Chondroitin Sulfate at the Plasma Membranes of Cultured Fibroblasts

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ABSTRACT We have previously shown that in confluent human fibroblast cultures chondroitin sulfate proteoglycan is a component of the fibronectin-containing pericellular matrix fibers. In the present work the distribution of chondroitin sulfate was studied in subconfluent cell cultures using antibodies that bind to a chemically defined carbohydrate fragment of chondroitinase ABC-modified chondroitin sulfate proteoglycan. Using immunofluorescence microscopy, we observed, in addition to the fibrillar matrix staining, chondroitin sulfate diffusely distributed at the cell surface. In indirect immunoferritin electron microscopy this staining corresponded to patchy binding of ferritin close (24 nm) to the outer aspect of the plasma membrane. The patchy organization appeared uniform in all cell surfaces. The cell surface chondroitin sulfate could not be removed from the plasma membrane by agents that dissociate electrostatic interactions. These data show that in fibroblasts chondroitin sulfate is a component of the outer aspect of the plasma membrane, and raise the possibility of an integral plasma membrane chondroitin sulfate proteoglycan.

The surfaces of cultured fibroblasts are composed of the plasma membrane and the pericellular matrix. The latter forms a fibrillar network, the major constituents of which are fibronectin (1, 2), procollagen α-chains (3, 4), and later collagen (5), other glycoproteins (6, 7), and sulfated proteoglycans (8, 9). The fibronectin fibers are in close association with cytoplasmic actin microfilaments at places (1, 10, 11). Such fibronexuses are preferentially located close to focal contacts or adhesions (12–16) between the cell and the growth substratum, particularly in low serum concentrations (11, 17). The role of fibronectin in fibroblast attachment, spreading and locomotion is widely accepted; how these functions relate to the genesis of matrix fibers is not clear. Heparan sulfate (HS)[1] has been hypothesized to stabilize the focal adhesion sites whereas chondroitin sulfate (CS) was suggested to destabilize them (18). Exogenous CS proteoglycans did, indeed, inhibit fibronectin-mediated cell adhesion to collagen (19). There is also in vitro evidence for differing functions of HS and CS in relation to fibronectin-collagen fibrillogenesis (20). These data have prompted us to study the distributions of external proteoglycans. We have previously shown that HS and CS proteoglycans are components of the pericellular matrix fibers, in firm association with the polypeptide backbone of the matrix (9). However, in isolated pericellular matrices, insoluble CS was also present beside the fibronectin-containing matrix fibers (9). Here we have defined the location of this cell surface component. Antibodies were produced against a chemically defined carbohydrate fragment of CS. This antigen, and unsaturated oligosaccharide, is generated by digestion of chondroitin-4-sulfate proteoglycan with chondroitinase ABC and remains covalently bound to the proteoglycan core (21).

MATERIALS AND METHODS

Cell Cultures: Human embryonic fibroblasts (HES) of local origin or murine 3T3 cells were grown on plastic dishes with or without glass coverslips, as previously described (1). The cells were used for the experiments at subconfluent densities, 5–48 h after subculture. For immunoenzymatic quantitation of cell surface chondroitin sulfate, HES cells were seeded into 96-well microtiter plates (Linbro/Flow, Hamden, CO) and were used 24 h after seeding.

Immunocytochemical Staining for Chondroitin Sulfate: CS was rendered antigenic to our antibody by treatment of living or fixed cell layers with chondroitinase ABC (cABC) or, for comparison, with chondroitinase AC (cAC) (Seikagaku Kogyo, Tokyo, Japan): live cells were briefly rinsed three times at 37°C with Dulbecco's minimum essential medium containing 2 mg/ml BSA, penicillin, and streptomycin (DME-BSA). The cultures were digested with 5-50 mU/ml of cABC or with 50-500 mU/ml of cAC in DME-BSA for 30 min at 37°C, rinsed three times with DME-BSA followed by Pi/NaCl (0.01 M sodium phosphate, 0.14 M NaCl, pH 7.4), and fixed with 4% paraformaldehyde or with 1% glutaraldehyde for immunofluorescence or immunoferritin staining, respectively. Alternatively, rinsed cell cultures were first fixed, rinsed,

[1] *Abbreviations used in this paper:* cABC, chondroitinase ABC; cAC, chondroitinase AC; CS, chondroitin sulfate; HS, heparan sulfate; HES, human embryonic fibroblasts; DME-BSA, Dulbecco's minimum essential medium–BSA.

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and subsequently digested with cABC for 30 min at 37°C, or for 16 h at 4°C. For comparison, in some experiments the enzyme treatment of fixed cell cultures took place in 0.1 M Tris-HCl, pH 8.0, containing 1 mM EDTA, 0.8 M *N*-ethylmaleimide, and 0.2 mM phenylmethylsulfonyl fluoride as proteinase inhibitors, for 16 h at 4°C. The two different digestion buffers gave identical immunofluorescence results. The digested, fixed cell cultures were then stained for the antigenic unsaturated oligosaccharides of CS using rabbit antiserum (21) diluted 1:100 in PBS for 30 min, and were subsequently rinsed three times for 10 min. The cultures were treated with antirabbit IgG-FITC (Wellcome, Beckenham, England; 1:20 in PBS) or with antirabbit IgG-ferritin (Cappel Laboratories, Cochranville, PA; 1:50 in Pi/NaCl) conjugates for 30 or 60 min, for immunofluorescence or immunoelectron microscopy, respectively. The rinsed samples on coverslips were mounted for immunofluorescence, observed and photographed as described (1). Immunoferritin-stained samples on plastic were fixed with 2% glutaraldehyde and processed for transmission electron microscopy (1). Thin sections were poststained with uranyl acetate and lead citrate and were observed and photographed in a Jeol 100 CX TEMSCAN electron microscope operated at 60 kV at the Department of Electron Microscopy, University of Helsinki, Finland.

Staining Controls: Preimmune serum of the same rabbit (bled at the time of first immunization) diluted 1:100 was allowed to stain cell cultures as above. A blocking control was carried out by adding to the diluted (1:10) immune antiserum 4-500 μg/ml of purified CS hexasaccharide, obtained from limited cABC digestion of rat chondrosarcoma proteoglycan (21), followed by 10-fold dilution of the blocked antiserum and immunofluorescence or immunoferritin staining as above. As a control for the blocking experiments the antiserum was treated with purified hexasaccharide produced as described (22) from rooster comb hyaluronic acid. Cell cultures were also fixed without chondroitinase treatment and immunofluorescence stained with the immune antiserum to show the specificity for chondroitinase digestion product of the results. Finally, two control experiments were carried out to eliminate artificial adsorption to the cell surface of antigenic digestion product: (*a*) living cell cultures were digested with cABC and the enzyme supernatant was collected and heated at 100°C for 5 min to inactivate the chondroitinase. Untreated cultures were then incubated in this oligosaccharide-containing solution for 30 min at 37°C, rinsed, fixed, and stained as described above. (*b*) Fixed, nondigested cell cultures were treated with purified CS hexasaccharide (see above) at 10 or 100 μg/ml followed by immunofluorescence staining.

Stability of Cell Surface Chondroitin Sulfate: Human fibroblast cultures were rinsed and kept in DME-BSA for 30 min at 37°C or at 4°C before fixation. Alternatively, the cell cultures were treated with cABC and then incubated in DME-BSA for 30 min at 37°C and subsequently fixed. To study the nature of bonds joining CS to the cell surface, rinsed cells were treated with 0.32 M NaCl or with 0.5–1.0 mg/ml of heparin (Medica, Helsinki, Finland) in DME-BSA for 15–30 min at 37°C or at 4°C, or with 0.5 or 5 mM EDTA in Pi/NaCl or DME-BSA, respectively, at 37°C or 4°C. The role of cytoskeletal elements was tested by treatment of cells with cytochalasin B or colchicine (23) in DME-BSA. The treated cell cultures were then briefly rinsed and fixed for immunofluorescence or immunoferritin electron microscopy.

To quantitate the possible release of CS from the cell surface by the above treatments, and to assess the specificity of the immunostaining for CS, we used enzyme immunoassay (24) briefly as follows: human embryonic fibroblasts grown in microtiter wells were treated as above, then fixed with 4% paraformaldehyde and treated with 5 or 50 mU/ml of cABC or 50 mU/ml of cAC for 45 min at 37°C in DME-BSA. After two washings with DME-BSA the cells were surface-treated with a saturating concentration of the anti-CS serum (1:100 dilution in Pi/NaCl containing 5 mg/ml BSA) or with the blocked antisera (see above) or the preimmune serum as specificity controls, for 6 h at 20°C. After two washings, the samples were treated with horseradish peroxidase-conjugated staphylococcal protein A (24) followed by washing and the peroxidase substrate. Three separate experiments were carried out and each time four parallel wells were used for each reagent tested.

RESULTS

Immunofluorescence Localization of Chondroitin Sulfate

Subconfluent cultures of HES or 3T3 cells were digested with cABC or cAC to render CS antigenic to our antiserum. The cells were enzyme treated alive or after fixation with paraformaldehyde. Irrespective of the sequence of fixation and enzyme treatment, cells stained with the CS antiserum showed a nearly uniform surface staining (Fig. 1, *a–c*). Upon focusing, however, brighter patchy or "hairy" fluorescence could be resolved on the dorsal cell surface (Fig. 1 *a*) of spindle-shaped cells (Fig. 1 *b*), or in mitotic cell pairs (Fig. 1 *c*). The fluorescence intensity varied between individual cells. Part of the difference could be correlated with cell shape: well-spread cells had the weakest apparent surface fluorescence (Fig. 1 *c*).

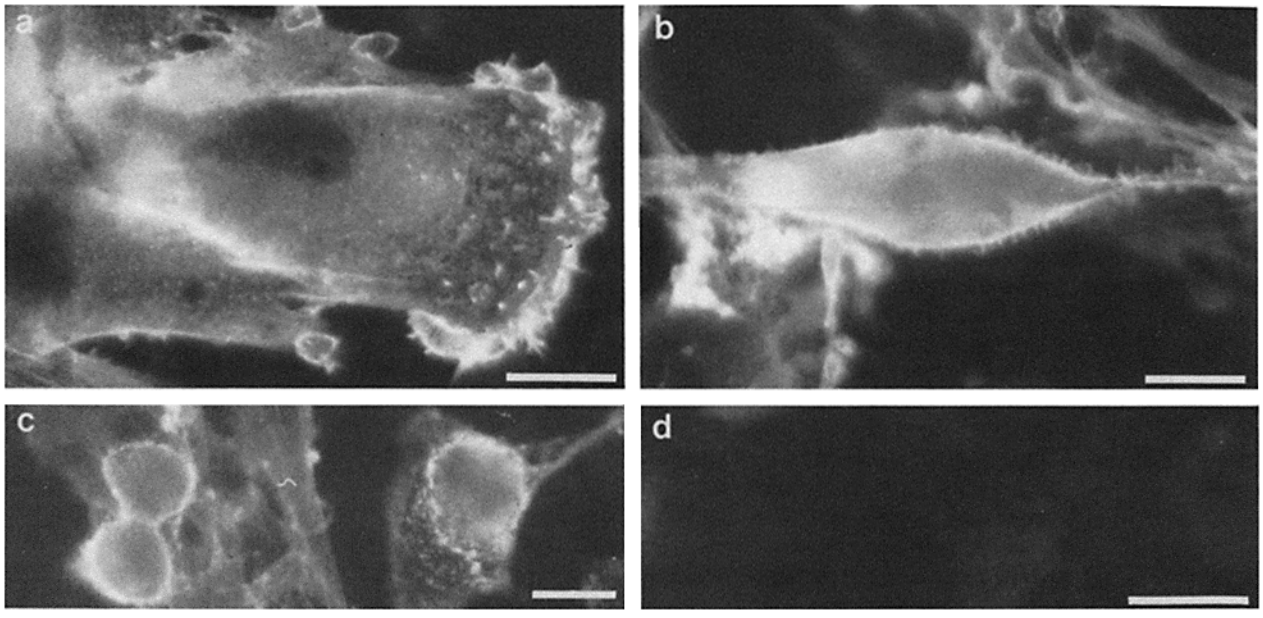

FIGURE 1 Immunofluorescence staining for cell surface CS. HES cells were fixed with paraformaldehyde 24 h after subculture, shortly digested with cABC and treated with rabbit immune antiserum against the unsaturated oligisaccharide of CS (*a–c*) or with the respective preimmune serum (*d*), followed by antirabbit IgG-FITC. Focusing on the dorsal surface of a locomotory fibroblast, *a* shows a uniform surface fluorescence interrupted by brighter dots and lamellae at the leading cell edge, to the right (× 720). A spindle-shaped cell (*b*) and a mitotic cell pair (*c*) are distinguished from the well spread cells of the background (× 670 and × 560, respectively). Pericellular fibrillar CS staining is also seen (*b* and *c*). (*d*) Control cells stained with preimmune serum (× 800). Bars, 20 μm.

This may, at least in part, be explained by the orientation of the fluorescent surface (Fig. 1, *a–c*). Weak fluorescence for CS was also found in pericellular matrix fibers, which could be double-stained with rhodamine-conjugated sheep antifibronectin (previously shown in reference 9). This matrix fluorescence was usually seen at the sites where local cell density approached confluence and seemed to appear in the matrix fibers later than that of fibronectin. 3T3 cells gave cell surface fluorescence very similar to that of HES (data not shown).

Specificity controls included staining of cABC-treated cells with preimmune serum, or staining of cells with the immune serum without prior enzyme treatment. These controls gave only negligible faint background fluorescence over the cell bodies and none in the pericellular matrix (Fig. 1*d*). Additionally, blocking controls were carried out in which the immune serum was mixed with various amounts of purified unsaturated hexasaccharide obtained from limited chondroitinase treatment of chondrosarcoma CS. Such blocking is known to inhibit the reactivity of our antiserum with chondrosarcoma CS proteoglycan (21). Here, 100 μg/ml of the hexasaccharide completely eliminated the cell surface fluorescence, whereas 4 μg/ml was partially active. Comparable attempts to block the staining using antibodies preadsorbed with unsaturated oligosaccharides from HA or with intact heparin did not diminish the immunofluorescence intensity (not illustrated, cf. Fig. 6). Finally, since antigenic oligosaccharides might have been solubilized and rebound during the chondroitinase treatment, another set of control experiments was carried out. For this purpose, cABC supernatants were collected from live-digested cell cultures, the enzyme activity was destroyed by boiling and untreated rinsed cell cultures were incubated in the antigenic digests. Alternatively, fixed cell layers were treated with antigenic purified hexasaccharide of CS. When such control samples were stained, no fluorescence above background was observed. These experiments excluded artificial binding from the digestion buffer to the surfaces of antigenic material.

Immunoferritin Electron Microscopy

The immunofluorescence results suggested presence of CS at the fibroblast surfaces, thus warranting further immunolocalization at the ultrastructural level. HES fibroblasts were treated with cABC before or after glutaraldehyde fixation and immunoferritin stained for surface CS. Preliminary experiments using immunoperoxidase staining at the light microscopic level showed that the used glutaraldehyde prefixation gave CS staining patterns identical to that obtained after paraformaldehyde prefixation (data not shown). After digestion of prefixed cell layers, ferritin was seen bound to the cell surface (Fig. 2). The mean distance of ferritin cores from the outer surface of the plasma membrane, as measured from 100 ferritin particles above a perpendicularly sectioned plasma membrane, was 23.8 nm. The same ferritin distribution was obtained whether fixed or unfixed cells were digested with cABC, and was present also when live-digested cells were immunostained and washed alive at 4°C and subsequently fixed. The amount of ferritin bound per length unit along the plasma membranes varied between different cells, but in a given cell appeared similar in different locations of the cell surface. The distribution of individual ferritin particles was, however, not random: the particles seemed to be arranged in patches. Since the observed ultrastructural patchy distribution of CS in the one dimension of sectioned cell surface could be derived from either patchy or reticular organization at the plasma membrane, it was important to find electron microscope sections tangential with and close to the outer surface of the membrane. In such micrographs ferritin was clearly seen arranged in patches, the organization of which was random in a given cell surface area (Fig. 3). Each "patch" comprised 1–30 ferritin particles and was separated from the adjacent one by a gap of 0–500 nm, average 100–200 nm (Fig. 3). The specificity controls, preimmune serum after cABC as well as immune serum without preceding enzyme treatment, gave virtually no binding of ferritin to the plasma membrane and only occasional binding of ferritin to the

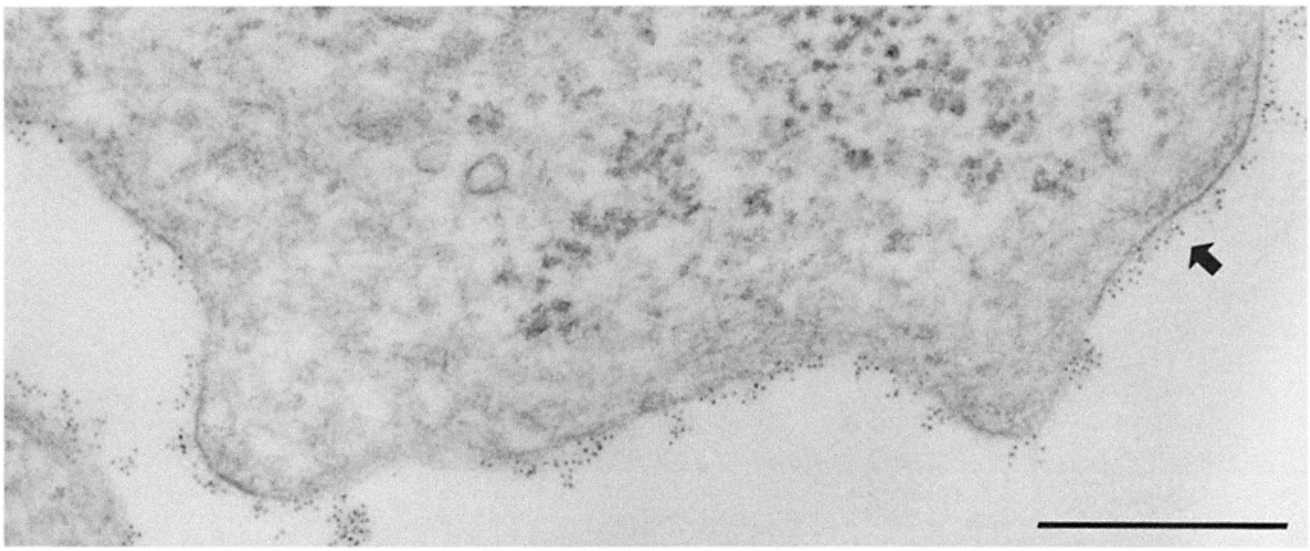

FIGURE 2 Immunoferritin electron microscope staining for CS at the surface of human fibroblasts. The cells were fixed with glutaraldehyde, digested with cABC, and treated with antiserum against the unsaturated oligosaccharide of CS, followed by ferritin-conjugated anti-IgG. Ferritin is bound close to the plasma membrane (arrow). Uranyl acetate-lead citrate poststaining. Bar, 500 nm. × 66,000.

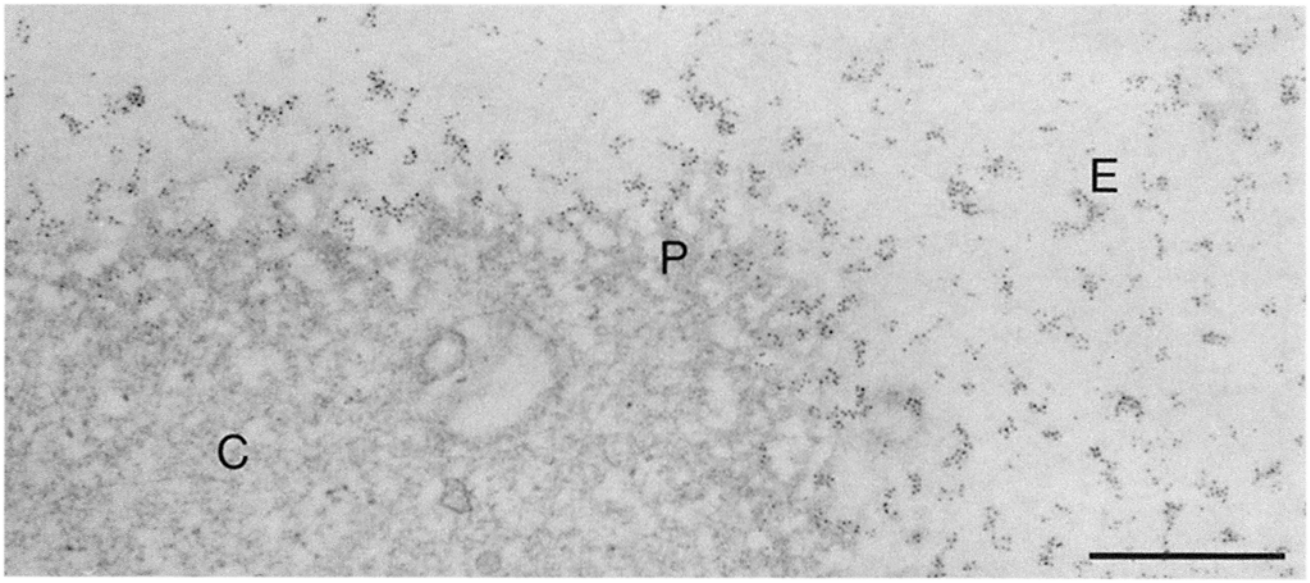

FIGURE 3 Immunoferritin staining for cell surface CS. The horizontal electron microscope section grazes along the upper cell surface including parts of the cortical cytoplasm (*C*), fuzzy zone indicating plasma membrane (*P*), and the extracellular space (*E*). Ferritin is distributed in patches of 1 to 30. Bar, 500 nm. × 52,000.

pericellular matrix. When the immune serum was adsorbed with 100 μg/ml of unsaturated hexasaccharide of CS, nearly all ferritin was removed from the plasma membrane (Fig. 4).

Stability of CS–Cell Surface Association

To study the nature of association between CS and the plasma membrane, HES fibroblasts were first incubated in DME-BSA for 30 min at 37°C or at 4°C. Whether the incubation took place before or after chondroitinase, the immunofluorescence results were qualitatively and quantitatively indistinguishable from those described above. The cell cultures were exposed to various reagents: EDTA treatment rounded up the cells, with concomitant apparent intensification of cell surface immunofluorescence. NaCl or heparin removed little or no CS from the plasma membrane. Colchicine gave results very similar to that of EDTA. Treatment of the cells with cytochalasin B caused dramatic "arborization" (23) of cell bodies with no apparent change in the degree of surface fluorescence. Estimations of plasma membrane immunofluorescence intensities after the various treatments of the cells are summarized in Table I. The inability of EDTA or heparin to deplete cell surfaces of immunoreactive CS was further documented by immunoelectron microscopy. EDTA-treated (Fig. 5) or heparin-treated (not illustrated) cellular plasma membranes bound ferritin conjugates at an apparently unaltered density.

Because of the semiquantitative nature of immunofluorescence, the ability of the chemicals to remove CS from the cell surface, and the specificity for CS of the antibody binding was further analyzed in situ in the cell cultures using an immunoenzyme assay. Cells grown in plastic microwells were digested with cABC or cAC, with or without prior treatment with EDTA, NaCl, heparin, colchicine, cytochalasin B, or Nonidet P-40, followed by immunoenzymatic quantitation of external CS. As summarized in Table II, NaCl, heparin, or colchicine had no effect on the amount of immunoreactive CS. Cytochalasin B and Nonidet P-40 at 0.01% diminished immunoreactivity slightly. EDTA detached 50–90% of the cells from the plastic, and Nonidet P-40 at 0.1% solubilized the cells. To exclude the remote possibility that the antibodies bound digestion products of HA (which is a substrate for the chondroitinases) the antiserum was pretreated with unsaturated HA oligosaccharides, and the binding inhibition was

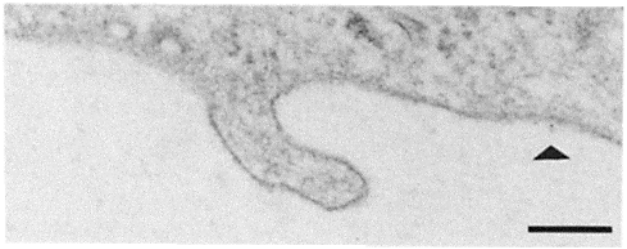

FIGURE 4 Blocking control for immunoferritin staining. The diluted immune antiserum was mixed with 100 μg/ml of purified unsaturated hexasaccharide obtained from cartilage CS and was then used in immunoferritin staining of cABC-treated human fibroblasts. One ferritin particle (arrowhead) is seen. Bar, 200 nm. × 56,000.

TABLE I

Stability of CS at the Cell Surface as Measured by Immunofluorescence Microscopy

Chemical	Estimated immunofluorescence intensity
cABC, 50 mU/ml	+++
cABC, 5 mU/ml	+++
cAC, 50 mU/ml	+
No enzyme	(+)
Preceding enzyme	
NaCl, 0.32 M	++(+)
Heparin, 1 mg/ml	++(+)
Colchicine, 10 M	+++
Cytochalasin B, 3 μg/ml	+++
EDTA, 0.5 mM	+++
Nonidet P-40, 0.1%	No cells
Nonidet P-40, 0.01%	++

Other treatments in MEM-BSA except for EDTA, which was in Pi/NaCl.

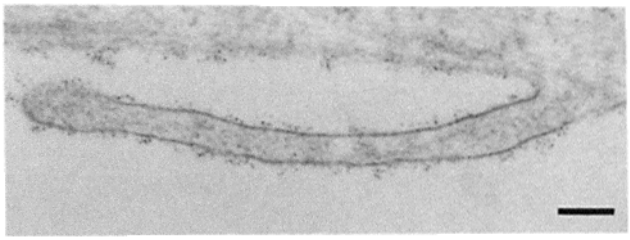

FIGURE 5 Stability of cell surface CS. HES fibroblasts were treated with 0.5 mM EDTA in Pi/NaCl for 30 min at 4°C, fixed, digested with cABC, and immunoferritin stained for CS. Ferritin decorates a microvillus and the cell surface. Bar, 200 nm. × 37,000.

TABLE II

Stability of CS at the Cell Surface as Measured by Enzyme Immunoassay

Chemical	Percentage from control	Significance
	(%)	(P)
cABC 50 mU/ml	100	
cABC 5 mU/ml	81	<0.05
cAC 50 mU/ml	48	<0.001
No enzyme	6	<0.001
Preceding cABC (50 mU/ml):		
NaCl, 0.32 M	98	ns
heparin, 1.0 mg/ml	99	ns
colchicine, 10 μM	102	ns
cytochalasin B, 3 μg/ml	81	<0.01
EDTA, 5 mM	66	<0.005 detachment of most cells
Nonidet P-40, 0.1%	63	<0.005 solubilization of cells
Nonidet P-40, 0.01%	87	<0.05

Cells treated with cABC (50 mU/ml) are indicated as 100% and serve as the comparison group. The percentages are calculated from absorbance ratios obtained in enzyme immunoassay. Background values obtained from preimmune serum were subtracted from the calculation. The figures are the mean of 15 microwells. Difference to comparison group is calculated using Student's *t* test. *ns*, not significant.

compared to that of the unsaturated CS oligosaccharides using immunoenzyme assay. As shown in Fig. 6, only CS oligosaccharides were able to block the antibodies. That this was not due to nonspecific electrostatic interaction is shown by the inability of heparin to inhibit the binding (Fig. 6).

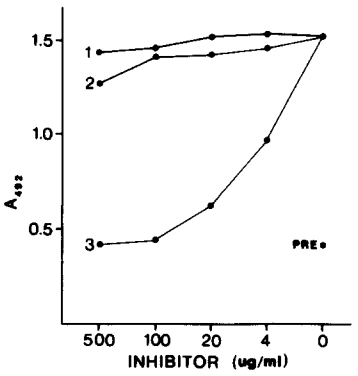

FIGURE 6 Blocking control by immunoenzyme assay. The immune antiserum diluted 1:10 was mixed with various concentrations of heparin (*1*), unsaturated hexasaccharide of hyaluronic acid (*2*), or unsaturated hexasaccharide of CS (*3*), kept overnight at 4°C, diluted further 1:10, and allowed to stain cABC-treated cell cultures in an indirect immunoenzyme assay. Background is shown as the absorbance using preimmune serum (*PRE*).

DISCUSSION

Cultured rat yolk sac tumor cells were recently shown to have a diffuse distribution of CS proteoglycan at the "cell surface" using immunofluorescence microscopy and antibodies raised to the protein domain (24). In the present work we show, using immunofluorescence and immunoferritin electron microscopy, that fibroblasts have CS at the outer surface of their plasma membrane. The antigen recognized by our antibodies is a chemically defined oligosaccharide generated by cABC specifically from the carbohydrate chains of chondroitin-4-sulfate-containing proteoglycans (21). As CS proteoglycans are major components secreted into the medium by fibroblasts, it was important to study the duration and quality of the CS-plasma membrane association. The antigenic CS could be removed neither by incubation of the cells for 30 min in fresh culture medium nor using EDTA, NaCl, or heparin. The results suggest a relatively long half-life at the plasma membrane, at least of a modified antigen, and indicate that forces stronger than those of ionic interaction join the molecule to the membrane. This again points to that the CS-containing molecule has strong, receptor-like interactions with the periphery of the plasma membrane, or, most interestingly, that it would itself occur as an integral plasma membrane component. There is evidence that hepatocytes have a relatively small molecular weight HS proteoglycan as an integral plasma membrane component (26). A small fraction of such cell surface HS proteoglycan could be removed by exogenous heparin (26, 27).

Although not shown, the external CS described in the present work conceivably belongs to a proteoglycan. Cultured fibroblasts seem to produce at least two types of CS proteoglycan (28); the larger contains CS types A and C, whereas the smaller is rich in CS type B (dermatan sulfate). The fact that cAC-treated cell surfaces reacted only weakly with our antiserum could be due to two reasons: (*a*) The antigenic molecules were mostly CS type B (dermatan sulfate), which contains chondroitin-4-sulfate, and is not a substrate of cAC. (*b*) The antibodies preferred cABC-digested cell surfaces over the cAC-digested ones because cAC is known to degrade CS down to a short linkage oligosaccharide, which reacts only minimally with our antiserum (21). Thus, although we convincingly showed that the reactive molecule at the plasma membrane was CS, it is not yet clear which of the CS types was responsible for the antibody binding.

We have previously shown that fibronectin, a pericellular matrix glycoprotein, is found at places in close association with the plasma membrane (1). Even then, using the same indirect immunoferritin procedure, the distance of ferritin particles from the lipid bilayer was longer than in the present case (24 nm) of cell surface CS. These data are permissive for the idea that the CS is part of a plasma membrane component. The CS-bound ferritin particles had a patchy distribution uniformly at all cell surfaces, quite unlike fibronectin, HS proteoglycan, and other pericellular matrix components. The simplest explanation for the observed CS-ferritin distribution would be lateral mobility at the plasma membrane. Such features are characteristic for a class of integral plasma membrane components.

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