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ORIGINAL ARTICLE

EXPERIMENTAL

Immune response to bovine pericardium implanted into α1,3-galactosyltransferase knockout mice: feasibility as an animal model for testing efficacy of anticalcification treatments of xenografts

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Abstract

OBJECTIVES: Glutaraldehyde (GA)-fixed xenografts are prone to calcification after implantation in humans and there is evidence that immune reaction to the Galα1,3-Galβ1,4GlcNAc-R (α-Gal) antigen may play a part in this process. The objectives of this study were to evaluate the immune response of α1,3-galactosyltransferase knockout (α-Gal KO) mice to bovine pericardium and to evaluate the effect of various anticalcification treatments on bovine pericardium using mouse subcutaneous implantation model.

METHODS: Bovine pericardial tissues were divided into eight groups according to the method of anticalcification treatments. Prepared tissues were subcutaneously implanted into the α-Gal KO and wild-type mice for 2 months, and anti-α-Gal antibodies were measured at 2 weeks and 2 months after implantation. Explanted tissues were examined by immunohistochemistry and calcium contents of the explanted tissues were measured.

RESULTS: Titres of IgM and IgG antibodies in the α-Gal KO mice increased significantly according to the duration of implantation, whereas titres of IgM and IgG antibodies in the wild-type mice increased until 2 weeks after implantation without further increase thereafter. Titres of IgG antibodies measured at 2 months after implantation were significantly higher in the α-Gal KO mice than in the wild-type mice. Immunohistochemistry revealed macrophages surrounding the pericardial tissues irrespective of the mouse type into which the tissues implanted, whereas T-cells could only be observed in the tissues implanted into the α-Gal KO mice. Except the high-concentration GA-treated group, calcium contents of anticalcification-treated groups were all significantly lower or tended to be lower than that of the control group, irrespective of the mouse type. Calcium contents of the control group were significantly higher in the α-Gal KO mice than in the wild-type mice.

CONCLUSIONS: Bovine pericardium implanted into the α-Gal KO mice caused significant increase in anti-α-Gal antibodies, showed some histologic evidences of chronic rejection and revealed a potential toward more calcification. These findings suggest a possible role of immune response in calcification of xenografts. High-concentration GA fixation alone did not prove to be an effective anticalcification treatment in mouse subcutaneous implantation model. α-Gal KO mouse subcutaneous implantation model might be a feasible animal model for testing efficacy of anticalcification treatments incorporating immunologic approach.

Keywords: Glutaraldehyde • Xenograft • Calcification • Knockout mice

INTRODUCTION

Xenografts are widely used for a variety of cardiovascular applications such as heart valve substitutes and patch materials. They are conventionally cross-linked with glutaraldehyde (GA) to impart tissue stability, reduce antigenicity and to maintain tissue sterility. However, GA-fixed xenografts are prone to dystrophic calcification after long-term implantation in humans, which is one of the limiting factors affecting the longevity of bioprostheses. The mechanism of calcification of GA-fixed xenografts is complex and not completely understood, but there are evidences that tissue phospholipids and free aldehyde groups of GA play an important role [1, 2]. Thus, various chemical methods to remove tissue phospholipids or free aldehyde groups have been investigated to prevent or delay the calcification of bioprostheses [3–7]. However, an ideal method which can prevent the calcification of xenografts completely has not yet been developed, which suggests that other factors may also play a role in the calcification process.

Another proposed mechanism responsible for the calcification of xenografts is an immune response to the xenografts [8–10].

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Modifications of fixation method using high-concentration GA proved to be effective in prevention of calcification, presumably by suppressing residual antigenicity of bioprosthetic tissues [11]. Galα1,3-Galβ1,4GlcNAc-R (α-Gal) is an unique carbohydrate structure, which has been evolutionarily conserved in most mammalian species except humans, apes and Old World monkeys [12]. Preformed xenoreactive antibodies against the α-Gal antigen are well known to cause hyperacute rejection of pig organs transplanted into humans. Recently, our group and others have suggested that these preformed anti-α-Gal antibodies might also play a role in calcification of currently used bioprosthetic heart valves [13–15]. Therefore, immunological approaches to prevent the calcification of xenografts, such as removing α-Gal epitopes from xenograft tissues or utilization of genetically manipulated α-Gal-deficient xenografts, may further improve durability of bioprostheses.

As an initial step for testing the efficacy of anticalcification treatments, a rat subcutaneous implantation model has widely been used. However, unlike the situation in humans, implanting porcine or bovine tissues into rats will not provoke anti-α-Gal immune response because both donor and recipient species have α-Gal epitopes. Therefore, it seems rational to use genetically manipulated α-Gal-deficient animals, such as α1,3-galactosyltransferase knockout (α-Gal KO) mice, as an animal model to mimic human immunologic environment [16]. To our knowledge, this animal model has not been used for testing the efficacy of anticalcification treatments. The objectives of this study were to evaluate the immune response of the α-Gal KO mice to bovine pericardium and to evaluate the effect of various anticalcification treatments on bovine pericardium using mouse subcutaneous implantation model.

MATERIALS AND METHODS

Experimental design

Bovine pericardial tissues were divided into eight groups according to the method of tissue preparation and the tissues were subcutaneously implanted into the α-Gal KO and wild-type mice for 2 months.

Group 1: conventional GA fixation (control group)
Group 2: high-concentration GA fixation
Group 3: conventional GA fixation + glycine
Group 4: high-concentration GA fixation in organic solvent + glycine
Group 5: decellularization + conventional GA fixation
Group 6: decellularization + high-concentration GA fixation
Group 7: decellularization + conventional GA fixation + glycine
Group 8: decellularization + high-concentration GA fixation in organic solvent + glycine.

Tissue preparation

Fresh bovine pericardium was obtained from local slaughterhouse, placed in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and immediately transported to our laboratory. On arrival, they were rinsed with normal saline and were freed of adherent fat. In Group 1, tissues were fixed in PBS-buffered 0.5% GA solution (pH 7.4) at room temperature for 14 days. In Group 2, tissues were initially fixed in 0.5% GA solution for 5 days. After initial fixation, they were consecutively fixed in 2% GA solution for 2 days and 0.25% GA solution for 7 days. In Group 3, tissues were fixed in 0.5% GA solution for 14 days. After fixation, tissues were treated in PBS-buffered 0.2 M glycine solution (pH 7.4) at 37°C for 48 h. After glycine treatment, tissues were treated in PBS-buffered 0.1 M $NaBH_4$ solution (pH 7.4) at room temperature for 24 h. In Group 4, tissues were initially fixed in 0.5% GA solution for 5 days. After initial fixation, they were consecutively fixed with 2% GA in organic solvent (67.5% ethanol + 2.5% octanol) for 2 days and 0.25% GA solution for 7 days. In Groups 5–8, initial decellularization process was performed as follows and post-decellularization treatments were identical as in Groups 1–4. Tissues were initially washed and treated with hypotonic-buffered solution at 4°C for 6 h and hypotonic-buffered solution containing 0.25% sodium dodecyl sulphate at room temperature for 14 h. These tissues were then washed and treated with hypertonic-buffered solution at 4°C for 8 h and isotonic-buffered solution at 4°C for 12 h.

Microscopic examination (preimplantation)

Representative tissue samples from each group were examined with light microscopy. Tissue samples were fixed in 10% formalin, embedded in paraffin wax and 2–4-μm-thick sections were stained with haematoxylin–eosin (H–E).

Mouse subcutaneous implantation

This study was approved by the Institutional Animal Care and Use Committee of the Clinical Research Institute, Seoul National University Hospital (IACUC No. 09-0017). This facility was accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

α-Gal KO ($n = 21$) and wild-type ($n = 19$) mice (C57BL/6, 6–7 weeks old, 18–27 g) were used. The α-Gal KO mice were obtained from the Center for Animal Resource Development, Seoul National University College of Medicine. After anaesthetizing and shaving, four subcutaneous pouches were created at dorsal area for each mouse. Treated pericardial samples ($8 \times 8\ mm^2$) were implanted into the pouches, and the wounds were closed with 6/0 nylon sutures. The mice were killed by CO_2 asphyxiation after 2 months and the pericardial samples were harvested.

Microscopic examinations (postimplantation)

Representative tissue samples (explanted bovine pericardium) from each group were examined with light microscopy. Harvested tissue samples were fixed in 10% formalin, embedded in paraffin wax and 2–4-μm-thick sections were stained with H–E and von Kossa. The structure of collagen fibres and the degree of inflammatory cellular reaction and calcification were examined.

Immunohistochemistry

Representative tissue samples (explanted bovine pericardium) from each group were stained for mouse macrophages and T-cells. The primary antibodies used were anti-mouse F4/80

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

Measurement of anti-α-Gal antibodies in mice

Anti-α-Gal serum IgM and IgG antibodies in the α-Gal KO ($n = 16$) and wild-type mice ($n = 10$) were measured before implantation and at 2 weeks and 2 months after implantation by enzyme-linked immunosorbent assay (ELISA). About 0.5–1.0 ml of blood per mouse was collected by infraorbital venous plexus sampling. Synthetic α-Gal epitopes linked to bovine serum albumin (α-Gal-BSA; Dextra, Reading, UK) were used as a solid-phase antigen. Serially diluted (1:40, 1:80, 1:160, 1:320, 1:640, 1:1280) serum samples were measured. The secondary antibody used were horseradish peroxidase-conjugated rabbit anti-mouse IgM (Jackson ImmunoResearch) or goat anti-mouse IgG (Jackson ImmunoResearch) at 1:5000 dilution. The colour reaction was developed with tetramethylbenzidine solution (PIERCE, Rockford, IL, 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

Harvested tissue samples (five samples for each group) were washed with normal saline, dried at 70°C for 24 h and weighed. Samples were then hydrolysed with 5.0 N HCl solution. Calcium content of the hydrolysate was measured colorimetrically by the *O*-cresolphthalein complexone method, as previously described [17], using an automatic chemistry analyser (Hitachi 7070, Japan). Calcium contents were expressed as μg mg^{-1} dry weight.

Statistical analysis

Calcium contents were expressed as medians with interquartile ranges. Graphs were expressed as mean or medians with 95% confidence intervals as appropriate. Comparison between groups was performed using Friedman, Wilcoxon signed ranks, Kruskal–Wallis or Mann–Whitney tests. Statistical significance was defined as $P < 0.05$.

RESULTS

Microscopic examination

Before implantation, light microscopy showed optimal preservation of collagen fibres that had multidirectional orientations and regular periodicity in all groups. In Groups 5–8, complete decellularization was apparent without significant alteration of the extracellular matrix (Fig. 1).

After implantation, extensive inflammatory cellular infiltrates disrupting collagen fibres were observed in all groups irrespective of the mouse type (Fig. 2). Calcium stain revealed some calcific deposits in high-concentration GA-treated group (Group 2), whereas no or rare calcific deposits were observed in other groups (Fig. 3).

Immunohistochemistry

F4/80 staining (macrophage staining) revealed macrophages which were in immediate contact with pericardium in all groups irrespective of the mouse type, suggesting that these

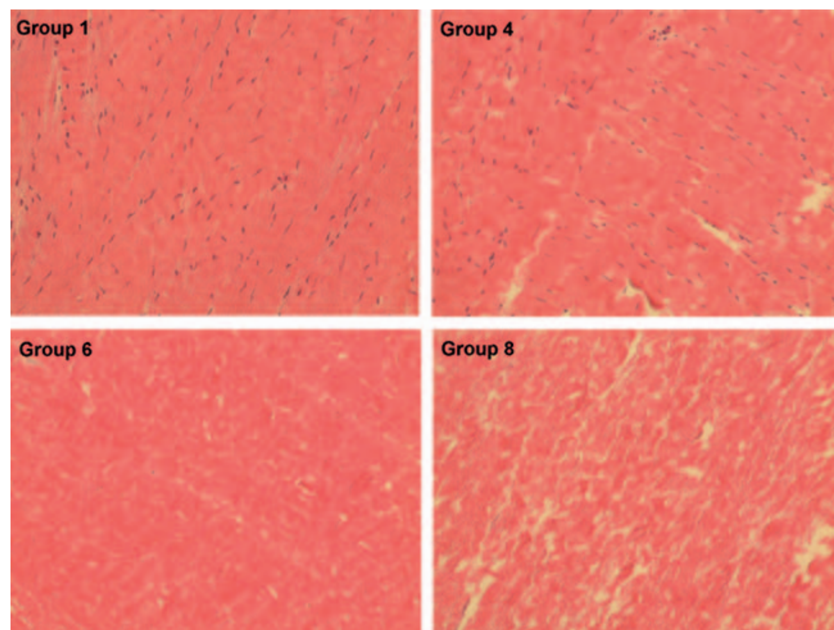

Figure 1: Light microscopy of unimplanted bovine pericardium (H–E stain, original magnification: ×100). Collagen fibres appear well preserved with a normally banded structure in all groups. In decellularized groups (Groups 6 and 8), there are no visible cells and no significant alteration of the extracellular matrix is observed.

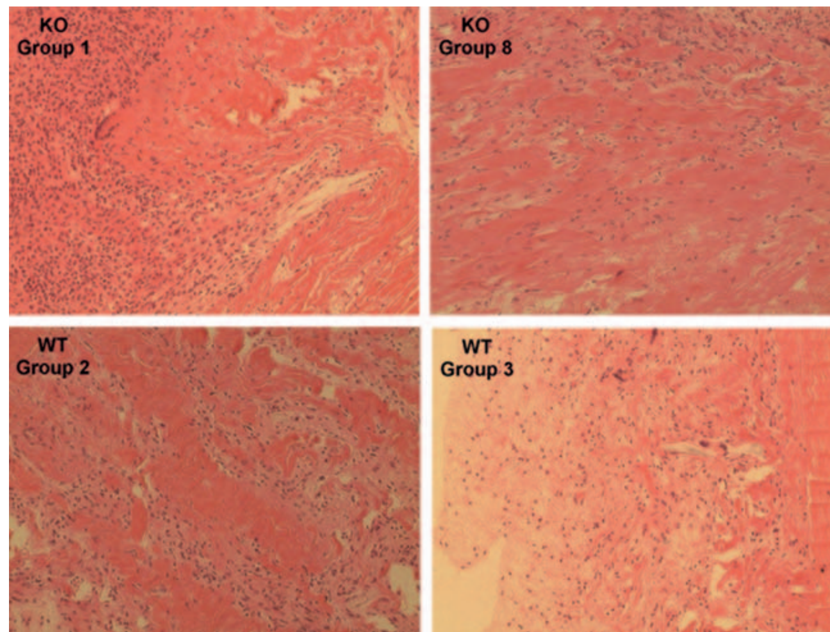

Figure 2: Light microscopy of harvested bovine pericardium (H–E stain, original magnification: ×100). Extensive inflammatory cellular infiltrates disrupting collagen fibres are observed in all groups irrespective of the mouse type. KO: α-Gal KO mouse; WT: wild-type mouse.

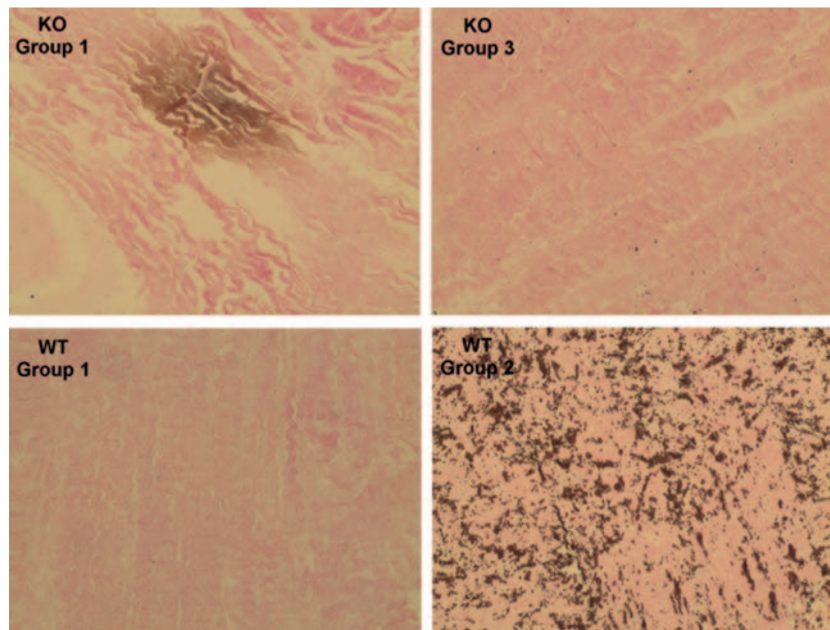

Figure 3: Light microscopy of harvested bovine pericardium (von Kossa stain, original magnification: ×100). Some calcific deposits are observed in high-concentration GA-treated group (Group 2), whereas no or rare calcific deposits are observed in other groups. KO: α-Gal KO mouse; WT: wild-type mouse.

macrophages were attacking the pericardial tissues. In pericardial tissues implanted into the α-Gal KO mice, a thick layer of non-stained inflammatory cells were observed in the outer surface of macrophages, whereas few non-stained inflammatory cells were observed in tissues implanted into the wild-type mice (Fig. 4). We could not find any noticeable differences between non-decellularized (Groups 1–4) and decellularized groups (Groups 5–8).

CD4 staining (T-cell staining) revealed T-cells surrounding the pericardial tissues implanted into the α-Gal KO mice. However, in the pericardial tissues implanted into the wild-type mice, no or rare T-cells were observed (Fig. 5). We could not find any noticeable differences between non-decellularized and decellularized groups.

Anti-α-Gal antibodies

Titres of anti-α-Gal IgM and IgG antibodies according to the mouse type and duration of implantation are depicted in Fig. 6. Titres of IgM and IgG antibodies in the α-Gal KO mice increased significantly according to the duration of implantation ($P < 0.001$ for both IgM and IgG, Fig. 7A). Titres of IgM and IgG antibodies in the wild-type mice increased until 2 weeks after implantation

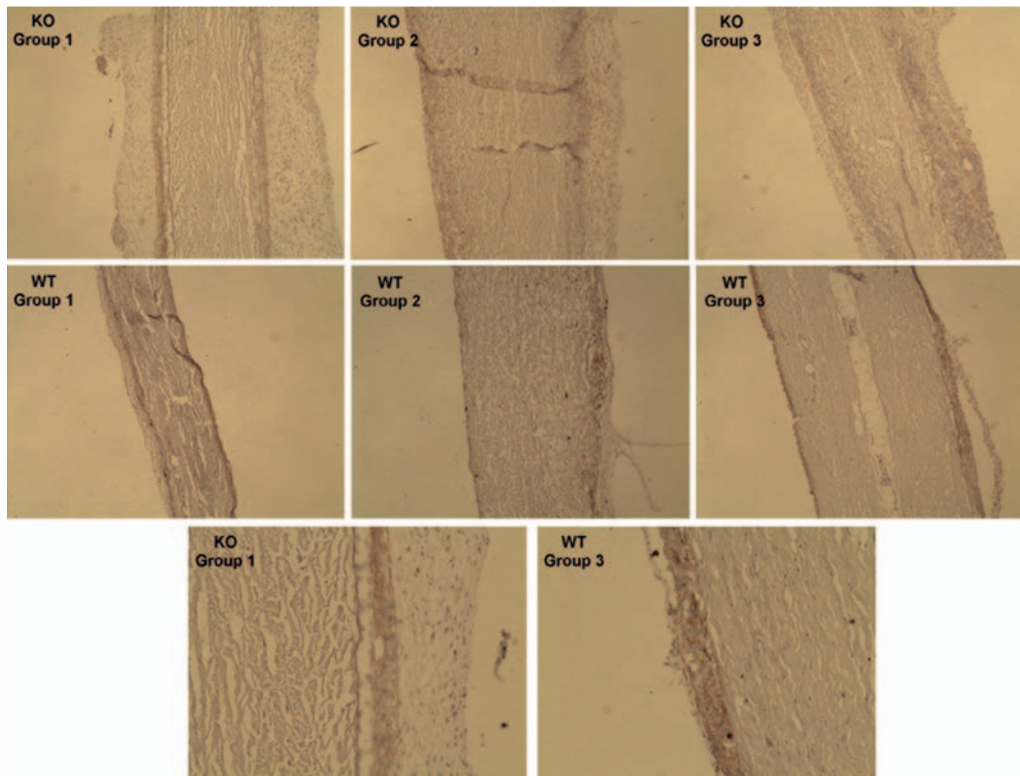

Figure 4: Representative immunohistochemistry of harvested bovine pericardium (macrophage stain). Macrophages (dark brown) are observed in immediate contact with pericardium in all groups irrespective of the mouse type (upper two panels, original magnification: ×40). In tissues implanted into the α-Gal KO mice, a thick layer of non-stained inflammatory cells are observed in the outer surface of macrophages, whereas few non-stained inflammatory cells are observed in tissues implanted into the wild-type mice (lower panels, original magnification: ×100). KO: α-Gal KO mouse; WT: wild-type mouse.

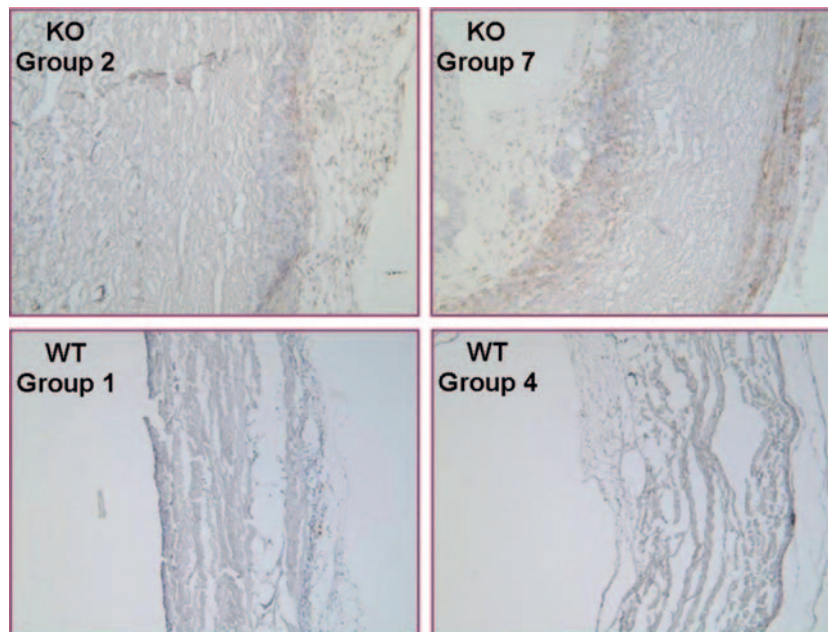

Figure 5: Representative immunohistochemistry of harvested bovine pericardium (T-cell stain, original magnification: ×100). In tissues implanted into the α-Gal KO mice, T-cells (dark brown) surrounding the pericardium are observed. In tissues implanted into the wild-type mice, no or rare T-cells are observed. KO: α-Gal KO mouse; WT: wild-type mouse.

($P = 0.005$ for both IgM and IgG) without further increase thereafter ($P = 0.445$ and 0.959, respectively, Fig. 7B). Titres of IgM and IgG antibodies measured before implantation were significantly higher in the α-Gal KO mice than in the wild-type mice ($P = 0.007$ and 0.002, respectively). There were no significant differences in titres of IgM and IgG antibodies measured at 2 weeks after implantation between the α-Gal KO and wild-type mice ($P = 0.660$ and 0.484, respectively). There was no significant

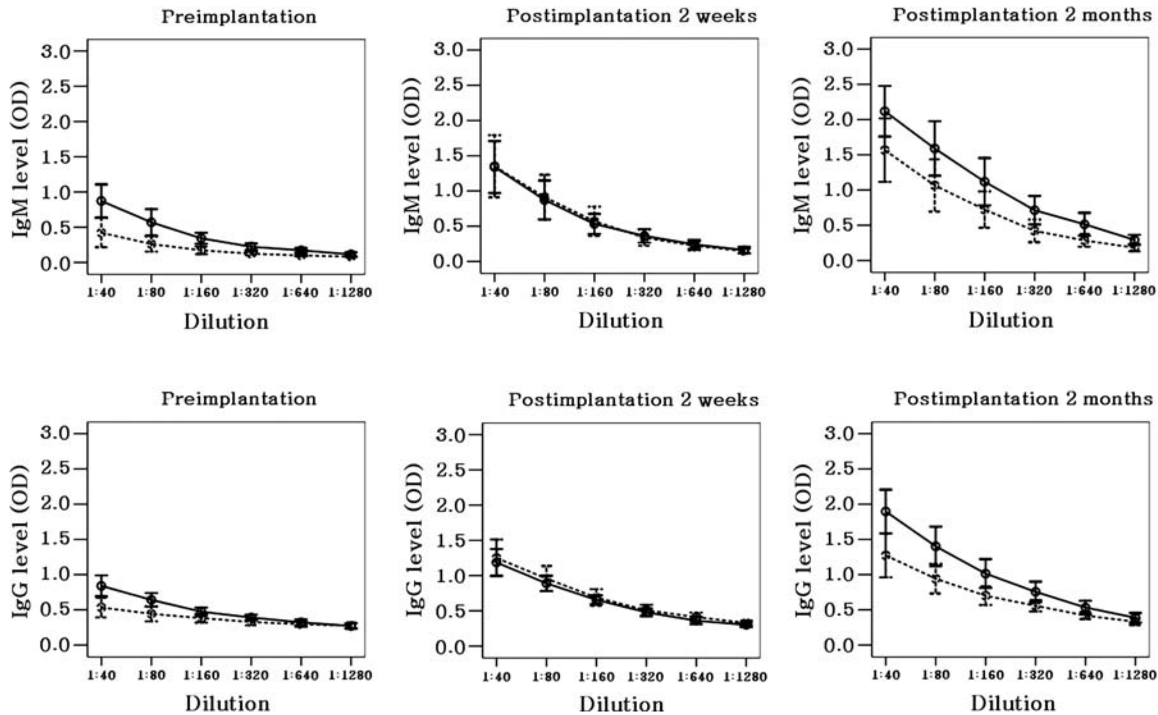

Figure 6: Titres of anti-α-Gal antibodies. Solid lines represent the α-Gal KO mice and dotted lines represent the wild-type mice. Circles represent mean values and error bars represent 95% confidence intervals. OD: optical density.

difference in titres of IgM antibodies measured at 2 months after implantation between the α-Gal KO and wild-type mice ($P = 0.150$). Titres of IgG antibodies measured at 2 months after implantation were significantly higher in the α-Gal KO mice than in the wild-type mice ($P = 0.002$, Fig. 7C). There were no differences in titres of anti-α-Gal antibodies at any time between non-decellularized and decellularized groups, irrespective of the mouse type.

Calcium analysis

Calcium contents of the bovine pericardium implanted into the wild-type mice were 0.00 μg mg^{-1} in all groups except for Groups 1 and 2, which contained 0.12 μg mg^{-1} (0.10–0.27 μg mg^{-1}) and 2.83 μg mg^{-1} (0.24–28.10 μg mg^{-1}), respectively. Except Group 2, calcium contents of anticalcification-treated groups were all significantly lower than that of the control group ($P < 0.05$). Calcium content of Group 2 tended to be higher than that of the control group ($P = 0.056$) and was significantly higher than those of all other groups ($P < 0.05$). Calcium contents of the bovine pericardium implanted into the α-Gal KO mice were 0.00 μg mg^{-1} in all groups except for Groups 1 and 2, which contained 0.77 μg mg^{-1} (0.20–2.80 μg mg^{-1}) and 23.97 μg mg^{-1} (3.67–105.30 μg mg^{-1}), respectively. Except Groups 2 and 7, calcium contents of anticalcification-treated groups were all significantly lower than that of the control group ($P < 0.05$). Calcium content of Group 2 was significantly higher than those of all other groups ($P < 0.05$). Calcium content of Group 7 tended to be lower than that of the control group ($P = 0.067$). Calcium contents of the control group were significantly higher in the α-Gal KO mice than in the wild-type mice ($P = 0.036$). There was no significant difference in calcium contents of Group 2 between the α-Gal KO and wild-type mice ($P = 0.465$).

DISCUSSION

GA-fixed bioprostheses fabricated from xenografts are widely used in cardiovascular surgery. However, durability of these bioprostheses is suboptimal, especially in children and young adults, owing to dystrophic calcification [18]. In an effort to prevent or delay the bioprosthetic calcification, various anticalcification strategies have been investigated [3–7] and are still being developed. However, an ideal method which can prevent the calcification of xenografts completely has not yet been developed. This is due to the complex, multifactorial and incompletely understood mechanism of the bioprosthetic calcification.

α-Gal is an unique carbohydrate structure which is present in most mammalian species except humans, apes and Old World monkeys [12]. Preformed antibodies against this α-Gal antigen are well known to cause hyperacute rejection of vascularized viable organs in pig-to-human xenotransplantation. However, hyperacute rejection is not a clinical issue when bioprostheses are implanted into humans, because these bioprostheses are not viable tissues. Rather, chronic rejection elicited by the α-Gal antigen can be an important mechanism responsible for bioprosthetic valve calcification. In fact, Stone *et al*. [19] and Galili *et al*. [20], in a model of transplanting porcine and bovine cartilage into cynomolgus monkey, demonstrated a significant increase in anti-α-Gal IgG antibodies and histological evidence of chronic rejection. Konakci *et al*. [14] demonstrated the presence of α-Gal antigens in the connective tissue of commercial porcine valves and a significant increase of naturally occurring cytotoxic IgM antibodies directed towards the α-Gal antigen in patients who underwent bioprosthetic valve replacement. Recently, our group has also demonstrated increased formation of anti-α-Gal antibodies in children who underwent commercial porcine heart valve replacement [13]. Although the exact mechanism by which the chronic rejection leads to the calcification of

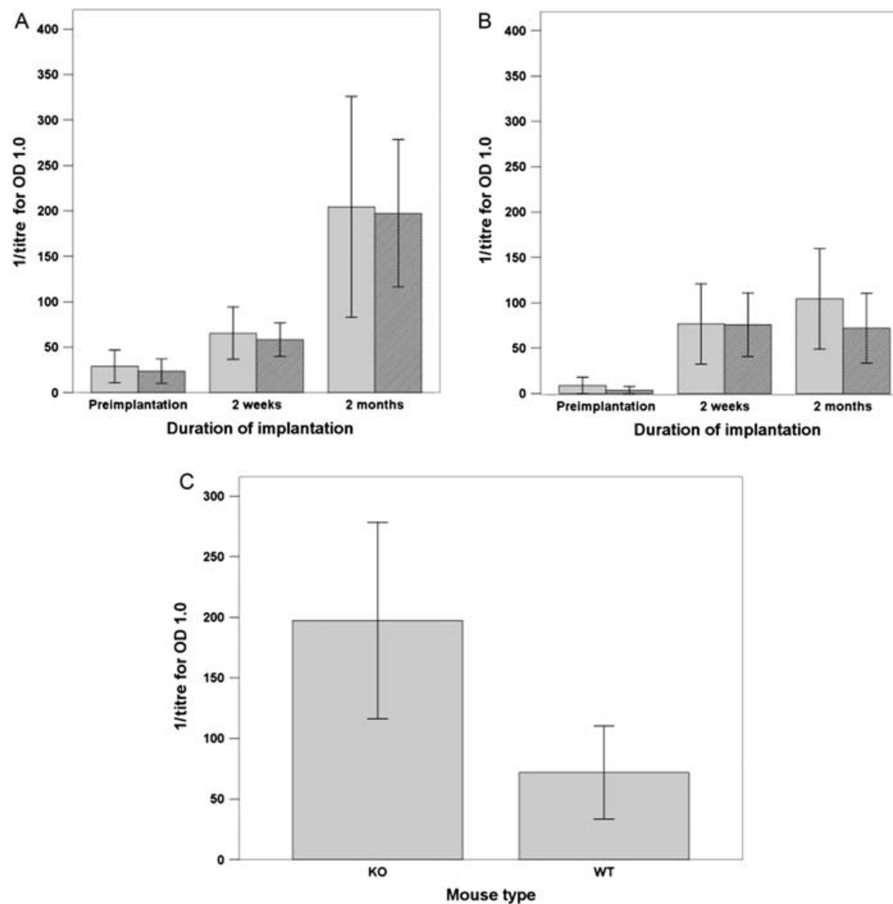

Figure 7: (**A**) Change of anti-α-Gal IgM (filled bars) and IgG (hatched bars) titres in α-Gal KO mice according to the duration of implantation. Titres of IgM and IgG increased according to the duration of implantation. (**B**) Change of anti-α-Gal IgM (filled bars) and IgG (hatched bars) titres in the wild-type mice according to the duration of implantation. Titres of IgM and IgG antibodies increased until 2 weeks after implantation without further increase thereafter. (**C**) Anti-α-Gal IgG titres measured at 2 months after implantation according to the mouse type. Titres of IgG antibodies were significantly higher in the α-Gal KO mice than in the wild-type mice. All graphs are expressed as mean with 95% confidence intervals. OD: optical density; KO: α-Gal KO mouse; WT: wild-type mouse.

bioprostheses is unknown, it is prudent to speculate that calcification may occur as a 'by-product' of the inflammatory process caused by immune reaction [9]. Therefore, immunologic approaches to prevent the calcification of xenografts, such as removing α-Gal epitopes from xenograft tissues or utilization of genetically manipulated α-Gal-deficient xenografts, may further improve durability of bioprostheses.

A rat subcutaneous implantation model has been widely used as a small-animal model for rapidly screening efficacy of anticalcification treatments. However, implanting porcine or bovine tissues into rats is a kind of concordant xenotransplantation in terms of α-Gal antigen because both donor and recipient species have α-Gal epitopes and, as such, will not provoke anti-α-Gal immune response. Therefore, it seems rational to use genetically manipulated α-Gal-deficient animals, such as the α-Gal KO mice, as an animal model to mimic human immunologic environment. We think that this is especially important if one plans to incorporate immunological approaches into anticalcification treatment. To our knowledge, this animal model has not been used for testing efficacy of anticalcification treatments.

One of the objectives of our study was to evaluate the immune response of the α-Gal KO mice to bovine pericardium using subcutaneous implantation model. Our data showed that titres of anti-α-Gal IgM and IgG antibodies in the α-Gal KO mice increased significantly according to the duration of implantation. Theoretically, we expected that anti-α-Gal antibodies would not be detected in the wild-type mice. However, interestingly, titres of anti-α-Gal IgM and IgG antibodies in the wild-type mice increased until 2 weeks after implantation without further increase thereafter. The reason for this phenomenon might be due to non-specific binding of mouse antibodies (other than anti-α-Gal antibodies) to ELISA plate, as pointed out by Galili *et al.* [20], or the differences in the fine specificity of natural anti-α-Gal antibodies recognizing various 'facets' of α-Gal epitope in its three-dimensional form [21]. Although there was no difference in titres of IgM antibodies, a marker for acute-phase humoral immune response, measured at 2 months after implantation between the α-Gal KO and wild-type mice, titres of IgG antibodies measured at 2 months after implantation were significantly higher in the α-Gal KO mice than in the wild-type mice, suggesting that there was a chronic humoral immune response to the α-Gal antigen in the α-Gal KO mice. Interestingly, we could not find differences in titres of anti-α-Gal antibodies between non-decellularized and decellularized groups, irrespective of the mouse type. Although decellularization can reduce residual antigenicity substantially, decellularization alone cannot completely remove α-Gal antigens [22].

Immunohistochemistry of the harvested bovine pericardial tissues revealed interesting findings. Macrophages were found in immediate contact with pericardium in all groups irrespective of the mouse type into which the tissues had been implanted. Of note, in pericardial tissues implanted into the α-Gal KO mice, a

thick layer of non-stained inflammatory cells were observed in the outer surface of macrophages, whereas few non-stained inflammatory cells were observed in tissues implanted into the wild-type mice. CD4 staining (T-cell staining) revealed T-cells surrounding the pericardial tissues implanted into the α-Gal KO mice, whereas no or rare T-cells were observed in the pericardial tissues implanted into the wild-type mice. Therefore, it is highly likely that the non-stained inflammatory cells which were observed in macrophage-stained tissues implanted into the α-Gal KO mice are T-cells. These immunohistochemistry findings strongly suggest that there was a chronic cellular immune response to the α-Gal antigen in the α-Gal KO mice [9, 19].

Calcium analysis of the harvested bovine pericardial tissues revealed several interesting findings. First, high-concentration GA fixation alone did not prove to be an effective anticalcification method. This is not surprising because residual free aldehyde groups or polymerized forms of GA are known to contribute to the calcification of bioprostheses [1, 2]. Although high-concentration GA fixation proved to be effective in prevention of calcification presumably by suppressing residual antigenicity of bioprosthetic tissue [11], we believe that post-fixation detoxification process is essential in order to neutralize the intrinsic calcification potential of high-concentration GA fixation [5]. All the other anticalcification treatments of our study proved to be effective in preventing calcification of bovine pericardium. Of note, high-concentration GA fixation after decellularization (Group 6) completely prevented calcification irrespective of the mouse type. Decellularization treatment might have compensated the intrinsic calcification potential of high-concentration GA fixation and prevented calcification. Second, calcium contents of the control group (conventional GA fixation) were significantly higher in the α-Gal KO mice than in the wild-type mice. Although drawing a firm conclusion from this finding is somewhat difficult owing to the small sample size, it is possible that chronic immune response to the α-Gal antigen, as evidenced by the immunologic results of our study, might have caused more calcification of the pericardial tissues in the α-Gal KO mice than in the wild-type mice. Finally, our mouse subcutaneous implantation model resulted in lower degree of calcification of control group tissues compared with the conventional rat subcutaneous implantation model [3, 4, 7]. This can be problematic in testing efficacy of anticalcification treatments because control group tissues should calcify enough to validate the difference in calcification between the control and experimental groups. One possible explanation of this phenomenon is differences in potential of inducing tissue calcification according to the species into which the tissues are implanted. Qiao *et al*. [23], in animal models for inducing atheromatous lesions using various mouse strains, reported that there were clear differences in the occurrence of arterial wall calcification among mouse strains, indicating a genetic component in calcification. Rajachar *et al*. [24], in a mouse subcutaneous implantation model using a C57BL/6 strain, reported low calcification of the conventional GA-fixed bovine pericardial tissue. Based on these studies, there is a possibility that the implantation duration of 2 months in our study might have been too short to induce sufficient calcification of the control group tissues. Therefore, in order to induce sufficient calcification of control tissues in an α-Gal KO mouse subcutaneous implantation model, further studies on the adequate duration of implantation seem necessary. Another possible solution to this problem might be pre-immunization of the α-Gal KO mice to increase anti-α-Gal antibody titres [16, 25] and consequently augment the immune response to xenograft tissues, although this is only a speculation.

In conclusion, bovine pericardium implanted into the α-Gal KO mice caused significant increase in anti-α-Gal antibodies, showed some histological evidence of chronic rejection and revealed a potential towards more calcification. These findings suggest a possible role of immune response in calcification of xenografts. High-concentration GA fixation alone did not prove to be an effective anticalcification method in mouse subcutaneous implantation model. α-Gal KO mouse subcutaneous implantation model might be a feasible animal model for testing efficacy of anticalcification treatments, especially those incorporating immunologic approach.

LIMITATIONS OF THE STUDY

Our experiment was a kind of pilot study for testing feasibility of α-Gal KO mouse as an animal model for screening effective anticalcification methods, and sample sizes of our experiment were small. In order to use α-Gal KO mouse as an animal model for anticalcification treatment, further studies on adequate duration of implantation for inducing sufficient calcification are needed.

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Conflict of interest: none declared.

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