Immunocytochemical Localization of Native Chondroitin-Sulfate in Tissues and Cultured Cells Using Specific Monoclonal Antibody

Zafira Avnur and Benjamin Geiger
Department of Chemical Immunology
The Weizmann Institute of Science
Rehovot 76100, Israel

Summary

Chondroitin-sulfate containing proteoglycan (CSPG) of the extracellular matrix (ECM) was visualized in chick tissues and cell cultures with a monoclonal antibody, CS-56. Cultured cells of various origins contained dense punctate layers of CSPG on both the substrate and the cell surface, as determined by immunofluorescent and immunogold staining. Under culture conditions the CSPG-containing matrix was usually excluded from stable cell-to-substrate focal contacts. The substrate-attached CSPG exhibited remarkable chemical stability but could be successfully removed by pronase or chondroitinases ABC and AC. Incubation of living cells with CS-56 antibodies resulted in the clustering of surface CSPG into patches, indicating that the surface-bound CSPG is free to move laterally along the plasma membrane. The unique properties of the CSPG-containing ECM revealed by CS-56 antibodies and their relationships to specific types of cell contacts are discussed.

Introduction

Many cellular activities depend on the interaction of cells with the surrounding extracellular matrix (ECM). Visualization of the spatial distribution of ECM components such as fibronectin, laminin, or collagen was usually performed using immunocytochemical methodologies such as immunofluorescence and immunoperoxidase labeling. The visualization of native GAGs by immunological techniques met serious difficulties due to the low immunogenicity of the GAG molecules. Some progress has nevertheless been made recently with the preparation of antibodies reactive with the protein cores of different PGs (Keiser, 1975; Wieslander and Heinegard, 1979; Vertel and Dorfman, 1979; Dorfman et al., 1980; Sugahara et al., 1981; Hassell et al., 1980) or with chondroitinase-treated PG (Hedman et al., 1982). In view of possible species and organ diversity of the protein cores, it was of interest to prepare specific immunological probes reactive with native GAGs and use them for the localization of these molecules in cells and tissues or to interfere selectively with GAG-mediated biological activities.

We have recently prepared monoclonal antibodies against substrate-attached materials of cultured chick fibroblasts. One of the hybridoma clones, denoted CS-56, produced antibodies that specifically stained the surface of cultured cells and the substrate between them. Solid phase radioimmunoassay indicated that these antibodies react specifically with chondroitin sulfate (CS). In this article we describe the spatial distribution of the CSPG and its relationships to specific cell-substrate contacts and to the cytoskeleton.

Results

Immunocytochemical Labeling of Cells and Tissues with CS-56 Monoclonal Antibody

Cultured chick fibroblasts and chondrocytes were extensively labeled with CS-56 antibodies, displaying staining patterns similar to those shown in Figure 1. At early stages of attachment of chick fibroblasts the distribution of the antigen was restricted to the areas underneath the cells (Figure 1A).

In all cells tested, the label associated with the substrate exhibited a fine punctate pattern and often could be visualized along the migratory pathways of motile cells (Figure 1D). We have noticed, however, that significant staining was also associated with the dorsal surfaces of cells. The fluorescence consisted of densely organized fine dots all over the cell surface with an increased density along the margins of the cells. After 2 hr of incubation (or more) the staining became broadly distributed on the substrate (Figures 1C and 1D). The labeling did not depend on the permeabilization of cells prior to fixation, though the cell-associated fluorescence appeared more extensive when the cells were pretreated with Triton X-100 before fixation. We have thus concluded that the antigen labeled by CS-56 antibodies is primarily extracellular.

Cultured chondrocytes produced large amounts of surface- and substrate-bound antigen, forming densely intermeshed networks and plaques (Figures 1E and 1F). The amounts of substrate-attached antigen produced by chondrocytes, as well as the fine pattern of labeling, were distinctly different from those detected near residual fibroblasts within the same cultures (insert in Figure 1F, see legend).

The antibodies exhibited very broad species and cell-type specificities. Among the cultured cells positively labeled were human foreskin fibroblasts, Chinese hamster ovary cells, baby hamster kidney cells, human HeLa and CCl6 cells, mouse 3T3 fibroblasts, melanoma line 6K-1735 and B-16, cultured human chondrosarcoma and osteosarcoma (obtained from Dr. R. Lotan at this Institute), and lines of polarized epithelium from dog kidney (MDCK) or bovine kidney (MDBK). In spite of some variability in the patterns and extent of labeling, the overall punctate staining in these cells was generally similar to that found in cultured chick fibroblasts.

Immunofluorescent labeling with CS-56 antibodies of frozen sections of a variety of chicken tissues, including intestine and muscle, indicated that the antigen was present predominantly in intercellular spaces associated with connective tissue (not shown). In the intestine, staining was observed along the basement membrane and in the lamina propria, while in skeletal and smooth muscles it was predominantly associated with the connective tissue surrounding the sarcolemma. Longitudinal sections of skeletal
Cells plated on glass coverslips were fixed with paraformaldehyde 30 min (A), 120 min (B-D), or 24 hr (E, F) after plating. Details of fixation, permeabilization, and immunofluorescent staining are as described (Avnur and Geiger, 1981). The CS-56 antibodies (ascites fluid) used here were diluted 1:50 in PBS and followed by rhodamine-labeled goat anti-mouse F(ab')2. It is notable that the staining is composed of dense arrays of dots found on both the substrate and the cell surface. The inset in F shows the substrate around a residual fibroblast detected in the chondrocyte culture. Bar represents 10 μm.
Localization of Chondroitin Sulfate in Cells and Tissues

Figure 2. Immuno-electron Microscopic Labeling of Cultured Chick Fibroblasts with CS-56 mcABs

Cells were fixed with glutaraldehyde and indirectly labeled with 1:20 dilution of the ascitic fluids of CS-56 and goat anti-mouse Fab' coupled to 5 nm gold particles. (A) survey photomicrograph showing CSPG on the dorsal cell membrane (arrowheads) and on the substrate (arrows). (B) and (C) photomicrograph taken at higher magnification showing immunogold label associated with the dorsal cell surface and with the substrate, respectively. The bar indicates 0.1 μm.

Muscle often revealed a fine striated pattern at the periphery of the sarcomers, similar to the reported "costameric" arrangement of several cytoskeletal proteins (Pardo et al., 1983).

At the electron microscope level extensive indirect labeling with CS-56 antibodies and secondary immunogold reagent (see Experimental Procedures) was observed both on the cell surface (Figures 2A and 2B) and on the substrate (Figures 2A and 2C). The immunogold labeling was generally clustered in both locations and apparently associated with amorphous material (see arrows), suggesting that the antigen was not an integral component of the membrane but rather attached to the membrane at the cell exterior. We have often noticed that the extent of
The Antigenic Specificity of CS-56 Antibodies

The cellular distribution of the antigen bound by CS-56 antibodies and its stability towards extraction suggested the possibility that it might be a proteoglycan of the extracellular matrix (ECM). Four major lines of evidence indicated that the epitope bound by CS-56 antibodies is associated with the GAG moieties of chondroitin sulfate-containing proteoglycan (CSPG). These indications included solid-phase radioimmunoassay with purified GAGs, sensitivity of the antigen to highly purified chondroitinases ABC and AC as well as to pronase, immunoprecipitation of 35S-sulfate containing materials from cells and culture medium, and high resistance of the antigen to chemical extractions.

Solid-phase radioimmunoassay with purified CS types A, B, or C was carried out in microtiter plates. The results shown in Figure 3 indicated that CS-56 antibodies reacted specifically with chondroitin sulfate of both types A and C but not with type B (dermatan sulfate). Moreover, affinity adsorption of CS-56 antibodies on CS A or CS C (but not on CS-B)-coated plates abolished completely their capacity to stain cultured cells. Additional determination of the reactivity of CS-56 antibodies with purified GAGs (kindly performed by Drs. G. Nicolson and F. Tresler, from the Cancer Center M. D. Anderson Hospital, Houston, TX) using, in an ELISA assay, HPLC-purified GAGs indicated that the antibodies also cross-reacted with heparan sulfate, but were negative for heparin. To exclude further the possibility that CS-56 antibodies react with residual protein stubs that may be left in the commercial GAG preparation used, we treated the CS solutions with 30 μg/ml pronase. We have found that this treatment had no measurable effect on the antigenic reactivity of the antigens. To characterize the cellular antigen we have carried out the radioimmunoassay directly on monolayers of viable cells. In performing such experiments on cells maintained in culture for variable periods of time, we have found that the level of antibody binding in plateau (antibody excess) was roughly proportional to the time of incubation of the assay fibroblasts following plating and that no binding of CS-56 was obtained unless cells were present. Furthermore, multiwell cultures of human fibroblasts were incubated with CS-56 antibodies at subsaturating dilutions in the presence of increasing concentrations of purified CS-C, CS-A, or CS-B. As shown in Figure 4A, the first two GAGs inhibited the binding of CS-56 to the cells while CS-B was not effective. It should be pointed out that neither CS-C nor CS-A had any inhibitory effect on the binding of other ECM antibodies (anti-fibronectin) to the same cells under the same assay conditions (Figure 4B). It should be added that CS-A or CS-C-coated dishes were used for an effective affinity chromatography of CS-56 antibodies. Incubation of CS-56 with such plates resulted in specific depletion of antibodies from the supernatant, leading to inability of the supernatant to bind to CS or to cultured cells. Inhibition experiments indicated that the antigen was also secreted into the culture medium. Thus when the antibodies were mixed with media of fibroblast cultures (2-3 days after plating) an effective inhibition of binding was obtained. The same type of binding and inhibition experiments carried out with different cell types yielded similar results. This corroborated the immunofluorescent data described above, which indicated that the CS-56 epitope was not species- or cell-type-specific.

Further characterization was made by the immunoprecipitation of the antigen from the medium and cell lysates of cultured 35S-sulfate-labeled chick fibroblasts. The cells were cultured for 16 hr in the presence of 35S-sulfate, then the medium was removed and the cells were washed with medium and lysed with RIPA (Geiger, 1979). The high molecular weight components in the medium were isolated by gel filtration through a short Sephadex G-50 column, and the cell extract and the fraction in the medium were subjected to immunoprecipitation with CS-56 antibodies. The results indicated that CS-56 could immunoprecipitate...
specifically 35S-sulfate-labeled material from both the culture medium and cell extracts of human foreskin fibroblasts. The radioactivities specifically immunoprecipitated with CS-56 in cell extract and medium were 2.1 × 10^4 and 1.9 × 10^4 cpm per 10^6 cells, respectively (immunoprecipitation with monoclonal antibodies to fibronectin was used here as control). This amounts to 7% and 21% of the total TCA-insoluble radioactivity in the two respective fractions. Moreover, we have found that incubation of cells with pronase (100 μg/ml) resulted in the release of immunoprecipitable, 35S-sulfate-containing material from both the culture medium and cell extracts of human foreskin fibroblasts. The radioactivities specifically immunoprecipitated with monoclonal antibodies to fibronectin was used here as control. This amounts to 7% and 21% of the total TCA-insoluble radioactivity in the two respective fractions. Moreover, we have found that incubation of cells with pronase (100 μg/ml) resulted in the release of immunoprecipitable, 35S-sulfate-containing material from the substrate. It was thus concluded that the cellular antigen bound by CS-56 antibodies contains sulfate moieties and can be released (but not destroyed) by pronase.

Table 1. Extraction of CSPG from Cultured Chick Fibroblasts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration</th>
<th>Substrate Bound CSPG</th>
<th>Cell Bound CSPG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detergents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP40 1%</td>
<td>1-16 hr</td>
<td>-</td>
<td>±b</td>
</tr>
<tr>
<td>Triton X-100 1%</td>
<td>1-16 hr</td>
<td>-</td>
<td>±b</td>
</tr>
<tr>
<td>NP40 1%</td>
<td>30-60 min</td>
<td>-</td>
<td>CR</td>
</tr>
<tr>
<td>High-Salt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M KI</td>
<td>30 min</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>3 M KCl</td>
<td>30 min</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>Denaturing Agents</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R M urea</td>
<td>1-16 hr</td>
<td>-</td>
<td>CR</td>
</tr>
<tr>
<td>+ 5 mM β-mercaptoethanol</td>
<td></td>
<td>-</td>
<td>CR</td>
</tr>
<tr>
<td>4 M guanidine HCl</td>
<td>1-48 hr</td>
<td>±</td>
<td>CR</td>
</tr>
<tr>
<td>8 M urea + 1.5 KCl</td>
<td>1-16 hr</td>
<td>-</td>
<td>CR</td>
</tr>
<tr>
<td>+ 1% Triton + 5 mM β-mercaptoethanol</td>
<td></td>
<td>-</td>
<td>CR</td>
</tr>
<tr>
<td>Enzymes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin 1-20 μg/ml</td>
<td>10-120 min</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chymotrypsin 1-20 μg/ml</td>
<td>10-90 min</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Pepsin 30-50 μg/ml</td>
<td>10-30 min</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>Pronase 5-100 μg/ml</td>
<td>10-30 min</td>
<td>±</td>
<td>CR</td>
</tr>
<tr>
<td>Chondroitinase AC 5 U/ml</td>
<td>30 min</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chondroitinase ABC 5 U/ml</td>
<td>30 min</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Heparitinase 5 U/ml</td>
<td>30 min</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Subconfluent cultures of chicken gizzard cells were treated as specified, then fixed and immunofluorescently labeled with CS-56 antibodies. Removal of CSPG is indicated by ±; partial removal by ±.
* Partial depletion of CSPG from cell surfaces was obtained after 2-10 min treatments; longer extractions resulted in complete detachment of cells.
* CR cells removed by the treatment.
* Pepsin in concentration of more than 30 μg/ml caused progressive cell detachment.

The Spatial Relationships between CSPG and Cell-Substrate Focal Contacts

The distribution patterns of substrate-attached CSPG in fibroblast cultures were often non-uniform both under the cells and outside their borders. Several lines of evidence, including extensive permeabilization and removal of cells from the substrate, suggested that the non-uniform labeling reflected a true variability in the distribution of CSPG and was not due to restricted penetration of the immune reagents (see Avnur and Geiger, 1981a). Comparison of the immunofluorescent patterns obtained with CS-56 antibodies to the interference-reflection microscopic (IRM) images of the same cells (Figures 5A and 5B, respectively) indicated that focal contacts were usually depleted from the substrate, as expected for cartilage CS (Hascall and Sajdera, 1969; Roden, 1980). More specific information was obtained from the enzymatic treatments (Table 1). Short incubation of the cells with either trypsin or chymotrypsin did not lead to the removal of the CSPG from either the substrate or from the surface of the cells that were left attached. Longer incubations with these enzymes (up to several hours) caused complete detachment of the cells from the substrate but still had no, or very limited, effect on the immunolabeling of the substrate-attached CSPG. Papain treatment was partially effective in removing CSPG from either the cells or the substrate, and a marked reduction in labeling was observed after treatment with pronase or chondroitinas ABC or AC. Highly purified heparitinase, which cleaves heparin and heparan sulfate GAGs, had very little effect on the distribution of the antigen on the substrate and partial removal of the antigen was noticed only after prolonged incubations (1 hr or more). It should be mentioned that for every enzyme treatment we have run parallel buffer controls, all of which had no detectable effect on the cells or substrate-bound CSPG.
Figure 5. Spatial Interrelationships between CSPG, Localized with CS-56 Antibodies (A and C), and Focal Contacts, Localized by Interference Reflection Microscopy (B) or by Labeling for Vinculin (D)

Arrows point to the same CSPG-free focal contacts. To correlate the labels for CSPG and vinculin (C and D) a positive transparency of vinculin labeling was matched with negative transparency of CSPG label and used for printing the picture in (E). Black areas in (E) represent vinculin-rich focal contacts on CSPG-free region (arrow). Vinculin-containing areas on top of CSPG matrix appear gray (arrowhead). In (F) the black areas represent focal contacts on CSPG-free areas and the dotted areas focal contact on top of CSPG matrix. Bar indicates 10 μm.
indicated that the CSPG was largely depleted from underneath the ends of stress fibers and sometimes from regions along their length (not shown). In order to correlate the spatial distribution of CSPG to focal contacts more accurately, we have double labeled the cultured fibroblasts for CSPG and vinculin.

In stationary cultured fibroblasts, the correlation between vinculin-containing sites and CSPG-free areas was more readily apparent (Figure 5, C–F). To compare precisely the distributions of CSPG (Figure 5C) and vinculin (Figure 5D), we have prepared positive transparencies of vinculin labeling and negative transparencies of immunofluorescent labeling for CSPG. The two transparencies were overlapped and used for printing of combined pictures such as the one shown in Figure 5E. CSPG-free areas that overlapped with vinculin-rich focal contacts appeared black in the compound picture while regions in which both were present appeared gray. To delineate clearly the spatial relationships between the two labels, we have added here a schematic drawing (Figure 5F) in which vinculin-rich focal contacts located over CSPG-free areas are marked in black while vinculin-rich focal contacts positioned on top of the CSPG-containing matrix are marked with dots. This analysis indicated that in certain regions of the cells essentially all focal contacts were positioned over CSPG-free sites. Exceptions to this were small (and probably new) vinculin-containing contacts in the same region, which might have been formed on top of the CSPG layer. In other regions of the same cells (see, for example, lower left part of the cell shown in Figure 5F), many of the focal contacts were located over a uniform or nearly uniform layer of CSPG. We have not observed an accumulation or "piling up" of CSPG near the edges of focal contacts as we previously reported for fibronectin (Avnur and Geiger, 1981a).

It thus appears that focal contacts are often devoid of CSPG labeling due either to the absence of secretory activity in these sites or to inaccessibility of these areas to CSPG released into the culture medium.

**Spatial Relationships between CSPG and Other ECM Components**

To determine directly the spatial relationships between CSPG and other defined protein components of the ECM, we have double-immunolabeled the same cultures with CS-56 antibodies and anti-fibronectin or anti-collagen (not shown). The results indicated that CSPG matrix displayed two patterns of distribution; the diffuse, substrate-attached CSPG was distinctly different from either fibronectin or collagen while some CSPG associated with fibrillary structures was closely related to both matrix proteins. The latter pattern was most abundant after prolonged incubation of the cells in culture.

**Antibody-Mediated Rearrangement of CSPG on the Surface of Cultured Fibroblasts**

Fixed cultured chick gizzard cells exhibited a dense uniform dotted labeling with CS-56 antibodies over their entire dorsal surfaces (see Figure 1B). We have noticed, however, that when living cells were incubated with these antibodies, the surface labeling appeared patchy. The redistribution of surface-bound CS was induced in several alternative ways. The cells were either incubated with CS-56 antibodies at 37°C or at 0–4°C for either 5 min or 30 min. At the end of incubation the cells were washed, fixed with formaldehyde, and labeled with rhodamine-conjugated goat anti-mouse IgG. Alternatively, we have exposed the cells to the two antibodies and examined the "indirect patches." Comparison of the distribution of surface CSPG after the various treatments indicated that the extent of patching increased with time and temperature. These effects could be best demonstrated by comparison of the two extreme conditions, namely 30 min incubation at 37°C and 5 min incubation of CS-56 antibodies alone at 0°C prior to fixation. The indirect patches obtained in cells treated with two layers of antibodies were usually larger and apparently formed more rapidly (The comparison of the various treatments is not shown here; nevertheless, the general appearance of the surface patches following incubation of the cells with CS-56 antibodies is shown in Figure 6).

Interestingly, at early stages of patching at both high and low temperatures, the patches often formed linear arrays along the cell surface. This "lining up" was reminiscent of the ligand-induced anchorage of surface receptors to actin filaments (Ash and Singer, 1976, Ash et al., 1977). To examine the relationships between the surface patches and the cytoskeleton, we have carried out double-labeling experiments in which CSPG was redistributed using antibodies coupled to one fluorophore, and actin, in the same cells, localized with phalloidin coupled to another fluorophore. As shown in Figure 6, the "lined-up" patches of CSPG were spatially related to stress fibers. This correlation could be best visualized in flat regions of the cells where individual stress fibers could be resolved. In these regions it was apparent that the linear assay of CSPG patches and actin-containing stress fibers were overlapping rather than interdigitated.

**The Effect of CS-56 Antibodies on the Biological Activity of CS**

The addition of CS-56 antibodies to cultures of chick, mouse, or human fibroblasts during plating had little effect on the rate of initial substrate attachment and the subsequent spreading. We have, however, noticed that addition of CS-C or CS-A to these cells during plating significantly increased the rates of cell attachment. This effect was dose-dependent and reached maximal values at CS-C concentrations of 0.3–0.9 mg/ml for human foreskin fibroblasts or mouse 3T3 cells. The addition of CS-56 antibodies to CS-C or CS-A prior to the incubation with the cells inhibited this CS-mediated enhancement of adhesion. The extent of inhibition obtained in different experiments or with other cell types was somewhat variable (possibly due to production and secretion of endogenous CS) but usually reached values of about 70% or more. The same effect
Figure 6. Spatial Relationships between Antibody-Clustered Surface CSPG (A) and Intracellular Actin Bundles (B)
The cells were incubated with CS-56 antibodies for 10 min at room temperature and then with rhodamine-labeled goat anti-mouse-F(ab')2 (5 min at 37°C). The cultures were then fixed and labeled with fluorescein-bound phalloidin. The arrowheads point to several matching arrays of CSPG and actin. The bar indicates 10 μm.

and essentially the same inhibition values were also obtained with CS-A (not shown).

Discussion

We have described here the distribution of CS-like material in tissues and cultured cells using a GAG-specific monoclonal antibody. Several lines of evidence were obtained in this study, indicating that CS-56 antibodies indeed react with proteoglycan and primarily with its chondroitin sulfate moieties. The antibody binds specifically to CS types A and C and, to some extent, to heparin sulfate. The former two GAGs also inhibit specifically the binding of the antibodies to cells. The protein content in the commercial preparations of CS used was found to be lower than 1% and the antigenicity was not sensitive to pronase. Second, CS-56 antibodies specifically precipitated 35S-sulfate labeled molecules from cells and culture medium. Finally, the cell- and substrate-associated antigen was susceptible to chondroitinase treatment (both ABC and AC). These enzymes are highly specific for CS and they essentially abolished the antigenic reactivity of CS or proteoglycan-bound CS. It should be pointed out that other GAGs, including hyaluronic acid, chondroitin, heparin, heparin sulfate, heparin sulfate, or keratin sulfate, are not cleaved by both enzymes (Yamagata et al., 1968). Moreover, the substrate-attached antigen is relatively stable towards purified heparitinase. This finding is interesting in view of previous reports on the presence of heparan sulfate on the cell surface and in basement membranes (Hasell et al., 1980; Kjellen et al., 1980; Kanwar and Farquhar, 1979; Hedman et al., 1982). In view of the reactivity obtained also with heparan sulfate in the ELISA test, we cannot exclude the possibility that the antibody may also bind to this GAG in cell cultures. Nevertheless, the extensive reactivity of the antibodies with CS and the pronounced sensitivity of the antigen to chondroitinases renders it likely that the primary reaction is indeed with CS.

Finally, the CS-like material showed remarkable stability towards extraction from either the substrate or the cell surface. The only chemical treatment that was even partially effective involved long guanidine-HCl treatment. This is in line with biochemical data indicating that proteoglycans (from cartilage) can be solubilized by 4 M GuHCl (Hascall and Sajdera, 1969; Roden, 1980). The exact molecular structure of the epitope on CS that is recognized by the antibodies is not yet known.

The specificity of CS-56 antibodies to intact CSPG renders them unique among the various PG-specific antibodies described in recent years. These antibodies were usually directed against the protein cores of PG (Keiser, 1975; Wieslander and Heingard, 1979; Vertel and Dorfman, 1979; Dorfman et al., 1980; Christner et al., 1980; Hassell et al., 1980; Sugahara et al., 1981; Baker et al., 1982), or were specific to chondroitinase ABC-digested CSPG (Hedman et al., 1982, 1983), and did not react with the native untreated GAGs. The former antibodies have often exhibited tissue and species specificity and were not used for the immunocytochemical localization of specific native GAGs in cells or tissues. The latter antibodies (Hedman et al., 1982, 1983) were used to show the spatial relationships between CSPG and other ECM components (see Discussion). In that respect, the antibodies described here reveal a CS-containing ECM, the structure and organization of which could not be observed before.

A broad survey of different cell lines and primary cultures
indicated that in essentially all cases CS-containing ECM was present. This was especially evident in cultures of chick chondrocytes, as shown in Figure 1 above. As pointed out, a large variety of additional adherent cells was tested, including fibroblasts, epithelial cells, neuronal (neuroblastoma) and glial lines, melanocytes, etc. All these cells produced and secreted similar CSPG-containing matrices. The CS-like material was produced and deposited on the substrate by both transformed and nontransformed cells, though differences in both pattern of distribution and apparent amounts were noted. A more detailed survey of the CS-containing ECM in such cell lines is in progress.

Immunofluorescent and immuno-EM labeling with CS-56 antibodies indicated that the CS-containing matrix had a fine granular substructure. Early after plating, CSPG appeared in sparsely spaced clusters ranging in size from about 0.2 to 0.5 \( \mu m \). With an increase in time of incubation, the apparent density of the CS-containing dots increased until they covered the entire substrate and substrate-attached materials. Immuno-EM labeling corroborated these observations and indicated that the immunogold particles were associated with aggregates associated with the substrate or with the cell surface. We would like to raise the possibility that these structures are related to PG aggregates formed by isolated cartilage PG (Lascall and Sajdera, 1969; Rosenberg et al., 1975).

Another feature of the CS-containing ECM was revealed by double immunofluorescent labeling with CS-56 antibodies in conjunction with either anti-fibronectin or anti-collagen. Recent experiments carried out in several laboratories have suggested that PG or GAGs may interact with these ECM proteins (Vertel and Dorfman, 1979; Rich et al., 1981; Stamatoglou and Keller, 1982; Klebe and Mock, 1982; Laterra et al., 1983a, and 1983b; Hedman et al., 1982). This conclusion was based on biochemical analyses, on the physiological effects of these components, or on direct immunocytochemical localization.

The experiments described here provided direct information on the spatial interrelationships of CSPG and the fibronectin-collagen network. (The latter two proteins did show extensive overlapping in their distribution, as described before (Vaheri et al., 1978; Furcht et al., 1980). It was observed that the two matrices were primarily independent, and only after prolonged culturing, when extensive fibronectin and collagen matrices were formed, CSPG showed apparent association with the two proteins (detailed documentation of this will be published elsewhere). This may explain the differences between our observations, which were based on relatively short incubations of the cells under culture conditions (between several hours and two days), and those of Hedman et al. (1982), who kept the cells for 5 days in culture prior to labeling. We propose that such associations are a result of deposition of the CSPG on the fibronectin- or collagen-coated substrate.

Comparison of CSPG distribution to focal contacts of chick fibroblasts indicated that often the latter were present "on top" of CSPG-free areas. This partial exclusion of CS-56 labeling from focal contacts could not be attributed merely to lack of accessibility of these regions to the antibodies since the same patterns were seen after extensive detergent permeabilization prior to fixation and could also be detected in regions from which cells moved away or detached during fixation. Such relationships could not be seen when cells apparently formed new contacts on a preexisting CSPG layer either during locomotion or during local rearrangements of its ventral surfaces. Immunofluorescent labeling of substrate-attached chondrocytes indicated that CSPG was largely absent from focal contacts of the residual fibroblasts while the carpet was essentially unperturbed under the chondrocytes; we have found that the latter formed only a few small substrate contacts with the underlying ECM.

On the basis of these observations we could draw several conclusions: CSPG is not an essential component of focal contacts, nor does it prevent their formation; secreted CSPG cannot penetrate into the cell-substrate gap of focal contacts and it is not released locally in these sites; unlike fibronectin, which is actively removed from the substrate by the cells and rearranged into cables (Avnur and Geiger, 1981a), substrate-attached CSPG seems to be tightly bound to exposed areas in the substrate and undergoes minimal rearrangement. This does not apply of course, to the fraction of CSPG that is deposited on fibronectin or collagen and may be rearranged together with these proteins.

Another point we have considered is related to the physiological effects of GAGs on cell attachment and spreading. In one series of experiments, we tried to modify cell attachment and spreading with CS-56 antibodies by the addition of the antibodies to cells during plating or following attachment and spreading. The effects of CS-56 antibodies on the rate of attachment of chick fibroblasts, 3T3 cells, or human fibroblasts were limited and only some rounding-up was reproducibly noticed. In view of the complexity of the ECM produced by the cells and the functional and spatial interrelationships between its various components, we regard these studies as difficult, if not impossible, to interpret in molecular terms.

The last aspect we will discuss here is related to the CS-like material on the dorsal surfaces of cultured cells. The membrane-bound antigen was similar in many respects to the ECM-bound CSPG. Both were sensitive to chondroitinases and were mildly affected by high-salt buffers or non ionic detergents. Moreover, both substrate and cell-bound CS had granular or punctate appearances. A major difference between the two populations of CS-like molecules was in their dynamic properties: unlike the CSPG on the substrate, which was essentially immobile, the membrane-bound molecules of viable cells were laterally mobile and underwent progressive patching in the presence of CS-56 antibodies as previously reported for a large variety of integral membrane proteins (for review see Schreiner and Unanue, 1976; Nicolson, 1976). The extent of patching, as visualized by immunofluorescent microscopy, depended on the extent of cross-linking and the
time and temperature of incubation. The surface patches formed at early stages were spatially related to actin, as documented before for several membrane components (Ash and Singer, 1976; Ash et al., 1977). These observations could be explained in either of two ways—by the presence of integral membrane component(s) with CS-like moieties or by the presence of a surface complex of CSPG bound to its membrane receptor(s). The immunoelectron microscopic data strongly support the latter possibility since the surface immunogold labeling was associated with membrane-bound materials of various shapes and did not seem to be restricted to the immediate vicinity of the plasma membrane.

The aspect under discussed here represent only a first step in the immunocytochemical characterization of native CSPG matrix in cells and tissues. We anticipate that the use of CS-66 antibodies, as well as antibodies to other ECM components, will improve our understanding of the spatial and temporal activities of PG-containing ECM and its role in the regulation of cellular interactions.

**Experimental Procedures**

**Preparation of Ventral Membranes**

Ventral membranes of chicken gizzard (CG) fibroblasts were prepared essentially as described (Avnur and Geiger, 1981b). Cells were incubated with 1 mM ZnCl₂ in 50 mM MES buffer, 5 mM MgCl₂, 1 mM EGTA, pH 6.0, then sheared with a stream of PBS (pH 7.2). In the preparation of ventral membranes for immunization we employed rigorous shearing, which left only few intact cells on the culture plate as verified by phase-contrast microscopy. The residual membranes were scraped from the plate with a moist policeman and used for immunization.

**Immunization**

Tim.e: 3-month-old BALB/c mice were injected in the first and second subcutaneously with ventral membrane suspensions (40 μg protein per mouse, emulsified in complete Freund's adjuvant). Injection was repeated twice in 3 week intervals and the formation of antibodies was monitored by solid-phase radioimmunoassay (see below).

**Cell Fusion and Growth**

Four days after the last (third) immunization, the spleens of two mice showing high antibody titers were removed and the cells from each individual spleen were fused with NSO myeloma line (Galfre and Milstein, 1981) at a ratio of 5:1, respectively, using 4% polyethylene glycol 1500 (Serva, Heidelberg, FRG) as described previously (Eshhar et al., 1979). Following fusion, cells were distributed into flat-bottom microtiter plates (96 wells each, Falcon, USA) at a concentration of 5 × 10⁴ viable myeloma cells/well.

The cells were cultured in HAT-containing medium (Littlefield, 1964) in a humidified incubator under an atmosphere of 8% CO₂ and 95% air. The growth medium was Dulbecco's modified Eagle's medium (DMEM, high glucose, Gibco USA) supplemented with 1 mM pyruvate, 2 mM glutamine, 10 U/ml penicillin, 20 μg/ml streptomycin, and 20% heat-inactivated horse serum (HS, purchased either from Gibco, USA or from Biolog, Israel). Screening of antibody activity in culture supernatants was carried out by solid-phase radioimmunoassay and immunofluorescence labeling.

From 900 culture wells (10 microtiter plates) we selected 86 clones that reacted specifically with cell culture. Over 50 of those lines showed specific reactivity with ECM components. Selected hybridoma lines showing unique distributions on the surface of cells or on the substrate were weaned out of HAT, cloned in agar, and propagated in ascites form in prystane-(i.e., 11.14-tetramethylpentadecane, Aldrich Chemical Co., USA)-injected BALB/c mice.

**Solid-Phase Radioimmunoassay on Substrate-Attached Cells**

Chicken gizzard cells or human foreskin fibroblasts were plated at confluence in flat-bottom 96-well culture plates (Falcon USA) and cultured for about 24 hr. The monolayers were washed twice with medium and 50 μl of the individual hybridoma culture fluids were added to each well. The cultures were further incubated for 30 min at 37°C and washed with medium. Subsequently, 25 μl of 125I-labeled purified goat anti-mouse Ig (containing 10⁶ cpm) diluted in culture medium was added to each well for 30 min at 37°C. After three washes 100 μl of 0.1 N NaOH was added to each well to lyse the cells, and the radioactivity in 90 μl aliquots was counted in a gamma scintillation counter.

**Characterization of Immunoglobulin Class of Monoclonal Antibodies**

The immunoglobulin (Ig) class and subclass of the different monoclonal antibodies secreted into the cultured medium was determined by double immunodiffusion in agar using class- and subclass-specific anti-mouse Ig antibodies (Melloy, Springfield, VA, USA). In this assay we have found exclusive reactivity of CS-56 antibody (in culture supernatant) with μ chainspecific anti IgM.

**Cells**

Cultures of chicken gizzard cells were prepared from 0-10 day embryos and maintained in culture in DMEM containing 10% fetal calf serum cultures. Cells were maintained in a humidified incubator under an atmosphere of 8% CO₂ and 95% air at 37°C. For the experiments described below we used cells of passages 2-8. Cells used for microscopic observation were plated on 18 or 22 mm square glass coverslips. NOC, nonproducing myeloma cells for fusion, were originally obtained from Dr. C. Milstein and kindly supplied to us by Dr. Z. Eshhar from our department. Different cell lines used in this study were obtained from the American Type Culture Collection (ATCC) and chick chondrocytes were kindly supplied by Dr. Z. Nevo of Tel Aviv University (Nevo et al., 1972).

**Enzymes**

TPOX transpeptidase, 209 U/mg, α-chymotrypsin (X3 crystallized), and papain 192 U/mg were obtained from Worthington Co. (Freehold, USA), and pronase was bought from Sigma. Chondroitinase AC, ABC, and heparitinase were from Seikagaku Kogyo Co. (Tokyo, Japan).

**Immunochemical Reagents**

Rabbit antibodies to vinculin and to human cold insoluble globulin (plasma fibronectin) were prepared as described earlier (Geiger, 1979; Avnur and Geiger, 1981a). Antibodies to rat tail tendon collagen were obtained from Dr. S. Fuchs of this department (Meez et al., 1973). Monoclonal antibodies denoted FN-54 react specifically with fibronectin of chick and mammalian cells. The antibodies were raised by the same procedure used for CS-56. All antibodies were purified on immunoadsorbents or the respective antigens immobilized on glutaraldehyde-activated Ultrogel ACA 22 (B&F, France), according to Ternynck and Avrameas (1976), or on activated sepharose 4B. Fluorescein- or rhodamine-labeled phalloidin was kindly provided by Dr. Fautsch, from the Max Planck Institute in Heidelberg.

Affinity-purified goat antibodies to rabbit or mouse IgG were coupled to lisamine modadine B-sulfonyl chloride and further chromatographed on DEAE cellulose (Brandtszag, 1973). The fraction containing 3 to 4 fluorescent per μg molecule was selected and stored until use at 4°C in the presence of 10 mM sodium azide.

**Immunofluorescent Microscopy**

Cultured cells on glass coverslips were fixed with 3% paraformaldehyde ether with or without previous detergent (Triton X-100) permeabilization (Avnur and Geiger, 1981a). Tissues were fixed with 3% paraformaldehyde and embedded in paraffin or directly frozen in liquid nitrogen-cooled isopentane. Sections of the tissue blocks (4-5 μm thickness) were prepared, postfixed with ethanol, and immunofluorescently labeled indirectly. Fluorescence microscopy was performed with a Zeiss photomicroscope III equipped with filter cells for double (fluorescein-modadine) immunofluorescence (Geiger and Singer, 1979). Interscience-reflection microscopy was performed in photomicroscope III as previously described (Geiger, 1979), with an extra polarizer and DIC slide together with an Antilex neofluor X63/1.25 objective.

**Immunoelectron microscopy**

Monolayer cultures of chick fibroblasts were fixed in 1% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2, stained with CS-56 antibodies for 30
min, and then with affinity-purified goat anti mouse F(ab')2 linked to colloidal gold particles of 5 nm diameter (Janssen Pharmaceutica, Beerse, Belgium). Second fixation was performed with 2% glutaraldehyde in 0.1 M cacodylate buffer, post-fixation with 1% osmium tetroxide in the same buffer, and staining with 1% Uranyl acetate in water. The cells were dehydrated in ethanol, flat embedded in open Polybed 812 (Polysciences, USA), and areas containing cells at proper densities were reembedded in epon. Ultrathin sections were cut at right angles to the plane of the substrate. The sections were rehydrated with uranyl acetate and lead nitrate and examined in a Philips EM-300 at 90 kV.

Solid-Phase Radioimmunoassay with GAG-Coated Plates

The wells of V-shaped microtitre plates (Cooke Labs, USA) were treated with 1% glutaraldehyde in H2O for 5 min. After three washes with PBS the wells were coated for 2 hr at room temperature with different concentrations of: chondroitin sulfate type A (from whale cartilage, C-4134, Sigma), chondroitin sulfate type B (from porcine skin, C-4259, Sigma), or chondroitin sulfate type C (from shark cartilage, C-4384, Sigma). After three washes with 1% BSA-PBS and 2 hr incubation in BSA-PBS, 50 µl of CS-GS antibodies in different dilutions (or equal amounts of irrelevant antibody) were added to each well and incubated for 1 hr at room temperature. After three washes with BSA-PBS, 50 µl of 125I-labeled purified goat anti-mouse IgG (~106 cpm) was added to each well, followed by an incubation for 1 hr at room temperature. After four washes the amount of bound radioactive antibodies was determined in a gamma counter.

Acknowledgments

We would like to express our gratitude to Dr. Z. Eshhar for his help and advice with the preparation of monoclonal antibodies, and to Ms. T. Volk for her assistance with electron microscopy. We are grateful to Drs. G. Brandtzaeg, P. Nicolson and R. Tresler from the Cancer Center M. D. Anderson Hospital, Houston, Texas, for their help in determining the specificity of our antibodies, and to Dr. F. Grinnell for a critical review of the manuscript. This study was supported by a grant from the Muscular Dystrophy Association.

This study was supported by a grant from the Muscular Dystrophy Association.

References


