

Removal of Alpha-Gal Epitopes from Porcine Aortic Valve and Pericardium using Recombinant Human Alpha Galactosidase A

It has been reported that the immune response due to α -Gal epitopes is an important factor in tissue valve failure. The elimination of the interaction between the natural anti-Gal antibodies and α -gal epitopes on the xenografts is a prerequisite to the success of xenografts in humans. Previously, we reported that the green coffee bean α -galactosidase could remove all α -Gal epitopes from cell surface of porcine aortic valve and pericardial tissue, but it has limitations on cost effectiveness. In this study we wanted to know whether the recently produced recombinant human α -galactosidase A has the same effective enzymatic activity as green coffee bean α -galactosidase in removing α -Gal epitopes from the same tissues. After treating fresh porcine aortic valve and pericardial tissue with recombinant α -galactosidase A, each sample was stained with *Griffonia simplicifolia* type I isolectin B4 indirect immunoperoxidase avidin-biotin technique. We then examined whether the α -Gal epitopes were reduced or abolished in each consecutive concentration of recombinant α -galactosidase A by comparing the degree of the *Griffonia simplicifolia* isolectin B4 staining. As a result, the recombinant α -galactosidase A could remove cell surface α -Gals on porcine aortic valve and pericardial tissue as effectively as green coffee bean α -galactosidase.

Key Words : *Alpha-Galactosyl Epitope; Alpha-Galactosidase; Bioprostheses*

Seongsik Park¹, Woong-Han Kim²,
Sun-Young Choi², and Yong-Jin Kim²

Department of Cardiothoracic Surgery¹, Dankook University Hospital, College of Medicine, Dankook University, Cheonan; Department of Thoracic and Cardiovascular Surgery², Seoul National University Hospital, Seoul National University College of Medicine, Seoul, Korea

Received : 3 December 2008
Accepted : 22 January 2009

Address for correspondence

Yong-Jin Kim, M.D.
Department of Thoracic and Cardiovascular Surgery,
Seoul National University Children's Hospital, 28
Yeongeon-dong, Jongno-gu, Seoul 110-460, Korea
Tel : +82.2-2072-3638, Fax : +82.2-745-5209
E-mail : kyj@plaza.snu.ac.kr

This study was supported by grants from Xonotransplantation research center and the Korea Health 21 Research & Development Project of the Ministry of Health, Welfare and Family Affairs (No. A040004-008).

INTRODUCTION

Tissue valves are considered as ideal human cardiac valve substitutes because they have excellent hemodynamic properties and chronic anticoagulants are not necessary. However, the structural failure after prolonged usage prevents widespread adaptation of the tissue valves, especially for young age groups (1). The reasons for the structural failure of the implanted tissue valves include repetitive mechanical wear, chronic inflammatory response by glutaraldehyde fixation, and pannus overgrowth. Recently, it has been found that the mammalian cell surface xenoantigen called α -Gal epitopes are still present on the commercially available, glutaraldehyde fixed tissue valves (2). Additionally it has been reported that patients who received these tissue valves exhibited increased levels of natural anti-Gal antibody titers against α -Gal epitopes (2). Therefore, it is believed that the animal immune response may play an important role in structural damage of the commercially available, glutaraldehyde fixed tissue valves.

The α -Gal epitopes on the mammalian cell surface trigger a strong immune response, especially on endothelial cells, resulting in a hyperacute rejection when we transplant mam-

malian organs to the primates (3). On the other hand, xenoantigen mediated immune response of the tissue valves were not seriously considered until now because the valvular tissue and pericardium of mammals were treated with glutaraldehyde to eradicate xenoantigenicity and potential risk of xenozoonosis before using them to make tissue valves. However, after anti-Gal immune response in glutaraldehyde treated, commercially available tissue valves was recently observed, there is a heightened concern in the immune response as a cause of tissue valve structural failure.

In porcine aortic valvular tissue and pericardial tissue, it became possible to stain α -Gal epitopes on the cell surface with *Griffonia simplicifolia* isolectin B4 (GS-IB4) immunohistochemical stain (4-6). And as green coffee bean α -galactosidase is known to degrade α -Gal epitopes at the site of Gala 1-3Gal chain (7-9), it is possible that it could remove α -Gal epitopes from those tissues. Previously we examined this possibility and reported that all α -Gal epitopes could be removed from porcine valvular and pericardial cell surfaces using green coffee bean α -galactosidase (10). Meanwhile, there are limitations in cost effectiveness using green coffee bean α -galactosidase in the commercial valve manufacturing process. There-

fore, we decided to investigate whether recently made, easily available recombinant human α -galactosidase A has same enzymatic activity as green coffee bean α -galactosidase, and whether the recombinant enzyme can effectively eradicate α -Gal epitopes on the cell surface of porcine aortic valve and pericardium. Additionally, we used standard indirect immunoperoxidase avidin-biotin technique in detecting α -Gals on cell surface instead of previously used immunofluorescent method.

MATERIALS AND METHODS

Staining α -Gal epitopes

The heart and pericardium of pigs, aged 6-12 months, were obtained from the local slaughter house and transported in 4°C normal saline bag to our facility. After removing the aortic valve and pericardial tissue, the tissue was thoroughly washed with phosphate buffered saline (PBS). Samples of the valve and pericardial tissue of 5 × 5 mm size were excised and washed with PBS 3 times, 5 min each. The samples were then immersed into 30% sucrose solution for preventing cell rupture during freezing process. Next, the frozen sample were sectioned and were mounted on the slides and fixed with cold acetone for 10 min. After thorough washing in PBS 3 times, for 5 min each, the sections were incubated on the slides in 1/500 diluted 500 mg/mL GS-IB4/biotin conjugates (Invitrogen, Carlsbad, CA, U.S.A.) at 37°C for 1 hr. Again sections were washed as above. After blocking with 100 mL of 1% bovine serum albumin (BSA)/PBS at 37°C for 1 hr, sections were washed as above. 100 mL of 5 mg/mL horseradishperoxidase (HRP) conjugated Streptavidin (HRP-SA) (Pierce Biotechnology, Rockford, IL, U.S.A.) were applied to the slides and incubated at 37°C for 1 hr. Thorough washing with PBS was followed. Finally 3,3'-diaminobenzidine (DAB) substrate (DAB kit, Vector Lab., Burlingame, CA, U.S.A.) was applied

on the tissue slide for 5 min and the slides were observed under mounting media. In this indirect immunoperoxidase avidin-biotin technique using DAB as a substrate, brown staining spots on the cell surface indicate GS-IB4 bound α -Gal epitopes.

Reacting with recombinant human α -galactosidase A

Recombinant human α -galactosidase A (Isu Abxis, Seoul, Korea) made from chinese hamster ovary mammalian cells were used in our experiment. 100 mM HEPES buffer solution at pH 5.0 was prepared, and used to make concentrations of recombinant α -galactosidase A of 1.0, 5.0, 10.0 unit/mL. The sliced aortic valve and pericardial tissue of pig of 5 × 5 mm size were incubated with each of the solution for 24 hr under 4°C. After 24 hr, the tissues were washed with PBS solution for 5 min 3 times, then immersed into 30% sucrose solution. After then α -Gal epitopes on the enzyme treated aortic valve and pericardial tissue were stained as described above.

RESULTS

The images representing the distribution of α -Gal epitopes on porcine aortic valve and pericardial tissue before and after treatment with recombinant α -galactosidase A were obtained via GS-IB4 conjugated indirect immunoperoxidase avidin-biotin staining technique. After removing α -Gal epitopes with consecutive incubations in 1.0, 5.0, 10.0 unit/mL of recombinant α -galactosidase A, we noted that different degrees of α -Gal epitopes were removed from the tissues according to tissue type and enzyme concentration.

An enzyme concentration of 1.0 unit/mL was not enough to completely eradicate the α -Gal epitopes from cell surface of the aortic valve (Fig. 1). At 5.0 unit/mL, nearly all α -Gal epitopes were removed from cell surface according to GS-IB4

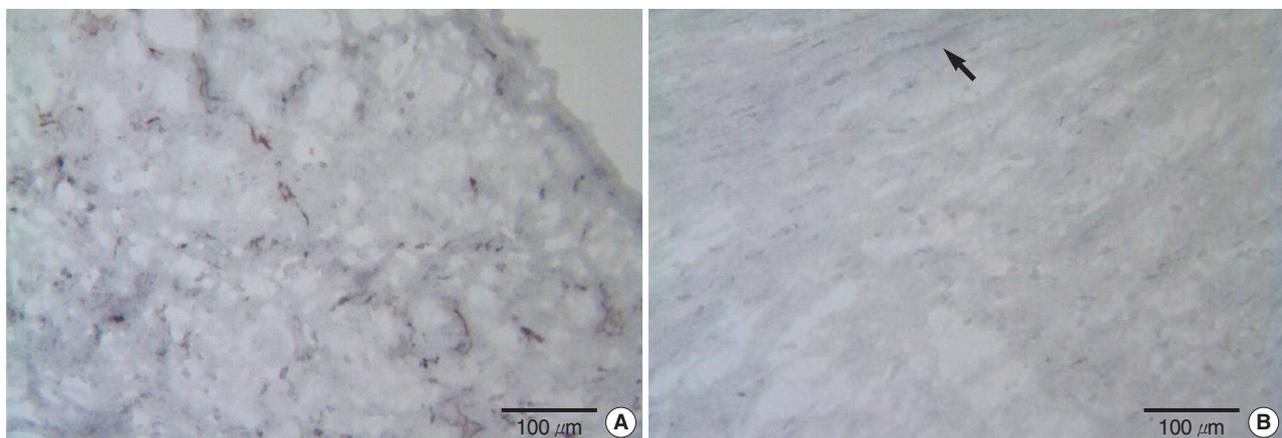


Fig. 1. Aortic valve of a pig ($\times 400$): (A) before treatment, (B) after treatment with 1.0 unit/mL of recombinant α -galactosidase A. Many α -Gal epitopes are still present on cell surface (arrow).

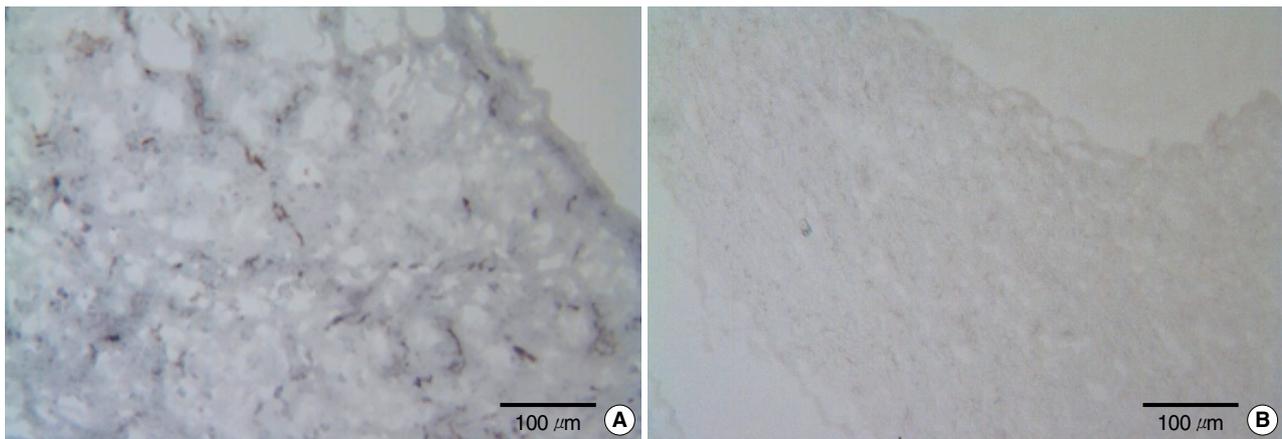


Fig. 2. Aortic valve of a pig ($\times 400$): (A) before treatment, (B) after treatment with 5.0 unit/mL of recombinant α -galactosidase A. α -Gal epitopes are nearly completely removed from cell surface.

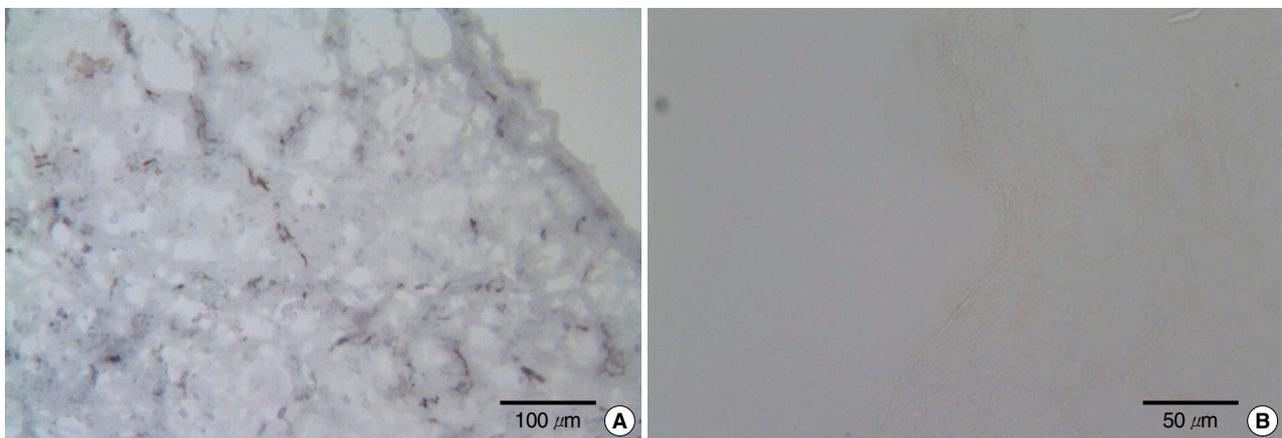


Fig. 3. Aortic valve of a pig ($\times 400$): (A) before treatment, (B) after treatment with 10.0 unit/mL of recombinant α -galactosidase A. No α -Gal epitopes on cell surface.

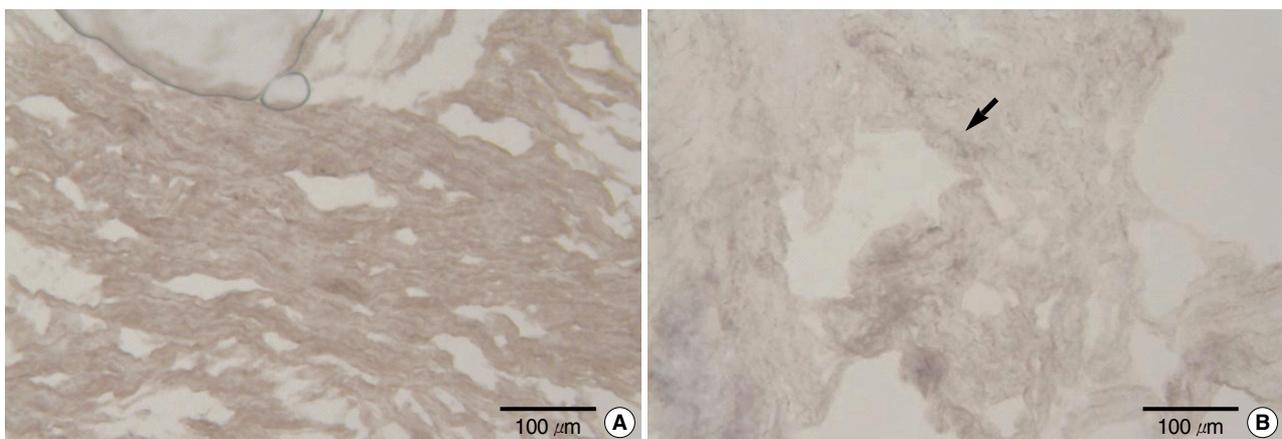


Fig. 4. Pericardium of a pig ($\times 400$): (A) before treatment, (B) after treatment with 1.0 unit/mL of recombinant α -galactosidase A. Many α -Gal epitopes are still present on cell surface (arrow).

staining (Fig. 2). There were no differences between 5.0 unit/mL to 10.0 unit/mL enzyme concentrations in removing α -

Gal epitopes from the cell surface of the porcine aortic valve (Fig. 3). Therefore, we could conclude that about 5.0 unit/mL

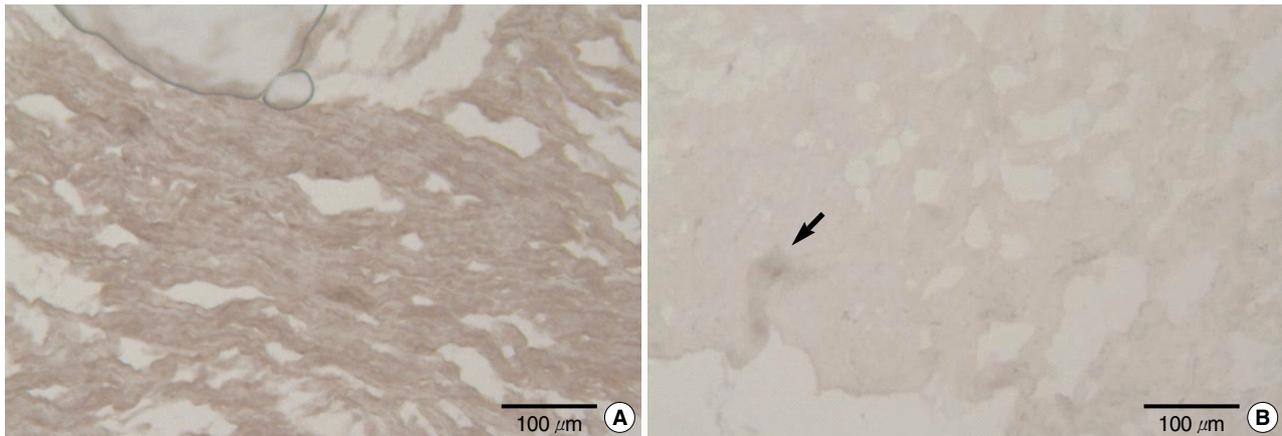


Fig. 5. Pericardium of a pig ($\times 400$): (A) before treatment, (B) after treatment with 5.0 unit/mL of recombinant α -galactosidase A. Some α -Gal epitopes are still present on cell surface (arrow).

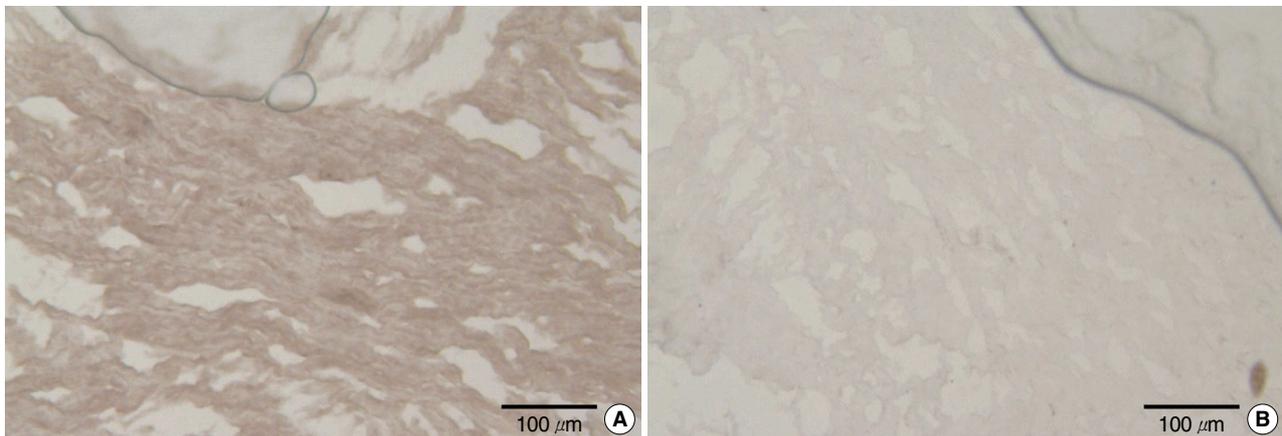


Fig. 6. Pericardium of a pig ($\times 400$): (A) before treatment, (B) after treatment with 10.0 unit/mL of recombinant α -galactosidase A. α -Gal epitopes are nearly completely removed from cell surface.

concentration of recombinant α -galactosidase A with reaction conditions of pH 5.0, temperature 4°C , 24 hr of incubation was enough to remove all α -Gal epitopes from cell surface of porcine aortic valve. On the other hand, in porcine pericardial tissue, enzyme concentration of 1.0 unit/mL (Fig. 4) and even 5.0 unit/mL (Fig. 5) were not enough to completely eradicate α -Gal epitopes from cell surface. At 10.0 unit/mL, all the α -Gal epitopes from the cell surface of pericardium were removed according to GS-IB4 staining (Fig. 6). Therefore, in porcine pericardial tissue, the more recombinant α -galactosidase A was needed to remove all α -Gal epitopes from cell surface than that needed in aortic valve. This finding is in accordance with the result of our previous study (10).

DISCUSSION

Improving the durability of the tissue valves are crucial, not only for the children-adolescent patients but also for the

increasing number of elderly patients receiving tissue valves in the aortic valve position. Recently, nearly 70% of aortic valve replacement in the patients older than 70 yr were done using tissue valves (11, 12). The tissue valve degeneration have been caused by degenerative calcification, glutaraldehyde related calcification (13), mechanical wear, and pannus overgrowth. Recently it was shown that α -Gal epitopes, responsible for hyperacute rejection in xenotransplantation, were still present on glutaraldehyde fixed, commercially available tissue valves. Also an increased number of anti-Gal antibodies in the patients who received those tissue valves was noted (2). Therefore, it is possible that the α -Gal-anti-Gal immune reaction may play a certain role in valve calcification and degradation after tissue valve replacement and removing α -Gals from the surface of the tissue valves can improve the durability of the tissue valves. Until now two methods of removing α -Gal epitopes from porcine tissues are available. One is enzymatic removal using α -galactosidase (7-9). The other is creating an α -1,3 galactosyltransferase deficient pigs (α -Gal

knockout pigs) with the recently well established animal cloning methods (14, 15). Using α -Gal knockout pigs is theoretically ideal and we can use organs and tissues without a fear of hyperacute rejection. But it is not feasible now. α -Gal degrading enzyme is easy to use and inexpensive. However, in the enzyme treated organs α -Gal reappears after a certain time (9). To remove α -Gal epitopes from cardiac valvular and pericardial tissues, enzymatic removal is feasible because no α -Gal reappearance occurs in such tissues. So, we used enzymatic method to remove α -Gals from cell surface of the tissues.

Green coffee bean α -galactosidase is a plant enzyme which eradicates the xenoantigenicity of α -Gal by cleaving Gal α 1-3Gal chain of α -Gal(Gal α 1-3Gal β 1-4GlcNAc) (7-9). Previously we reported that α -Gal epitopes could be removed from porcine aortic valve and pericardial tissue using green coffee bean α -galactosidase (Sigma, St. Louis, MO, U.S.A.) (10). But green coffee bean α -galactosidase is not cost effective for the commercial valve manufacturing process.

Fabry disease, which exhibits X-linked genetic trait, is a congenital human α -galactosidase A deficiency representing early peripheral neuropathy, late chronic renal failure, coronary artery disease and cerebral vascular disease. It is well known that enzyme replacement therapy (ERT) using recombinant human α -galactosidase A is helpful for those patients (16, 17). Recently, recombinant human α -galactosidase A with Chinese hamster ovary mammalian cell expression system was developed for ERT in Fabry disease. This enzyme cleaves exactly the same site of α -Gal as green coffee bean α -galactosidase (10), which can also applied in removing α -Gal epitopes from cell surface of porcine aortic valve and pericardial tissue. In aortic valve, the recombinant enzyme of 5.0 unit/mL concentration could remove almost all α -Gals under reaction condition of pH 5.0, temperature 4°C, 24 hr of incubation. In pericardial tissue, 10.0 unit/mL concentration of the recombinant enzyme was needed.

There are several methods which can detect α -Gal epitopes in the tissues. First and most popular is detecting α -Gals with Griffonia simplicifolia type I isolectin B4 which highly specifically bind to cell surface α -Gals (4-6). Others are using monoclonal antibody M86 made by Galili (18), using FITC labeled anti-immunoglobulin M, anti-immunoglobulin G which are species specific secondary antibodies derived from anti-Gal immunoglobulin M and anti-Gal immunoglobulin G, respectively (7). We previously reported that all three methods of α -Gal detection technique must be simultaneously used to prove complete removal of α -Gals from cell surface (10). But in this study we intended to prove only that recombinant human α -galactosidase A can just as effectively remove α -Gal epitopes from porcine aortic valve and pericardial tissue as green coffee bean α -galactosidase. In other words, we did not intend to prove the completeness of removal of α -Gals from cell surface of those tissues. So, we did not use the three methods simultaneously. In this study we used stan-

dard indirect immunoperoxidase avidin-biotin technique instead of previously used FITC labeled immunofluorescent method in detecting GS-IB4 bound to α -Gals on cell surface. We used DAB (DAB kit, Vector Lab., Burlingame, CA, U.S.A.) as a substrate for the indirect immunoperoxidase avidin-biotin staining method and as a result, the cell surface α -Gals exhibit brown colors. In this way we could examine the distribution of α -Gals on cell surface more precisely and avoid autofluorescence issues of FITC.

In this study, we conclude that α -Gal epitopes can be removed from the porcine aortic valve and pericardial tissue using the recombinant human α -galactosidase A as effectively as using green coffee bean α -galactosidase. Thus with this recombinant enzyme, α -Gal deficient, more durable tissue valves can be made more economically in the future.

REFERENCES

- Jamieson WR, Rosado LJ, Munro AI, Gerein AN, Burr LH, Miyagishima RT, Janusz MT, Tyers GF. *Carpentier-Edwards standard porcine bioprosthesis: primary tissue failure (structural valve deterioration) by age groups. Ann Thorac Surg 1988; 46: 155-62.*
- Konakci KZ, Bohle B, Blumer R, Hoetzenecker W, Roth G, Moser B, Boltz-Nitulescu G, Gorlitzer M, Klepetko W, Wolner E, Ankersmit HJ. *Alpha-Gal on bioprosthesis: xenograft immune response in cardiac surgery. Eur J Clin Invest 2005; 35: 17-23.*
- Oriol R, Ye Y, Koren E, Cooper DK. *Carbohydrate antigens of pig tissues reacting with human natural antibodies as potential targets for hyperacute vascular rejection in pig-to-man organ xenotransplantation. Transplantation 1993; 56: 1433-42.*
- Goldstein IJ, Blake DA, Ebisu S, Williams TJ, Murphy LA. *Carbohydrate binding studies on the Bandeiraea simplicifolia I isolectins. Lectins which are mono-, di-, tri-, and tetravalent for N-acetyl-D-galactosamine. J Biol Chem 1981; 256: 3890-3.*
- Goldstein IJ, Winter HG. *The Griffonia simplicifolia I-B4 isolectin. A probe for alpha-D-galactosyl end groups. Subcell Biochem 1999; 32: 127-41.*
- Kirkeby S, Moe D. *Binding of Griffonia simplicifolia I isolectin B4 (GS1 B4) to α -galactose antigens. Immunol Cell Biol 2001; 79: 121-7.*
- LaVecchio JA, Dunne AD, Edge AS. *Enzymatic removal of alpha-galactosyl epitopes from porcine endothelial cells diminishes the cytotoxic effect of natural antibodies. Transplantation 1995; 60: 841-7.*
- Stone KR, Ayala G, Goldstein J, Hurst R, Walgenbach A, Galili U. *Porcine cartilage transplants in the cynomolgus monkey: III Transplantation of α -galactosidase treated porcine cartilage. Transplantation 1998; 65: 1577-83.*
- Luo Y, Wen J, Luo C, Cummings RD, Cooper DK. *Pig xenogeneic antigen modification with green coffee bean alpha-galactosidase. Xenotransplantation 1999; 6: 238-48.*
- Park S, Kim WH, Kim KH, Lee CH, Choi SY, Lee C, Oh SS, Kim KC, Kim YJ. *Removal of alpha-Gal epitopes in aortic valve and pericardium of pig using green coffee bean α -galactosidase. Kor J Thorac Cardiovasc Surg 2008; 41: 12-24.*

11. Treasure T. *Rethink on biological aortic valves for the elderly. Lancet* 1999; 354: 964-5.
12. The United Kingdom Heart Valve Registry Report 1997. *London: UK Heart valve registry, Imperial College School of Medicine, Hammersmith Hospital, 1999.*
13. David TE, Ivanov J. *Is degenerative calcification of the native aortic valve similar to calcification of bioprosthetic heart valves? J Thorac Cardiovasc Surg* 2003; 126: 939-41.
14. Phelps CJ, Koike C, Vaught TD, Boone J, Wells KD, Chen SH, Ball S, Specht SM, Polejaeva IA, Monahan JA, Jobst PM, Sharma SB, Lamborn AE, Garst AS, Moore M, Demetris AJ, Rudert WA, Bottino R, Bertera S, Trucco M, Starzl TE, Dai Y, Ayares DL. *Production of (α)1,3-galactosyltransferase deficient pigs. Science* 2003; 299: 411-4.
15. Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, Ayares DL. *Targeted disruption of the alpha1,3-galactosyltransferase gene in cloned pigs. Nat Biotechnol* 2002; 20: 251-5.
16. Pisani A, Spinelli L, Sabbatini M, Andreucci MV, Procaccini D, Abbaterusso C, Pasquali S, Savoldi S, Comotti C, Cianciaruso B. *Enzyme replacement therapy in Fabry disease patients undergoing dialysis: effects on quality of life and organ involvement. Am J Kidney Dis* 2005; 46: 120-7.
17. Wanner C. *Fabry disease model: a rational approach to the management of Fabry disease. Clin Ther* 2007; 29 (Suppl A): S2-5.
18. Galili U, Latemple DC, Radic MZ. *A sensitive assay for measuring alpha-Gal epitope expression on cells by a monoclonal anti-Gal antibody. Transplantation* 1998; 65: 1129-32.