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Using an *in vitro* xenoantibody-mediated complement-dependent cytotoxicity model to evaluate the complement inhibitory activity of the peptidic C3 inhibitor Cp40

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ABSTRACT

Simple and reliable methods for evaluating the inhibitory effects of drug candidates on complement activation are essential for preclinical development. Here, using an immortalized porcine aortic endothelial cell line (iPEC) as target, we evaluated the feasibility and effectiveness of an *in vitro* xenoantibody-mediated complement-dependent cytotoxicity (CDC) model for evaluating the complement inhibitory activity of Cp40, a potent analog of the peptidic C3 inhibitor compstatin. The binding of human xenoantibodies to iPECs led to serum dilution-dependent cell death. Pretreatment of the human serum with Cp40 almost completely inhibited the deposition of C3 fragments and C5b-9 on the cells, resulting in a dose-dependent inhibition of CDC against the iPECs. Using the same method to compare the effects of Cp40 on complement activation in humans, rhesus and cynomolgus monkeys, we found that the inhibitory patterns were similar overall. Thus, the *in vitro* xenoantibody-mediated CDC assay may have considerable potential for future clinical use.

1. Introduction

When dysregulated or inadvertently triggered by diseased, injured or foreign cells, the complement system can contribute to a wide spectrum of inflammatory disorders [1]. As a consequence, there is a growing interest in complement therapeutics, particularly in strategies that interfere at the activation of the central complement component C3 [2]. Compstatin, a C3-targeted complement inhibitor, was originally discovered by screening a phage-displayed random peptide library [3]. This peptide specifically binds to native primate C3 and potently inhibits complement activation *via* all major initiation pathways [4]. Cp40 is a novel analog of compstatin that shows higher serum stability, 5000-fold stronger binding affinity for C3, and improved pharmacokinetic properties when compared to compstatin [1,5,6]. In a clinically relevant study on paroxysmal nocturnal hemoglobinuria (PNH), Cp40 was found to effectively protect PNH erythrocytes from both intravascular and extravascular hemolysis *in vitro*, thereby showing potential therapeutic advantage over the established anti-C5 therapy [5]. In another preclinical study, Cp40 was able to inhibit complement dysregulation *in vitro* in C3 glomerulopathy and may therefore offer a novel therapeutic option for affected patients [7]. In addition, Cp40 has been shown to be a potent inhibitor of complement activation in several *in vivo* and *ex vivo* animal models, such as a primate model of hemodialysis-induced complement activation [8], a ligature-induced periodontitis model in nonhuman primates (NHP) [9], and a xenogeneic model of interactions between human whole blood and porcine endothelium [10]. This experimental evidence suggests that Cp40 has strong potential as a therapeutic agent for clinical use [4].

Whereas the plasma levels of Cp40 and C3 during *in vivo* studies can be monitored using analytical methods to estimate the drug-to-target ratio, sensitive *ex vivo* methods are desired to experimentally confirm the inhibitory efficacy of Cp40 during treatment. The first method described for measuring the inhibitory effect of compstatin on complement activity was based on a hemolytic model in human serum [3]. After the incubation of rabbit erythrocytes and normal human serum pretreated with compstatin, the percentage of red cell lysis was determined by measuring the optical density of supernatant at 414 nm and normalizing the results by considering 100% lysis to be equal to lysis occurring in the absence of the peptide. However, the lack of available standard rabbit erythrocytes and the indirect evaluation by OD value

Abbreviations: α-Gal, Galα1-3Gal; CDC, complement-dependent cytotoxicity; FACS, fluorescence-activated cell sorter; Gmean, geometric mean fluorescence intensity; HINHS, heat-inactivated normal human serum; HINMS, heat-inactivated normal monkey serum; iPEC, SV40-immortalized porcine aortic endothelial cell line; NHS, normal human serum; NHP, nonhuman primate; PI, propidium iodide.

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limit the repeatability and accuracy of this method. Within the past decade, an ELISA-based assay was established to quantify the inhibitory effect of Cp40 and other compstatin derivatives on complement activation [11–13]. With this method, complement is activated by antibody–antigen complexes *via* the classical pathway, and the deposition of C3b is detected by ELISA. Although this method avoided the individual differences inherent in targeting primary cells, OD values were also used as the indicator in this method. The development of a simple and reliable method that can directly evaluate the effects of Cp40 and other complement inhibitors in a clinically relevant context would therefore be valuable for efficacy monitoring.

It has been demonstrated that the majority of preformed natural antibodies contained in human or NHP sera can bind to the Galα1-3Gal (α-Gal) epitope expressed on porcine endothelial cells (PECs), resulting in the activation of complement *via* the classic pathway and subsequent rapid cell death [14–18], which can be sensitively and accurately detected by flow cytometry *via* propidium iodide (PI) staining [17,19]. Given that the xenoantibody-mediated cytotoxicity to PECs is well defined as being complement-dependent, the *in vitro* cell death model may be useful for evaluating the complement inhibitory activity of compstatin and its derivatives.

In the present study, with the use of an SV40-immortalized porcine aortic endothelial cell line iPEC as a target and human or NHP sera as sources of xenoreactive natural antibodies and complement, we have tested the feasibility and effectiveness of the porcine cell lysis model in evaluating the complement inhibitory activity of Cp40 in human serum and have also compared the effects of Cp40 in different primate species.

2. Materials and methods

2.1. Cell line and cell culture

The SV40-immortalized porcine aorta-derived endothelial cell line iPEC was a gift from Dr. J. Holgersson (Karolinska Institute, Huddinge, Sweden). iPECs were maintained in low-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclone, China) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, USA). The cells were cultured in cell culture flasks at 37 °C in a 5% CO_2 atmosphere before experimentation.

2.2. Blood and serum preparations

Fresh non-anticoagulated human blood, without any restriction of blood type, was obtained from eight healthy volunteers who had given informed consent in accordance with the Helsinki Protocol and had received no medication for at least 10 days. Complement-active normal human serum (NHS) was collected, pooled, and stored at −80 °C to maintain its complement activity. Pooled normal serum samples from cynomolgus monkeys (n = 8) and rhesus monkeys (n = 8) were obtained separately, following the same procedure as for humans. The donor monkeys (3–5 years old; weight, 3–7 kg) were purchased from Guangzhou Landao Biotechnology Corporation and South China Primate Research Center, Guangzhou, China. They were kept in the primate facility at the Experimental Animal Centre of Tongji Medical College according to the University's Research Animal Resources guidelines. As negative controls, pooled normal serum of each species was heated at 56 °C for 30 min and is referred to as heat-inactivated normal serum.

2.3. Pretreatment of serum samples with CP40

Cp40 (dTyr-Ile-[Cys-Val-Trp(Me)-Gln-Asp-Trp-SAR-His-Arg-Cys]-mIle-NH_2) [17,19], a potent compstatin analog, was prepared by solid-phase peptide synthesis [6] and used to block complement activation. Pooled normal serum samples from either humans, cynomolgus monkeys, or rhesus monkeys were separately pretreated for 1 h at 37 °C with various final concentrations of Cp40 (from 1.25 μg/ml to 1280 μg/ml) prior to incubation with iPECs.

2.4. Detection of α-Gal expression on iPECs by FACS

Staining for α-Gal was performed as described previously [20]. In brief, iPECs (2×10^5) were incubated with fluorescein isothiocyanate (FITC)-conjugated *Griffonia simplicifolia* isolectin B4 (GS-IB4) (5 μg/ml, Sigma-Aldrich Corp., USA) for 30 min at 4 °C. Cells incubated with PBS alone served as a negative control. The stained cells were washed twice and then analyzed by flow cytometry (FACSCalibur, BD Biosciences).

2.5. Binding of xenoreactive antibodies present in human serum

To detect the binding of preformed anti-pig xenoreactive IgG and IgM to iPECs, 2×10^5 non-fixed single cells were incubated with heat-inactivated normal human serum (HINHS, 1:5 dilutions) at 4 °C for 30 min. After two washes with PBS, the cells were incubated with FITC-conjugated rabbit anti-human IgG or IgM (1:100, Zhongshan Biotechnology Co. Ltd., Beijing, China) at 4 °C for 30 min in the dark. iPECs incubated with secondary antibody alone served as negative controls. The stained cells were analyzed by flow cytometry. The geometric mean fluorescence intensity (Gmean) was used to evaluate the degree of xenoantibody binding to iPECs.

Binding of human xenoreactive IgG and IgM to iPECs was also detected by immunofluorescent staining. In brief, iPECs (5×10^4/well) were cultured overnight in 48-well plates with 200 μl of DMEM. Cells were washed four times with PBS, then incubated with 20% HINHS or PBS (negative control) at 37 °C for 20 min. After being washed, the cells were then incubated with FITC-conjugated rabbit anti-human IgG or IgM (1:50, Zhongshan Biotechnology Co. Ltd., Beijing, China) at 37 °C for 30 min in the dark. Fluorescence was visualized with a fluorescence microscope (Nikon ECLIPSE TE2000-U, Japan).

2.6. Detection of C3b/iC3b, C4b/iC4b, and C5b-9 deposited on iPECs

After incubation with 20% pooled NHS at 37 °C for 20 min, the deposition of C3- and C4-derived opsonins, and C5b-9 onto iPECs was detected by flow cytometry and immunofluorescent staining, using methods similar to those we described previously [20]. Cells incubated with HINHS were used as negative controls. To detect deposition of C3c- and C4c-containing fragments (*i.e.*, C3b/iC3b and C4b/iC4b, respectively), cells were incubated for 30 min with FITC-conjugated rabbit anti-human C3c and anti-human C4c antibody, respectively (1:100 for FACS and 1:50 for immunofluorescent staining, Zhongshan Biotechnology, Beijing, China). To detect terminal complement complex (*i.e.*, C5b-9) deposition, cells were incubated for 30 min with mouse anti-human C5b-9 primary antibody (1:100 for FACS and 1:50 for immunofluorescence, clone aE11, DAKO Corporation, CA, USA), followed by the Dlight 488-conjugated goat anti-mouse secondary antibody (1:100, Zhongshan Biotechnology, Beijing, China) for 30 min; all incubations were done at 4 °C for FACS and 37 °C for immunofluorescent staining. The stained cells were analyzed by flow cytometry. Gmean was used to evaluate the degree of complement deposition. Fluorescence was visualized with a fluorescence microscope (Nikon ECLIPSE TE2000-U, Japan).

2.7. Antibody-mediated complement-dependent cytotoxicity (CDC) assay

iPECs (100 μl, 2×10^5) were added to 100 μl of various diluted or pretreated serum samples. Incubation was carried out at 37 °C for 30 min and stopped with 3 ml FACS buffer (PBS containing 2% FBS and 0.02% azide). iPECs incubated with 20% or 40% heat-inactivated normal