The Dynamic Structure of the Pericellular Matrix on Living Cells

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Abstract. Although up to several microns thick, the pericellular matrix is an elusive structure due to its invisibility with phase contrast or DIC microscopy. This matrix, which is readily visualized by the exclusion of large particles such as fixed red blood cells is important in embryonic development and in maintenance of cartilage. While it is known that the pericellular matrix which surrounds chondrocytes and a variety of other cells consists primarily of proteoglycans and hyaluronan with the latter binding to cell surface receptors, the macromolecular organization is still speculative. The macromolecular organization previously could not be determined because of the collapse of the cell coat with conventional fixation and dehydration techniques. Until now, there has been no way to study the dynamic arrangement of hyaluronan with its aggregated proteoglycans on living cells. In this study, the arrangement and mobility of hyaluronan–aggrecan complexes were directly observed in the pericellular matrix of living cells isolated from bovine articular cartilage. The complexes were labeled with 30- to 40-nm colloidal gold conjugated to 5-D-4, an antibody to keratan sulfate, and visualized with video-enhanced light microscopy. From our observations of the motion of pericellular matrix macromolecules, we report that the chondrocyte pericellular matrix is a dynamic structure consisting of individual tethered molecular complexes which project outward from the cell surface. These complexes undergo restricted rotation or wobbling. When the cells were cultured with ascorbic acid, which promotes production of matrix components, the size of the cell coat and the position of the gold probes relative to the plasma membrane were not changed. However, the rapidity and extent of the tethered motion were reduced. Treatment with *Streptomyces* hyaluronidase removed the molecules that displayed the tethered motion. Addition of hyaluronan and aggrecan to hyaluronidase-treated cells yielded the same labeling pattern and tethered motion observed with native cell coats. To determine if aggrecan was responsible for the extended configuration of the complexes, only hyaluronan was added to the hyaluronidase-treated cells. The position and mobility of the hyaluronan was detected using biotinylated hyaluronan binding region (b-HABR) and gold streptavidin. The gold-labeled b-HABR was found only near the cell surface. Based on these observations, the hyaluronan–aggrecan complexes composing the cell coat are proposed to be extended in a brush-like configuration in an analogous manner to that previously described for high density, grafted polymers in good solvents.

BETWEEN the plasma membrane and the extracellular matrix lies the pericellular matrix. Intermingled in this transition zone are the ectodomains of integral membrane glycoproteins, proteoglycans, and glycolipids, as well as hyaluronan and a variety of extracellular glycoproteins and proteoglycans (Hedman et al., 1982; Kjellan and Lindahl, 1991). An enlarged pericellular matrix, or cell coat, occurs during certain stages of embryonic development and within the first few days of plating for a variety of cell types, including fibroblasts and chondrocytes. The cell coat on cultured chondrocytes can be considered an in vitro model for the pericellular and/or territorial matrices of cartilage (Goldberg and Toole, 1984; Hauselman et al., 1992). The functions of the cell coat or pericellular matrix are not well-defined but appear to include maintenance of cell separation and protection against compression. Within the pericellular matrix, the potency of growth factors are modulated and extracellular matrix macromolecules are modified and assembled. The existence of the pericellular matrix has not been fully appreciated partially because of its invisibility by phase contrast and DIC microscopy. This invisibility is due to the low ratio of organic material to water resulting from the hydration of the proteoglycans, a major constituent (Hun-

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ziker and Schenk, 1987). Electron microscopy has shown that the matrix is an open structure (Hunziker and Schenk, 1987; Lee et al., 1993; Wight et al., 1991), but the exact structure is an enigma as the cell coat can be 10 or more microns thick, while a random coil of hyaluronan, the other major constituent, is only 500 nm in diameter when in solution (Laurent and Fraser, 1992; Laurent, 1989). An arrangement of the hyaluronan–proteoglycan complexes on the cell surface has been suggested (Toole, 1991), but it does not incorporate the extended structure of the aggregate (Hunziker and Schenk, 1987) formed by hyaluronan with the aggregating proteoglycans, aggrecan, or versican, depending on cell type. There has been speculation that hyaluronan chains on the cell surface are extended (Heinegard and Pimentel, 1992; Laurent and Fraser, 1992), but confirmation has been difficult as the cell coat tends to collapse with fixation and dehydration for conventional histochemistry and electron microscopy (Clarris and Fraser, 1968). Cryotechnical tissue preservation appears to preserve proteoglycan structure in cartilage sections (Hunziker and Shenck, 1987), but the density of these and other matrix macromolecules in the tissue prohibit the determination of the configuration of individual hyaluronan aggrecan complexes.

The concept that long polymers attached to a surface could extend outward in a brush-like configuration has been described for the behavior of linear, neutral polymers in good solvents (de Gennes, 1987). In the de Gennes grafted polymer model, when polymers are attached at one end to a surface, they have two possible conformations depending on the number of polymers in a given area. At low density, the polymers collapse down in a mushroom-like configuration. At high density, the polymers extend out in a brush-like configuration. It is possible that hyaluronan molecules, when attached to receptors on the cell surface, behave in an analogous manner. The large proteoglycans complexed with the hyaluronan provide the increase in density necessary for extension. That aggrecan molecules are necessary for an extended configuration is supported by the observation by Knudson (1993) that hyaluronan added alone to *Streptomyces* hyaluronidase-stripped cells will not produce a pericellular matrix that excludes large particles; aggrecan must also be added. Additional evidence is provided by the minimal sized matrix produced by nanomelic chondrocytes that have a genetic defect interfering with aggrecan secretion (Vertel et al., 1993).

The difficulties with preservation of the cell coat can be avoided by using live cells, as is routinely done with the particle exclusion assay. In this assay, fixed red blood cells are layered over the cells growing as a monolayer. The extent or size of the cell coat can then be measured. To obtain information on the arrangement of the macromolecules in the cell coat, nanovid microscopy (Geerts et al., 1987) can be used. This approach of using 30- to 40-nm colloidal gold probes combined with video-enhanced light microscopy has been used to characterize the mobility of molecules in the plasma membrane (Kucik et al., 1990; Lee et al., 1993; Sheetz et al., 1989). Colloidal gold probes are especially well suited for tagging macromolecules in the cell coat because of the gold's small size relative to the large matrix macromolecules (see Fig. 1). In this study, we have used colloidal gold labeling and video-enhanced light microscopy to directly observe the location and movements of the hyaluronan–aggrecan complexes in the pericellular matrix of living cells. We have found that the complexes do extend out from the cell, and they "wobble" about their tether point, presumably, the cell surface receptor. That this extension requires aggrecan was tested with reconstitution experiments where hyaluronan and aggrecan were added back to *Streptomyces* hyaluronidase-stripped cells (Knudson, 1993; Knudson and Knudson, 1991). From these observations, we propose that hyaluronan–aggrecan complexes composing the cell coat are in an extended or brush-like configuration similar to that described by de Gennes for polymers grafted to a surface (de Gennes, 1987). The value of this configuration for probable functions of the cell coat is discussed.

Materials and Methods

Tissue Culture

Chondrocytes were isolated from bovine articular cartilage as previously described (Kuettner et al., 1982). The chondrocytes were plated on 22-mm^2 coverslips at low density (2×10^5 cells/coverslip), and maintained in Ham's F-12 nutrient mixture containing 10% fetal bovine serum. For some cultures, 25 μg/ml ascorbic acid was added to the medium, which was changed daily. Chondrocytes were always used within 7 d of plating.

Particle Exclusion Assay

To determine cell coat size, fixed horse red blood cells (Knudson and Knudson, 1991) were layered onto cells growing in a monolayer as previously described (Lee et al., 1993) and viewed with DIC video microscopy. DIC images viewed with 20× and 40× objectives were recorded and analyzed with an Image 1 image processor (Universal Imaging, West Chester, PA). The thickness of the matrix (the distance from the red blood cells to the plasma membrane of the chondrocyte) was measured for the three most representative regions of the coat on each cell.

Colloidal Gold Probes

30- to 40-nm colloidal gold particles (Ted Pella Inc., Irvine, CA), were conjugated by a previously described procedure (Lee et al., 1991) to an antibody to keratan sulfate, 5-D-4, purified by monoQ and FPLC, and to streptavidin (Sigma Chemical Co., St. Louis, MO).

Reconstitution of the Pericellular Matrix

Freshly isolated chondrocytes were plated as above 20 to 48 h before use. After three rinses with Hanks balanced salt solution, 8 U *Streptomyces* hyaluronidase (Fluka) in Ham's F-12 nutrient mixture containing 1% bovine serum albumin was placed onto each coverslip and the cells were incubated for one hr at 37°C. The addition of hyaluronan (12 μg/ml in Ham's F-12) (human umbilical cord; Sigma Chemical Co.) and aggrecan (2 mg/ml in Ham's F-12) (A1D1D1 fraction prepared from bovine nasal cartilage by 4 M guanidine HCl extraction and subsequent fractionations with CsCl density gradient centrifugation) was essentially as previously described (Knudson, 1993) except that the hyaluronan and aggrecan were added sequentially with a 1-h incubation each and rinsed in between, rather than being added together. To shorten the hyaluronan on the cells, sheep testicular hyaluronidase (Sigma Chemical Co.) was used at 0.2 U per coverslip for 15 min. Biotinylated hyaluronan-binding region (b-HABR)[1] (gift from M. Bayliss, Kennedy Institute of Rheumatology, London) contained only the G1 domain of aggrecan and was used at 0.4 mg/ml.

Assessment of Mobility of Gold 5-D-4–labeled Macromolecules

The colloidal gold was visualized using video-enhanced brightfield microscopy and then analyzed as previously described (Lee et al., 1991). Briefly,

1. *Abbreviations used in this paper*: gold 5-D-4, colloidal gold conjugated to the antibody 5-D-4; b-HABR, biotinylated hyaluronan-binding region.

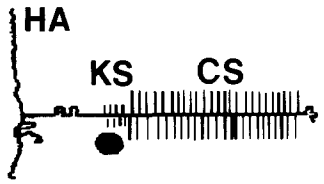


Figure 1. The structure of an aggrecan molecule attached to a portion of a hyaluronan molecule (*HA*) is shown with a 30-nm gold particle (black ellipsoid) drawn to approximately the same scale. Gold-5-D-4 likely binds to the region rich in keratan sulfate chains (*KS*). (*CS*, chondroitin sulfate-rich region). A single hyaluronan molecule typically has multiple aggrecan molecules bound.

analog- and digital-contrast enhanced images were recorded at video rate in series of 200 to an optical disk recorder (Panasonic). After further enhancement, the centroid of each gold particle in each image of the series was located using the Object Measurement Mode of Image 1. From these centroids, a trajectory for the two-dimensional motion of each gold particle was computed. To obtain an approximate measure of the rapidity of movement, two-dimensional diffusion coefficients were computed from the slope of mean square displacement versus time interval plots derived from the trajectories. Extent of movement refers to the area covered by each trajectory.

Results

The molecular mobility of the hyaluronan-aggrecan complex was visualized with video-enhanced microscopy of colloidal gold conjugated to the antibody 5-D-4 (gold-5-D-4), which recognizes epitopes on keratan sulfate glycosaminoglycans (Caterson et al., 1983), a component of the aggrecan molecule (Fig. 1). The gold-5-D-4 labeled some, but not all cells, consistent with previous observations that there are at least two populations of chondrocytes obtained from articular cartilage that differentially synthesize keratan sulfate–containing proteoglycans (Aydelotte et al., 1992). Additional support for the specificity of the gold label is provided by gold probes conjugated to antibodies to type II collagen and to fibronectin. These probes gave a different labeling pattern and labeled macromolecules that moved very slowly or not at all (not shown). The gold 5-D-4 attached to molecules in the pericellular matrix moved in a rapid, but restricted manner. Within a short observation time, some gold 5-D-4 particles were observed to repeatedly move from out in the cell coat to various places near the plasma membrane and back again, but always moving in a restricted area (Fig. 2). Other gold 5-D-4 particles were more restricted in their motion and did not approach the cell surface. The gold particles appeared to be attached to tethers. The center of the motion, or the anchor site of the tether, was usually on or near the plasma membrane. The tethered molecular complex could not be visualized by DIC microscopy; however, immunofluorescence confirmed that 5-D-4 binds to molecules that extend out from the cell surface (Fig. 3).

To obtain an approximate index for the rapidity of motion, an average, apparent diffusion coefficient for the two dimensional diffusion of the gold was computed. An approximation is necessary because we were observing the projection of three dimensional motion on a two dimensional plane. The diffusion coefficient for the gold attached to labeled molecules in the pericellular matrix was calculated from plotted trajectories (Lee et al., 1991; 1993) (Fig. 4) and was 1.8×10^{-9} cm²/s. The trajectories for the gold on the labeled molecules covered an average area of 2.3 ± 2.2 μm^2 (mean $\pm$ SD).

That the necessary density for extension of the hyaluronan–aggrecan complexes was present in the measurements made above is supported by experiments done with chondrocytes cultured with the vitamin, ascorbic acid, to promote production of matrix components (Kao et al., 1990; Murad et al., 1981). As determined with the particle exclusion assay (Fig. 5), for cells grown with ascorbic acid the average thickness of the cell coat (6.0 ± 2.6 μm, mean $\pm$ SD) was not increased over that for chondrocytes grown without ascorbic acid (6.2 ± 2.4 μm). However, growth with ascorbic acid appeared to produce an increase in the density of the pericellular matrix. Both the rapidity of movement and the area covered were reduced (2.8×10^{-10} cm²/s and 0.4 ± 0.4 μm^2, respectively). Thus, a minimum density necessary for extension of the hyaluronan–aggrecan complexes can be achieved with cells cultured without ascorbic acid. These studies indicate that collagen fibrils are not necessary for extension.

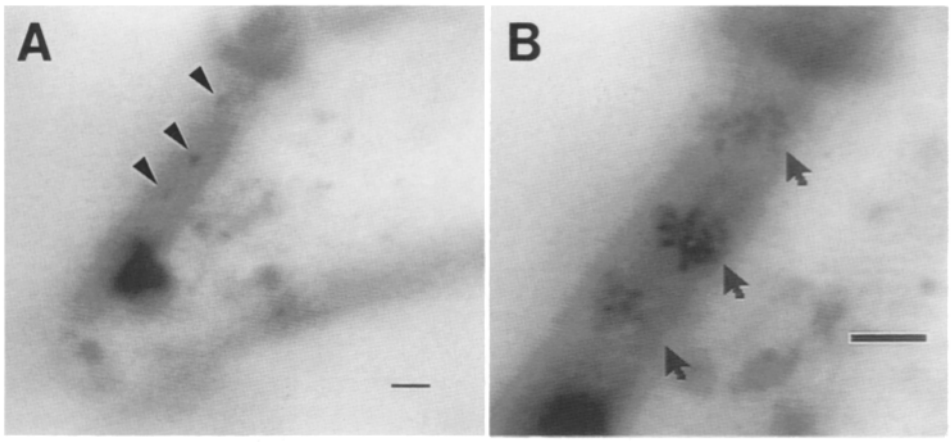
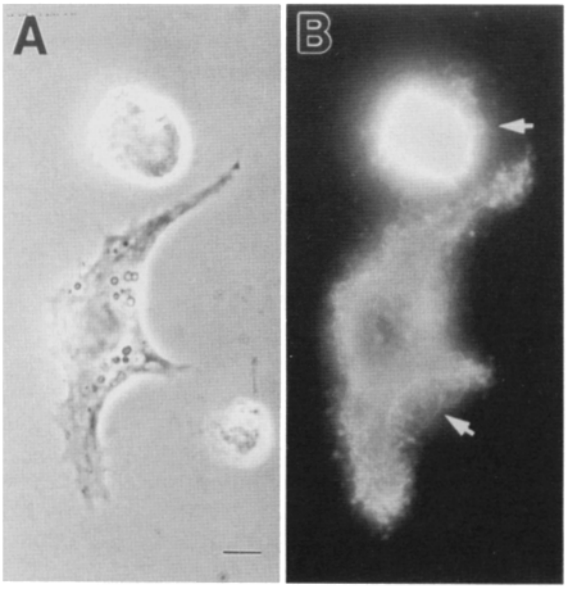


Figure 2. The mobility of hyaluronan–aggrecan complexes in the pericellular matrix of a bovine articular chondrocyte cultured without ascorbic acid. (*A*) Brightfield video micrograph of a region of a cell labeled with gold 5-D-4. Arrowheads indicate three gold 5-D-4 particles in the pericellular matrix. (*B*) A composite of brightfield video images of the same cell collected over 4.1 s. The arrows indicate the probable attachment sites of the tethered molecules to the plasma membrane. The black spots above the arrows show the cumulated positions of the gold 5-D-4 particles shown in *A*. The center particle moved in a range extending from out in the cell coat to near the plasma membrane. The apparent diffusion coefficients for the two dimensional movement of the gold particles are: 2.8×10^{-9} cm²/s (*lower*), 3.3×10^{-9} cm²/s (*middle*), and 4.2×10^{-9} cm²/s (*upper*). Bars, 2 μm.



Collagen fibrils were present on cells cultured with ascorbic acid as determined by DIC microscopy, immunofluorescence, and biochemical means (Lee, G. M., and B. Johnstone, unpublished data). They were not detected on cells cultured without ascorbic acid.

An estimate of the average distance from the cell surface to the gold particle was obtained from the tethered molecules

Figure 3. Hyaluronan–aggrecan complexes extend out from the cell surface with one end attached to the cell surface. When labeled by immunofluorescence for keratan sulfate using the 5-D-4 antibody, the pericellular matrix can be seen to contain long thin strands (*arrows*) extending out from the cell surface. These labeled strands are probably hyaluronan–aggrecan complexes. Phase contrast (*A*) and corresponding fluorescence (*B*) micrographs, of two bovine articular cartilage chondrocytes, one round and one spread, from an 8-y-old animal. The pericellular labeling is especially evident on chondrocytes from older specimens as they produce more keratan sulfate (Thonar and Kuettner, 1987) on their aggrecan molecules. Bar, 10 μm.

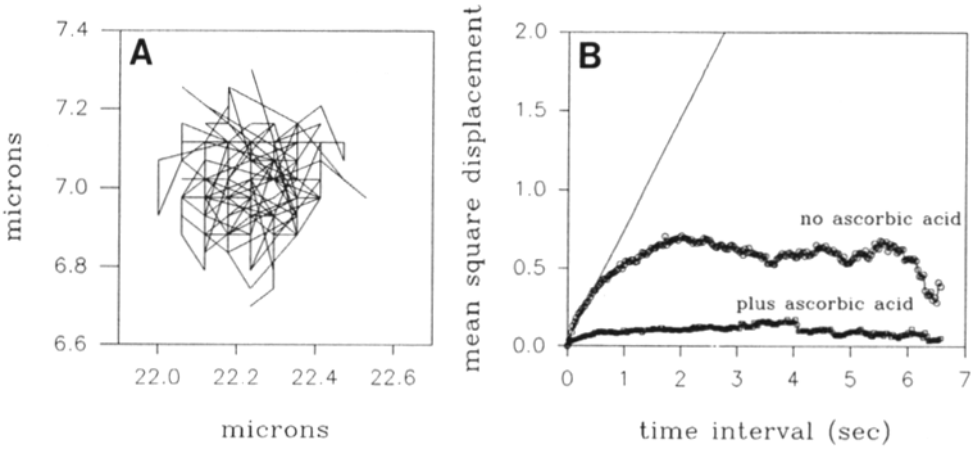


Figure 4. (*A*) An example of a trajectory of a gold-5-D-4–labeled molecular complex tethered to the cell surface. The trajectory reflects 7 s of motion. The frequent crossing over is indicative of restricted motion. (*B*) The averaged mean square displacement (*MSD*) plots are shown for the tethered movement of the gold 5-D-4 on cultures grown with (□) and without ascorbic acid (○). The straight line represents the theoretical plot for unrestricted random motion at the same diffusion coefficient as that for the cells without ascorbic acid. The experimental plots bend and then level off at a fairly early time point consistent with the motion being restricted to a small area (Lee et al., 1993). The leveling of the plot at a lower MSD value for the cells cultured with ascorbic acid indicates that the tethered motion was restricted to a smaller area. The slope of the linear region of the line near the origin was used to compute the diffusion coefficient (Lee et al., 1991).

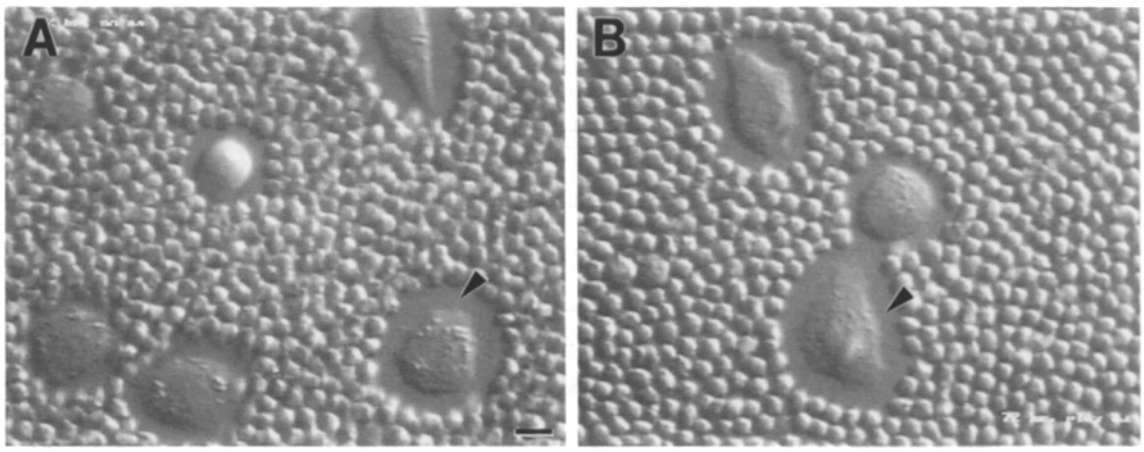


Figure 5. The particle exclusion assay is used to outline the edges of the pericellular matrix surrounding living chondrocytes. DIC video micrographs of chondrocytes growing as a monolayer and overlaid with fixed red blood cells. The pericellular matrix fills the clear area (*arrowhead*) between the fixed red blood cells and the live chondrocytes. There was no difference in average coat size for chondrocytes cultured without (*A*) and with (*B*) ascorbic acid. Bar, 10 μm.

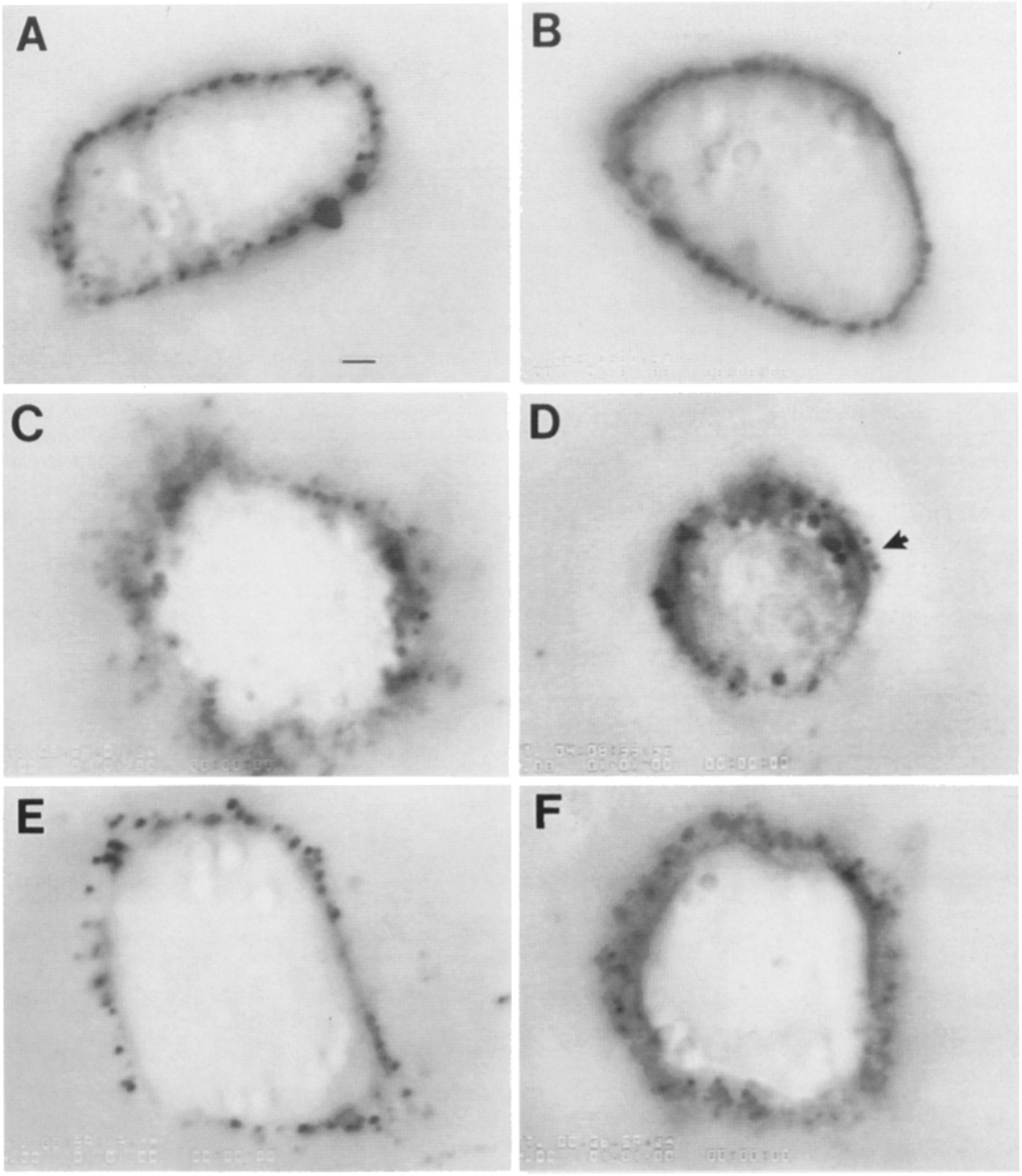


Figure 6. Reconstitution of the matrix on *Streptomyces* hyaluronidase-stripped cells shows that the hyaluronan–aggrecan complex is the source of the observed tethered molecules and that aggrecan is necessary for hyaluronan to be in an extended configuration as diagrammed in Fig. 8 *B*. Brightfield micrographs are shown of cells that were especially well-labeled with gold-5-D-4 (*A–D*) and with gold–streptavidin (*E* and *F*). (*A*) Hyaluronidase only. The gold 5-D-4 labels a cell surface component but there is no pericellular matrix labeling. (*B*)Hyaluronidase followed by addition of hyaluronan. The hyaluronan did not affect the cell surface labeling nor did it bind the gold-5-D-4. (*C*) Hyaluronidase followed by hyaluronan and then aggrecan molecules. The matrix of the cell is heavily labeled with gold 5-D-4 which displayed rapid tethered motion. (*D*) Cells were treated with testicular hyaluronidase after the addition of hyaluronan and prior to the addition of aggrecan molecules. The testicular hyaluronidase cleaved the hyaluronan molecules resulting in reduced labeling and shorter tethers. Arrow indicates three gold particles in the pericellular matrix which displayed a tethered-type motion. (*E*) After the addition of hyaluronan, biotinylated HABR was added. The gold streptavidin is found near the cell surface indicating that the hyaluronan is collapsed on the cell surface rather than being in an extended configuration. These gold particles moved more slowly and in a much more restricted manner than when aggrecan was present. (*F*) After the addition of hyaluronan, aggrecan molecules were added with the biotinylated HABR. The gold–streptavidin is found away from the surface in the pericellular matrix indicating that the aggrecan molecules cause the hyaluronan molecules to extend outward. The gold-labeled complexes moved with a rapid tethered motion similar to that observed with the native matrix and with gold-5-D-4 labeling of the reconstituted matrix. Bar, 2 μm.

which extended out from the edge of the cell as visualized with the microscope. The distance from the gold particle to the cell surface averaged 3.1 μm without ascorbic acid and 1.9 μm with ascorbic acid. The longest distances measured were 8.4 μm and 6.3 μm, without and with ascorbic acid, respectively. The molecular weight of hyaluronan molecules in articular cartilage ranges between 300 and 600 kD (Holmes et al., 1988) although newly synthesized chains can be much larger (Hardingham et al., 1992). A 1,000-kD molecule would be ~2-μm long (Wight et al., 1991) and a 5,000 kD molecule about 10-μm long (Hardingham et al., 1992). Although the position of the gold-labeled aggrecan relative to the outer end of the hyaluronan molecule could not be ascertained, the measured tether lengths are consistent with the previously reported range of lengths for hyaluronan molecules and thus support the proposal that the hyaluronan molecules are extended in the brush configuration. The average tether length is less than the average thickness (6.0 μm) of the pericellular matrix measured by the particle exclusion assay. This difference is probably due to most of the labeled aggrecan molecules not being at the end of the hyaluronan molecules. However, some (7%) were found 6 to 8 μm from the cell surface. The low number may also be due to the difficulty in recognizing gold particles at the end as tethered particles because they would be only briefly in focus due to their three dimensional movement taking them out of the depth of field for the microscope objective.

That the motion is tethered and is due to the hyaluronan-aggrecan complex is shown in matrix reconstitution experiments modeled after those done by Knudson (1993). Treatment of the chondrocytes with *Streptomyces* hyaluronidase before the addition of gold 5-D-4 resulted in cell surface labeling (see below) but little to no detectable pericellular matrix labeling or tethered motion (Fig. 6 *A*). The addition of hyaluronan after hyaluronidase treatment and before addition of gold-5-D-4 produced the same results (Fig. 6 *B*). However, the addition of hyaluronan followed by purified aggrecan molecules prior to gold-5-D-4 addition yielded many cells with little surface labeling but heavy labeling in the pericellular matrix (Fig. 6 *C*). The reduction in cell surface labeling is probably due to competition for the gold-5-D-4 by the aggrecan molecules that are more abundant. The labeled pericellular matrix molecules showed rapid tethered motion similar to that observed with low density cultures grown without ascorbic acid (Fig. 4). The average apparent diffusion coefficient computed from the two dimensional trajectories of the gold-5-D-4 on these cells was 2.2×10^{-9} cm^2/s. When the cells with hyaluronan added were treated

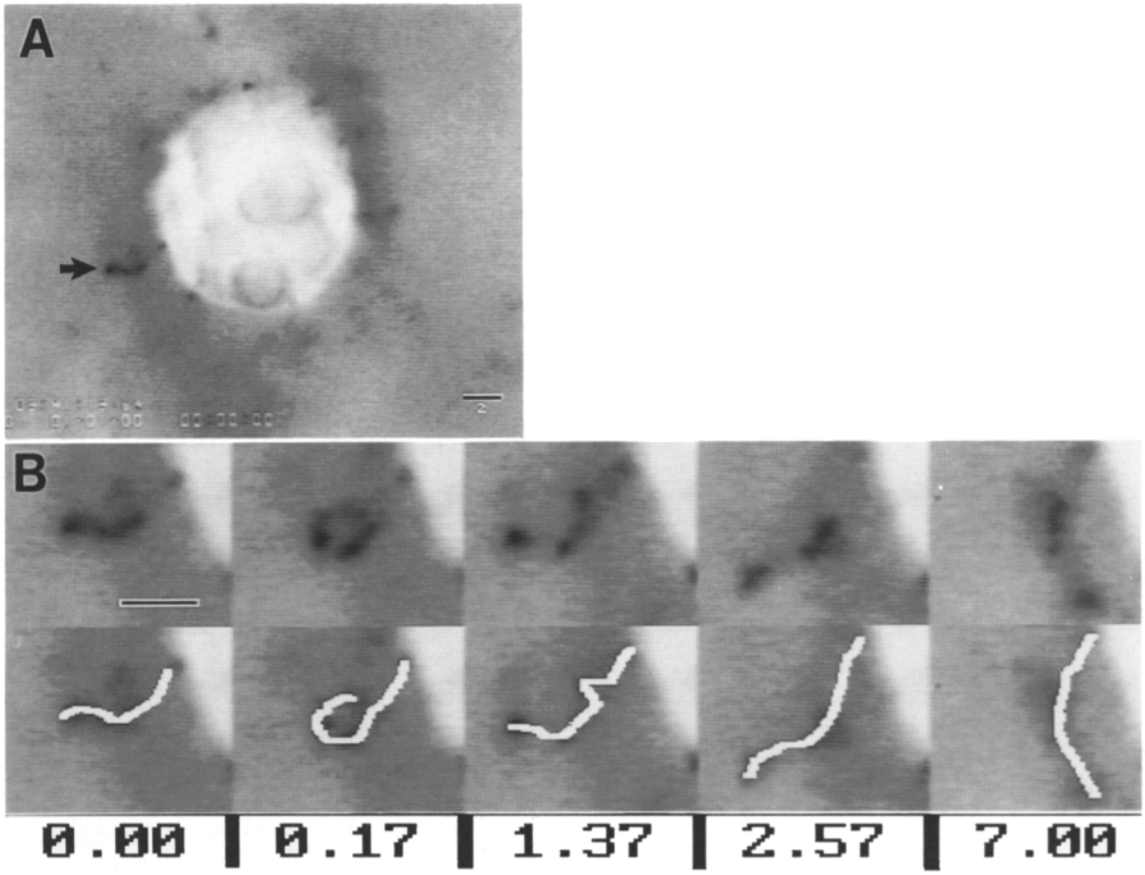


Figure 7. Hyaluronan–aggrecan complexes with multiple gold labels extend out from the cell surface. The dynamic nature of the complex is shown by the rapid bending and moving in three dimensions. (*A*) Brightfield image of a cell with a reconstituted matrix showing a complex (~4.6-μm long) labeled with seven to eight gold particles (*arrow*). (*B*) Series of micrographs showing a sample of the conformations the labeled complex underwent. These video images were selected on the basis of having the most gold particles in focus (the three-dimensional movement resulted in the particles going in and out of focus). The time intervals, in seconds, between the micrographs are shown. Below the series are line drawings to indicate the probable location and shape of the hyaluronan backbone. Bars, 2 μm.

with testicular hyaluronidase to shorten the hyaluronan prior to adding aggrecan (Fig. 6 *D*), the gold label was much closer to the cell surface (the tether was shorter), but the tethered motion was still present.

To determine if aggrecan molecules were necessary for the extension of hyaluronan away from the cell surface, chondrocytes stripped with hyaluronidase were allowed to bind hyaluronan and then b-HABR was added. HABR consists of the G1 domain of aggrecan. The hyaluronan-b-HABR complexes were visualized with 40-nm gold-streptavidin. As shown in Fig. 6 *E*, the gold label appeared near or on the cell surface and only occasional slight tethered motion was observed. Thus the hyaluronan without aggrecan molecules is more compact and far less dynamic. When aggrecan molecules were added with the b-HABR, the gold-streptavidin was away from the cell surface and showed the same tethered motion as observed with gold 5-D-4 labeling of hyaluronan-aggrecan complexes (Fig. 6 *F*).

The cell surface labeling observed with gold 5-D-4 after hyaluronidase treatment (Fig. 6, *A* and *B*) is due to either a keratan sulfate-containing proteoglycan bound to a receptor or to a membrane protein with a keratan sulfate chain attached. In addition to aggrecan, bovine chondrocytes produce at least one other keratan sulfate-containing proteoglycan, fibromodulin (Plaas et al., 1990). Fibromodulin could be the labeled molecule as an antibody to fibromodulin labeled the cell surface of many, but not all, cultured chondrocytes (not shown). To our knowledge, a keratan sulfate-containing membrane protein has not been previously described. Using immunoprecipitation with gold 5-D-4, the isolation and characterization of the keratan sulfate-containing surface molecule is currently being undertaken.

In some instances, long macromolecular complexes labeled with multiple gold probes were observed. Multiple gold particles on single complexes were infrequently observed because the labeling density of the gold 5-D-4 was kept low in order to be able to track the movements of individual gold particles. Theoretically, the number of aggrecan molecules per hyaluronan can range from 2 up to 800 depending on the length of the hyaluronan and the ratio of the two molecules (Hardingham et al., 1992). With the reconstituted pericellular matrix, multiple gold-5-D-4 particles were occasionally observed to move as a highly flexible unit (Fig. 7). Hyaluronans with multiple gold-labeled aggrecans extended out from the cell surface. The observed rapid bending and flexing is consistent with hyaluronan being a flexible polysaccharide. This is in contrast to fibrils composed of proteins, such as actin, tubulin, and fibronectin, which slowly flex (Gittes et al., 1993; Lee, G. M., unpublished observations). The high degree of flexibility indicates that the hyaluronan-aggrecan complex is free to move and is not cross-linked with other complexes in the reconstituted matrix.

Discussion

We have shown that gold 5-D-4-labeled aggrecan-hyaluronan complexes in the cell coat extended out from the cell surface and were highly mobile. The outward extension was dependent on the presence of aggrecan molecules. Several observations support the conclusion that the motion is tethered rather than entirely random or confined to corrals. As shown by immunofluorescence, 5-D-4-labeled molecules appear as individual projections from the cell surface. Individual gold 5-D-4 particles appeared to move as if on a tether. The trajectories of the gold label were confined to small regions and thus were not freely diffusing. Complexes that were multiply labeled and attached at one end to the cell surface could be directly observed to move in a tethered fashion. Additional information supporting the conclusion of tethered motion include the demonstration that hyaluronan is bound to receptors on the cell surface of chondrocytes (Knudson, 1993) and that electron microscopy of rotary shadowed aggregates shows an extended conformation for the complex (Buckwalter et al., 1984; Morgelin et al., 1992)

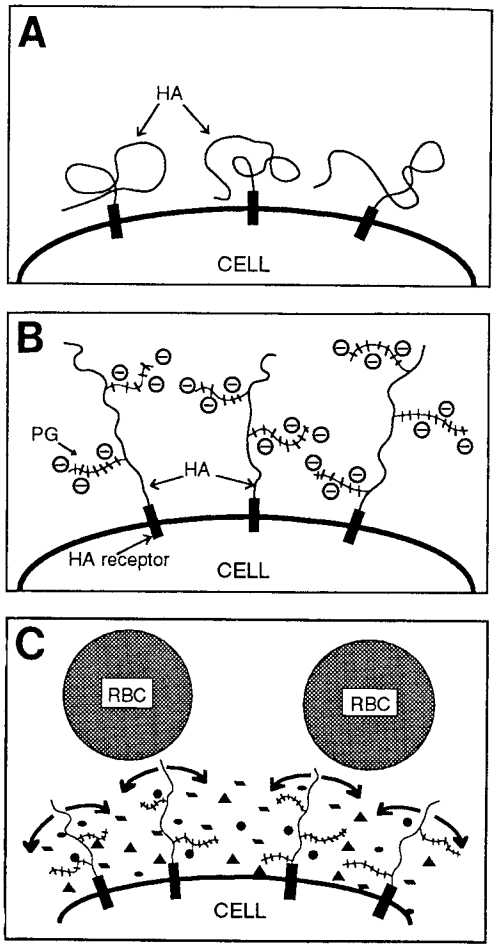


Figure 8. Adaptation of the de Gennes model (de Gennes, 1987) for grafted polymers to the structure of the pericellular matrix. (*A*) With hyaluronan alone, the polysaccharide chains collapse onto the cell surface forming the "mushroom" configuration that grafted polymers are predicted to assume at low density. (*B*) Diagram of hyaluronan (*HA*)-aggrecan (*PG*) complexes on the cell surface. The negative charge repulsion (⊖) of the glycosaminoglycan chains on the proteoglycans has the same effect as increased polymer density proposed by de Gennes. The result is that the complexes straighten and extend outward from the surface. (*C*) The brush configuration of the hyaluronan-aggrecan complexes combined with tethered motion (*curved arrows*), due to thermal fluctuations, causes large particles, such as red blood cells (*RBC*), to be kept away from the cell surface, but smaller particles (*closed symbols*) can readily enter.

rather than the network-forming polymer observed for hyaluronan alone (Scott et al., 1991).

From these observations, we are proposing a model for the arrangement of macromolecules within the cell coat which incorporates the principles of the de Gennes' grafted polymer model (de Gennes, 1987). The possible shapes that can be assumed by a long polymer attached to a surface has been previously described where the density of the polymer on the surface determines the shape the polymer will assume. A version of the model adapted for hyaluronan-aggrecan complexes in the cell coat is shown in Fig. 8 (*A* and *B*). At low density, the polymer has a "mushroom" shape (Fig. 8 *A*). At high density, the polymers extend outward forming a "brush" (Fig. 8 *B*). In a dynamic version, each polymer in the "brush" configuration would be restricted to moving within a cone shape with the movement being most restricted near the membrane (the vertex of the cone) (Fig. 8 *C*). Motion would be less restricted at the end distal to the cell (the directrix of the cone) with the extent of the motion depending on the packing density of the surrounding matrix macromolecules. With the hyaluronan–aggrecan complex, there is an additional layer of complexity added by the long, highly charged aggrecan molecules attached to the hyaluronan molecules. Thus a lower density of "grafted" hyaluronan–aggrecan complexes would be required to produce a "brush". The combination of the extended polymer and the rapid tethered motion also explains how the low density matrix produced by cells cultured without ascorbic acid could exclude large particles such as fixed red blood cells. The extension of the hyaluronan molecules would allow more freedom of movement than would occur if the hyaluronan–aggrecan complexes were coiled on the cell surface. This freedom of movement is consistent with the rapid tethered motion observed for gold-labeled hyaluronan–aggrecan complexes.

The observations of multi-labeled complexes (Fig. 7) provide additional support for the "brush" configuration of the hyaluronan-aggrecan complex. The extended molecular complex appears to be highly dynamic and flexible. However, it was not ascertained whether the hyaluronan is fully extended or in an extended random coil. This determination requires precise determination of the length of the hyaluronan molecules produced by the chondrocytes or added to hyaluronidase-stripped chondrocytes in the reconstitution assays. Additionally, there was some variability between cells as to the mobility of the hyaluronan–aggrecan complexes and the size of the cell coat. Thus, there may be transition states between a loose, extended random coil and full extension.

The brush conformation explains the lack of significant difference in average cell coat size for cells grown with and without ascorbic acid. This lack of effect on coat size was somewhat surprising considering that it has been well-established that culture with ascorbic acid promotes synthesis of matrix components (Kao et al., 1990; Murad et al., 1981). With the brush conformation, the thickness of the cell coat is a function of the average length of the hyaluronan molecule. The concentrations of hyaluronan and aggrecan should not be determinants of coat thickness as long as there is some minimum concentration of hyaluronan and sufficient aggrecan present to cause extension of the hyaluronan. Additional increases in aggrecan concentration, above that necessary for extension, would only result in an increase in the density of the cell coat, not a change in the conformation of the hyaluronan molecule. Thus culture with ascorbic acid results in a denser matrix but not a larger one.

The cell coat, or enlarged pericellular matrix, has several functions. One is to maintain separation of embryonic cells during certain stages of development (Toole, 1991). This separation of cells with coats is analogous to a prediction for the behavior of opposing brushes (de Gennes, 1987) which says that opposing brushes will not interdigitate. In articular cartilage, an important function of the pericellular matrix is to protect the cell from compressive forces exerted by joint movement. This resistance to compression is thought to be due to osmotic pressure produced by the hydrated proteoglycans (for example see Comper, 1991). de Gennes (1987) has predicted that compression of polymers will increase osmotic pressure. If the pericullar matrix in vivo has a similar structure to the cell coat in vitro, a brush configuration for the hyaluronan-aggrecan complexes would enhance the resistance to compression provided by the hydration of the proteoglycans.

The cell coat may also be involved directly and indirectly in ECM assembly. The expression of a cell coat in vitro is directly related to the production of ECM in vivo (Knudson and Toole, 1985). Chondrocytes from nanomelic chickens produce normal type II collagen but do not secrete aggrecan resulting in a greatly reduced volume occupied by the ECM (Vertel et al., 1993). Thus, maintenance of cell separation appears to allow assembly of a more extensive ECM than if the cells were touching. Additionally, it is possible that an extended conformation of the hyaluronan-aggrecan complexes provides a scaffold for the assembly of ECM fibrils. Chondroitin sulfate, the major glycosaminoglycan on aggrecan and versican, is noncovalently bound to fibronectin in the pericellular matrix of fibroblasts (Hedman et al., 1982). Thus the cell coat may serve to retain newly secreted collagen and fibronectin prior to assembly. Assembly of collagen and fibronectin into fibrils could be facilitated by extended hyaluronan–aggrecan complexes that are sufficiently mobile and flexible to accommodate the formation of large fibrils.

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