# Hyaluronan Receptor-directed Assembly of Chondrocyte Pericellular Matrix

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*Abstract.* Initial assembly of extracellular matrix occurs within a zone immediately adjacent to the chondrocyte cell surface termed the cell-associated or pericellular matrix. Assembly within the pericellular matrix compartment requires specific cell–matrix interactions to occur, that are mediated via membrane receptors. The focus of this study is to elucidate the mechanisms of assembly and retention of the cartilage pericellular matrix proteoglycan aggregates important for matrix organization. Assembly of newly synthesized chondrocyte pericellular matrices was inhibited by the addition to hyaluronan hexasaccharides, competitive inhibitors of the binding of hyaluronan to its cell surface receptor. Fully assembled chondrocyte pericellular matrices were displaced using hyaluronan hexasaccharides as well.

When exogenous hyaluronan was added to matrix-free chondrocytes in combination with aggrecan, a pericellular matrix equivalent in size to an endogenous matrix formed within 30 min of incubation. Addition of hyaluronan and aggrecan to glutaraldehyde-fixed chondrocytes resulted in matrix assembly comparable to live chondrocytes. These matrices could be inhibited from assembling by the addition of excess hyaluronan hexasaccharides or displaced once assembled by subsequent incubation with hyaluronan hexasaccharides. The results indicate that the aggrecan-rich chondrocyte pericellular matrix is not only on a scaffolding of hyaluronan, but actually anchored to the cell surface via the interaction between hyaluronan and hyaluronan receptors.

THE initial assembly of the chondrocyte extracellular matrix most likely occurs near the cell surface within a zone termed the cell-associated or pericellular matrix. How this assembly is regulated by the chondrocytes and how the matrix itself is anchored to the cell surface is largely unknown. Hyaluronan, a high molecular weight matrix polysaccharide, has a central role in the organization of the extracellular matrix of cartilage as the backbone of the cartilage proteoglycan aggregate (Hascall and Heinegard, 1974; Christner et al., 1977). In the pericellular matrix of chondrocytes where it is an essential matrix component, hyaluronan may not only serve a structural role, but other functions as well.

Chondrocytes in culture exhibit large pericellular matrices extending from the plasma membrane which can be readily visualized by a particle exclusion assay (Clarris and Fraser, 1968; Goldberg and Toole, 1984; Knudson and Toole, 1985). Small particles when added to low density cultures settle onto the culture dish, and are excluded from a distinct zone surrounding the chondrocytes. This zone or "coat" defines the chondrocyte pericellular matrix. A major component of this matrix is the large, chondroitin sulfate-rich aggregating proteoglycan, termed "aggrecan" (Doege et al., 1991). Treatment of chondrocytes with a small amount of *Streptomyces* hyaluronidase, which specifically degrades hyaluronan (Ohya and Kaneko, 1970; Harrison et al., 1986), removes this pericellular matrix (Goldberg and Toole, 1984; Knudson and Toole, 1985; McCarthy and Toole, 1989) suggesting that the majority of the aggrecan is in the form of aggregates with hyaluronan. The focus of this study is the mechanism of retention of these proteoglycan aggregates in order to elucidate the interactions important to establish and maintain chondrocyte pericellular matrix organization.

Many cells, including chondrocytes (Knudson and Toole, 1987; McCarthy and Toole, 1989; Toole et al., 1989; Yu et al., 1992), have specific cell surface binding proteins, or receptors, for hyaluronan. Hyaluronan receptors present on chondrocytes have properties similar to hyaluronan receptors reported on other cell types such as SV-40 transformed 3T3, BHK, and human bladder carcinoma cells (Underhill and Toole, 1979; Underhill, 1989; Nemec et al., 1987). These hyaluronan receptors are a family of non-integrin, hydrophobic membrane proteins, termed the hyaladherins (Toole, 1991). Studies have also suggested that hyaluronan receptors are related or identical to the CD44 family of lymphocyte homing receptors (Aruffo et al., 1990; Culty et al., 1990). The hyaluronan receptors are grouped together as a family based on their similar physical and functional properties which include: (*a*) a high binding affinity for hyaluronan ($K_d \cong 10^{-9}$ M); (*b*) a high degree of specificity for hyaluronan (when receptor is assayed on non-extracted, intact membranes); (*c*) binding affinity for hyaluronan that increases

with increase in ionic strength; (*d*) binding that is stable to mild fixation of the receptor with glutaraldehyde; and lastly (*e*) binding that can be competed by hyaluronan oligosaccharides with a minimum size of six monosaccharides. These and other properties help to distinguish this family of hyaluronan binding proteins/receptors from other hyaluronan binding proteins including aggrecan and link protein.

One important property shared by all the CD44-like cell surface hyaluronan receptors is the minimum size of hyaluronan oligosaccharide required to effectively compete for the binding of native hyaluronan to its receptor, which is a hyaluronan hexasaccharide ($HA_6$)[1]. This property is helpful in differentiating the specific binding of hyaluronan to the receptor from the aggregation of hyaluronan with other matrix macromolecules, in particular aggrecan and link protein, which require a minimum sequence of 10–12 monosaccharides for competition (Hascall and Heinegard, 1974; Christner et al., 1977; Hardingham et al., 1992). Incubation of cells with $HA_6$ has been shown to displace pre-bound $^3$H-hyaluronan from cell surfaces (Nemec et al., 1987), to compete with binding when added together with $^3$H-hyaluronan to intact cells (Underhill and Toole, 1979) or isolated cell membranes (Underhill et al., 1983), and in this study to inhibit assembly of newly synthesized chondrocyte pericellular matrices. A large proportion of endogenous hyaluronan can be displaced from the cell surface by exogenous hyaluronan or $HA_6$; proteoglycans are also displaced along with the hyaluronan (Knudson and Toole, 1987; Knudson, C. B., L. J. Coombs, and K. E. Kuettner, 1991. *Ortho Trans.* 15:467–468; Hua, Q., C. B. Knudson, and W. Knudson, 1992. *Trans. Orthop. Res. Soc.* 17:29). As shown in this paper, $HA_6$ can displace fully assembled chondrocyte pericellular matrices.

This study characterizes the roles of hyaluronan, aggrecan, and cell surface hyaluronan receptors in the assembly, organization, and maintenance of the chondrocyte pericellular matrix. Two cell types are used as model systems for study; embryonic chick tibial chondrocytes (Kim and Conrad, 1977; Knudson and Toole, 1985, 1987) and adult rat chondrocytes, derived from the well-characterized Swarm rat chondrosarcoma (Kimura et al., 1979; Sun et al., 1986). The results indicate that the aggrecan-rich chondrocyte pericellular matrix is anchored to the cell surface via the interaction between hyaluronan and hyaluronan receptors. Therefore, the hyaluronan receptors may direct the assembly of the chondrocyte pericellular matrix.

## Materials and Methods

All reagents, unless specified otherwise, were purchased from Sigma Chemical Co. (St. Louis, MO). Tissue culture samples were from Gibco BRL (Grand Island, NY).

### Chondrocytes and Culture Conditions

Embryonic chick chondrocytes were released from stage 38 tibiae (Hamburger and Hamilton, 1951), zone II cartilage segments (Kim and Conrad, 1977), by trypsin (type II)/collagenase (type II; Worthington, Freehold, NJ) treatment (Knudson and Toole, 1985). Rat chondrosarcoma chondrocytes from long-term continuous cell cultures were obtained from Dr. James H. Kimura (Henry Ford Hospital, Detroit, MI). The two chondrocyte types were cultured in DME (4.5 g/l glucose) containing 10% FBS (Hyclone, Logan, UT), 1% penicillin/streptomycin solution, 50 μg/ml ascorbic acid and 2 mM glutamine, at 37°C in 5% $CO_2$/95% air. Chondrocytes were also grown in suspension cultures over poly-2-hydroxyethylmethacrylate coating (polyhema; Aldrich, Milwaukee, WI) in complete medium. To coat the surface, 35-mm tissue culture dishes containing 2 ml of 0.06% polyhema in ethanol were incubated to dryness at 37°C.

### Particle Exclusion Assay

The particle exclusion assay followed a protocol described previously for cells in monolayer culture (Knudson and Toole, 1985). The culture medium was removed and replaced with 750 μl of a suspension of formaldehyde-fixed RBCs ($10^8$/ml) in PBS/0.1% BSA. The particles settled in 10 min, and the cells were then observed by phase-contrast microscopy. To visualize the pericellular matrix assembled by chondrocytes in suspension culture, cells were transferred to 6-well flat-bottom tissue culture plates and spun at 500 *g* for 15 min in an Omnifuge microtiter plate holder before the addition of particles.

### Morphometric Analysis of Matrix to Cell Area

A matrix/cell ratio, defined as the ratio of the area delimited by the perimeter of the pericellular matrix to the area delimited by the plasma membrane, was determined by tracing the matrix and cell perimeters on a digitizing tablet using Sigma Scan software (Jandel Scientific, Corte Madera, CA; Knudson and Toole, 1985). The matrix/cell ratio was ~1.0 if no detectable pericellular matrix was present. The values given represent the mean matrix/cell ratio and the 95% confidence range from the mean. Experimental groups were compared with regard to control by a *t* test.

### Enzymatic Removal of Pericellular Matrix

For studies on assembly of newly synthesized pericellular matrix, the endogenous pericellular matrices were removed by adding 20 μl of a 100 U/ml solution of *Streptomyces* hyaluronidase (type IX) directly to 24-h chondrocyte cultures, containing complete culture medium with 10% FBS, and incubating the cells for 1 h at 37°C, followed by HBSS washes. After this treatment, the cells were "matrix-free" by the particle exclusion assay. Following *Streptomyces* hyaluronidase treatment, some cells were fixed in 1% glutaraldehyde (EMS, Fort Washington, PA) in PBS, for 5 min, and then rinsed with PBS/1% BSA.

### Matrix Assembly and Displacement Studies

The influence of $HA_6$ or high molecular weight hyaluronan on matrix assembly was studied. $HA_6$ can compete for the binding of hyaluronan to its receptor but cannot support proteoglycan aggregate formation. Chondrocytes were made matrix-free with *Streptomyces* hyaluronidase. Reassembly of the endogenous pericellular matrix was monitored in complete medium or medium containing hyaluronan (250 μg/ml), or $HA_6$ (25–125 μg/ml).

The ability of exogenous hyaluronan and $HA_6$ to displace the endogenous matrix made by chondrocytes in suspension culture over polyhema, or chondrocytes grown as attached cells in monolayer, was also determined. Cells grown under these culture conditions for 2 to 48 h were incubated for 2 to 3 h more in the presence or absence of hyaluronan or $HA_6$ before the particle exclusion assay.

### Preparation and Addition of Exogenous Macromolecules

To test the ability of chondrocytes to assemble a pericellular matrix with exogenous macromolecules, hyaluronan and aggrecan were added to matrix-free live or glutaraldehyde-fixed cells. Initially, aggrecan derived from several sources was tested; monomer preparations from the Swarm rat chondrosarcoma were gifts from Drs. J. Kimura and T. Glant (Henry Ford Hospital, Detroit, MI and Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL, respectively), and, proteoglycan monomers derived from adult bovine articular or nasal cartilages were gifts from Dr. E. Thonar (Rush-Presbyterian-St. Luke's Medical Center). Subsequently, proteoglycan was extracted from rat chondrosarcoma tumor homogenate according to Faltz et al. (1979) and isolated by dissociative cesium chloride equilibrium centrifugation in 4.0 M guanidine HCl with protease inhibitors at a starting density of 1.5 g/ml (Hascall et al., 1976) for 50 h at 100,000 *g* at 10°C.

1. *Abbreviations used in this paper*: CMF-PBS, Calcium- and magnesium-free PBS; $HA_6$, hyaluronan hexasaccharides; polyhema, poly-2-hydroxyethylmethacrylate.

The bottom 1/4th of the gradient, density >1.6 g/ml, was collected and recentrifuged. The bottom 1/4th of the second gradient was collected (DlDl fractions), dialyzed, and lyophilized. The DlDl fractions were incubated with *Streptomyces* hyaluronidase to degrade small concentrations of hyaluronan found within these preparations. The proteoglycans were recovered by another dissociative equilibrium centrifugation.

$HA_6$ were prepared by digestion of hyaluronan (grade I) with testicular hyaluronidase (type I-S) with 172 USP/NFU hyaluronidase/mg hyaluronan (Knudson et al., 1984; Kimura et al., 1979; Hascall and Heinegard, 1974). The hyaluronan oligosaccharides were separated on a 2.5 × 118 cm column of Bio-gel P30 in 0.5 M pyridinium acetate (Solursh et al., 1980; Knudson and Knudson, 1991).

In parallel experiments, the glycosaminoglycan chondroitin sulfate was added to chondrocytes cultures. Chondroitin sulfate (grade III) was pretreated with *Streptomyces* hyaluronidase, boiled, precipitated in 1.3% potassium acetate in 95% ethanol. Chondroitin sulfate hexasaccharides were prepared by testicular hyaluronidase digestion, purified by Bio-gel P-30 chromatography as above.

Additions to matrix-free chondrocytes were DME containing either: (*a*) no additional components; (*b*) 2.0 mg/ml proteoglycan monomer; (*c*) 12 μg/ml hyaluronan; (*d*) 2.0 mg/ml proteoglycan monomer plus 12 μg/ml hyaluronan (determined empirically as optimal, data not shown); or (*e*) 2.0 mg/ml proteoglycan monomer, 12 μg/ml hyaluronan, and 100 μg/ml $HA_6$.

The hyaluronic acid binding region of DlDl proteoglycan was separated from the chondroitin sulfate-rich fragment by dissociative cesium chloride gradient centrifugation as above, after clostripain digestion (Caputo et al., 1980). Reduction/alkylation of proteoglycan was by treatment with 20 mM DTT followed by addition of iodoacetamide (Hardingham et al., 1976). The products of chondroitinase ABC digestion (Hascall and Heinegard, 1974), were separated by Sephacryl S-300 chromatography in PBS. The proteoglycan core protein, containing chondroitin-4-sulfate stubs, was detected using 9-A-2/E9 antibody (ICN Biomedicals, Inc., Irvine, CA). Residual chondroitinase activity was detected in later eluting fractions by $^3$H-hyaluronan degradation assays (Knudson and Toole, 1987). Modified proteoglycan was lyophilized, dissolved in DME at 10 ng/ml, and added to matrix-free chondrocytes at the same concentration as the native proteoglycan.

The ability of aggrecan to associate with chondrocytes in monolayer culture was tested by adding $^3$H-aggrecan to matrix-free chondrocytes or to chondrocytes with an endogenous pericellular matrix. The $^3$H-aggrecan (Byers et al., 1987) was added at 1 mg/ml (62,000 cpm/mg) serum-free DME per $10^5$ cells for 2 h at 37°C. Cells were then rinsed three times with serum-free DME; the radioactivity was released from the culture dishes with 1% SDS.

### *Proteoglycan Biosynthesis in the Presence of Hyaluronan Hexasaccharides*

Matrix-free chondrocytes were incubated with 25 μCi/ml of [$^{35}$S]sulfuric acid (43 Ci/mg; ICN Biomedicals) in the presence or absence of 250 μg/ml $HA_6$. The media fractions were collected at 24 and 48 h and analyzed by descending chromatography (Knudson et al., 1984). Chondroitinase ABC-sensitive radioactivity at the origin of the strip represented incorporation into chondroitin sulfate proteoglycan. Samples were also run on a Sepharose CL-2B column in 0.5 M sodium acetate, 0.1% Triton X-100, pH 6.8, with and without pre-aggregation with hyaluronan for 24 h (Sandy et al., 1989). The percentage of macromolecular [$^{35}$S]sulfate-labeled material eluting at the $V_0$ of the column represents the capacity of the radiolabeled proteoglycan to form stable aggregates with hyaluronan.

## *Results*

### *Inhibition of Matrix Assembly*

Our model system for matrix assembly uses chondrocytes in monolayer (Fig. 1, *a* and *b*) or suspension culture. The matrix of these chondrocytes was removed with *Streptomyces* hyaluronidase, and then reassembly of newly synthesized pericellular matrices was followed from 2 h up to 5 d. This matrix assembly was monitored in the presence of $HA_6$ to test the hypothesis that receptor-bound hyaluronan, that exhibits competition via a hexasaccharide, functions to anchor the chondrocyte pericellular matrix. Chondrocytes stripped of their pericellular matrix with hyaluronidase reassembled a small pericellular matrix by 3 h and a typical matrix (Fig. 1, *c* and *d*) by 24 h. Fig. 1, *e* and *f*, shows that matrix assembly on both chick embryo chondrocytes and rat chondrocytes was inhibited by the presence of 0.25 mg/ml $HA_6$ in the medium. In the extended presence of 0.25 mg/ml $HA_6$, no matrix was detected after 5 d of culture (data not shown). In these studies, the $HA_6$ were added at time zero, and there were no further additions to the cultures.

Chondrocytes that had no matrix after a 24-h incubation in the presence of $HA_6$ (as in Fig. 1, *e* and *f*), were rinsed twice in HBSS and then incubated in fresh medium. As shown in Table I, after 4 h a small pericellular matrix was observed on these chondrocytes and by 24 h following the wash out of $HA_6$, pericellular matrix assembly was similar in size to chondrocytes in culture for 24 h.

There was a dose-dependent inhibition of matrix assembly by $HA_6$ as shown in Table II. Addition of hyaluronan did not inhibit matrix assembly. The time course for matrix assembly on chondrocytes in monolayer culture following *Streptomyces* hyaluronidase treatment in the presence or absence of exogenous hyaluronan was nearly identical (Table III). In the presence of chondroitin sulfate or its hexasaccharides pericellular matrix assembly was similar to controls for chick chondrocytes, although rat chondrocyte matrix size was decreased somewhat ($P < 0.02$). The concentration of chondroitin sulfate in these cultures has a viscosity nearly equal to that of the hyaluronan solution (Forrester and Lackie, 1981).

### *Displacement of Fully Assembled Pericellular Matrix*

When chondrocytes in monolayer culture for 20 h (Fig. 2, *a* and *c*) were subsequently incubated for an additional 2 h in medium containing $HA_6$, the entire pericellular matrix was displaced (Fig. 2, *b* and *d*; see also Table IV). Although we had shown previously that hyaluronan could displace radiolabeled hyaluronan and chondroitin sulfate from the chondrocyte cell surface (Knudson and Toole, 1987), addition of hyaluronan did not displace the fully assembled endogenous pericellular matrix (Table IV). Also the addition of chondroitin sulfate, chondroitin sulfate hexasaccharides, or proteoglycan monomer did not displace the fully assembled endogenous pericellular matrix (Table IV). Exogenous aggrecan monomer may also be incorporated into a hyaluronan-anchored pericellular matrix since the matrix size was increased in the presence of aggrecan ($P < 0.001$). Chondroitin sulfate hexasaccharides did not displace the matrix, indicative of the specificity of the hyaluronan receptor.

### *Matrix Assembly and Displacement on Chondrocytes Grown in Suspension Culture*

Chondrocytes were grown in suspension over polyhema-coated plastic for 48 h, then centrifuged at 500 *g* for 15 min onto plastic for immediate analysis by the particle exclusion assay. 90% of the cells adhered were viable by trypan blue exclusion, and exhibited a pericellular matrix (Fig. 3, *a* and *c*). If cells from 48 h suspension cultures were incubated for an additional 3 h in the presence of $HA_6$ and then visualized, the endogenous matrix was displaced (Fig. 3, *b* and *d*).

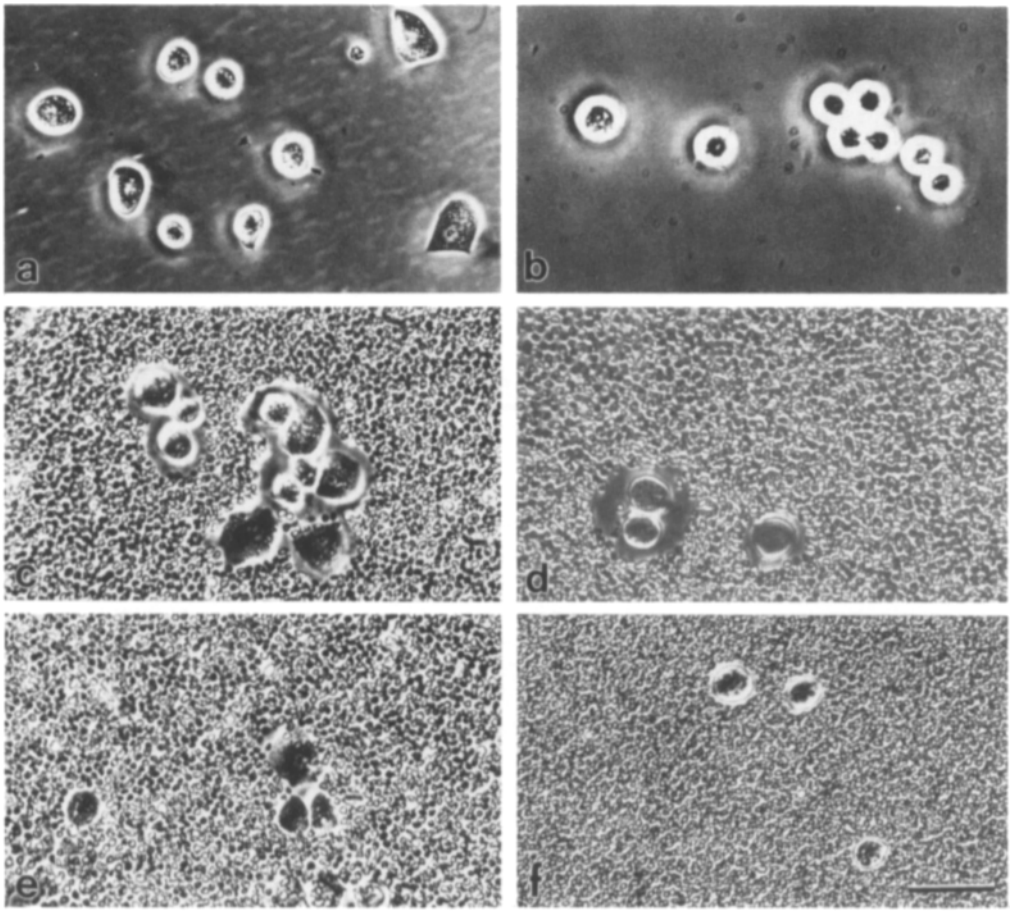


*Figure 1.* Chondrocyte pericellular matrix assembly; inhibition by hyaluronan hexasaccharides. Chondrocytes grown in monolayer were well-spread 24 h following plating (*a* and *b*). These chondrocytes, stripped of matrix with hyaluronidase, reassembled a pericellular matrix in complete medium after 24 h as observed with the particle exclusion method (*c* and *d*). However, if chondrocytes were stripped of matrix and then incubated with 0.25 mg/ml $HA_6$ no matrix was observed after 24 h (*e* and *f*). *a*, *c*, and *e* show embryonic chick chondrocytes; *b*, *d*, and *f* show rat chondrocytes. Bar, 50 $\mu$m.

### *Matrix Assembly with Exogenous Hyaluronan and Aggrecan*

To determine which molecules are required for pericellular matrix assembly, exogenous aggrecan, hyaluronan, and $HA_6$ were added in combinations to matrix-free rat and chick chondrocytes. No matrix assembled for up to 2 h when hyaluronan alone (Fig. 4, *c* and *d*) was added. Addition of $^3$H-hyaluronan to these cells resulted in specific, saturable binding (chick chondrocytes $K_d \approx 6 \times 10^{-9}$ M; Knudson and Toole, 1987; rat chondrocytes $K_d \approx 10^{-10}$ M; McCarthy and Toole, 1989), but hyaluronan alone was not sufficient for matrix assembly. No matrix assembled for up to 2 h when aggrecan alone (Fig. 4, *e* and *f*) was added. A large pericellular matrix assembled within 30 min when both hyaluronan and aggrecan were added to matrix-free chick or rat chondrocytes (Fig. 4, *g* and *h*). No endogenous matrix was detected in control cultures at this time point, or by 2 h (Fig. 4, *a* and *b*). The presence of $HA_6$ prevented the assembly of hyaluronan and aggrecan into a pericellular matrix; no matrix was observed after 2 h (Fig. 4, *i* and *j*), 4 or 48 h (data not shown).

Proteoglycan monomer incorporation into a hyaluronan-anchored pericellular matrix was further studied by adding $^3$H-aggrecan to monolayer cultures of chondrocytes either immediately following matrix removal by *Streptomyces*

*Table I. Morphometric Analysis of Matrix Recovery Following "Wash out" of $HA_6$*

| | 0* | 4‡ | 24§ |
|---|---|---|---|
| Chick chondrocytes | | | |
| + $HA_6$ | 1.07 ± .01 | 1.33 ± .20 | 1.89 ± .24 |
| Medium alone | 2.07 ± .23 | 2.30 ± .27 | 2.91 ± .37 |
| Rat chondrocytes | | | |
| +$HA_6$ | 1.22 ± .04 | 1.75 ± .13 | 2.52 ± .24 |
| Medium alone | 3.00 ± .20 | 4.13 ± .51 | 4.54 ± .62 |

* (0 h) Represents matrix assembly in the presence or absence of $HA_6$ (200 $\mu$g/ml) for 24 h.
‡ (4 h) Represents matrix assembly in fresh medium for 4 h following 24 h culture in the presence or absence of $HA_6$.
§ (24 h) Represents matrix assembly in fresh medium for 24 h following 24 h culture in the presence or absence of $HA_6$.

*Table II. Chondrocyte Pericellular Matrix Assembly in the Presence of Glycosaminoglycans and Oligosaccharides*

| Condition | Chick chondrocytes | Rat chondrocytes |
|---|---|---|
| Medium alone | 2.65 ± 0.41 | 3.29 ± 0.29 |
| Hyaluronan | 2.97 ± 0.21 | 3.29 ± 0.20 |
| $HA_6$ (0.05 mg/ml) | 1.70 ± 0.15 | 2.08 ± 0.11 |
| $HA_6$ (0.1 mg/ml) | 1.67 ± 0.20 | 1.47 ± 0.08 |
| $HA_6$ (0.15 mg/ml) | 1.27 ± 0.18 | 1.30 ± 0.10 |
| $HA_6$ (0.2 mg/ml) | 1.09 ± 0.03 | 1.16 ± 0.06 |
| $HA_6$ (0.25 mg/ml) | 1.07 ± 0.01 | 1.22 ± 0.05 |
| Chondroitin sulfate | 2.74 ± 0.39 | 2.69 ± 0.19 |
| $ChSO_4$ hexasaccharides | 2.46 ± 0.23 | 2.44 ± 0.18 |

Matrix-free chondrocytes were incubated for 24 h in medium containing the glycosaminoglycan listed under condition.

*Table III. Morphometric Analysis of Matrix Assembly*

| | 3 h | 5 h | 18 h | 24 h |
|---|---|---|---|---|
| Chick chondrocytes | | | | |
| + Hyaluronan | 1.46 ± .12 | 1.58 ± .17 | 2.33 ± .28 | 2.93 ± .42 |
| Medium alone | 1.50 ± .15 | 1.77 ± .27 | 2.40 ± .34 | 2.63 ± .42 |
| Rat chondrocytes | | | | |
| + Hyaluronan | 1.23 ± .04 | 1.46 ± .10 | 2.57 ± .26 | 3.15 ± .22 |
| Medium alone | 1.38 ± .12 | 1.67 ± .20 | 2.48 ± .26 | 3.00 ± .31 |

Matrix-free chondrocytes were incubated in medium with or without hyaluronan and matrix assembly monitored at 3, 5, 18, and 24 h.

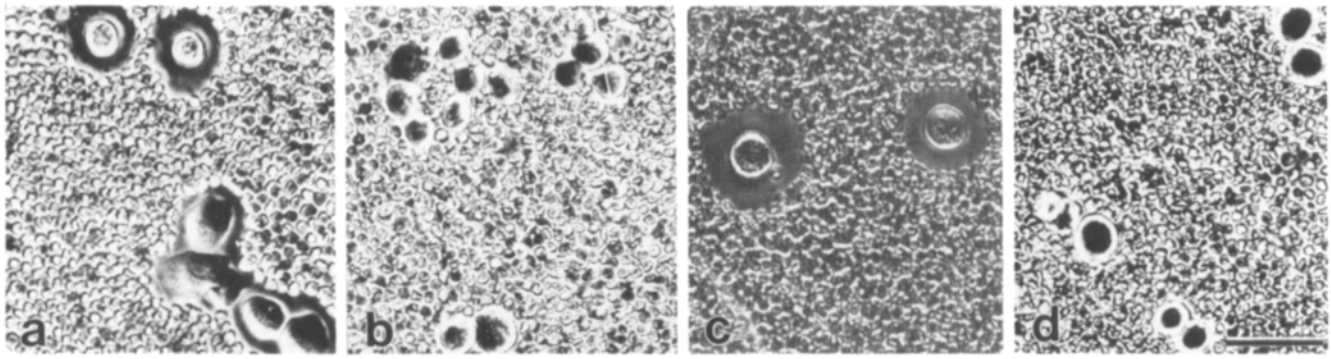


*Figure 2.* Matrix displacement by exogenous hyaluronate hexasaccharides. Chondrocytes in monolayer culture for 20 h, exhibit a pericellular matrix (*a*, chick; *c*, rat). These chondrocytes were incubated for 2 h more in medium containing $HA_6$ which resulted in matrix displacement (*b*, chick; *d*, rat). Bar, 50 μm.

hyaluronidase, or after 24 h of matrix regrowth. Aggrecan did not bind to the monolayers of matrix-free chondrocytes, but exogenous aggrecan did become associated with the monolayers of chondrocytes with fully assembled pericellular matrices (Table V).

### *Specificity of $HA_6$ Displacement of Matrix*

Exogenous pericellular matrices were established on matrix-free chondrocytes using exogenous link-stabilized aggrecan/hyaluronan aggregates. The cells assembled a pericellular matrix with the exogenous components within 90 min (matrix/cell ratio = 2.5 ± 0.2). When chondrocytes with a reassembled link-stabilized pericellular matrix (90 min) were subsequently incubated with fresh media containing $HA_6$ for 2 h, the pericellular matrix was displaced (matrix/cell ratio = 1.12 ± 0.15). Since link protein-stabilized aggrecan/hyaluronan aggregates cannot be disrupted by hyaluronan or hyaluronan oligosaccharides (Hascall and Heinegard, 1974), these results support that matrix displacement occurred via competition of $HA_6$ for hyaluronan/hyaluronan receptor binding.

*Table IV. Displacement of Endogenous Matrix*

| Condition | Chick chondrocytes | Rat chondrocytes |
|---|---|---|
| 2 h cultures | | |
| Medium alone | 2.58 ± 0.22 | 2.31 ± 0.20 |
| Hyaluronan | 2.59 ± 0.36 | 2.32 ± 0.41 |
| $HA_6$ | 1.04 ± 0.03 | 1.04 ± 0.03 |
| Chondroitin sulfate | 2.72 ± 0.43 | 2.44 ± 0.30 |
| $ChSO_4$ hexasaccharides | 2.23 ± 0.23 | 2.12 ± 0.21 |
| Aggrecan | 3.63 ± 0.33 | 3.09 ± 0.32 |
| 20 h cultures | | |
| Medium alone | 3.08 ± 0.31 | 3.24 ± 0.74 |
| $HA_6$ | 1.03 ± 0.03 | 1.09 ± 0.08 |
| Chondroitin sulfate | 3.30 ± 0.39 | 3.31 ± 0.31 |
| $ChSO_4$ hexasaccharides | 3.08 ± 0.45 | 3.18 ± 0.35 |

Matrix-free chondrocytes were grown in monolayer culture for 2 or 20 h. At these time points, the medium was removed and replaced with medium alone or medium containing hyaluronan, $HA_6$, chondroitin sulfate, chondroitin sulfate ($ChSO_4$), hexasaccharides, or rat chondrosarcoma proteoglycan monomer, and all cultures were incubated for an additional 2 h.

### *Domain Structure of Aggrecan Required for Pericellular Matrix Assembly*

Aggrecan from bovine nasal and bovine articular cartilage were compared and found to support matrix assembly with hyaluronan on rat chondrocytes as effectively as rat chondrosarcoma aggrecan (matrix/cell ratios = 3.06 ± 0.30; 3.40 ± 0.21, respectively). Modified rat chondrosarcoma aggrecan was added to matrix-free chondrocytes to determine the domain structure of aggrecan required for matrix assembly; the following results are shown in Table VI. If hyaluronan and reduced/alkylated aggrecan were added, no matrix assembled; this suggests that matrix assembly is mediated via the interaction of the hyaluronic acid binding region of aggrecan with hyaluronan. However, if either the hyaluronic acid binding region or the chondroitin sulfate-rich region, obtained by clostripain digestion of aggrecan followed by cesium chloride gradient centrifugation, were added in the presence of hyaluronan, no matrix assembled. If hyaluronan and chondroitinase ABC pre-treated aggrecan monomer (which stills aggregates with hyaluronan, but has only stubs of chondroitin sulfate) were added, no matrix assembled.

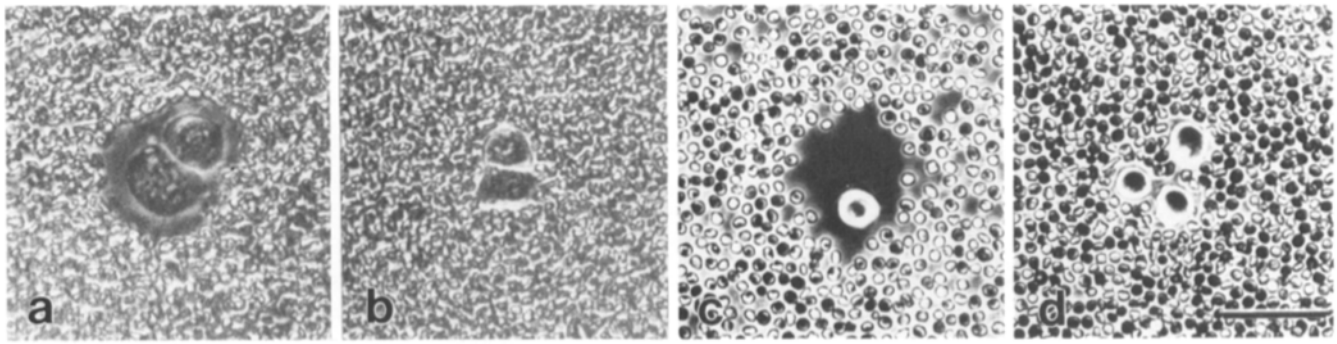


*Figure 3.* Chondrocytes in suspension culture; matrix assembly and displacement. Chondrocytes were grown in suspension for 48 h over 0.06% polyhema coating, transferred to six-well plates and spun at 500 *g* for 15 min; the particle exclusion assay revealed the pericellular matrix. (*a*) Matrix formed on chick chondrocytes in suspension. (*b*) Chick chondrocytes cultured in suspension for 48 h to which $HA_6$ were added for 3 h more; the matrix was displaced. (*c*) Matrix formed on rat chondrocytes in suspension. (*d*) Rat chondrocytes cultured in suspension for 48 h to which $HA_6$ were added for 3 h more; the matrix was displaced. Bar, 50 μm.

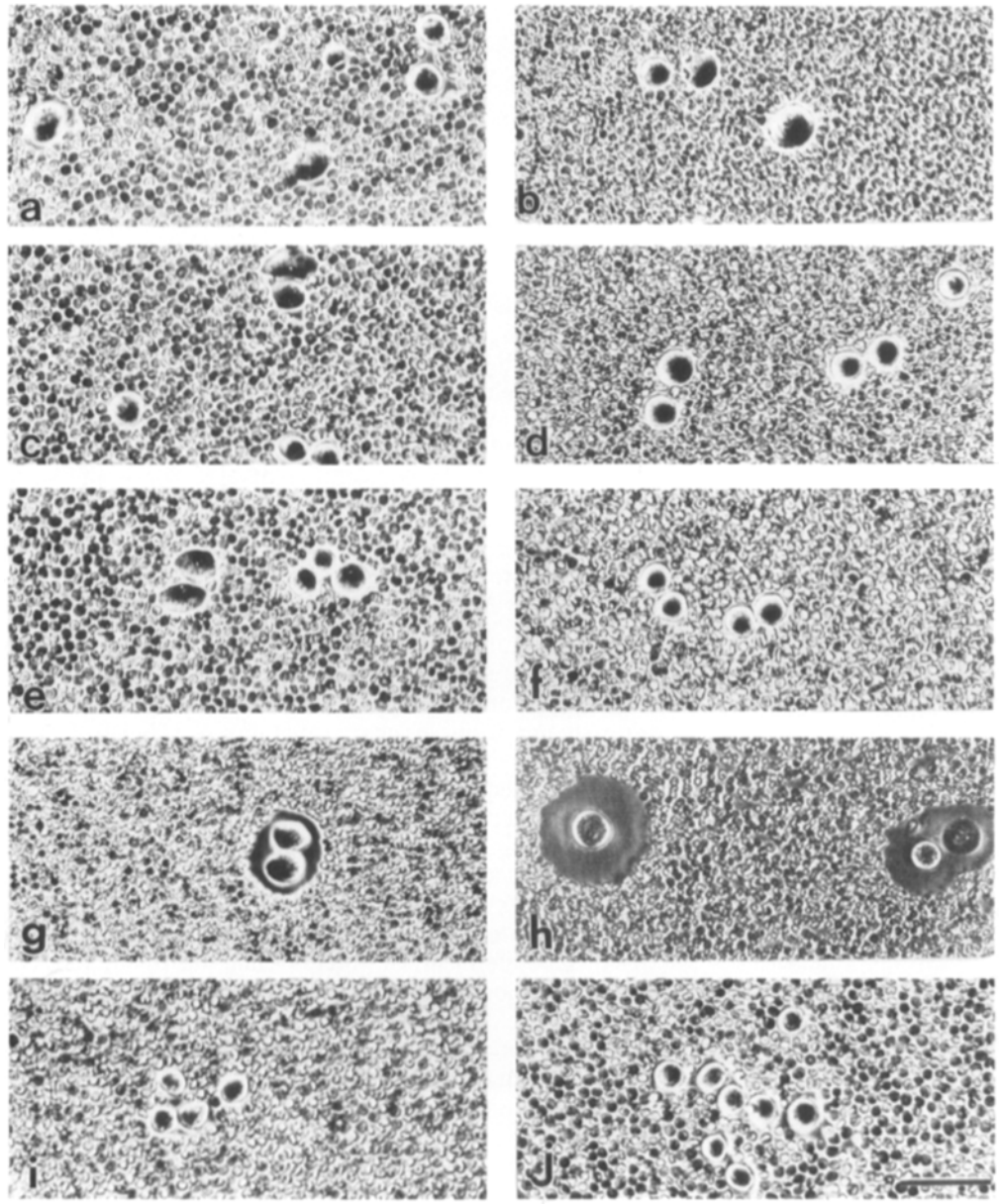


*Figure 4.* Matrix assembly with exogenous macromolecules. *Streptomyces* hyaluronidase-treated chick (*a*, *c*, *e*, *g*, and *i*) and rat (*b*, *d*, *f*, *h*, and *j*) chondrocytes were incubated with either: medium alone for 2 h (*a* and *b*); medium containing hyaluronan for 2 h (*c* and *d*); medium containing aggrecan for 2 h (*e* and *f*); medium containing hyaluronan plus aggrecan for 30 min (*g* and *h*); or medium containing hyaluronan plus aggrecan plus $HA_6$ for 2 h (*i* and *j*). Only cells incubated in the presence of both hyaluronan and aggrecan exhibited significant pericellular matrices (*g* and *h*) and the inclusion of $HA_6$ inhibited matrix formation with these macromolecules (*i* and *j*). Bar, 50 μm.

*Table V. Association of Exogenous $^3$H-Aggrecan with Chondrocyte Monolayers*

| Time following *S.* hyaluronidase treatment | cpm bound per $10^5$ cells | |
|---|---|---|
| | Chick chondrocytes | Rat chondrocytes |
| (*h*) | | |
| 0 | 220 ± 32 | 415 ± 7 |
| 24 | 5,400 ± 215 | 6,780 ± 485 |

Monolayer chondrocyte cultures were pre-treated with *Streptomyces* hyaluronidase, and incubated in serum-free medium containing $^3$H-aggrecan (*O h*), or allowed to regrow a matrix for 24 h in complete medium, and then incubated with $^3$H-aggrecan (*24 h*).

### *Pericellular Matrix Assembly on Glutaraldehyde-fixed Chondrocytes*

To exclude the possibility that exogenous matrix components stimulated endogenous matrix synthesis and assembly, studies were performed on fixed, non-living cells. It is known from previous studies that hyaluronan receptors are stable to mild fixation with glutaraldehyde and still bind $^3$H-hyaluronan with specificity and affinity comparable to live cells (Nemec et al., 1987). After incubation of matrix-free glutaraldehyde-fixed chondrocytes in serum-free medium containing hyaluronan and aggrecan, a pericellular matrix assembled (Fig. 5, *a* and *b*). Pericellular matrices on fixed chondrocytes did not form without the addition of the exogenous hyaluronan and aggrecan. Addition of equivalent concentrations of hyaluronan without aggrecan or, aggrecan without hyaluronan to the fixed chondrocytes did not result in pericellular matrix assembly. Addition of excess $HA_6$ inhibited matrix assembly in the presence of exogenous hyaluronan and aggrecan.

The persistence or stability of these exogenous pericellular matrices on fixed chondrocytes was determined. The cells were rinsed to remove the hyaluronan and aggrecan still in solution and not bound to the cells, and fresh medium with or without $HA_6$ added. After 24 h, these fixed chondrocytes in medium alone, retained the matrix (Fig. 5, *c* and *d*). However, if the fresh medium contained $HA_6$, the matrix formed by exogenous hyaluronan and aggrecan on fixed chondrocytes was displaced (Fig. 5, *e* and *f*). Also, the matrix formed by exogenous hyaluronan and aggrecan on fixed chondrocytes was removed by treatment with *Streptomyces* hyaluronidase (matrix/cell ratio = 1.03 ± 0.02).

*Table VI. Requirement for Intact Aggrecan Plus Hyaluronan for Matrix Assembly with Exogenous Macromolecules*

| Condition | Chick chondrocytes | Rat chondrocytes |
|---|---|---|
| + HA | 1.02 ± 0.02 | 1.01 ± 0.02 |
| + PG | 1.01 ± 0.02 | 1.21 ± 0.05 |
| + HA + PG | 2.39 ± 0.33 | 2.74 ± 0.26 |
| + HA + PG + $HA_6$ | 1.07 ± 0.03 | 1.07 ± 0.04 |
| + HA + HABR | 1.03 ± 0.03 | 1.02 ± 0.02 |
| + HA + CS-R | 1.04 ± 0.03 | 1.05 ± 0.03 |
| + HA + R/A-PG | 1.04 ± 0.02 | 1.05 ± 0.03 |
| + HA + C'ase-PG | 1.06 ± 0.04 | 1.03 ± 0.03 |

Matrix-free chondrocytes were incubated for one hour with medium plus the components indicated. *PG*, aggrecan from rat chondrosarcoma tumor; *HABR*, hyaluronic acid binding region of aggrecan; *CS-R*, the chondroitin sulfate-rich region of aggrecan (minus the hyaluronic acid binding region); *R/A-PG*, aggrecan following reduction alkylation to inactivate the hyaluronan bindng activity; *C'ase-PG*, aggrecan following chondroitinase ABC removal of chondroitin sulfate chains.

To insure the absence of the hyaluronan receptor, chondrocytes were treated with 0.25% trypsin for 60 min, rinsed, and fixed with glutaraldehyde. No binding of $^3$H-hyaluronan to cells is detected following trypsin treatment (Knudson and Toole, 1987; Nemec et al., 1987). After incubation of trypsin-pretreated, fixed chondrocytes in suspension with optimal concentrations of hyaluronan and aggrecan, no pericellular matrix assembled (matrix/cell ratio = 1.10 ± 0.04), whereas matrix assembly did occur on fixed, non-trypsin treated chondrocytes (Fig. 5, *a* and *b*).

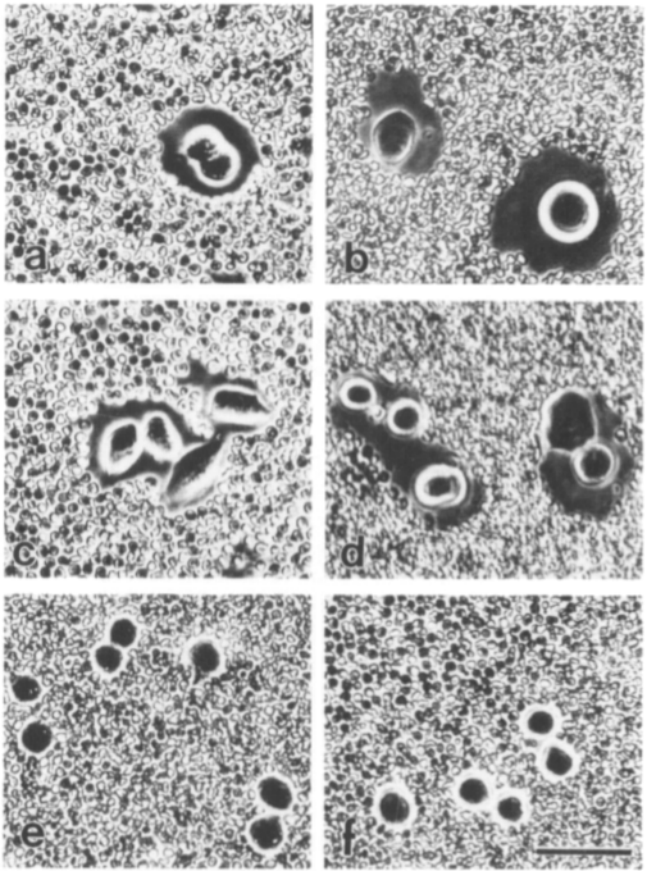


*Figure 5.* Assembly of a pericellular matrix with exogenous macromolecules on matrix-free, glutaraldehyde-fixed chondrocytes. Chick (*a*, *c*, and *e*) and rat (*b*, *d*, and *f*) chondrocytes, which were trypan-blue inclusive, were incubated in DME containing hyaluronan and aggrecan; matrix assembly was observed at 24 h (*a* and *b*). This medium was removed and replaced with new DME for 24 h or DME containing $HA_6$ for 3 h. Cells in DME alone, representing non-specific shedding into the culture medium, retained the matrix (*c* and *d*). Incubation of cells in DME containing $HA_6$ resulted in the displacement of the pericellular matrix from the fixed chondrocytes (*e* and *f*). Bar, 50 μm.

*Table VII. Capacity of Proteoglycan Recovered from the Medium to Aggregate with Hyaluronan*

| Time | $HA_6$ | Chick chondrocytes | Rat chondrocytes |
|---|---|---|---|
| (*h*) | | (%) | (%) |
| 24 h | + | 34 | 24 |
| 24 h | − | 13 | 14 |
| 48 h | + | 45 | 71 |
| 48 h | − | 44 | 70 |

Values represent the percentage of macromolecular $^{35}S$-labeled material eluting at the $V_0$ of Sepharose CL-2B; that is the capacity of $^{35}S$-proteoglycan to form stable aggregates with exogenous hyaluronan. Labeled media from chondrocytes cultured in the presence or absence of $HA_6$ for 24 or 48 h were analyzed.

### *Aggregation Competence of Newly Synthesized Proteoglycans*

To test whether aggrecan synthesized in the presence of $HA_6$ has the same capacity to aggregate with hyaluronan, $^{35}S$-proteoglycans obtained from the medium of chondrocytes cultured in the presence or absence of $HA_6$ were compared. The capacity of the $^{35}S$-proteoglycan to form stable aggregates with hyaluronan was determined as the percentage of proteoglycan eluting at the $V_0$ of a Sepharose CL-2B column after aggregation with 6% exogenous hyaluronan for 24 h. In the presence of $HA_6$ the percentage of aggregating proteoglycans at 24 h was slightly increased for both cell types; however, equivalent percentage of aggregating proteoglycans were found in the medium at 48 h (Table VII).

## *Discussion*

Although many matrix macromolecules within cartilage are capable of cell-free self-assembly, the hypothesis of this study is that initial assembly occurs within a zone immediately adjacent to the chondrocyte cell surface termed the cell-associated or pericellular matrix. The three components of the matrix aggregate in cartilage–hyaluronan, aggrecan and link protein—are synthesized by the chondrocytes. Of these three, only aggrecan and link protein pass through the RER and Golgi compartments, where cytoplasmic interactions could occur (Hardingham et al., 1992). The current model for hyaluronan biosynthesis is of a plasma membrane enzyme complex, by which newly elongating hyaluronan is extruded to the extracellular matrix (Prehm, 1989; Ng and Schwartz, 1989). Thus aggrecan and link protein can only interact with hyaluronan extracellularly. Previous studies indicated specific interaction between the hyaluronan acid binding region of aggrecan and hyaluronan at the chondrocyte cell surface (Sommarin and Heinegard, 1983), but aggrecan receptors have not been detected. Also, newly secreted aggrecan is converted, in the extracellular matrix, to a form with a higher finding affinity for hyaluronan (Sandy and Plaas, 1989; Sandy et al., 1989). The control or catalysis of cartilage matrix assembly may therefore require specific cell–matrix interactions, which are in turn, mediated via membrane binding proteins or receptors. The interactions of matrix macromolecules with cell surface receptors would allow macromolecules to be clustered in proportions and concentrations which assure optimal assembly in the pericellular compartment. The pericellular matrix, once assembled, may then serve as a nucleating template for territorial or interterritorial matrix organization.

Extensive pericellular matrices, of varying thicknesses, have been visualized surrounding a wide variety of eukaryotic cells in culture (Laurent and Fraser, 1992). The principal feature known concerning the structural composition of these pericellular matrices is that they are dependent on hyaluronan. In previous studies, however, we observed that cells of embryonic limb mesenchyme could turn on, or off, the expression of pericellular matrices without change in the levels of their cell surface hyaluronan, suggesting that other components are required for matrix formation (Knudson and Toole, 1985). The removal of the chondrocyte pericellular matrix by treatment with a dilute solution of *Streptomyces* hyaluronidase, led to the hypothesis that a network of hyaluronan is the scaffold for the chondrocyte pericellular matrix, to which other matrix macromolecules are bound (Knudson and Toole, 1985, 1987). The in vivo presence or function of extensive pericellular matrices which surround many cell types in culture is not well established. Clearly, the expression of pericellular matrices around chondrocytes correlates well with their primary cellular function; to generate and organize an extracellular matrix capable of withstanding load forces. The interactions of chondrocytes with the extracellular matrix may provide a mechanism for the cells to respond to the composition and/or mechanical properties of this surrounding matrix. The degree of extracellular matrix surrounding chondrocytes in vivo also seems to correlate well with the expression of pericellular matrices in vitro, suggesting that these structures do in fact exist in vivo (Knudson and Toole, 1985).

To determine that these cell-associated matrices visualized by the particle exclusion assay were not an artifact of monolayer culture, we developed techniques to analyze chondrocytes grown in suspension culture. Via a modified cytospin technique, cells in suspension were centrifuged ("splatted") onto a plastic well assay surface. The cells were found to exhibit pericellular matrices similar to those observed on chondrocytes grown on monolayer culture. Thus, cells in suspension also elaborate a pericellular matrix which can be visualized in our two-dimensional assay by the rapid splatting of cells before visualization. With this method it was now possible to determine whether chondrocytes elaborate such a matrix in vivo. In a preliminary study (Knudson, C. B. 1991. *J. Cell Biol.* 115:297), chondrocytes were isolated from the 12-d embryonic chick tibiae by a 10-min treatment of the cartilage with purified collagenase P (Boehringer Mannheim Biochemicals, Indianapolis, IN), in medium containing 20% FBS. The freshly isolated single cells were diluted, splatted onto the six-well plastic culture surface and visualized by the particle exclusion assay. The newly isolated chondrocytes exhibited prominent pericellular matrices (matrix/cell ratio = $3.21 \pm 0.20$) similar in size to the cultured chondrocytes. This matrix was displaced by subsequent 3-h incubation in medium with $HA_6$.

The first conclusion of this study is that the ability to assemble a pericellular matrix requires the expression of cell surface hyaluronan binding sites or "receptors" and the interaction of hyaluronan with these receptors. This conclusion was drawn because the pericellular matrices could be prevented from assembling or, displaced from the cells once assembled, via the addition of an excess of $HA_6$, competitive inhibitors of the binding of hyaluronan to its receptor. These

results suggest that the pericellular matrix is not only on a scaffolding of a hyaluronan network but actually anchored to the chondrocyte cell surface via hyaluronan/hyaluronan receptor interactions. Exogenous hyaluronan did not inhibit matrix assembly or displace intact matrices. High molecular weight hyaluronan added exogenously does bind to matrix-free chondrocytes (Knudson and Toole., 1987; McCarthy and Toole, 1989), and thus may be incorporated into the matrix, exchanging for endogenous receptor-bound hyaluronan and supporting proteoglycan aggregate formation and retention. However, in the presence of $HA_6$, when potentially all cell surface hyaluronan receptors are occupied with hexasaccharides which are too small to mediate proteoglycan aggregate formation, the assembly and retention of the pericellular matrix is inhibited. Thus receptor-bound hyaluronan appears to serve as the pericellular matrix anchor.

When added to pre-chondrogenic micromass cultures established from embryonic chick limb bud mesoderm, $HA_6$ inhibits chondrogenesis, a process believed to be mediated via hyaluronan receptors (Toole, 1991; Maleski, M. P., and C. B. Knudson. 1992. *Trans. Orthop. Res. Soc.* 17:105). A mAb (IVd4) was generated by Toole and his colleagues (1991), directed against a hyaluronan receptor present in embryonic chick tissues. Analogous to the $HA_6$, this antibody blocks binding of $^3$H-hyaluronan to hyaluronan receptors (Banerjee and Toole, 1991), inhibits assembly of newly synthesized pericellular matrices on chondrocytes (Yu et al., 1992) and inhibits chondrogenesis of micromass cultures (Toole, 1991).

To determine the components minimally required to establish a chondrocyte pericellular matrix, namely a matrix that can exclude particles, purified exogenous macromolecules were added back to matrix-free chondrocytes. Only when exogenous hyaluronan was added in combination with intact aggrecan did a pericellular matrix assemble. The presence or absence of keratan sulfate on the aggrecan did not affect matrix assembly. These results suggest that the matrix observed by the particle exclusion assay depends on the space occupied by the chondroitin sulfate chains of the aggrecan. However, the aggrecan must interact with hyaluronan to be retained at the chondrocyte cell surface; the hyaluronan being anchored to the cell surface via the hyaluronan receptor, and displaceable by $HA_6$. The second conclusion of this study is that hyaluronan and intact aggrecan, decorating the hyaluronan in aggregate form, are essential for matrix formation. Addition of exogenous link-stabilized hyaluronan/aggrecan aggregates also supported matrix assembly and, more importantly, the link-stabilized matrix could still be displaced by $HA_6$. This also supports the conclusion that the displacement of the cell-associated, proteoglycan aggregate pericellular matrix by $HA_6$ is via competition with hyaluronan/hyaluronan receptor interactions and not hyaluronan/aggrecan interactions. This later interaction occurs between aggrecan and hyaluronan oligosaccharides of a decasaccharide size or larger, with specific structural requirements (Hascall and Heinegard, 1974; Christner et al., 1977).

To determine whether the addition of exogenously added macromolecules promoted matrix assembly by stimulation of endogenous matrix production, nonliving chondrocytes mildly fixed by treatment with 1% glutaraldehyde were assayed. Addition of hyaluronan and aggrecan to fixed chondrocytes in the absence of serum resulted in pericellular matrix assembly comparable to live chondrocytes. These matrices were stable for 24 h without continuous exposure to the soluble macromolecules. Therefore the third conclusion from our current results is that the formation of a pericellular matrix on chondrocytes can be obtained with as few as two components, the addition of exogenous hyaluronan and an aggregating type proteoglycan and that serum components are not required. No matrix formed if trypsin pre-treated cells were used demonstrating that matrix assembly is mediated via a cell surface-associated protein(s).

In previous studies, to substantiate the role of the hyaluronan receptor in matrix assembly exogenous hyaluronan and aggrecan were added to other cultured cell types (Knudson and Knudson, 1991). The cell types chosen were ones which never exhibit pericellular matrices in culture, synthesize little hyaluronan or proteoglycan, yet do express hyaluronan receptors similar to those expressed on chondrocytes. Addition of hyaluronan plus aggrecan to two different human bladder carcinoma cell lines or to SV-40–transferred 3T3 cells, resulted in the assembly of chondrocyte-like pericellular matrices. As with the chondrocytes, hyaluronan, or aggrecan alone were unable to support matrix assembly; $HA_6$ inhibited matrix assembly or displaced matrices once assembled; and glutaraldehyde-fixed tumor cells still supported matrix assembly. Addition of hyaluronan and aggrecan to matrix-deficient tumor cells which did not express hyaluronan receptors (B16 melanoma and RT-4 papilloma) were unable to assemble pericellular matrices. In addition, conditioned medium from rat fibrosarcoma cells (cells which exhibit pericellular matrices) also supported matrix assembly, suggesting that other hyaluronan-aggregating proteoglycans (e.g., versican, hyaluronectin; see Toole, 1991) may also support matrix assembly.

The influence of hyaluronan on cell physiology probably depends on its hydrodynamic properties and its interaction with the cell surface (Laurent and Fraser, 1992). Inhibition of chondrocyte proteoglycan biosynthesis in response to exogenous hyaluronan has been reported for several cell culture systems (Wiebkin and Muir, 1973; Solursh et al., 1974, 1980; Handley and Lowther, 1976; Bansal et al., 1986) although the results have reflected undetermined variability (reviewed in Mason et al., 1989). In our studies, exogenous hyaluronan did not inhibit matrix assembly or displace intact matrices. Also, total incorporation into $^{35}$S-proteoglycans was not altered significantly in the presence of $HA_6$. In the presence of $HA_6$, there was an increased accumulation of newly synthesized proteoglycan in the medium compartment, which was competent to aggregate with hyaluronan. This suggests that a decrease in the synthesis of aggregating proteoglycans was not responsible for the blocked matrix assembly in the presence of $HA_6$, but rather than the retention of aggrecan by receptor-bound hyaluronan in the pericellular matrix was blocked in the presence of $HA_6$. These were 24-h experiments, and the long-term influence of matrix displacement via $HA_6$ on the chondrocyte phenotype is currently under study. Additionally, through the interaction of these matrix receptors with extracellular components, chondrocytes may fine tune their patterns of matrix biosynthesis and/or turnover, as has been previously suggested by several investigators (Wiebkin and Muir, 1973; Solursh et al., 1974, 1980; Handley and Lowther, 1976; Bansal et al., 1986; Morales and Hascall, 1988).

Matrix assembly and retention at cell surfaces is important for the health of any tissue, cartilage in particular. Chondro-

cyte interactions with extracellular matrices are important in the maintenance of tissue integrity, and for matrix assembly in repair and morphogenesis. These interactions are mediated via binding proteins/receptors for extracellular matrix molecules. Little is known about the distribution of integrins on chondrocytes. Anchorin CII is the major site to anchor chondrocytes in their collagen extracellular matrix (Mollenhauer et al., 1984). In their studies, reassembly of the chondrocyte pericellular matrix was correlated with the intensity of anchorin CII immunoreactivity on the cell surface (Mollenhauer et al., 1984). Receptors with anchoring functions may also transduce information from the extracellular matrix influencing cell proliferation, differentiation, and physiology. The relationship between the interactions of the collagens and the proteoglycan aggregates with the chondrocyte surface is under investigation to further our understanding of the mechanisms used by chondrocytes to interact with the extracellular environment in healthy and diseased cartilage.

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