Receptor-mediated adhesive and anti-adhesive functions of chondroitin sulfate proteoglycan preparations from embryonic chicken brain

Heidemarie Ernst, Mary K. B. Zanin, David Everman and Stanley Hoffman*
Medical University of South Carolina, Department of Medicine, Division of Rheumatology and Immunology, 171 Ashley Avenue, Charleston, SC 29425, USA
*Author for correspondence

SUMMARY

Chondroitin sulfate proteoglycans inhibit the adhesion of cells to extracellular matrix proteins that otherwise permit adhesion. Although proteoglycans are widely assumed to act by masking the other protein in a mixed substrate, recent studies suggest that proteoglycans inhibit adhesion through mechanisms initiated by their binding to specific cell surface receptors. To explore this issue, we developed a purification scheme to isolate proteoglycan aggregates, monomers, and core proteins. Two distinct adhesion assays were used to study the interaction of these proteoglycan preparations with human foreskin fibroblasts: the gravity assay in which cell attachment is stabilized by cell spreading, and the centrifugation assay in which spreading does not play a role. All proteoglycan preparations mediate adhesion in the centrifugation assay but not in the gravity assay. In the centrifugation assay, proteoglycan aggregates and monomers are considerably more active than other extracellular matrix proteins while proteoglycan core proteins are at least as active as other extracellular matrix proteins. Proteoglycan core proteins bind to cell-associated hyaluronic acid, but not to integrins. Using mixed substrates in the gravity assay, all proteoglycan preparations inhibited cell attachment to fibronectin and vitronectin but not to collagen I and laminin. Although proteoglycan aggregates and monomers are more active than core proteins in inhibiting adhesion in the gravity assay, core proteins are still clearly active. A variety of control experiments suggest that the inhibition of cell attachment by proteoglycans is mediated through the specific interactions of proteoglycans with cell surface receptors, resulting in the inhibition of cell spreading. These results suggest at least two molecular mechanisms for proteoglycan-fibroblast interactions, one involving the chondroitin sulfate on the proteoglycan and an as yet unidentified receptor, the other involving the proteoglycan core protein and cell-associated hyaluronic acid.

Key words: proteoglycan, anti-adhesion, hyaluronic acid, restrictin

INTRODUCTION

Chondroitin sulfate proteoglycans (CSPGs) can inhibit cell attachment to normally adhesive extracellular matrix (ECM) proteins coated on plastic (Rich et al., 1981; Knox and Wells, 1979; Yamagata et al., 1989). It is widely believed that this repulsive or ‘anti-adhesive’ function may result from the masking of the adhesive substrate by the proteoglycan, possibly due to a direct interaction between the proteoglycan and the other ECM protein (Rich et al., 1981). Recently, however, evidence has accumulated that large, extracellular CSPGs can bind to specific cell surface receptors. Ng-CAM and NCAM on embryonic brain cells serve as receptors for neurocan and phosphacan, but not aggregan (Grumet et al., 1993). The glycosyl transferase, N-acetylgalactosaminyltransferase (GalNAcPTase), on retinal cells serves as a receptor for a CSPG with a 250 kDa core protein (likely to be neurocan), but does not bind CSPGs with 400 kDa core proteins (Balsamo et al., 1995). Moreover, this interaction indirectly inhibits N-cadherin-mediated adhesion. These observations support the idea that large, extracellular CSPGs may inhibit cell-ECM adhesion through transmembrane signals initiated by their binding to cell surface receptors.

Four large (core protein >200 kDa), extracellular CSPGs have been cloned and sequenced. The cartilage CSPG, aggregan, was sequenced from rat (Doege et al. 1987), human (Doege et al., 1991), and chicken (Chandresekaran and Tanzer, 1992). Subsequently, two structurally related CSPGs were sequenced: versican from human fibroblasts (Zimmermann and Ruoslahti, 1989) or embryonic chicken limb bud (Shinomura et al., 1993) and neurocan from rat brain (Rauch et al., 1992). These CSPGs contain a series of structural domains found in other proteins: near the N terminus they contain an immunoglobulin domain and tandem repeat(s) homologous to the hyaluronic acid (HA)-binding region of link protein (Neame et al., 1985); at the C terminus they contain EGF-like and lectin-like domains as well as a complement regulatory protein-like sequence. The central regions of the molecules are unique and contain attachment sites for chondroitin sulfate (CS) and other oligosaccharides. With the exception of the
To isolate CSPGs, 14-day embryonic chicken brains were sonicated to isolate CSPG aggregates, monomers, and core proteins. We used two distinct assays to compare the effects of these CSPG preparations on adhesion, the gravity assay in which cell attachment is stabilized by cell spreading and the centrifugation assay in which spreading does not play a role. All CSPG preparations mediate adhesion in the centrifugation assay but not in the gravity assay; CSPG aggregates and monomers are particularly active, mediating adhesion at 200-fold lower molar concentrations than does fibronectin. These experiments reveal at least two molecular mechanisms for CSPG-fibroblast interaction, one involving the CS on the CSPG and an as yet unidentified receptor, the other involving the CSPG core protein and cell-surface-associated HA. As part of mixed substrates, all CSPG preparations inhibit cell attachment in the gravity assay to fibronectin and vitronectin but not to collagen I and laminin. Again, aggregates and monomers are more active than core proteins, although core proteins are clearly active. A variety of experiments strongly suggest that the specific interaction of CSPGs with cell surface receptors results in the inhibition of cell spreading and consequently, the inhibition of cell attachment in the gravity assay.

MATERIALS AND METHODS

Purification of CSPG aggregates, monomers and core proteins

To isolate CSPGs, 14-day embryonic chicken brains were sonicated in PBS in the presence of protease inhibitors (N-ethylmaleimide (10 mM), benzamidine (5 mM), leupeptin (50 μg/ml), aprotinin (1 μg/ml), pepstatin A (5 μg/ml), and PMSF (2 mM)) using a Braun-Sonic 2000 U ultrasonic generator. The homogenate was clarified by ultracentrifugation (45 minutes, 35,000 rpm, Beckman Ti 45 rotor, 4°C). CsCl was added to the supernatant to a final concentration of 0.5 g/ml (w/v) and the sample was spun for 18 hours at 45,000 rpm at 20°C in a Beckman VAC50 rotor. The densest 8 ml fraction was collected (CsCl fraction 1), dialyzed, and lyophilized.

To prepare CSPG aggregates, CsCl fraction 1 was fractionated by HPLC gel filtration on a 7.5 mm× 60 mm G4000SW column (TosoHaas) in buffer A (20 mM sodium phosphate, pH 6.3, 0.15 M NaCl). Fractions with kav values of 0.25-0.35 contained 250 kDa core proteins (PG250 core). As part of the preparation migrated with a lower apparent molecular mass than does fibronectin. The identity of this material migrating with kav values of 0.1-0.2 contained 400 kDa core proteins (PG400 core). The sample was lyophilized, resuspended in 20 mM Tris-HCl (pH 8.2) and treated with chondroitinase. The sample was lyophilized, resuspended in buffer B, and fractionated by HPLC gel filtration on a G4000SW column in buffer B. Typically, fractions with kav values of 0.1-0.2 contained 400 kDa core proteins (PG400 core) and fractions with kav values of 0.25-0.35 contained 250 kDa core proteins (PG250 core).

CSPG monomers and core proteins were isolated from hyaluronidase-treated CsCl fraction 1. Following addition of dry guanidine-HCl (0.5 g/ml), the sample was fractionated on a G4000SW column in buffer B (4 M guanidine-HCl, 0.1 M sodium phosphate, pH 6.3). Fractions from each run with kav values of 0-0.43 were pooled, dialyzed, and lyophilized. This fraction is referred to as monomer mix because it contains a mixture of CSPG monomers (and restrictin, see Results). Aliquots (50 μg) of monomer mix were injected into rabbits and IgG and Fab’ fragments prepared from immune and non-immune sera (Brackenbury et al., 1977).

To separate restrictin from CSPG monomers, monomer mix was fractionated by rate-zonal sedimentation on sucrose gradients (60, 40, 35, 25, 20, 15, and 10% sucrose in PBS; 27,000 rpm for 24 hours at 25°C in a Beckman SW28 rotor). The gradients were fractionated into 25 ml fractions. Typically, restrictin was present in fractions 7-14, CSPG monomers containing 400 kDa core proteins (PG400 monomer) were present in fractions 13-18, and CSPG monomers containing 250 kDa core proteins (PG250 monomer) were present in fractions 15-20.

To isolate CSPG monomers, sucrose gradient fractions 15-20 were pooled, dialyzed, lyophilized, resuspended in buffer B and fractionated by HPLC gel filtration on a G4000SW column in buffer B. Individual fractions were dialyzed; aliquots were treated with chondroitinase (chondroitin ABC lyase; Seikagaku America, Inc.) and resolved by SDS-PAGE to identify their core proteins. Typically, fractions with kav values of 0.1-0.1 contained PG400 monomer while fractions with kav values of 0.15-0.25 contained PG250 monomer.

To isolate CSPG core proteins, the material from sucrose gradient fractions 15-20 was resuspended in 20 mM Tris-HCl (pH 8.2) and treated with chondroitinase. The sample was lyophilized, resuspended in buffer B, and fractionated by HPLC gel filtration on the G4000SW column in buffer B. Typically, fractions with kav values of 0.1-0.2 contained 400 kDa core proteins (PG400 core) and fractions with kav values of 0.25-0.35 contained 250 kDa core proteins (PG250 core).

Cell culture

Primary monolayer cultures of foreskin fibroblasts were established in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 μg/ml gentamycin and maintained at 37°C in 10% CO₂. For adhesion experiments, confluent fibroblasts between third and fifth passage were harvested using low concentrations of trypsin (20 μg/ml) and EDTA (1 mM) in serum-free Hanks’ balanced salt solution containing 10 mM Hepes, pH 7.5. Cells were separated from debris by centrifugation through BSA cushions (Friedlander et al., 1988).

Gravity assay

Test proteins were applied as 3 μl dots in a circular array near the center of a 35-mm non-tissue culture-treated plastic dish (Friedlander et al., 1988). Following a one-hour incubation, the solutions were aspirated and the plates were washed three times and blocked for one hour with 10 mg/ml of BSA in PBS. This solution was replaced with 2 ml of serum-free DMEM containing 1.5×10⁵ human foreskin fibroblasts. Plates were incubated for one hour at 37°C in 10% CO₂. Unbound cells were removed by gentle submersion of the dishes in PBS; bound cells were fixed in 3.7% formaldehyde. The bound and spread cells in a 1 mm² area were counted using an Axiovert 35 microscope (Zeiss) with a 10× objective and an eyepiece reticle.

Centrifugation assay

A 40 μl sample of each protein was placed in individual wells of a 96-well U-bottom microtest assay plate (Friedlander et al., 1988). Wells were washed and blocked as described above. Then 200 μl of serum-free DMEM containing 12,500 foreskin fibroblasts was added to each well and the plate centrifuged at 250 g for two minutes in a Sorvall RT6000D centrifuge. The pattern of cells in each well was observed immediately and interpreted in terms of a balance between...
centrifugal force and cell-substrate adhesion. On a non-adhesive substrate such as BSA, centrifugal force predominates and the cells form a pellet at the bottom of the well. Where cell-substrate adhesion is strong, cells bind to the substrate in a uniform distribution on the sides and at the bottom of the well. At intermediate levels of adhesion, a balance between centrifugal force and adhesion is achieved which results in cells being distributed in a ring with a region of cell binding inside. The stronger the adhesion, the greater the diameter of the ring. In some experiments, adhesion was perturbed by treating cells as indicated.

**Adsorption of ECM molecules to plastic**

Proteins and CSPG preparations were radioiodinated using Chloramine T (Friedlander et al., 1988), then 40 μl aliquots in triplicate containing only labeled or a mixture of labeled and unlabeled molecules were incubated for one hour in non-tissue culture treated Immunolon Removawell Strips (Dynatech Laboratories). Subsequently, the wells were washed three times with 200 μl of 1% BSA in PBS. Bound radioactivity was determined by counting the washed wells in a Beckman Gamma 4000 counter. Using the known specific radioactivity of the labeled protein solutions, the amount of adsorbed protein was calculated.

**Binding of radiolabeled 4B4 to fibroblasts**

The mAb 4B4 was radioiodinated using chloramine T (Friedlander et al., 1988). A 50 ng sample of labeled 4B4 was incubated with 5×10^5 foreskin fibroblasts in 150 μl of binding medium (20 mg/ml of BSA in Hapes-buffered Hanks’ balanced salt solution, pH 7.5) or binding medium supplemented with 5 μg of either unlabeled 4B4, PG aggregate, or monomer mix. After 15 minutes on ice, 1 ml of binding medium was added to each sample and the cells were pelleted and washed several times with binding medium. Labeled 4B4 bound to the final cell pellet was quantified using a Beckman Gamma 4000 counter.

**RESULTS**

We have isolated CSPG aggregates, monomers, and core proteins from 14-day embryonic chicken brains and analyzed their ability to mediate cell-substrate adhesion using two different assays. In the gravity assay, as cells come into contact with ECM proteins such as fibronectin during a 60-minute incubation, cell spreading leads to a dramatic increase in the area of the cell surface in contact with the substrate and thereby enhances the strength of adhesion. For ECM proteins that inhibit cell spreading (such as cytotactin (Hoffman et al., 1994) or the CSPGs described in this study), adhesion in the gravity assay is weak at best. In contrast, in the centrifugation assay cell spreading cannot play a role in strengthening adhesion because the entire assay takes place in only two minutes. In this assay, ECM proteins that inhibit cell spreading can nevertheless provide strong adhesion.

**CSPG preparations**

The composition and purity of the CSPG preparations used in these studies are shown in Fig. 1. PG aggregate contains CSPGs with 400 and 250 kDa core proteins that can be resolved by SDS-PAGE on 6% gels only after chondroitinase treatment (compare lanes 1 and 2); other minor and smaller CSPG core proteins can also be distinguished. In addition, a major 190 kDa polypeptide is present in the preparation that can be observed both before and after chondroitinase treatment. N-terminal sequence analysis (Table 1) indicates that this protein is restrictin (Nörenberg et al., 1992). Like cartilage proteoglycan aggregates, brain PG aggregate also contains link protein (lane 10) and HA (lane 12).

One step in the purification of CSPG monomers and core proteins is the generation of a fraction (monomer mix) that contains CSPG monomers and restrictin. Monomer mix is indistinguishable from PG aggregate when analyzed by SDS-PAGE except that it lacks link protein (lane 11) and HA (lane 13). From this material, we isolated highly purified monomers (PG250 monomer and PG400 monomer, lanes 3-6), chondroitinase-treated core proteins (PG250 core and PG400 core, lanes 7,8), and restrictin (lane 9). The core proteins present in purified monomers can be resolved by SDS-PAGE on 6% gels only after chondroitinase treatment (compare lane 3 with lane 4 and lane 5 with lane 6). Prior to treatment, they remain in the stacking gel (not shown) or are present at the top of the resolving gel. In addition to its major 400 kDa component, PG400 core also contains a smaller amount of core protein that migrates near the top of the resolving gel (lane 8).

**Characterization of PG aggregate**

Most CSPG monomers present in the 14-day embryonic chicken brain do not appear to be in CSPG aggregates. During the purification of PG aggregate (see Materials and Methods), the material is fractionated on a G6000PW gel filtration
Table 1. Identification of the 190 kDa polypeptide as restrictin

<table>
<thead>
<tr>
<th>190 kDa polypeptide</th>
<th>RLEVTXEPAERPAVDEGXLAN 22</th>
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<tbody>
<tr>
<td>Restrictin</td>
<td>32 RLEVTPAPAERPAVDEGGGLAN 53</td>
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</table>

To determine the N-terminal sequence of the 190 kDa polypeptide, monomer mix was resolved by SDS-PAGE, transferred to PVDF (Matsudaira, 1987) in 25 mM N-ethylmorpholine formate (pH 8.3), detected by Coomassie Blue staining, and sequenced by automated Edman degradation using a Beckman/PORTON Model LF3000 gas phase sequencer by Dr Liane Mende-Müller (Protein/Nucleic Acid Shared Facility of the Medical College of Wisconsin). Numbering for restrictin starts at the translation initiation site (Nörenberg et al., 1992). The signal peptide was predicted to be 33 amino acids long; however, our data indicate that it is 31 amino acids long.

column. Only about 25% of the monomers loaded on the column migrate in the high molecular mass PG aggregate fraction, the remaining 75% migrate indistinguishably from hyaluronidase-treated monomers (data not shown). We cannot rule out the possibility that a higher percentage of CSPG monomers are present in aggregates in vivo but that these aggregates have broken down during purification.

To demonstrate that the PG aggregate preparation indeed represents CSPG aggregates, untreated or hyaluronidase-treated PG aggregate was analyzed by gel filtration. The components of untreated aggregates (PG400 monomer, PG250 monomer, restrictin, link protein) all chromatograph together with a $k_{av}$ of 0.36 on a G6000PW HPLC column. In contrast, following hyaluronidase treatment, all of these components migrate more slowly through the column. The two CSPG monomers and restrictin each have $k_{av}$ values of 0.68 while link protein has a $k_{av}$ of 0.88. The ability of the isolated CSPG monomers and core proteins to bind to HA was confirmed in ELISA-like assays using immobilized HA as described by Fryer et al. (1992). The association of restrictin with CSPG aggregates appears to involve a direct interaction between restrictin and the CSPGs (our unpublished observations).

CSPGs mediate strong adhesion in the centrifugation assay but not in the gravity assay

In the gravity assay, no cell attachment to CSPG aggregates, monomers, or core proteins was observed, even when high concentrations (>100 µg/ml) of CSPGs were used to coat the plastic surface (not shown). In striking contrast, CSPGs were more potent mediators of adhesion in the centrifugation assay than other ECM proteins (Table 2). Strong adhesion (++) to fibronectin, vitronectin, or laminin was observed down to concentrations of 10 µg/ml. Brain CSPGs gave strong adhesion at even lower concentrations; 0.1 µg/ml for PG aggregate and 0.03 µg/ml for monomer mix, PG400 monomer, or PG250 monomer. PG400 core and PG250 core were not as effective as CSPGs bearing CS, but were still as effective as fibronectin, vitronectin, and laminin. PG400 core and PG250 core were also similar to these ECM proteins in that at optimal concentrations the area of adhering cells was always larger (++++) than that measured at optimal concentrations using the forms of CSPG still bearing CS (++). We also observed strong adhesion at low concentrations of aggrecan (0.3 µg/ml). The adhesion of fibroblasts to CSPGs in the centrifugation assay (Table 2) differs from the adhesion of neurons to CSPGs (Grumet et al., 1993) in that neurons bind to brain CSPGs but not to aggrecan in the centrifugation assay.

Whereas all CSPG preparations tested mediated fibroblast adhesion in the centrifugation assay, none allowed for cell spreading during a 60-minute incubation following centrifugation (not shown). In contrast, cells did spread following centrifugation onto substrates that also mediate spreading in the gravity assay.

To evaluate whether the very different concentration dependencies for adhesion to fibronectin, PG400 monomer, PG250 monomer, PG400 core, and PG250 core might result from the differential ability of these proteins to adsorb to plastic, the concentration of these proteins that adsorb to the wells was determined (not shown). These data substantiate and, in fact, enhance the difference between fibronectin and CSPGs in their ability to mediate adhesion in the centrifugation assay. Strong adhesion to CSPG monomers occurs at molar concentrations which are at least 200-fold lower than those necessary for fibronectin. Strong adhesion to CSPG core proteins occurs at molar concentrations which are up to 3-fold lower than the concentration of fibronectin required for strong adhesion.

Cell-surface receptors for CSPGs

To determine whether integrins acts as cell-surface receptors for CSPGs, fibroblasts were incubated with antibodies against...
Table 3. Centrifugation assay: reversal of adhesion by various agents

<table>
<thead>
<tr>
<th>Substrate, µg/ml</th>
<th>Control</th>
<th>α5</th>
<th>β1</th>
<th>EDTA</th>
<th>CS</th>
<th>HAse</th>
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<tr>
<td>FN, 10</td>
<td>+++</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>FN, 3</td>
<td>+++</td>
<td>–</td>
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<td>–</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>FN, 1</td>
<td>+</td>
<td>–</td>
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<td>PG400 Mono, 0.1</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>++</td>
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<tr>
<td>PG400 Mono, 0.03</td>
<td>++</td>
<td>++</td>
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<td>PG400 Mono, 0.01</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>PG250 Mono, 0.1</td>
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<td>++</td>
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<td>PG250 Mono, 0.03</td>
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<td>PG250 Core, 1</td>
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Centrifugation assays were performed as described in Materials and Methods. Adhesion is expressed as described in Table 2. To analyze the influence of mAbs against human integrin subunits on the binding of fibroblasts to the indicated substrates, cells were incubated for 15 minutes on ice in the presence of 10 µg/ml of these mAbs before addition to the wells and subsequent centrifugation. mAbs tested include: anti-β1 (4B4) from Coulter; and anti-α2 (P1E6), anti-α5 (P1B5), anti-α4 (P4C2), anti-α5 (P1D6), anti-β2 (P4H9), and anti-β1 (3E1) from Gibco-BRL. Only the data obtained with the anti-α5 and the anti-β1 antibodies are presented as none of the other antibodies tested had any effect. To test the effects of 5 mM EDTA, 2 units/ml of hyaluronidase (HAse), or 50 µg/ml of chondroitin sulfate (CS) on cell binding, cells were incubated with these reagents for 15 minutes either on ice (EDTA, CS) or at 37°C (HAse) before addition to the wells.

Because CSPGs can interact with HA to form aggregates, we evaluated the possibility that they might also interact with cell-surface HA. Pretreatment of cells with hyaluronidase blocked adhesion to core proteins in the centrifugation assay (Table 3), implying that the cores interact with cell-surface HA. This inhibition of adhesion was not observed when only the CSPG-coated substrate was hyaluronidase treated (not shown), indicating that the HA involved in adhesion in these experiments is on the cell surface and not in the CSPG preparations. Hyaluronidase treatment of cells had no effect on their adhesion to fibronectin or to CSPG monomers. The apparent lack of effect of hyaluronidase treatment on adhesion to CSPG monomers is not surprising. The hyaluronidase-sensitive adhesion mechanism requires that the substrate be coated using a 10 µg/ml solution of core proteins; CSPG monomers participate in an additional more avid hyaluronidase-insensitive mechanism which requires only 0.03 µg/ml of CSPG monomer.

Further evidence that core proteins mediate adhesion by a specific mechanism is provided by experiments showing that soluble core proteins inhibit adhesion to immobilized core proteins in a dose-dependent manner, but do not inhibit specific integrin subunits. Although antibodies against the β1 and α5 integrin subunits inhibited adhesion to fibronectin in the centrifugation assay, they had no effect on adhesion to PG250 monomer, PG400 monomer, PG250 core, or PG400 monomer (Table 3). Similarly, antibodies against other integrin subunits had no effect on adhesion to these CSPG fractions (not shown). As adhesion to integrins routinely depends on the presence of divalent cations, we also tested the effect of EDTA on cell adhesion. As expected, adhesion to fibronectin in the presence of EDTA was completely eliminated. However, EDTA treatment had no effect on adhesion to monomers and core proteins (Table 3). Thus, it is unlikely that integrins are cell-surface receptors for these CSPGs.

CSPGs appear to mediate adhesion through at least two molecular mechanisms, one involving only the core proteins, and the other dependent on the presence of CS on CSPG monomers (Table 2). To examine whether the latter mechanism involves a cell surface receptor for CS, we performed centrifugation assays in the presence of up to 50 µg/ml of soluble CS (Table 3). The presence of soluble CS had no effect on adhesion to PG400 monomer and PG250 monomer (as well as to fibronectin and to PG250 core and PG400 core) suggesting that the mechanism of fibroblast adhesion to CSPG monomers is more complex than an interaction between CS and a CS receptor. For example, this mechanism may involve both protein and CS or be very sensitive to the spacing, orientation, or structure of CS molecules.

Fig. 2. Centrifugation assay: inhibition of fibroblast binding to immobilized PG400 core or PG250 core by soluble core proteins. centrifugation assays were performed as described in Materials and Methods. Wells were coated using 10 µg/ml solutions of fibronectin (○), PG250 core (■), or PG400 core (▲). Human foreskin fibroblasts (12,500 in 200 µl DMEM containing the indicated amounts of soluble PG250 core (A) or soluble PG400 core (B) were incubated for 10 minutes on ice and 5 minutes at 37°C before addition to the wells and centrifugation.
adhesion to fibronectin (Fig. 2). Soluble PG250 core was more effective in preventing adhesion to immobilized PG250 core than to immobilized PG400 core (upper graph), whereas soluble PG400 core affected binding to immobilized PG250 core and PG400 core to about the same degree (lower graph). Soluble core proteins had no effect on adhesion to immobilized monomers (data not shown), further supporting the idea that CSPG monomers participate in a more avid adhesion mechanism involving a cell surface receptor which does not interact with core proteins.

**Mixed substrates: CSPGs inhibit cell spreading and thereby inhibit cell attachment in the gravity assay**

When CSPGs are mixed with other ECM proteins that normally promote cell attachment in the gravity assay, their presence either inhibits adhesion or has no effect. For example, monomer mix inhibited cell spreading and attachment to fibronectin (Fig. 3, compare a and b) or to mAb 4B4 which is specific for the human β1 integrin subunit (Fig. 3, compare c and d), but had no effect on attachment to collagen I (Fig. 3, compare e and f). The lack of effect of monomer mix on adhesion to collagen I was not due to an insufficient concentration of CSPG; even at 10-fold higher doses of CSPG no inhibition of cell spreading and attachment was observed (not shown).

Detailed results obtained with mixed substrates are presented in Fig. 4. Among the CSPG preparations used, data are shown only for PG400 monomer and PG400 core. Data obtained using PG aggregate and PG250 monomer were essentially indistinguishable from PG400 monomer data; data obtained with PG250 core were essentially indistinguishable from PG400 core data. The presence of PG400 monomer, PG400 core, or restrictin each inhibited cell attachment and spreading on fibronectin (Fig. 4A), but with very different concentration dependencies. Strong inhibition of attachment and spreading was obtained using 0.8 μg/ml of PG400 monomer; although strong inhibition of spreading was obtained with 0.8 μg/ml of PG400 core, strong inhibition of attachment was not obtained until 5 μg/ml was used. Restrictin was even less active than PG400 core; strong inhibition of spreading was obtained at 2 μg/ml and strong inhibition of attachment was obtained at 5 μg/ml. Similar results were obtained with vitronectin (Fig. 4B) and with 4B4 (Fig. 4C). Strong inhibition of attachment and spreading was obtained with 0.8-2.0 μg/ml of PG400 monomer. Whereas strong inhibition of spreading was obtained with 0.8-2.0 μg/ml of PG400 core, strong inhibition of attachment was only observed with 5 μg/ml of PG400 core. The fact that low concentrations of CSPG (or restrictin) inhibit only cell spreading while higher concentrations are required to inhibit both cell spreading and attachment suggests that inhibition of cell attachment is a secondary result of the inhibition of cell spreading. Finally, little inhibition of attachment or spreading on laminin (not shown) or collagen I (Fig. 4D) was observed in the presence of either PG400 monomer or core.

These results strongly suggest that the ability of PG aggregate to inhibit cell spreading and attachment is not due to the HA, link protein, or restrictin present in the preparations. Purified CSPG monomers lack these molecules yet are as active at inhibiting adhesion; purified restrictin has relatively weak activity. In addition, HA even at high doses (100 μg/ml) does not inhibit cell spreading and attachment (not shown). CSPG core proteins are somewhat less active than CSPG monomers but are clearly active, indicating that a substantial portion of the ability of CSPGs to inhibit cell spreading and attachment resides in their core proteins.

To determine whether purified aggrecan has a similar ability to inhibit cell spreading and attachment in mixed substrates, we tested commercial preparations of bovine nasal aggrecan in the gravity assay (not shown). As with brain CSPGs, aggrecan aggregate and monomer inhibited adhesion to fibronectin, vitronectin, and mAb 4B4 but had little effect on collagen I and laminin.

We also examined mixed substrates in the centrifugation assay. As expected, because each component of the mixed substrates mediates adhesion on its own, no inhibition of adhesion to other ECM proteins was caused by the presence of the various CSPG preparations (not shown). Nevertheless, as in the gravity assay, the presence of each CSPG prepara-
tion blocked the spreading of cells on normally permissive substrates during a 60 minute incubation following centrifugation (not shown).

**Specificity of the anti-adhesive effects of CSPGs**

The results of Figs 3 and 4 suggest that CSPGs do not block cell attachment by masking interactions between ECM proteins and their cell-surface receptors. If CSPGs non-specifically mask ECM proteins, then they should block the adhesion of fibroblasts to collagen I and laminin as well as to fibronectin, vitronectin, and mAb 4B4. To further support the idea that CSPGs do not non-specifically mask substrates permissive for cell spreading and attachment, we tested the ability of anti-monomer mix Fab' fragments to reverse the ability of monomer mix to inhibit cell attachment to fibronectin and mAb 4B4. If CSPGs inhibit cell attachment by masking the substrate, then the decoration of the CSPGs with Fab' fragments should lead to greater masking and inhibition. If CSPGs inhibit cell attachment by binding to cell-surface receptors and thereby triggering an inhibition of cell spreading, then the addition of Fab' fragments might block the binding of CSPGs to their receptors and reverse the inhibition of cell spreading and attachment. In fact, anti-monomer mix Fab' fragments almost completely reversed the inhibition of cell spreading and attachment while non-immune Fab' fragments had no effect (Table 4). These data strongly support the idea that CSPGs inhibit cell spreading and attachment by binding to cell-surface receptors.

We were also concerned with the possibility that CSPGs might inhibit cell spreading and attachment by directly binding to the permissive molecules and thereby preventing their interaction with cell surface receptors. While it is conceivable that the inhibition of adhesion to fibronectin or vitronectin is mediated in part by interactions of the CSPGs with the ECM proteins, it is improbable that CSPGs react with mAb 4B4. To evaluate this possibility, we tested the ability of soluble proteins to block the binding of radioiodinated mAb 4B4 to cells. Whereas excess unlabeled mAb 4B4 decreased binding >90%, a similar amount of PG aggregate or monomer mix had no effect (not shown). This result suggests that these CSPGs do not interact with 4B4 and thereby block its binding to the β1 integrin subunit.

To prove that CSPGs do not inhibit adhesion by interfering with the adsorption to plastic of the other protein present, we prepared mixtures between radioiodinated and unlabelled proteins and quantified protein adsorption (not shown). These experiments revealed that in the presence of 2 µg/ml of the various CSPG preparations, the concentration of fibronectin or vitronectin...
vitronectin on the plastic surface was marginally reduced (up to 26%). This small decrease in adsorption should not affect cell attachment to fibronectin or vitronectin; when one-half as concentrated solutions of radioactive fibronectin or vitronectin were used in the absence of CSPG, about 40% less protein adsorbed. This amount of fibronectin or vitronectin still provided for optimal cell spreading and attachment (not shown). These experiments also revealed that strong inhibition of cell spreading occurs at 1:10 molar ratios of CSPG to fibronectin or vitronectin on the substrate. This observation further supports the idea that CSPGs cannot be blocking cell spreading and attachment through a specific one-to-one interaction with the other protein on the substrate.

These various results strongly suggest that the anti-adhesive effect of CSPGs is not due to the non-specific masking of the interaction of the other protein on the substrate with its cell-surface receptors, to a direct specific interaction with the other protein, or to an inhibition of its adsorption to the substrate.

**DISCUSSION**

We have isolated CSPG aggregates, monomers, and core proteins from 14-day embryonic chicken brains and used these preparations to analyze the ability of CSPGs to mediate cell-substrate adhesion for foreskin fibroblasts or to inhibit their adhesion to other ECM proteins. These studies have provided several novel observations:

1. Restrictin co-purifies with brain CSPGs. This fact and other unpublished observations suggest that restrictin, like the related protein tenascin (Hoffman and Edelman, 1987), binds directly to certain CSPGs.

2. CSPGs mediate adhesion in the centrifugation assay (which does not involve cell spreading), but not in the gravity assay (in which cell spreading stabilizes cell attachment). The observation that soluble CSPG core proteins specifically and dose-dependently inhibit adhesion to immobilized CSPG core proteins indicates that adhesion occurs through specific interactions between CSPGs and cell-surface receptors. Putative cell surface receptors for CSPGs include cell-associated HA which binds to CSPG core proteins, and an as yet unidentified receptor whose ligand includes the CS portion of CSPGs.

3. Using mixed substrates in the gravity assay, the presence of CSPGs inhibits cell spreading and attachment to fibronectin, vitronectin, or monoclonal anti-β1 integrin antibody 4B4, but not to laminin or collagen I. CSPGs do not inhibit adhesion as part of mixed substrates in the centrifugation assay, although they still inhibit cell spreading (either alone or as part of a mixed substrate). These observations strongly suggest that the inhibition of cell attachment by CSPGs results from their inhibition of cell spreading.

4. PG aggregate, PG250 monomer, and PG400 monomer all mediate adhesion in the centrifugation assay at extraordinarily low concentrations on the substrate (e.g. 200-fold lower concentration than fibronectin); PG250 core and PG400 core are slightly more active than fibronectin. PG aggregate, PG250 monomer, and PG400 monomer are also more effective than PG250 core or PG400 core in inhibiting cell spreading and attachment as part of mixed substrates in the gravity assay. Restrictin is less active than these core proteins.

5. The inhibition of cell attachment by CSPGs in the gravity assay is not due to the non-specific masking of the other protein on the substrate, the direct specific interaction of the CSPGs with the other protein, or to an inhibition of the other protein’s adsorption to the substrate. For example, the inhibition by CSPGs of adhesion to mAb 4B4 and fibronectin is reversed by the addition of Fab’ fragments of anti-CSPG antibodies suggesting that these antibodies block CSPG-receptor interactions that inhibit cell spreading and attachment. If CSPGs inhibited cell attachment through non-specific masking, then the decoration of the CSPGs with Fab’ fragments should lead to greater masking and inhibition. The idea that the inhibition of cell attachment and spreading by CSPGs does not involve specific interactions with the other protein on the substrate is supported by the observations that CSPGs inhibit attachment and spreading on mAb 4B4 but do not interact with this antibody and that CSPGs block cell attachment and spreading at molar concentrations one-tenth as high as the other protein on the substrate.

These studies support the idea that large, extracellular CSPGs bind to specific cell-surface receptors. The fact that treating cells with hyaluronidase inhibits adhesion to core proteins in the centrifugation assay indicates that one of these receptors is cell surface-associated HA. Related observations have been reported for the interaction of aggrecan with neural crest cells and chondrocytes. Aggrecan inhibits the migration of neural crest cells on a variety of ECM proteins (Perris and Johansson, 1990). This inhibitory effect is reversed in the presence of hyaluronan oligosaccharides or following pre-treatment of the cells with hyaluronidase suggesting that the interaction of aggrecan with cell-surface HA is a prerequisite for the inhibition of cell migration. The assembly of pericellular matrices by chondrocytes requires the expression of cell-surface HA receptors, the interaction of HA with these receptors, and the presence of intact aggrecan decorating the HA (Knudson, 1993).

Although hyaluronidase treatment of cells inhibits adhesion...
to core proteins in the centrifugation assay, it does not reverse the core protein-mediated inhibition of cell spreading and attachment on fibronectin in the gravity assay (not shown). This observation suggests the existence of another cell-surface receptor capable of binding to core proteins that mediates the inhibition of cell spreading and attachment on fibronectin.

The fact that CSPG aggregates and monomers are more active than CSPG core proteins in mediating adhesion in the centrifugation assay or in inhibiting adhesion as part of mixed substrates in the gravity assay suggests that cells have a CSPG receptor that requires the presence of CS on the molecules. This receptor is not simply a CS receptor, given that soluble CS does not inhibit cell adhesion to CSPG aggregates or monomers in the centrifugation assay. This result suggests that the ligand for this putative receptor includes both CS and another part of the molecule or is highly sensitive to the orientation and spacing of the CS.

We have identified an additional CSPG receptor on retinal cells (Balsamo et al., 1995). PG250 monomer and PG250 core bind to the cell surface glycosyl transferase, GalNaCPTase. This interaction indirectly inhibits N-cadherin function by promoting the tyrosine phosphorylation of β-catenin and thereby uncoupling N-cadherin from the cytoskeleton. Unlike the present study in which we find PG400 monomer to be as active as PG250 monomer and PG400 core to be as active as PG250 core, PG400 monomer and PG400 core did not inhibit N-cadherin-mediated adhesion in retinal cells. Likewise, PG400 monomer or PG400 core did not interact with GalNaCPTase. Because GalNaCPTase is also present on foreskin fibroblasts (not shown), it is possible that it may serve as a receptor for PG250 monomer and PG250 core in these cells as well. Nevertheless, it is unlikely to be the critical CSPG receptor for inhibiting cell-ECM adhesion in foreskin fibroblasts unless it also recognizes PG400 monomer and PG400 core in the context of these cells. In summary, we have evidence for several distinct CSPG receptors on foreskin fibroblasts. Presently, we do not know whether one of these receptors is predominantly responsible for the inhibition of cell attachment and spreading by CSPGs or whether several cooperate in this process.

Our results agree with those of Yamagata et al. (1989) in that we find that CSPG monomers are considerably more active than CSPG core proteins in inhibiting adhesion to other ECM proteins. Our results differ in that we can detect activity in core proteins whereas they detect no activity. We also observe inhibition of adhesion to fibronectin and vitronectin, but not to laminin and collagen I, whereas they observe inhibition of adhesion to all of these proteins. These distinctions may be due to any of several differences between the studies. We used foreskin fibroblasts whereas they used BHK cells primarily and also chick embryo fibroblasts, melanoma cells, and CHO cells.

For adhesion assays, we harvest cultured cells with only one-fifth the concentration of trypsin that they use. We perform our adhesion assays on multiple test spots within a 35 mm dish whereas they use individual wells of a 96-well plate for each test. We used either what turned out to be a mixture of CSPGs from embryonic chicken brain (see below) or bovine nasal aggre can while they used PG-M (versican) from chick embryo fibroblasts. Any of these differences may affect the results; it is even possible that versican isolated from brain differs in activity from versican isolated from fibroblasts.

As discussed by Balsamo et al. (1995), N-terminal sequence analyses of CNBr fragments derived from PG400 core and PG250 core have revealed that both preparations are mixtures of known CSPGs. PG400 core contains phosphacan, versican, and aggrecan whereas PG250 core contains aggrecan, phosphacan, and neurocan. Therefore, despite the difference in their appearance on SDS gels, it is not surprising that PG400 core and PG250 core have similar activities in our experiments. To allow us to study these CSPGs further, we have recently succeeded in preparing antibodies against synthetic peptides chosen from the known sequences of chicken aggrecan (Chandrasekaran and Tanzer, 1992) and versican (Shinomura et al., 1993) and from sequences that we obtained from chicken neurocan and phosphacan. These antibodies will play a central role in our future studies on the distribution and function of individual CSPGs and their interactions with specific cell surface receptors.

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