Expression of human α-galactosidase leads to reduction of major xenoepitope Galα(1,3) Gal in NIH 3T3 cell[1]

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ABSTRACT

AIM: To examine the effects of the expression of α-galactosidase on the expression of the major xenoepitope Gal α(1,3) Gal (G antigen) in NIH 3T3 cell. **METHODS:** The expression levels of G antigen and H antigen and binding of human natural antibodies (IgG and IgM) and complement (C3c) to NIH 3T3 cells were analyzed by flow cytometry. Western blot was employed to further determine the expression of glycoproteins of G antigen. Cytolysis assay with normal human serum was performed by MTT assay. **RESULTS:** In transfectants, Western blot showed that the binding of human IgG to glycosylated proteins located on the cell membrane was decreased, even abrogated totally. Together with the reduced binding of Gs-IB4 (*Griffonia simplicifolia*) to transfectants, the stable expression of human α-galactosidase effectively inhibited Galα(1,3) Gal, Gal epitope synthesis in NIH 3T3 cell. As a result, the xenoreactivities of human IgG, IgM, and C3c were reduced by 73.4 %, 22.3 % and 47.9 %, respectively, while the cell lysis mediated by human XNA and complements was decreased by 42.4 %. **CONCLUSION:** The stable expression of human α-galactosidase in NIH 3T3 cell strongly inhibits the expression of Gal epitopes, resulting in abrupt reduction in xenorejection induced by human serum.

INTRODUCTION

α-Galactosidase is a lysosomal enzyme and widely present in a variety of species with various molecular weight. It can cleave the terminal α-*D*-galactosyl residues from glycosphingolipids and glycoproteins[1-4]. Detailed studies have been done on its important functions. First, the deficient activity of α-galactosidase results in Fabry disease, an X-linked recessive disorder which is pathologically characterized by the lysosomal accumulation of the enzyme's primary globotriaosylceramide and galabiosylceramide. Progressive substrate deposition results in ischemia and infarction of the heart, kidney, and/or brain[5-8]. Second, α-galactosidase can convert B erythrocytes to blood group O under conditions that neither impair their viability *in vitro* nor survival ability normally after transfusion to individuals of groups O, A, and B[9,10].

Xenotransplantation is actively pursued as a potential solution to the current acute shortage of donor organs for human beings. A major obstacle to xenotransplantation is the presence of xenoreactive natural antibodies (XNA) in human beings that react with mol-

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ecules on foreign cells from animals, particularly foreign endothelial cells in vascularized organs, which are known as xenorejection. It has been demonstrated that most of the xenorejection is caused by the carbohydrate epitope Gal α(1,3) Gal (Galα1-3Galβ-4GlcNAc-R). The treatment of porcine endothelial cells with α-galactosidase *in vitro* resulted in reduction of the xenorejection induced by human natural antibodies[11]. These studies imply a great potential applications of α-galactosidase in overcoming xenorejection for xenotransplantation.

In this study, the cDNA of human α-galactosidase was cloned from human liver tissue and consistently expressed in NIH 3T3 cells under the control of CMV promoter to study the influence of human α-Galactosidase on Gal α(1,3) Gal and consequent xenoreactivity in NIH 3T3 cells.

MATERIALS AND METHODS

Cloning of human α-galactosidase cDNA RNA of human hepatic cells was extracted with Trizol reagents (GIBCO) and identified by electrophoresis on 1.2 % agarose gel. cDNA was obtained by reverse transcription-polymerase chain reaction technique (RT-PCR). The PCR reaction was performed with sense primer (P1: 5' gcgaattccatgcagctga ggaacccagaactaca 3') and anti-sense primer (P2: 5' ggcggccgcttaaagtaagtct-tttaatgacatctgcat 3') at 95 ºC 30 s, 57 ºC 30 s, and 72 ºC 1 min 20 s for 30 cycles.

Expression vector The expression vector used in this study was the CMV immediate early promoter containing pcDNA3. The recombinant plasmid pcDNA3-Galase was constructed by subcloning a stretch of cDNA fragment coding for human α-galactosidase with both ends of *Eco*RI and *Not*I into the corresponding sites of pcDNA3, down stream the site of CMV promoter sequence which controls the expression of human α-galactosidase. Control experiments were performed using the empty vector, pcDNA3.

Transfection of NIH 3T3 cells with plasmid pcDNA3-Galase Plasmid pcDNA3-Galase was purified by Qiagen kit. Transfection was performed essentially by calcium phosphate as described. Briefly, cells were plated at a density of 1×10^6 cells per 60 mm dish. After 24 h, media were removed 3 h before transfection. Plasmid 10 μg in 500 μL reaction system was used per transfection. The cells were incubated for 24 h at 37 ºC and then the media were renewed for continuing incubation. G418 1 g/L was added into media for getting stable cell lines 2 d later. Cell clones were obtained and analyzed 15 d later.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) RNA was extracted with Trizol reagent (GIBCO) according to the manufacturer's instruction and identified by electro-phoresis. PCR was performed as described previously. GAPDH mRNA was co-detected with primer 1 (5' acgacccccttcattgacc 3') and primer 2 (5' agacaccagtagactccacg 3'). The concentration of resultant PCR products of GAPDH mRNA were referred to normalize the expression level of human α-galactosidase in the host cells. Relative intensity of PCR products was estimated by a Molecular Image FX System (Bio-Rad).

Flowcytometric analysis Single cell suspensions were prepared at a density of 1×10^6. For IgM, IgG, and C3c complement binding assays, cells were incubated in 200 μL 10 % normal human serum (NHS) at 4 ºC overnight and then incubated with FITC-conjugated rabbit anti-human IgM, IgG or C3c antibody(DAKO) at 1:50 dilution at 37 ºC for 30 min. For C3c complement binding analysis, edetic acid 10 mmol/L was added to 10 % NHS to inhibit complement activation. FITC-conjugated Gs-IB4 (Sigma) and UEA-I (Ulex europaeus-I) (Sigma) were incubated at 4 ºC for 30 min to analyze G antigen and H antigen.

Western blot Cell membranes were prepared as following. Cells were pelleted by centrifugation and resuspended in a lysis buffer (Tris-HCl 5 mmol/L, edetic acid 5 mmol/L, egtazic acid 5 mmol/L). Large cellular debris were removed by centrifugation at 500×*g* for 5 min. The cell membrane in supernatant was then pelleted by centrifugation at 10 000×*g* for 30 min.

Samples were run on 10 % SDS-PAGE and then transferred to nitrocellulose membrane. The membrane was first incubated with normal human serum (1:10 dilution) and subsequently with HRP-conjugated rabbit anti-human IgG antibody (1:500 dilution). Finally membrane was developed with a chemiluminescent detection kit (ECL Western blot Detection kit). Protein bands on the film were scanned and their intensities were quantified by a Molecular Image FX System (Bio-Rad).

Cell lysis assay Cell lysis assay with normal human serum was performed with Thiazolyl Blue (MTT, Sigma). Briefly, cells transfected with pcDNA3-Galase were seeded in 96-well plate at 1×10^7/L. Media were removed 48 h later and cells were incubated at 37 ºC for 30 min, mixed with 50 mL of normal human serum

(serial dilutions). DMEM 200 μL containing MTT 15 μL was added and incubated at 37 ºC for 4 h. Then 50 μL lysis buffer (10 % SDS-5 % isobutanol-0.01 mol/L HCl) was added into wells and reacted at 37 ºC overnight. The absorbance at 570 nm/630 nm was detected with Universal Microplate Reader.

Statistical analysis The data were presented as mean±SD. Statistical difference was assessed by *t*-test.

RESULTS

Cloning of human α-galactosidase cDNA A 1.3 kb cDNA coding for human α-galactosidase from human hepatic cells was prepared by RT-PCR (Fig 1) and its sequence was identified by DNA sequencing technique. The cDNA fragment was cloned into pcDNA3 at the *EcoR*I-*Not*I site to construct the recombinant plasmid pcDNA3-Galase (Fig 1).

Expression of human α-galactosidase in NIH 3T3 cells Human α-galactosidase could be stably expressed in NIH 3T3 cells transfected with pcDNA3-Galase. Its expression was examined by semi-quantitative RT-PCR. In mock the result was negative, whereas the transfectants got higher expression of the interest product (Fig 2).

Expression of human α-galactosidase inhibited the appearance of Galα(1,3)Gal epitope on NIH3T3 cell membrane Galα(1,3)Gal is a terminal disaccharide on glycoproteins and glycolipids and mostly distributes on cell surface. In this study, Western blot was employed to detect the expression of Galα(1,3) Gal epitope on the membrane of mock and transfectants. Compared with controls, the glycoprotein bands equal to about 98 kDa and 56 kDa were totally diminished

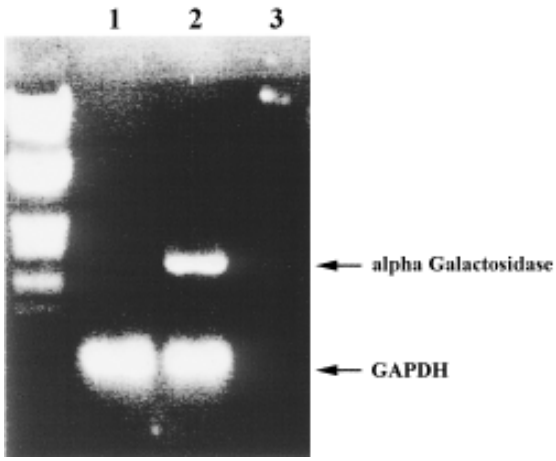

Fig 2. Semi-quantitative RT-PCR showed that human α galactosidase was highly expressed in the transfectants. GAPDH was selected as intrinsic standard. Lane 1, 2, and 3 are the samples from mock, alpha galactosidase transfectants and negtive control.

and the densities of the bands equal to about 107 kDa, 88 kDa, 40 kDa, and 37 kDa were decreased in transfectants, whereas the bands equal to about 80 kDa did not change (Fig 3). The results indicated that the biosynthesis Gal epitope was inhibited by human α-galactosidase.

The cells were immunostained with FITC-conjugated Gs-IB4 lectin which is special for the terminal Galα(1,3) Gal residues (G antigen) or FITC-conjugated UEA-1 which is special for terminal α-*L*-fucose residuses (H antigen). All the human α-galactosidase transfectants reacted weakly to Gs-IB4, while strongly to UEA-1 compared with controls (Fig 4). G antigen was decreased approximately by 57.4 %, whereas H anti-

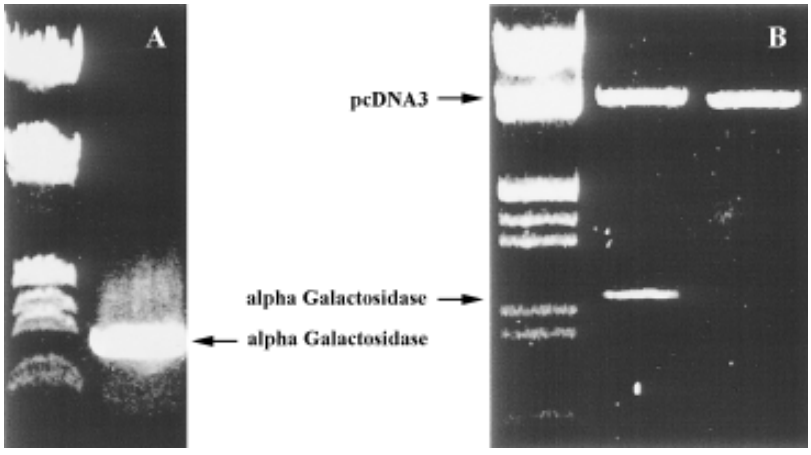

Fig 1. cDNA of alpha galactosidase (1.3 kb) was obtained from human hepatic cell by RT-PCR (A) and inserted into the EcoR1-Not1 site of pcDNA3. B is the digestion of recombinant plasmid pcDNA3-Galase.

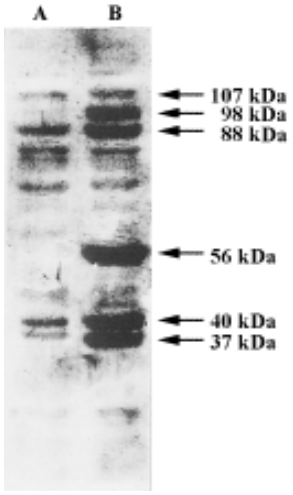

Fig 3. Western blot showed the membrane glycosylated protein changed (arrow) obviously in the transfectants of α galactosidase. A and B present the samples from alpha galactosidase transfectants and mock.

gen increased 6 fold.

Human α-galactosidase reduced the xeno-rejecting reactivity of human natural antibodies and inhibited antibody-mediated lysis Controls and transfectants were examined by flowcytometric analysis for their ability to bind human natural antibodies. Compared with controls, the fluorescence obviously subsided in transfectants (Fig 5). The positive counts for IgG, IgM, and C3c were reduced by 73.4 %, 22.3 %, and 47.9 %, respectively.

To assess the functional significance of the observed reduction, we further analyzed the complement-mediated lysis (Fig 5). The results indicated that the transfectants were much more sustaining to human serum-mediated cell lysis compared with mock.

DISCUSSION

The urgent shortage of donor organs forces scientists to throw highlights on xenotransplantation. From the respects of physiology and economics, porcine organs offer a potential source to xenografts for human beings. However, such trans-species transplantation results in immunological rejections, primarily mediated

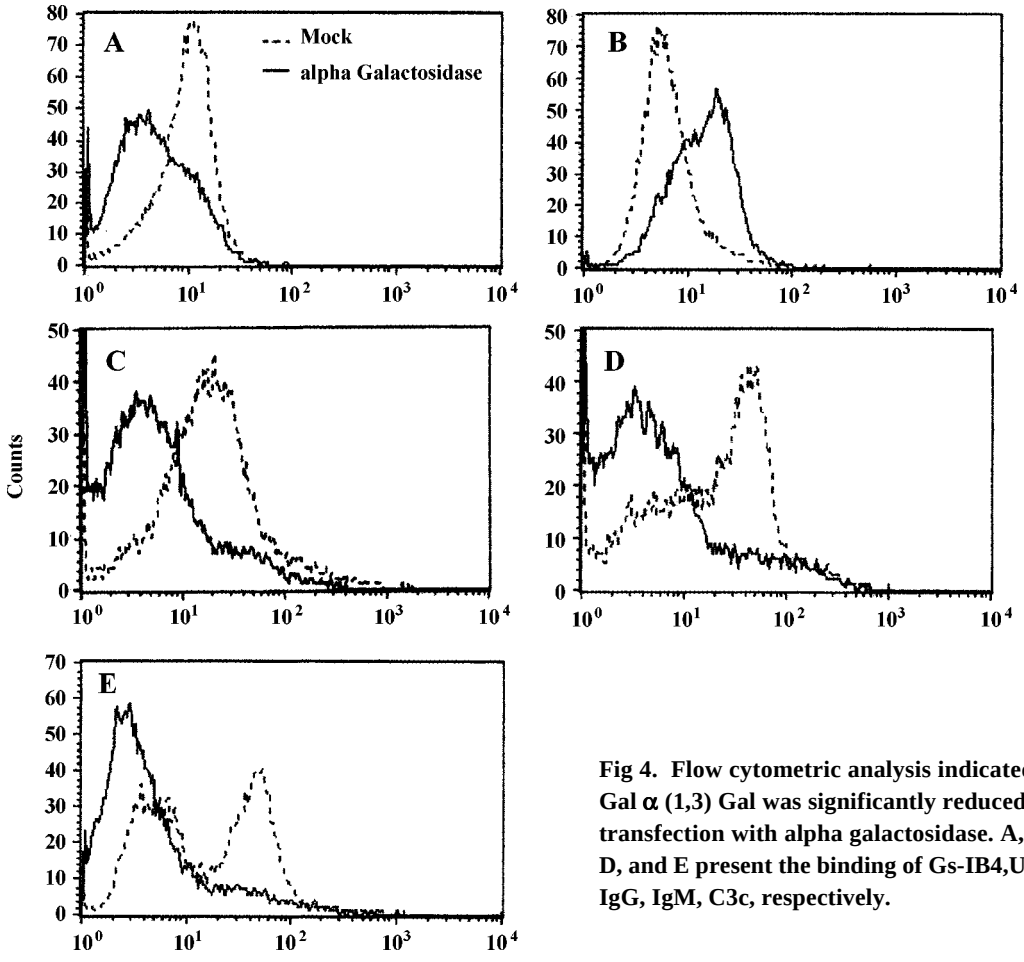

Fig 4. Flow cytometric analysis indicated that Gal α (1,3) Gal was significantly reduced after transfection with alpha galactosidase. A, B, C, D, and E present the binding of Gs-IB4,UEA-I, IgG, IgM, C3c, respectively.

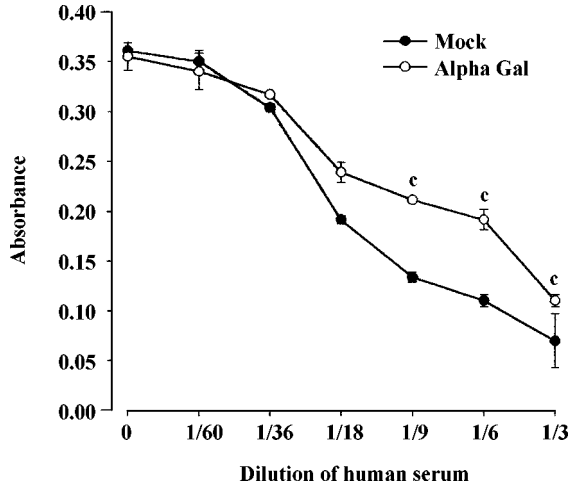

Fig 5. Cell lysis mediated by human serum is inhibited by alpha galactosidase. *n*=3. Mean±SD. $^{c}P<0.01$ *vs* Mock.

by the interaction of the human natural anti-Gal antibody with Gal epitopes abundantly expressed in swine and mice as well as other species[12-14]. Since Gal epitopes are synthesized by the glycosylation enzyme α(1,3) galactosyltransferase (α 1,3 GT), a lot of studies have been done to reduce the expression of α-gal epitopes by raising transgenic animals with glycosyltransferases competing with α(1,3) GT or by eliminating this epitope with knock-out technique. The expression of Gal epitopes can be markedly reduced in α1,2 fucosyltransferase [α(1,2) FT] transgenic mice[15-17]. In that case, expression of α-gal is down-regulated by enzyme competition between the α(1,3)GT and α(1,2)FT for the common *N*-acetyl lactosamine acceptor in the trans Golgi network. These studies implied that Gal epitopes were not required for mouse development and raised the hope of successfully inhibiting Gal epitope expression. In this study, human α galactosidase was steadily transfected into NIH 3T3 cells. The results demonstrate that the expression of human α galactosidase can effectively inhibit Galα(1,3)Gal expression and, in turn, the immunorejection induced by human XNA, implying a great and practical potency of human α galactosidase for gene therapy in xenotransplantation.

Generally speaking, anti-Gal antibody, a natural polyclonal antibody which constitutes approximately 1 % of circulating immunoglobulins in human beings, apes and Old World monkeys[18], mainly consists of IgG isotype as well as IgM and IgA isotypes[8,19], while a few people think they are mainly IgM isotype[20] . Many studies indicate that IgG and IgM play different roles in immunorejections. Typically, the immunorejection is divided into three phases, namely hyperacute rejection (HAR), delayed xenograft rejection (DXR), and T-cell mediated rejection. HAR, characterized pathologically by interstitial hemorrhage and diffuse thrombosis, begins immediately after xenotransplantation or perfusion with foreign serum. This process inevitably destroys the graft within minutes to a few hours. Studies proved that HAR was mainly mediated by IgM[21]. Binding of anti-Gal IgM to porcine cells induces, in general, complement-mediated lysis of cells[22,23]. On the other hand, some results indicate that it is IgG, but not IgM, mediates HAR in hepatic xenograft[24]. Furthermore, the human and primate immune systems react vigorously against the porcine α-galactosyl epitope by producing a great deal of high affinity anti-Gal IgG molecules, which are likely to be detrimental to the xenograft via mechanisms other than complement-mediated lysis. During DXR, IgG fixed to endothelial cell can bind NK cells via the FcγIII(CD16) receptor, whereas the role of IgM appears to be less significant[25]. In our study, flowcytometric analysis showed that the binding of human IgG decreased by 73.4 %, three times higher than those of IgM (22.3 %), whereas the binding of C3c reduced by 47.9 %. This result indicated that the stable expression of α-galactosidase in NIH 3T3 cell exerted more prominent inhibition on the xenoreactivities mediated by human IgG than those mediated by IgM. And it may also offer an evidence to confirm that IgG is the main part of anti-Gal antibody. Combining the decrease in the binding of C3c, the inhibitory effect of α-galactosidase implies that the expression of α-galactosidase may be a potential solution for the immunorejection mediated by IgG and IgM as well as complement system during hyperacute rejection and delayed xenograft rejection.

In conclusion, the stable expression of human α-galactosidase in NIH 3T3 cell strongly inhibits the expression of Gal epitopes, resulting in abrupt reduction in xenorejection induced by human serum. However, recent study[26] shows that expression of Gal epitopes in porcine organs is many fold higher than mice organs, which raises the question whether pigs can properly develop without expression of Gal epitopes. According to our results in this study and former study[27], the stable expression of human α-galactosidase strongly inhibits, but not totally abrogates the expression of Gal epitopes and xenoreactivity mediated by human XNA. Therefore, more efforts are required before the application of human α-galactosidase in xenotransplantation.

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