High-level Expression of Human α-1,2-fucosyltransferase Gene in Transgenic Mice Enhances Heart Function in *ex vivo* Model of Xenograft Rejection

Peihuan Li[1], Bingqian Liu[2], Yudong Wu[2], Lixia Zhang[3], Guangsan Li[3]

1. *Department of Pathology, Institute of Hematology, Chinese Academy of Medical Sciences and Peking Union Medical College, Tianjin 300052, China*
2. *Department of Urology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China*
3. *Institute of Genetics and Development Biology, Chinese Academy of Sciences, Beijing 100080, China*

Abstract: Background. Hyperacute rejection (HAR) of discordant xenotransplantation is the consequence of binding of natural antibodies to galactose-α-1,3-galactose (Gal) of vascular endothelium of the xenograft that activates complement system. Expression of human α-1,2-fucosyltransferase (HT) eliminating α-Gal antigen in donor organs has proven to be a promising approach to dealing with HAR. The aim of the paper was to investigate the effect of expressing human *HT* gene *in vivo* on α-Gal antigen and the role of human HT in overcoming HAR. **Methods.** Transgenic mice were produced by microinjection of gene construct for the enzyme human HT. PCR and Southern blot were used to screen the positive transgenic mice. Real-time PCR analysis was used to detect the level of human *HT* mRNA expression in transgenic mice. Expressions of H antigen and α-Gal antigen on peripheral blood mononuclear cells (PBMCs) of transgenic mice were detected by Flow Cytometry (FCM). In addition, the hearts of transgenic mice were perfused *ex vivo* with 12% human plasma by using a modified Langendoff apparatus, and the effect on cardiac function was determined. **Results.** 1176 injected and surviving zygotes were implanted into the oviducts of pseudopregnant foster mothers. Integration rate of human *HT* gene was 10.2% (14/137). Expression of human *HT* mRNA was detected in the heart, liver, kidney and muscle of transgenic mice, and expression rate was 78.6% (11/14). FCM analysis showed high-level expression of H antigen and with reduction of α-Gal antigen on PBMCs in transgenic mice. When perfused *ex vivo* with 12% human plasma, hearts from transgenic mice showed prolongation in survival time, compared to normal controls. **Conclusion.** A transgenic mice model with overexpression of human *HT* was successfully established. The transgene was integrated and transmitted into chromosome of transgenic mice. The present study suggests that the expression of human *HT* gene can reduce expression of the α-Gal antigen, and be effective in prolonging survival of xenograft and overcoming HAR. [Life Science Journal. 2006;3(4):27-32] (ISSN: 1097-8135).

Keywords: xenotransplantation; α-1,2-fucosyltransferase; hyperacute rejection; transgene

Abbreviations: α-Gal: galactose-α-1,3-galatose; HAR: hyperacute rejection; HT: α-1,2-fucosyltransferase; FCM: flow cytometry; PBMCs: peripheral blood mononuclear cells; XNAs: xenoreactive natural antibodies

1 Introduction

Notable improvements in the success of organ transplantation have created a severe imbalance between organ supply and demand. Xenotransplantation using porcine organs is currently viewed as a possible solution to overcome the worldwide shortage of donor organs for transplantation[1,2]. However, hyperacute rejection (HAR) must be overcome before organs can be transplanted between disxordant species[3]. HAR is primarily mediated by the binding of xenoreactive natural antibodies (XNAs) to the galactose-α-1,3-galatose (α-Gal) on vascular endothlium of the xenograft followed by complement activation, and is initiated within minutes of reperfusion[3]. Therefore, elimination of this interaction will be highly beneficial to overcoming HAR. The α-Gal antigen is synthesized by the enzyme α-1, 3-galactosyltransferase (α-1, 3-GT). Human α-1,2- fucosyltransferase (HT) was shown to efficiently compete with α-1,3-GT for the same substrate, N-acetyl lactosamine, impeding the transfer of the temrminal galactose residue that gives rise to the α-Gal antigen[4]. Furthermore, HT generates fucosylated residues that are universally tolerated[5]. So we can utilize the expression

of HT to down-regulate α-Gal antigen expression.

This study was designed, with a transgenic mice model, to investigate the effects of expressing the human HT on α-Gal antigen. In addition, function of human HT expression was studied by using an *ex vivo* heart perfusion model.

2 Materials and Methods

2.1 Animals

Kunming mice were purchased from Institute of Genetics and Development Biology, Chinese Academy of Sciences.

2.2 Chemicals and reagents

Restriction endonucleases *Not* I and *Pvu* I, Trizol, and RT-PCR kit were purchased from Invitrogen Inc. (USA). DIG-High Prime DNA labeling and detection starter kit I and nylon membranes were purchased from Roche (Switzerland). PCR kit was purchased from TaKaRa (Japan). Fluorescein isothiocyanate (FITC) -conjugated UEA I and GS-IB4 were purchased from Sigma (USA). Red blood cell lysate was purchased from BD Company (USA). Proteinase K was purchased from Merck (Germany).

2.3 Plasmid

The pRc-CMV plasmid containing full-length sequences of human *HT* cDNA was offered by Dr. Hiroshi Kimura (Department of Forensic Medicine and Human Genetics, Kurume, Japan). The pCMV-MCS plasmid was purchased from Merck (Germany).

2.4 Production of transgenic mice

Gene construct used to generate transgenic mice has been described previously[6]. The pCMV-MCS-HT recombinent plasmid was indetified by enzyme digests. It consists of CMV promoter, β-globin intron, *hHT* cDNA and hGH polyadenylation signal (Figure 1). Transgenic mice were generated as described by Gordon[7]. Transgene construct was microinjected into pronuclei of one-cell embryos with dosage of 5 μg/ml. The injected and surviving zygotes were reimplanted into the oviducts of pseudopregnant female mice and allowed to develop to term.

Figure 1. Schematic diagram of the target transgene construct

2.5 PCR

Genomic DNA of mice was extracted from the tail[8,9], and were screened for integration of human *HT* gene by PCR with the following primers: up-stream: 5′-AAC GTG CTG GTC TGT GTG CG-3′; down-stream: 5′-CTC CGA TGT GGC ACC TTT CA-3′. PCR cycling parameters were: initial denaturation at 95 ℃ for 10 minutes, 30 cycles (denaturation at 95 ℃ for 40 seconds, anneal at 60 ℃ for 40 seconds, extension at 72 ℃ for 50 seconds), and a final extension at 72 ℃ for 10 minutes. Positive PCR reaction using the primer generate a 915 bp fragment. For Southern blot analysis, mouse genomic DNA was digested with restriction enzyme *Eco*RI/*Bam*HI, product was subjected to electrophoresis in a 1.0% agarose gel and transferred to nylon membrane according to the manufacturer's instruction. Hybridization was performed with a DIG labeled human *HT* cDNA probe under stringent condition according to the protocol of DIG DNA labeling and detection kit.

2.6 RT-PCR

Total RNA was isolated by using Trizol Reagent as described by the manufacturer. Expression of human *HT* mRNA was evaluated by RT-PCR from total mRNA samples of heart, liver, kidney and spleen. First strand cDNA was prepared by reverse transcription using AMV reverse transcriptase and used for PCR. The primers designed according to the sequence of cDNA of human *HT* gene: up-stream: 5′-GAC TTT CTT CCA CCA TCT CC-3′; down-stream: 5′-TAA TGC CCA CCC ACT CG-3′. The expected PCR products is 544 bp. The β-actin primers: up-stream: 5′-CCA ACT GGG ACG ACA TGG AG-3′; down-stream: 5′-AGG TCC AGA CGC AGG ATG GC-3′. The products is 300 bp. Reaction condition: denaturation at 96 ℃ for 2 minutes; then for 30 cycles (denaturation at 94 ℃ for 30 seconds, annealing at 65 ℃ for 60 seconds), extension at 72 ℃ for 60 seconds; and a final extension at 72 ℃ for 7 minutes.

2.7 Flow cytometry analysis of α-Gal and H antigen

Mice blood was collected by orbital eye-bleed and erythrocytes lysed by incubation in red blood cell lysate. Peripheral blood leukocytes were collected by centrifugation and washed 3 times in phosphate-buffered saline, containing 2% heat-inactivated febovine serum and NaN_3, and then incubated with FITC-conjugated lectins GS-IB4 and UEAI for expression of α-Gal and H antigen respectively on ice for 30 minutes, washed twice in Hanks' balanced salt solution. The expression on peripheral blood mononuclear cells (PBMCs) was analyzed by Flow cytometry (FCM) as described[10].

2.8 *Ex vivo* isolated perfused heart model

Mouse hearts were perfused on a Landendorff

apparatus essentially as reported[11]. Mice were anethetized with pentobarbitone sodium, and the hearts were removed and immersed 20 ml of ice-cold modified heparin-containing Krebs-Henseleit buffer to arrest cardiac activity. Hearts were then attached to a 21-gauge cannula via the aortic root, connected to the perfusion apparatus and perfused in a retrograde manner. Heart rate and force of contraction were monitored using PowerLab 4.0 software at 5 minutes intervals, and heart work was calculated as the product of heart rate multiply force of contraction. After a 20-minute period of stabilization perfused with K-H buffer, pooled human plasma was added at 5 minutes intervals to final concentration of 12%.

2.9 Statistical analysis

All data were expressed as mean ± standard deviation and analyzed by SPSS 11.0 software, statistical significance was determined by Student's test, significance was defined as a P value <0.05.

3 Results

3.1 Identification of human *HT* transgene construct

The length of the resulting construct pCMV-MCS-HT plasmid was about 5.6 kb containing 1.85 kb human *HT* cDNA. The electrophoresis assay showed: 1.1 kb and 4.5 kb in the *Eco*RI/*Bam*HI digest, and 2 fragments containing 2.85 kb and 2.75 kb in the *Not*I digest, and 3 fragments containing 2.85 kb, 1.4 kb and 1.35 kb in the *Pvu*I/ *Not*I digest (Figure 2), indicating that human *HT* cDNA was correctly inserted and linked.

3.2 Sequence data

Sequence data showed that the sequenced fragment was the same as human *HT* in the Genebank, and no ATG was present in the 60 bp vector fragment between CMV promoter and human *HT* cDNA on the recombinment plasmid.

3.3 Screening of transgenic mice

Tail genomic DNA from G_0 mice was screened by PCR, which amplify a 915 bp fragment from transgenic mice but not from non-transgenic mice littermates (Figure 3). Southern blot revealed the same results of PCR (Figure 4).

3.4 Transgenic efficiency of transgenic mice

To create transgenic mice, a total 1,300 fertilized embryos was microinjected with purified human *HT* transgene construct. 1,176 surviving injected embryos were reimplanted into 55 pseudopregnant foster mice, and 43 of whom gave birth to 137 mice. Pregnancy rate was 78.2% (43/55), and birthrate was 11.6% (137/1176). Among 137 G_0 mice, transgene integration was confirmed by PCR and Southern blot in 11 mice. Integration efficiency was 8.0% (11/137).

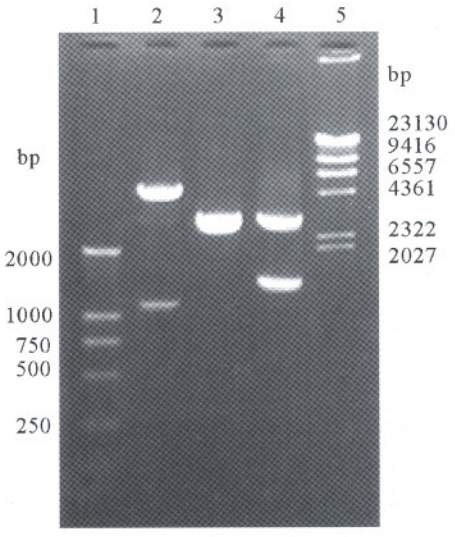

Figure 2. Identification of recombinant pCMV-MCS-HT plasmid by enzyme digestion
Lane 1: DL2000 Marker; Lane 2: Plasmid digested with *Eco*RI/*Bam*HI(1.1 kb and 4.5 kb); Lane 3: Plasmid digested with *Not*I (2.85 kb and 2.75 kb); Lane 4: Plasmid digested with *Pvu* I / *Not* I (2.85 kb, 1.4 kb and 1.35 kb); Lane 5: λ-*Hind* III Marker

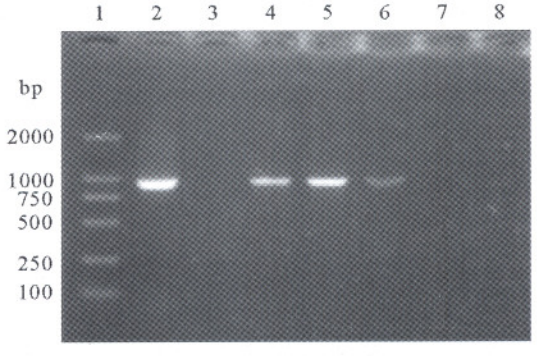

Figure 3. Identification of transgenic mice by PCR
Lane 1: DL2000 Marker; Lane 2: pCMV-MCS-HT plasmid as positive control; Lane 4,5,6: positive mice; Lane 3,7: negative mice; Lane 8: normal mice as negative control

3.5 Expression of human *HT* mRNA in transgenic mice

RT-PCR anslysis was used to determine *hHT* RNA expression in various tissues derived from control and transgenic mice. Total RNA was isolated from heart, liver, kidney and spleen. Human *HT* mRNA expression was detected in 8 mice among 11 G_0 transgenic mice, and expression was positive in organs of transgenic mice including

heart, liver, kidney and spleen, no specific PCR products were observed in any of the non-transgenic littermate. Endogenously-expressed β-actin was used as a control to confirm the integrity of all samples tested by PCR (Figure 5).

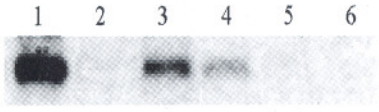

Figure 4. Identification of transgenic mice by Southern blot
Lane 1: pCMV-MCS-HT plasmid; Lane 3 and Lane 4: positive mice; Lane 2 and Lane 5: negative mice; Lane 6: normal mice as negative control

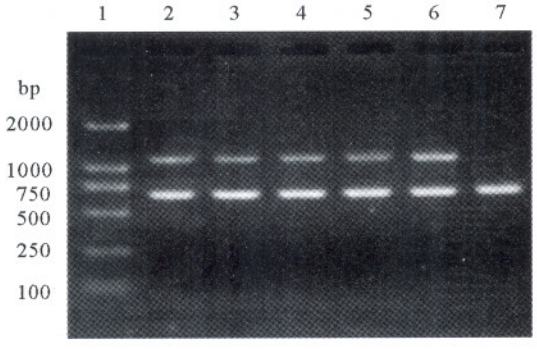

Figure 5. The expression of human *HT* gene in transgenic mice detected by RT-PCR
Lane 1: DL2000 Marker; Lane 2: positive control; Lane 3: heart; Lane 4: liver; Lane 5: kidney; Lane 6: muscle; Lane 7: negative control

3.6 FCM analysis

H antigen and α-Gal antigen expression was evaluated by FCM on PBMCs. Control mice cells showed only background staining for H antigen, but cells from transgenic mice expressed high levels of this antigen. Staining with GS-IB4 for α-Gal revealed a fourfold reduction in mean fluorescence intensity of transgenic mice cells relative to controls (Figure 6).

3.7 *Ex vivo* heart perfusion

Six hearts from control and transgenic mice were removed quickly respectively. The hearts were attached to a modified Langendorff perfusion apparatus, then perfused with 12% human plasma[11]. Heart work from control mice dropped sharply to below 20% of maximum within 20 minutes of plasma addition. In contrast, hearts from transgenic mice were still functioning at 30% of maximum after 60 minutes perfusion. 100% work is defined as heart work measured immediately before time 0 (Figure 7).

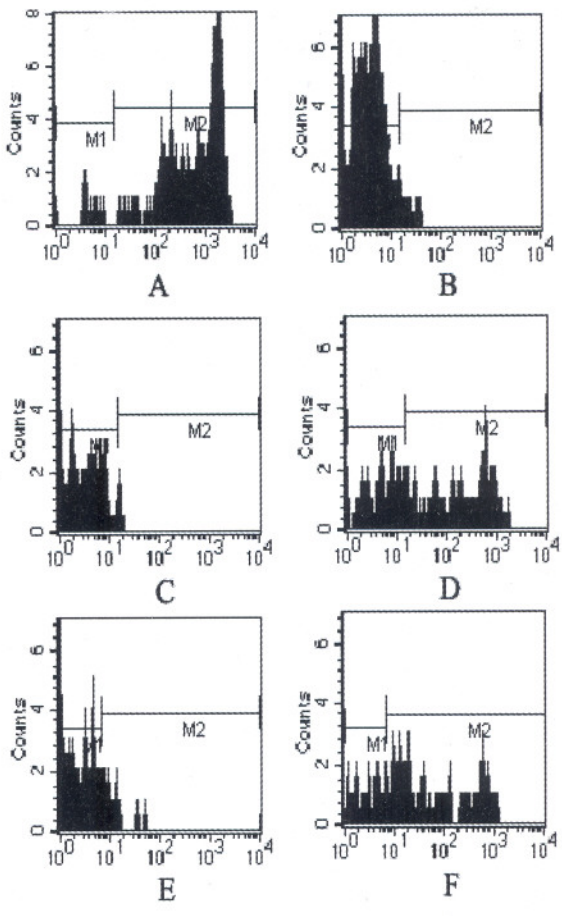

Figure 6. Expression of various levels of H and Gal antigen.
A: Gal of normal mice; B: H of normal mice; C: Gal of human; D: H of human; E: Gal of transgenic mice; F: H of transgenic mice

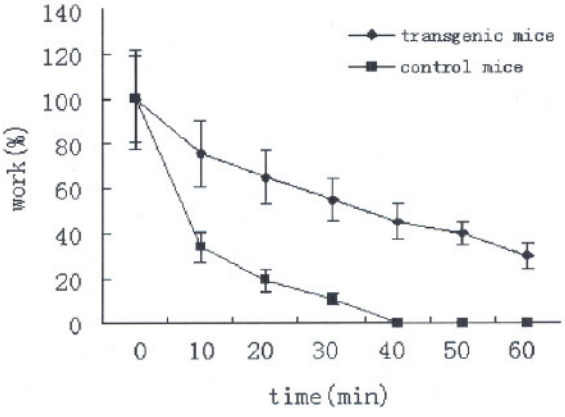

Figure 7. Function of mice hearts perfused *ex vivo* with human plasma

4 Discussion

Rapid improvement in the success of organ

transplantation have created an obvious imbalance between organ supply and demand. The serious shortage of human organs available for allotransplantation has stimulated people to look at the possibility of using animals as donors. Xenotransplantation using pig organs is currently regarded as a feasible approach to solving the problem, but the immediate barrier to the transplantation of vascularized pig organ to primates is HAR[12,13].

It is now clear that the binding of XNAs to α-Gal on vascular endothelium of xenograft is key mediator of HAR[14], therefore elimination of this interaction will be highly beneficial to overcoming HAR. The strategies could be classified as recipients or donors directed. The disadvantages of the former are that their effect is transient, and impose an additional burden on the recipient. Therefore genetic engineering of the donor organ has greater potential for effectively inhibiting xenograft rejecting with reduced risk to recipient. Transgenic expression of human complement regulation proteins (CRPs) in donor organs has significantly prolonged the survival of xenografts[15]. However, expression of CRPs without eliminating xenogeneic natural antibody reactivity may not provide sufficient protection for clinical application.

α-Gal is a major antigen involved in HAR of xenotransplantation that is synthesized by the enzyme α-1,3-galactosyltransferase. Removal of α-Gal antigen from pigs would prevent HAR, and prolong survival of xenograft[4,16]. The production of GT-KO mice and pigs has been reported recently, these GT-KO animals would, by definition, be deficient in α-Gal antigen, theoretically leaving no target for human natural antibodies. Some investigators suggest, however, that eliminating the α-Gal antigen would expose underlying "cryptic" oligosaccharide determinanta against which human may also have preformed antibody[17]. So, an alternative transgenic approach was developed that based on the competition between α-1,3-GT and human HT for a common substrate – N-acetyllactosamine, transgenic expression of human HT is currently viewed as the feasible strategy to reduce α-Gal expression in pigs[18]. The strategy has been to significantly reduce Gal expression on a variety of cells in transgenic mice and pigs. However its ability at prologing xenograft survival is yet to be demonstrated.

Thus, we obtained transgenic mice by microinjection of human *HT* transgene construct, and to investigate the effect of expressing human HT *in vivo* on α-Gal antigen and the role in overcoming HAR. α-Gal was expressed in all tissues and organs of normal mice, so we chosed CMV promoter to achieve ubiquitous expression of human HT. The result suggested that human *HT* mRNA was expressed in most organs of transgenic mice including heart, liver, kidney and spleen. According to expression of H antigen and α-Gal on PBMCs was the same as on vacular endothelium[19], we evaluted expression of them on PBMCs. FCM analysis showed that overexpression of human HT could greatly reduce the amount of α-Gal antigen on PBMCs.

In the study, we tested the ability of human HT expressed in transgenic mice to protect organs against HAR when perfused *ex vivo* with human plasma. As expected, the cardiac function of hearts from control mice perfused with 12% human plasma dropped sharply to 20% of maximum work within 20 mintutes from plasma addition and stopped beating at the 35th minute. In constrast, the function of human HT transgenic hearts was only reduced by 50% – 65% after 20 minutes, and was maintained approximately 35% after 60 minutes. Therefore, as far as HAR concerned, the results would suggest that the human *HT* expression is effective in protecting against XNAs and complement mediated HAR.

However, FCM analysis showed that there was still α-Gal expression on PBMCs of transgenic mice. It is suggested that overexpression of human *HT* gene would be unlikely to totally eradicate α-Gal. Whether transgenic expression of human *HT* is sufficient to avoid HAR and whether residual α-Gal is related to subsequent acute vascular rejection (AVR) of xenotransplantation is not clear and is under examination. And xenotransplantation is related to many factors[20], so a combination of human HT transgene approach, together with the transgenic expression of human complement inhibitory proteins, may lead to murine heart entirely resistant to HAR. The present study provide a helpful technique for the further research of transgenic pigs.

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Correspondence to:
Bingqian Liu
Department of Urology
The First Affiliated Hospital
Zhengzhou University
Zhengzhou, Henan 450052, China
Telephone: 86-371-6686-1002
Email: liubq76@yahoo.com.cn

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