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, displayed concentration-dependent prolongation of plasma and fibrinogen solution clotting times. Under factor XIIIa catalyzed cross-linking conditions and maximum C1qI concentrations, minor amounts of clot formed displaying complete γ-γ dimer formation but virtually no α-polymer formation. The anticoagulant effect was undiminished by its binding to C1q, by increased ionic strength, and by $CaCl_2$, but was abolished by incubation of C1qI with chondroitinase ABC. ^{125}I-labeled C1qI bound to immobilized fibrinogen, fibrin monomer, fibrinogen plasmic fragments D_1 and E, and fibrin polymers. Occupancy on the E domain required uncleaved fibrinopeptides together with another structure(s), and it did not decrease binding of thrombin to fibrinogen. Occupancy on the D domain did not decrease the fibrinogen binding to fibrin monomer. We conclude that the E domain occupancy impaired fibrinopeptide cleavage, and occupancy on the D domain impaired polymerization, both steric hindrance effects. C1qI binding to fibrinogen explains at least in part the well-known fibrin(ogen) presence in immune complex–related lesions, and the fibrinogen presence in vascular basement membranes and atheromata. We postulate that fibrin binding by resident basement membrane proteoglycans provides dense anchoring of thrombus, substantially enhancing its hemostatic function. (*J. Clin. Invest.* 1994. 93:303–310.) Key words: anticoagulant • C1q inhibitor • fibrin • fibrinogen • proteoglycan

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 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.

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 [fibrin(ogen)]. Initial results disclosed binding to and an anticoagulant property against fibrin(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. Plasmic fragments D_1 and E were isolated and their concentrations were measured by procedures previously applied (11). Plasma coagulability was measured by adding ~ 50–200 μg of ^{125}I-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. ^{125}I-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,

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

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

μ = 0.15 contained factor XIII 5–40 nM, 2–30 mM $CaCl_2$, 0.1 U/ml human thrombin, and Kunitz pancreatic trypsin inhibitor KPTI (FBA Pharmaceuticals, West Haven, CT) 100 U/ml. In some experiments, clots were obtained by dilution of preformed fibrin monomer solutions with this buffer (11) in the presence or absence of C1qI. Under these conditions factor XIIIa activity measured by the monodansyl cadaverine assay (13) was unaffected by C1qI (n = 2). Chondroitinase ABC, which cleaves galactosaminoglycan side chains from C-4SP, and chondroitin sulfate A (a core-free C-4SP carbohydrate side chain component) were used as supplied (Sagakaku America Inc., Rockville, MD). Fibrinopeptide measurements were performed by HPLC (14). For binding measurements, C1qI was added to solutions containing 3–5 μg/ml of A + B peptides (each in roughly equal concentrations), vortex-mixed, and filtered after 5–10 min via a 10,000 mol wt cutoff (Millipore Corp., Bedford, MA) filter. This removed C1qI which otherwise appeared as a single protein peak near the start of the HPLC acetonitrile elution.

C1qI isolation and related analyses. C1qI was isolated essentially as described (6), and its electrophoretic (SDS-PAGE) bands could not be stained with Coomassie Brilliant Blue dye. It was subfractionated by size exclusion chromatography to obtain the active moiety (15). The C1qI concentration was determined by using a Bosch and Loemb (Leica Inc., Buffalo, NY) refractometer; thereafter, the calculated extinction coefficient A (1%, 1 cm at 280 nm) of the active chromatographic isolate, 2.8 (range 2.6–3.0, n = 3) was used. The isolation procedure can be summarized as follows. Serum containing 1 mM EDTA, pH 7.5, μ = 0.16, was incubated at 37°C for ~ 10 min to dissociate C1q from C1r and C1s. This was diluted to a final concentration of μ = 0.04, followed by stirring at 4°C for at least 20 min to precipitate C1q. The centrifuged (4°C) precipitate was washed thrice in buffer containing 5 mM EDTA to remove contaminants, and allowed to dissolve in 0.7 M NaCl overnight. Material remaining insoluble was discarded, and the remaining solution was dialyzed against 100 mM EDTA, pH 5, μ = 0.078. The precipitate was removed by centrifugation (16,000 g, 30 min, 4°C), washed twice in cold buffer excess, and dissolved in 300 mM NaCl, 5 mM EDTA, pH 7.5. This solution, further centrifuged to remove insoluble material, was dialyzed in barbital buffer, μ = 0.5, pH 8, and 1 mM each of $CaCl_2$, and $MgCl_2$. The solution was subjected to Con A-Sepharose (Pharmacia-LKB, Piscataway, NJ) chromatography, harvesting the inhibitor in the unbound eluate (and C1q by subsequent elution with 10% methyl-α-D-mannopyranoside, Sigma Chemical Co.). The unbound fraction was subjected to HPLC (14) exclusion chromatography (TSK columns, Beckman Instruments, Inc., Palo Alto, CA), pH 7.4, μ = 0.15. The protein peak displaying inhibition against the C1q hemolytic activity amounted to ~ 15% of total protein and was examined for its effect on fibrinogen clotting. Possible contaminant Con A contributing to the effects on fibrin(ogen) was excluded by one C1qI preparation that was exposed overnight to solid phase (Sepharose-bound, Sigma Chemical Co.) anti–Con A IgG antibody. Its inhibition against fibrin gelation was undiminished by this exposure. For C1qI digestion, chondroitinase ABC was added to a solution of radiolabeled or unlabeled C1qI, 5–20 μg/ml, pH 8, to achieve 30 mU/ml and incubated overnight, 37°C. The mixture also contained 10 μg/ml pepstatin, 20 μg/ml leupeptin (Calbiochem Corp.), 1 mM benzamidine, and 2 mM o-phenanthroline (Sigma Chemical Co.). The mixture was heated at 90° for 1 min and tested for fibrinogen clotting effects either directly or after dialysis against the testing buffer. This heat exposure of untreated C1qI control did not decrease its fibrin binding or its effect against fibrin polymerization (n = 2). Lack of degradation of fibrin was ascertained by demonstrating no change in the polymerization rate, clot turbidity, and its SDS-PAGE bands (11).

Assay of C1qI activity. Solutions containing C1qI were tested by a hemolytic assay using C1q-deficient or C1q-depleted (C1qD, Quidel, San Diego, CA) serum as described (16, 17). Briefly, isolated C1q (5–10 μg/ml) was mixed with various concentrations of C1qI in a final volume of 300 μl of veronal buffer, pH 7.5, adjusted to μ = 0.15 with NaCl, containing $CaCl_2$ 1.5 mM, $MgCl_2$ 5 mM, and 0.1% gelatin). After 30 min at 37°C, 10 μl of C1qD serum and 200 μl of 1.5×10^{-8}/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 assayed spectrophotometrically at 412 nm. The C1qI activity was computed as percentage of the C1q 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 C1qI 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 C1qI 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 C1qI and there was no cross-reactivity with C1q and with Con A. The isolated IgG neutralized the capacity of C1qI to inhibit the C1q hemolytic activity.

Radioiodination and measurements. ^{125}I-labeling was carried out (11) as described, and specific activities were > 60×10^3 cpm/ng 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 aliquants 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 C1q bound to each microwell, calculated from a 1:50 mixture of (^{125}I-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 P-PACK 50 nM or hirudin 5 U/ml to neutralize thrombin, or PMSF 100 nM, to neutralize batroxobin.

Results

Investigations of whole C1qI

In pilot experiments, several C1qI 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 C1qI 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 ($\times 10^{-3}$/s (see Methods). A similar inhibition was demonstrated by soluble fibrin monomer induced to polymerize (n = 4). Addition of C1qI to plasma in further investigations resulted in invariable delay in clotting times which was concentration dependent, demonstrable in either heparin or citrate-anticoagulated plasma, in the presence (Table I) or absence of $CaCl_2$, and whether clotting was induced by thrombin, batroxobin, or ancrod (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

C1qI (μg/ml)	5	8	26	Control
Thrombin time (s)	16.8	26.6	>300	6.4
n	4	2	6	7

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 $CaCl_2$ 67 mM.

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 $CaCl_2$ 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, ^{125}I-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 × 10^3 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 C1q (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 heterogeneous than the parent material (Fig. 2 *B*), and it was subjected to more extensive investigations. By ^{125}I-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 $CaCl_2$ 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 an other. Comparison of the calculated slopes of maximal C1qI inhibition before and following its preincubation with fibrinogen

2. Other subfractions obtained by the gel sieving chromatographic procedure also displayed some inhibition against fibrin gelation, but because their activity against C1q was minimal or absent these were not investigated further. However, this effect along with their clear binding shown by the autoradiograms implied that the anticoagulant effect was not restricted to this chromatographic subfraction.

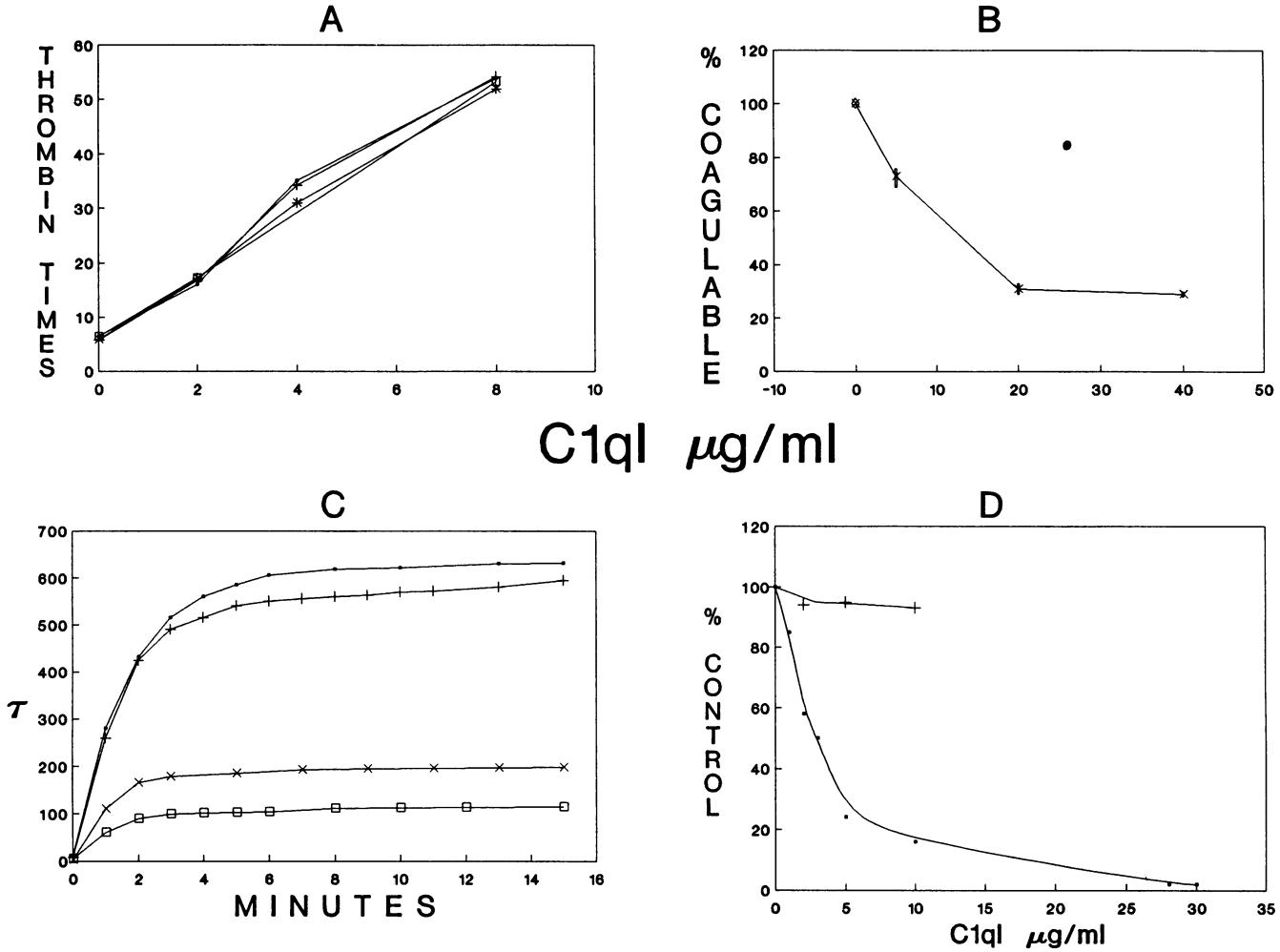

Figure 1. Anticoagulant effects of C1qI. (*A*) Plasma thrombin times, shown in seconds, of plasma from four donors whose fibrinogen concentrations were 2.2 (○), 2.47 (+), 2.62 (□), and 2.74 (*) g/liter, respectively. Each line graph reflects values from the untreated plasma and from plasma to which three different C1qI concentrations shown had been added. (*B*) Decreased coagulability of fresh citrated plasma, fibrinogen 2.48 g/liter, to which ~ 200 ng ^{125}I-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 (τ = absorbance, 350 nm, shown $\times 10^3$) 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.

Fibrin(ogen) binding studies. SDS-PAGE autoradiograms of ^{125}I-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 C1q 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 fibrin(ogen). 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 reacted 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

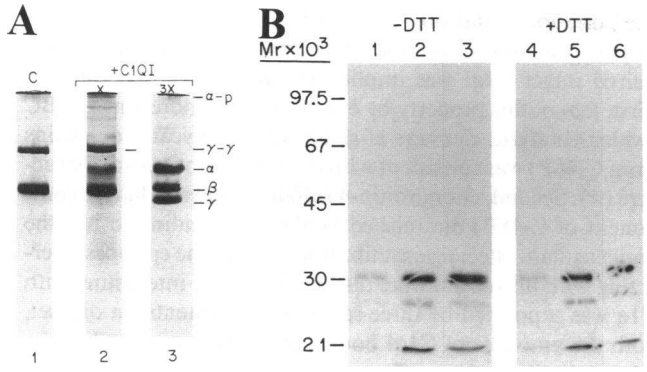

Figure 2. (*A*) PAGE-SDS comparisons of partly coagulable, gel *2*, and incoagulable, gel *3*, fibrin obtained at two different concentrations of C1qI, 3 and 9 μg/ml shown as × and 3×, respectively, and obtained under maximum cross-linking conditions. Gel *1*, fibrin clot control; β chains of fibrin, α-p, α polymers; γ-γ, γ-γ dimers. Clotting conditions were 2 μM fibrinogen, 40 nM XIIIa, and 5 mM $CaCl_2$, 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 ^{125}I-C1qI 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 kD was 28 kD. Identical amounts of C1qI 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 C1qI, 47% bound to C1q, 42% to fibrinogen, and 11% to albumin; for mol/mol fibrinogen and C1q estimates see Table II and legend.

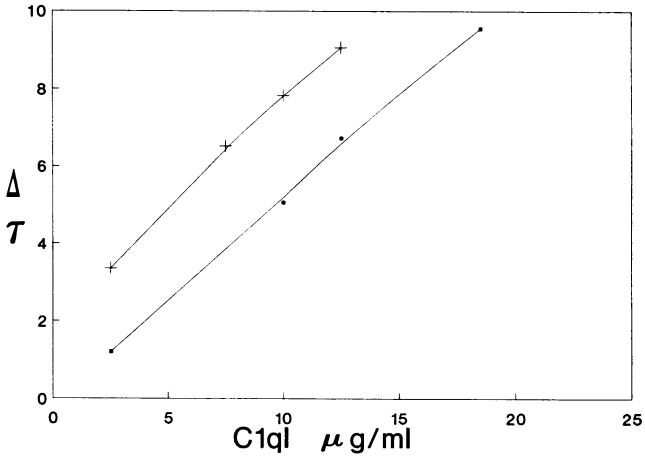

Figure 3. Inhibition of polymerization of (2 μM) fibrin monomer by C1qI in the presence (x) and absence (□) of (1.5 μM) fibrinogen at different C1qI concentrations (abscissa). Δτ, change in maximum turbidity (extent of decreased absorbance, 350 nm, shown ×10^3). The experiments were carried out at 37°C, pH 7.4, μ = 0.15.

against the 30-kD band, in Western blot experiments (not shown).[3] Preincubation of C1qI 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 C1qI bound to fibrinogen whether or not it had been pretreated with C1q or with chondroitin sulfate, respectively. This intimated that native C1qI structure was required for its binding to fibrinogen, and that the C1q and fibrinogen binding sites on C1qI were distinct. In further analyses, C1qI exposure to chondroitinase ABC decreased its binding to fibrinogen to that of albumin controls (n = 6; not shown).

Comparison of C1qI binding by immobilized fibrinogen, monomeric desAA fibrin, and desAA/desBB fibrin (Table II), disclosed C1qI binding to all three forms but in respectively decreased amounts. Also, fibrin (1 μM) clots immersed overnight in 6.7 μM C1qI bound 68% of C1qI (n = 4), indicating that C1qI binding sites remained available in polymerized fibrin. These results led to assessment of binding to isolated plasmic fragment E of fibrinogen. Fragment E also bound C1qI (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 Bβ chains (18) precluding B peptide assessments; however, a role of the uncleaved B peptide was clearly indicated by comparisons of C1qI binding to dessAA and desAA/desBB fibrin monomers (Table III), and by results indicating cleaved B peptide bound to liquid phase C1qI (vide infra). To explore the C1qI binding to the intact E domain, immobilized fibrinogen containing bound ^{125}I-C1qI was exposed to thrombin (1 U/ml, overnight). This resulted in no release of C1qI (n = 6). Also, ^{125}I-thrombin was exposed to fibrinogen containing bound C1qI (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 D_1 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 D_1 or fibrinogen bound by solid-phase fibrin monomers were undiminished following binding of C1qI to such monomers (n = 6). This was consistent with the lack of

Table II. Amounts of C1qI Bound to Fibrin Monomers and Polymers, and Isolated Plasmic Fibrinogen Fragments D_1 and E

		Fibrin monomer		Fragment		
Fibrinogen	Fibrin clot	desAA	desAA/desBB	D_1	E	E-Thr.
		mol/mol				
6.7	5.3	5.6	3.7	1.9	3.5	< 1

Shown are means (range <8% above and below each mean) from at least triplicate measurements using varying ratios of radiolabeled/unlabeled C1qI, corrected for albumin controls. Not shown were C1q-positive controls disclosing 16 mol C1qI bound/mol C1q. Amounts in fibrin polymers were computed from synerized fibrin (1 μM) clots and their supernatants described in Methods, using a C1qI 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 C1qI 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 other 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 C1qI

C1qI (μg/ml)	3	10	20	40
		%		
A Peptide	87	57	40	0
B Peptide	94	80	53	18

Values reflect unbound peptides calculated as percentage of A and B peptide controls from HPLC measurements. The C1qI + 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 C1qI.

binding of C1qI to the fibrin E domain and with other results (*vide supra*) disclosing additive inhibition of fibrin polymerization by fibrinogen and C1qI.

Binding to fibrinopeptides. Preliminary attempts to assess possible effects of C1qI on A and B peptide release failed ($n = 4$), and this was further explored by preparing mixtures of cleaved A and B peptides with C1qI in molar excess. These yielded no detectable free peptides by HPLC measurements. For example, a solution containing ~ 10 μg C1qI/ml, 2 μg of A, and 2 μg of B/ml yielded no free A or B peptides ($n = 3$). That is, when such solution mixtures were applied whole or after microfiltration to remove C1qI (see Methods), no free peptides were detectable in the filtrate, in sharp contrast to control solutions lacking C1qI. Moreover, the amounts of free peptides were inversely proportional to the C1qI concentration (Table III). Also, using a single A and B peptide solution mixture higher C1qI concentrations were required to remove peptide B than those which removed peptide A or AP ($n = 3$), peptide AP requiring the lowest concentrations of C1qI for removal (not shown). Calculated from such data, a single mole of C1qI 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.

Discussion

The most important findings of the foregoing investigations relate to the capacity of C1qI to bind to fibrin(ogen) either free and in complex with C1q and to impair fibrin gelation in plasma. The plasma results indicated that C1qI 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 C1qI (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 μg/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 C1qI could bind no additional fibrinogen. Absent a self-association by C1qI 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 C1qI 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 C1q was supported by three series of experiments. In one set, both the amounts of C1qI bound to fibrinogen as well as the related anticoagulant effect remained undiminished by its preincubation with C1q 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 C1qI. Finally, neither the anticoagulant property of C1qI nor its binding to fibrinogen were diminished by increased ionic strength in sharp contrast with its binding to C1q which is abolished by high ionic strength (4).

The results imply two independent but concerted mechanisms of action by C1qI. One mechanism involves binding of C1qI to the fibrinogen E domain; at least 2 mol of C1qI bound to the E domain before but not after fibrinopeptides have been cleaved, assuming no self-associating C1qI complexes. Fibrinopeptide cleavage was more difficult to assess. Failure to release bound C1qI by thrombin suggested impaired fibrinopeptide cleavage. However, even under maximum C1qI 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 γ-γ 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 assayed owing to their binding to C1qI. It is unclear whether multiple epitopes or a single C1qI epitope bound fibrinopeptides. Differences in binding of each fibrinopeptide by C1qI suggested that similar C1qI sites bound fibrinopeptides but with different affinities owing to peptide structure differences. The dessAA data (Table III) intimated that one mol of C1qI bound to desAA, two to fibrinogen and none to the desAA/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 C1qI occupancy. This raises the possibility that fibrinogen-immobilized C1qI itself bound thrombin, consistent with the demonstrated failure of thrombin to release any bound C1qI. C1qI 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 C1qI and that C1qI bound elsewhere within the D domain and inhibited by steric hindrance. This was supported by (*a*) C1qI binding to fibrin monomers and to D_1, (*b*) the incorporation of C1qI in polymerized fibrin gels, (*c*) the capacity of fibrinogen or of

fragment D_1 to inhibit fibrin polymerization in the presence of C1qI, and (*d*) the additive inhibition of polymerization by fibrinogen and C1qI (Fig. 3). Additional evidence of steric hindrance was provided by use of C1qI 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 C1qI, 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 C1qI exist whether or not it is bound to C1q/antibody complexes, C1qI 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 C1qI effect. That is to say, neither the anticoagulant effects nor the binding of C1qI 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 C1qI 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 enzymic 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 immunoassay (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 C1qI 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 C1qI 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 intimated 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.

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