A Unique Property of a Plasma Proteoglycan, the C1q Inhibitor
An Anticoagulant State Resulting from Its Binding to Fibrinogen

Dennis K. Galanakis and Berhane Ghebrehiwet
Blood Bank, University Hospital, State University of New York, Stony Brook, New York 11794

Abstract

The C1q inhibitor, C1qI, an ~ 30-kD circulating chondroitin-4-sulfate proteoglycan, is a disulfide-linked dimer. It is secreted by monocytes (4, 6), and its presence in human plasma (4) is the low sulfated chondroitin-4-sulfate proteoglycan (C-4SP), which has been shown to bind to fibrinogen/fibrin [fibrinogen/fibrin (ogen)]. Initial results disclosed binding to and an anticoagulant property against fibrinogen (ogen) by C1qI. This formed the subject of the present investigations.

Methods

Fibrinogen, fibrin, and plasmic fragments. Fibrinogen was isolated and its concentration was determined as described (10). Fibrin monomer preparation and polymerization (11) and SDS-PAGE (12) were performed as described. Polymerization rates were computed from the time course of clot absorbance at 350 nm, and clot turbidity maxima were obtained as described (11). Batroxobin and human thrombin were kind gifts from Dr. K. Stocker (Pentapharm Ltd., Basel, Switzerland) and Dr. J. Jesty (Health Sciences Center, SUNY, Stony Brook, NY), respectively. Hirudin (Sigma Chemical Co., St. Louis, MO) and d-phenylalanyl-prolyl-arginyl chloromethyl ketone (P-PACK) (Calbiochem Corp., La Jolla, CA) were used as provided. Plasma fragments D₁ and E were isolated and their concentrations were measured by procedures previously applied (11). Plasma coagulability was measured by adding ~ 50–200 μg of 125I-labeled fibrinogen (11) per milliliter of plasma, and by obtaining thrombin (1 U/ml)-induced clots, at ambient and then at 4°C temperatures for 1 h and several hours, respectively. The counts per minute (cpm) of synerized clots and their supernatants (> 90% of original volume) were obtained in a γ counter. 125I-labeled C1qI (vide infra) clot binding was obtained either by incorporation before clotting or by immersing the clot in the C1qI solution overnight. To obtain cross-linked clots, the buffer, Tris-HCl, pH 7.4.

Glycosaminoglycans (GAG)¹ are present in blood in concentrations of ~ 5 μg/ml (2). One fourth or fewer of these circulate in free form, and the remainder are bound to unidentified proteins. GAG heterogeneity (3), their presence in numerous sites (4) such as cell membranes and basement membranes, and their release from cells in culture (3, 5) suggest multiple sites of origin. The major form in human plasma (2, 4) is the low sulfated chondroitin-4-sulfate proteoglycan (C-4SP), which consists of repeating disaccharides of glucuronic acid-N-acetyl-galactosamine, generally bound to the hydroxyl group of a serine residue of a core peptide to give the sequence (disaccharide)nGlcUA-Gal-Gal-Xyl-O-Ser. These repeating disaccharides are sulfated to a variable degree, a variation that modifies interactions (5) with other molecules. The discovery that a polyanionic molecule(s) was associated with isolated C1q (6), the first component of complement, led to its identification (4) as a heterogeneous population of C-4SP that displayed potent inhibition of the hemolytic activity of C1q (4), whether it was isolated from serum (4, 6) or from lymphocyte membranes (7). The known interaction of fibrin with tissue glycosaminoglycans (8, 9), and the increased circulating concentrations of proteoglycans in certain pathologic states (2) led us to examine the possible interactions between C1qI and fibrinogen/fibrin [fibrinogen (ogen)]. Initial results disclosed binding to and an anticoagulant property against fibrinogen (ogen) by C1qI. This formed the subject of the present investigations.

Introduction

Fibrinogen is a plasma glycoprotein containing three pairs of polypeptide chains Aα, Bβ, and γ interlinked by disulfide bridges which are clustered in three regions, the central or E and two outer or D domains (1). It is converted to fibrin monomer by cleavage of two small amino-terminal peptides, A and B, by thrombin—A from its Aα chain and B from its Bβ chain, respectively. Fibrin monomers spontaneously polymerize to form the gel matrix, which is further stabilized by the formation of covalent links catalyzed by factor XIIIa. Polymerization is initiated by the binding of one fibrin E domain to the D domains of two other fibrin molecules thus forming a two-molecule-thick strand, the protofibril.

Address correspondence to Dr. Dennis K. Galanakis, Blood Bank, University Hospital, SUNY, Stony Brook, NY 11794.

Received for publication 12 March 1993 and in revised form 22 July 1993.


¹Abbreviations used in this paper: C-4SP, chondroitin-4 sulfate proteoglycans; GAG, glycosaminoglycans; KPTI, Kunitz pancreatic trypsin inhibitor; P-PACK, d-phenylalanyl-prolyl-arginyl chloromethyl ketone.

Proteoglycan Anticoagulant 303
After 30 min at 37°C, 10 μl of ClqD serum and 200 μl of 1.5 × 10^{-4}/ml sheep erythrocytes sensitized with specific antibody were added and incubated for a further 60 min at 37°C; 1 ml of cold buffer was then added to stop the reaction and the hemoglobin released in the supernatant was assessed spectrophotometrically at 412 nm. The ClqI activity was computed as percentage of the Clq control hemolytic activity.

Production and isolation of antibody. Monospecific polyclonal antibody was raised in rabbits by multiple subcutaneous and deep intramuscular injections of ~ 100–200 μg/ml ClqI suspended in complete Freund's adjuvant which had been diluted with incomplete adjuvant 1:2. The second set of injections was given 2 wk later by the same route, with ClqI suspended in complete/incomplete adjuvant ratio of 1:4. Subsequent injections were made in incomplete adjuvant at 2 wk intervals; 6 wk after the fourth injection date a final injection was given and the rabbits were bled 10 d later. IgG was isolated using the Immunopure A/G IgG purification kit (Pierce Chemical Co., Rockford, IL) according to the manufacturer's procedure, and was assayed by radial immunodiffusion. A single precipitin arc was shown against ClqI and there was no cross-reactivity with ClqI and with Con A. The isolated IgG neutralized the capacity of ClqI to inhibit the Clq hemolytic activity.

Radiiodination and measurements. 125I-labeling was carried out (11) as described, and specific activities were > 60 × 10^3 cpm/μg protein. Autoradiograms and densitometry were obtained on dried polyacrylamide gels by using X-ray film (Eastman Kodak Co., Rochester, NY) and a (Pharmacia-LKB) laser densitometer equipped with a recorder, respectively.

Binding assays. These were carried out using buffer, pH 7.4, μ = 0.15, containing KPTI 100 U/ml throughout. Fibrinogen solution, 50 μg/ml, pH 7.4, was added to polystyrene (Immulon Removawell, Dynatech Laboratories, Alexandria, VA) miniature test tubes (i.e., removable microwells or microplates) in 60- or 70-μl aliquots and left covered in a moist chamber overnight at ambient temperature or for 2 h at 37°C. The solution was then replaced with human serum albumin 50 μg/ml for at least several hours; it was then removed and the tube washed thrice with excess buffer; the desired ligand was then added and after incubation this washing procedure was repeated, but the final wash was performed five times and the buffer contained 0.05% Tween 20. Residual wash liquor was carefully removed using a micropipette. Under these conditions 1.4 pm of fibrinogen and 0.4 pm Clq bound to each microwell, calculated from a 1:50 mixture of (125I-labeled/unlabeled protein, n = 7). To prepare fibrin monomer thrombin 0.1 U/ml or batroxobin 0.5 U/ml, containing KPTI, 200 U/ml, was left for several or more hours. This was then replaced for 30–60 min with enzyme neutralizing buffer (50 μg/ml albumin) containing PPACK 50 nM or hirudin 5 U/ml to neutralize thrombin, or PMSF 100 nM, to neutralize batroxobin.

Results

Investigations of whole ClqI

In pilot experiments, several ClqI isolates separated from C1q by the Con A chromatographic procedure prolonged the thrombin clotting times of plasma and of isolated fibrinogen solutions. For example, one ClqI isolate (at 38 μg/ml, pH 7.4) decreased the thrombin (0.2 U/ml) induced polymerization rate of fibrin (2 μM, pH 7.4) from 2.6 to 1.38 (×10^{-3}/s) (see Methods). A similar inhibition was demonstrated by soluble fibrin monomer induced to polymerize (n = 4). Addition of ClqI to plasma in further investigations resulted in variable delay in clotting times which was concentration dependent, demonstrable in either heparin or citrate-anticoagulated plasma, in the presence (Table I) or absence of CaCl2, and whether clotting was induced by thrombin, batroxobin, or ancorod (not shown). Clots formed and were partly or fully soluble in urea (n = 4). SDS-PAGE analyses (n = 2) disclosed the
Table I. Effect of Three Different Concentrations of a Single Preparation of Whole or Unchromatographed C1qI on Thrombin Times of Plasma

<table>
<thead>
<tr>
<th>C1qI (μg/ml)</th>
<th>5</th>
<th>8</th>
<th>26</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin time(s)</td>
<td>16.8</td>
<td>26.6</td>
<td>&gt;300</td>
<td>6.4</td>
</tr>
<tr>
<td>n</td>
<td>4</td>
<td>2</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

Values are given as means with n = number of determinations. The clotting mixtures contained 70% citrated plasma in Tris-HCl 10 mM, pH 7.4, NaCl 150 mM, and CaCl2 67 mM.

The expected decrease in cross-linking by plasma factor XIIIa, in that when exhaustively washed in (4°C) buffer most of the unreduced material migrated as fibrin(ogen) monomer with minor amounts migrating as fibrin dimers and even lesser or trace amounts as oligomers and larger polymers thus explaining the clot solubility in urea. Nevertheless, maximal inhibitory concentrations of C1qI at physiologic temperatures yielded some amount of plasma clot, although minor compared to controls. Thrombin-treated fibrinogen solutions yielded no visible clot unless incubated further at melting ice temperatures when in the presence of CaCl2 a minor granular precipitate formed. When examined in EDTA-containing buffer (not shown) thrombin-induced clot turbidity tended to approach that of non-C1qI controls, but the delayed onset induced by C1qI was invariably unaffected by EDTA (n = 5). In binding assays, 125I-labeled C1qI bound to immobilized fibrinogen and to C1q, in amounts at least severalfold higher than those bound to albumin or IgG controls (n = 2), and this difference was most marked the wash buffer contained Tween 20 (see Methods, n = 5). In a single set of experiments, for example, values of bound C1qI (3.5 μg applied per microwell) were 29, 113, and 157 cpm × 10^4 for albumin, fibrinogen, and C1q, respectively (range < 10% from each of these means, n = 6). Autoradiograms of C1qI disclosed the expected size heterogeneity (4), in that six major and at least three minor or trace amount bands ranging from 21 to ~ 200 kD along with material not entering a 10% gel were shown. This electrophoretic band pattern was indistinguishable from those displayed by eluates from microwells containing C1qI (n = 2) or fibrinogen (n = 1). In other experiments, immobilized fibrinogen that contained bound C1qI did not bind additional fibrinogen, (n = 4), implying that all binding sites on C1qI had been occupied.

Investigations of the C1qI active moiety

Enzyme-induced fibrin polymerization. The discovery (15) that the inhibition of the C1q hemolytic activity was attributable to a chromatographic subfraction displaying electrophoretic bands present in the parent material led to experiments that determined an anticoagulant property (2) (Fig. 1 A) indistinguishable from that of the parent material. This chromatographic isolate was electrophoretically much less heterogenous than the parent material (Fig. 2 B), and it was subjected to more extensive investigations. By 125I-labeled clot assay, the amounts of coagulable fibrinogen/fibrin in plasma (Fig. 1 B) decreased as concentrations of C1qI were increased. In fibrinogen (1 μM, pH 7.4) solutions, the thrombin clotting times were prolonged by the presence of C1qI (1 μg/ml), from controls of 34.2 s (range 33.8–35.6 s, n = 3) to 71.8 s (69.3–76.8). Also, thrombin-induced clot turbidity decreased progressively with increasing C1qI concentrations (Fig. 1 D), and its onset (not shown) was also progressively delayed. Limiting the C1qI concentrations to permit clot formation as shown in Fig. 1 C enabled rate measurements of this effect. Using conditions similar to those of Fig. 1 C in separate experiments, the rate of rise of clot turbidity was decreased by C1qI from 15 to 4 × 10^-3/s. In other experiments, comparisons between ionic strengths of 0.15 and 0.3 disclosed no changes in the extent of inhibition by C1qI (n = 3).

In further investigations, the decreased polymerization rate, as well as the turbidity maxima of such clots (Fig. 1 D), were normalized when the C1qI solution had been treated with chondroitinase ABC (see Methods, n = 2), consistent with its known proteoglycan structure (6). Moreover, preincubation of C1qI with either a polyclonal anti-C1qI antibody (n = 2, see Methods, not shown) or with C1q (Fig. 1 C) did not diminish its anticoagulant effect.

Polymerization of preformed fibrin monomer. Solubilized fibrin (0.5–3 μM) induced to polymerize (11) invariably displayed a decreased maximum turbidity in the presence of C1qI and this was also a concentration-dependent effect. In a typical experiment, fibrin added to buffer was > 95% coagulable but in the presence of > 15 molar excess of C1qI fibrin failed to form a gel at physiologic, ambient, or melting ice temperatures. Although several or more moles C1qI/mole fibrinogen were required for marked inhibition of gelation, appreciable inhibition by turbidimetric assay could be shown with as little as 0.3 mol/mol fibrin (n = 3). The presence of factor XIIIa and CaCl2 had no effect on this incoagulability. Fibrin incoagulable in the presence of C1qI formed a finely granular precipitate at melting ice temperatures (n = 3). Analyzed further, this precipitate amounted to approximately half (n = 2) of total protein and readily dissolved on rewarming. As was the case with plasma clots, electrophoretic analyses disclosed that the cryoprecipitate consisted of fibrin monomer, minor amounts of dimers (~ 680 kD), and of trace amounts of oligomers which migrated into the 3.5% gel. These oligomers were identical in size (reduced and unreduced) to those of partially crosslinked fibrin controls. Moreover, the cryoprecipitate supernatant contained only monomeric fibrin. Thus by these analyses, no covalent crosslinking of C1qI to fibrin could be shown.

Fibrin polymerization in the presence of fibrinogen. Numerous attempts to neutralize the C1qI inhibition of fibrin polymerization by preincubation of C1qI with fibrinogen failed to disclose such an effect. For example, the polymerization rate of 3 μM fibrin of 2.3 × 10^-2/s was decreased to 1.5 and 1.3 × 10^-2/s by fibrinogen (0.5 μM) and by C1qI (9 μM), respectively. When the two inhibitors were both present in the solution the rate was further decreased to 0.3 × 10^-2. Similarly, additive inhibition was shown in other experiments (n = 5), irrespective of the molar ratio of one inhibitor to another. Comparison of the calculated slopes of maximal C1qI inhibition before and following its preincubation with fibrinogen
Figure 1. Anticoagulant effects of C1qI. (A) Plasma thrombin times, shown in seconds, of plasma from four donors whose fibrinogen concentrations were 2.2 (○), 2.47 (+), 2.62 (□), and 2.74 (△) g/liter, respectively. Each line graph reflects values from the untreated plasma and from plasma to which three different C1qI concentrations had been added. (B) Decreased coagulability of fresh citrated plasma, fibrinogen 2.48 g/liter, to which ~200 ng 125I-labeled fibrinogen/ml had been added prior to addition of human thrombin. Amounts shown in percentage of total reflect mean and range (n = 3) and were calculated from cpm of the synerized clots and of the clot free liquor. Plasma controls containing C1qI 30 μg/ml and lacking added thrombin formed no insoluble gel (not shown). (C) Lack of effect of C1q on the C1qI inhibition of fibrin polymerization, pH 7.4, μ = 0.16, 37°C. Thrombin induced turbidity (r = absorbance, 350 nm, shown ×10^3) of fibrinogen (1.3 μM) control (○) is compared with that containing C1qI (x, 16 μg/ml), C1q (+, 84 μg/ml), and C1q preincubated (90 min) with C1qI (□). (D) Correction of the effect of C1qI (○) on the maximum clot turbidity, by C1qI preincubation with chondroitinase ABC (+). Turbidity maxima (ordinate; see Methods) are expressed as percentage of clots obtained in the absence of C1qI. Fibrinogen, 2.8 μM, 35°C, was clotted with thrombin, 0.1 U/ml, and buffer controls, pH 7.4, μ = 0.16, contained inactivated chondroitinase as detailed in methods.

were parallel (Fig. 3), indicating the two inhibitors acted independently against fibrin polymerization. These results were consistent with those from binding experiments (vide infra) and are considered further in the discussion section.

Fibrinogen binding studies. SDS-PAGE autoradiograms of 125I-C1qI disclosed that, like the parent material, the amounts bound to C1q as well as those bound to fibrinogen were approximately three- to fivefold higher than those bound to either albumin or IgG controls (n = 5). Similarly, the autoradiograms disclosed that eluates from either immobilized fibrinogen or C1qI disclosed the same electrophoretic bands, a major 30-kD and minor bands of 28 and 21 kD (Fig. 2 B), thus establishing that the same molecular species possessed the two distinct inhibitory properties, one against C1q and another against fibrinogen. The presence of EDTA (2 mM) resulted in no change in C1qI binding to either fibrinogen (n = 6) or fibrin (n = 6). Also, the amounts of C1qI bound to fibrinogen (n = 6) did not change when the ionic strength was increased from 0.15 to 0.5, consistent with similar results (vide supra) on its inhibition of fibrin polymerization, but in sharp contrast to its known failure to bind to C1q at high ionic strength (4). In further experiments, the polyclonal IgG antibody reared only

3. These minor (21 and 28 kD) proteoglycan bands appeared as fainter bands in autoradiograms of the parent material (not shown), and were increased in the chromatographic isolate as shown; their band distribution among the three proteins was similar averaging 30%, 15%, and 55% of bound C1qI. Accordingly, for calculating molar amounts bound, a 27-kD M_r mean was employed. Failure of the smaller two bands to react with the polyclonal antibody (i.e., which neutralized
Figure 2. (A) PAGE-SDS comparisons of partly coagulable, gel 2, and incoagulable, gel 3, fibrin obtained at two different concentrations of C-1q, 3 and 9 μg/ml shown as × and 3×, respectively, and obtained under maximum cross-linking conditions. Gel 1, fibrin clot control; β chains of fibrin, α-α, α polymers; γ-γ, γ-γ dimers. Clotting conditions were 2 μM fibrinogen, 40 mM XIa, and 5 mM CaCl₂, 0.1 U thrombin/ml, 50 U KPTI/ml, pH 7.4, μ = 0.15, incubated at ambient temperature overnight. (B) Comparison of autoradiograms of 125I-C1q eluates from microwell immobilized human albumin, lanes 1 and 4, C1q, lanes 2 and 5, and fibrinogen, lanes 3 and 6; lanes 1–3 reflect unreduced and lanes 4–6 reduced material. The band between that of 30 and that of 21 kDa was 28 kDa. Identical amounts of C1q were applied (3.5 μg/microwell) and each lane reflects amounts bound to four microwells and eluted by the urea-SDS sample buffer. Calculated from a densitometric scan, of the total bound C1q, 47% bound to C1q, 42% to fibrinogen, and 11% to albumin; for mol/mol fibrinogen and C1q estimates see Table II and legend.

against the 30-kD band, in Western blot experiments (not shown). Preincubation of C1q with either chondroitin-4-sulfate (i.e., a polysaccharide component of C-4SP, n = 2) or C1q in molar excess (n = 4) did not diminish the amount of C1q bound to fibrinogen; but did not it had been pretreated with C1q or with chondroitin sulfate, respectively. This intimated that native C1q structure was required for its binding to fibrinogen, and that the C1q and fibrinogen binding sites on C1q were distinct. In further analyses, C1q exposure to chondroitinase ABC decreased its binding to fibrinogen to that of albumin controls (n = 6; not shown).

Comparison of C1q binding by immobilized fibrinogen, monomeric desAA fibrin, and desAA/desBB fibrin (Table II), disclosed C1q binding to all three forms but in respectively decreased amounts. Also, fibrin (1 μM) clots immersed overnight in 6.7 μM C1q bound 68% of C1q (n = 4), indicating that C1q binding sites remained available in polymerized fibrin. These results led to assessment of binding to isolated plasmatic fragment E of fibrinogen. Fragment E also bound C1q (Table II), but this was virtually abolished by prior exposure of fragment E to thrombin, indicating peptide A was necessary for binding. Fragment E contains decreased amounts of intact ββ chains (18) precluding B peptide assessments; however, a role of the uncleaved B peptide was clearly indicated by comparisons of C1q binding to desAA and desAA/desBB fibrin monomers (Table III), and by results indicating cleaved B peptide bound to liquid phase C1q (vide infra). To explore the C1q binding to the intact E domain, immobilized fibrinogen containing bound 125I-C1q was exposed to thrombin (1 U/ml, overnight). This resulted in no release of C1q (n = 6). Also, 125I-thrombin was exposed to fibrinogen containing bound C1q (n = 6), and the amounts of thrombin bound, did not differ from those bound to fibrinogen controls whether or not thrombin had been inactivated by P-PACK.

Binding to fragment D₄ was also demonstrated (Table II). This and the lack of binding to thrombin treated E suggested that binding to fibrin monomer reflected binding to the intact D domain. In related investigations, the amounts of either radiolabeled fragment D₄ or fibrinogen bound by solid-phase fibrin monomers were undiminished following binding of C1q to such monomers (n = 6). This was consistent with the lack of

Table II. Amounts of C1q Bound to Fibrin Monomers and Polymers, and Isolated Plasmatic Fibrinogen Fragments D₄ and E

<table>
<thead>
<tr>
<th>Fibrinogen</th>
<th>Fibrin clot</th>
<th>Fibrin monomer</th>
<th>Fragment</th>
</tr>
</thead>
<tbody>
<tr>
<td>desAA</td>
<td>desAA</td>
<td>desAA/desBB</td>
<td>D₄</td>
</tr>
<tr>
<td>mol/mol</td>
<td>6.7</td>
<td>5.3</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Shown are means (range <8% above and below each mean) from at least triplicate measurements using varying ratios of radiolabeled/unlabeled C1q, corrected for ambin controls. Not shown were C1q-positive controls disclosing 16 mol C1q bound/mol C1q. Amounts in fibrin polymers were computed from synerized fibrin (1 μM) clots and their supernatants described in Methods, using a C1q concentration of 7 μM. All others reflect amounts bound by microwell immobilized ligand as described in Methods. Abbreviations: fibrin monomer desAA, fibrin lacking both A peptides; fibrin monomer desAA/desBB, fibrin lacking both A and both B peptides; E-Thr, fibrinogen fragment E treated with thrombin before C1q exposure.

C1q inhibitory activity, vide supra] suggested they were species with no inhibitory activity against C1q. Alternatively, they were inhibitory but lacked the antigen epitope possibly reflecting either fragments of a larger (e.g., 30 kD) species or unrelated forms. Nevertheless, they typically did not stain with Coomassie Blue and were assumed to be C-4SP in accord with the known characterization of the parent material (4).
Table III. Fibrinopeptide Binding to ClqI

<table>
<thead>
<tr>
<th>ClqI (μg/ml)</th>
<th>3</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A Peptide</td>
<td>87</td>
<td>57</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>B Peptide</td>
<td>94</td>
<td>80</td>
<td>53</td>
<td>18</td>
</tr>
</tbody>
</table>

Values reflect unbound peptides calculated as percentage of A and B peptide controls from HPLC measurements. The ClqI + peptide mixture was assayed after microfiltration described in Methods. Estimated from these and related measurements, not shown, at least 1 mol peptide A, and 0.7 mol peptide B were bound by 1 mol of ClqI.

The binding of ClqI to the fibrin E domain and with other results (vide supra) disclosing additive inhibition of fibrin polymerization by fibrinogen and ClqI.

\[ \text{Binding to fibrinopeptides: Preliminary attempts to assess possible effects of ClqI on A and B peptide release failed (n = 4), and this was further explored by preparing mixtures of cleaved A and B peptides with ClqI in molar excess. These yielded no detectable free peptides by HPLC measurements. For example, a solution containing \sim 10 \mu g ClqI/ml, 2 \mu g of A, and 2 \mu g of B/ml yielded no free A or B peptides (n = 3). That is, when such solution mixtures were applied whole and after microfiltration to remove ClqI (see Methods), no free peptides were detectable in the filtrate, in sharp contrast to control solutions lacking ClqI. Moreover, the amounts of free peptides were inversely proportional to the ClqI concentration (Table III). Also, using a single A and B peptide solution mixture higher ClqI concentrations were required to remove peptide B than those which removed peptide A or AP (n = 3), peptide AP requiring the lowest concentrations of ClqI for removal (not shown). Calculated from such data, a single mole of ClqI displayed a capacity to bind at least 1 mol of peptide A and 0.7 mol of B from the same mixture. Since the amounts of AP peptide were minor (i.e., < 20% of A) this was not separately calculated.}\\

\[ \text{Discussion}\\
\]

The most important findings of the foregoing investigations relate to the capacity of ClqI to bind to fibrin(ogen) either free and in complex with ClqI and to impair fibrin gelation in plasma. The plasma results indicated that ClqI binding to fibrinogen was selective among other proteins, shown by the binding of much lower amounts to isolated albumin and IgG. Moreover, the circulating form of (i.e., C1qrs complex) does not bind ClqI (7). Because most circulating proteoglycans are protein bound (2, 4) our results suggest that fibrinogen is a major binding protein in normal plasma; anticoagulant effects by such proteoglycans could not be expected owing to their low concentrations (< 5 \mu g/ml [2]), their estimated maximum binding of 6.7 mol/mol fibrinogen (vide supra), the at least 20-fold molar excess of fibrinogen, and some amounts bound by other proteins. That the same molecule(s) expressed the two inhibitory effects was established by the identical electrophoretic bands of eluates from fibrinogen and from C1q. Also, fibrinogen bound ClqI could bind no additional fibrinogen. Absent a self-association by ClqI that may mask such epitopes, this implied a single set of epitopes for fibrin(ogen) binding (i.e., one for E and one for D domain sites, respectively). A related conclusion, that the fibrinogen interacting epitope required intact ClqI was implied by two sets of experiments. First, loss of this property by exposure to chondroitinase ABC (which catalyzes cleavage of galactosaminoglycan side chains from C-4SP) was consistent with its known proteoglycan structure (4). Second, chondroitin-4-sulfate (a polysaccharide component of C-4SP) diminished neither the binding to nor the anticoagulant effect against fibrinogen. That the epitopes interacting with fibrinogen were distinct from that interacting with ClqI was supported by three series of experiments. In one set, both the amounts of ClqI bound to fibrinogen as well as the related anticoagulant effect remained undiminished by its preincubation with ClqI under conditions which neutralized the C1q hemolytic activity. In an other set, our polyclonal IgG antibody abolished its capacity to neutralize the C1q hemolytic activity, but it had no effect on the anticoagulant property of ClqI. Finally, neither the anticoagulant property of ClqI nor its binding to fibrinogen were diminished by increased ionic strength in sharp contrast with its binding to ClqI which is abolished by high ionic strength (4).

The results implicate two independent but concerted mechanisms of action by ClqI. One mechanism involves binding of ClqI to the fibrinogen E domain; at least 2 mol of ClqI bound to the E domain before but not after fibrinopeptides have been cleaved, assuming no self-associating ClqI complexes. Fibrinopeptide cleavage was more difficult to assess. Failure to release bound ClqI by thrombin suggested impaired fibrinopeptide cleavage. However, even under maximum ClqI inhibition some fibrin monomer formed in both plasma and fibrinogen solutions (i.e., insoluble material in plasma, and granular aggregates in isolated fibrinogen solutions showing \gamma-\gamma dimers, could be harvested by prolonged incubation at melting ice temperatures). This implied that some cleavage of fibrinopeptides occurred, however slow or partial, although these were not asayed owing to their binding to ClqI. It is unclear whether multiple epitopes or a single ClqI epitope bound fibrinopeptides. Differences in binding of each fibrinopeptide by ClqI suggested that similar ClqI sites bind fibrinopeptides but with different affinities owing to peptide structure differences. The dessAA data (Table III) indicated that one mol of ClqI bound to desAA, two to fibrinogen and none to the dessAA/dessBB fibrin E domain. This along with the capacity for binding nearly one B and one A peptide implied two distinct binding epitopes for the E domain. The apparent higher affinity for the AP peptide suggests subterminal carbohydrate structures are involved rather the terminal sialic acid residues. Also of interest, neither native nor P-PACK neutralized thrombin binding to fibrinogen was diminished by ClqI occupancy. This raises the possibility that fibrinogen-immobilized ClqI itself bound thrombin, consistent with the demonstrated failure of thrombin to release any bound ClqI. ClqI clearly requires uncleaved A and to a lesser extent B for its occupancy of the E domain, and this makes it difficult to envision unhindered binding of thrombin to at least the catalytic site on E.

A second mechanism, emerged from the fibrin polymerization and binding studies, both supporting the conclusion that D domain polymerization sites were not occupied by ClqI and that ClqI bound elsewhere within the D domain and inhibited by steric hindrance. This was supported by (a) ClqI binding to fibrin monomers and to D,, (b) the incorporation of ClqI in polymerized fibrin gels, (c) the capacity of fibrinogen or of
fragment D$_1$ to inhibit fibrin polymerization in the presence of C1q, and (d) the additive inhibition of polymerization by fibrinogen and C1q (Fig. 3). Additional evidence of steric hindrance was provided by use of C1q concentrations which allowed partial insoluble gel formation. All γ chains formed γ-γ dimers (Fig. 2 A) indicating complete oligomer or protofibril formation. However, little or no α-polymers formed, shown in Fig. 2 A, indicating progression to more complex polymer forms could not be demonstrated. This implied that occupancy of fibrin E by the D domains of two other monomers (i.e., linear polymerization) was not impaired, notwithstanding that it may have occurred slowly. However, lateral or α chain dependent polymerization was either prevented or possibly occurred by misalignment which prevented formation of covalent crosslinks containing α chains.

Unimpaired thrombin binding along with the potent effect against fibrin polymerization by C1q, raises possible in vivo implications. It may, for example, provide an enhancement of the anticoagulant state of the relatively self-contained inflammatory or tissue repair microenvironments in the presence of saturated inhibitors such as AT III. Even where limited concentrations of fluid phase C1q exist whether or not it is bound to C1q/antibody complexes, C1q does bind to fibrinogen and to fibrin serving to further limit thrombogenesis. Clearly, such complexed fibrin(ogen) still binds thrombin serving to further enhance the anticoagulant state. Furthermore, such binding to immune complexes may explain the long known presence of fibrin(ogen) in immune complex-related pathologic lesions. Reported binding of C1q to fibrinogen (19) appears to be independent of the C1q effect. That is to say, neither the anticoagulant effects nor the binding of C1q could be diminished by its preincubation of or in the presence of molar excess of C1q (vide supra). In that report a different C1q isolation procedure was used and it is unclear whether a possible effect by undesorbed C1q played a role, if any. Whether or not membrane intercalated or other solid-phase proteoglycans bind fibrinogen is an open question. A report on thrombomodulin (20), a thrombin inhibitor residing on endothelial cell membranes, is of interest in this regard, in that enzymatic removal of the sulfated GAG moiety of rabbit thrombomodulin abolished its anticoagulant activity against thrombin fibrinogen clotting, intimating an effect similar to that of the present proteoglycan.

Among other possible in vivo roles suggested by the results, one relates to cell receptor binding of fibrinogen. Certain fluid-phase chondroitin sulfate proteoglycans reportedly (5) inhibit receptor interaction with ligand, particularly as it relates to extracellular matrix proteins, or they may inhibit cell-cell interaction (21). Conversely, membrane intercalated proteoglycans mediate cell-matrix and cell-cell interactions; melanoma cells, for example, failed to interact with the extracellular matrix when their chondroitin sulfate proteoglycan synthesis was blocked (22), leading the authors to suggest a cooperative role between cell receptors and such membrane proteoglycans. Since fibrinogen and fibrin are native occupants of such extracellular sites early on during thrombus repair, their interaction with cell membrane proteoglycans may play a role in receptor mediated cell transit within the thrombus.

A mechanism for the presence of fibrinogen in atherosclerotic lesions (23–26) can be formulated from our results. During early stages of the endothelial lesion or loss, exposed basement membrane being rich in proteoglycans (5) can bind fibrinogen from circulation thus preventing its fibrinopeptide release even though it does not prevent thrombin binding (present studies). Thus, fibrinogen remains in atheromata (23–26) and in the intima adjoining such or other conditions (23, 26). Similarly, a possible role by such proteoglycans in thrombus formation and function can also be postulated. By tight binding to the basement membrane proteoglycans, the fibrin polymer is densely anchored, and this enhances its (noncovalent) stability and hemostatic effectiveness. Finally, the present findings raise the possibility of fibrinopeptide binding by proteoglycans in blood or tissues thus making them inaccessible to the widely used fluid-phase measurements. Assuming such fluid phase binding does not impair their immunassays (27), binding by solid-phase proteoglycans, may influence their circulating levels and possibly account for the very short half life of fibrinopeptides.

The possible relationship of C1q to circulating proteoglycans, which are increased in some disorders (2) and display anticoagulant properties in others (28–32), is speculative, in that attempts to assay for a C1q inhibitory activity were not made. Nevertheless, among this heterogeneous group chondroitin proteoglycans have been described (28, 34). Most glycosaminoglycans identified as circulating anticoagulants enhanced the antithrombin III activity and thus were directed against thrombin; when examined further, evidence for heparan (29), and keratan (29, 34) sulfate proteoglycans and for hyaluronic acid (30) was obtained in different patients. A chondroitin sulfate proteoglycan (28) differed from the others by impairing the thrombin release of fibrinopeptides, suggesting some similarity to the present form. These and other reported cases implied association with substantial neoplastic proliferation (28–34) and with parenchymal tissue destruction (31, 32). Even so, keratan sulfate along with dermatan sulfate are known to be increased in mucopolysaccharide storage diseases but are not associated with a coagulopathy (33). Increased circulating levels of proteoglycans occur in suramin treated patients (34) and among these, undersulfated chondroitin sulfate forms not unlike the present proteoglycan have been described. Along with abnormal accumulation of proteoglycans in the liver of suramin-treated rats (35), these findings intimates inhibition of enzymes which degrade proteoglycans. Thus, catabolic processes, as well as cell proliferation and tissue destruction appear to modulate their circulating levels in health and disease.

Acknowledgments

The authors wish to thank Dr. B. Coller for his generous support and Yuqun Hong for excellent technical assistance.

The foregoing studies were supported in part by grants CA-4107 from the National Cancer Institute and 900696 from the American Heart Association.

References