Resistance to anti-xenogeneic response by combining α-Gal silencing with HO-1 upregulation☆☆

Min Zhu a, Wei Zhang b, Fang Liu b, Lu Wang a, Bin Liu a, Dong Chen a, Xue-Hai Zhu a, Wei-Jie Zhang a, Thomas E. Ichimc, Zhi-Shui Chena, Ping Zhou a, Shi Chena,⁎, Gang Chen a,⁎

a Key Laboratory of Organ Transplantation, Ministry of Education and Health, Institute of Organ Transplantation, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China
b Medistem Laboratories, San Diego, CA 92122, USA

c Corresponding authors. Institute of Organ Transplantation, Tongji Hospital, 1095 Jiefang Avenue, Wuhan, 430030, China. Tel.: +86 27 83662655; fax: +86 27 83662892.
E-mail addresses: schen@tjh.tjmu.edu.cn (S. Chen), gchen@tjh.tjmu.edu.cn (G. Chen).

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ABSTRACT

Background: A major barrier to clinical xenotransplantation is preformed xenoreactive natural antibodies (XNA) found in higher primates which react to Galα(1,3)Gal (α-Gal) epitopes found on lower species. Accommodation of organs to xenogeneic recipients involves upregulation of cytoprotective genes and resistance to complement dependent cytotoxicity (CDC).

Methods: To develop methods of increasing these organ-protective effects, we established an in vitro CDC model utilizing human serum as the source of XNA and porcine endothelial cells (pEC) as targets.

Results: Using this system we demonstrated that downregulation of α-Gal epitopes by siRNA silencing of α1,3-galactosyltransferase (α-GT) led to marginal protection from CDC while α-Gal silencing combined with Griffonia simplicifolia isoelectin B4 (GS-IB4), a lectin that specifically binds to α-Gal epitopes, led to complete protection. Interestingly, α-Gal silencing and GS-IB4 mediated effects were not associated with inhibition of XNA binding to cells, but with significant decreased E-selectin expression and cytoprotective gene HO-1 upregulation. PI3K inhibitor LY294002 could block the elevation of HO-1 protein expression and reverse the protective effect of α-Gal silencing and GS-IB4 against CDC.

Conclusion: These data support the use of combination approaches targeting independent accommodation mechanisms to synergistically enhance donor organ survival in a xenogeneic setting.

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1. Introduction

Allografts transplanted into ABO-incompatible individuals or xenografts of a vascularized organ between discordant species usually undergo an immediate rejection response known as hyperacute rejection (HAR). The Galα(1,3)Gal (α-Gal) epitope on porcine endothelial cells (ECs), synthesized by the glycosylation enzyme α1,3-galactosyltransferase (α-GT), is believed to be the major target for preformed xenoreactive natural antibodies (XNA) which are responsible for HAR [1,2]. The α-Gal epitope is homologous to the ABO blood group antigens, both belonging to the histocompatibility carbohydrate antigen family. Under certain conditions, resistance to antibody-mediated rejection termed “accommodation” has been observed in clinical allo-transplantation where ABO-mismatched organs have been demonstrated to survive in rare cases [3–5]. Such “accommodation”, however has not been observed in pig-primate xenotransplantation models to date.

The greater difficulty in achieving accommodation of discordant xenografts compared to ABO-incompatible allografts may be due to two differences: 1) the titer of preformed anti-Gal antibody in primates is four to five fold higher than that of preformed anti-A and anti-B antibody [6]; 2) the number of α-Gal epitopes per cell expressed on pig aortic endothelial cells has been reported to be 1 to 2 × 10⁶ [7], while only 0.8 to 1.2 × 10⁶ A1 or 0.6 to 0.8 × 10⁶ B epitopes per cell are present on human red blood cells [8]. Reducing the titer of anti-Gal antibodies or down-modulation of α-Gal epitopes on endothelial cells may facilitate the development of accommodation. We have previously reported that gene silencing via specific small interfering RNA (siRNA) targeting the α1,3GT gene could effectively reduce the expression of α-Gal on porcine ECs and
confer significant protection against human anti-α-Gal antibody/complement-mediated injury. However, in vitro accommodation with complete resistance to antibody/complement-mediated lysis could not be achieved, which may be due to residual expression of α-Gal or the presence of non-Gal epitopes [9].

Based on small animal models of accommodation, several groups have found that anti-apoptotic and anti-inflammatory genes such as heme oxygenase-1 (HO-1), Bcl-xL, Bcl-2 and A20 expressed on xenograft ECs play an important role in induction of accommodation [10–12]. Moreover, Soares et al. [13] reported that accommodation could not be induced in mice lacking HO-1, suggesting that HO-1 is essential to ensure accommodation. HO-1 expression is upregulated in most cell types exposed to different pathological conditions including antibody-mediated stimuli [14–16]. Additionally, it has been reported that stimulation of ECs by crosslinking of the α-Gal with the lectin Griffonia Simplicifolia I (GS-1) can result in upregulation of HO-1 in a dose-dependent manner [17]. Although HO-1 mediated cytoprotective effects against cytotoxicity induced by XNA have been well studied both in vitro and in vivo [13,18], the exact molecular mechanism and signal transduction pathways remain to be elucidated.

The aim of the present study was to address the question whether various α-Gal expression levels could confer porcine ECs different resistant ability to Ab/complement-mediated lysis. Furthermore, we developed a new in vitro accommodation model of porcine ECs by combined α-Gal silencing with Griffonia Simplicifolia IB4 (GS-IB4), which was used to elucidate signaling pathways responsible for HO-1 mediated cytoprotection.

2. Materials and methods

2.1. Cell line and culture conditions

The porcine endothelial cell line, PED was a generous gift from Dr. J. Holgersson (Karolinska Institute, Huddinge, Sweden) and was maintained in Dulbecco’s minimum essential medium (Invitrogen Corporation, Paisley, U.K.) with 10% heat-inactivated fetal calf serum (FCS, HyClone Laboratories, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were maintained in a 5% CO₂/95% air atmosphere at 37 °C.

2.2. siRNA transfection

The specific siRNA duplexes targeting α1,3GT, designated SiRNA-1, were the same as the ones used in our previous study [9]. The sense and antisense sequences were the following: sense: 5′-GAAGAAGACGCCUAAUGCGAAdTdT-3′, antisense: 5′-UGCCUAAUGCUCUUSCUUCdTdT-3′, respectively. For transfection experiments, cells in a 6-well plate (50–70% confluence) were transfected with RibolJuicer™ (Novagen Inc., Darmstadt, Germany) according to the manufacturer’s protocol. To achieve variable extent of depletion of α1,3GT gene, SiRNA-1 was prepared and transfected at a final concentration of 2 nM, 5 nM and 15 nM (per ml of media). At the indicated time points, the cells were ready for further experiments as described below.

2.3. Complement-dependent cytotoxicity (CDC)

Forty-eight hours post transfection, PEDs were transferred to flat bottom 96-well plates (Costar, Corning Inc., U.S.A.) at a density of 10⁴ cells/plate and incubated overnight. Target cells were then exposed to increasing dose of GS-IB4 (0.5, 2 and 8 μg/ml) for 4 h–16 h with or without pretreatment for 15 min by inhibitor of PI3K, LY294002 (50 μM). The viability of PEDs after GS-IB4 treatment was routinely checked by the method of Malassagne with minor modifications [19]. After labeling with ⁵¹Cr (2 μCi/well, Amersham Pharmacia Biotech) and washing, cells were incubated with 20% or 40% normal human serum (NHS, human AB serum pooled from Wuhan municipal blood bank, China) at 37 °C for 4 h. One hundred microliters of supernatant was collected from each well and gamma counts (Hefei Zhongjia Scientific Instruments Corp., Hefei, China) were performed. Heat-inactivated NHS (HINHS) was used as negative control. Spontaneous release was measured in target cells incubated with media alone. Maximum release was measured by treatment of target cells with 1% NP-40. Data are presented as percentage of specific lysis and calculated as follows: 100×(specific release–spontaneous release)/(maximum release–spontaneous release).

2.4. Flow cytometric analysis

Different α-Gal expression pattern on the cell surface of PEDs by SiRNA-1 was analyzed by fluorescence activated cell sorter (FACS) as described previously [20]. In brief, 5 × 10⁴ of appropriate parental PEDs and transfectants cells were harvested and resuspended in phosphate-buffered saline with 0.1% bovine serum albumin (BSA). FITC-conjugated GS-IB4 (2 μg/ml, Sigma, St. Louis, MO) was added to each cell suspension and incubated for 45 min at 4 °C. Human immunoglobulin and complement binding to parental PEDs and transfectants treated with various dose of GS-IB4 were also assayed. Resuspended cells were incubated with 20% HINHS at 4 °C for 1 h, washed and then incubated with 1 μg of FITC-conjugated anti-human Ig (IgG, IgM, Cappel, West Chester, PA, U.S.A.). Assays for C3 binding to parental cells were performed following the same methodology, using 20% NHS and a FITC-conjugated anti-human C3c antibody (1:40, Dako, Glostrup, Denmark). E-selectin expression on the cell surface of PEDs after treatment by SiRNA-1 and GS-IB4 was also measured in a similar manner by using anti-human E-selectin (Abcam, Cambridge, UK, cross-react with pig [21]). Cells incubated with second antibody only served as a negative control. Samples were counted on a flow cytometer (FACSCalibur, Becton Dickinson). Analysis was performed using the Cell Quest program from BD Sciences.

2.5. RNA isolation and RT-PCR amplification

Total RNA was isolated from treated PEDs using Trizol reagent (Life Technologies, Rockville, MD). 2 μg of total RNA was reverse-transcribed by incubating with oligo (DT), and avian myeloblastosis virus reverse transcriptase (Takara, Tokyo) at 42 °C. PCR amplifications were conducted using the following primer sequences: forward, 5′-CTCAGGGAGGAATGACC-3′; reverse 5′-GTGCGGCAATTGGGAAGAGG-3′ for HO-1, forward, 5′-GTGCAGATTCAAGGAA-3′; reverse, 5′-TGTCACGCTGCCACTTCAT-3′ for β-actin, generating 341-bp and 241-bp fragments, respectively. The MasterCycler (Eppendorf, Germany) was used for the PCR amplifications with the following settings: 33 cycles at 94 °C for 30 s, 63 °C for 30 s, and 72 °C for 30 s and a final elongation step at 72 °C for 10 min. Subsequently, amplified products were electrophoresed on 2% agarose gel stained with ethidium bromide and were photographed under ultraviolet light. The expression level was normalized with the level of β-actin bands.

2.6. Western blot analysis

PEDs were harvested for total protein analysis in RIPA buffer (0.15 M NaCl, 1% NP40, 0.01 M deoxycholate, 0.1% SDS, 0.05 M Tris–HCl pH 8.0, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) supplemented with the complete protease inhibitor cocktail (Roche Diagnostics). 20 μg of cellular proteins was resolved on a 12% SDS/PAGE and electroblotted onto nitrocellulose membranes (TransBlot, Bio-Rad). HO-1 was detected using an anti-HO-1 antibody (Santa Cruz Biotechnology). Total and activated/phosphorylated forms of Akt were detected using rabbit polyclonal Ab directed against the total or phosphorylated forms of Akt (Cell Signaling, MA). Primary Abs were detected using HRP-
conjugated goat anti-mouse or rabbit IgG secondary Ab (Zhongshan Biotechnology, Beijing). Peroxidase activity was visualized using the ECL assay (SuperSignal West Pico, Pierce).

2.7. Data analysis

All results are presented as mean±standard deviation. Statistical analyses were evaluated by one-way ANOVA tests among the treatments. Student t-test was used when appropriate. \( p < 0.05 \) was considered significant.

3. Results

3.1. SiRNA-1 induced suppression of \( \alpha \)-Gal expression protects PEDs against CDC

To avoid siRNA non-specific effects on gene expression and induction of immune activation at a relative high concentration mentioned by previous articles [22,23], PEDs were transfected with relatively low concentrations of SiRNA-1 ranging from 2 to 15 nM. Subsequently, \( \alpha \)-Gal epitope expression was assessed by FACS analysis. After 48 h, \( \alpha \)-Gal expression was approximately 67–86% down-regulated in the SiRNA-1 transfected PEDs compared with parental PEDs (Fig. 1A). In contrast, mock-transfected PEDs showed no major differences in \( \alpha \)-Gal expression (Fig. 1B). The inhibition of Ab/complement-mediated lysis as a result of knockdown of \( \alpha \)-Gal expression was determined with 20% NHS or 40% NHS as the source of XNA and complement. The incubation of xenogeneic cells in the presence of NHS serves as an established model of xenoreactivity and accommodation thereof. PEDs that were transfected with increasing amount of SiRNA-1 for 48 h were protected from cytotoxicity by human Ab/complement in a concentration-dependent fashion; maximal protection was obtained at 15 nM SiRNA-1 (Fig. 1C). HINHS displayed only background cytotoxic activity to all cells, due to inactivation of complement components. These results indicate that introduction of SiRNA-1 into PEDs caused specific interference with \( \alpha \)-Gal expression and resistance to CDC in a concentration-dependent manner.

3.2. Complete inhibition of CDC towards PEDs pretreated with SiRNA-1 and various doses of GS-IB4

To investigate whether protection from CDC induced by suppression of \( \alpha \)-Gal can be augmented by co-treatment with the \( \alpha \)-Gal binding-GS-IB4 lectin, \(^{51}\text{Cr} \) release assays were performed on treated PEDs. SiRNA-1 targeting cells at 15 nM following exposure to increasing dose of GS-IB4 lectin (0.5, 2 and 8 \( \mu \)g/ml) for four h evoked almost complete resistance of CDC by SiRNA-1 plus low dose of GS-IB4 (0.5, 2 and 8 \( \mu \)g/ml). The cytolysis results are expressed as the mean percent of specific lysis±SD of three independent experiments (B) GS-IB4 alone, however, only up to 24 \( \mu \)g/ml began to achieve a significant protective effect against CDC. (C) The white arrows represent cellular gaps formation. The single asterisk (*) and double asterisks (**) indicate significant difference from control cells with \( p < 0.05 \) and \( p < 0.01 \), respectively. Scale bar equals 20 \( \mu \)m.

Fig. 1. Effect of \( \alpha \)-Gal knockdown by specific siRNA on \( \alpha \)-Gal activity. (A) The reduction of the \( \alpha \)-Gal epitope on PEDs surface by various concentrations of SiRNA-1 (2, 5 and 15 nM) was analyzed using FITC-GS-IB4 isolectin. (B) Parental and mock-transfected PEDs showed almost similar mean fluorescence intensity (MFI). (C) PEDs transfected with increasing amount of SiRNA-1 was protected from CDC in a concentration-dependent fashion. HINHS displayed only background cytotoxic activity to all cells.

Fig. 2. Induction in vitro accommodation of PEDs by SiRNA-1 and low dose of GS-IB4. (A) Almost complete resistance of CDC by SiRNA-1 plus low dose of GS-IB4 (0.5, 2 and 8 \( \mu \)g/ml). The cytolyis results are expressed as the mean percent of specific lysis±SD of three independent experiments (B) GS-IB4 alone, however, only up to 24 \( \mu \)g/ml began to achieve a significant protective effect against CDC. (C) The white arrows represent cellular gaps formation. The single asterisk (*) and double asterisks (**) indicate significant difference from control cells with \( p < 0.05 \) and \( p < 0.01 \), respectively. Scale bar equals 20 \( \mu \)m.
complete protection from CDC. As shown in Fig. 2A, at 2 μg/ml, GSIB4 exhibited the maximum protective effect with SirNA-1. Beyond 8 μg/ml, however, there was not a significant reinforcement tendency (Fig. 2A). Interestingly, GSIB4 alone, only up to 24 μg/ml, began to achieve a significant protective effect against CDC (Fig. 2B). Furthermore, the protective effect was able to successfully maintained PEDs treated with SirNA-1 and GSIB4 in the presence of 20% or 40% NHS for up to 20 h at 37°C. No visible morphological changes were observed by fluorescence microscopy. The PEDs treated with GSIB4 alone remained as a monolayer, with no evidence of detachment or retraction (Fig. 2C). In contrast, simultaneously with the changes in cellular shape, the parental PEDs monolayer retracted and lost contact, forming cellular gaps until complete detachment occurred (Fig. 2C).

3.3. Binding of human XNA and complement to PEDs is strongly reduced by SirNA-1 but not GSIB4

Parental PEDs and transfectants incubated with various doses of GSIB4 (0.5, 2 and 8 μg/ml) were treated with 20% pooled NHS for FACs determination of XNA binding. SirNA-1 (15 nM) transfected compared with control parental PEDs, showed a diminished reactivity to human natural antibodies and complement in NHS (Fig. 3). Interestingly, GSIB4 treatment did not affect the binding of human XNA and complement to either control parental PEDs or sirNA-transfected PEDs (Fig. 3).

3.4. Dose-dependent inhibition of E-selectin expression on sirNA-transfected PEDs treated with GSIB4

Up-regulated expression of adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 is associated with EC activation, as commonly observed following the vascular prothrombotic and inflammatory changes in xenorejection. Therefore, we compared E-selectin expression on parental and SirNA-1 (15 nM) transfected PEDs after incubation with 20%HINHS. As shown in Fig. 4, when compared with control parental PEDs, SirNA-1 pretreatment did not alter the expression of E-selectin on PEDs, which suggested that α-Gal knockdown could not inhibit the activation of PEDs when incubating with HINHS. However, GSIB4 treatment before incubation with 20%HINHS could significantly inhibit the expression of E-selectin on PEDs after incubation with 20% NHS (Fig. 4). As shown in Fig. 4, when compared with control parental PEDs, SirNA-1 (15 nM) pretreatment did not alter the expression of E-selectin on PEDs, which suggested that α-Gal knockdown could not inhibit the activation of PEDs when incubating with HINHS (Fig. 3). Interestingly, GSIB4 treatment did not affect the binding of human XNA and complement to either control parental PEDs or sirNA-transfected PEDs (Fig. 3).

3.5. GSIB4 induces HO-1 expression in Gal knockdown PEDs through Akt signaling pathway

To study the mechanism of the resistance of PEDs pretreated with SirNA-1 in combination with GSIB4 to Ab/complement-mediated lysis, the cytoprotective gene HO-1 was analyzed for mRNA and protein expression in SirNA-1 (15 nM) transfected PEDs treated with GSIB4 in the presence of 20% NHS (Fig. 3). GSIB4 at a concentration of 8 μg/ml almost caused a complete inhibition of the expression of E-selectin when compared with negative controls.

3.6. The PI3K/Akt pathway is necessary for PED protection from Ab/complement-mediated lysis

To analyze the effect of the PI3K/Akt pathway on the resistance of PEDs to XNA and complement induced cytolysis in NHS. SirNA-1 (15 nM) transfected PEDs were pretreated for 15 min with 50 μM LY294002, an inhibitor of PI3K, and then stimulated with 8 μg/ml of GSIB4 for 4 h. As shown in Fig. 5D, the PI3K inhibitor significantly blocked the elevation of HO-1 protein expression induced by GSIB4 in SirNA-1 transfected PEDs, indicating that GSIB4 upregulates HO-1 expression, at least in part, through the PI3K/Akt pathway.

Discussion

Accommodation is a method of graft self-protection against Ab/complement-mediated injury. Several strategies have been proposed to induce xenograft accommodation, including decrease of either the titer or the specificity of the xenoreactive antibodies, down-modulation of expression or the nature of antigenic epitopes on graft endothelial cells, and induction of an active process which results in a protected state against antibody deposition and complement activation [26]. With regard to antigen expression level, however, the relationship between α-Gal expression and accommodation remains relatively unclear. This study highlighted the role of α-Gal expression on accommodation induction, displaying that various levels of α-Gal expression achieved by different concentrations of specific siRNA targeting α1,3GT confer dose-dependent resistance of porcine ECs to antibody/complement-mediated lysis in vitro. It has been demonstrated that there were quantitative differences of α-Gal expression on porcine and murine organs [27,28]. The reactivity of primate XNA to rat EC antigens was weaker overall than to pig EC antigens [27]. In our model system, a relatively low expression of α-Gal was achieved by treatment with specific siRNA, creating a favorable environment for accommodation induction, which was consistent with the phenomena that induction of accommodation was easier in rodent models than in pig-to-primate. Our results are also in line with previous reports that
different expression levels of α-Gal on pancreas and heart cause different outcomes in transplantation of these organs from rats to α1,3GT knockout mouse recipients [29].

Furthermore, since we found that in vitro accommodation with complete resistance to antibody/complement-mediated lysis could not be achieved by only down-modulating α-Gal expression, which may be due to residual α-Gal expression and non-Gal epitopes, we developed a novel strategy by additionally up-regulating a protective gene, HO-1 on porcine ECs. As a specific ligand of the α-Gal epitope, GS-IB4 at a concentration of 25 μg/ml has been reported to induce porcine ECs resistance to CDC through upregulation of HO-1 [30]. In this study, compared with SiRNA-1 treated alone, the combination therapy of SiRNA-1 and low concentrations of GS-IB4 dramatically inhibited CDC towards PEDs, achieving up to maximum protection of porcine ECs against humoral destruction. Although as much as 80% of α-Gal epitopes was reduced by RNAi, we found that PEDs resistance to CDC was still accompanied with high expression of E-selectin, which usually serves as a marker of EC activation. When pretreated with low concentrations of GS-IB4, however, these SiRNA-1 transduced PEDs were observed to have significant decreased E-selectin expression, demonstrating that inhibition of ECs activation by GS-IB4 might be associated with the in vitro accommodation of porcine ECs. It has been reported that overexpression of HO-1 in ECs significantly inhibits the ability of TNF-α to induce the expression of E-selectin [31]. We now demonstrate that E-selectin downregulation in PEDs treated with GS-IB4 might be related with HO-1 upregulation.

We found that when crosslinking α-Gal by GS-IB4 prior to incubation with NHS, SiRNA-1 transduced PEDs caused an active defense against CDC, associated with GS-IB4 dose-dependent increase in both HO-1 mRNA and protein expression. Interestingly, there was
of the Gal-knockout pig which still expresses the α-Gal epitope as a result of compensatory synthesis by iGb3 synthase [38,39].

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