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Spatial Interrelationships between Proteoglycans and Extracellular Matrix Proteins in Cell Cultures

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Immunolabelling of cultured cells for chondroitin-sulfate proteoglycan (CSPG), in conjunction with antibodies to fibronectin, collagen and laminin, revealed the spatial interrelationships between the different matrix components. CSPG was organized in two major forms. Fibronectin-independent dotted patterns of CSPG were detected on the substrate and cell surfaces at early stages after plating. At later stages, however, significant overlapping was found between the two extracellular matrix components. Immunoelectron microscopic examination indicated that the CSPG was organized as granules of varying sizes which were associated with the cell surface, the substrate, or with the periphery of the fibronectin network. © 1985 Academic Press, Inc.

Most cells, in intact tissues and in culture, are attached to an extracellular matrix (ECM, for review see [1–3]). Epithelial cells are associated with the basement membrane, fibroblastic cells are usually embedded in a pericellular mesh of fibrils and tissue culture cells usually grow on a substrate which is coated by various ECM components. Many studies in the last few years have indicated that the matrix or its various isolated components provide not only adhesive surfaces for cells to grow on but has far-reaching effects on the rate of cell growth, motility, morphogenesis and differentiation (e.g. [1–7]). Within the ECM several glycoproteins and proteoglycans were identified and it was proposed that the different constituents interact with each other in a rather complex fashion. Among the ECM constituents we may mention fibronectin, collagen, laminin, and other 'adhesive' proteins as well as heparan sulfate- and chondroitin sulfate-containing proteoglycans [3, 9–13]. In vitro experiments indicated that some of the ECM components can interact with each other [14–16] and double immunofluorescent labelling for collagen and fibronectin indicated that the two largely coincided [17]. Less defined were the correlations between the latter two proteins and proteoglycans. The poor antigenicity of PG, especially of their glycosaminoglycan (GAG) moieties rendered it difficult to localize these molecules in tissue and cell cultures. Nevertheless, recent studies suggested that fibronectin and chondroitin sulfate-containing proteoglycan (CSPG) are largely superimposed.

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This was carried out with antibodies reactive against chondroitinase-treated CSPG [18, 19].

We have recently been successful in isolating CS-reactive antibodies, specific for the GAG portion of native CSPG [20]. In the present study we show that the distribution of extracellular CSPG is primarily and predominantly independent of the matrices formed by fibronectin and collagen or by laminin. Only after long periods in culture (several days) which involved extensive and active reorganization of the matrix by the cells, a partial, yet significant coincidence between fibronectin and CSPG was evident at the light and electron microscope level.

MATERIALS AND METHODS

Cells

Cultured chicken gizzard cells were prepared from 8–10-day-old chicken embryos and maintained in culture in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Biolab, Israel) and antibiotics. The cells consisted predominantly of fibroblasts and smooth muscle cells. For fluorescence microscopy the cells were plated on 18 mm square coverslips (Corning, USA).

Immunochemical Reagents

Antibodies to fibronectin were prepared in rabbits or mice. Rabbits were injected with purified plasma fibronectin and the antibodies were affinity-purified as previously described [21]. Monoclonal antibodies to fibronectin (FN-54) were raised by injection of substrate-attached membranes of cultured chick fibroblast and the reactive clone selected by radio-immunoassay and immunofluorescent labelling as described [20]. The specificity of the antibodies was verified by radio-immunoassay on purified fibronectin, and immunoblotting analysis on the purified protein and crude cell lysates. Antibodies to collagen (from rat tail tendon) were kindly supplied by Dr S. Fuchs from this department [22]. Antibodies to laminin were prepared and kindly supplied by Dr V. P. Terranova of the National Institute of Dental Research, Bethesda, Md. [23]. Monoclonal antibodies to CSPG (CS-56) were prepared and characterized as detailed elsewhere [20]. Briefly, these antibodies reacted with CS types A and C (not with CS-B) and showed some cross-reactivity with heparan sulfate. The antigen was highly sensitive to chondroitinases and was also removed efficiently from tissue culture substrate with pronase (but not with trypsin, chymotrypsin or papain). In addition, these antibodies precipitated ^{35}S-labelled material from extracts of [^{35}S]sulfate-labelled cells. Secondary antibodies for indirect labelling were isolated on the proper immunoadsorbents and coupled to a fluorophore as described [24]. Gold-conjugated antibodies against mouse or rabbit IgG were obtained from Janssen Pharmaceutica (Beerse, Belgium). In all experiments involving double immunolabelling at both the light microscopic level and EM level we have verified that the two secondary antibodies were exclusively reactive only with the respective antibody and absorbed the antibodies on a heterologous IgG column when necessary.

Immunofluorescent- and Immunoelectron-microscopic Labelling

Fixed or fixed-permeabilized cells were immunofluorescently labelled indirectly and observed in a Zeiss photomicroscope III equipped with selective filters for fluorescein and rhodamine. For immuno-EM labelling cells were cultured on 35-mm Falcon tissue culture dishes, and fixed with 3% paraformaldehyde in PBS for 30 min. The cells were then incubated with the primary antibodies, washed and further incubated with the secondary, gold-conjugated antibodies. After extensive rinsing, the cells were refixed with 2% glutaraldehyde, in 0.1 M cacodylate buffer, pH 7.2, post-fixed in osmium tetroxide, dehydrated and embedded in Epon (Polybed 812, Polysciences, Warrington, Pa., USA). Sections were cut perpendicular to the plane of the substrate and examined in a Philips EM 410 at 80 or 100 kV.

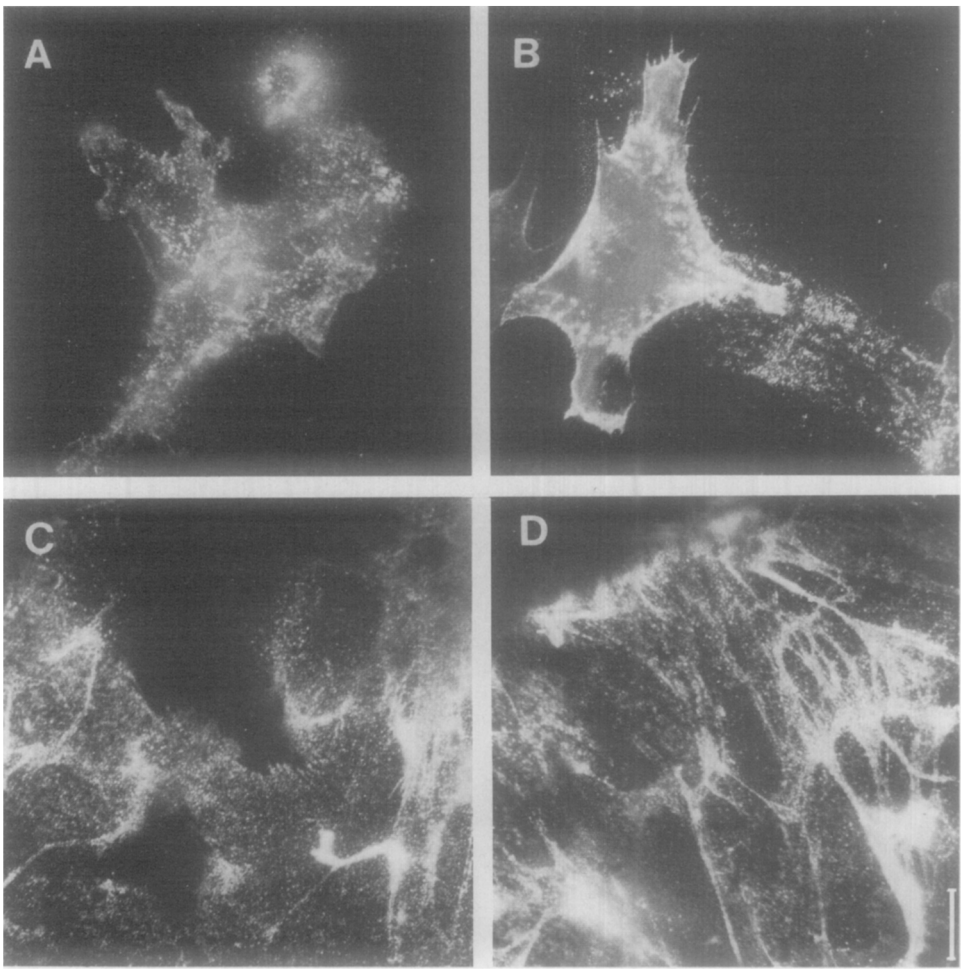

Fig. 1. Distribution of CSPG in chick gizzard fibroblast cultures as visualized by indirect immunofluorescent labelling with CS-56 monoclonal antibodies. The cells were fixed at various times after plating: (*A*) 30 min; (*B*) 2 h; (*C*) 24 h; (*D*) 5 days. Note that the labelling was initially distributed as a granular 'carpet' over the cells and substrate, while at later stages filamentous patterns became apparent. Bar, 10 μm.

RESULTS

Two Patterns of Distribution of CSPG in Cultured Chick Fibroblasts

Immunofluorescent labelling of cultured chicken gizzard cells for CSPG using CS-56 antibodies pointed to the presence of two patterns of distribution. Initially CSPG produced by the cells was deposited on the substrate in the vicinity of the cells (fig. 1*A*) or along the migratory pathways of motile cells (fig. 1*B*). The substrate-attached CSPG was uniformly distributed on the substrate around the cells displaying a dense dotted pattern.

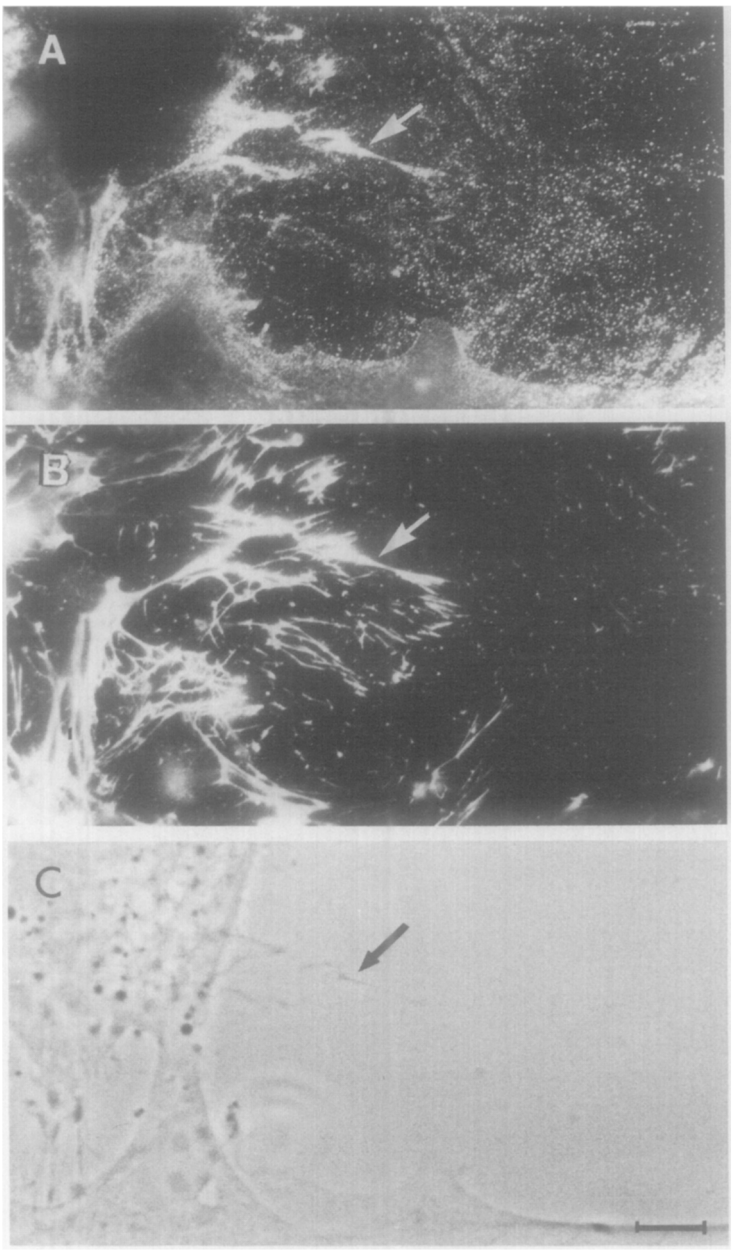

Fig. 2. Double immunofluorescent labelling of cultured chicken gizzard cells 24 h after plating for (*A*) CSPG; (*B*) fibronectin. The same field photographed with phase-contrast optics is shown in (*C*). Note that no fibronectin labelling is associated with the dotted, substrate-attached CSPG, while the filamentous CSPG is superimposed on fibronectin cables (*arrow*). Nevertheless, many of the fibronectin filaments are apparently devoid of CSPG. Bar, 10 μm.

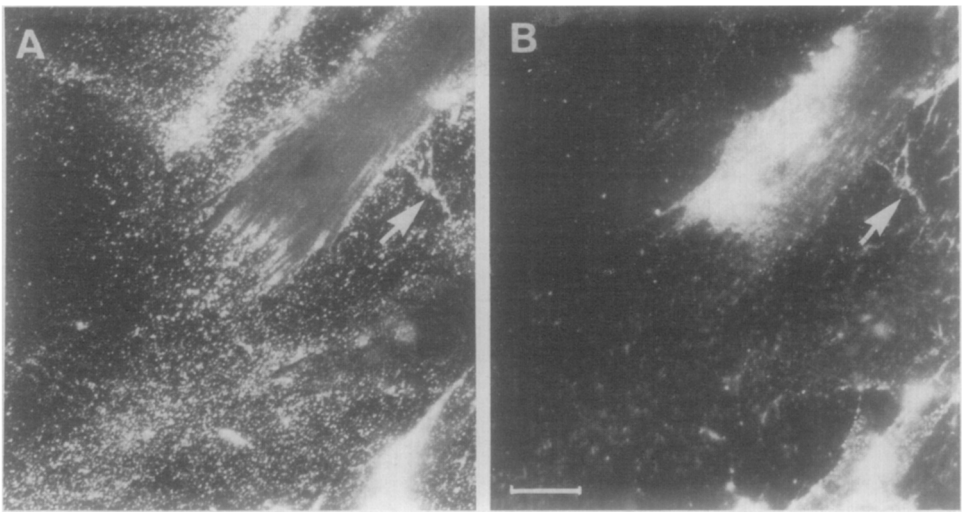

Fig. 3. Double immunofluorescent labelling of chicken gizzard cells 24 h after plating for (*A*) CSPG; (*B*) collagen. The dotted pattern of CSPG does not strictly overlap the dotted collagen patterns on the substrate, while both components are associated with the ECM filaments (*arrow*). Bar, 10 μm.

Only at later stages, namely after one or several days in culture, a second pattern of CSPG was apparent. This was characterized by positively labelled filaments which gradually became the predominant form (fig. 1 *C, D*).

Spatial Relationships between CSPG and Other ECM Components

Double immunofluorescent labelling for CSPG (using CS-56 antibodies) and for fibronectin (using affinity-purified rabbit antibodies) pointed to remarkable differences between the two (fig. 2). The uniform cell- or substrate-attached layer of CSPG was apparently devoid of fibronectin, while both components were associated with the filaments (fig. 2, *arrows*). After prolonged incubation in culture (3–5 days) extensive co-localization of CSPG and fibronectin was found at the fluorescence-microscope level. It should be pointed out, however, that this co-distribution was only partial and that while all the CSPG cables were superimposed on fibronectin, not all the filaments of the latter contained CSPG.

Double labelling for CSPG (fig. 3 *A*) and collagen (fig. 3 *B*) provided additional information. Both matrix components exhibited a dotted pattern on the substrate or on the cell surface, as well as filamentous pattern in both locations. Comparison of the labelling patterns indicated that the staining of the filaments was similar for the two components, while the substrate-attached dots were distinct and not overlapping. Double labelling for collagen (fig. 4 *A*) and fibronectin (fig. 4 *B*) indicated that the two maintained close mutual spatial relationships.

The distribution of laminin and its relationships to CSPG were examined in cultured chick fibroblasts and bovine mammary gland epithelial (BMGE) cells

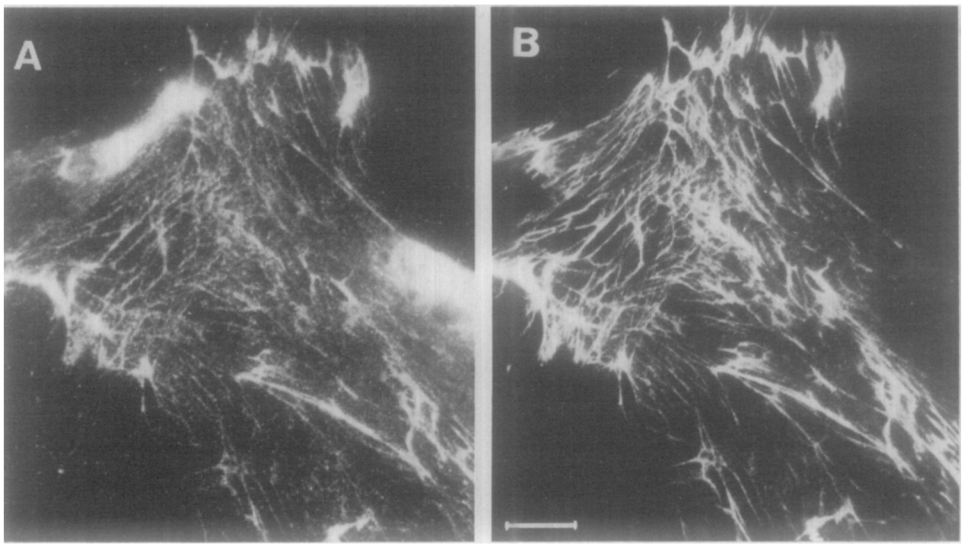

Fig. 4. Double immunofluorescent labelling of chicken gizzard cells (24 h after plating) for (*A*) collagen; (*B*) fibronectin. An area rich with ECM filaments was selected to show the extensive (though incomplete) coincidence of the two labels. Bar, 10 μm.

[25]. While there was some variability in the patterns observed, most cells showed surface-associated labelling with no apparent labelling of the dots or short cable of CSPG (fig. 5*A*,*B*). It is noteworthy that the extensive labelling for CSPG in fig. 5*A* is associated with the substrate in an area from which the cell has detached prior to, or during the time of fixation and immunolabelling. This area contains nearly no detectable laminin.

High-resolution Localization of CSPG and Fibronectin Using Immunoelectron Microscopy

Immunogold labelling of fixed chick fibroblast cultures for CSPG and fibronectin using monoclonal antibodies CS-56 and FN-54 pointed to pronounced differences between the two, both after short or long incubation periods.

CSPG was most abundantly present on the substrate, packed into densely labelled particles or aggregates. In most cells similar structures, with variable packing densities, were also found on the ventral or dorsal cell surfaces. Often those aggregates were rather elongated and apparently 'bridged' the gap between the ventral cell surface and the substrate (fig. 6*A*,*B*).

It should be emphasized that careful examination of many cells and electron micrographs indicated that the labelling for CSPG was absent from focal contacts. This aspect was specifically examined and discussed elsewhere [20].

The distribution of fibronectin was distinctly different. This protein was bound primarily to the cell surfaces or associated with linear cables present near or at

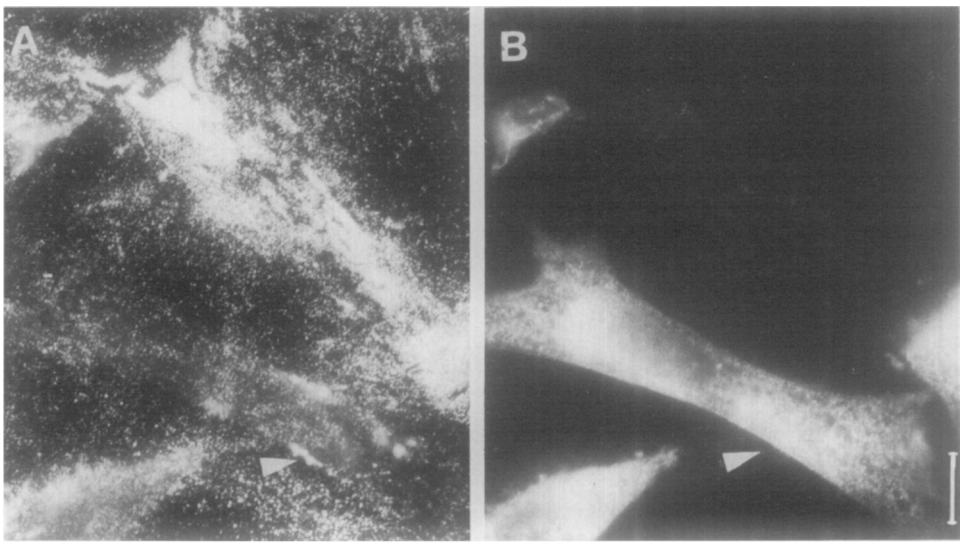

Fig. 5. Double immunofluorescent labelling of chick fibroblasts, 24 h after plating, for (*A*) CSPG; (*B*) laminin. The staining for the latter protein was mainly associated with the cell surface and absent from the CSPG dots and short filaments (*arrowheads*). Bar, 10 μm.

some distance away from the substrate (fig. 6*C*). The cell-associated fibronectin was organized in distinct filaments (fig. 6*D*) which were often present next to submembrane bundles of microfilaments, forming a fibronexus-like structure [26]. The ECM-associated fibronectin was also attached to collagen fibrils present in these cultures. As shown in fig. 6*C*, the fibronectin was not uniformly present along the collagen mesh, but rather interacted with the fibrils peripherally.

Most useful was the double immunolabelling for CSPG and fibronectin using 5-nm and 20-nm gold particles coupled to the anti-mouse and anti-rabbit Ig, respectively. Fig. 7*A* shows that even after a long period of incubation in culture (5 days), CSPG (*arrow*) and fibronectin (*double arrowhead*) showed remarkable spatial segregation. The former was abundantly present on the substrate, while the latter was associated mostly with the cells. However, in line with the immunofluorescent data, we have also observed significant co-localization of the two ECM components, both near the substrate and on or near the cells (fig. 7*B–D*). In all these locations the CSPG was packed into particles or aggregates and was apparently associated with the fibronectin cable peripherally and periodically. It is important to emphasize that while the CSPG was often bound to the fibronectin network, it was most commonly associated with the cells or the substrate in the absence of fibronectin, and vice versa. This observation suggested that each of these ECM components has its own and independent mechanism of anchorage to cells and substrates.

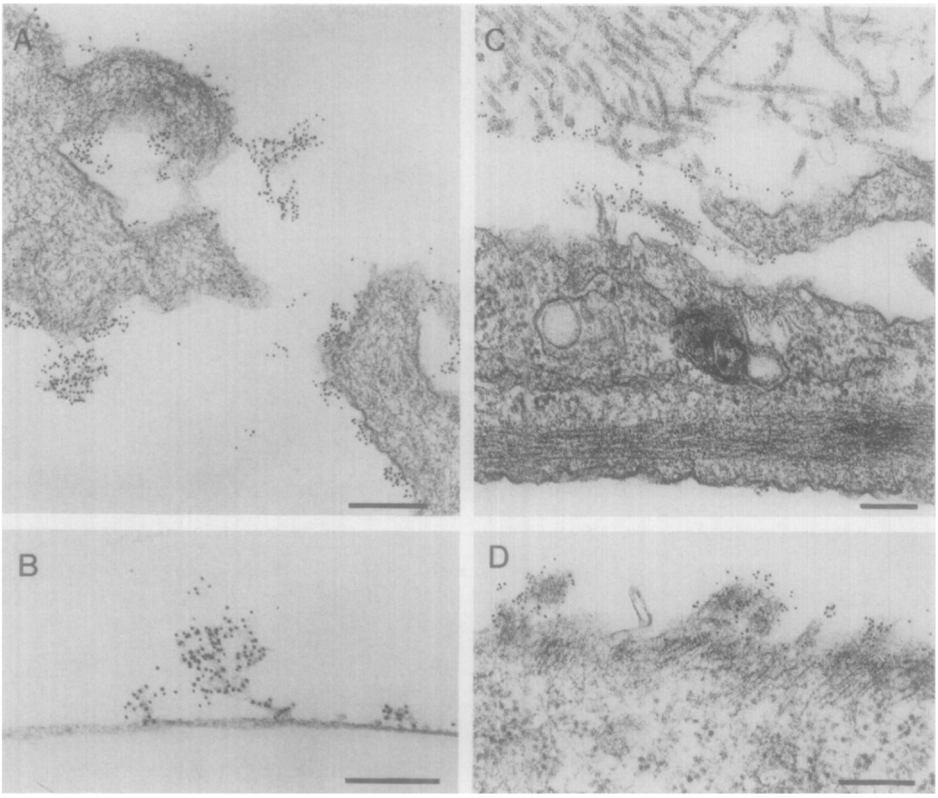

Fig. 6. Immunoelectron microscopic labelling for CSPG, (*A*, *B*) using CS-56 antibodies; (*C*, *D*) for fibronectin, using FN-54 monoclonal antibodies. In both cases the secondary antibody was goat anti-mouse Fab, conjugated to 5-nm gold particles. Note that the CSPG labelling was primarily associated with granules both on the (*A*) cells; (*B*) on the substrate. Fibronectin was present mostly on or between the cells, often associated with filamentous structures. These filaments were occasionally attached to (*C*) collagen arrays; or (*D*) to the cell surface. In the latter case, we have noticed that microfilaments were often abundant in the subplasmalemmal area subjacent to the fibronectin filaments. Bar, 0.1 μm.

DISCUSSION

Numerous studies carried out for over two decades have pointed out to the cardinal effects of the ECM on a variety of functions in cells and tissues (for review see [1–7]). These intercellular meshes direct or even determine the morphogenesis of cells, their assembly into tissues, the rate and orientation of their movement and, in many cases, their growth and differentiation. Recent studies on the structure of the ECM have shed light on its physico-chemical properties and molecular composition. Studies on the latter aspect have revealed several molecules which co-assemble to form the ECM network. These include fibronectin, different collagens, laminin and various proteoglycans. In spite of the wealth of information on the differential effect of each component on cell adhesion, very

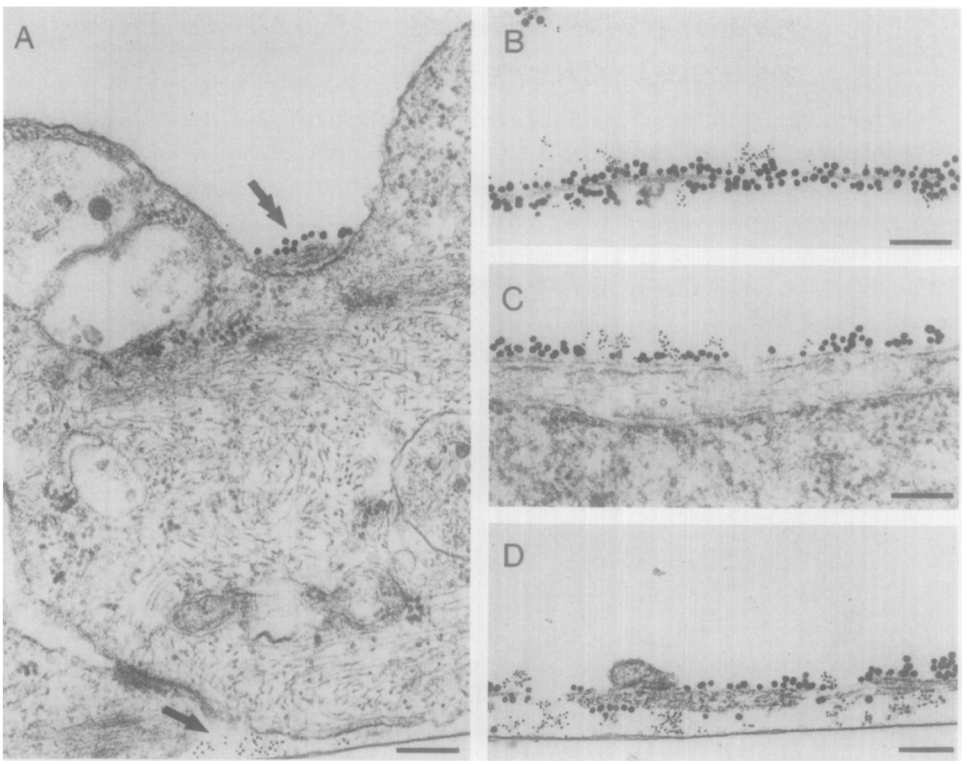

Fig. 7. Double immunoelectron microscopic labelling of 5-day culture, of chick gizzard fibroblasts for CSPG (using CS-56 monoclonal antibody) and fibronectin (using affinity-purified rabbit antibodies) in conjunction with 5-nm gold-conjugated to goat anti-mouse Fab and 20-nm gold-conjugated to goat anti-rabbit Ig. Note that fibronectin (*double-headed arrow*) is abundant mostly on the (*A*) cell surfaces, while CSPG (*single-headed arrow*) is present mostly on the substrate. Areas where both antigens were present are shown in (*B–D*). These include (*B*) 'free' cables; (*C*) surface-bound matrix; (*D*) substrate-attached filaments. Note that the CSPG represented by the 5-nm gold particles is usually present at the periphery of the fibronectin cables and possibly mediates their association with the substrate. Bar, 0.2 μm.

little is known on their fine molecular organization in the native ECM. Chemical analyses have indicated that purified fibronectin contains specific binding sites for collagen and proteoglycans [14–16, 27–31] and that collagen alone can also bind to the latter molecule [15, 16]. These results have raised the possibility that secreted ECM components may readily bind to each other and to the existing intercellular network. If indeed the distribution of proteoglycan was largely directed by the fibronectin–collagen networks (or vice versa) one may expect to find nearly homogeneous distribution of these components along the matrix.

The results presented in this paper strongly suggest that this is not the case. We have focused here predominantly on the localization of CSPG and various ECM proteins, mainly fibronectin. In this study we have used a unique monoclonal

antibody, which reacts specifically with CSPG. This monoclonal antibody was raised by immunization with substrate-attached membranes of chicken fibroblasts and its detailed characterization was described elsewhere [20]. It is worthwhile pointing out here that, although CS-56 antibodies also cross-reacted weakly with heparan sulfate, the fluorescent labelling was highly sensitive to pure chondroitinases ABC and AC and relatively resistant to heparitinase [20]. This and additional controls described previously were the basis for our conclusion that the immunofluorescence observed is predominantly due to CSPG.

Double labelling experiments have pointed out the marked differences, both temporal and spatial, between the CSPG and the fibronectin matrices. At the light microscope level we have noticed that CSPG primarily formed uniform arrays of dots over the surface of the substrate and the cells. Electron-microscopic examination indicated that these dots corresponded to amorphous particles or granules usually measuring 50–300 nm, which were extensively labelled by the antibodies. The exact molecular nature of these structures is not entirely clear, nevertheless their dimensions suggest that they might represent either monomers similar to those found in cartilage [32] or aggregates of the smaller CSPG typical of other tissues [33–35]. After long periods of incubation a significant and increasing proportion of CSPG was associated with fibronectin cables, though even at these late stages electron microscopy indicated that the intermixing between the two was limited; CSPG was either associated with fibronectin-free areas on the cell surface or on the substrate or was, alternatively, periodically attached to the surface of fibronectin-rich filaments. We may thus conclude that the primary binding sites for CSPG on the substrate or on cells are independent of fibronectin and that the binding of the latter was largely independent of CSPG.

This view was further supported by additional experiments (part of which were reported previously [20]) in which cultured cells were exposed to different hydrolytic enzymes. We have shown that short trypsin treatment, which effectively removed fibronectin filaments, had a limited effect on CSPG. Only after a relatively long period of culturing (5 days), a significant proportion of the CSPG became trypsin-sensitive. Treatment with purified clostridial collagenase had no significant effect on the distribution of CSPG. Removal of the latter by chondroitinases AC or ABC had little effect on the distribution of fibronectin.

The association of CSPG (and possibly heparan sulfate PG, which cross-react with CSPG [20]) with ficronectin cables, appears to be a secondary process affected by the active reorganization of the matrix by the cells. We have shown a few years ago, that cells cultured on fibronectin layer actively remove the protein from tight cell–substrate contacts and either pile it into conspicuous cables or endocytose it. We have proposed that it is through this process that the typical patterns of the fibronectin ECM are generated [21]. This view has recently been supported by the results of several laboratories [36, 37]. The observation reported by us earlier, as well as the present report suggest that CSPG may be reorganized along fibronectin via the same mechanism. Namely, that a fraction of CSPG

which is bound to fibronectin is reorganized along with the 'underlying' matrix protein. This is in line with the common presence of CSPG on top of the fibronectin layer (fig. 7), the apparent absence of CSPG from stable cell–substrate focal contacts [20], and the fact that essentially all the 'fibrillary' CSPG coincided with fibronectin, while many of the fibronectin arrays were devoid of labelling for CSPG (figs 2, 7).

In conclusion, the results presented here suggest that the ECM in fibroblast cultures contains distinct molecular microdomains enriched with either proteoglycan or with fibronectin and collagen. This 'mosaic' structure appears to be actively modulated and reorganized by the cells. It is yet to be determined whether similar spatial segregation and reorganization processes also exist in intact tissues.

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