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BLOOD GROUP ANTIGEN TARGETING PEPTIDE SUPPRESSES THROMBOTIC MICROANGIOPATHY IN RENAL GLOMERULAR CAPILLARIES AFTER ABO-INCOMPATIBLE BLOOD REPERFUSION

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Abstract

Background. Antibody-mediated rejection (AMR) after ABO-incompatible kidney transplantation (ABO-I KTx) is a major barrier to the success of transplantation. The advent of immunosuppressive therapy has markedly improved graft survival in ABO-I KTx. However, compare with a normal KTx, clinical conditions during ABO-I KTx are difficult to control due to over-immunosuppression. To reduce the immunosuppression we try to develop the blood group antigen-neutralizing therapy.

Methods. We screened an ABO blood group antigen targeting peptide (BATP) by peptide library displayed T7 phage screening. After screening, a hemagglutination (HA) inhibition assay and ELISA assay was used to analyze the blood group antigen blocking effect of the BATP. We also tested the inhibitory effect of anti-blood group Ab binding in normal human kidney tissues blocked with BATP.

Results. We identified six peptide sequences. BATP efficiently suppresses hemagglutination of red blood cells caused by anti-ABO blood group antibodies and binding of these antibodies to ABO histo-blood group antigens on kidney tissue.

Conclusions. These data indicating that blood group A/B-antigen on RBCs and on kidney tissues may neutralize by BATP. This approach may enable the development of novel blood group antigen neutralizing therapy to overcome the challenges of ABO-I KTx.

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Key words: Blood group antigen targeting peptide (BATP); ABO-incompatible kidney transplantation (ABO-I KTx)

Kidney transplantation (KTx) is the best treatment for chronic kidney failure. However, the donor population in Japan is 10-fold smaller than in the US and European countries. Donor deficiencies also the case in Western countries[1, 2, 3)]. To solve the chronic shortage of kidney donors, it is necessary to rapidly increase the number of potential donor and recipient combinations using ABO-I KTx. Although the use of ABO-I donor kidneys is a possible solution to the shortage of donor kidneys for transplantation, natural antibodies (Abs) and *de novo* Abs against ABO histo-blood group antigens are a major barrier to successful ABO-I KTx. Recently, effective antibody removal and several immunosuppressive treatments have improved graft survival, especially in kidney transplantation[4, 5, 6, 7, 8)]. However, graft failure

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caused by antibody-mediated rejection (AMR) or serious infections due to over-immunosuppression still occurs in some patients.

It has also been reported that the preoperative anti-A/B Ab titer has been correlates with long-term graft survival in ABO-I KTx[9]. Furthermore, in ABO-I liver transplantation, recipients with high anti-blood group Ab titers are also at very high risk of graft failure[10, 11]. Therefore, ABO incompatibility in organ transplantation remains a high risk factor for AMR, despite the progress in effective treatments. As the effect of ABO incompatibility have not been overcome completely, development of a novel treatment against AMR is therefore of considerable importance.

AMR results from complement activation and procoagulation following an antigen–Ab reaction and AMR associated with the ABO histo-blood group antigen and Ab as well as complement and coagulation factors. Clinically manifested treatments are being developed to control of Ab and complement as well as coagulation. These treatment include Ab removal by plasmapheresis. Use of splenectomy, antimetabolites and anti-CD20 mAb for the suppression of Ab production; use of IVIG for complement inhibition; and use of anticoagulant and antiplatelet drugs for suppression of microthrombi formation. However, direct modification or blocking of ABO blood group antigens in grafts is not yet available for clinical use and has been reported in only a few papers. The methods reported include neutralization of blood group A antigen by monoclonal anti-A Ab fab fragment[12], removal of blood group A/B antigen in baboon kidney by *in vivo* and *ex vivo* administration of endo-β-galactosidase (ABase) [13] and neutralization of preformed anti-A/B antigen antibodies in baboons by intravenously infusion of ABO blood group trisaccharide carbohydrate epitope[14] were reported. In this study, we used a peptide-displaying phage system to screen for a ABO histo-blood group antigen targeting peptide that had the ability to suppress AMR.

Peptide-displaying phage technology provides a method for identifying short peptide sequences specific to a target. This technology may also provide a means of identifying peptide sequences that mimic specific carbohydrate or antibody epitopes. As antibody drugs are still expensive and sometimes cause serious side effect, a specific peptide that functions in antibody mimicry provides us with a practical biological tool and potent alternative to antibody-based therapy. Several sequences have served as reagents to inhibit interactions between carbohydrate-binding proteins and their ligands have been reported[15, 16, 17, 18]. We identified a blood group A/B antigen targeting peptide, using a 7-mer random peptide library displayed T7 phage. After the 4th round of screening, we achieved significant enrichment of A and B trisaccharide binding phage clones (Fig. 1a, b). We picked twelve phage clones from the A and B trisaccharide binding phage pools after the 4th round of screening and determined six peptides sequences (Fig. 1c), that contained two common sequences (RPRNPNK and SPARRPR) identified from both A and B trisaccharide binding phage clones. Three peptide sequences (ASNKRPR, RPRNPNK and SPARRPR) contained RPR (Arg-Pro-Arg) motif. We therefore expected that RPR motif was important to recognize both A and B trisaccharide structure.

A hemagglutination (HA) inhibition assay was used to analyze the ABO blood group antigen blocking effect of the peptide (Fig. 1d). Red blood cells (RBCs) treated with the seven peptides had decreased HA activity ($2^{2\text{-}3}$ of the control, blood group A RBC) and ($2^{1\text{-}3}$ of the control, blood group B RBC). Although these peptides had a weak inhibitory effect on HA activity, we found that the RPRNPNK peptide had the strongest inhibitory effect of all the peptides tested. The RPRNPNK peptide may

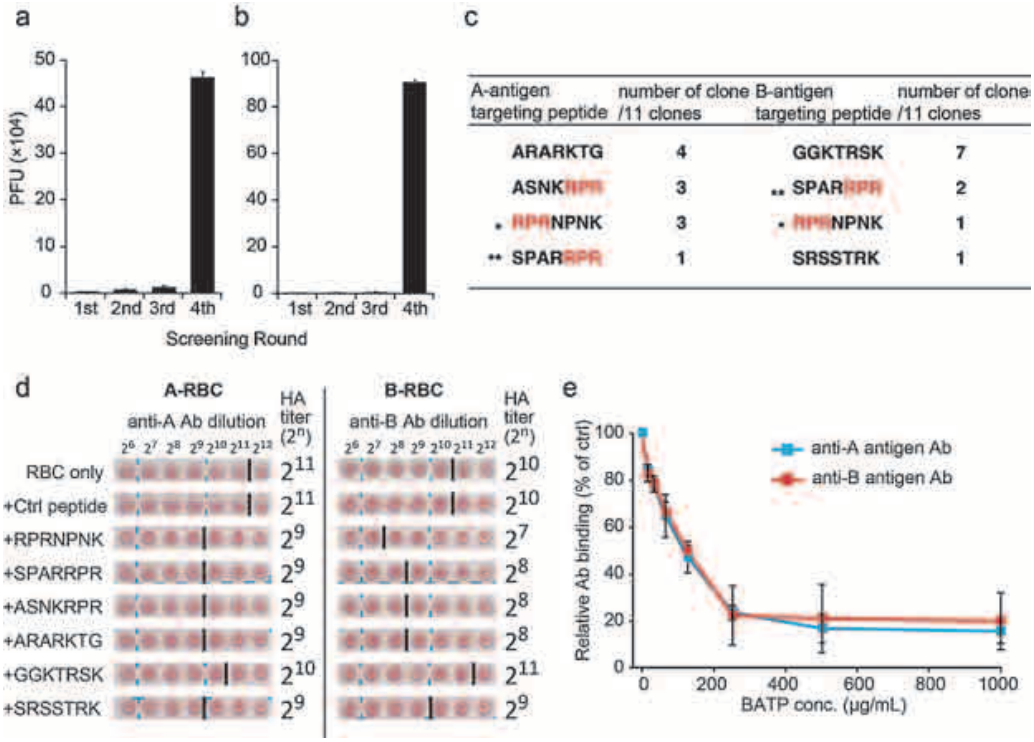

Figure 1. Screening of A or B trisaccharide-BSA binding peptide and characterization of BATP. (a) Screeing of A trisaccharide-BSA binding phage. (b) Screening of B trisaccharide-BSA binding phage. Both screening procedures were performed for 4 rounds. (c) Peptide sequence of enriched phage clone identified from the A and B phage clone pool after the 4th round of screening. (d) HA inhibition assay. An HA assay was performed by the microtitration method. Each titer was determined after incubation for 2 h of Anti-A/B Abs with 500 μg/mL BATPs blocked A or B RBC. RBC without BATP exhibited the maximum titer of 2^{11} (type A RBC) and 2^{10} (type B RBC) respectively. Data are obtained form a representative experiment repeated three times. (e) Inhibitory effect of BATP on the binding of anti-A/B Abs to A/B antigen. Blood group A or B trisaccharide-BSA coated wells were blocked with BATP at the indicated concentrations for 1 h at RT. Anti-A or anti-B Abs (1:1000 dilution in TBST) were added to each well and incubated for 1 h at RT. Using anti-rabbit IgM conjugate with HRP, changes in the antigen–antibody interaction were monitored using a Microplate reader. The level of Ab binding without BATP was tconsidered to be 100%. The results are expressed as mean ± SD of three independent experiments.

bind to blood group A and B antigens on RBCs and inhibit antigen-antibody interaction. We therefore selected RPRNPNK (designated as the blood group antigen targeting peptide; BATP) for use in subsequent experiments.

An ELISA assay was used to analyze the specificity and affinity of BATP against A- and B-trisaccharide BSA (Fig. 1e). We first investigated the dose-dependent inhibitory effects of BATP on anti-A/B Ab binding to A- and B-trisaccharide BSA. The addition of more than 200 μg/mL of BATP decreased the binding of anti-A/B Abs to A- and B-trisaccharide BSA to approximately 20% of the levels seen in controls). The IC_{50} value of BATP was also shown to be 66.6 μM for anti-A Ab and 111.6 μM for anti-B Ab.

To examine whether BATP inhibited binding of anti-A or anti-B Abs binding to A and B histo-blood group antigen on normal kidney tissue, we tested the inhibitory effect of anti-blood group antibody binding in A or B normal human kidney tissues (Fig. 2). The staining intensity of glomerular capillaries as well as preritubular and microvascular endothelium in type A and B kidney sections was markedly reduced by BATP-blocking treatment. This suggest that BATP masked A and B histo-blood group antigen on

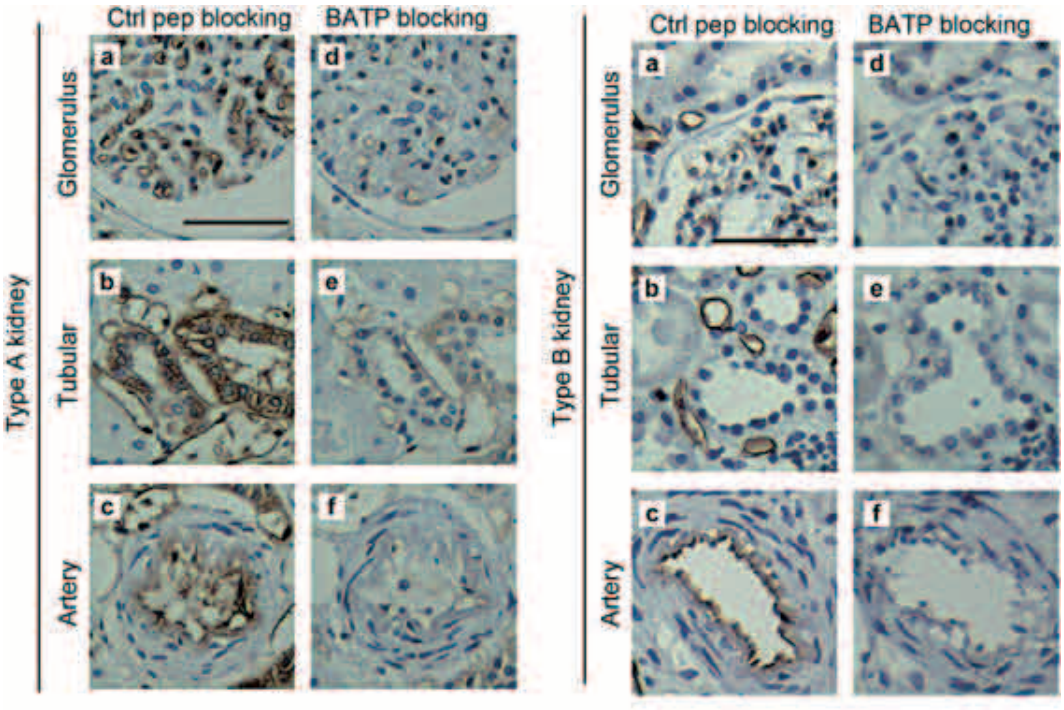

Figure 2. Blood group A or B antigens were masked using control peptide (a, b and c) or BATP (d, e and f) in immunohistochemical analyses of human kidney tissues. Either control peptide (a, b and c) or BATP (d, e and f) was added to the kidney sections of a blood group-A or B individual at a concentration of 200 μg/ml for 30 min at RT. Subsequently, anti-A Ab or anti-B Ab diluted 1:1000 were added and incubated for 1 h at RT. HRP conjugated anti-mouse IgM was incubated for 45 min at RT. Color was developed using 3,3-diaminobenzidine tetrahydrochloride for 1 min and the sections were counterstained with hematoxylin.

glomerular capillaries as well as peritubular and microvasucular endothelium. It was reported that ABO histo-blood group antigen on kidney tissue are different from ABO blood group antigen on RBC due to differences in carrie proteins[19]. Therefore, as shown in Figure.2, BATP may recognize A and B trisaccharide epitopes and inhibit ABO histo-blood group antigen-antibody interaction by masking their trisaccharide structure.

These experiments strongly suggest that peptide binding to ABO blood group antigen is an anti-blood group A and B Ab epitope mimetic that behaves like an anti-A or B Ab. Blood group A/B-antigen on RBCs and on kidney tissues may be neutralized by BATP. These findings imply a lack of reaction between ABO histo-blood group antigens and *de novo* synthesized anti-A/B histo-blood group antibodies that may contribute to long-term graft survival without rejection. There is evidence that this accommodation state is established within the two weeks after transplantation[20, 21, 22]. We therefore speculate that blocking of A and B antigens in donor organs by BATP administration during the first two weeks after transplantation (i.e., until accommodation probably occurs), is a reasonable and practical strategy. *Ex vivo* perfusion of the donor organ during cold storage, and prior to transplantation is also possible. Neutralization of blood group antigen by BATP may represent one strategy to overcome the challenge of ABO-I KTx. This alternative approach using a peptide may also be useful for minimizing Ab removal and anti-B cell immunosuppression as an adjuvant therapy in ABO-incompatible kidney. Currently, we do not have the data on *ex vivo* and *in vivo* peptide

stability and systemic side effect. Further studies on ABO-I KTx using animal model such as baboon[13, 14] are required to establish ABO antigen blocking therapy using BATP.

METHODS

Construction of T7 phage-displayed 7-mer peptide (X7) library. Libraries were constructed using the T7Select 415-1b vector as outlined in the T7Select System Manual (Novagen). Briefly, random oligonucleotide insert DNA was synthesized in the following format (insert for the X7 library shown): 5'-AACTGCAAGCTTTTA-(MNN)7-ACCACCACCAGAATTCGGATC CCCGAGCAT-3', (where N is a hand-mixed equimolar ratio of each nucleotide, and M is a hand-mixed equimolar ratio of both adenine and cytosine nucleotides). The amino acid translation of the complementary nucleotide sequence is: MLGDPNSGGGX7. The insert DNA was incubated with a complementary extension primer (5'-ATGCTCGGGGATCCGAATTCTGGT-3'), Klenow enzyme (TaKaRa), and dNTPs (Invitrogen) to form the complementary DNA strand. This reaction was digested with EcoRI and HindIII (NEB), followed by phenol/chloroform extraction and ethanol precipitation using standard techniques. The purified fragments were then ligated into the predigested T7Select 415–1b vector using a DNA ligation kit Mighty Mix containing T4 DNA ligase (TaKaRa). This method inserts the randomized oligonucleotide library DNA in-frame after amino acid 348 of the capsid 10B gene. The ligation reactions were incubated for 16 h at 16°C, subsequently subjected to *in vitro* packaging, and immediately followed by phage titration using a plaque assay. The remaining *in vitro* packaging solution was amplified once using BL21 until lysis. The lysate was centrifuged, titered, and frozen at –80°C in 0.5 M NaCl as glycerol stock.

Library biopanning procedure. Aliquots (100 μL) of Tris-bufferd saline (TBS) containing 10 μg Blood group A- or B-trisaccharide BSA (Dextra) were added to the wells with high binding capacity (BD Falcon) and incubated at 4°C overnight. The wells were washed three times with 300 μL of TBS and blocked for 1 hr at 4°C with 5% BSA and 5% normal goat serum (NGS) in TBS. The BSA/NGS-blocked blood group A- or B-trisaccharide BSA-coated wells were washed with 200 μL of TBS containing 0.5% Tween 20 three times, and then 100 μL of library phage was applied to each well subsequently. The plates were incubated at room temperature for 30 min with orbital shaking at 250 rpm. Followed by washing ten times with 200 μL of TBS containing 0.5% Tween 20. Bound phages were eluted by incubation with 100 μL of TBS containing 100 mM of blood group A or B trisaccharide per well at room temperature for 20 min with orbital shaking at 250 rpm. The eluate was collected, and 10 μL was removed for titering by plaque assay. The remaining eluted phage was amplified in 20 mL of freshly prepared BL21 in a baffled shaker flask by incubating at 37°C until visual lysis or for 3 h. The inoculated phages were centrifuged at 3400 rpm at 4°C for 15 min, with 100 μL of the supernatant being subjected to the next round of biopanning. The 2nd, 3rd and 4th rounds of biopanning were carried out in the same manner. For each round, the input number of PFU was held constant (as determined by the plaque forming assay) in order to keep the ratio of phage particles to target molecules approximately constant at 3.0×10^{10} PFU throughout the biopanning process.

Clone isolation and DNA sequence analysis. In the plate forming assay, the eluted clones were plated at a concentration of approximately 75 PFU per 100-mm plate to ensure well-isolated plaques. Each plaque was lifted with an inoculation

needle and placed into 500 μL of BL21 in a plastic test tube. The tubes were incubated at 37°C with orbital shaking at 250 rpm until visual lysis or for 3 h. The NaCl concentration of the lysate was adjusted to 0.5 M, followed by centrifugation at 3400 rpm at 4°C for 15 min. An 450 μL aliquot of clarified lysate was transferred to a 1.5 mL tube and stored at 4°C. The following components were used for PCR: Phire Hot Start DNA Polymerase (Finzyme), sterile molecular biology grade water, T7 Up primer (10 μM in TE buffer, pH 8.0), and T7 Down primer (10 μM in TE buffer, pH 8.0). The primers were synthesized on a 1 μM scale and cartridge purified. The sequences were as follows: T7 Up: 5'-AGCGGACCAGATTATCGCTAA-3', and T7 Down: 5'-AACCCCTCAAGACCCGTTTA-3'. A 1 μL of clarified phage lysate was added to each tube, and the T7 insert amplified in a 30-cycle PCR. The PCR product was electrophoresed and purified using QIAquick gel extracrtion Kit (QIAGEN) and sequenced with the T7 Up primer at the Sigma Genosys facility. The DNA sequence and translated amino acid sequence were analyzed using Geneious Pro ver. 5.0.3 (Biomatters Ltd.).

Inhibitory effect of BATPs on hemagglutination (HA) of human RBCs. RBCs of blood group A and B were isolated from healthy volunteers. Anti-A (Z2A), anti-B (Z5H-2) and anti-H (87-N) monoclonal antibodies were purchased from Santa Cruz biotechnology. An HA assay was performed using the following microtitration method. Anti-A/B Abs (100 μg/mL, 35 uL) were diluted in serial 2-fold steps and mixed with an equal volume of a 0.5% human RBCs pre-incubated for 30 min at RT with 500 ug/mL control peptide or BATPs (ARARKTG, ASNKRPR, RPRNPNK, SPARRPR, RMSRKLP, GGKTRSK and SRSSTRK). After incubation at RT for 2 h, the reciprocal HA titer was determined as 2^n.

Inhibitory effect of BATP on the binding of anti-A/B Abs to A/B antigen measured by ELISA. Blood group A- or B-trisaccharide BSA (5 μg/well) coated wells were washed three times with 300 μL of TBS and blocked overnight at 4°C with 5% BSA/NGS in TBS. The wells were then blocked with 100 μL of 0–500 μg/mL BATP (RPRNPNK) and incubated at RT for 1h. The wells were washed with 300 μL of TBST, followed by the addition of 100 μL of anti-A (Z2A) or anti-B (Z5H-2) Abs (1:1000 dilution in TBST) and incubated at RT for 1h. The wells were washed with 300 μL of TBST, and subsequently, 100 μL of HRP-labeled goat anti-mouse IgM Ab (1:10000 dilution in TBST) was added, followed by incubation at RT for 30 min. After the secondary Ab reaction, 100 μL of tetramethylbenzidine (TMB) peroxidase substrate (Funakoshi) was added and incubated at RT for approximately 3 min. The reaction was stopped with 100 μL of 1 N HCl, and then the OD measured at 450 nm.

Human normal kidney tissues. The human normal kidney tissues were obtained from renal tumor patient who underwent radical nephrectomy at the Department of Urology, Hirosaki University Hospital (Hirosaki, Japan). After the routine radical nephrectomy for the patient with renal tumor, a minor portion of normal kidney was removed, which was subjected to *ex vivo* perfusion of BATP experiment. Informed consent was obtained from all patients prior to the initiation of the study. This study was approved by the Ethics Committee of Hirosaki University, Faculty of Medicine. Likewise, the study was performed in accordance with the Guidelines of the Declaration of Helsinki.

Immunohistochemistry. The normal part of the kidney was fixed in formalin for hematoxylin–eosin and immunohistochemical staining. The deparaffinized sections were then exposed to 3% hydrogen peroxidase for 5 min. After washing

with PBS, the expression of ABO histo-blood group antigen was examined using anti-A (Z2A) or anti-B (Z5H-2) or anti-H (87-N) monoclonal Ab and HRP-labeled anti-mouse IgM Ab or Alexa488-anti-mouse IgM Ab. The sections were then counterstained with haematoxylin, and appropriate mounted. To examine the blood group antigen blocking effect, 200 μg/mL of control peptide or BATP (RPRNPNK) was added to the kidney sections, followed by incubation for 30 min at 25° C before the 1st antibody staining.

Statistical Analyses. Results are expressed as mean ± standard deviation. Student's t test was used to determine the significance of difference between the groups. A value of $P<0.05$ was considered statistically significant.

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