 10) were measured. The secondary antibodies used were horseradish peroxidase-conjugated rabbit anti-mouse IgM (Jackson ImmunoResearch) or goat anti-mouse IgG (Jackson ImmunoResearch) at 1 : 10 000 dilution to detect anti α-Gal serum IgM and IgG antibodies, respectively. The colour reaction was developed with tetramethylbenzidine solution (BD Biosciences, San Diego, CA, USA), and absorbance was measured in an ELISA reader at 450 nm. The titre of anti-α-Gal antibody was defined as the reciprocal of serum dilution, which yields 1.0 optical density.

Calcium analysis

After harvesting the tissue samples (32 samples for each group), those were washed with normal saline and dried at 70°C for 24 h. Samples were then hydrolyzed using 5.0 N HCl solution. Calcium content of the hydrolysate was analysed using an automatic chemistry analyser (Hitachi 7070, Japan) and expressed as μg/mg dry weight.

Statistical analysis

Statistical analysis was performed with the SPSS software package (version 20.0; SPSS, Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation. A *P*-value of <0.05 was considered statistically significant. Each group consisted of four mice. This was the smallest number of sample size for adequate statistical analysis. For antibody measurements, the data of porcine and bovine tissues were combined according to the tissue type to overcome this limitation. For tissue studies, however, each group included 32 segments of implanted tissue because four segments of tissue were implanted into each mouse. Comparisons between groups were performed using the Mann–Whitney *U* and Kruskal–Wallis tests. Wilcoxon signed-rank test and repeated-measures analysis of variance were used to assess the significance of differences at each time interval within the groups and between groups.

RESULTS

Mechanical and chemical tests for porcine and bovine tissues before implantation

Mechanical test. There was no significant difference in tensile strength between the porcine and bovine pericardia (10.8 ± 2.7 vs 13.8 ± 5.2 MPa, $P = 0.158$). However, mean elongation at break was significantly smaller in the porcine pericardium than in the bovine pericardium (50.0 ± 8.7% vs 74.8 ± 4.5%, $P < 0.001$; Table 1).

Thermal stability test. There was no significant difference in shrinkage temperature between porcine and bovine pericardia (82.3 ± 0.8 vs 82.6 ± 0.1°C, $P = 0.310$).

Pronase test. Resistance towards pronase degradation in Groups 1–6 was 89.1 ± 7.6%, 95.1 ± 1.8%, 90.3 ± 5.3%, 93.7 ± 3.3%, 89.1 ± 2.4% and 89.1 ± 3.0%, respectively. There was no significant difference in resistance towards pronase degradation among the groups of tissues ($P = 0.061$).

Histological study

H–E staining showed inflammatory cellular infiltrates disrupting collagen fibres 90 days after implantation in all groups. The von Kossa staining revealed heavy calcific deposits, regardless of species or tissue type. The tissues implanted into the α-Gal KO mice generally showed more severe calcification than those implanted into the wild-type mice (Fig. 1).

Immunohistochemistry

F4/80 staining showed macrophages that were in close contact with implanted tissues in all groups, irrespective of the mouse type. A thick layer of non-stained inflammatory cells was observed in the outer surface of macrophages in the tissues implanted into the α-Gal KO mice, whereas few non-stained inflammatory cells were observed in the tissues implanted into the wild-type mice. CD4 staining revealed T-cells surrounding the tissues implanted into the α-Gal KO mice; however, few T-cells were observed in the tissues implanted into the wild-type mice. Aortic wall tissues showed more inflammatory cells than other tissues, regardless of the type of staining or mouse type (Fig. 2).

Enzyme-linked immunosorbent assay

The titres of anti-α-Gal IgM and IgG antibodies in both the wild-type and α-Gal KO mice increased early after implantation in all groups of tissues (Table 2). The IgM antibody titres of the α-Gal KO mice tended to be higher until 30 days after implantation, and IgG titres of the α-Gal KO mice tended to be higher until 60 days after implantation. Anti-α-Gal IgM and IgG titres of the wild-type mice tended to be higher than those of pre-implantation until 90 days after implantation. When the data of antibody titres were combined regardless of tissue type and species, this tendency was clearer (Table 3). The antibody titres of the α-Gal KO mice were increased higher than those of the wild-type mice (wild-type vs α-Gal KO mice; IgM titres at 30 days, 0.71 ± 0.27 vs 1.07 ± 0.48, $P = 0.004$; IgG titres at 30 days, 0.81 ± 0.07 vs 1.28 ± 0.79, $P = 0.017$; IgG titres at 60 days, 0.54 ± 0.16 vs 1.43 ± 1.10, $P = 0.045$). In the α-Gal KO mice, the IgM titres were increased until 30 days after implantation, whereas the IgG titres were increased until 60 days after implantation. The anti-α-Gal IgM antibody titre increments of the α-Gal KO mice according to time sequence were significantly larger than those of the wild-type mice until 30 days after implantation ($P = 0.010$). Anti-α-Gal IgG antibody titre increments of the α-Gal KO mice according to time sequence were

Table 1: Tensile strength and elongation at tensile strength of porcine and bovine pericardia

	N	Thickness (mm)	Peak load (*N*)	Tensile strength (Mpa)	Elongation at tensile strength (%)
Porcine pericardium	12	0.19 ± 0.04	9.65 ± 1.90	10.8 ± 2.7	50.0 ± 8.7
Bovine pericardium	12	0.55 ± 0.07	37.49 ± 14.25	13.8 ± 5.2	74.8 ± 4.5
P-value	–	<0.001	<0.001	0.158	<0.001

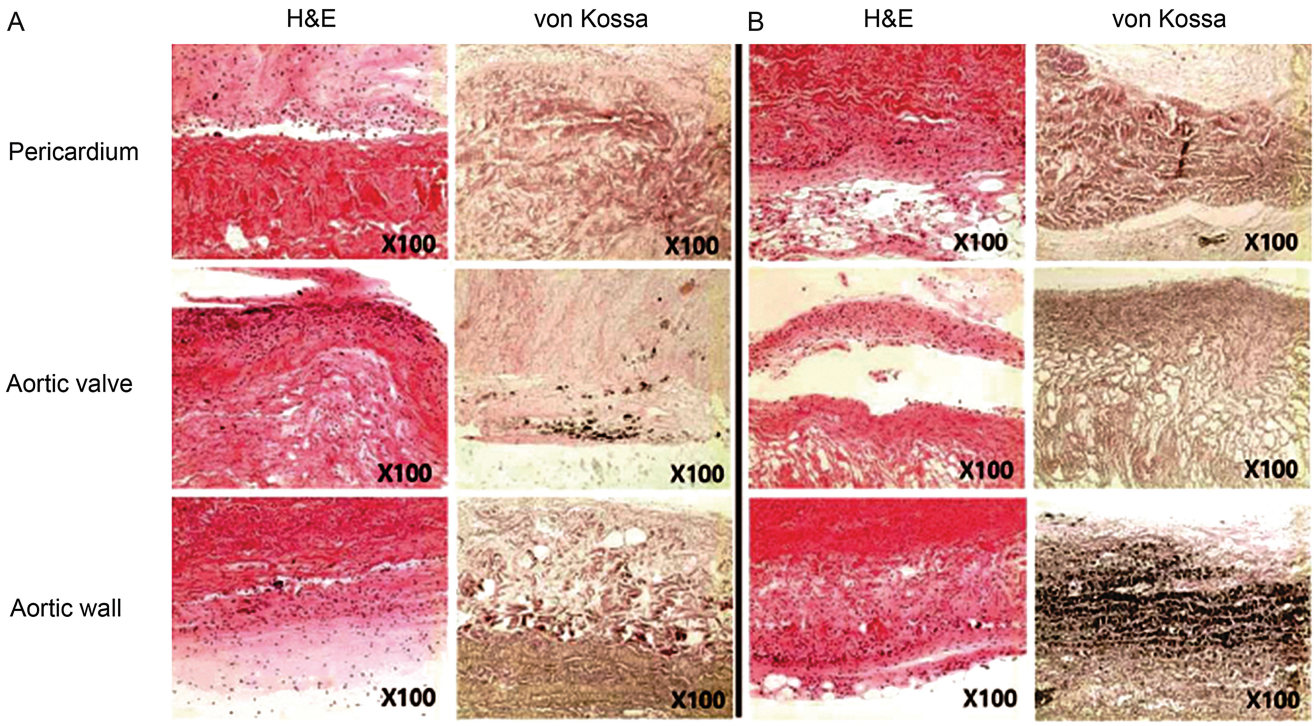

Figure 1: Light microscopic findings of harvested tissues from the wild-type (**A**) and α-Gal KO mice (**B**). Same tissue types showed similar microscopic features regardless of species. All tissues underwent H&E and von Kossa staining (×100). H&E staining revealed extensive inflammatory cellular infiltrates disrupting collagen fibres in all tissues irrespective of the mouse type. von Kossa staining demonstrated more calcific deposits in the α-Gal KO mice than in the wild-type mice. H&E: haematoxylin–eosin; KO: knock-out.

significantly larger than those of the wild-type mice until 60 days after implantation ($P = 0.002$).

Calcium analysis

Calcium contents were significantly higher in all groups of tissues implanted into the α-Gal KO mice than the wild-type mice (Table 4).

DISCUSSION

The present study revealed three main findings. First, there were no significant differences in mechanical and chemical properties between the GA-treated porcine and bovine pericardia, although the bovine pericardia showed greater elasticity than the porcine. Secondly, IgM titres of the α-Gal KO mice were significantly higher than those in the wild-type mice at 30 days after implantation, whereas the IgG titres of the α-Gal KO mice continued to be higher until 60 days after implantation. Thirdly, calcium contents of the tissues implanted into the α-Gal KO mice were significantly higher than those implanted into the wild-type mice regardless of tissue type.

GA-fixed bioprostheses made of porcine or bovine tissue are widely used in cardiovascular surgery, because the mechanical properties of porcine and bovine pericardia have been shown to have no significant differences [6]. Of variables reflecting characteristics of bioprosthetic tissues such as tensile strength, thickness and elongation at tensile strength, tensile strength is the representative mechanical property. The present study also showed a similarity in tensile strength between the porcine and bovine pericardia.

In the present study, GA-fixed porcine and bovine pericardia showed no significant differences in mechanical and chemical properties, although bovine pericardium was more elastic than porcine pericardium. The difference in elasticity may be related to differences in their morphological characteristics. In manufacturing the bioprosthetic valve, naturally grown porcine heart valves and pericardial-based bovine heart valves have been used.

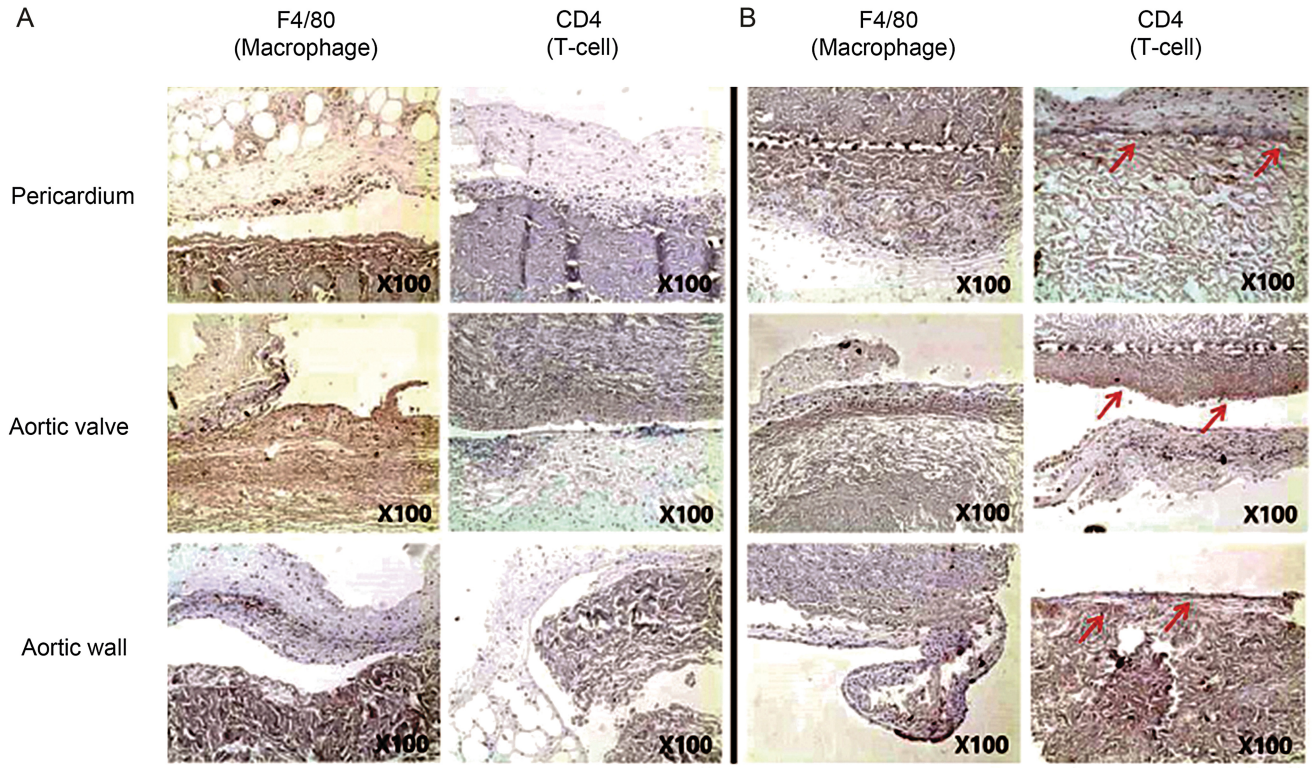

Figure 2: Immunohistochemical study of harvested tissues from the wild-type (**A**) and α-Gal KO mice (**B**). Same tissue types showed similar microscopic features regardless of species. All tissues underwent macrophage and T-cell staining (×100). F4/80 staining showed macrophages in close contact with tissues irrespective of the mouse type. CD4 staining revealed T-cells (red arrows) encircling tissues implanted into the α-Gal KO mice. However, few T-cells were observed in tissues implanted into the wild-type mice.

Although bioprostheses have the clinical advantage of not requiring lifelong anticoagulation, they ultimately fail due to dystrophic calcification. In order to reduce calcification, several methods have been investigated including detoxification with amino acid post-fixation, decellularization with surfactants, removal of phospholipids with organic solvents and fixation with cross-linking agents other than GA [7]. Although the mechanisms leading to degradation of the implanted bioprosthesis are not yet fully understood, xenoreactive immune response has been regarded as playing an important role in the degeneration process. Xenoantigen may initiate a cascade of immune responses because bioprosthesis implantation is a form of xenotransplantation. Recently, xenoreactive Galα1,3-Galβ1–4GlcNAc-R (α-Gal) epitopes and anti-α-Gal antibodies have been found to be important factors in tissue valve failure [2, 8, 9].

The mouse subcutaneous implantation model has been widely used for screening the efficacy of anticalcification treatments in a short period. However, implanting porcine or bovine tissues into mice is a type of concordant xenotransplantation because both donor and recipient species have α-Gal epitopes, and will not provoke an anti-α-Gal immune response. Humans do not express the α-Gal epitope and human serum contains high titres of antibodies (IgG and IgM) against the α-Gal epitope, probably due to continual exposure after birth to intestinal bacterial flora that have α-Gal epitopes [10]. To simulate immune response against xenoantigen in humans, genetically manipulated α-Gal KO mice lacking the α-Gal epitope and containing high titres of antibodies against the α-Gal epitope were used in previous studies [10–12]. In those studies, the α-Gal KO mice were used to mimic the human immunological environment. Therefore, the findings of this study could be extrapolated to humans.

In the present study, titres of anti-α-Gal IgM and IgG antibodies in the α-Gal KO mice increased early after implantation of all tissue types. However, statistical significance was not reached in most of the subgroups, probably because of the small number of samples in each group. Because the mechanical and chemical properties between the GA-fixed porcine and bovine tissues showed similarity, we combined the ELISA results of porcine and bovine tissues on the assumption that bioprosthetic tissues treated with GA may produce similar immune reaction. Theoretically, anti-α-Gal antibodies should not be detected in the wild-type mice. Increased titres of anti-α-Gal IgM and IgG antibodies in the wild-type mice in the present study may be due to non-specific binding of mouse antibodies other than anti-α-Gal antibodies or due to differences in the fine specificity of natural anti-α-Gal antibodies recognizing various 'facets' of an α-Gal epitope in its 3D form [13, 14]. The increment in anti-α-Gal IgM antibody titres was significantly larger until 30 days after implantation, and the increment in anti-α-Gal IgG antibody titres was significantly larger until 60 days after implantation in the α-Gal KO mice than in the wild-type mice. IgM antibodies represent a marker for acute humoral immune response and IgG antibodies represent a marker for chronic humoral immune response. Our results suggest that more chronic humoral immune response took place in the α-Gal KO mice than in the wild-type mice. Immunohistochemistry of the harvested tissues revealed macrophages to be in close contact with the tissues irrespective of the mouse type. In addition, T-cells were found in the tissues implanted into the α-Gal KO mice,

Table 2: Titres of anti-α-Gal IgM antibodies according to the tissue type implanted and mouse type at pre-implantation and 30, 60 and 90 days after implantation and of anti-α-Gal IgG antibodies according to the tissue type implanted and mouse type at pre-implantation and 30, 60 and 90 days after implantation

Tissues	Mouse type	*P*-value	Pre-implantation	30 days	60 days	90 days
Titres of anti-α-Gal IgM						
Pericardium	Wild-type		0.57 ± 0.22	0.72 ± 0.25	0.56 ± 0.32	0.78 ± 0.28
		P		*0.263*	*0.889*	*0.012*
	α-Gal KO		0.34 ± 0.25	0.98 ± 0.51	0.37 ± 0.29	0.59 ± 0.34
		P		*0.025*	*0.889*	*0.069*
		**P*	*0.065*	*0.328*	*0.195*	*0.279*
Aortic valve	Wild-type		0.39 ± 0.27	0.57 ± 0.34	0.57 ± 0.27	0.57 ± 0.27
		P		*0.327*	*0.161*	*0.093*
	α-Gal KO		0.77 ± 0.21	1.32 ± 0.48	0.35 ± 0.26	0.47 ± 0.27
		P		*0.050*	*0.017*	*0.093*
		**P*	*0.010*	*0.007*	*0.065*	*0.645*
Aortic wall	Wild-type		0.48 ± 0.30	0.84 ± 0.15	0.55 ± 0.34	0.83 ± 0.15
		P		*0.012*	*0.401*	*0.036*
	α-Gal KO		0.49 ± 0.35	0.92 ± 0.41	0.39 ± 0.35	0.69 ± 0.28
		P		*0.069*	*0.779*	*0.161*
		**P*	*0.878*	*0.505*	*0.574*	*0.382*
Titres of anti-α-Gal IgG						
Pericardium	Wild-type		0.16 ± 0.05	0.81 ± 0.08	0.57 ± 0.09	0.68 ± 0.08
		P		*0.012*	*0.012*	*0.012*
	α-Gal KO		0.05 ± 0.03	1.09 ± 0.47	0.82 ± 0.54	0.11 ± 0.07
		P		*0.012*	*0.012*	*0.050*
		**P*	*<0.001*	*0.130*	*0.959*	*<0.001*
Aortic valve	Wild-type		0.21 ± 0.05	0.82 ± 0.08	0.52 ± 0.11	0.69 ± 0.10
		P		*0.012*	*0.012*	*0.012*
	α-Gal KO		0.06 ± 0.09	0.75 ± 0.78	0.70 ± 0.47	0.08 ± 0.10
		P		*0.036*	*0.012*	*1.000*
		**P*	*0.007*	*0.798*	*0.798*	*<0.001*
Aortic wall	Wild-type		0.12 ± 0.03	0.81 ± 0.06	0.54 ± 0.24	0.68 ± 0.25
		P		*0.012*	*0.017*	*0.012*
	α-Gal KO		0.08 ± 0.04	2.00 ± 0.53	2.76 ± 0.66	0.11 ± 0.11
		P		*0.012*	*0.012*	*0.674*
		**P*	*0.021*	*0.002*	*<0.001*	*0.002*

Italic values represent each *P* values of the data.
**P*-values comparing the antibody titres of post-implantation with those of pre-implantation.
***P*-values comparing antibody titres of the wild-type mice with those of the α-Gal KO mice.

Table 3: Titres of anti-α-Gal IgM antibodies according to the mouse type at pre-implantation and 30, 60 and 90 days after implantation and of anti-α-Gal IgG antibodies according to the tissue type implanted and mouse type at pre-implantation and 30, 60 and 90 days after implantation

Mouse type	*P*-value	Pre-implantation	30 days	60 days	90 days
Titres of anti-α-Gal IgM					
Wild-type		0.48 ± 0.27	0.71 ± 0.27	0.56 ± 0.28	0.73 ± 0.26
	P		*0.004*	*0.253*	*<0.001*
α-Gal KO		0.53 ± 0.32	1.07 ± 0.48	0.37 ± 0.29	0.58 ± 0.30
	P		*0.001*	*0.086*	*0.475*
	**P*	*0.592*	*0.004*	*0.023*	*0.083*
Titres of anti-α-Gal IgG					
Wild-type		0.17 ± 0.06	0.81 ± 0.07	0.54 ± 0.16	0.68 ± 0.15
	P		*<0.001*	*<0.001*	*<0.001*
α-Gal KO		0.07 ± 0.06	1.28 ± 0.79	1.43 ± 1.10	0.10 ± 0.09
	P		*<0.001*	*<0.001*	*0.166*
	**P*	*<0.001*	*0.017*	*0.045*	*<0.001*

Italic values represent each *P* values of the data.
**P*-values comparing the antibody titres of post-implantation with those of pre-implantation.
***P*-values comparing antibody titres of the wild-type mice with those of the α-Gal KO mice.

Table 4: Calcium contents of tissues implanted into the wild-type and α-Gal KO mice

Mouse type	Porcine			Bovine		
	Pericardium	Aortic valve	Aortic wall	Pericardium	Aortic valve	Aortic wall
Wild-type						
N	10	8	15	13	13	12
Calcium (μg/mg)	4.72 ± 1.75	3.05 ± 1.04	2.13 ± 1.48	1.02 ± 0.53	3.18 ± 3.41	6.21 ± 5.56
α-Gal KO						
N	10	13	15	14	13	14
Calcium (μg/mg)	27.76 ± 22.73	15.90 ± 6.98	29.76 ± 30.71	5.97 ± 1.40	30.55 ± 66.69	21.65 ± 17.77
**P*-value	<0.001	<0.001	<0.001	<0.001	<0.001	0.002

**P*-values comparing calcium contents of tissues between the wild-type and α-Gal KO mice.

whereas few T-cells were observed in the tissues implanted into the wild-type mice. It seemed that cellular immune response as well as humoral immune response occurred more actively in the α-Gal KO mice than in the wild-type mice. Calcium levels in the implanted tissues were significantly higher in the α-Gal KO mice than in the wild-type mice regardless of the tissue type. Chronic immune response to the α-Gal antigen may have caused more severe calcification of the tissues in the α-Gal KO mice.

In this study, the calcium content was higher in tissues implanted into the α-Gal KO mice than that implanted into the wild-type mice. In addition, calcification level of porcine aortic valve implanted into the α-Gal KO mice was higher than that of bovine pericardium implanted into the α-Gal KO mice. Therefore, it was suggested that when using porcine aortic valve as a bioprosthesis, more vigorous tissue preparation would be required to decrease immunological tissue deterioration and calcification.

In conclusion, the present study results suggest that α-Gal epitopes play an important role in both the humoral and cellular immune response, and eventually in calcification, of xenogeneic tissues. Removal of α-Gal antigenicity could suppress deterioration and improve durability of bioprostheses. In addition, the choice of a xenogeneic tissue with a low degree of antigenicity and calcification is important in preventing the degenerative failure of bioprostheses.

LIMITATIONS OF THE STUDY

There are limitations to the present study that must be recognized. First, the number of sample sizes was relatively small due to the limited supply of animals, and four segments of tissues were implanted into each mouse to overcome this limitation. This experimental design may cause a bias. Secondly, tissues were treated only with GA, and other anticalcification methods such as decellularization or detoxification were not applied. If additional decellularization, detoxification or treatment with organic solvents was performed, the relationship between α-Gal xenoantigen and immune reaction could be defined more clearly. Thirdly, mechanical and chemical tests were only performed on fixed tissue that was not implanted. Therefore, those test results would reflect the quality of fixation and did not include the effects of immune reaction. Fourthly, the tissues to be implanted were not in direct contact with blood. However, this study was performed in mouse models. In addition, the immune response does not necessarily require direct contact with blood. Our expectation was that subcutaneous tissue implantation in mice might reflect an *in vivo* immune response against the xenogeneic tissues.

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