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Design, Structure, Function and Application of Compstatin

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Abstract. A phage-displayed random peptide library was used to identify a cyclic peptide that binds reversibly to C3 and inhibits the activation of the complement system. Studies of overlapping peptides indicated that the active site of the parent peptide was located at the 13-residue cyclic segment in the N-terminal region, which is named Compstatin. The structural characteristics and the contribution of each residue to the binding of Compstatin to C3 were examined. Analysis of Ala substitution analogues suggested that Val^3, Gln^5, Asp^6, Trp^7 and Gly^8 contribute significantly to the inhibitory activity of Compstatin. The solution structure of a major conformer of Compstatin was determined using restraints derived from 2D NMR data and a hybrid distance geometry-restrained simulated annealing methodology. The structure of Compstatin contains a Type I β-turn spanning the segment Gln^5-Asp^6-Trp^7-Gly^8. A hydrophobic clustering of the side chains of residues Val^3, Val^4 and Trp^7 was observed. The structural and inhibitory activity data indicate that the β-turn and specific side chain interactions are critical for preservation of the conformational stability of Compstatin and they are also significant for maintaining the functional activity of Compstatin. The therapeutic potential of Compstatin was examined in *in vitro* and *ex vivo* models. Compstatin was shown to be active in a model of extracorporeal blood circuitry that represents the clinically important *in vitro/in vivo* interface encountered in bypass surgery and blood dialysis. The peptide also showed a promise in a xenotransplantation model.

1. Introduction

1.1. The complement system

The complement system is an important line of immunological defense against pathogenic organisms [1-5]. However, its inappropriate activation may lead to host cell damage. Complement is implicated in several disease states, and evidence for its involvement has been obtained directly from the examination of patient tissues and from animal models of disease. Complement-mediated tissue injury has been reported in a wide variety of diseases, including experimental allergic neuritis, type II collagen-induced arthritis, myasthenia gravis, hemolytic anemia, glomerulonephritis, immune complex-induced vasculitis and multiple sclerosis. Inappropriate complement activation has also

been implicated as a pathologic factor in adult respiratory syndrome, stroke, heart attack and burn injuries. Complement-mediated tissue injury has also been found to be a consequence of bioincompatibility situations such as those encountered in patients undergoing dialysis or cardiopulmonary bypass. The synthetic dialyzer membrane surfaces and the surfaces of extracorporeal vascular plumbing can function as complement activators, and activation products are found in patients upon assay. The post-bypass syndrome, characterized by symptoms of hypotension and dyspnea, may arise directly from complement activation as a result of circulation of blood through the extracorporeal oxygenation circuits during surgery.

Control of complement activation will have to be achieved if xenotransplantation is ever to become an approach for organ transplantation or some human gene therapies. Foreign organs or cells are rejected within minutes after transplantation. Allografts are rejected in a hyperacute manner that is mediated by both the classical (involving natural antibody) and alternative pathways of complement activation within the graft.

1.2. The role of C3 in complement activation and regulation

The complement system is comprised of more than thirty protein components that undergo protein-protein interactions. Functional protein fragments and complexes are generated by sequential and specific proteolytic processing events, several of which involve the pivotal molecule known as C3. The complement component C3 plays a central role in the activation of the classical, alternative and lectin complement pathways. It is multi-functional, and various derivatives of the C3 molecule interact specifically with approximately 25 ligands [1,2]. The C3 molecule and its derivatives are unique in that this single entity possesses a rich architecture that provides a multiplicity of a diverse ligand binding sites.

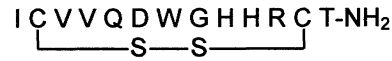
The tissue injury that results from complement activation is directly mediated by the membrane attack complex, C5b-C9, and indirectly by the generation of anaphylatoxic peptides, C3a and C5a, which induce damage through their effects on neutrophils [6] and mast cells [7]. The complexity of the process indicates that there are several control points to consider for therapeutic regulation. Natural *in vivo* regulation is provided by both plasma and membrane proteins. The primary plasma protein inhibitors are factor H and C4-binding protein, and the primary regulatory membrane proteins located on cell surfaces are complement receptors 1 (CR1), decay-accelerating factor (DAF), membrane cofactor protein (MCP) and CD59 [8]. These proteins, except CD59, prevent the release of the anaphylatoxic peptides C3a and C5a by inhibiting the C3 and C5 convertases (multi-subunit proteases), by promoting dissociation of the multisubunit complexes and/or by inactivating the complexes by proteolysis (catalyzed by factor I). CD59 on the other hand prevents the formation of membrane-attack complex and protect homologous cells from complement-mediated lysis. Several pharmacological agents that regulate or modulate complement activity have been identified by *in vitro* assay but most have been shown *in vivo* to be toxic or of low activity [9-12].

1.3. Use of a phage-displayed random peptide library to identify a complement inhibitor

Within the last ten years random peptide libraries have proved to be a useful tool in identifying the peptide epitopes recognized by particular MoAbs as well as mimetics of ligands for various proteins [13]. A phage-displayed random peptide library has been used to search for C3-interactive peptides and a peptide, named Compstatin, has been discovered

that binds to C3 and prevents its further activation by both the classical and alternative pathways [14].

Compstatin is a small molecular weight disulfide bonded cyclic peptide of 1551 Da with sequence:



There is an evidence that this peptide is significantly active *in vitro* and *ex vivo*. A peptide structure may not be the optimal chemical form for a therapeutic agent for *in vivo* since the delivery, uptake, transport, and metabolism of this particular peptide are currently unknown and can influence *in vivo* efficacy. In order to permit the future design of a peptide-mimetic or a drug with the most efficacious activity *in vivo*, the three-dimensional (3D) solution structure of Compstatin was determined using two-dimensional (2D) NMR spectroscopic analysis and a hybrid distance geometry-restrained simulated annealing calculation [15]. The structural information derived from this study of Compstatin can be used to design informative analogs in order to gain insight into the role of conformation in eliciting biological activity. The 3D structure defined in this way provides the basis for rational drug design involving alternative chemistries that may yield more efficacious drugs.

2. Design of Compstatin

2.1. Identification of Compstatin

In the past, various approaches have been used to identify complement inhibitors, although with a limited success [16-22]. In our laboratory, we have used a phage-displayed random peptide library to identify peptides that bind C3 and inhibit complement. In this approach it has been hypothesized that binding of a peptide to C3b might affect the interaction of C3b with other complement proteins or a peptide could functionally mimic other C3-binding proteins that regulate complement and in turn produce complement inhibition. Indeed, such an inhibitory C3-binding peptide was isolated using a phage-displayed random peptide library, which inactivates complement at a concentration approximately twice that of human C3 in normal human serum [14]. A 27-mer library of complexity ~ 2 X 10^8 has been used for screening. To isolate C3-binding phage particles from the library, phages were affinity purified on a microtiter plate coated with C3b. Three rounds of biopanning resulted in isolation of C3b-binding clones; 14 of 16 clones bound to C3b. All positive clones were grown, and their DNA was isolated and subjected to dideoxy sequencing. Interestingly, all positive clones showed an identical sequence, suggesting that the clone was specific and was amplified during each step of biopanning [14]. Two factors were critical in isolating this peptide. First, stringent binding conditions that would select phages with high affinity were used. It is well known that protein-protein interactions in the complement system, and particularly the interaction of C3b with other known proteins, are usually of low affinity. In order to study the interaction of C3b with other complement proteins, buffers of half-physiologic ionic strength were used, and to select high affinity phages, buffers of physiologic ionic strength were used. Second, a 27-mer phage display library was selected because it was expected that long peptides would form secondary structures more favorable to binding [14].

In an attempt to demonstrate the specificity of this peptide, an ELISA was performed in which its binding to C3 and C3 fragments was inhibited by aggregated C3 [14]. The binding assay was done with a positive and a negative clone. The positive clone bound to C3, C3b and C3c, but not to C3d, and its binding was inhibited by aggregated C3. This indicated that its binding to C3 and to various C3 fragments is specific and that its binding site is located in the C3c region of C3 [14]. To confirm these results, a peptide corresponding to the sequence of the phage-displayed peptide (Peptide I, Table 1) was synthesized and its binding to C3 was examined in an ELISA. As expected, the peptide bound to C3 and C3b but not to C3d. It is interesting that this peptide also failed to bind to C3c, suggesting that the binding site of the peptide is buried when C3c is present in its native conformation [14].

Table 1: Amino acid sequences and inhibitory activity of C3 binding peptide and its analogs

Peptide/ Clone	Amino acid sequence[a]	Mass spectral analysis Expected	Observed	Classical Pathway[b] IC_{50} (μM)	Alternative Pathway[c] IC_{50} (μM)
Clone	**SS-ICVVQDWGHHRCTAGHMANLTSHASAI-SR**				
I.	I*CVVQDWGHHRC*TAGHMANLTSHASAI	2913	2919	65	19
II	ICVVQDWGHHRCTAGHMANLTSHASAI	3021	3018	>300	>300
III	RATAGHMANLTSHASAI	1709	1708	>300	>300
IV	I*CVVQDWGHHRC*T **(Compstatin)**	1552	1551	63	12
V	ICVVQDWGHHRCT	1660	1664	>600	>300
VI	*CVVQDWGHHRC*	1340	1339	N.D.	33
VII	*CAVQDWGHHRC*	1311	1309	N.D.	1200
VIII	*CVAQDWGHHRC*	1311	1309	N.D.	67
IX	*CVVADWGHHRC*	1282	1281	N.D.	910
X	*CVVQAWGHHRC*	1296	1297	N.D.	257
XI	*CVVQDAGHHRC*	1224	1223	N.D.	182
XII	*CVVQDWAHHRC*	1353	1352	N.D.	>1200
XIII	*CVVQDWGAHRC*	1273	1272	N.D.	15
XIV	*CVVQDWGHARC*	1273	1272	N.D.	74
XV	*CVVQDWGHHAC*	1254	1255	N.D.	70

[a] * denotes oxidized cysteine

[b] Classical pathway activity was determine using EA lysis assay.

[c] Alternative pathway activity was determined using Er lysis assay

2.2. Inhibition of complement activation by Compstatin

The synthetic peptide corresponding to the phage-displayed peptide (Peptide I, Table 1) was tested for ability to inhibit complement activation [14]. Normal human serum was used as the source of complement. Inhibition of the alternative pathway was determined by measuring the lysis of rabbit erythrocytes in the presence of MgEGTA, and lysis of sheep erythrocytes coated with antibody was used as an indicator of classical pathway inhibition (both are standard assays for measuring the complement activity, [22]). The peptide inhibited both the classical and alternative pathways, with IC_{50} value of 65 and 19 μM, respectively (Table 1). The concentration of the peptide required to inhibit the classical pathway was higher than that required to inhibit the alternative pathway, conceivably because the alternative pathway is more sensitive to activation and deposition of C3 on the target cells.

2.3. Identification of Compstatin residues critical for its complement inhibitory activity

During the experiments discussed above, it was discovered that reduction and alkylation of peptide I destroyed its inhibitory activity (Peptide II, Table 1), strongly suggesting that oxidation of cysteines is important in maintaining the stable and preferred structure of the peptide [14]. Various analogs have been examined to determine the region/residues of the peptide involved in inhibition of complement. Results obtained are summarized in Table 1. The purity and identity of all the peptides were critically monitored by matrix-assisted laser desorption mass spectrometry (MALDI-TOFMS) [23]. In all cyclic peptides formation of a disulfide bond was confirmed mass spectrometricaly using a mass shift assay and involving reaction of thiols with p-hydroxy mercuribenzoic acid [24].

To identify the minimal region of peptide I (Table 1) that is required for interaction with C3, two overlapping peptides were synthesized and their activity was measured in the classical and alternative pathway-mediated hemolytic assays [14]. These two deletion analogues were the cyclic 13-amino acid N-terminal peptide named Compstatin (peptide IV, Table 1) and the linear 17-mer C-terminal peptide (peptide III, Table 1). The N-terminal cyclic peptide peptide IV (Compstatin) retained the functional activity of the larger peptide and inhibited the classical as well as the alternative pathway at concentrations similar to those of peptide I (63 and 12 μM, respectively). No inhibitory activity was detected in peptide III. As expected, reduction and alkylation of Compstatin destroyed its inhibitory activity (peptide V). Compstatin contains two flanking amino acid residues outside the constrained region. To further reduce the size of Compstatin, these two residues were deleted (peptide VI, Table 1). This resulted in a 2.8-fold reduction in the activity of the peptide, indicating the importance of these residues in enhancing the inhibitory activity of the peptide [14].

To identify the contribution of each residue in maintaining the biological activity of the peptide a series of nine peptides was synthesized in which each residue of the 11-membered ring, except for the two cysteines, was systematically substituted with alanine (peptides VII-XV, Table 1) [15]. Replacement of Val^4, His^9, His^{10} or Arg^{11} resulted in minimal change in the functional activity, suggesting that these residues do not contribute significantly to the interaction with C3. Replacement of Val^3, Gln^5, Asp^6 or Trp^7 reduced the activity of the peptide from 6 to 36-fold as compared to peptide VI (Table 1). These residues are clustered together in the N-terminal half of the peptide. Replacement of Gly^8 with Ala dramatically reduced the activity of the peptide by more than 100-fold, suggesting that the side chain of the Ala might sterically hinder the binding of the peptide to C3. From

these results it was inferred that the side chains of Val^3, Gln^5, Asp^6, Trp^7 and Gly^8 contribute significantly to the binding and biological activity of the peptide.

3. Structure of Compstatin

3.1. NMR Studies of Compstatin

The three-dimensional structure of a major conformer of Compstatin in solution, detectable by 2D NMR methods was solved [15]. Proton resonance assignments were made using procedures reviewed by Wóthrich [25]. First, correlation spectroscopy through three-bond scalar J-couplings was used. 2D Total Correlation Spectroscopy (TOCSY), 2D Double Quantum Filter Correlation Spectroscopy (DQF-COSY) and 2D Double Quantum (DQ) experiments were used in combination, for unambiguous spin system identification. All thirteen spin systems of Compstatin were identified [15].

Cross relaxation experiments that provide Nuclear Overhauser Enhancement (NOE) correlations through dipolar (through space) couplings, such as 2D NOE Spectroscopy (NOESY) and 2D Rotating Frame NOE Spectroscopy (ROESY) experiments were used for sequential assignments [15]. In cases where particular cross peaks were not observed in regular NOESY or ROESY spectra because they were suppressed by the water presaturation scheme utilized in these pulse sequences, 2D Jump-and-Return NOE Spectroscopy (J-R NOESY) experiments were used [15]. No conformational exchange cross peaks were found in the 2D ROESY spectra of Compstatin. NMR spectra were collected at 5^O C and 10^O C and pH 6 [15].

3.2. Structure calculations

The availability of several short, medium and long range NOEs from NOESY and ROESY spectra is critical for the solution structure determination which is based on the $1/r^6$ dependence of the Nuclear Overhauser Effect, where r is the inter-nuclear distance. An NOE buildup sequence consisting of five NOESY experiments with mixing times between 50-400 ms was used in order to establish the optimal mixing time for quantitation of a NOESY experiment and to eliminate spin diffusion effects [15]. A list of distance restraints was generated mainly from the NOE volumes of the 150 ms NOESY spectrum [15].

Additional lists of scalar $^3J_{NH-H\alpha}$-coupling constant restraints and backbone dihedral angle restraints were created [15]. $^3J_{NH-H\alpha}$-coupling constants were extracted from the DQF-COSY experiment and they were used to solve the Karplus equations [26] in order to provide ϕ-dihedral angle restraints. The fitting methodology of Kim and Prestegard [27] was applied to DQF-COSY cross peaks for evaluation of J-coupling constants. Stereo-specific assignments for H^β-methylene protons were made qualitatively by inspection of $^3J_{H\alpha-H\beta}$ couplings in the DQF-COSY spectrum and intra-residue NOE intensities. χ_1-dihedral angle restraints were also extracted from the combined use of NOESY and DQF-COSY spectra [15].

A total of 136 NOE-derived distance restraints, 7 ϕ-dihedral angle and 2 χ_1-dihedral angle restraints and 12 $^3J_{NH-H\alpha}$-coupling constant restraints were used for structure calculations [15]. The Compstatin structures were calculated using the program X-PLOR 3.851 [28]. The hybrid distance geometry-restrained simulated annealing and refinement protocol [29] was utilized using distance and ϕ- and χ_1-dihedral angle restraints. Further

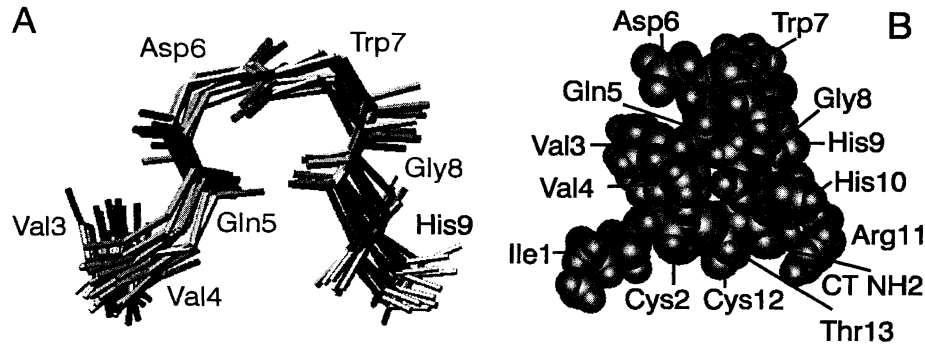


Fig 1: (A) A family of the 10 lowest energy structures of Compstatin. Only the backbone, the backbone amide hydrogens and carbonyl oxygens, of the best-defined region between residues 3-9 is shown. The Type I β-turn segment consists of residues Gln^5-Asp^6-Trp^7-Gly^8. (B) A space-filling model of the lowest energy structure of Compstatin.

direct $^3J_{NH-H\alpha}$-coupling constant refinement [30] was performed using distance χ_1- but not ϕ-dihedral angle restraints.

Figure 1A shows the backbone of residues 3 to 9 of the ensemble of the 10 lowest energy structures out of 21 accepted final refined structures of Compstatin [15]. Residues 3 to 9 comprise the best defined segment of Compstatin and they all participate in intra-residue, sequential and long range NOE contacts. The remaining of the residues show only intra-residue and sequential NOEs but not long range NOEs. The acceptance criteria were no NOE violation > 0.3 Å, no dihedral angle violation > 5°, no bond violation > 0.05 Å, no angle violation > 5° and no improper angle violation > 5° and no $^3J_{NH-H\alpha}$-coupling constant violation > 1.2 Hz [15]. The average RMSD of the ensemble of the family of the 21 structures of Compstatin is 0.6 Å for the backbone heavy atoms and 1.2 Å for all heavy atoms [15]. A turn segment is observed in the region between residues Gln^5-Gly^8.

The cyclic peptide Compstatin forms a type I β-turn comprising the residues Gln^5-Asp^6-Trp^7-Gly^8 (Fig. 1A). The general criteria for the presence of a β-turn are a $C^\alpha(1)$-$C^\alpha(4)$ distance < 7 Å, a C=O(1)-N(4) distance 2-5 Å (with the possibility of the presence of a hydrogen bond) and that the central residues are not helical [31-33]. More specifically a type I -turn is characterized by dihedral angles $(\phi_2,\psi_2)=(-60°,-30°)$ and $(\phi_3,\psi_3)=(-90°,0°)$ with a variation of ±30° associated with them [31,33-35]. In the case of the ensemble of the family of the 21 calculated structures of Compstatin these distances are $C^\alpha(1)$-$C^\alpha(4)$=4.7±0.2 E, C=O(1)-N(4)=3.3±0.4 Å and the dihedral angles are $(\phi_2,\psi_2)=(-65°\pm13°, -26°\pm8°)$ and $(\phi_3,\psi_3)=(-108°\pm12°,-14°\pm3°)$.

Since peptides in solution are present as ensembles of interconverting conformers, the population of the β-turn of Compstatin was examined. The $^3J_{NH-H\alpha}$-coupling constant of the second residue of the β-turn (Asp^6) was modeled as a population weighed average between a β-turn and an extended conformer. It was estimated that a β-turn conformer with a 42-63% population is present in Compstatin [15]. The calculated structure was critically examined for consistency and was found to be in agreement with the NMR parameters such as characteristic NOE connectivities, $^3J_{NH-H\alpha}$-coupling constants, chemical shifts and temperature dependence of chemical shifts [15].

Figure 1B shows a space-filling model of the lowest energy structure of Compstatin. Long range spatial contacts of the rings of His^9 and Trp^7 with Gly^8, Gln^5 and Asp^6 and of the methyls of Val^3 and Val^4 with Gln^5 and Asp^6 are consistent with observed NOEs [15]. A clustering of hydrophobic side chains of Val^3, Val^4 and Trp^7 was observed [15].

In the Ala scanning experiments described above it was found that residues Val^3, Gln^5, Asp^6, Trp^7 and Gly^8 are critical for maintaining the inhibitory activity of Compstatin. Interestingly four out of these five residues (Gln^5-Asp^6-Trp^7-Gly^8) comprise the Type I β-turn and the fifth residue (Val^3) is a critical residue in the formation of the hydrophobic cluster of Compstatin. From the four residues that comprise the Type I β-turn it seems that the two end-residues, Gln^5 and Gly^8, that are somehow buried as shown by a solvent accessibility calculation [15], are more essential for Compstatin activity. Also replacement of Gly^8 with Ala causes a dramatic total loss of the inhibitory activity of Compstatin (Table 1). It appears that Gly is the most favorable residue at the fourth position of Type I β-turns because it releases steric hindrance and stabilizes the turn structure [33]. However, the role of Val^3 in peptide binding is not obvious. As discussed above a hydrophobic clustering of side chains of Val^3, Val^4 and Trp^7 is present in Compstatin. Interaction between the side chain of Val^3 with Gln^5 and Asp^6 is observed in the calculated structure [15]. These interactions might be important in stabilizing the β-turn. The same residues that are involved in side chain and backbone interactions that stabilize the -turn are also important for the functional activity of Compstatin. It is possible that disruption of the turn structure and/or the hydrophobic cluster can cause loss of the structural stability and affect the inhibitory activity of Compstatin.

It is possible that Compstatin undergoes structural reorientation upon binding to C3. A comparison between the structures of the free and bound Compstatin could yield significant insight into the C3-Compstatin recognition process.

A BLAST [36] data base search was performed but no peptide or protein segment with the sequence of Compstatin or peptide VI (Table 1) was found. The sequence segment VVQDWG was found to be part of the protein haloalkane dehalogenase where it is located at the active site cavity and it is part of a nucleophile elbow [37]. However, the lack of the two end disulfide-bonded cysteines does not allow us to make a direct comparison with Compstatin.

4. Function of Compstatin

4.1. Proposed mechanism of complement inhibition by Compstatin

The data obtained from the hemolytic assays showed that a higher concentration of Compstatin was required to inhibit the classical pathway than to inhibit the alternative pathway [14]. This finding pointed to study the effect of the peptide on the early phases of complement activation, which are more susceptible to activation and deposition of C3 onto target cells. In this assay, various concentrations of the peptide were mixed with normal human serum (NHS) containing MgEGTA and ^{125}I-labeled C3; complement was then activated by adding zymosan particles. After incubation, the reaction mixtures were analyzed on a SDS-PAGE gel and the conversion of C3 to iC3b was quantitated [14].

The results showed a concentration-dependent inhibition of C3 cleavage (iC3b formation), with an IC_{50} of 10 μM, which correlated well with the concentration required to inhibit 50% of the hemolytic activity (12 μM) [14]. The inhibition observed could either be

due to C3 convertase (C3b,Bb)-mediated cleavage of C3 to C3b or to inhibition of factors H- and I-mediated conversion of C3b to iC3b. The second possibility was ruled out when inactivation of C3b by factors H and I was studied in a fluid-phase assay. When purified C3b was incubated with factor H and factor I in the presence of various concentrations of the peptide, the peptide had no effect on the cleavage of C3b to iC3b. Thus, from these data it was inferred that the primary site of inhibition by Compstatin involved either the activation of C3 or the formation of the C3 convertase, C3b,Bb [14]. These results were further confirmed by reconstituting the alternative pathway using purified complement components. In this fluid-phase assay, purified C3 was incubated with various concentrations of the peptide. Thereafter, factors B and D were added together with MgEGTA to initiate the activation process. Reaction mixtures were analyzed on a SDS-PAGE gel. It was clear from the results that Compstatin inhibited the conversion of C3 to C3b. Thus, to dissect the effect of Compstatin on C3 activation as opposed to C3b,Bb formation, C3b, factor B and factor D were incubated together with MgEGTA in the presence or absence of Compstatin. Compstatin showed no inhibition of factor B cleavage, suggesting that this peptide has no effect on the interaction of C3b with factor B or on C3b,Bb formation [14]. In human serum, C3 convertase (C3b,Bb) is stabilized by properdin, resulting in the formation of C3b,Bb,P. Binding of Compstatin to C3 could inhibit the interaction of C3 with properdin, which could inhibit the stabilization of C3 convertase and, in turn, inhibit the cleavage of C3. A competition ELISA was used to study the effect of Compstatin on properdin binding to C3. Compstatin failed to inhibit properdin binding to C3, indicating that the observed inhibition of C3 cleavage was not due to disruption of properdin-stabilized C3 convertase, C3b,Bb,P [14].

The reversibility of complement inhibition by Compstatin was also assessed [14]. Purified C3 was incubated with the peptide (100μM) at 37^{O} C for 1 hr, spin-dialyzed on a G-25 column, diluted 5-fold and further incubated at 37^{O} C for 15 min. The hemolytic activities of C3 before and after the dialysis were then compared. The hemolytic activity of treated C3, which was 2% of the untreated C3, was restored to 85% after dialysis followed by incubation at 37^{O} C for 15 min, suggesting that the observed inhibition is reversible.

The question that arose was whether the binding of Compstatin to native C3 hinders the access to the C3a/C3b cleavage site. It is known that the first cleavage of C3 by trypsin and the C3b,Bb-mediated cleavage of C3 occur at the same site (Arg^{726}-Ser^{727}). Keeping this in mind, C3 was incubated with peptide VI (a trypsin resistant analog of Compstatin in which Ala was substituted for Arg) for 15 min. The reaction mixture was then incubated either with trypsin or with factors B, D and MgEGTA and subjected to electrophoresis to measure the cleavage as described above. The peptide inhibited the C3 convertase-mediated cleavage of C3, but did not inhibit the cleavage by trypsin, suggesting that binding of the peptide to C3 does not cause a local steric effect [14]. Together these results suggest that Compstatin reversibly binds to native C3 to inhibit its activation, and this inhibition is not due to sterically hindered access to the C3a/C3b cleavage site.

5. Application of Compstatin

5.1. Complement inhibition by Compstatin in a model for cardiopulmonary bypass circuits

To test the potency of Compstatin in a clinically relevant situation, its effect on complement in tubing loops was evaluated as a model for cardiopulmonary bypass (CPB)

circuits [Nilsson, B., Hong, J., Larsson, R., Elgue, G., Nilsson-Ekdahl, K., Sahu, A., and Lambris, J.D., unpublished results]. In these experiments, freshly drawn blood containing minimal amount of heparin was rotated for 1 hr at 37^{O} C and thereafter analyzed for blood cell counts, hemolysis, C3a concentration, terminal complement complex (sC5b-9) concentration, binding of C3/C3b fragments to PVC tubing, and expression of CR3 (CD11b) on polymorphonuclear leukocytes (PMNLs). Compstatin corresponding to 4-, 8-, 12- and 36-fold (180 μM) molar excesses (compared to the C3 concentration in blood) was used. Blood cell counts, including platelets (approx 230 X 109/L) remained stable during the incubation, and no hemolysis was noted, suggesting that the peptide was not toxic to the cells. Generation of C3a and sC5b-9, binding of C3/C3b fragments to PVC tubing, and expression of CR3 on PMNLs were all completely inhibited, even at only a 4-fold molar excess of Compstatin [Nilsson, B., Hong, J., Larsson, R., Elgue, G., Nilsson-Ekdahl, K., Sahu, A., and Lambris, J.D., unpublished results]. As a result of the inhibition of complement activation, the expression of CD11b on PMNs and the binding of these cells to the biomaterial surface was almost completely lost. These results strongly suggest a potential therapeutic use for Compstatin in diseases involving complement-mediated damage and in situations in which complement is activated.

5.2. Prevention of hyper-acute rejection in a porcine-to-human ex vivo kidney xenotransplantation model by complement inhibition using Compstatin

Hyperacute rejection in discordant kidney xenotransplantation was studied *ex vivo* using a porcine-to-human perfusion model [38]. In each of six experiments the two kidneys from one pig were perfused in parallel with heparinized human blood to which was added either 45 mg Compstatin or equivalent amounts of control peptide or albumin. The kidneys in the control group were rejected after 45-120 (median 90) minutes showing macroscopical changes consistent with hyperacute rejection as previously shown in this model [38]. Addition of Compstatin significantly prolonged the survival to 270-450 (median 380) minutes, which is the time limit of this perfusion model. The macroscopical changes were markedly less than in the control group. Deposition of complement activation products in the kidney were virtually abrogated in the Compstatin group compared with the controls [Fiane, A.E., Mollnes, T.E., Videm, V., Hovig, T., Hogasen, K., Mellbye, O.J., Spruce, L., Moore, W.T., Sahu, A., Lambris, J.D., unpublished results]. It was concluded that the C3 binding peptide efficiently reduces complement activation and hyper-acute rejection in this porcine-to-human model and may be of significant clinical interest in future xenotransplantation study.

Acknowledgments

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