Fibrinogen interaction with complement C3: a potential therapeutic target to reduce thrombosis risk

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Methods

Patient recruitment

We recruited 12 patients with type 1 diabetes (T1DM) and 12 age and sex matched controls with enough blood samples collected to allow purification of fibrinogen and C3 from each individual sample. Ethical approval for the study was provided by the Leeds East, National Research Ethics Service (NRES) committee and informed consent was obtained from participants in accordance with the Declaration of Helsinki.

We additionally tested a total of 24 stored plasma samples from high vascular risk individuals, including 11 with a history of ischaemic heart disease and 13 with a history of type 2 diabetes. All these samples were collected after appropriate ethical approval.

Protein purification and turbidimetric analysis

Fibrinogen and complement C3 were purified from each plasma sample as previously described (7). Briefly fibrinogen was purified by affinity chromatography using a calcium dependant IF-1 monoclonal antibody and C3 was purified by precipitation with polyethylene glycol 4000, anion exchange chromatography using Mono Q 5/50 GL (GE Healthcare, Bucks, UK) and finally gel filtration using Superose 12 10/300 GL (GE Healthcare).

Clots were formed from each purified fibrinogen (0.22 mg/ml [0.65 μM]) in the presence/absence of C3 (0.11 mg/ml [0.6 μM]) from the same individual following the addition of a lysis mixture containing 1.25 μg/ml plasminogen (Enzyme Research

Laboratories, Swansea, UK) and 2.2 µg/ml FXIII in tris buffered saline (TBS), pH 7.4 and subsequently an activation mix containing tPA (Tecnoclone, Vienna, Austria), at 50 ng/ml, $CaCl_2$ 2.5 mM and thrombin (Calbiochem, Germany), 0.12 U/ml (all final concentrations). Measurements were taken at 340nm every 24 seconds for 6 hours in a plate reader as previously described (7). Time from full clot formation to 50% lysis was calculated and presented as clot lysis time throughout this work.

Peptide Microarray

A peptide microarray chip containing overlapping 15 aa peptides of the entire fibrinogen molecule (PEPperPRINT, Germany) was synthesised and used to screen against pooled C3 (0.8 to 8 µg/ml) purified from 6 healthy individuals. Fibrinogen peptide chips were incubated with C3 and, after extensive washing, bound protein was detected using anti-C3 antibody (LifeSpan Biosciences Inc, USA). Control peptides framing the peptide arrays were stained as internal quality control to confirm peptide microarray integrity.

Quantification of spot intensities and peptide annotation were performed with PepSlide® Analyzer (PEPperPRINT). Using a validated software algorithm, intensity maps were generated for each assay and averaged spot intensities were plotted against the linked fibrinogen sequence from the N- to the C-terminus. The intensity plots were correlated with peptide and intensity maps as well as with visual inspection of the microarray scan to identify peptides and consensus motifs that interacted with C3.

Non-antibody synthetic proteins (Affimers)

Affimers are small proteins developed locally and are composed of a scaffold protein that constrains two variable conformational 9 amino acid loops. The variable region has great diversity (>3x10^{10} random peptides) and have the capability of binding "target" protein as previously described (11;12).

Panning and protein production

A phage display library of these Affimer proteins, comprising 1.3 x10^{10} random variable clones, was screened against human fibrinogen to identify high affinity binders as follows: Commercial human fibrinogen (Calbiochem) was further purified using IF-1 monoclonal antibody (13), biotinylated using EZ-link NHS-SS-biotin (Pierce Biotechnology, Rockford, Illinois, USA) and successful biotinylation was confirmed by ELISA using streptavidin-conjugated horseradish peroxidase (HRP). Biotinylated fibrinogen was then added to streptavidin coated wells (ThermoFisher, UK) for 1 hour followed by addition of the Affimer phage display library with repeated panning steps undertaken as described (12). Briefly, Affimers were incubated with fibrinogen for 2.5 h, after which wells were washed 10 times, followed by elution of the bound phage and expression in *Escherichia coli* ER2738 electrocompetent cells (Lucigen, USA). Colonies were grown on LB agar plates overnight followed by inoculation with M13K07 helper phage in order to generate a new library for the second round of panning. In order to select specific Affimers capable of targeting fibrinogen–C3 interactions, competitive elution with C3 (0.5mg/ml) for 20 min was applied in the fourth round of panning. Individual ER2738 colonies were selected and confirmed to be specific binders to fibrinogen by phage ELISA as previously described (12) .

To rule out interference from the phage and to generate high quantity of the protein, Affirmers of interest were produced by subcloning the coding regions into pET11 vectors. Affimers of interest were then expressed in *E. coli* BL21 (DE3) with subsequent purification as previously described (12).

Affimers and fibrinolysis

Purified experiments

A total of 0.65uM µM fibrinogen (Calbiochem) was incubated with the relevant Affimer (0.163 mg/ml or 5:1 molar concentration of Affimer:fibrinogen) at room temperature for 30 min followed by the addition of C3 at 0.6 µM and Factor XIII 2.2 µg/ml. A lysis mix containing plasminogen (final concentration 1.25 µg/ml) and TBS was added followed by an activation mix containing tPA at 50 ng/ml, $CaCl_2$ 2.5 mM and thrombin, 0.12 U/ml (all final concentrations). Measurements were taken at 340nm every 12 seconds for 1 hour as described (7). In all experiments, an Affimer scaffold with no insert was used as a control.

Plasma experiments

Plasma samples were mixed with increasing concentrations of Affimer A6 and control (ranging from 0:10 – 50:10 Affimer:fibrinogen molar ratio). Turbidimetric experiments using plasma conditions were subsequently conducted as previously described (14).

Molecular modelling

In order to identify potential sites of ligand binding on C3, the molecular modelling software Autoligand was employed to scan the whole protein (15). Molecular modelling was used to predict binding sites of any peptide sequences identified from the microarray screening and Affimer work used the webserver Pepsite2® (http://pepsite2.russelllab.org). Pepsite2 predicts the binding site of each residue in a peptide (up to 10 aa in length) to a known protein surface using pre-determined preferred binding environments for each residue. The result is an approximation of the peptide structure bound to the protein surface. Images were viewed and produced using Pymol (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC) (16).

Mass Spectrometry

This was performed to investigate potential mechanisms for the difference in the anti-fibrinolytic activity of C3 from patients with diabetes compared with healthy controls. Purified C3 from 6 T1DM and 6 healthy controls was digested by trypsin and analysed by matrix-assisted-laser-desorption/ionisation-time-of-flight-mass spectrometry (MALDI-TOF/TOF) as previously described (17). Briefly, C3 was incubated with aqueous ammonium bicarbonate (50 mmol l^{-1}) and 0.2% w/c trypsin for 24 h at 37°C. The resulting tryptic peptides were desalted and concentrated utilizing the $ZipTip_{C18}$ technology (Millipore, USA) using 0.1% trifluoroacetic acid (TFA) and 80% acetonitrile (ACN). The eluate was spread onto the (MALDI) target plate (MTP-AnchorChip 400/384; Bruker-Daltonic, Germany) using α-cyano-4-

hydroxycinamic acid (205 mg ml^{-1}) as matrix with mass spectrometric analyses carried out using MALDI-TOF/TOF mass spectrometer (Ultraflex III; Bruker-Daltonic, Germany). MS/MS fragments were analysed using Lift-option of the mass-spectrometer. Calibrated and annotated spectra were subjected to the database search Swiss-Prot (http://www.expasy.org/) utilizing the software tool “Bruker Bio-Tool 3.2 and the “Mascot 2.2 search engine” (Matrix Science Ltd, London, UK). Results were entered into the database and subsequently used empirically determined factors to assign a statistical weight to each individual peptide match.

To compare potential differences between *in vivo* and *in vitro* C3 glycation, we undertook *in vitro* glycation of the protein as previously described (18). Briefly, 1 ng/ml C3 solution was mixed with 0.5 mol/l D-glucose solution to reach a final concentration of 1.5 nmol/l. After an incubation period of 4 h, the reaction mixture was diluted with water and dialyzed against PBS. Afterwards, the post-translational modifications of C3 were validated by mass-spectrometry as described above.