

Pericellular Coat of Chick Embryo Chondrocytes: Structural Role of Hyaluronate

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ABSTRACT Chondrocytes produce large pericellular coats in vitro that can be visualized by the exclusion of particles, e.g., fixed erythrocytes, and that are removed by treatment with *Streptomyces* hyaluronidase, which is specific for hyaluronate. In this study, we examined the kinetics of formation of these coats and the relationship of hyaluronate and proteoglycan to coat structure. Chondrocytes were isolated from chick tibia cartilage by collagenase-trypsin digestion and were characterized by their morphology and by their synthesis of both type II collagen and high molecular weight proteoglycans. The degree of spreading of the chondrocytes and the size of the coats were quantitated at various times subsequent to seeding by tracing phase-contrast photomicrographs of the cultures. After seeding, the chondrocytes attached themselves to the tissue culture dish and exhibited coats within 4 h. The coats reached a maximum size after 3–4 d and subsequently decreased over the next 2–3 d. Subcultured chondrocytes produced a large coat only if passaged before 4 d. Both primary and first passage cells, with or without coats, produced type II collagen but not type I collagen as determined by enzyme-linked immunosorbent assay. Treatment with *Streptomyces* hyaluronidase (1.0 mU/ml, 15 min), which completely removed the coat, released 58% of the chondroitin sulfate but only 9% of the proteins associated with the cell surface. The proteins released by hyaluronidase were not digestible by bacterial collagenase. Monensin and cycloheximide (0.01–10 μ M, 48 h) caused a dose-dependent decrease in coat size that was linearly correlated to synthesis of cell surface hyaluronate ($r = 0.98$) but not chondroitin sulfate ($r = 0.2$). We conclude that the coat surrounding chondrocytes is dependent on hyaluronate for its structure and that hyaluronate retains a large proportion of the proteoglycan in the coat.

In cartilage, chondrocytes lie within lacunae that are in turn surrounded by a territorial matrix that exhibits intense basophilia compared with the interterritorial matrix more distant from the cells. The lacunar area contains a narrow pericellular region of matrix, the ultrastructure of which differs from the adjacent territorial matrix (1–3). This pericellular matrix is 1–3- μ m thick, whereas the territorial matrix is often much larger, up to 50- μ m thick (2). The pericellular and territorial matrices form the interface between the chondrocyte surface and the bulk of the tissue, i.e., the interterritorial matrix, and thus are presumed to be important in control of matrix composition and turnover. They differ considerably in structure from the interterritorial matrix (2–5), and vary in structure according to site and physiological state of the cartilage (4–6). Two types of experimental evidence have indicated that hyaluronate may play an important role in the assembly of

these matrices. First, hyaluronate-proteoglycan interactions have been found to mediate the retention of proteoglycan at the cell surface of chondrocytes (7–9). Second, hyaluronate interaction with the chondrocyte surface has been shown to influence proteoglycan synthesis and secretion (8, 10–12).

Surrounding many types of cells in culture is a large pericellular coat, 10–20- μ m thick, which can be visualized by exclusion of particles, e.g., fixed erythrocytes (13–15). Hyaluronate is known to play a role in the structure of these coats because low concentrations of *Streptomyces* hyaluronidase, an enzyme that degrades only hyaluronate (16), remove these coats (15) and drugs that inhibit hyaluronate synthesis prevent their formation (17, 18). We have found that chick embryo chondrocytes in culture make very large, *Streptomyces* hyaluronidase-sensitive coats that may then provide an in vitro model for the pericellular and/or territorial matrices. In this

publication we examine the kinetics of formation of these coats and the relationship of hyaluronate and proteoglycan to their structure.

MATERIALS AND METHODS

Cell Cultures

Chick chondrocytes preparation and culture conditions were based upon previously published techniques (19). The tibia from 12-d-old chick embryos (stage 37) were dissected free of muscle and extraneous connective tissue. The cartilaginous portion of the diaphysis was removed from within the periosteum and rinsed in 0.02 M Tris-0.15 M NaCl buffer, pH 7.5, and then with enzyme solution, 0.5% collagenase, 0.2% trypsin in the same buffer. The cartilage was digested three times with this enzyme solution for 30 min at 37°C. The cells were pelleted by centrifugation and washed once with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin-streptomycin. The cells were cultured in the same medium at 37°C in 5% CO₂/95% air. Ascorbate (50 µg/ml) was added to the cultures every 2 or 3 d. The drugs monensin (Calbiochem-Behring Corp., La Jolla, CA) and cycloheximide (Sigma Chemical Co., St. Louis, MO), were dissolved in ethanol before addition to cultures. The 3T3 cells were grown in the same medium and under the same conditions as described for the chondrocytes.

DNA Content

The DNA content of cultures was quantitated by the intercalated fluorescence of ethidium bromide (18, 20). Sperm whale DNA (Sigma Chemical Co.) was used as a standard. 1 µg of DNA was the lower limit of quantitation.

Visualization of Coats

A suspension of formalin-fixed erythrocytes (0.8 ml, 10⁸ cells/ml in 0.1% BSA in PBS) was added to a 35-mm dish of chondrocytes and the erythrocytes were allowed to settle for 10–20 min as described previously (15). The cultures were observed with a phase-contrast microscope and photographed. The coats were removed with 1 mU/ml of *Streptomyces* hyaluronidase (Miles Laboratory Inc., Elkland, IN) at 37°C for 30 min.

Morphometric Analysis of Coats

We determined areas within the coat perimeter (i.e., the area from which erythrocytes are excluded) and the cell perimeter, termed "coat + cell area" and "cell area," respectively, by tracing photomicrographs on a digitizing graphics tablet connected to an Apple computer. Relative coat size or thickness is defined by the "coat/cell ratio," i.e., the ratio of the area within the coat perimeter (the coat + cell area) to the area within the cell perimeter (cell area). A ratio of 1.0 would thus signify no detectable coat. Randomly chosen fields were photographed for these measurements.

Glycosaminoglycan Synthesis

The incorporation of [³⁵S]sulfate and [³H]acetate into glycosaminoglycans of the cell surface and the medium was measured. After 24 h incorporation with 100 µCi/ml [³⁵S]sulfate or 20 µCi/ml [³H]acetate, the medium was removed, then the cell layer was washed twice and treated with 0.25% trypsin, 0.02% EDTA in calcium- and magnesium-free PBS at 37°C for 15 min. The cell surface material (trypsinate) was separated from the cells by centrifugation. The medium and trypsinate were digested with 1 mg/ml of protease for 24 h at 50°C followed by boiling to inactivate the protease. Because of the large amount of free isotope in the medium, the macromolecules in this fraction were precipitated with 3 vol of 1.3% potassium acetate in 95% ethanol (-20°C, 24 h) prior to assay. From each preparation 0.2-ml aliquots were incubated with or without 2 U of *Streptomyces* hyaluronidase at 50°C or 0.1 U chondroitinase ABC at 37°C for 24 h. The labeled glycosaminoglycans were precipitated with 0.02 ml of a 10% cetylpyridinium chloride solution in the presence of 0.4 ml of carrier (0.5 mg/ml hyaluronate and 0.25 mg/ml chondroitin sulfate). After 1 h of incubation at 37°C, the samples were centrifuged in a Beckman microfuge (10,000 g, 5 min [Beckman Instruments, Inc., Palo Alto, CA]). The pellets were washed twice with 0.05% cetylpyridinium chloride, 0.05 M NaCl, then dissolved in 0.15 ml of methanol. We measured the radioactivity in the solutions by adding 1.45 ml of scintillation cocktail into the microfuge tubes and counting the tubes in carrier vials in a Beckman LS-8100.

The amount of labeled hyaluronate was determined from the difference in radioactivity in the nondigested sample and the *Streptomyces* hyaluronidase-

digested sample. Labeled chondroitin sulfate was the difference between the *Streptomyces* hyaluronidase-digested and the chondroitinase ABC-digested samples.

Protein Synthesis

NONCOLLAGENOUS PROTEIN: Cultures were incubated for 24 h with [³H]proline (20 µCi/mol, 10 µCi/ml) and the medium and cells were harvested. BSA was added to aliquots of each to give a concentration of 0.1%, and protein was then precipitated with trichloroacetic acid (10% final concentration) at 4°C for 3 h. The samples were centrifuged in a microfuge for 3 min (10,000 g) and the pellet was washed twice with 10% trichloroacetic acid. The pellet was dissolved in 0.1 ml of 0.2 N NaOH then acidified with 0.02 ml of 1 N acetic acid. Radioactivity in the microfuge tube was measured as described for glycosaminoglycan synthesis (above). The trypsinate was separated from the cells by centrifugation and the radioactivity of an aliquot of the supernatant was used as a measure of the residual protein at the cell surface.

COLLAGEN: Metabolically labeled collagen was quantitated in the cell surface trypsinate and PBS incubation medium from the above cultures by the susceptibility of trichloroacetic acid precipitable material to purified, protease-free bacterial collagenase. The difference between the nondigested sample and the digested sample was considered collagenous. The samples with trypsin were boiled before digestion with collagenase. Also, *N*-ethylmaleimide was added to all samples to prevent possible nonspecific degradation. Data were corrected for the 5.4 times greater proline content of collagen compared with the average content of other proteins (21).

Identification and Quantitation of Collagen Types

SDS PAGE: Cultures were incubated with fresh medium containing ascorbate and [³H]proline (20.7 µCi/mol, 10 µCi/ml medium). After 24 h the medium was removed and the macromolecules were precipitated with ammonium sulfate (45% final concentration) and then centrifuged (1,500 g, 15 min). The pellet was dissolved in 0.5 M acetic acid and dialyzed against the same at 4°C. After 2 h, pepsin (0.1 mg/ml final concentration) was added to the sample, which was then digested and dialyzed at 4°C for 24 h. The sample was dialyzed against 0.01 M sodium phosphate, pH 7.4, for 24 h. Aliquots were electrophoresed on SDS polyacrylamide slab gels (6% acrylamide) as described by Laemmli (22). The gels were dried and placed in Enhancer™ and the autoradiographs were developed. Radioactive rat tail type I collagen obtained from Dr. C. Biswas (Tufts University School of Medicine) was used as a standard.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA):¹ The relative inhibition of color production in an ELISA assay was used to calculate the amounts of type I and II collagens in culture medium. Conditions for coating plates and concentrations of primary and secondary antibodies were optimized according to Rennard and co-workers (24, 25). Chick type I and II collagens and rabbit polyclonal antibodies against chick type I and II collagens were obtained from Drs. T. Linsenmayer and T. Schmid (Tufts University School of Medicine) (23). 50% inhibition was obtained at 200–600 ng/ml for the homologous collagen and 300 times more was needed for 50% inhibition with the heterologous collagen. The second antibody used was goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Chemical Co.). Microwell plates (Dynatech Laboratories, Inc., Alexandria, VA) were read with a Bio-Tek EIA reader (EI 307; Bio-Tek Instruments, Inc., Burlington, VT). Standard curves were constructed with the ALLFIT program (26) modified for Apple computers (Biomedical Computing Technology Information Center, Nashville, TN) for best fit parameters for a sigmoid curve. The computed collagen concentration of diluted medium samples was calculated by substituting into the sigmoid equation the absorption value of the sample obtained in the ELISA assay and the fit parameters obtained from ALLFIT based upon the standard collagens. Only values >15% and <85% inhibition (i.e., ± 2 SD) were considered significant.

Proteoglycan Monomer Size

Cultures were incubated with [³⁵S]sulfate (100 µCi/ml) for 24 h. The medium was removed and precipitated with 3 vol of 1.3% potassium acetate in 95% ethanol at -20°C for 24 h. The samples were centrifuged (1,500 g, 15 min) and the pellets were dissolved in 4 M guanidine-HCl, 0.05 M sodium acetate, pH 5.8, plus the protease inhibitors, 5 mM benzamidine-HCl, 100 mM 6-amino-hexanoic acid, and 10 mM EDTA. Samples were first passed through a Sepharose CL-6B column equilibrated with the above buffer to remove unincorporated sulfate. The macromolecular materials were pooled and aliquots were then passed through a Sepharose CL-2B gel filtration column in the above buffer (27).

¹ Abbreviations used in this paper: ELISA, enzyme-linked immunosorbent assay.

Release of Cell Surface Macromolecules with Hyaluronidase

Chondrocytes were incubated with fresh serum-containing medium plus 50 $\mu\text{g}/\text{ml}$ ascorbate and 100 $\mu\text{Ci}/\text{ml}$ of either [^{35}S]sulfate or [^3H]proline. After 24 h the cells were washed five times with PBS then incubated with either PBS or PBS containing *Streptomyces* hyaluronidase (1 mU/ml) at 37°C for 30 min. The incubation medium was then removed from the cell layer and centrifuged at 10,000 g . The supernatant was either analyzed for chondroitin sulfate by susceptibility to chondroitinase ABC digestion or for proteins by precipitation with trichloroacetic acid as described above. The remaining cell surface macromolecules were released with 0.25% trypsin and also analyzed as described above.

RESULTS

Pericellular Coats of Chondrocytes

Chick embryo chondrocytes produced large pericellular

coats that can be visualized by exclusion of particles, e.g., fixed erythrocytes (Fig. 1a). These coats were removed by treatment with small amounts of *Streptomyces* hyaluronidase (Fig. 1b), which is specific for hyaluronate (16). At days 3–4 of culture, >95% of the cells displayed these coats with a mean thickness of 12 μm (\pm 2 SD).

Since chondrocytes are labile in their expression of extracellular matrix, we checked that the culture conditions under which maximum coat formation was observed also allowed expression of typical chondrocyte characteristics. First, the cells were seen to have the rounded to polygonal shape typical of chondrocytes (Fig. 1a). Second, these chondrocytes produced three to four times more chondroitin sulfate than hyaluronate (Table IC), and the chondroitin sulfate was present in large proteoglycan monomers. When chromatographed in 4 M guanidine-HCl on Sepharose CL-2B, these monomers

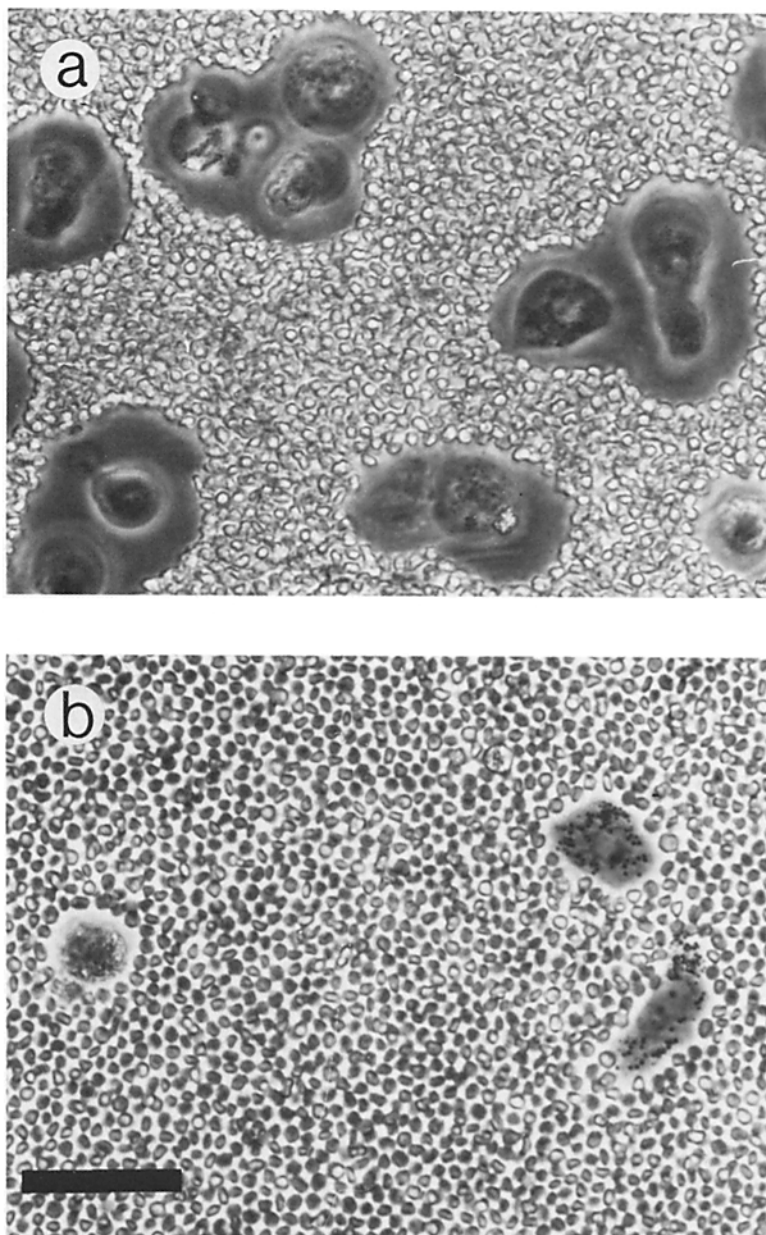


FIGURE 1 Pericellular coats of chick embryo chondrocytes visualized by exclusion of formalin-fixed erythrocytes. Phase-contrast photomicrographs taken 15 min after the addition of erythrocytes. (a) Untreated chondrocytes. (b) Chondrocytes treated with *Streptomyces* hyaluronidase (1 mU/ml, 30 min, 37°C). Bar, 50 μm .

TABLE I
Matrix Macromolecule Synthesis by Chondrocytes in Primary Culture

	Days in culture		
	2	3	4
A Coat/cell ratio	4.1 ± 0.4	5.1 ± 0.6	2.9 ± 0.2
B Cell surface GAG*			
Chondroitin sulfate	0.30 ± 0.04	0.24 ± 0.01	0.08 ± 0.1
Hyaluronate	0.05 ± 0.01	0.06 ± 0.01	0.01 ± 0.003
C Medium GAG*			
Chondroitin sulfate	56 ± 2	77 ± 4	134 ± 10
Hyaluronate	13 ± 4	26 ± 4	41 ± 6
D Medium collagen†			
Type II	0.32 ± 0.02	0.64 ± 0.06	1.10 ± 0.06
Type I	<0.1	<0.1	<0.1

Macromolecule synthesis and coat/cell ratio were determined as described in Materials and Methods. Synthesis of glycosaminoglycan (GAG) was measured by the incorporation of [³H]acetate and collagen by ELISA.

* cpm × 10⁶/μg DNA.

† μg/μg DNA.

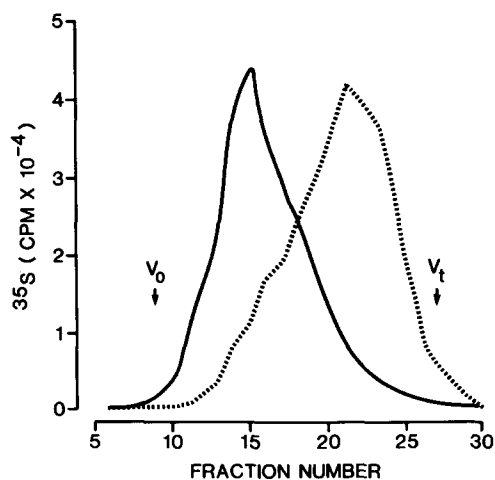


FIGURE 2 Gel chromatography of proteoglycan monomers on Sepharose CL-2B. ³⁵S-labeled media from chondrocyte (-) and 3T3 cell (-) cultures were precipitated in ethanol and then dissolved in 4 M guanidine-HCl plus protease inhibitors. We removed unincorporated [³⁵S]sulfate by passing through a gel filtration column before chromatography on CL-2B.

were shown to have a K_{av} of 0.3 (Fig. 2), similar to the value obtained by others for chondrocytes (27). In comparison, 3T3 cells were shown to produce much smaller proteoglycans with a K_{av} of 0.7. The third criterion used was the type of collagen synthesized. SDS PAGE revealed only one band in the region of collagen alpha-chains (Fig. 3). This suggested strongly that type II, not type I, collagen was being produced (28). In addition, no band was observed in the collagen gamma-chain region in the gel performed under nonreducing conditions, indicating that no type III collagen was produced. The identity of the type II collagen was confirmed by ELISA (Table I), which showed the presence of type II and not type I collagen. Thus we conclude that the cells in these cultures were differentiated chondrocytes.

Chondrocyte Spreading and Coat Formation

To characterize the kinetics of coat formation, we measured two parameters. These were the area within the cell perimeter (cell area), which is a measure of the degree of spreading, and the area within the coat perimeter (coat + cell area). The ratio of these two parameters (coat/cell ratio) provides a relative

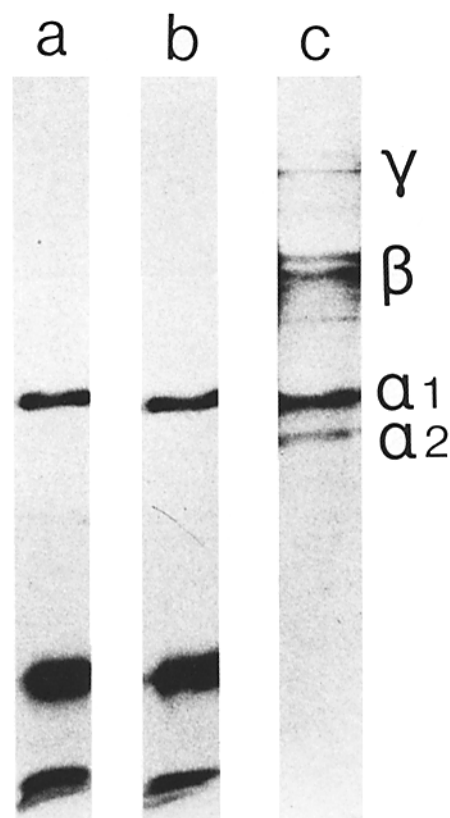


FIGURE 3 Autoradiograph of an SDS gel of proteins from pepsin-digested medium of chondrocyte culture after incubation with [³H]proline. Lane a, after reduction; lane b, without reduction; lane c, standard type I collagen from rat tail tendon.

estimate of the coat thickness.

When primary cultures were observed 1 h after we seeded cells onto tissue culture dishes, the chondrocytes were seen to have attached to the substratum but no significant coats were visible (Fig. 4a). After 4 h (Fig. 4b) coats were clearly evident around most of the chondrocytes, but the cells had not yet spread to any extent. Quantitation by morphometry confirmed that the initial rate of coat formation was greater than that of cell spreading (Fig. 5, inset). Over the next 3 d the coats continued to increase in size, giving rise to coat/cell ratios that changed from approximately 2 at 4 h to approxi-

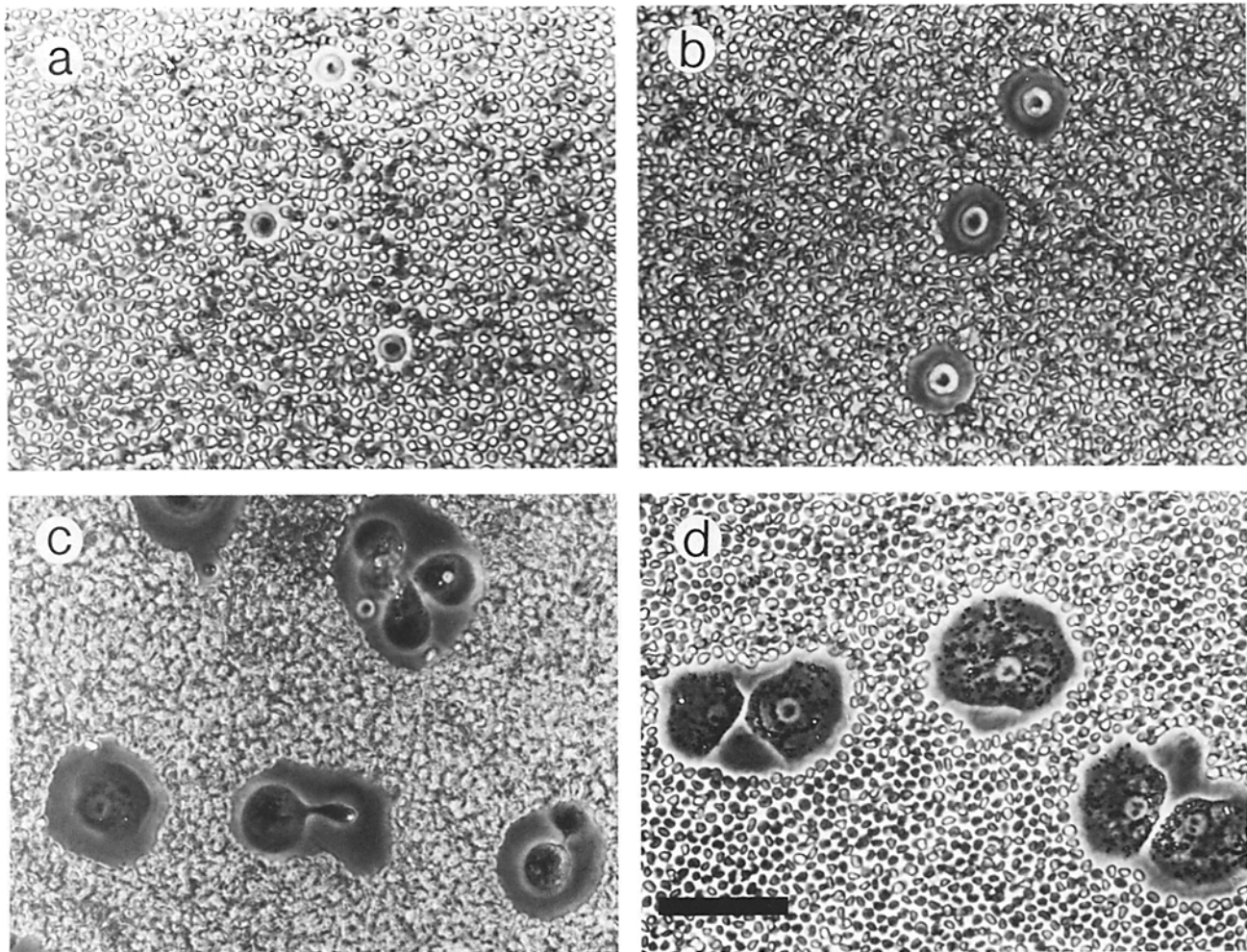


FIGURE 4 Photomicrographs of the time course of chondrocyte spreading and pericellular coat formation. After releasing the chondrocytes from the cartilage matrix, we plated the cells in serum-containing medium. Cultures were removed from the incubator at various time intervals and coats were visualized by addition of fixed erythrocytes. Phase-contrast photomicrographs of the primary cultures of chondrocytes were taken at the following times after plating of the cells: (a) 1 h; (b) 4 h; (c) 2 d; and (d) 6 d. Bar, 50 μm .

mately 4 at 3 d (Fig. 6). Also, the cells spread within these newly forming coats (Figs. 4c and 5). After 4 d the size of the coats decreased but the cells continued to spread until day 5–6, after which virtually no coat was visible (Figs. 4d and 5). Thus the coat/cell ratio approached 1.0 by day 7–10 of culture (Fig. 6).

When the chondrocytes were subcultured, large coats were seen only if the cells were passaged before 4 d (Fig. 6). These chondrocytes, subcultured prior to 4 d, spread more quickly than the chondrocytes in primary cultures. Also they formed and lost their coats more rapidly (Fig. 6). Therefore, the size of the chondrocyte coat is both time and passage dependent.

Synthesis of extracellular matrix macromolecules also changed with time in culture and with passage. The amount of hyaluronate and chondroitin sulfate produced per 24 h increased with time during primary culture (Table IC). Only a small portion of these glycosaminoglycans was retained at the cell surface but this proportion decreased with time (Table IB) in parallel with the decrease in coat size (Table IA). The level of type II collagen remained relatively high throughout this period; no type I collagen was detected (Table ID). Glycosaminoglycan and collagen synthesis were also examined at a fixed interval of subculture (3 d) after passage at

various times of primary culture (2, 3, and 4 d). It was found that the later the day of initiation of the subculture, the higher the synthesis of hyaluronate and chondroitin sulfate and the lower the synthesis of type II collagen; no type I collagen was detected. Once more, a small proportion of each glycosaminoglycan was retained at the cell surface and this proportion varied for each glycosaminoglycan with time of passage (data not shown). Correlation coefficients between these various metabolic parameters and coat size were calculated. No significant correlation was found for any of these parameters except cell surface glycosaminoglycans ($P < 0.5$).

Release of Cell Surface Macromolecules with Hyaluronidase

To determine whether proteoglycans and collagen are retained in the coat by interaction with hyaluronate, we treated the cells with *Streptomyces* hyaluronidase and measured the macromolecules released as a consequence of degradation of the pericellular coat. The cells were labeled for 24 h with either [^3H]proline or [^{35}S]sulfate. After washing the cells with PBS to remove secreted macromolecules and unincorporated isotope, we incubated the monolayers for 0.5 h with or

without *Streptomyces* hyaluronidase. The residual cell surface material was then released with trypsin. In a parallel experiment, we found that the hyaluronidase treatment completely removed the coat. The hyaluronidase treatment caused the release of ~60% of ^{35}S -labeled cell surface glycosaminoglycans and ~10% of the [^3H]proline-labeled proteins (Table II). Greater than 90% of the ^{35}S -labeled glycosaminoglycans released was chondroitin sulfate. Virtually none of the released protein was collagen. Because >90% of the proteins were retained on the cell surface after hyaluronidase treatment, it is unlikely that the release of the chondroitin sulfate was due to a protease contaminant in the hyaluronidase. It is probable that the small amount of protein that is released is comprised mainly of the link protein and core protein associated with the released chondroitin sulfate-proteoglycan.

Inhibition of Coat Formation by Monensin and Cycloheximide

Since much higher levels of chondroitin sulfate than hyaluronate were found to be associated with the chondrocyte

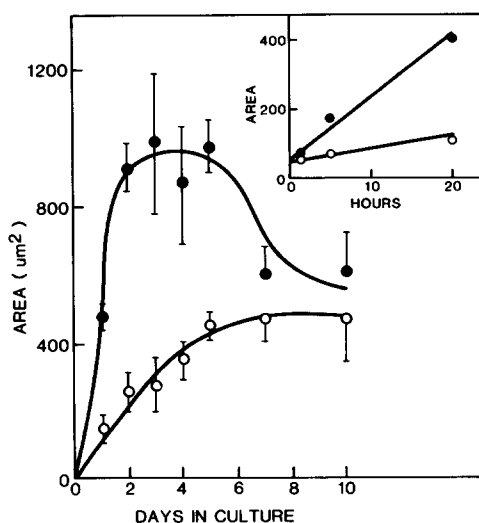


FIGURE 5 Morphometric measurements of the time course of chondrocyte spreading and coat formation. Coats were visualized at various times after plating of the cells as described in Fig. 4. The cell areas (O) (representative of cell spreading) and coat + cell areas (●) were measured by tracing randomly selected photographs of the cultures on a digitizing graphics tablet. (inset) Values from one embryo preparation (30 to 50 measurements for each value). Values with SE bars were from three embryo preparations (60 to 150 measurements for each value).

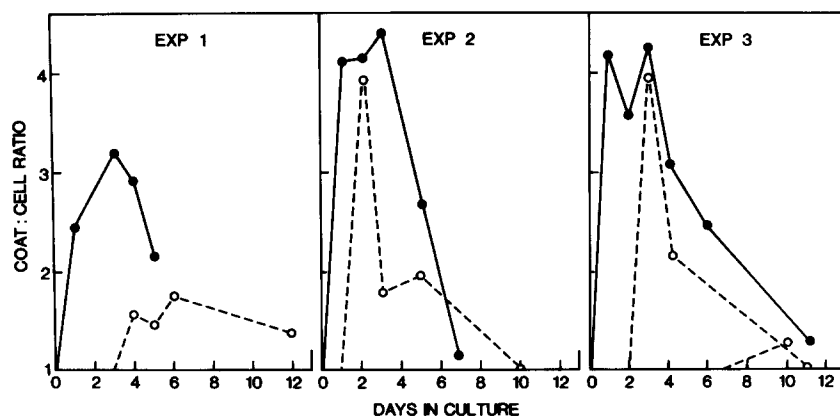


FIGURE 6 Time course of changes in coat/cell ratios for chondrocytes in primary cultures (●) and first passage cultures (O). The coat + cell and cell areas were quantitated as described in Fig. 5; the ratio of these parameters was then plotted against days in culture. Each experiment (EXP) was from a different preparation of chick embryos. The chondrocytes were subcultured on day 3 in EXP 1, day 1 in EXP 2, and days 2 and 7 in EXP 3.

surface (Table IB), we investigated whether the structure of the coat was dependent upon chondroitin sulfate-proteoglycan in addition to hyaluronate. Because enzymes that degrade chondroitin sulfate also degrade hyaluronate, we approached this problem pharmacologically, exposing the chondrocytes to various concentrations of monensin and cycloheximide. In parallel experiments we measured coat size (Fig. 7) and incorporation of isotopic precursor into cell surface glycosaminoglycans (Fig. 8). We found that both monensin and cycloheximide inhibited coat formation and glycosaminoglycan synthesis in a dose-dependent manner. However, monensin was more potent in inhibiting both coat formation (Fig. 7) and hyaluronate synthesis (Fig. 8) than cycloheximide. Both cycloheximide and monensin inhibited chondroitin sulfate synthesis at a much lower concentration than needed to inhibit hyaluronate synthesis (Fig. 8) and coat formation (Fig. 7). At 0.1 μM , chondroitin sulfate synthesis was almost completely inhibited by both drugs. However, at this dose monensin caused only a 50% inhibition of hyaluronate synthesis and coat formation whereas cycloheximide caused virtually no inhibition. Comparison of cell surface hyaluronate synthesis and coat size showed that they were linearly correlated ($r = 0.98$), but no significant correlation ($r = 0.2$) was found for chondroitin sulfate (Fig. 9).

DISCUSSION

In this study, we have shown that chondrocytes in early primary cultures exhibit prominent pericellular coats that are equivalent in size to the pericellular plus territorial matrices surrounding chondrocytes in vivo (2, 3). The integrity of these coats is dependent on hyaluronate and does not appear to be dependent on proteoglycan. This was shown in two ways; first, by the sensitivity of the coats to low concentrations of *Streptomyces* hyaluronidase (Fig. 1), and second, by the differential inhibition of coat formation by drugs that block hyaluronate and chondroitin sulfate-proteoglycan synthesis at different doses (Figs. 7 and 8).

Although hyaluronate is responsible for coat integrity, a major cell surface component of the chondrocytes is chondroitin sulfate-proteoglycan (Table IB). We have shown here that 50–60% of this proteoglycan is released from the chondrocyte surface by treatment with *Streptomyces* hyaluronidase (Table II). This finding agrees with past studies by other investigators (7, 8) showing that addition of hyaluronate to chondrocytes in culture caused displacement of newly synthesized proteoglycan from the chondrocyte surface. It has also been shown that hyaluronate associated with the chondrocyte

TABLE II
Effect of Hyaluronidase on the Release of Cell Surface Macromolecules

	Released cpm ($\times 10^{-3}$)			
	-Hase	+Hase	-Hase	+Hase
[35 S]Sulfate	Total GAG		Chondroitin sulfate	
Inc. med.	20 \pm 2	189 \pm 6	11 \pm 2	157 \pm 11
Trypsinate	282 \pm 26	112 \pm 11	262 \pm 21	100 \pm 9
% Released by Hase	56		57	
[3 H]Proline	Total protein		Collagen	
Inc. med.	23.1 \pm 4.6	32.0 \pm 2.0	0.3 \pm 0.1	0.3 \pm 0.1
Trypsinate	110.4 \pm 6.4	89.2 \pm 6.3	0.4 \pm 0.1	0.6 \pm 0.3
% Released by Hase	9		0	

Hase, *Streptomyces hyaluronidase* (1 mU/ml); GAG, glycosaminoglycan; Inc. med., incubation medium (PBS). % released by Hase:

$$100 \times \left[\frac{\text{Inc. med. (+Hase)}}{\text{Trypsinate (+Hase) + Inc. med. (+Hase)}} - \frac{\text{Inc. med. (-Hase)}}{\text{Trypsinate (-Hase) + Inc. med. (-Hase)}} \right]$$

Values are means \pm SE from three separate cultures. Chondrocytes were labeled for 24 h with either 100 μ Ci/ml [35 S]sulfate or 100 μ Ci/ml [3 H]proline. Cell layers were washed twice with PBS buffer and incubated with or without hyaluronidase for 0.5 h at 37°C, and incubation medium was collected and centrifuged. The cells were released by trypsin (0.25%) and separated from the trypsin by centrifugation. Chondroitin sulfate and collagen were quantitated by susceptibility to digestion by specific enzymes as described in Materials and Methods.

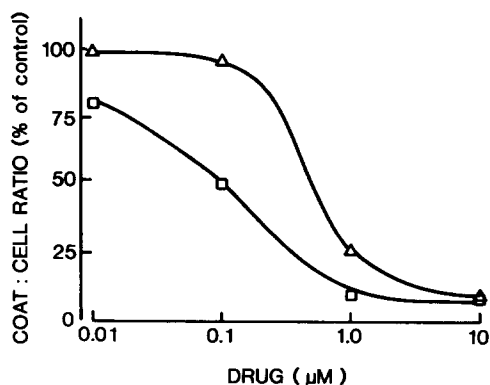


FIGURE 7 Effects of monensin and cycloheximide on chondrocyte coat formation. After 2 d in culture, chondrocytes were exposed to various concentrations of monensin (□) and cycloheximide (Δ) for 48 h. The coat/cell ratios were determined as in Fig. 6.

surface is able to bind exogenously added proteoglycan (9). These various studies indicate that proteoglycan aggregate formation takes place in the pericellular (and/or territorial) matrix of chondrocytes, and that the aggregates are held to the chondrocyte surface via cell surface-bound hyaluronate. Currently we are attempting to determine the mode of interaction of hyaluronate with the chondrocyte surface.

Although it is clear that much of the proteoglycan at the chondrocyte surface is retained via interaction with hyaluronate, there is also a considerable proportion (40–50%) that seems to be held by a different mechanism since it remains bound to the chondrocytes after digestion with sufficient *Streptomyces hyaluronidase* to remove their pericellular coats (Table II). Previous studies with cells other than chondrocytes have provided precedent for at least three other possible mechanisms for binding of proteoglycans to the surface of cells. First, specific interactions of glycosaminoglycan chains with binding proteins on the surface of various cell types have been demonstrated for heparan sulfate (29, 30) and for hyaluronate (31, 32). Such interactions can occur directly with

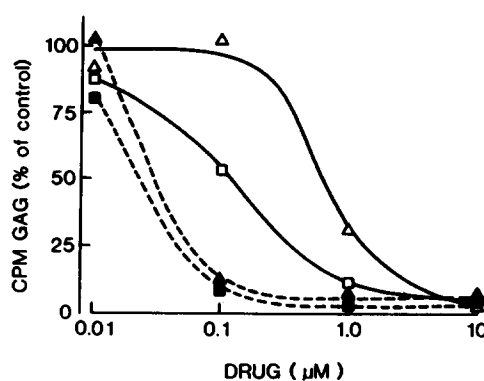


FIGURE 8 Effects of monensin and cycloheximide on cell surface glycosaminoglycan synthesis. The chondrocytes were exposed to the drugs with the same regimen as in Fig. 7. For the last 24 h of exposure to the drugs, [3 H]acetate (100 μ Ci/ml) was added to the medium and incorporation into hyaluronate, and chondroitin sulfate in the trypsin fraction from washed cells was measured as described in the text. Effect of cycloheximide on synthesis of cell surface hyaluronate (Δ) and cell surface chondroitin sulfate (▲). Effect of monensin on synthesis of cell surface hyaluronate (□) and cell surface chondroitin sulfate (■). Values are the means from triplicate cultures.

plasma membrane receptors (33) or indirectly through other cell surface-bound proteins such as fibronectin (34). Second, fibroblast proteoglycans have been found to bind via their protein core to receptors on the surface of fibroblasts (35). Third, evidence has been obtained for direct intercalation of a hydrophobic region of the protein core of heparan sulfate proteoglycan in the plasma membrane of various cell types (36–38). Binding of some of the proteoglycan to the chondrocyte surface by a mechanism analogous to any of these would explain its retention after *Streptomyces hyaluronidase* treatment of the cells (Table II).

A puzzling feature of the pericellular coats described herein is their transient nature. We have shown that the coats begin to form within a few hours but take 3–4 d to reach full size

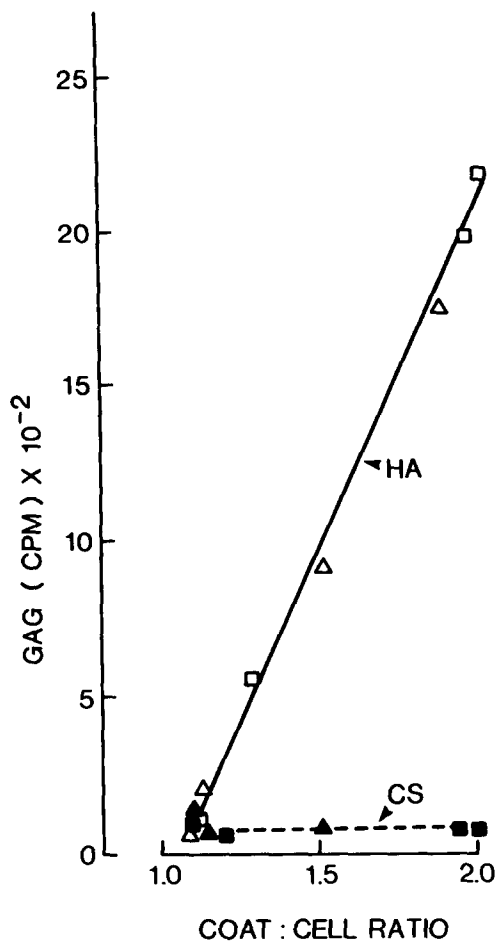


FIGURE 9 Correlation of cell surface glycosaminoglycan (GAG) synthesis and coat size. The values for glycosaminoglycan synthesis are from Fig. 8 and those for coat/cell ratio are from Fig. 7. Open symbols are hyaluronate (HA) and closed symbols are chondroitin sulfate (CS).

(Figs. 4 and 5). During this time, the chondrocytes spread on the culture substratum while encased in their coats. Subsequently, however, the coat diminishes in size until, at 7–10 d, it is no longer discernible (Figs. 4 and 5). If the chondrocytes are subcultured early in the primary culture, the coat forms again but is lost more rapidly than if left in primary culture. Regardless of the time of passaging, the pericellular coat diminishes after 4–5 d of “total” culture time. If subcultured at or subsequent to this time, the resulting coats are much smaller than in the primary (Fig. 6). We have found that the kinetics of formation and decay of pericellular coats differs considerably for different cell types. For example, coats around rat fibrosarcoma cells (18) reform each time the cells are subcultured, but they form and decay more rapidly than those of the chondrocytes described in the present study. The physiological significance of the formation and loss of coats is not known. However, it is possible that this turnover may reflect the differentiated state of the cells. It is known that chondrocytes in culture gradually change their phenotypic properties (39–42) unless cultured under stringent conditions including extremely high cell densities (43–46). To observe the pericellular coats by the method employed in this study, it was necessary to culture the chondrocytes at subconfluent densities. Thus it is possible that the decrease in size of the coats with time and passaging may be a sign of impending

dedifferentiation. Consistent with this possibility, we observed that the synthesis of type II collagen decreased greatly in the subcultures performed late in primary culture, a condition where only small coats formed (Fig. 6).

In conclusion, we have shown that chick embryo tibia chondrocytes exhibit large pericellular coats whose structural integrity is dependent on hyaluronate. Although proteoglycan appears to be a major component of these coats, it is not necessary for their integrity and is in large part retained in the coats via interaction with hyaluronate. It will be of considerable interest to determine in more detail the structure and interactions of the components of these coats, the changes that occur in these components during chondrogenesis and osteogenesis, and the mechanism of retention of hyaluronate and proteoglycan at the plasma membrane of chondrocytes.

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REFERENCES

- Weiss, C., L. Rosenberg, and A. J. Helfet. 1968. An ultrastructural study of normal young adult human articular cartilage. *J. Bone Jt. Surg. Am.* Vol. 50:663–674.
- Stockwell, R. A. 1979. *Biology of Cartilage Cells*. Cambridge University Press, Cambridge. Chapt. 3. 32–80.
- Poole, A. R., I. Pidoux, A. Reiner, and L. Rosenberg. 1982. An immunoelectron microscope study of the organization of proteoglycan monomer, link protein, and collagen in the matrix of articular cartilage. *J. Cell Biol.* 93:921–937.
- Eisenstein, R., N. Sorgente, and K. E. Kuettner. 1971. Organization of extracellular matrix in epiphyseal growth plate. *Am. J. Pathol.* 65:515–534.
- Eisenstein, R., S.-E. Larsson, N. Sorgente, and K. E. Kuettner. 1973. Collagen-proteoglycan relationships in epiphyseal cartilage. *Am. J. Pathol.* 73:443–456.
- Farnum, C. E., and N. J. Wilsman. 1983. Pericellular matrix of growth plate chondrocytes: a study using postfixation with osmium-ferrocyanide. *J. Histochem. Cytochem.* 31:765–775.
- Kimura, J. H., T. E. Hardingham, V. C. Hascall, and M. Solorsh. 1979. Biosynthesis of proteoglycans and their assembly into aggregates in cultures of chondrocytes from the Swarm rat chondrosarcoma. *J. Biol. Chem.* 254:2600–2609.
- Solorsh, M., T. E. Hardingham, V. C. Hascall, and J. H. Kimura. 1980. Separate effects of exogenous hyaluronic acid on proteoglycan synthesis and deposition in pericellular matrix by cultured chick embryo limb chondrocytes. *Dev. Biol.* 75:121–129.
- Sommarin, Y., and D. Heinegard. 1983. Specific interaction between cartilage proteoglycans and hyaluronic acid at the chondrocyte cell surface. *Biochem. J.* 214:777–784.
- Toole, B. P. 1973. Hyaluronate and hyaluronidase in morphogenesis and differentiation. *Am. Zool.* 13:1061–1065.
- Wiebkin, O. W., and H. Muir. 1973. The inhibition of sulfate incorporation in isolated adult chondrocytes by hyaluronic acid. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 37:42–46.
- Handley, C., and D. A. Lowther. 1976. Inhibition of proteoglycan synthesis by hyaluronic acid in chondrocytes in cell culture. *Biochim. Biophys. Acta.* 444:69–74.
- Clarris, B. J., and J. R. Fraser. 1968. On the pericellular zone of some mammalian cells in vitro. *Exp. Cell Res.* 49:181–193.
- McBride, W. H., and J. B. Bard. 1979. Hyaluronidase-sensitive halos around adherent cells. Their role in blocking lymphocyte-mediated cytotoxicity. *J. Exp. Med.* 149:507–515.
- Underhill, C. B., and B. P. Toole. 1982. Transformation-dependent loss of the hyaluronate-containing coats of cultured cells. *J. Cell. Physiol.* 110:123–128.
- Ohya, T., and Y. Kaneko. 1970. Novel hyaluronidase from *Streptomyces*. *Biochim. Biophys. Acta.* 198:607–609.
- Goldberg, R. L., and B. P. Toole. 1984. Hyaluronate coat formation and cell spreading in rat fibrosarcoma cells. *Exp. Cell Res.* 151:258–263.
- Goldberg, R. L., and B. P. Toole. 1983. Monensin inhibition of hyaluronate synthesis in rat fibrosarcoma cells. *J. Biol. Chem.* 258:7041–7046.
- Kim, J. J., and H. E. Conrad. 1977. Properties of cultured chondrocytes obtained from histologically distinct zones of the chick embryo tibiotarsus. *J. Biol. Chem.* 252:8292–8299.
- Prasad, A. S., E. du Mouchelle, D. Koniuch, and D. Obertas. 1972. A simple fluorometric method for the determination of RNA and DNA in tissues. *J. Lab. Clin. Med.* 80:598–602.
- Peterkofsky, B., and R. Diegelmann. 1971. Use of a mixture of proteinase-free collagenases for specific assay of radioactive collagen in the presence of other proteins. *Biochemistry.* 10:988–993.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680–685.
- Linsenmayer, T. F., Gibney, E., and C. D. Little. 1982. Type II collagen in the early embryonic chick cornea and vitreous: immunochemical evidence. *Exp. Eye Res.* 34:371–379.
- Rennard, S. I., R. Berg, G. R. Martin, J. M. Foidart, and P. Gehron-Robey. 1980. Enzyme-linked immunosorbent assay (ELISA) for connective tissue components. *Anal. Biochem.* 104:205–214.
- Rennard, S. I., G. R. Martin, and R. G. Crystal. 1982. Enzyme linked immunosorbent assay (ELISA) for connective tissue proteins: type I collagen. *In* *Immunocytochemistry of the*

- Extracellular Matrix. H. Furthmayr, editor. CRC Press, Inc., Boca Raton, FL. 1:237-252.
26. De Lean, A., P. J. Munson, and D. Rodbard. 1978. Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.* 235:E97-E102.
 27. De Luca, S., A. I. Caplan, and V. C. Hascall. 1978. Biosynthesis of proteoglycans by chick limb bud chondrocytes. *J. Biol. Chem.* 253:4713-4720.
 28. Benya, P. D., and M. E. Nimni. 1979. The stability of the collagen phenotype during stimulated collagen, glycosaminoglycan, and DNA synthesis by articular cartilage organ cultures. *Arch. Biochem. Biophys.* 192:327-335.
 29. Kraemer, P. M. 1977. Heparin releases heparan sulfate from the cell surface. *Biochem. Biophys. Res. Commun.* 78:1334-1340.
 30. Kjellen, L., A. Oldberg, and M. Hook. 1980. Cell surface heparan sulfate. Mechanisms of proteoglycan-cell association. *J. Biol. Chem.* 255:10407-10413.
 31. Underhill, C. B., and B. P. Toole. 1979. Binding of hyaluronate to the surface of culture cells. *J. Cell Biol.* 82:475-484.
 32. Underhill, C. B., and B. P. Toole. 1980. Physical characteristics of hyaluronate binding to simian virus-transformed 3T3 cells. *J. Biol. Chem.* 255:4544-4549.
 33. Underhill, C. B., G. Chi-Rosso, and B. P. Toole. 1983. Effects of detergent solubilization on the hyaluronate-binding protein from membranes of simian virus 40-transformed 3T3 cells. *J. Biol. Chem.* 258:8086-8091.
 34. Ryser, H. J.-P., N. Morad, and W. C. Shen. 1983. Heparin interaction with cultured cells: possible role of fibronectin in uncoupling surface binding and endocytosis. *Cell Biology International Report.* 7:923-930.
 35. Glossl, J., R. Schubert-Prinz, J. D. Gregory, S. P. Damle, K. Von Figura, and H. Kresse. 1983. Receptor-mediated endocytosis of proteoglycans by human fibroblasts involves recognition of the protein core. *Biochem. J.* 215:295-301.
 36. Kjellen, L., I. Pettersson, and M. Höök. 1981. Cell-surface heparan sulfate: an intercalated membrane proteoglycan. *Proc. Natl. Acad. Sci. USA.* 78:5371-5375.
 37. Rapraeger, A. C., and M. Bernfield. 1983. Heparan sulfate proteoglycans from mouse mammary epithelial cells. A putative membrane proteoglycan associates quantitatively with lipid vesicles. *J. Biol. Chem.* 258:3632-3636.
 38. Norling, B. Glimelius, and A. Wasteson. 1981. Heparan sulfate proteoglycan of cultured cells: demonstration of a lipid- and a matrix-associated form. *Biochem. Biophys. Res. Commun.* 103:1265-1272.
 39. Mayne, R., M. S. Vail, P. M. Mayne, and E. J. Miller. 1976. Changes in type of collagen synthesized as clones of chick chondrocytes grow and eventually lose division capacity. *Proc. Natl. Acad. Sci. USA.* 73:1674-1678.
 40. Von der Mark, K., V. Gauss, H. Von der Mark, and P. Muller. 1977. Relationship between cell shape and type of collagen synthesized as chondrocytes lose their cartilage phenotype in culture. *Nature (Lond.)* 267:531-532.
 41. Benya, P. D., S. R. Padilla, and M. E. Nimni. 1977. The progeny of rabbit articular chondrocytes synthesize collagen types I and III and type I trimer, but not type II. Verification by cyanogen bromide peptide analysis. *Biochemistry.* 16:865-872.
 42. Oegema, T. R., and R. C. Thompson. 1981. Characterization of hyaluronic acid-dermatan sulfate proteoglycan complex from dedifferentiated human chondrocyte cultures. *J. Biol. Chem.* 256:1015-1022.
 43. Solursh, M., and S. Meier. 1974. Effect of cell density on the expression of differentiation by chick embryo chondrocytes. *J. Exp. Zool.* 187:311-322.
 44. Hascall, V. C., T. R. Oegema, M. Brown, and A. I. Caplan. 1976. Isolation and characterization of proteoglycans from chick limb bud chondrocytes grown in vitro. *J. Biol. Chem.* 251:3511-3519.
 45. Oakes, B. W., C. J. Handley, F. Lisner, and D. A. Lowther. 1977. An ultrastructural and biochemical study of high density primary cultures of embryonic chick chondrocytes. *J. Embryol. Exp. Morphol.* 38:239-263.
 46. Kuettner, K. E., V. A. Memoli, B. U. Pauli, N. C. Wrobel, E. J.-M. A. Thonar, and J. C. Daniel. 1982. Synthesis of cartilage matrix by mammalian chondrocytes in vitro. II. Maintenance of collagen and proteoglycan phenotype. *J. Cell Biol.* 93:751-757.