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A novel recombinant fibrinogenase of Agkistrodon acutus venom protects against hyperacute rejection via degradation of complements

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ABSTRACT

Hyperacute rejection (HAR) is a main barrier in xenotransplantation, which is mediated by the combination of natural antibody to the xenograft and complement activation. Current therapies have focus on the inhibition of complement by development of complement inhibitor and transgenic animal organ. Here, we investigated the effects of rFII, a recombinant fibrinogenase from Agkistrodon acutus venom, on complement and HAR. The degradation effect of rFII on complement was tested by SDS-PAGE, CH50 examination, ELISA Kit and cofocal immunofluorescence microscopy in vitro and in vivo. An ex-vivo rat-to-human perfusion model and a vivo guinea-pig-to-rat heat HAR model were used to determine the protection of rFII against HAR. Our investigation indicated that rFII could significantly degrade human C5, C6, and C9, decrease the activity of complement, and inhibit the MAC deposition on HUVECs membrane in vitro. In addition, serum levels of C1q, C3 and C4 in rat were gradually reduced after infusion of rFII. Importantly, in an ex vivo rat-to-human perfusion model, the survival of rat hearts perfused with human serum treated with rFII (83.36 ± 16.63 min) were significantly longer than that of hearts perfused with fresh human serum(15.94 ± 4.75 min). At the time of 15 minutes after perfusion, functions of hearts added with 50 ug/ml rFII sustained well with heart rates at 283 ± 65.32 beats/minute and LVDP at 13.70 ± 5.45 Kpa, while that of hearts perfused with fresh human serum were severely damaged by HAR with heart rates at 107.77 ± 40.31 beats/minute and LVDP at 1.01 ± 0.83 Kpa. We also found that rFII significantly decreased the levels of C1q, C3 and C4 in human fresh serum perfusate. In a vivo guinea-pig-to-rat heat HAR model, the survival of rat hearts treated with rFII were significantly longer than that of hearts perfused with normal saline; and relieved heart damage by complete activation. Our finding demonstrates the anti-complement property of rFII and its protection against HAR, indicating that rFII might be as a potential therapeutic agent for xenotransplantation.

1. Introduction

Clinical transplantation is the only effective therapy for end-stage organ failure. Unfortunately, many patients do not receive this therapy owing to the severe shortage of suitable human organ donors [1]. This supply-demand imbalance could be corrected by transplanting organs from other species (xenografts) into human. The most major barrier to xenotransplantation, however, is hyperacute rejection (HAR) to the organ graft.

HAR is mediated by the binding of preexisting of xenoreactive natural antibodies(XNAs) to antigens expressed on the donor organ endothelia, which active complement and finally lead to thrombosis and destruction to the graft [2,3]. The antibody and complement system have been shown to play a central patho-physiologic role in HAR and to contribute to the inflammation and

Abbreviations: ADP, adenosine diphosphate; ATII, antithrombin; CH50, complement hemolytic activity 50; CVF, cobra venom factor; HAR, hyperacute rejection; HUVEC, human umbilical vein endothelial cell; HR, heart rate; IgM, immunoglobulin M; LVDP, left ventricular developed pressure; LSCM, laser scanning confocal microscopy; MAC, membrane attack complex; rFII, recombinant fibrinogenase of Agkistrodon acutus venom; SCR1, soluble human complement receptor type1; XNAs, xenoreactive natural antibodies.

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organ injury associated with transplantation [4,5]. Furthermore, increasing evidence suggested that coagulation and thrombotic disorders were associated with HAR as well, which was characterized by the formation of platelet adhesion, aggregation, thrombosis, and endothelial dysfunction resulting from activation of the coagulation pathway [6,7]. Thus, interventions made to overcome HAR mainly concentrated on prevention of complement activation or removal xenoreactive natural antibodies [1,8] and therapeutic strategies to prevent intravascular thrombosis after xenotransplantation have received increasing attention as well. Despite these advances, development of an effective drug that has therapeutic role against HAR is still in urgent.

Our previous results revealed that rFII, a novel recombinant fibrinogenase from Agkistrodon acutus venom could inhibit the platelet aggregation and directly degrade the microvascular thrombosis without activating intrinsic plasminogen [9–11]. Based on the hypothesis that inhibition of both complememnts and coagulation cascade could be a clue to avoid HAR, an exploration for the possible therapeutic potential of rFII against HAR is therefore essential.

Here we demonstrated that rFII cleavaged and decreased the activity of complements *in vitro*, reduced the serum level of complements *in vivo* and protected against HAR in a rat heart *ex vivo* perfusion model and a guinea pig-to-rat heart HAR model, indicating that it might be an potential drug candidate against HAR.

2. Materials and methods

2.1. Reagents, cell cultures and animals

The recombinant fibrinogenase II(rFII) from Agkistrodon acutus venom was prepared as we previously described [11].

Human Umbilical Vein Endothelial cells (HUVECs) were obtained from the American Type Culture Collection (Manassas, USA). Cells maintained in DMEM (Invitrogen, USA) supplemented with 10% FBS and a humidified atmosphere of 5% CO_2 at 37 °C.

All animal experiments were conducted in accordance with the National Guide for the Care and Use of Laboratory Animals and were approved by Sun Yat-sen University Animal Care and Use Committee (Guangzhou, China). Adult male S-D rats (250–300 g) and adult male guinea-pigs (300–350 g) were supplied by the Experimental Animal Center of Zhongshan Medical College, Sun Yat-san University.

2.1.1. Degradation effect of rFII on human complement in vitro

Human complement C_5, C_6 and C_9 (Sigma Aldrich, USA) were incubated with 10 ug/ml rFII at 37 °C for 2 h. Then samples were analysed by 12% SDS-PAGE.

2.1.2. Complement hemolytic activity (CH50) assay

The complement hemolytic activity (CH50) was detected as previously described [12]. The red cells of guinea-pig were diluted to the concentration of 2% by the buffer (NaCl17g, Na_2HPO_4 11 g, KH_2PO_4 0.27 g, 10% $MgCl_2$ 2 ml and distilled water 280 ml). Then the dilutions were incubated with isovolumic hemolysin at 37 °C for 0.5 h. The SD rat serums were treated with or without rFII for different times (2 h, 6 h and 12 h). We established four groups: low-dose (10 μg/ml rFII) group, medium-dose(20 μg/ml rFII) group, high-dose(50 μg/ml rFII) group and normal group. The appropriate series of treated or untreated rat serum were added into a series of reaction tubes containing the buffer and sensitized guinea-pig red cells. After incubation at 37 °C for 1 h, the tubes were centrifuged to remove unlysed cells, and the optical density of supernatant was measured at 540 nm. The quantity of complement required for 50% hemolysis is defined as CH50 value. Data were expressed as the percent change in CH50 at selected time point which was calculated using the following formula: (CH50 value at the selected time point/pre-injury baseline value × 100%).

2.1.3. Degradation effect of rFII on serum complement in vivo

Rats were infused with rFII for 6 h at the speed of 1 ml/h through the right femoral vein. Four different groups were established: normal group: saline was infused; rFII groups: 0.1 mg/kg, 0.2 mg/kg and 0.5 mg/kg. The plasma of rats were collected in tube at pre-infusion (0 h) and post-infusion (2 h, 4 h and 6 h) time points and stored at −20 °C until assayed. The concentrations of C_{1q}, C_3 and C_4 in animal plasma was determined using ELISA Kit (R&D, USA).

2.1.4. Ex-vivo rat hearts perfused with human serum supplemented with rFII

This working heart–perfusion model used for hyperacute rejection was prepared as previously described [13]. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg). Heparin (200IU/100 g) was injected via the inferior vena cava, and the heart was quickly removed to ice-cold Krebs-Henseleit buffer (K-H buffer) containing (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.75, $MgSO_4$ 1.2, glucose 11, EDTA 0.5, $NaHCO_3$ 25. Following aortic cannulation, hearts were perfused in the *ex vivo* Langendorff mode at a constant flow rate of 15 ml/min with oxygenated (95%O_2 and 5% CO_2) perfusate at 37 °C. Two electrodes were put on the left ventricle and the right atrium to monitor heart rate (HR) and left ventricular developed pressure (LVDP).

Human blood was obtained from healthy volunteers after informed consent, separated into serum and cellular components by centrifugation. The hearts of 60 male SD rats (200–300 g) were explanted. Administration was started after 30-min equilibration period. Six groups were established: serum-control group was perfused with fresh human serum and served as a positive control; decomplementation group was perfused with the decomplemented human serum which was performed by heating of the serum at 56 °C for 30 minutes to inactivate the complement activity as negative controls. rFII-treated groups (low-, medium-, and high-dose rFII) were given the human serum which incubated with different doses of rFII(10 ug/ml, 20 ug/ml and 50 ug/ml) for 6 h at 37 °C before perfusion. The additional isolated hearts, which were given neither treated- nor untreated- human serum, were infused with K-H buffer.

The experiments were terminated when hearts failed to pump against the afterload column of 55 mm Hg and the survival time of perfused hearts was recorded. The serum were collected in tube before and after experiments and stored at −20 °C The concentrations of C_{1q}, C_3 and C_4 in human serum were determined using ELISA Kit (R&D,USA).

2.1.5. Guinea-Pig-to-Rat heart transplantation in vivo

S-D rat underwent a heterotopic guinea-pig-heart transplantation into the abdomen as previously described [13]. The transplant was performed through a lower midline abdominal incision, implanting the guinea-pig heart by anastomosis of the donor aorta to the recipient abdominal aorta and of donor pulmonary artery to the recipient inferior vena cava. Treatments were started 6 h before heterotopic heart transplantation through femoral vein. Four groups were established, rFII-treated groups (low-, medium-, and high-dose rFII) were given different doses of rFII(0.1 mg/kg, 0.2 mg/kg, and 0.5 mg/kg) for 6 h. Normal group: saline was infused. Guinea-pig heart xenografts were removed at the time of rejection or death for histological study with H/E staining, and immunohistochemically for immunoglobulin C_3.