Transplant Immunology 19 (2008) 202–208

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

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

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ARTICLE INFO

Article history:
Received 16 March 2008
Received in revised form 5 June 2008
Accepted 9 June 2008

Keywords:
Accommodation
RNA interference
Endothelial cell
Xenotransplantation

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 isolectin 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.

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 histocompatiblity 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 2×10^7 [7], while only 0.8 to 1.2×10^6 A1 or 0.6 to 0.8×10^6 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

Abbreviations: α1,3GT, α1,3-galactosyltransferase; α-Gal, Galα(1,3)Gal; CDC, complement dependent cytotoxicity; ECs, endothelial cells; FACS, fluorescence activated cell sorter; FCS, fetal calf serum; GS-IB4, griffonia simplicifolia isolectin B4; HINHS, heat-inactivated normal human serum; HO-1, heme oxygenase-1; NHS, normal human serum; RNAi, RNA interference; siRNA, small interfering RNA; XNA, xenoreactive natural antibodies.

☆ This work was supported by National High Technology Research and Development Program (863 program) of China (2003AA205009) to S.C., Research Fund for the Doctoral Program of Higher Education (RFDP, 20040487077) to S.C. and National Natural Science Foundation of China (30600571) to M.Z.

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doi:10.1016/j.trim.2008.06.002

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-I) 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% CO_2/95% 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′-GAAGAAGACGCUAUAGGCAdTdT-3′, antisense: 5′-UGCCUAUAGCGUCUUCUUCdTdT-3′, respectively. For transfection experiments, cells in a 6-well plate (50%–70% confluency) were transfected with RiboJuice™ (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 10^4 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 staining with 0.1% neutral red, and more than 95% viability was obtained. To address the functional significance of combined RNAi and GS-IB4 treatment, CDC was determined in a chromium release assay according to the method of Malassagne with minor modifications [19]. After labeling with ^{51}Cr (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 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, 5×10^5 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 C3c binding from human serum 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′-CTGGAGGAGGAGATTGAGC-3′; reverse 5′-TTGGCGACATTGGGGAAAG-3′ for HO-1, forward, 5′-GTGCGGGACATCAAGGAGAA-3′; reverse, 5′-TGTCCACGTCGCACTTCAT-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 desoxycholate, 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-