Specific Regulation of Procoagulant Activity on Monocytes

INTRINSIC PATHWAY INHIBITION BY CHONDROITIN 4,6-DISULFATE*

Maria P. McGee, Hoa Teuschler, Narayanan Parthasarathy, and Williams D. Wagner

From the Departments of Medicine and Comparative Medicine, Bowman Gray School of Medicine, Winston-Salem, North Carolina 27157

Hypercoagulability of blood, monocytic infiltration, and changes in pericellular and extracellular matrix glycosaminoglycans (GAGs)1 are observed in atherosclerosis, inflammation, and neoplasia. In the present studies, monocyte procoagulants and different GAGs including chondroitin sulfate (CS) A, CSB, CSC, CSD, CSE, and heparan sulfate, were tested either in clotting assays with whole plasma or in chromogenic assays with purified coagulation proteases. Procoagulant activity in plasma was inhibited by three of the seven GAGs, including heparan sulfate, CSE, and CSB. In contrast, activity of purified coagulation protease was inhibited only by CSE, and the inhibition was observed with intrinsic (factor VIIIa/IXa) but not extrinsic (tissue factor/factor VII) components. Reciprocal titration experiments with enzyme and substrate and Scatchard type analyses were consistent with concentration-dependent inhibitory interactions between CSE and sites on both factor VIIIa and IXa. On purified phospholipids, CSE concentration resulting in half-maximal inhibition (KΙ) was 5 ng/ml for interaction with factor IXa and >500 ng/ml for interaction with factor VIIIa. The Kι values were lower for reactions on purified lipid than for reactions on monocyte surfaces and for reactions on resting than on endotoxin-stimulated monocytes. Experiments with CSE oligosaccharides of defined size indicated that the smallest CSE fragment capable of inhibitory activity was composed of 12–18 monosaccharide units. Collectively, these results indicate that factor X-activating reactions are inhibited by GAGs expressed on monocyte membranes. Inhibition is specific with respect to the structure of both the GAG and the activating protease. Lack of inhibition by added CSA, CSB, and CSC in contrast to CSE strongly suggests a direct role of 4,6-di-O-sulfated N-acetylgalactosamine GAG structures in the inhibition of intrinsic pathway protease. These findings also suggest potential pharmacologic use of CSE as specific anticoagulant in the management of prothrombotic states mediated by intrinsic pathway coagulation reactions.

Mononuclear phagocytes constitute one of the major components in the cellular infiltrates that characterize atherosclerotic, neoplastic, and chronic inflammatory lesions (Ross, 1993; McGee et al., 1978; Rickles et al., 1988). These lesions are also associated with localized blood coagulation reactions and both qualitative and quantitative changes in GAGs1 content (Wagner and Salisbury, 1989; McGee et al., 1990; Wilcox et al., 1989; Dietrich et al., 1993).

Monocytes and macrophages can accelerate membrane-dependent coagulation reactions via expression of TF (tissue factor) and membrane assembly sites for coagulation protease complexes. Membrane TF interacts with plasma factor VII with high affinity (KΙ, approximately 10⁻⁹ M), forming functional protease complexes that cleave plasma coagulation factors IX and X and generate the serine esterases, factors IXa and Xa (Bach et al., 1986). Factor IXa in turn assembles on acidic phospholipids with coagulation factor VIIIa (Kι, approximately 10⁻⁹ M) to form the “intrinsic pathway” protease that also generates factor Xa (Ahmad et al., 1989). Thus, this protease complex constitutes a seemingly redundant amplifying loop for activation of factor X to Xa. However, both the extrinsic and intrinsic coagulation proteases are essential for normal hemostasis, as evidenced by the coagulation deficiencies exhibited by patients with single genetic defects in either plasma factor VII, VIII, or IX. (Brigg and Nossel, 1961; Malar et al., 1982).

Chondroitin sulfates are the predominant GAGs comprising the proteoglycans produced by monocyte/macrophages (Owens and Wagner, 1992; Kolset et al., 1983; McQuillan et al., 1989; Uhlin-Hansen et al., 1989; Edwards et al. 1995). Transition from monocyte to macrophage upon exposure to differentiating stimuli is often accompanied by the appearance on the membrane and pericellular spaces of an oversulfated chondroitin sulfate containing 4,6-di-O-sulfated N-acetylgalactosamine residues (i.e. CSE). Whereas previous studies address the importance of CSE during cell differentiation, no information is available on the effects of this unique GAG on procoagulant activity. In the present study we demonstrate that CSE specifically modulates the rate of factor X-activating reaction on monocyte/macrophage membranes.

MATERIALS AND METHODS

Cells and Procoagulant Lipid Surfaces—Human blood monocytes, the human monocytoid cell line THP-1, and human brain homogenates were used as sources of natural procoagulant surfaces. Lipid extracts from rabbit brain (Sigma) were used as a source of procoagulant lipids essentially devoid of either protein or sugars. The THP-1 cells were maintained in culture at 37°C, 5% CO₂ atmosphere in RPMI 1640 media supplemented with 10% fetal calf serum and 2 × 10⁻⁵ M β-mercaptoethanol. Cells were propagated by serial passage at 3–5-day intervals. Before use in kinetic experiments, cells were washed with serum-free medium, M-199 (Life Technologies, Inc.) containing 0.5% low protein serum replacement (Sigma) and 2 µg/ml endotoxin (Escherichia coli Serotype 0127:88, Sigma), and incubated at 5 × 10⁶/ml in slanted polypropylene tissue culture tubes. The pattern of GAG synthesis by THP-1 cells is very similar to that of human, rat, and mouse

1 The abbreviations used are: GAG, glycosaminoglycan; CS, chondroitin sulfate; HS, heparan sulfate; TF, tissue factor.

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†To whom correspondence should be addressed: Dept. of Medicine, Rheumatology Section, Medical Center Blvd., Winston-Salem, NC 27157-1089.
### RESULTS AND DISCUSSION

Inhibition of Monocyte Procoagulant Activity by GAGs—Procoagulant activity was induced on either blood monocytes or THP-1 cells by incubation in M-199 serum-free media supplemented with 0.4% low protein serum replacement and endotoxin (400 mg/ml fed 20 h). As described previously (McGee et al., 1994), under these conditions both cell types express procoagulant activity with similar time courses. Activity increased rapidly during the first 4 h of culture, afterward slowly reaching maximum levels at 18–20 h. The increase in procoagulant activity was primarily due to expression of TF on the cytoplasmic membrane. Expression of lipid cofactor sites for assembly of IXa-VIIa is constitutive in monocytes and changes little upon incubation with inflammatory stimuli. The possibility that added GAGs can modify the levels of procoagulant activity was investigated using one-step clotting assay with recalified human plasma as substrate. Chondroitin sulfate A, B, C, D, and E and HS were incorporated in reaction mixtures at concentrations ranging from 0 to 134 μg/ml. Activity was primarily due to expression of TF on the cytoplasmic membrane. Clotting times were measured in mixtures containing either cells or rabbit brain lipid extracts as a source of tissue factor. Intrinsic pathway procoagulant was measured in mixtures containing either cells (monocytes or THP-1) or human brain homogenates as a source of tissue factor. Intrinsic pathway procoagulant was measured in mixtures containing either cells (monocytes or THP-1) or human brain homogenates as a source of tissue factor. Intrinsic pathway procoagulant was measured in mixtures containing either cells (monocytes or THP-1) or human brain homogenates as a source of tissue factor. Intrinsic pathway procoagulant was measured in mixtures containing either cells (monocytes or THP-1) or human brain homogenates as a source of tissue factor. Intrinsic pathway procoagulant was measured in mixtures containing either cells (monocytes or THP-1) or human brain homogenates as a source of tissue factor.

#### TABLE I

| GAG  | Procoagulant activity | µg/ml | milliliters/ml |
|------|-----------------------|-------|----------------|
| CSA  |                        | 167   | 513            |
| CSB  |                        | 83    | 500            |
| CSC  |                        | 42    | 500            |
| CSD  |                        | 21    | 628            |
| CSE  |                        | 10    | 500            |
| HS   |                        |       |                |

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Inhibition of Intrinsic Pathway Protease as a Function of Lipid Concentration—

Dependence of Inhibition on Size of CSE Polymer—Analysis by size exclusion chromatography indicated considerable size heterogeneity in the CSE preparation, Fig. 3. The elution profile included the range of CSE sizes described for monocyte CSs (Edwards et al., 1993; Uhlin-Hansen et al., 1993). Based on peak elution profile, the estimated average molecular weight was 88,000, consistent with a distribution coefficient, $k_{av}$ of 12–18 monosaccharide units, inhibition may be

Further evidence for inhibitory GAGs expressed on the monocyte membrane was obtained by removing surface proteoglycans using mild trypsinization (0.05% Trypsin and 0.53 mM EDTA solution, Life Technologies, Inc.). Intrinsic pathway protease complexes were assembled on cells, either after a limited, 0.5-min trypsin treatment or after a 10-min trypsin treatment. Cells in both preparations were subjected to the same extent of physical manipulation except for the length of incubation in the presence of trypsin. As previously reported, trypsinization effectively removes CSE- and HS-containing proteoglycans from the monocyte surface. (Edwards et al., 1995). Rates of factor X activation on cell trypsinized for 10 min were 9.8 ± 0.8 nM factor Xa/min and significantly faster than rates on cells subjected to limited trypsinization (4.0 ± 0.2 nM factor Xa/min). This result is also consistent with membrane expression of inhibitory GAGs by the monocyte.

Monocytes and monocytoid cell lines are known to increase synthesis and expression of CSE upon incubation in vitro in the presence of inflammatory stimuli. (Kolset et al., 1983; Uhlin-Hansen et al., 1989; Uhlin-Hansen et al., 1993; Edwards et al., 1995). To determine if this incubation-dependent increase in CSE levels is reflected on intrinsic pathway protease activity, inhibition of factor X activation with CSE was measured on either human monocytes or THP-1 incubated with endotoxin for 48 h at either 37 or 4–8 °C. The concentrations of CSE required to achieve half-maximal inhibition were significantly (p < 0.05) increased (2.7-fold for three independent experiments) on cells incubated at 37 °C as compared with cells incubated at 4–8 °C. Again, results are consistent with baseline regulation of intrinsic protease by membrane GAGs on macrophages.

Fig. 2. Inhibition of intrinsic pathway protease on cells and purified lipid surfaces. Initial rates of factor X activation were measured in reaction mixtures without GAGs and mixtures with 6 μg/ml CSE. Concentration phospholipid membranes supplied either as rabbit brain lipid extract or as intact THP-1 cells was as indicated on the abscissa. Intrinsic pathway components and other reagents were as indicated in the legend to Fig. 1. Inhibition, (I) was calculated as 1 – R/R, where R and R are the initial rates measured in mixtures with and without GAG, respectively. Apparent inhibition decreased with cell membrane concentration (●) but not with the sugar-free, protein-free lipid extract concentration (○).
correlated with polysaccharide size. Among the small oligosaccharides obtained upon fragmentation of CSE by partial hydrazinolysis/high pH nitrous acid treatment and subsequent fractionation on Bio-Gel P-6 chromatography, oligosaccharides with 12-20 monosaccharide units at 10 μg/ml mediated inhibition that was 25% of the inhibition mediated by intact CSE at the same concentration (see Table I). Additional chromatography of the CSE fragments in Sepharose 6B suggested that the average size of the smaller oligosaccharide with inhibitory activity, correspond to an molecular weight of approximately 6500, consistent with a $K_{av}$ of 0.77.

Mechanisms of Intrinsic Pathway Protease Inhibition by Chondroitin Sulfate E—Reaction components assembled either on cells or on purified lipid were titrated with CSE over three different concentration ranges. (Table I). Apparent kinetic parameters ($K_i$ and $I_{max}$) varied with the concentration range, suggesting that inhibition values reflect more than one type of molecular interaction. Determination of relative values of apparent $K_i$ was obtained using complete titrations with CSE concentrations ranging from 0.008 to 158 μg/ml. Using Scatchard type plots, values of $K_i$, approximated from the slope of the two extreme linear segments were 5 and 1000 ng/ml (Fig. 4, insets A and B, respectively). Although there are inherent uncertainties in the analysis of multiple equilibria from kinetic data, these results identified at least two different types of inhibitory interactions with affinities differing at least by 100-fold. These results, however, do not exclude the possibility of additional interactions of intermediate affinity or that the observed heterogeneity reflects the wide range of polymer sizes in the CSE preparation.

Based on these results, further investigations of GAG-protein interactions were approached using multiple titration experiments with reaction components. For these experiments, each one of the reactants was alternatively used as a titrating component, whereas the inhibitor and the rest of the reactants were maintained at constant concentrations. The rationale of this approach is that the component containing binding sites for CSE should be displaced by the inhibitor from the functional protease complex and that the empirical value of the kinetic parameter ($K_{sat}$), that is, concentration of component resulting in half-maximal rates, will increase in reactions with CSE as compared with reactions without the inhibitor. Simi-

**TABLE I**

| CSE concentration range | Cells | Lipids |
|-------------------------|-------|--------|
| μg/ml | $K_i$ | $I_{max}$ | μg/ml | $K_i$ | $I_{max}$ |
| 158.7–2.4 | 8.4 ± 1.40 | 0.94 ± 0.04 | 0.75 ± 0.10 | 0.92 ± 0.02 |
| 39.7–2.5 | 1.2 ± 0.13 | 0.89 ± 0.01 | 0.51 ± 0.13 | 0.60 ± 0.01 |
| 15.8–0.6 | 0.7 ± 0.24 | 0.52 ± 0.04 | 0.13 ± 0.02 | 0.45 ± 0.01 |

**TABLE II**

| Monosaccharide units | Inhibition |
|----------------------|------------|
| 6 | 0.076 |
| 8 | 0.021 |
| 12 | 0.025 |
| 12-18 | 0.250 |
| unfractionated | 0.740 |

**FIG. 4.** Parameters of intrinsic protease inhibition on purified lipid extracts; Scatchard analyses. Rates of factor X activation were measured in mixtures with and without CSE at concentrations ranging from 8 to 1.6 $\times$ 10$^7$ ng/ml. Other reagents and determination of inhibition were as indicated in the legend to Fig. 2. The ratios between inhibition (I) and CSE concentration ([CSE]) are plotted versus I. The slopes of the two extreme linear segments calculated by linear regression correspond to $K_{1} = 5$ ng/ml and $K_{2} = 1000$ ng/ml. The results are consistent with two independent sites $n_{0}$ and $n_{2}$ resulting in 0.57 and 0.43 fractional inhibition, respectively.
using lipid extracts from rabbit brain as a source of protein-free and sugar-free lipid surface.

The concentration ranges for the titrating component were: factor X, 0.1–300 nM; factor IXa, 0.05–8 nM; and factor VIII and sugar-free lipid surface.

The inhibition increased only when either factor IXa or factor VIII was increased but not when both were increased. (Fig. 5, A, B, and C). To further test the hypothesis and to determine the component with the high affinity site for CSE, titrations were repeated at CSE concentrations below the $K_i$ estimated for the low affinity site. In these experiments, increasing the concentration of VIIIa while maintaining IXa constant had little effect on the inhibition level, particularly at low concentrations of VIIIa. In contrast, increasing factor IXa while maintaining factor VIIIa concentration fixed resulted in a marked and progressive increase in the inhibition. Therefore, the results exclude the possibility that CSE inhibits the factor X-ac-

| Varied component | $K_{IX}\cdot K_{IX}$ | $V_{att}/V_{att}$ |
|------------------|----------------------|------------------|
| Lipid Cells      | Lipid Cells          |
| X                | 0.24 ± 0.15          | 0.29 ± 0.06      |
| VIIIa            | 0.134 ± 0.020        | 0.16 ± 0.015     |
| IXa              | 0.56 ± 0.12          | 0.17 ± 0.09      |
| VIIIa/IXa        | 0.89 ± 0.025         | 0.20 ± 0.020     |

**TABLE IV**

Titrations with intrinsic protease components: ratio between kinetic parameters of inhibited and noninhibited reactions

Kinetic parameters ($K_{IX}$, $V_{att}$, $K_{IX}$, and $V_{att}$) were determined from the initial rates of factor X activation in reaction mixtures with (subscript I) and without CSE, respectively. Parameters were calculated from rectangular hyperbolas fitted to the data by least square methods. Rates were measured at fixed concentrations of all reactants except one. Fixed concentrations were: factor IXa, 0.85 nm; factor VIIIa, 1 nm; and factor X, 150 nm. Surface for protease assembly were either cells (1 x 10^6/ml) or rabbit brain lipid extract (60 nM lipid phosphorous) suspended in Tris buffer, pH 7.3, 0.15 M NaCl, 5 mM CaCl$_2$, and 0.5 mg/ml ovalbumin. Chondroitin sulphate E was at 40 g/ml. Results are expressed as the ratios between kinetic parameters of reactions with CSE and kinetic parameters of reactions without CSE. Mean ± S.D. of three independent sets of determinations. Reaction rates were also measured at fixed concentrations of all components except IXa and VIIIa, which were added at increasing concentrations while maintaining a constant molar ratio between the two.
Monocyte Procoagulants and Glycosaminoglycans

Glycosaminoglycans are known to interact with and modulate the activity of many biologically important proteins, including coagulation inhibitors such as AT III (Marcum et al., 1992) and heparin cofactor II (Pratt et al., 1993), and growth factors such as fibroblast growth factor and hepatocyte growth factor (Lyon and Gallagher, 1994). Available evidence indicates that the interaction of GAGs with these proteins depends on the sequence and size of their oligosaccharide units. For example, chondroitin sulfates A, B, and C have common disaccharide units of hexuronic acid (glucuronic acid in CSA and CSE; iduronic acid in CSB) and N-acetylgalactosamine-O-sulfate. However, only CSB is known to bind to AT III and heparin cofactor II (Maimone and Tollefsen, 1990). Similarly, commercial heparin and heparan sulfates have common disaccharide units of hexuronic acid (glucuronic acid/iduronic acid) and N-acetyl (or N-sulfated) glucosamine O-sulfates, but their protein-binding characteristics are different. In this regard, the binding sites for proteins have been mapped to specific regions comprising a few oligosaccharide units (Gallager et al., 1992). The consensus from these studies is that the minimum oligosaccharide length required for binding is 5–7 disaccharide units but that the size required to elicit biological activity after binding is larger. Therefore, it is not surprising that in the present functional studies oligosaccharides longer than dodecasaccharides were required for activity. Heparin has been reported to inhibit intrinsic but not extrinsic or common proteases (Barrow et al., 1994). The saccharide sequence(s) required to mediate this inhibition was/were not determined. Because heparin sequences are heterogeneous with respect to sugar composition and sulfate substitutions, it is possible that the inhibition observed was mediated by O-sulfate groups as shown for CSE in the present studies. Alternatively, intrinsic protease components may have sites for interaction both with CSE and with N-sulfated sequences in commercial heparin. Whereas heparan sulfate GAGs are present in most tissues, heparin sequences such as those in commercial anticoagulant preparations are almost exclusively found in mast cell granules. Anticoagulant effects of CSE mediated primarily by acceleration of heparin cofactor II interaction with thrombin have been reported (Scully et al., 1986). The apparent $K_d$ for the interaction was in the μg/ml range, approximately 2 orders of magnitude higher than the values obtained in the present studies for the interaction between CSE and the intrinsic pathway protease.

Considered together, the variety of different molecular interactions increasingly being described in the regulation of blood coagulation by GAGs (Bourin and Lindahl, 1993) points to complex physiochemical mechanisms and underlines the importance of considering structural requirements for each interaction independently rather than generalizing from studies with heparin.

Intense efforts in coagulation research are currently being directed to identify specific inhibitors and modulators of blood coagulation that may control hypercoagulability while maintaining the vital hemostatic function of the coagulation system. Because of the existence of alternate routes for factor Xa generation, it is reasonable to expect that specific inhibition of intrinsic pathway protease would result in less hemorrhagic complications than inhibition of the initiating complex, TF/FVIIa, or the prothrombinase complex. In this regard, studying the response of monocyte procoagulants to GAGs is of importance not only for characterization of coagulation reactions in a biologically relevant microenvironment but also for identification of inhibitors with potential use in pharmacologic control of hypercoagulable states associated with atherosclerosis, inflammation, and cancer.

The results of the present studies indicate specific modulation of the intrinsic pathway protease but not of extrinsic protease by chondroitin sulfate E. The results are consistent with modulation of baseline procoagulant activity by GAGs expressed on monocytes. Added GAGs in the extracellular medium further inhibited reactions in a manner that was strictly dependent on the spatial distribution of charges on the polysaccharide. Multiple functional titrations and Scatchard-type analysis provided kinetic evidence consistent with a hypothetical model of inhibition mediated by interaction between CSE and at least two independent sites: one on factor Xa and another on VIIIa. In addition, results suggest that this GAG may provide a useful anticoagulant to manage prothrombotic states associated with activity of the intrinsic pathway amplifying loop.

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Fig. 6. Inhibition as a function of protease component concentration; low inhibitor concentration regime. Rates of factor X activation of lipid membranes were measured in mixtures with and without CSE at 50 ng/ml. Concentrations of either VIIIa or IXa were increased as indicated on the abscissa of A and B, respectively.
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