Chondroitin Sulfate Anticoagulant Activity Is Linked to Water Transfer
Relevance to Proteoglycan Structure in Atherosclerosis

Maria McGee, William D. Wagner

**Objective**—Changes in chondroitin sulfate (CS) proteoglycan (PG) during atherosclerosis are associated with chronic inflammatory changes and increased incidence of thrombosis. To explore how glycosaminoglycan changes could influence the thrombogenicity of atherosclerotic lesions, water-transfer reactions were examined during activation of antithrombin by CS.

**Methods and Results**—Advanced type IV atherosclerotic lesions prone to thrombosis contained CSPG (versican) with undersulfated CS relative to CS of the adjacent healthy aorta. Approximately 11% of the CS disaccharide in versican from healthy arteries was oversulfated, but this proportion decreased markedly to 3% in atherosclerotic lesions. Oversulfated CS functionally bound antithrombin with a dissociation constant of $3.3 \times 10^{-9}$ mol/L. Measured by osmotic stress (OS) techniques with a 26 Å probe, the reaction was linked to transfer of $2.5 \times 10^{-9}$ mol water per mole of coagulation factor Xa inhibited. Under OS, the anticoagulant efficiency of CS was $1.3 \times 10^{-1}$·s$^{-1}$, 5- and 15-fold higher than heparan sulfate efficiency measured under OS and standard conditions, respectively.

**Conclusions**—Decreased sulfation of high molecular weight CSPG in the advancing atherosclerotic lesions may predispose the lesions to thrombosis by disrupting osmotic regulation, limiting avidity for antithrombin and decreasing activation efficiency. (Arterioscler Thromb Vasc Biol. 2003;23:1921-1927.)

**Key Words:** atherosclerosis ■ osmotic stress ■ antithrombin ■ chondroitin sulfate proteoglycan ■ coagulation

Protoglycans (PGs) interact with hyaluronic acid, glycolipids, glycoproteins, insoluble fibrillar proteins, and associated plasma proteins, forming a gel matrix that regulates cell microenvironments. PGs consist of core proteins covalently attached to linear pendant glycosaminoglycan (GAG) chains expressing various degrees of sulfation. The fixed negative charges of the sulfate groups influence the partitioning of ions, spatial osmotic gradients, and the hydration volume of the fibrillar meshwork. The extracellular gel matrix also functions as a biological sieve at which macro-molecules interact with GAGs to modulate a variety of processes, including cell proliferation, cell adhesion, cell motility, and blood coagulation. Pressure gradients that control hydration may influence the interaction of GAGs with plasma proteins.

Antithrombin is a prototypical GAG-regulated protein and the most important inhibitor of coagulation proteases. Heparan sulfate (HS) and other GAGs, abundant in blood vessels, including dermalan sulfate and chondroitin sulfate (CS), have anticoagulant activities. The heparin sequences with high affinity for antithrombin are extremely rare in sulfated PGs other than serglycins in mast cell granules. Consequently, studies of heparin-antithrombin interactions primarily relate to pharmacological anticoagulation but provide less insight into the physiological regulation of antithrombin by vascular GAGs.
and suggests the possibility that anticoagulant activity of GAGs in the vasculature is influenced by water-transfer reactions in the gel matrix.5–8

The volume and direction of water transfer characterizing specific macromolecular interactions can be measured in isolated systems by the technique of osmotic stress (OS).13,23–27 For interactions between arterial GAG and antithrombin, analysis of the water-transfer component has obvious biological importance because it is a significant factor in regulation of matrix hydration and in the thrombogenic potential of vascular lesions.

Previous analyses of healthy and atherosclerotic human aortas4,20,21 as well as healthy and osteoarthritic joints22 have demonstrated disease-related changes in the amount and sulfation patterns in the GAGs. Other studies also suggest a role of PG changes in the thrombotic complication of atherosclerosis.28–30 The present study focuses on versican structure and explores the possibility that osmotic forces influence the activation of antithrombin by CS sequences present in arteries. Results show that under OS, oversulfated CS sequences can activate antithrombin with efficiencies exceeding those measured with HS. The relevance of the findings in blood coagulation control is further explored by analyses of the CS sulfation pattern of high molecular weight CSPG (versican) isolated from healthy and atherosclerotic human arteries.

Methods

Isolation and Characterization of Versican From Human Arteries

PGs were extracted from type IV lesions and adjacent normal aorta from autopsy cases fully described previously.20 Intimal preparations were made by stripping vessels under ×10 magnification, and atherosclerotic lesions were characterized according to the American Heart Association classification scheme as type IV. PGs were extracted in buffer containing 4 mol/L guanidine HCl in 0.05 mol/L sodium acetate (pH 4.5) and protease inhibitors as described previously.4,20,21 The versican component was identified and isolated on the basis of its physicochemical properties, including charge and size, by using chromatography on DEAE-Sephacel followed by Sepharose CL-4B, respectively. (The first chromatographic procedure separates HSPGs and hyaluronic acid, eluted with 0.15 to 0.35 mol/L NaCl, from the versican/decorin/biglycan fraction eluted with 1.0 mol/L NaCl.) The subsequent chromatography of the highly charged fraction on Sepharose CL-4B separates versican from the smaller-sized decorin and biglycan (Figure 1). Recovery of extracted PG after chromatographic procedures was determined by sampling aliquots and measuring hexuronic acid. Total isolated PG was 487±47 and 421±80 μg per gram wet aorta for normal tissue and atherosclerotic plaque, respectively. For structural analysis of the CS chains, versican core protein was removed by digestion with papain. CS (2 μg hexuronic acid) was digested with chondroitinase ABC to produce unsaturated disaccharides. Samples were chromatographed in buffer containing 70% acetonitrile/methanol (vol/vol) and 30% of 0.5 mol/L ammonium acetate, pH 5.8, on a 250×4.6-mm Partisil–10-PAC column (Whatman). Typical elution profiles are illustrated in Figure 2A through 2E. Peaks detected at 232 nm were identified by comparison with retention times for monosulfated and disulfated disaccharide standards obtained from Seikagaku American, Inc. The sulfation patterns of standards corresponded to those in the CS species CSA, CSC, CSB, and CSE (Table 1). Percentage compositions were calculated from the sum of the peak areas. Values are the mean of at least 3 determinations per sample.

OS Technique

OS technology and its applications to rate reaction processes have previously been described and validated.23–27 Differences in water activity between hydration spaces of reactants and bulk solution are induced with inert cosolutes that are excluded from the hydration spaces at and near the surface of the reactants. The reactants are osmotically stressed toward a more dehydrated conformation; consequently, the reaction energetics change. According to classic thermodynamic reasoning, this enables measurement of the work and volume of water transfer during the reaction.23,24 In the present studies, the effect of OS on anticoagulant activity of HS and HS was determined from reaction rates and equilibrium parameters in antithrombin reaction mixtures equilibrated with various concentrations of inert polymers: polyethylene glycol (PEG), polyvinylpyrroldione (PVP), or dextran.

Measurement of GAG-Induced Antithrombin Activity

Antithrombin activity was determined from factor Xa (fXa) decay rate in reaction mixtures with GAGs.17 Rates were calculated from the residual concentration of proteolytically active fXa in 6 consecutive samples taken at 5- to 60-second intervals after adding fXa to antithrombin-GAG mixtures. Standard mixtures were equilibrated in buffer (25 mmol/L Tris, pH 7.4, containing 0.25 mg/mL ovalbumin) and incubated at 33°C in a total initial volume of 300 μL. Samples, 40 μL each, were diluted immediately in hexamethrine bromide, and fXa concentration was determined by amidolytic assay with methoxy carbonyl-d-cyclohexylglycyl-arginine-p-nitroanilide-acetate substrate.33 Osmotically stressed mixtures were identical but included cosolute polymers at various concentrations. Salts were at either 0.075N or 0.15N NaCl with or without 0 to 15 mmol/L CaCl2, depending on the experiment. Baseline control mixtures were prepared and processed like reaction mixtures but contained either no antithrombin and/or no GAG. Pseudo–first-order rate coefficient (k∞) values were determined by fitting a single exponential to concentration/time data points with the use of the computer program StatView (SAS).
Parameters $K_{\text{eq}}$ (GAG concentration giving half-maximal rate) and $V_{\text{max}}$ (rate at saturation) were determined from functional titration of antithrombin activity with either CS or HS. The data were analyzed according to schemes previously validated for the interaction between antithrombin and heparin.\(^{15}\)

### Table 1. Disaccharide Composition of Chondroitin Sulfate Prepared From High Molecular Weight Arterial Proteoglycan

<table>
<thead>
<tr>
<th></th>
<th>$\Delta$Di-6S</th>
<th>$\Delta$Di-4S</th>
<th>$\Delta$Di-2,4-diS</th>
<th>$\Delta$Di-4,6-diS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal aorta</td>
<td>55.0±7.0</td>
<td>34.0±4.3</td>
<td>2.0±0.4</td>
<td>9.1±3.1</td>
</tr>
<tr>
<td>Type IV lesions</td>
<td>67.8±8.5</td>
<td>28.7±9.7</td>
<td>0.7±0.3</td>
<td>2.9±0.9</td>
</tr>
</tbody>
</table>

Values are expressed as percentage of total disaccharides and are means±Sem

$\Delta$Di-6S indicates 2-acetamido-2-deoxy-3-O-(4-deoxy-4-sulpho-D-galactose) (primary disaccharide component of CS); $\Delta$Di-4S, 2-acetamido-2-deoxy-3-O-(4-deoxy-D-galactose) (primary disaccharide component of CSA); $\Delta$Di-2,4-diS, 2-acetamido-2-deoxy-3-O-(4-deoxy-2-O-sulpho-D-galactose) (primary disaccharide component of CS); $\Delta$Di-2,4,6-diS, 2-acetamido-2-deoxy-3-O-(4-deoxy-4-sulpho-D-galactose) (primary disaccharide component of CSE).

$K_d$ is the dissociation constant for the antithrombin-GAG interaction, $K_t$ is the dissociation constant for the ternary complex between antithrombin-GAG and $fXa$, and $k$ is the first-order rate constant for the stabilization of the inhibitory antithrombin protease complex. With antithrombin in excess of $fXa$, but low relative to $K_d$, $fXa$ activity decays exponentially with a $k_{obs}$ value that increases with GAG, giving a titration curve with $K_{eq}=K_d$.

### GAGs and CSE Fractionation

To define the GAG species (among those identified in arterial tissues) with anticoagulant activity, highly purified preparations obtained from Seikagaku Kogyo Co Ltd were used in kinetic studies. These included HS from bovine kidney, CSC and CSD from shark cartilage, CSA from sturgeon notochord, and CSE from squid cartilage. According to the manufacturer’s data, preparations gave a single band after electrophoresis in cellulose acetate; CSE had a sulfate/hexosamine ratio of 1.5; and sulfate groups, both with axial and equatorial orientation, were located at the C-4 and C-6 positions of galactosamine.\(^{34}\) The CSE preparation was further fractionated by gel filtration. Five milligrams of CSE was loaded on a Sepharose 6B column (68×0.9 cm), eluted with 0.2 mol/L NaCl, and collected into 2-mL aliquots. The average molecular weight distribution was determined from the distribution coefficient of elution volume,\(^{19}\) and concentration was determined by hexuronic acid measurements.\(^{36}\) Aliquots were pooled into 4 fractions with approximate average molecular weights of >100, 80, 46, and 21 kDa.

### Results

#### Decreased Oversulfated CS in Versican From Atherosclerotic Lesions

Extracts from healthy tissues and type IV lesions yielded 216 and 173 μg hexuronic acid per gram tissue of versican-CS, respectively. This compared with 181 and 71 μg, respectively, from HSPG in the same samples. Sulfation patterns corresponding to CSA, CSC, CSB, and CSE species were found in healthy tissue and in type IV lesions at the proportions indicated in Table 1. The data indicate a significant content of disulfated disaccharides in high molecular weight CSPG isolated from human aorta. Normalized on a wet tissue basis, the oversulfated CS corresponds to 19 and 6 μg hexuronic acid per gram wet tissue.
in healthy and type IV lesions, respectively. Reductions in oversulfated species found in type IV lesions amounted to losses of 65% in disaccharides containing 2,4-disulfated N-acetylgalactosamine and to losses of 68% in disaccharides containing 4,6-disulfated N-acetylgalactosamine.

Anticoagulant Effect of CS Is Enhanced by OS
Under dehydrating OS, the rate of reactions linked to water binding is slowed, whereas the rate of reactions linked to water release is accelerated. The possibility that CS has anticoagulant activity and whether OS influences this activity was explored by using functional antithrombin assays. Pure CS preparations with sulfation patterns corresponding to CS species in arterial versican were compared with HS. The effect of CSA, CSC, CSD, CSE, and HS on antithrombin (150 nmol/L) activity was determined from the rate of fXa (18 nmol/L) activity decay measured in standard solutions or in solutions osmotically stressed with PEG 8000 at 0.5-atm pressure. Because the local distribution of monovalent and divalent salt ions in a GAG-rich microenvironment may vary widely,16 reaction rates were measured at different salt concentrations. Initially, the concentration of each GAG was 5 μmol/L, close to values of rough estimates for total GAG concentration in hydrated human aorta made from total hexuronic acid determinations in extracts. Increases in the rate of fXa inhibition by antithrombin were observed with HS and CSE but not CSA, CSC, and CSD. OS and calcium significantly increased the rates of reactions with either CSE or HS.

Functional parameters for the interaction between antithrombin and either CSE or HS were derived from complete titrations of antithrombin with GAGs. Increasing GAG concentration increased the velocity of the fXa inhibition by antithrombin, approaching a maximum. The parameters V_{sat} and K_{1/2} were determined by fitting the quadratic form of the binding isotherm to titration data by using the program Tablecurve (SAS).15 Results are illustrated in Figures 3A and 3B, and kinetic parameters from titrations under various conditions are summarized in Table 2.

OS and CaCl2 induced the value of kinetic parameters with either HS or CSE. V_{sat} was always higher with HS than with CSE, whereas the effects of OS were more marked with CSE than HS. Relative reaction efficiencies approximated from the second-order rate coefficient, i.e., V_{sat}/K_{1/2}, increased between 2- and 10-fold with OS. The magnitude of the increase was larger for CSE than HS. The inhibition reaction with CSE in the presence of calcium under low ionic strength was 15-fold more efficient than it was with HS under standard conditions.

Volume of Water Transfer Linked to Antithrombin Activation Reaction
The increase in inhibition rate and in affinity evidenced by the increase in V_{sat} and decrease in K_{1/2}, respectively, indicates that functional interactions among GAGs, antithrombin, and fXa are linked to net water transfer from reactants to bulk and are thus favored by the dehydrating potentials prevailing in the interstitial spaces. To determine the volumes of water transferred, the change in reaction rate with OS was measured. In these experiments, concentrations of reactants were constant with GAGs maintained near K_{1/2} (2.1 and 3.3 μmol/L nominal concentration of CSE and HS, respectively), whereas OS varied between 0- and 0.5-atm pressure by including increasing concentrations of PEG 8000. At these GAGs, concentration-measured changes in reaction rates with OS reflect effects in GAG-antithrombin interaction affinity and fXa inhibition rate. The change in the free energy of activation (∆G‡) was calculated from k_{obs}, and the volume of water transfer was derived from the slope of ∆G‡ versus -Δπ plots. The slope value was determined by fitting straight lines to data points. Results are shown in Figure 4. The volumes of water transfer measured in reactions with either CSE or HS were not significantly different.
respectively (nominal concentration). OS from 0- to 0.5-atm pressure was induced with PEG 8000. In these reactions in which CSE was more homogeneous than the unfractionated preparation, water transfer was also from reactant to bulk, and the transfer volume was of similar magnitude for each size range. The calculated volumes were 2319±464, 2227±455, 2195±460, and 2159±406 mol water per mole of fXa inhibited for reactions with CSE fractions with molecular weights of >100, 70 to 90, 33 to 60, and 12 to 30 kDa, respectively.

### Water-Transfer Volumes Measured With Polymers of Different Size and Chemical Structure

The volume transferred during the reaction was also measured by using PEG with molecular weight ranging from 0.3 to 8 kDa, corresponding to molecular radii \( \approx 5 \) to \( 26 \) Å.\(^3\)&\(^7\) These experiments were designed to explore increasingly larger volumes of the intermolecular spaces defined by the ternary complex and subsequent antithrombin-fXa approach.\(^1\) Because the excluded spaces increase with the size of the probe, transferred volumes should be correlated with probe sizes up to sizes similar to the intermolecular spaces defined by the reactants in the encounter complex.

In addition, to ensure that rate changes observed with PGE reflect OS effects exclusively, measurements were repeated with dextran and PVP as alternative probes. These polymers have different physical and chemical characteristics and thus are expected to be different with respect to nonosmotic effects, such as changes in density and dielectric constant of solutions. In these experiments, reaction mixtures contained antithrombin at 100 nmol/L, either CSE or HS at 2.0.1 and 3.3 \( \mu \)mol/L nominal concentration, respectively, and fXa at 14 nmol/L. Results are presented in Table 3. The volumes measured increased with polymer size up to \( \approx 17 \) Å radius. The direction of the transfer and the magnitude of the volume transferred were essentially independent of the chemical nature of the stressing polymer. The effect was primarily osmotic, as confirmed in reactions stressed with dextran and PVP, yielding volumes not significantly different from volumes determined with PEG.

### Discussion

The initiating event in most acute coronary syndromes is rupture of vulnerable atherosclerotic plaques.\(^3\)&\(^8\)-\(^4\) In the

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**TABLE 2. Functional Parameters for Antithrombin Interaction With Either CSE or HS: Effect of Osmotic and Ionic Conditions**

<table>
<thead>
<tr>
<th>GAG Solution</th>
<th>Standard</th>
<th>Osmotic Stress (0.5 atm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_{1/2}, \mu\text{mol/L} )</td>
<td>( V_{sat}, \text{s}^{-1} \times 10^1 )</td>
</tr>
<tr>
<td>CSE in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 N NaCl</td>
<td>3.3±1.9</td>
<td>5.3±1.3</td>
</tr>
<tr>
<td>0.15 N NaCl, 2 mmol/L CaCl(_2)</td>
<td>2.7±0.8</td>
<td>5.7±0.7</td>
</tr>
<tr>
<td>0.075 N NaCl</td>
<td>4.3±2.8</td>
<td>6.1±1.8</td>
</tr>
<tr>
<td>0.075 N NaCl, 2 mmol/L CaCl(_2)</td>
<td>1.3±0.4</td>
<td>13.8±0.1</td>
</tr>
<tr>
<td>0.15 N NaCl, 5 mmol/L CaCl(_2)</td>
<td>4.8±0.8</td>
<td>13.2±0.1</td>
</tr>
<tr>
<td>HS in</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 N NaCl</td>
<td>4.0±0.6</td>
<td>48±4</td>
</tr>
<tr>
<td>15 N NaCl, 2 mmol/L CaCl(_2)</td>
<td>6.9±0.9</td>
<td>97±5</td>
</tr>
<tr>
<td>0.075 N NaCl</td>
<td>6.6±0.8</td>
<td>78±4</td>
</tr>
<tr>
<td>0.075 N NaCl, 2 mmol/L CaCl(_2)</td>
<td>9.2±1.8</td>
<td>204±19.4</td>
</tr>
</tbody>
</table>

Antithrombin (137 nmol/L) was titrated with either CSE or HS. Parameters, \( K_{1/2} \) (concentration giving half maximal inhibition rate) and \( V_{sat} \) (maximal inhibition rate at saturating GAG concentration), were determined from fXa decay rates measured under standard or OS conditions with NaCl and CaCl\(_2\) at the indicated concentrations.

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**Figure 4. Change in free energy of activation with OS during antithrombin activation by either HS or CS.** Antithrombin activation by either CSE (solid line) or HS (dotted line) was determined in fXa inhibition assays at the different levels of OS indicated. \( \Delta G \) was determined from the change in \( K_{1/2} \). \( \Delta G \) is plotted vs the osmotic pressure increments induced with PEG 8000. The slopes of lines fitted to data points by linear regression were 1.3±0.2 and 1.1±0.1 kcal \( \cdot \) mol\(^{-1} \) \cdot \) atm\(^{-1} \) for CSE and HS, respectively.
present study, vulnerable plaques classified as type IV atherosclerotic lesions according to the American Heart Association classification of atherosclerosis were examined. Analyses of arterial CSPG (versican) of normal and atherosclerotic lesions demonstrated heterogeneous composition that included disaccharides found in CSA, CSC, CSB, and CSE. A significant decrease in the proportion of the oversulfated species, CSE, was found in the vulnerable plaque compared with adjacent normal tissue. In pure form, CSE had significant anticoagulant activity and bound antithrombin with affinities similar to those of HS. The functional interaction had a strong electrostatic component(s), and the reaction efficiency was increased by calcium. More important, under OS, the efficiency of the functional interaction between CSE and antithrombin increased markedly and surpassed by ≈15 fold that of HS under standard conditions. These findings indicate that CSE species found in arterial tissues can contribute significantly to coagulation control.

The observation that the anticoagulant activity of GAGs was linked to water transfer has important biological implications. This finding suggests that anticoagulant activity in vivo may respond to the same physicochemical factors that maintain the hydration volume of the extracellular and pericellular gel matrix. In living tissues, the interstitium is maintained in a relatively dehydrated state. The mechanisms that maintain this dehydrated state are complex but depend on GAGs. Removing GAGs enzymatically from excised gels also abolishes the swelling response of excised tissues, including arteries. In general, the hydration volume of gels is maintained by a balance of forces that include the elasticity of the various polymer components, their chemical affinities, the fixed charges, and the osmotic forces generated by ionized solutes. An increasing body of experimental evidence in artificially stressed systems indicates that partial dehydration can influence the rate of biologically important reactions. The macromolecular interactions that control and determine the evolution of atherosclerotic lesions must be subjected to the dehydrating forces prevailing in vivo. Because these forces are intrinsically connected to mechanical and structural factors responding to the amounts and spatial distribution of GAGs in intact tissues, changes in structure and distribution of GAGs are expected to influence hydration volumes. Together with quantitative and qualitative changes in versican, the histological abnormalities consistently found in atherosclerotic tissues, such as diffuse thickening and disorganization of fibrillar elements in the intima, point to deregulation of local water homeostasis.

The CS in versican from normal and atherosclerotic arteries structurally corresponded to hybrid GAG molecules. The major disaccharide was monosulfated at the C-6 position of N-acetylgalactosamine. Oversulfated disaccharide sequences (primarily sulfated at the C-4 and C-6 position of N-acetylgalactosamine) were found to constitute ≈11% of the total disaccharide in versican. A key observation was that versican in advanced lesions had markedly reduced levels (by 68%) of the oversulfated disaccharides present in the GAG species with anticoagulant activities. Together with the low frequency of high-affinity heparin sequences in the vessel wall, the measured anticoagulant activity of CSE is relevant to the prothrombotic potential of type IV lesions. Because the estimated CSE content approaches 20% of total HS amounts in healthy arterial tissues, relative dehydration in vivo could shift the binding avidity of antithrombin from HS to CSE. In healthy arteries and during early disease progression, CSE may decrease thrombogenicity by significantly contributing to the inhibition of coagulation proteases. In advanced type IV lesions, structural changes in CS may translate to functional differences, diminishing the anticoagulant reserves of the extracellular matrix. A further understanding of oversulfated artery CS changes may lead to better therapies for the thrombotic complication of atherosclerosis.

The dehydrating energy stored in the interstitium influences all macromolecular interactions therein. Therefore, the net overall effect on the coagulation systems depends on the water-transfer energetics of procoagulant and anticoagulant...
mechanisms. Clearly, more research is needed to completely understand this important aspect of coagulation kinetics.

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