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Human xenoreactivity is reduced in mice bearing porcine antisense α(1,3) galactosyltransferase cDNA[1]

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KEY WORDS epitopes; galactosyltransferases; heterologous transplantation; fluorescent antibody technique; antisense DNA; reverse polymerase chain reaction; Southern blotting; swine

ABSTRACT

AIM: To explore the effect of antisense α(1,3) galactosyltransferase α(1,3) GT cDNA on production of Gal α (1,3) Gal (Gal epitope) xenoantigen *in vivo*. **METHODS**: Transgenic mice bearing the porcine antisense α(1, 3) GT cDNA (nt 1α – 640) were generated by pronuclei microinjection method. The integration of transgene was identified by PCR and Southern-blot analysis. The expression of murine α(1,3) GT was characterized by RT-PCR. Morphology of the spleen was examined by histological technique. Gal epitope was detected by immunofluorescent analysis. Binding of human natural xenoantibodies (IgM and IgG) and complement (C3c) to cells from mice was determined by flow cytometric assay. **RESULTS**: Transgenic mice bearing the porcine antisense α(1,3) GT cDNA were born healthy and developed normally. However, necrosis occurred in the spleen of some mice heterozygous for transgene. Cell surface Gal epitope in transgenic heterozygotes was evidently reduced. Substantially less (30 % – 60 %) xenoantibodies in human serum bound to cells from a variety of tissues of transgenic heterozygotes compared with wild-type controls. Consequentially, human complement activation on cells from these mice was reduced by 40 % – 50 %. **CONCLUSION**: Human xenoreactivity could be effectively reduced by inhibiting the expression of α (1,3) galactosyltransferase with an antisense gene.

INTRODUCTION

The current shortage of human organs available for transplantation has stimulated a re-evaluation of xenotransplantation as a potential solution. Pigs are currently viewed as the preferred donors for clinical xenotransplantation because of their similarity in many aspects to humans[1,2]. However, transplants from pigs to primates are rapidly rejected by a complement-mediated process termed hyperacute rejection (HAR)[3]. This process is initiated by the binding of xenoreactive natural antibodies (XNA) to carbohydrate structures present on the donor vascular endothelium, followed by activation of the host complement and coagulation cascades, leading to interstitial hemorrhage, intravascular coagulation, and ischemic necrosis[4].

The major xenoantigen on murine and porcine cells and tissues which is recognized by naturally occurring xenoantibody in human plasma is galactose α(1,3) galactose (Gal epitope), a terminal disaccharide on glycoproteins and glycolipids[5-7]. The α(1, 3) galactosyltransferase enzyme α(1,3) GT; EC 2.4.1.51 that forms the Gal epitope is present in all mammals with the exception of humans, apes, and Old World monkeys, in which the gene has been inactivated by frame shift and nonsense mutations[8]. As a result, humans develop high-titer anti-αGal antibodies (both IgM and IgG) against the Gal epitope, probably due to continual exposure to Galα(1, 3) Gal on the surface of enteric bacteria and other pathogens[9].

The importance of the Gal epitope to xenograft rejection was initially demonstrated by carbohydrate competition studies, in which only carbohydrates containing terminal α-galactose residues could inhibit the binding of human XNA to porcine endothelial cells[6,7,10,11]. In other

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experiments, transfection of COS cells, which are derived from Old World monkey and therefore lack the Gal epitope, with α(1,3) GT cDNA rendered them sensitive to human serum-mediated cytotoxicity[11]. Most significantly, human serum that had been absorbed with COS cells expressing the α(1,3) GT no longer reacted with porcine endothelial cells.

Genetic modification of the donor is currently considered as the preferred option to overcome HAR. Strategies aimed at limiting complement activation include the transgenic expression of human complement regulatory molecules such as CD 46 (MCP), CD 55 (DAF), and CD 59 on the vascular endothelium[12,13]. Unfortunately, it would appear that complement regulation alone will be insufficient to prevent HAR[14,15]. Other strategies depend on modifying the Gal epitope from the donor. Recently, Gal knockout mice (Gal KO) have been generated[2], in which the α(1,3) GT gene had been inactivated by homologous recombination. Hearts from these mice exhibited prolonged survival compared with wild-type control when perfused *ex vivo* with human plasma, confirming that Gal α(1,3) Gal antigen plays a critical role in xenograft rejection. However, the present unavailability of porcine embryonic stem cells precludes the application of this approach to the pig, thus prompting investigation of alternative strategies to reduce Gal epitope. The most successful of these to date has been the transgenic expression of human α(1,2) fucosyltransferase [α(1,2) FT; EC 2.4.1.69], which compete with α(1,3) GT for the same substrate *N*-acetylactoamine and caps it with the non-immunogenic fucose. Expression of α(1,2) FT in mice[3,16] and pigs[3,17] caused decreased expression of the Gal epitope, and consequently in partial protection from human complement-mediated damage *in vitro*. On the other hand, specifically inhibiting the expression of α(1,3) GT with antisense RNA generated from an inverted gene may be another attractive method to achieve the same goal. Here we described the application of this approach in reducing human xenoreactivity.

MATERIALS AND METHODS

Production of transgenic mice Porcine α(1,3) GT cDNA (1.2 kb) was obtained by RT-PCR employing whole RNA extracted from the spleen of pig, subsequently verified by sequencing analysis (data not shown). A 640 bp fragment from the 5′ terminal of the α(1,3) GT cDNA [nucleotides: (+) 1 – (+) 640; GenBank Accession: L36152] was cloned, in the inverted orientation under the control of human cytomegalovirus (HCMV) promoter/enhancer, into the *EcoR* I and *Xho* I site of pcDNA3 vector (Fig 1a). This construct, linearized with *Nru* I and *Tth111* I and then gel-purified, was microinjected into the pronuclei of fertilized eggs of (C57BL/6J) F1 hybrid mice (Jackson Laboratory). Founders and their progenies and age-matched wild-type control mice were housed in groups (< 4 per cage) in temperature- and humidity-controlled environment with a 12 h-light/12 h-dark rhythm.

Genotypic characterization of transgenic mice For Southern-blot analysis, 10 μg genomic DNA extracted from mouse tail was digested with *Nde* I and *Xho* I, fractioned by electrophoresis on 1.0 % gel, and transferred to a nylon membrane (Amersham), followed by hybridization with a randomly [^{32}P]-labeled *EcoR* I – *Xho* I fragment excised from the transgenic construct (Fig 1a). After hybridization, blots were washed and exposed against an X-ray film for 24 h.

For nest PCR analysis, the primers (P2: 5′ AAGAATTCTGGTCTTCATGCGCATCATGCT 3′; P3: 5′ TAACTCGAGCATGAGGAGAAAATAATG 3′; underlined sequences contain *EcoR* I and *Xho* I site, respectively) were designed to match nucleotides of α(1,3) GT cDNA. Another primer (P1: 5′ TGGAGTTCCGCGTTACATAACTTACG 3′) is located in the upstream of the CMV promoter region. With P2-P3 and P1-P3 primer pairs, respectively, amplification was carried out as follows: 94 ℃ for 45 s, 48 ℃ for 45 s, and 72 ℃ for 60 s; 34 cycles.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis Kidney was removed from 2-month-old wild-type and heterozygotic transgenic mice after cervical dislocation. Whole RNA was extracted with Trizol reagent (GIBCO) according to the manufacturer, then identified by formaldehyde-electrophoresis. Before reverse transcription, RNA samples were thoroughly treated by incubating with RNA-Free DNase I (5 MU/g RNA; Promega) for 45 min at 37 ℃. PCR was performed with the P3 primer above mentioned and another P4 primer (5′ TGCTGGATGTGGGCCAGG 3′) located outside the corresponding region to the porcine α(1,3) GT (nt 1 – 640) in the murine cDNA [GenBank Accession: M26925; P4 primer location: nt (+) 984 – (+) 1002]. GAPDH mRNA was co-detected with primers (5′ ACGACCCCTTCATTGACC 3′; 5′ AGACACCAGTAGACTCCACG 3′) which matched from (+141) to (+344) of murine GAPDH cDNA. Resultant PCR products were referred to normalize the

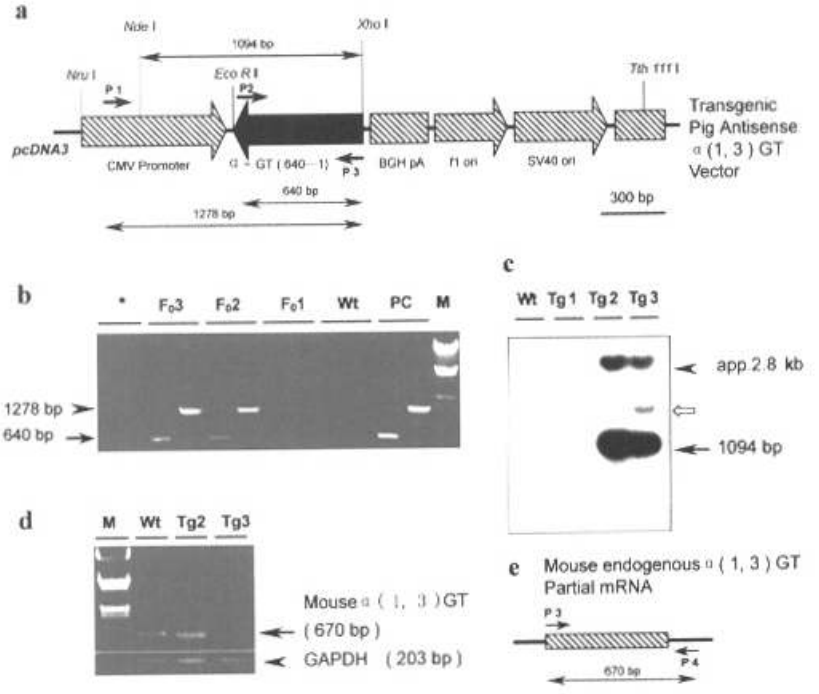

Fig 1. a. Schematic illustration of the porcine antisense α(1,3) GT transgene construction. b. Nest PCR identification of the integration and integrity of the porcine antisense α(1,3) transgene in independent transgenic founders. Primer positions and the predicated size of PCR products are indicated in Fig 1a. M, λ/*Hind* III & *EcoR* I; PC, positive control (plasmid); Wt, wild-type mouse; F0, Founder mouse. Asterisk (*) indicates the mouse bearing the truncated transgene which is absent of the CMV region. c. Southern-blot analysis of the transmission of transgene. Genomic DNA 10 μg extracted from tails was digested with *Nde* I and *Xho* I, electrophoresed, blotted, and hybridized with [^{32}P]-labeled porcine α(1,3) GT cDNA. Lower arrow shows the predicted band, whose location and size are indicated in Fig 1a; upper arrowhead indicates the approximately 2.8 kb band resulting from asymmetrical digestion of the tandem copies of transgene; middle hollow arrow indicates the band unexpectedly yielded with unknown mechanism. d. Semi-quantitative RT-PCR analysis of the murine α(1,3) GT expression in the kidney of transgenic heterozygotes raised from two independent founders (Tg2 and Tg3). GAPDH was served as an internal standard. Note the murine α(1,3) GT expression in the Tg3 mice was obviously reduced. e. Strategy of the RT-PCR described in Fig 1d. Shadow box represents the corresponding region to the porcine α(1,3) GT cDNA (nt 1 – 650) indicated in Fig 1a.

expression level of murine α(1,3) GT. Relative intensity of PCR fragments was estimated with a Molecular Image FX System (Bio-Rad).

Histological and immunofluorescent analysis

For histological analysis, 2-month-old wild-type and heterozygotic transgenic mice were anesthetized with sodium pentobarbital, and subsequently perfused intracardially with 4 % paraformaldehyde in 0.1 mol/L phosphate-buffer. After perfusion, spleens were removed and then dehydrated through up-graded ethanol, and then cleared with xylene, finally embedded in paraffin. Sections 5 μm-thick were cut on microtome (Leitz) and stained with haematoxylin and eosin according to the standard methods, then examined and photographed under Leitz microscope.

For immunofluorescent detection, after perfusion procedure described above, a variety of tissues were surgically removed and then immersed in 30 % sucrose in Tris buffered-saline (TBS), subsequently frozen in OCT and 20 μm-thick cryosections were cut and air-dried onto the gelatin-coated slides. In staining procedure, sections were first blocked with 10 % goat serum containing 1 % bovine serum albumin and 0.2 % Triton X-100 in TBS, and then incubated with fluorescein isothiocyanate (FITC)-conjugated IB_4 lectin (isolated from *Griffonia simplicifolia*; Sigma) in TBS containing $CaCl_2$ 0.01 mol/

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L at 4 ℃ for 90 min, and subsequently intensively rinsed with TBS, then finally cover-slipped with TBS buffered-glycerol. Slides were examined and photographed under Olympus fluorescence microscope.

Flow cytometric analysis Single-cell suspensions were prepared from a variety of tissues by mechanical isolation and subsequent filtration through a hole-size optimized nylon membrane. Before staining procedure, erythrocytes were lysed by treatment with NH_4Cl 0.168 mol/L. For IgM and IgG binding assay, cell samples were incubated with 200 μL of diluted normal human serum (NHS; 2.5 % for IgM, and 1.25 % for IgG) at 37 ℃ for 10 min, and intensively washed, and then incubated with FITC-conjugated rabbit anti-human IgM antibody (specific for μ chain) and FITC-conjugated rabbit anti-human IgG antibody (specific for γ chain, DAKO) at 1:50 dilution at 4 ℃ for 45 min, respectively, subsequently washed again, finally suspended in 400 μL PBS buffer and analyzed by FACScan assay (Becton Dickinson).

For C3c complement binding assay, cells were incubated in 200 μL 10 % NHS at 37 ℃ for 10 min, washed, then incubated with FITC-conjugated rabbit anti-human C3c antiboby (DAKO) at 1:50 dilution at 4 ℃ for 30 min. Cells incubated with NHS containing edetic acid 10 mmol/L (to inhibit complement activation), and cells incubated with anti-C3c antibody alone, were served as control for non-specific antibody deposition. IgM, IgG, and C3c binding were quantified by flow cytometry and expressed as mean channel fluoresence (MCF) of triplicate samples.

Statistical analysis Experimental values were expressed as $\bar{x} \pm s$. Statistical differences between groups were assessed by analysis of variance and t-test.

RESULTS

Generation of transgenic mice Linearized DNA construct carrying the porcine α(1,3) GT cDNA (*nt 1 – 640*), in the inverted orientation under the control of CMV promoter, was introduced into the C57BL/6J F1 hybrid mice germ line by microinjection method (Fig 1a). To characterize the integration and integrity of the transgene, nest PCR analysis was performed (Fig 1b). With the primer pairs (P2 and P3, indicated in Fig 1a) specifically designed to α(1,3)GT cDNA, 4 mice out of 58 newborns yielded the predicted 640 bp fragment. Further amplification using the primer (P1, indicated in Fig 1a) located in the upstream of CMV element and the reverse primer matched with α(1,3) GT cDNA (P3) demonstrated that only 3 mice yielded the expected 1278 bp fragments. It meant that these three mice termed founder mice carried the intact transgene, whereas the 1278 bp fragment-absent one bore a truncated copy. All founder mice were bred with wild-type mice to generate heterozygotes, which were identified by Southern-blot analysis. As shown in Fig 1c, offspring (Tg2 and Tg3) raised from two independent founders yielded the predicted 1094 bp fragment and another approximately 2.8 kb fragment resulting from asymmetrical digestion of tandem copies of transgene by *Nde* I and *Xho* I endonuclease. Neither fragment could be observed in wild-type control and all 17 progenies (Tg1) derived from the other founder, which indicated that the transgene was probably excluded in Tg1 mice during generation transmission. It was previously reported that CMV promoter could facilitate the expression of the target gene, under its control, in the kidney in most transgenic models[18,19], therefore, we determined the mRNA level of endogenous murine α(1,3) GT, by semi-quantitative RT-PCR analysis, to indirectly evaluate the expression of porcine antisense α(1,3) GT transgene in the kidney. Employing the identical P3 primer and another P4 primer (Fig 1e) located outside the corresponding region to the porcine α(1,3) GT cDNA (nt 1 – 640), murine α(1,3) GT mRNA yielded a specific band (Fig 1d), whereas RNA derived from the transgene could not be amplified. The resultant 670 bp fragment was subsequently convinced by sequencing analysis to possess 100 % similarity to the murine α(1,3) GT mRNA (data not shown). Quantitative analysis demonstrated that the amounts of murine α(1,3) GT mRNA was apparently reduced in Tg3 mice compared with wild-type mice, which possibly resulted from the inhibition of the transcription of endogenous gene by RNA transcribed from its antisense copy. However, no reduction in the amounts of endogenous α(1,3) GT mRNA could be observed in Tg2 mice. It indicated that the transgene was transcribed by varying efficiency with poorly known mechanism, probably associated with the insertional position, in mice from independent founders[20].

Necrosis occurred in the spleen of some transgenic mice Transgenic mice bearing the porcine antisense α(1,3) GT cDNA were born health, and developed normally. Histological examination demonstrated that no any abnormality was detected in all examined organs except spleen. Apparently, necrosis occurred in partial region of the spleen from some transgenic mice. In contrast sharply with wild-type mice, spleens from

these mice exhibited decreased density of cells and increased necrosis foci (Fig 2). Since the phenotype was observed in heterozygotic progenies raised from both independent founders, it should not result from an insertional mutation.

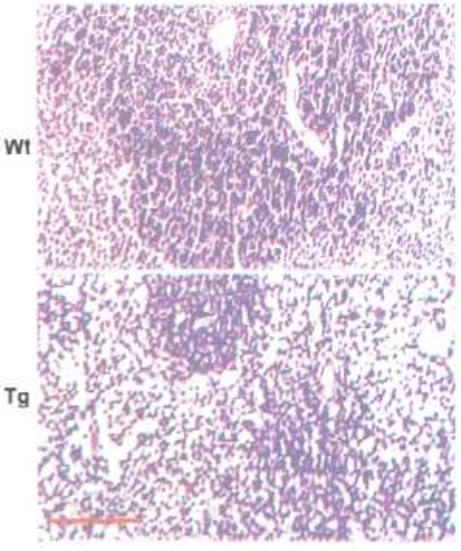

Fig 2. Histological appearance of the spleen. Photomicrograph shows the apparent necrosis in the spleen of transgenic mice bearing the porcine antisense α(1,3) GT cDNA. Samples taken from 20-sections of the wild-type (Wt, $n = 4$ mice) and transgenic mice (Tg, $n = 5$ mice). Scale bar represents 100 μm. HE staining paraffin-embedded 5 μm thick-sections. ×150.

Cell surface Gal epitope decreased in transgenic mice As shown in Fig 3, in contrast to tissues from wild-type mice, which were strongly stained, tissues from transgenic mice exhibited moderate intensity of IB4-reactivity. It indicated that the Gal α(1,3) Gal was partially eliminated, though not thoroughly, in transgenic mice compared with wild-type mice. The reduction in the amounts of Gal epitope possibly suggested the down-regulation of the expression of α(1,3) GT protein due to the inhibitory effect of antisense RNA.

Cells from transgenic mice exhibited attenuated reactivity to human serum Cells prepared from a variety of tissues were incubated with diluted human serum, and the bound xenoreactive antibodies were detected by FITC-conjugated anti-human IgM and IgG antibody and assayed by flow cytometric analysis. Median channel fluorescence was used to compare levels

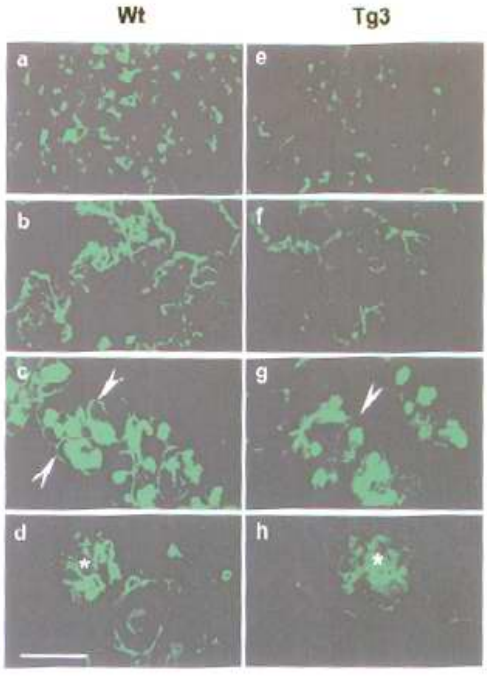

Fig 3. Immunofluorescent detection of the cell surface Gal epitope in the spleen (a, e), lung (b, f), pancreas (c, g), and kidney (d, h) from wild-type (Wt, left column) and transgenic mice (Tg3, right column) with Gal α(1,3) Gal-specific IB_4 lectin. Arrowheads in c and g point to the membrane of the pancreatic acinar cells; asterisks in d and h indicate the glomerulus. Scale bar represents 50 μm in a, b, c, d, e, f, and g (×140) and 100 μm in d and h (×280), respectively. Cryosections 20 μm thick.

of antibody binding. Statistical analysis demonstrated that the binding of XNA (both IgM and IgG) in human serum to various kinds of transgenic cells was reduced by 30 % – 60 % depending on cell types, relative to wild-type cells (Fig 4a, 4b). It was attributed reasonably to the reduction in the amounts of Gal epitope, the major xenoantigen recognized by XNA. We further determined the deposition of C3c, a component of activated complement[2], on the cell surface. Similarly, less C3c (40 % – 50 % depending on cell types) was deposited on a variety of cells from transgenic mice than cells from wild-type mice (Fig 4c). It indicated that complement activation was attenuated due to the reduction in the binding of xenoantibodies to cells.

DISCUSSION

The immediate barrier to the transplantation of

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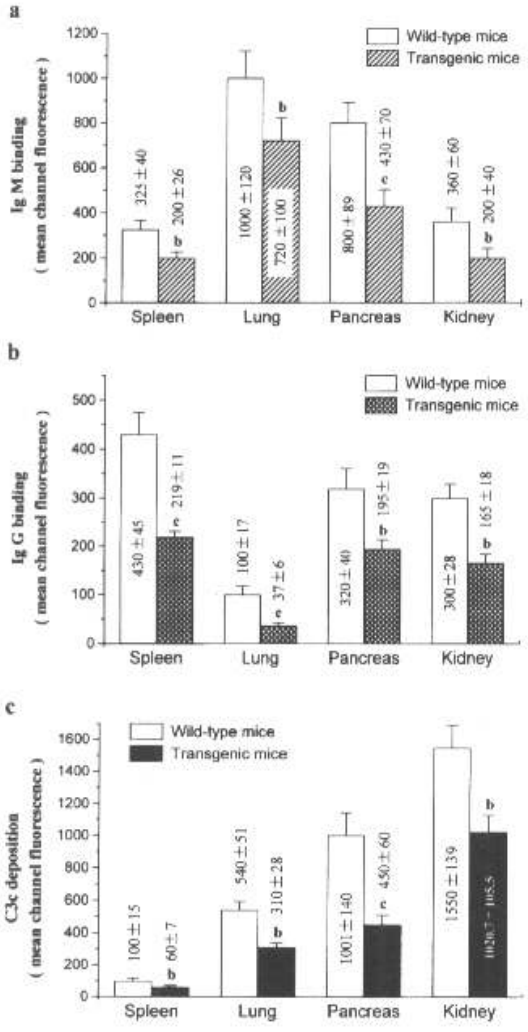

Fig 4. Flow cytometric analysis of the binding of human serum IgM (a), IgG (b) xenoantibodies, and C3c complement (c) on cells prepared from spleen, lung, pancreas, and kidney of wild-type and transgenic mice (Tg3 heterozygotes). Dilution of normal human serum is 2.5 % for IgM, 1.25 % for IgG, and 10 % for C3c, respectively. $n = 4$ mice (twice in each separate experiment). $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* wild-type mice.

vascularized pig organs to primates is hyperactuate rejection, which is initiated by the binding of XNA and activation of complement cascade[3,4]. The major target of human XNA in pigs is the Gal epitope synthesized in the trans-Golgi by α(1,3) GT[8,21]. Inactivating the α(1,3) GT gene by gene targeting has been proposed as a means of totally and permanently depleting the Gal epitope, thereby reducing the involvement of XNA. However, gene knockouts currently are not technically feasible in pigs. Since transgenic pigs can be generated and bred, we have focused our interests on the strategy of antisense RNA inhibition. The concept of inhibiting a specific gene by an antisense gene has been used effectively in a number of cell transfection experiments[22-24]. Successful, though relatively few, application in animals also have been reported[24-26]. As a first step, we have generated mice bearing the porcine antisense α(1,3) GT cDNA, and have preliminarily evaluated the xenoresponse of human plasma to cell from these mice for further study with pigs.

Construct for microinjection harbors a fragment of porcine α(1,3) GT cDNA containing 5′ terminal untranslated sequence and partial coding region, in the inverted orientation under the control of CMV promoter. We previously convinced that transfection of NIH3T3 cells with this construct significantly reduced the constitutive expression of Gal epitope, and further reduced the sensitivity of these cells to human serum-mediated cytotoxicity (unpublished data). With this construct, three founders bearing the various copies of intact transgene were generated at a relatively low integration ratio (5.2 %). In the process of breeding heterozygotes, the transgene was found to be excluded in the progenies derived from one founder, suggesting that selective pressure operated against these mice during development. Although developing normally, increased necrosis occurred in the spleen of some transgenic heterozygotes raised from two independent founders. The cause of this pathologic abnormality and its potential consequence remains to be further explored. It was noteworthy that our mice have not developed cortical cataracts, occurring in the α(1,3) GT knockout mice and may present ethical and animal husbandry problems[2].

The amounts of murine α(1,3) GT mRNA was considerably reduced in the heterozygotic mice derived from one founder compared with wild-type mice, which indicated that the inhibition of the expression might occur in transcription process. It is currently considered that antisense RNA (minus-strand RNA complementary to particular RNA) inhibits the expression of the corresponding gene by various mechanisms, such as interfering with transcription, down-regulating the stability of mRNA, inhibiting translation[22-26]. Hence, although the progenies from another founder mouse showed no reduction in the level of endogenous α(1,3) GT mRNA, it did not mean the failure to inhibit the production of α(1,3) GT protein.

We particularly determined the expression of Gal

epitope in a variety of tissues from transgenic heterozygotes, raised from the founder in which endogenous α(1, 3) GT mRNA was evidently down-regulated. As expected, there was a substantial reduction in the level of Galα(1,3) Gal though considerable amounts of this carbohydrate structure still remained. It was attributed to the introduction of porcine antisense α(1,3) GT cDNA into the mice. Presumably, Gal epitope would be further inhibited in the mice homozygous for transgene. The reduction in the amounts of Gal epitope was functionally significant, as evidenced by less (30 % – 60 %) binding of naturally occurring IgM and IgG present in human serum to a variety of cells from transgenic mice than to cells from wild-type mice. Furthermore, the activation of complement was consequentially reduced by 40 % – 50 % depending upon cell types. In the future study, we will employ *ex vivo* model of heart perfusion with human serum to investigate whether hearts from mice bearing porcine antisense α(1,3) GT cDNA would function longer than those from normal mice.

It should be pointed out that the porcine antisense α (1,3) GT cDNA fragment employed in this study only possesses 74.7 % similarity in nucleotide sequence to the corresponding region of murine α(1,3) GT mRNA. It would, theoretically, inhibit the expression of α(1,3) GT more effectively in pigs than in mice. In addition, while anti-Galα(1,3) Gal antibodies represent at least 60 % of human anti-mouse xenoantibodies, they are estimated to account for 80 % – 90 % human anti-pig xenoantibodies[27,28]. It implied that inhibiting the expression of α(1,3) GT would reduce the binding of XNA and consequent complement activation more significantly in pigs than in mice. Although eliminating the XNA binding and complement deposition by the approach of antisense RNA inhibition is not complete, it may substantially reduce the immunosuppressive requirements imposed on the recipient in xenotransplantation.

In summary, the expression of Gal epitope was reduced in transgenic mice bearing porcine antisense α(1,3) GT cDNA, consequentially, binding of XNA present in human serum to cells from these mice and subsequent complement activation was reduced. It suggested that constitutive expression of antisense gene for α(1,3) GT may be a potential strategy to attenuate Galα(1,3) Gal xenoantigen in pig tissues for xenotransplantation.

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反义猪 α(1,3)半乳糖转移酶转基因小鼠的人鼠异种反应性降低[1]

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关键词　表位；半乳糖转移酶类；异种移植；荧光抗体技术；反义 DNA；逆转录聚合酶链反应；DNA 印迹法；猪

目的：研究体内 α(1,3)半乳糖转移酶 α(1,3) GT 反义抑制降低 Galα(1,3) Gal (Gal 表位)异种抗原的作用. **方法**：原核显微注射法产生转基因小鼠，PCR 和 Southern-blot 鉴定转基因的整合，RT-PCR 检测小鼠 α(1,3) GT 的表达，组织学染色检查脾脏形态，免疫荧光检测 Gal 表位，流式细胞仪分析人血清中异种天然抗体(IgM 和 IgG)和补体(C3c)对鼠源细胞的结合. **结果**：猪反义 α(1,3)半乳糖转移酶转基因小鼠发育正常，但部分杂合体小鼠脾脏有细胞坏死特征. 转基因小鼠细胞表面 Gal 表位数量明显减少. 与野生型对照相比，转基因小鼠多种组织的细胞对人血清异种天然抗体的结合降低 30 % – 60 %，对补体的结合降低 40 % – 50 %. **结论**：通过反义基因抑制 α(1,3)半乳糖转移酶的表达能有效降低人异种反应性.

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