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Removal of blood group A/B antigen in organs by *ex vivo* and *in vivo* administration of endo-ß-galactosidase (ABase) for ABO-incompatible transplantation

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ABSTRACT

Background: ABO incompatibility in organ transplantation is still a high risk factor for antibody-mediated rejection, despite the progress in effective treatments. We have explored the possibility of using the enzyme to remove the blood type A/B antigen in organs.
Methods: Recombinant endo-ß-galactosidase (ABase), which releases A/B antigen, was produced in *E. coli* BL-21. Human A/B red blood cells (RBC) were digested with ABase, and subjected to flow cytometric analysis after incubation with human sera. Purified recombinant ABase was intravenously administered to a baboon. Biopsies were taken from kidney and liver before and 1, 4 and 24 h after *in vivo* administration. Excised baboon kidneys were perfused with cold UW solution+/−purified recombinant ABase and preserved at 4 °C. Biopsies were taken before and 1 and 4 h after *ex vivo* perfusion. The change in A/B antigen expression was analyzed by immunohistochemical study.
Results: ABase removed 82% of A antigen and 95% of B antigen in human A/B red blood cells, and suppressed anti-A/B antibody binding and complement activation effectively. ABase was also found to remain active at 4 °C. *In vivo* infusion of ABase into a blood type A baboon demonstrated a marked reduction of A antigen expression in the glomeruli of kidney (85% at 1 h, 9% at 4 h and 13% at 24 h) and the sinusoids of liver (47% at 1 h, 1% at 4 h and 3% at 24 h) without serious adverse effects. After *ex vivo* perfusion and cold storage of excised baboon kidney (blood type B) with ABase, the expression levels of B antigen in glomeruli were reduced to 49% at 1 h and 6% at 4 h.
Conclusions: This alternative approach might be useful for minimizing antibody removal and anti-B cell immunosuppression as an adjuvant therapy in ABO-incompatible kidney, liver and possibly heart transplantation.

1. Introduction

Living donor transplantation has been promoted to alleviate the chronic shortage of organ donors. However, donor and recipient combinations with ABO blood type incompatibilities (i.e., A to O or B, B to O or A, AB to O, A or B) have been unavoidable hurdles, because the donor is generally limited to the relatives of recipients. Such an ABO-incompatible transplantation was originally documented to be associated with poor prognosis because preformed anti-A/B antibody (Ab) would elicit Ab-mediated rejection (AMR) [1].

Recent progress in effective treatments such as Ab removal with (double filtration) plasmapheresis, splenectomy, anti-CD20 monoclonal Ab, intravenous immunoglobulin (IVIG) and potent immunosuppression has improved graft survival particularly in kidney transplantation [2–6]. However, graft failure due to refractory AMR or serious infection caused

Abbreviations: Ab, antibody; AMR, antibody-mediated rejection; Ag, antigen; ABase, endo-ß-galactosidase; EndoGalC, endo-ß-galactosidase C; Gal Ag, Galα1-3Galß1-4GlcNAc; MFI, mean fluorescence intensity; RBC, red blood cells.

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by overimmunosuppression is still observed in some recipients. Furthermore, hepatic necrosis or bile duct complications caused by severe rejection and thrombotic microangiopathy have been indicated as a reason for unfavorable graft prognosis in ABO-incompatible liver transplantation [7–9], although graft survival is gradually improving [10–12]. Thus, since the obstacle of ABO incompatibility has not been overcome completely, development of a new strategy against AMR would be valuable.

AMR is considered to be the result of antigen (Ag)–Ab reaction, activation of complement and procoagulation. Four main factors, (i) Ag, (ii) Ab, (iii) complement and (iv) coagulation, are associated with AMR. All treatments developed so far are measures against (ii) Ab, (iii) complement and (iv) coagulation, e.g., Ab removal, splenectomy, antimetabolites and anti-CD20 monoclonal Ab for the control of Ab, IVIG for inhibition of complement, and anticoagulant and antiplatelet drugs for suppression of microthrombi formation. However, no attempt has been made to modify blood type A/B Ag in the grafts directly, although neutralization of blood type A Ag by monoclonal anti-A Ab was reported [13]. Recently, several bacterial exoglycosidases which can effectively cleave blood type A/B Ag, have been successfully identified for the production of universal red blood cells (RBC) [14,15]. We have focused our attention on another type of specific enzyme which digests A/B Ag and explored the possibility of removing A/B Ag from the graft using this enzyme. We preliminarily reported the potential value of the enzymatic digestion of A/B Ag as an alternative strategy for overcoming ABO incompatibility [16].

2. Objective

Our objective in this study was to reveal the feasibility of removing blood type A/B Ag in organs by *in vivo* and *ex vivo* administration of recombinant enzyme.

3. Materials and methods

3.1. Production of endo-ß-galactosidase (ABase) which digests blood group A and B Ag

Two types of recombinant proteins of endo-ß-galactosidase, which were originally identified from *Clostridium perfringens*, were produced in *E. coli* BL-21 as described previously [17,18]. ABase was prepared for releasing A/B Ag and endo-ß-galactosidase C (EndoGalC) for releasing Galα1-3Galß1-4GlcNAc (Gal Ag), which is known to be highly immunogenic in xenotransplantation, and has a carbohydrate structure resembling the A/B Ag. ABase cleaves Galß1-4GlcNAc linkage in blood type A [GalNAcα1-3(Fucα1-2) Galß1-4GlcNAc] and in blood type B [Galα1-3(Fucα1-2) Galß1-4GlcNAc] [17] (Fig. 1).

Briefly, after cloning of ABase, an expression plasmid with a C-terminal His tag was constructed in the pET-15b vector eabC without signal peptide. This recombinant gene was transformed into *E. coli* BL-21 cells. The enzyme produced in the cells as a soluble protein fraction was purified over a nickel-nitrilotriacetic acid column (QIAGEN GmbH, Hilden, Germany). Finally, 5 mL of purified recombinant ABase was obtained at the concentration of 3.6 mg/mL with the specific activity of 1500 U/mg. One unit of the enzymatic activity was defined as the amount of the enzyme required to hydrolyze 1 μmol of the substrate per min.

3.2. Flow cytometric analysis of Ag expression

The inhibitory effect of ABase treatment on Ag expression, Ab binding and complement activation was examined. Human A/B RBC were digested with ABase and subjected to flow cytometric analysis after incubation with human sera.

After human blood type A RBC ($n=3$) and B RBC ($n=3$) were treated with dimethyl suberimidate solution, 1% suspensions of RBC were prepared [18,19]. Fifty μL of human blood type A or B RBC were incubated with 10-fold diluted recombinant ABase for 90 min at 37 °C. After washing, human RBC were reacted with mouse anti-human blood type A or B monoclonal Ab (Ortho-Clinical Diagnostics, Raritan, NJ) (×200, 100 μL) for 30 min at 4 °C and then with FITC-labeled goat anti-mouse IgM Ab (American Qualex, San Clemente, CA) (×100, 100 μL) for 30 min at 4 °C. The stained RBC were subjected to flow cytometric analysis using EPIX XL (Beckman Coulter, Inc., Miami, FL). The mean fluorescence intensity (MFI) was used to quantitate the expression level of blood type A, B and Gal Ag. Digestion level was expressed as a percentage of blood type A or B Ag expressed on RBC after incubation in the absence of ABase, which was calculated as follows,

Percentage of digestion level=[(MFI in the absence of ABase−MFI in the presence of ABase)/(MFI in the absence of ABase−MFI with FITC-labeled second Ab alone)]×100.

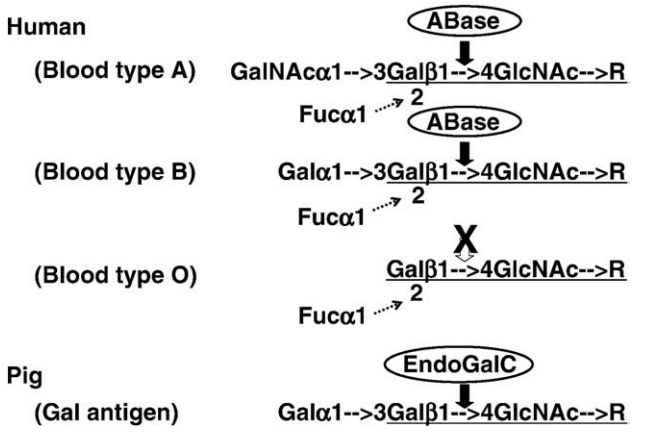

Fig. 1. Digestion of blood group A/B and Gal antigens by two types of endo-ß-galactosidases (ABase and EndoGalC). Endo-ß-galactosidase (ABase) cleaves Galß1-4GlcNAc linkage in human blood type A [GalNAcα1-3(Fucα1-2) Galß1-4GlcNAc] and in blood type B [Galα1-3(Fucα1-2) Galß1-4GlcNAc], but not in blood type O [Fucα1-2 Galß1-4GlcNAc], as indicated by arrows, while endo-ß-galactosidase C (EndoGalC) cleaves Galß1-4GlcNAc linkage in pig Gal antigen [Galα1-3Galß1-4GlcNAc].

3.3. Flow cytometric analysis of Ab binding and C3d deposition

Fresh blood type O sera were pooled from three healthy human volunteers and frozen at −80 °C to preserve endogenous complement activity until used. Heat-inactivated (for 30 min at 56 °C) sera were used for analysis of Ab binding. RBC with and without enzyme (ABase) digestion were incubated with 50% blood type O sera (100 μL) diluted with phosphate-buffered saline containing 0.2% bovine serum albumin (PBS/BSA) for 30 min at 37 °C. After washing, RBC were reacted with FITC-labeled anti-human IgG/IgM (DAKO, Glostrup, Denmark) (×30, 100 μL) for 30 min at 4 °C and then subjected to flow cytometric analysis.

The inhibitory effect of enzyme treatment on complement activation was also evaluated by the change of C3d deposition. After RBC were incubated with 50% human sera in the presence of complement activity for 15 min at 37 °C, RBC were reacted with FITC-labeled rabbit anti-human C3d Ab (DAKO, Glostrup, Denmark) (×100, 100 μL) for 30 min at 4 °C and then applied to flow cytometric analysis. The percentage of the control level (in the absence of enzyme) was calculated based on MFI to evaluate the inhibitory effect of enzyme treatment on Ab binding and C3d deposition.

3.4. Animal

Two female baboons (*Papio anubis*) weighing 12–13 kg with blood type A and B were used for an *in vivo* and *ex vivo* administration test,