Cells and Materials, Volume 8, 1998 (Pages 135-149) 1051-6794/98$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

INTERACTIONS OF THE CHONDROCYTE WITH ITS PERICELLULAR MATRIX

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(Received for publication November 21, 1997 and in revised form March 15, 1998).

Abstract

In addition to having an effect on cell physiology, the pericellular matrix is a major site of matrix assembly and remodeling. The ability to isolate chondrons, chondrocytes with their native pericellular matrix, has facilitated studies of pericellular matrix structure, function and remodeling. In response to environmental cues, the chondrocyte builds a new pericellular matrix, alters the existing pericellular matrix, or simply exits its pericellular matrix. Chondrocytes likely use "inside-out" and "outside-in" signaling mechanisms as illustrated by the remodeling of the pericellular matrix in an adhesion-dependent manner. Interactions with the matrix, including direct manipulation of collagen fibrils and rebuilding of a new matrix, are mediated through integrins and CD44.

Key Words: Osteoarthritis, integrins, CD44, chondron, extracellular matrix.

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Introduction

Most cell types have a pericellular matrix which consists of extracellular matrix (ECM) molecules closely associated with the plasma membrane. For many cell types, this region is very rich in glycoproteins, glycolipids, proteoglycans and hyaluronan. Hence, the term "glycocalyx." Chondrocytes produce a pericellular matrix that is rich in hyaluronan and proteoglycans and, in adult cartilage, type VI collagen (C.A. Poole *et al*., 1984; 1991a; 1992). In the middle and deep zones of articular cartilage, this pericellular matrix is separated from the territorial matrix by a fibrous capsule (C.A. Poole *et al*., 1987). The chondrocyte with its pericellular matrix and surrounding capsule (when present) forms a structural unit in cartilage and is termed a "chondron" (C.A. Poole *et al*., 1987). The pericellular matrix also contains type II collagen but lacks the large type II collagen fibrils found in the interterritorial matrix (C.A. Poole *et al*., 1984). Within the pericellular matrix and in the capsule, large, thick aggregates of type VI collagen can be found, especially in osteoarthritic cartilage (C.A. Poole *et al*., 1987; Ronziere *et al*., 1990). With confocal microscopy, type VI collagen was observed to have a laminar arrangement in some chondrons (Lee, unpublished observations). Other molecules found in the pericellular matrix of adult articular cartilage include type IX collagen (C.A. Poole, 1997; C.A. Poole *et al*., 1988b), fibronectin (Glant *et al*., 1985), laminin (Durr *et al*., 1996), perlecan (SundarRaj *et al*., 1995), and a small proteoglycan, biglycan (Miosge *et al*., 1994). Another small proteoglycan, decorin, has been described as present in the pericellular matrix (A.R. Poole *et al*., 1986; 1996; C.A. Poole *et al*., 1993) and as excluded from the pericellular matrix in human articular cartilage (Miosge *et al*., 1994). The difference in localization of decorin is probably due to the age and species of the donor rather than to fixation and tissue preparation. Bianco *et al*. (1990) found similar labeling patterns for frozen and paraffin-embedded sections.

That the extracellular matrix can have a major impact on a cell's phenotypic expression and on its response to cytokines is now well established (for reviews, see: Adams and Watt, 1993; Boudreau *et al*., 1995; Juliano and Haskill,

1993). There have also been numerous studies on the effects of extracellular matrix molecules on the chondrocyte's response to cytokines (Qi and Scully, 1997), production of cytokines (Homandberg *et al.*, 1997; Yonezawa *et al.*, 1996), and synthesis of proteoglycans (Homandberg and Hui, 1994). Considering that, by definition, the pericellular matrix is the region of contact between the cell surface and the extracellular matrix, it is logical that the composition of this region has a strong influence on cellular activity. Molecules that interact with the chondrocyte surface must pass through the pericellular environment. Growth factors or cytokines can be retained, activated or inactivated by matrix molecules (Iozzo, 1997; Taipale and Keski-Oja, 1997). Newly secreted matrix molecules also undergo modification in the pericellular matrix. Procollagen molecules are cleaved prior to assembly into stable collagen fibrils within the pericellular matrix (Lamande and Bateman, 1993; Trelstad and Silver, 1981). Assembly of matrix aggregates typically occurs within 20 μm of the cell surface (Trelstad and Silver, 1981). The presence of the native pericellular matrix also affects the rate of proteoglycan synthesis (Kelley *et al*., 1996).

The converse of the ECM affecting the chondrocyte is the cell actively modifying its pericellular matrix. Why and how this is done is the subject of this review article. Why refers to changes in the pericellular matrix with development and differentiation, replacement after cell isolation, and in response to disease states such as osteoarthritis. How includes a variety of methods including new synthesis; selective, localized degradation; altered adhesion (inside-out signaling); and direct physical manipulation.

The ability to isolate chondrons (Lee *et al*., 1997b; C.A. Poole *et al*., 1988a) has greatly facilitated studies of the structure, composition and function of the pericellular matrix (Kelley *et al*., 1996; Larson *et al*., submitted; Lee *et al*., 1997a,b; C.A. Poole, 1997 and references therein). C.A. Poole *et al*. (1988a) developed a method for isolating chondrons using a mechanical extraction technique that involves homogenization. More recently, Lee *et al*. (1997b) have developed a method for isolating chondrons using enzymatic digestion. The enzymatic digestion, which uses dispase and collagenase, yields chondrons because the chondron in articular cartilage is defined by type VI collagen (C.A. Poole *et al*., 1992), which is resistant to digestion by collagenase (Kielty *et al*., 1993) and, apparently, by dispase. The two methods yield chondrons with similar morphology and that contain the principle constituents: types II and VI collagen and aggrecan. The mechanical technique retains fibronectin and bits of interterritorial matrix, while the enzymatic technique loses fibronectin but gives much greater yields of viable chondrons (Lee *et al*., 1997b). Within a day of isolation, chondrocytes have begun to synthesize fibronectin, and by 2 to 3 days there is considerable fibronectin in the pericellular matrix (Lee *et al*., 1997b). Chondrons have been isolated from adult bovine, canine, human and porcine articular cartilage. Dispase-collagenase digestion of cartilage from immature animals yields chondrocytes with only a few percent having a distinct rim of pericellular matrix (Lee, unpublished observations).

Changes in the pericellular matrix in development and differentiation

Numerous *in vitro* studies have shown that the addition of ECM molecules to cells *in vitro* has a significant effect on the differentiation state of the cells (for a review, see Adams and Watt, 1993). What is not as widely appreciated is that *in vivo* cells synthesize and secrete their surrounding pericellular matrix. Thus the cell, in response to appropriate extracellular signals and in response to existing ECM, alters or remodels its pericellular matrix. For instance, hyaluronan has a major effect on pericellular matrix size, and its presence and synthesis and thus the size of the pericellular matrix is modulated with the developmental stage of chondrocytes in embryonic cartilage (Knudson and Toole, 1985; Pavasant *et al*., 1996). Expression of type VI collagen, a major component of the adult pericellular matrix, is also regulated as the chondrocyte proceeds through different developmental stages. Quarto *et al*. (1993) found that in 13-day-old chick embryo tibiae articular cartilage, but not other cartilages, expressed type VI collagen. Quarto *et al*. (1993) further found a dramatic increase in mRNA levels for type VI collagen when embryonic chick chondrocytes were placed in suspension culture followed by a rapid fall prior to expression of cartilage-specific genes. Adhesion of cells to specific ECM molecules plays a major role in tissue morphogenesis (for review, see, Gumbiner, 1996). An example in chondrocytes is that different sets of integrins and their corresponding ECM ligands are present in articular cartilage, growth plate and meniscal cartilage of human embryonic knee (Salter *et al*., 1995).

Alterations of the pericellular matrix in arthritis

In osteoarthritis, there is an earlier and greater incidence of degraded type II collagen in the pericellular matrix than in the interterritorial matrix (Dodge and Poole, 1989; Hollander *et al*., 1995; Hwang *et al*., 1992; Pelletier *et al*., 1983). As determined by semiquantitative immunocytochemistry using an antibody to the stromelysin cleavage site, cleaved aggrecan is increased in the pericellular matrix during inflammatory arthritis (Singer *et al*., 1995). Although type II collagen is being degraded in this area, there is an increase in proteoglycans (Mitchell and Shepherd, 1981), type X collagen (von der Mark *et al*., 1992; Walker *et al*., 1995) and type VI collagen (Ronziere *et al*., 1990). The pericellular matrix is also enlarged around many chondrons in osteoarthritic cartilage as determined by examination of cartilage sections (C.A. Poole *et al*.,

1991b; C.A. Poole, 1997) and by measurement of isolated chondrons (Lee *et al*., 1997a). As determined by quantitative immunofluorescence microscopy of chondrons prepared as whole mounts, the densities of keratan sulfate (5D4 immunolabeling) and types II and VI collagen were similar for normal sized and enlarged chondrons (Lee *et al*., manuscript in preparation). Thus, the enlargement is due to increased matrix synthesis and/or deposition rather than to increased hydration. That alterations in the composition of the pericellular matrix occur in osteoarthritis are further reflected in the effects of the pericellular matrix on chondrocyte metabolism *in vitro*. Significant differences in the rate of cell division were observed between chondrons and isolated chondrocytes obtained from osteoarthritic cartilage but not between chondrons and chondrocytes from normal cartilage (Lee *et al*., 1997b).

Rebuilding and retention of a new pericellular matrix *in vitro*

A new pericellular matrix is quickly formed *in vitro*, but retention of this matrix is dependent on cell shape, whether isolated from superficial, middle or deep layers of cartilage, and on culture conditions. When chondrocytes are isolated by procedures involving pronase and collagenase (Kuettner *et al*., 1982), the pericellular matrix is completely removed. In monolayer culture, the chondrocytes develop a new hyaluronidase-sensitive matrix within 4 hours of culture (Goldberg and Toole, 1984). This hyaluronan-containing pericellular matrix peaks in size by day 4, but then the coat disappears by day 7 as the chondrocyte phenotype changes (Goldberg and Toole, 1984). Goldberg and Toole (1984) found no type I collagen synthesized through day 4, but other authors have reported a decline in type II collagen synthesis and an increase in type I with prolonged monolayer culture (Archer *et al*., 1990; Bonaventure *et al*., 1994; Benya *et al*., 1988; von der Mark *et al*., 1977). Embryonic chicken chondrocytes with prominent stress fibers do not label with an antibody to chondroitin sulfate and express type I instead of type II collagen (Mallein-Gerin *et al*., 1991). This work has recently been repeated in our laboratory with chondrocytes from adult human cartilage (Lee, unpublished data).

A new pericellular matrix or proteoglycan halo is also formed in three-dimensional agarose or alginate cultures (Archer *et al*., 1990; Aydelotte and Kuettner, 1988; Loredo *et al*., 1996). This matrix is evident within a day of culture and generally continues to expand with time in alginate culture (Figs. 1a and 1b). The stability of the matrix with time depends on the cell source and culture conditions. Aydelotte and Kuettner (1988) reported a decline in the percentage of superficial zone, but not deep zone, bovine chondrocytes positive for alcian blue staining when cultured in agarose for 12 days. When cultured in suspension over agarose for 21 days, both superficial and deep zone human chondrocytes showed an increase in the percent positive for keratan sulfate and hyaluronan binding region (Archer *et al*., 1990). The pericellular halo formed by chondrocytes in alginate bead culture is very different from the morphology of the pericellular matrix on freshly isolated chondrons (compare Figs. 1b and 1c). However, when chondrons are maintained in alginate bead culture for 2 weeks, the native pericellular matrix is retained with the same overall morphology as freshly isolated chondrons (Figs. 1c and 1d).

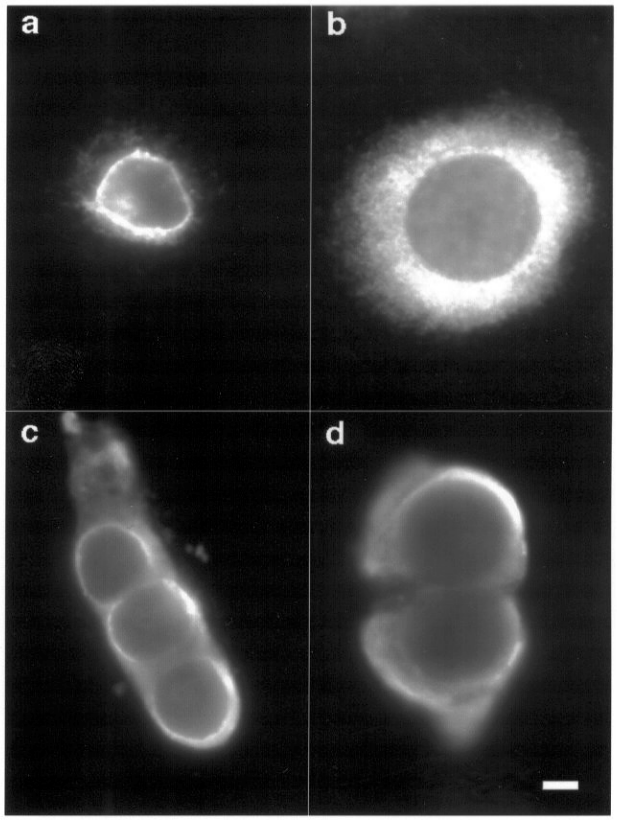

Figure 1. The structure of the pericellular matrix produced *in vitro* differs from the native pericellular matrix. Chondrocytes (1a and 1b) and chondrons (1c and 1d) were maintained in alginate bead culture for one day (1a and 1c) and one month (1b and 1d) and immunolabeled for keratan sulfate with the antibody 5D4. The pericellular halo produced by the chondrocytes has a diffuse, punctate appearance while that of the chondrons is more compact and uniform. Conventional fluorescence micrographs; all are at the same magnification. Bar = 5 μm.

Methods

Cell isolation and culture

Cartilage was obtained from osteoarthritic human knees at the time of joint replacement surgery as surgical

waste. The cartilage from the whole joint was pooled and minced. When chondrocytes were compared to chondrons, the minced cartilage was divided prior to enzymatic digestion. Chondrocytes were isolated by digestion with 1320 PUK/ml pronase (Boehringer-Mannhiem, Indianapolis, IN, and Calbiochem-Novabiochem Corp., San Diego, CA) for one hour followed by 0.4% collagenase (CLS-2, Worthington, Biochemical Corp., Lakewood, NJ) for 3 hours as previously described (Kuettner *et al*., 1982). Chondrons were isolated by digestion with 0.3% dispase (a neutral protease classified as an amino-endo peptidase produced by *Bacillus polymyxa*) (GIBCO BRL, Rockville, MD) plus 0.2% collagenase (CLS-2, Worthington) in phosphate buffered saline (PBS) for 5 hours as previously described (Lee *et al*., 1997b). After filtering through a 70 μm nylon mesh cell strainer (Falcon/Becton Dickinson Labware, Franklin Lakes, NJ) and centrifugation at 400 x g for 6 minutes, the cells were counted using a hemocytometer. For monolayer culture, chondrocytes and chondrons were plated on 22 mm^2 coverslips in 35 mm dishes. For alginate bead culture, they were embedded in alginate as previously described (Guo *et al*., 1989; Hauselmann *et al*., 1994: Lee *et al*., 1997b). For pellet culture, aliquots of 600,000 cells were centrifuged in 15 ml polypropylene culture tubes and cultured without disturbing the pellet. Cultures were maintained in Opti-MEM (modified Eagle's medium) with GlutaMax (GIBCO) containing 2% serum, 25 μg/ml ascorbate-2-PO_4, and 2.7 mM $CaCl_2$.

DTAF labeling

To determine how the native pericellular matrix is remodeled *in vitro*, chondrons were labeled with DTAF, (6-({4,6-dichlorotriazin-2-yl]amino)-fluorescein) (Sigma, St. Louis, MO), which is a reactive dye that covalently binds to proteins, especially collagens (Davison and Galbavy, 1985) and to polysaccharides (Molecular Probes catalog, Eugene, OR). The DTAF was made up fresh as a 1 mg/ml solution in 0.2 M Na bicarbonate, also freshly prepared. The alginate beads were washed with 0.15 M NaCl, the DTAF added, the beads agitated briefly (less than one minute), and then the DTAF was removed, and DMEM (Dulbecco's modified Eagle's medium) added to inactivate any unbound DTAF. The beads were rinsed in 0.15 M NaCl. By labeling the native pericellular matrix of chondrons before culture, modifications in the original matrix could be readily assessed using fluorescence microscopy. The DTAF label covalently attaches to the pericellular matrix and thus provides a stable tag. We have found that it is detectable after 18 days culture in alginate beads and has no effect on cell viability. Since chondrocytes without a pericellular matrix do not appear fluorescent, the DTAF either does not bind directly to the plasma membrane or labeled membrane proteins are rapidly turned over. Exodus of the chondrocyte from its native pericellular matrix

Chondrons cultured for 3 days in alginate bead culture were labeled with DTAF. After dissolving the beads in 50 mM NaCl 55 mM Na citrate solution as described (Lee *et al*., 1997b), the labeled chondrons were plated onto untreated glass coverslips in the presence of 2% serum. The chondrons were examined with fluorescence microscopy after 24, 48 and 72 hours and 1 week of monolayer culture.

Collagen fibril manipulation by monolayer cultures

Type II collagen from chick sterna (Sigma, prepared by pepsin digestion according to Trentham *et al*., 1977) was prepared as a 2 mg/ml stock solution by dissolving at 4°C in Ham's F-12 containing 25 mM HEPES {N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)} (TCM, tissue culture medium) (Sigma) or in 0.02% acetic acid and then adding 10X PBS to 1X. The stock collagen was either directly added at a 1:20 dilution to the culture medium bathing the cells or diluted 1:20 with TCM and allowed to assemble at 37°C for 2 hours to overnight before adding to the cells. To determine the role of integrins in fibril bending, the β1 integrin blocking antibody, 4B4 (Coulter Immunology, Hialeah, FL) was applied at a 1:100 dilution in tissue culture medium to the chondrocytes for 30 minutes at 37°C. Additional antibody was added at 1:100 to preformed chick type II collagen fibrils which were then added to the chondrocytes. Following a 2 hour incubation at 37°C, the chondrocytes were rinsed in tissue culture medium and fixed in 3.7% formaldehyde in PBS for 20 minutes. The collagen fibrils were labeled for immunofluorescence as described below. Using video-enhanced light microscopy, the total number of fibril bends on a chondrocyte was determined for 50 chondrocytes. Only chondrocytes with collagen fibrils on the surface were included. The assay was done in duplicate.

Labeling for immunofluorescence

Isolated chondrocytes and chondrons were fixed in alginate beads in cold dimethyl sulfoxide:methanol (2:8 v/v). After rinsing in PBS, the beads were dissolved as above. Melted 1% Seakem gold agarose (at 42°C) was added to the alginate-cell suspension. The agarose with cells was cast into a thin film and cut into 6 mm squares. The agarose-cell films were handled as free-floating sections for labeling. Monolayer cultures were fixed in 3.7% formaldehyde in PBS and rinsed 3 times in PBS prior to labeling. Primary antibodies were H4C4 to CD44 (Prepared by J.E.K. Hildreth and J.T. August and obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA), and 5D4 to keratan sulfate (Caterson *et al*., 1983). The secondary antibodies were Cy3 labeled donkey anti-mouse and Texas red donkey anti-mouse (Jackson Immunoresearch Laboratories, Inc.,

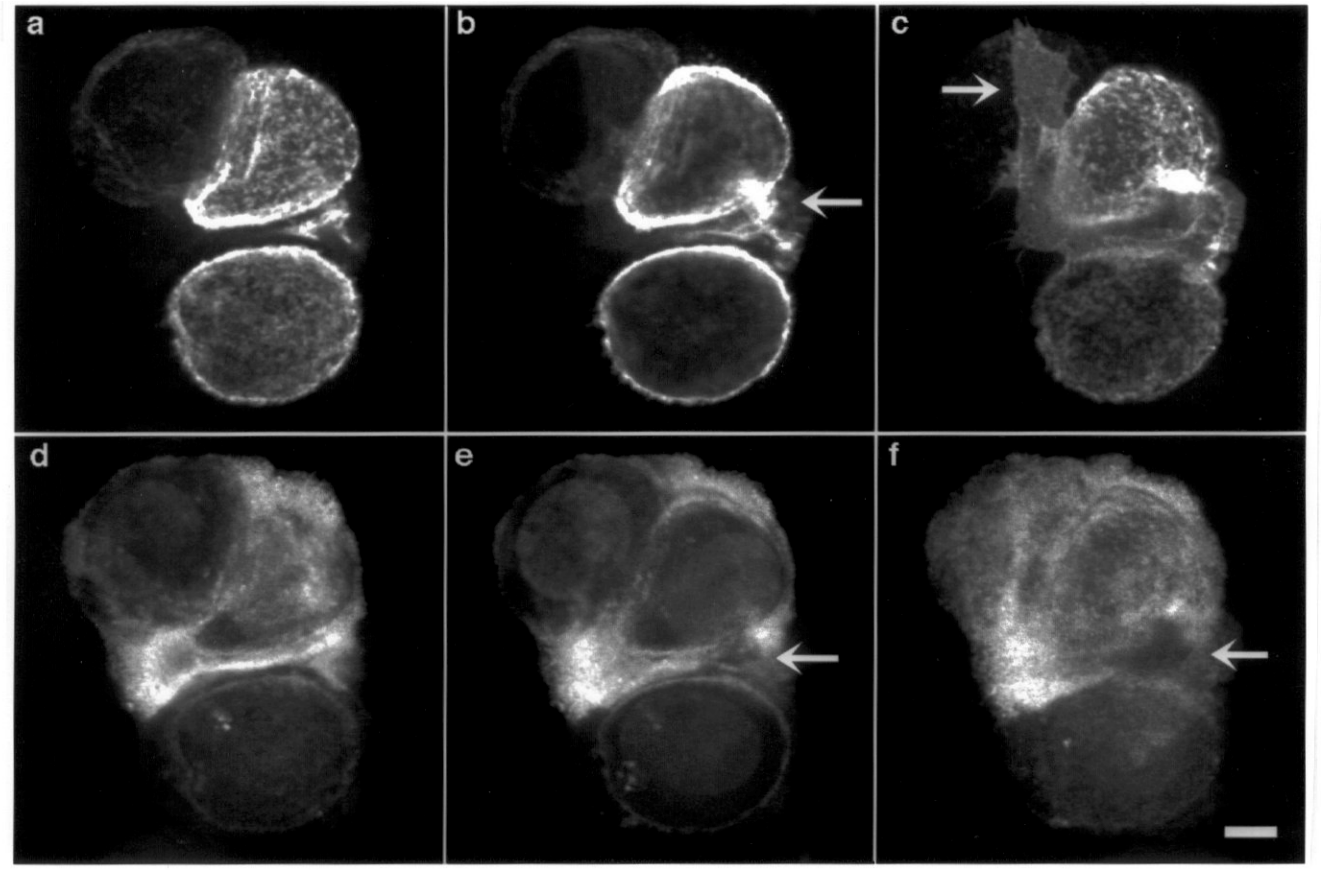

Figure 2. A three-chondron cluster after 12 hours in monolayer culture showing the exodus of a chondrocyte from its pericellular matrix. The chondrons were labeled with DTAF (2d, 2e, and 2f) to reveal the pericellular matrix prior to plating and fixed and double labeled with an antibody to CD44 (2a, 2b, and 2c) to reveal the plasma membrane after plating. The images are at three focal levels: near the dorsal surface (2a and 2d), midway (2b and 2e), and at the substratum (ventral surface; 2c and 2f). The arrows in Figures 2b and 2c indicate the extension of a lamellipodium from the center chondrocyte. The arrows in Figures 2e and 2f indicate an opening in the pericellular matrix through which the lamellipodium was extended. Confocal fluorescence micrographs. Bar = 5 μm.

West Grove, PA). Hoechst dye 33342 (Sigma) was added at 10 μg/ml with the secondary antibodies to stain the nuclei and thus facilitate selecting cells that were viable at the time of fixation. The films and monolayer cultures were mounted in PBS:glycerol (50:50 v:w) containing 0.01% sodium azide and sealed with clear nail polish. Specimens were examined with an Olympus IX-70 fluorescence microscope (Olympus America Inc., Melville, NY) and images collected with an intensified newvicon camera equipped with manual gain and offset controls and digitized with a Scion AG-5 board (Scion Corp., Frederick, MD) in a Macintosh computer using NIH-Image software. Confocal micrograps were obtained using a LEICA confocal microscope (LEICA, Inc., Deerfield, IL).

Results

Interactions of the chondrocyte with its native pericellular matrix

Chondrons in monolayer culture Fresh preparations of enzymatically isolated chondrons contain only a small proportion of chondrocytes without a pericellular matrix (Lee *et al*., 1997b). With time in monolayer culture, the pericellular matrix was shed as the chondrocytes spread. After 12 hours in monolayer culture, chondrons had begun to adhere, and there were only a few spread cells. By 24 hours, fully spread chondrocytes with no associated pericellular matrix were present. By 48 hours, spread cells were abundant, and by 72 hours, spread cells were predominant. After a week in culture, the only intact chondrons remaining were found in areas of very high cell density where the chondron was

attached to adjacent cells rather than to the substratum. These experiments were repeated many times with different chondron preparations, with and without DTAF labeling. The results were always the same: when chondrons were plated in monolayer culture, the chondrocytes attached and spread on the substratum and the native pericellular matrix was lost.

To determine how the chondrocyte separates from its pericellular matrix in monolayer culture, confocal microscopy was used. Chondrons were labeled with DTAF, plated for 12, 24 and 48 hours, and then fixed and labeled with an antibody to CD44 to show the plasma membrane. At the early time points, rounded chondrocytes with surrounding pericellular matrices were found with lamellipodia attached to the substratum. An example is shown in Figure 2 (on previous page), where a chondrocyte has extended a narrow pseudopod through its pericellular matrix. The region where the pseudopod contacted the substratum has spread, forming a lamellipodium typical of a motile cell. Intact pericellular matrices without chondrocytes could not be found, but the presence of occasional remnants of pericellular matrix associated with spread chondrocytes indicates that the pericellular matrix was partially degraded as well as shed.

Chondrons in alginate culture In three-dimensional culture, chondrocytes also extend lamellipodia through their pericellular matrices but only after several days of culture. Chondrons were embedded in alginate beads immediately after isolation and then labeled with DTAF the following day (see Methods). The chondrons were maintained in alginate culture for 18 days in Opti-MEM medium with 2% serum. Chondrons were fixed at days 4, 6, 10 and 18, immunolabeled for CD44 and examined by confocal microscopy. During the 18 day culture period, the DTAF label was retained throughout the pericellular matrix including adjacent to the chondrocyte although there was some redistribution as bands of increased fluorescence intensity were noted between chondrons in a cluster (Fig. 3f). Immunolabeling for CD44 to show the plasma membrane revealed that, starting at day 6, many of the chondrocytes had extended processes out through the pericellular matrix, and by day 18, over 60% of the chondron clusters had lamellipodia spread over the surface of the pericellular matrix (Fig. 3). Unlike the monolayer cultures where the chondrocyte had a rigid substratum to adhere to, the pericellular matrix in the alginate bead cultures was retained with the same shape as when isolated, and the body of the chondrocyte remained within the center of the pericellular matrix.

Chondrons in pellet culture In pellet culture where the chondrons have been centrifuged and then cultured without disturbing the pellet, the chondrocytes also extend pseudopodia through the pericellular matrix and exit the matrix. In contrast to monolayer and alginate bead culture, in the pellet cultures, the pericellular matrix was not degraded but was left as an empty shell. This is illustrated in Figure 4 which shows transmission electron micrographs of a pellet cultured for 2 months. Figure 4a shows empty chondrons and chondrocytes in various stages of crawling out of their pericellular matrix. Examples of a chondrocyte attached to

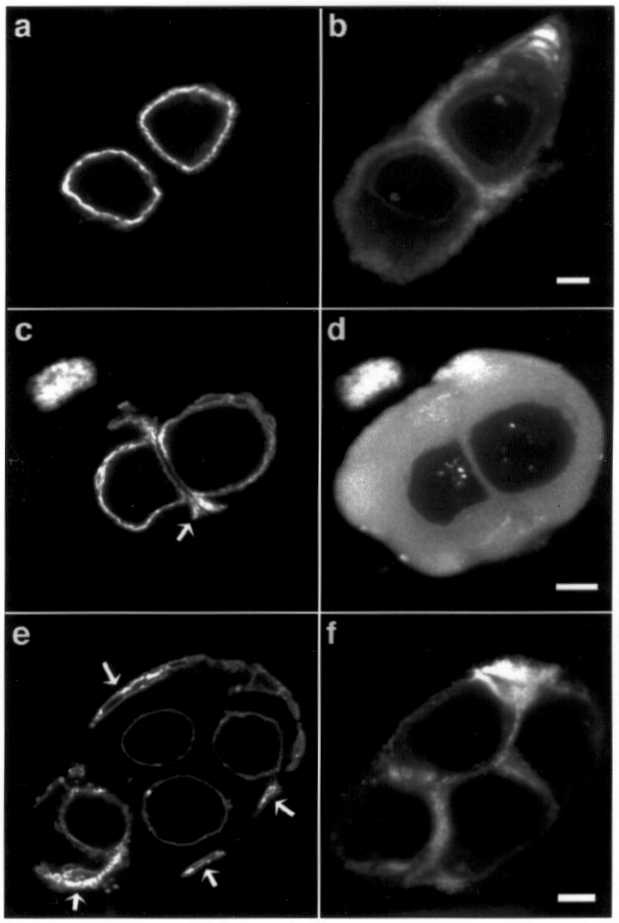

Figure 3. Chondrons maintained in alginate bead culture for one day (3a and 3b), 6 days (3c and 3d) and 18 days (3e and 3f). The chondrons were labeled with DTAF (3b, 3d and 3f) on day 1 of culture and with an antibody to CD44 (3a, 3c and 3e) after fixation. Initially the chondrocytes were rounded within the chondron (3a and 3b) but after a few days of culture cell processes could be seen extending away from the chondrocytes (arrow in 3c). After longer culture times, chondrons could be found where lamellipodia (arrows in 3e) had extended and enveloped the entire chondron cluster, but the pericellular matrix was not degraded. The differences in the DTAF labeling are mainly due to variability between chondrons and do not reflect changes with time except with chondron clusters where the fluorescence was consistently concentrated between chondrons (3f). Confocal fluorescence micrographs. Bars = 5 μm.

the outer edge of the matrix and of a chondrocyte with processes extending through the pericellular matrix are shown in Figure 4b. In other pellet cultures, the pericellular matrix retained its original shape and the appearance of immunofluorescence labeling for type VI collagen remained unchanged throughout a 2 month culture period (Larson *et al.*, submitted). The shape of the elongated chondrocyte in Figure 4b is reminiscent of the shape of chondrocytes in the superficial layer of articular cartilage and of chondrocytes surrounding cell clusters grown in alginate (Aydelotte and Kuettner, 1988) or over agarose (Archer *et al.*, 1990).

Interaction of the chondrocyte with added collagen fibrils

Another way in which chondrocytes alter their surrounding pericellular matrix is by direct manipulation of extracellular matrix fibrils. To examine this, chondrocytes isolated from adult human and bovine articular cartilage were plated as monolayers. After 2 to 6 days in culture, type II collagen in solution was added to the culture medium. The collagen polymerized into fibrils which were acquired and arranged by the chondrocytes. An example of a chondrocyte that has wrapped itself in the added collagen fibrils is shown in Figure 5. Time-lapse video microscopy of the chondrocytes revealed that they pulled the fibrils onto the cell surface, moved the fibrils with filopodia, and bent the fibrils lying on the cell surface (Lee and Loeser, 1999). Preincubation of the chondrocytes with a blocking antibody to $\beta 1$ integrin, significantly reduced the number of bends found in the collagen fibrils on the dorsal cell surface (Lee and Loeser, 1999). This suggests that bending of the collagen fibrils is mediated through the $\beta 1$ family of integrins. The $\alpha 1\beta 1$ integrin has been shown to be a collagen receptor on chondrocytes from adult cartilage and is present *in vivo* (Loeser *et al.*, 1995).

Discussion

A review of the literature and the results presented here indicate that chondrocytes actively modify their pericellular matrix in a situation-dependent manner. The modifications include synthesis of new matrix material, modulation of the adhesion of specific receptors, matrix degradation, and direct manipulation of matrix fibrils. In three different culture systems, monolayer, alginate bead and pellet, chondrocytes respond to their environment by altering their surrounding pericellular matrix and/or altering their position relative to the matrix. The action of the chondrocyte on the matrix is dependent on the stimuli received. In monolayer culture, the chondrocyte adheres to a rigid substratum which appears to activate a signaling pathway(s) that results in the cell releasing its hold on the pericellular matrix and the cell releasing enzymes which degrade the surrounding pericellular matrix. In alginate bead culture, where the only available surface for adhesion is that surrounding the chondron, the chondrocyte extends

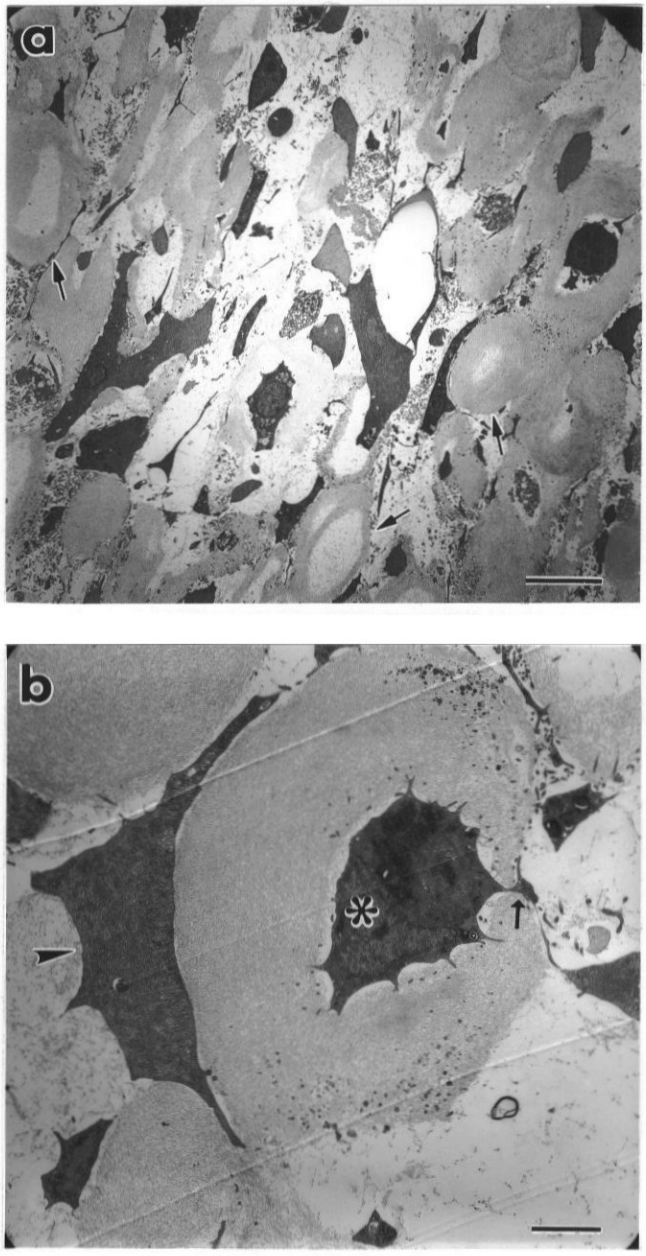

Figure 4. Transmission electron micrographs of chondrons in pellet culture for 2 months: (a) low magnification showing intact chondrons and empty pericellular matrices (arrows); (b) higher magnification of a different area showing a chondrocyte remaining within its native pericellular matrix (asterisk) that has extended a pseudopod to the outer edge of the pericellular matrix (arrow). Another chondrocyte (arrowhead) is spread on the outer surface of the matrix. The pericellular matrix has retained its original shape. Bars = 10 μm (in a), and 3 μm (in b).

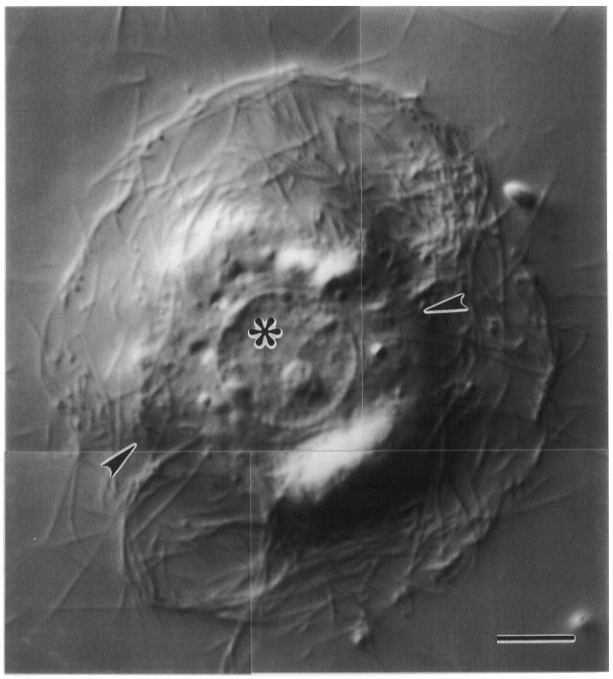

Figure 5. A rounded chondrocyte, in monolayer, cultured overnight in culture medium containing 100 μg/ml chick type II collagen. The added collagen assembled into fibrils which were then pulled in by the chondrocyte and arranged on the cell surface. The cell edge is indicated by arrowheads and the nucleus by an asterisk. There was considerable variability between chondrocytes in the extent of collagen fibril acquisition and arrangement. This is one of the many cells that had surrounded itself with fibrils. Video-enhanced digital interference contrast microscopy. Bar = 5 μm.

and spreads a lamellipodium over the surface but does not leave or degrade its pericellular matrix. In the three-dimensional environment of the pellet culture, the chondrocyte releases adherence to and leaves the pericellular matrix without significantly degrading it. The details of the mechanisms of stimulation and signaling which result in the release of the matrix from the cell surface and the selective degradation of the pericellular matrix have not been elucidated. The following sections will attempt to address these issues by reviewing what is known about chondrocytes and incorporating what is known from studies with other cell types to fill in the gaps.

Receptors

Integrins are heterodimeric transmembrane glycoproteins which have been found to be important receptors for ECM proteins and mediate cellular interactions with the pericellular matrix in many diverse tissues (Hynes, 1992). Integrin stimulation occurs during binding of ECM proteins and generates intracellular signals, which can modulate gene expression referred to as "outside-in signaling" (Hynes, 1992; Schwartz *et al*., 1995). Also, the binding affinity of integrins for ECM proteins can be modulated by intracellular signals ("inside-out signaling"). More than 20 different integrin receptors have been described resulting from the combination of 9 types of β subunits (designated β1-9) with 14 types of α subunits (Hynes, 1992). Adult articular chondrocytes express several different integrins including α1β1, α3β1, α5β1, αvβ3, and αvβ5 (Salter *et al*., 1992; Loeser *et al*., 1995; Woods *et al*., 1994). Unlike normal adult articular chondrocytes, fetal chondrocytes and chondrosarcoma cells can express α2β1 (Durr *et al*., 1993; Enomoto *et al*., 1993; Holmvall *et al*., 1995). The α1β1 and α2β1 integrins can serve as receptors for types II and VI collagen while the α5β1 integrin serves as a fibronectin receptor (Durr *et al*., 1993; Enomoto *et al*., 1993; Holmvall *et al*., 1995; Loeser *et al*., 1995; Loeser, 1997). Chondrocyte integrin expression can be modulated by cell culture and by treatment with growth factors such as IGF (insulin-like growth factor)-1 and TGF (transforming growth factor)-β (Loeser *et al*., 1995; Loeser, 1997). Expression of α1β1 by adult bovine chondrocytes was stimulated by cell culture in medium containing 10% serum and in serum-free medium with IGF-1 but decreased by treatment with TGF-β. Changes in levels of integrins correlated with changes in adhesion to ECM proteins such that serum and IGF-1 stimulated adhesion to type VI collagen while TGF-β decreased chondrocyte adhesion to type VI collagen, a major component of the pericellular matrix in adult chondrocytes. Expression of α5β1 was stimulated by 10% serum, IGF-1, and TGF-β and this resulted in increased adhesion to fibronectin. Thus, growth factor exposure can affect how a chondrocyte interacts with its pericellular matrix by altering integrin expression. The observed exodus of the chondrocyte from its pericellular matrix would likely require changes in integrin affinity which have not been well studied with chondrocytes but have been noted with other cell types particularly in migrating cells. This is discussed further below.

Annexin V (formerly anchorin cII) binds to types II and X collagen and is found on the surface of chondrocytes (Mollenhauer *et al*., 1984; von der Mark and Mollenhauer, 1997). The annexins form Ca^{2+} channels (Demange *et al*., 1994) which may be important in Ca^{2+} uptake by matrix vesicles (Kirsch and Pfaffle, 1992; von der Mark and Mollenhauer, 1997). Although annexin V binds type II collagen, there has been no mechanism proposed for regulating the binding. Whether annexin V plays a role in the chondrocyte's interaction with the pericellular matrix remains to be determined.

CD44 is a transmembrane protein that serves as a hyaluronan receptor in chondrocytes (Knudson *et al*., 1996). CD44 has been shown to be responsible for binding of hyaluronan in the formation of the large pericellular matrix halos seen *in vitro* with the particle exclusion assay (Knudson *et al*., 1993; 1996). The extracellular domain is quite variable between cell types and can be glycosylated or have chondroitin or heparan sulfate attached (Naot *et al*., 1997). The cytoplasmic domain is involved in regulation of ligand binding affinity (Isacke, 1994) and is described in more detail below. When CD44 has a glycosaminoglycan side chain added, it binds other ligands beside hyaluronan including collagen, fibronectin, laminin and chondroitin sulfate (Isacke, 1994; Naot *et al*., 1997 and references therein). CD44 is produced by chondrocytes isolated from normal and osteoarthritic human cartilage (Salter *et al*., 1996). Because of its abundance and uniform distribution on the cell surface of chondrocytes, immunofluorescence of CD44 serves as a useful marker of the plasma membrane (Fig. 3).

Inside-out signaling is used by cells to regulate adhesion of receptors to extracellular matrix molecules. The mechanism involves modifications to the cytoplasmic domain that reduce or enhance binding affinity to the receptor's ligand. The modifications are controlled through tyrosine kinase signaling cascades. Unfortunately, there is little information published at this time on the details of inside-out signaling in chondrocytes. Our work showing the release of the pericellular matrix by chondrons in monolayer culture demonstrates that inside-out signaling is used by chondrocytes (Fig. 2). There is substantial evidence from work with other cell types that CD44 and integrins are involved in intracellular signaling. The cytoplasmic domain must be present on CD44 for hyaluronan binding (Liu *et al*., 1996). With some cell types, cytochalasin treatment, which results in actin fibril disassembly, reduces or eliminates hyaluronan binding (Liu *et al*., 1996), but in others it has no effect on hyaluronan binding but blocks cell-cell adhesion (Isacke, 1994). Phorbol esters, which activate protein kinase C, induce binding in some cell types that would not otherwise bind hyaluronan (Isacke, 1994; Liu *et al*., 1996). CD44 has been shown to associate with the ERM (ezrin, radixin, and moesin) proteins in cultured fibroblasts (Tsukita *et al*., 1994) and this association can be disrupted in living cells by treatment with C3 exotransferase, a potent inhibitor of Rho (Hirao *et al*., 1996). Rho is a Ras-related, GTP (guanosine triphosphate)-binding protein that regulates adhesion and stress fiber formation in cultured cells (Burridge and Chrzanowska-Wodnicka, 1996). Hirao *et al*. (1996) did not report on whether treatment with C3 exotransferase has an effect on hyaluronan binding, but they have shown a direct link of CD44 with a major intracellular signaling protein and others have shown that the cytoplasmic domain can have a major effect on hyaluronan binding. The loss of the pericellular matrix halo by chondrocytes in monolayer culture (Goldberg and Toole, 1984) demonstrates that chondrocytes also regulate hyaluronan binding.

A cell can control its adhesiveness to specific matrix molecules and to other cells by regulating the number of integrins on the cell surface (reviewed in Kim and Yamada, 1997). This was reported for the effect of growth factors on chondrocytes (Loeser, 1997) and in osteoarthritis (Lapadula *et al*., 1997; Loeser *et al*., 1995). Alternative splicing, usually in the cytoplasmic domain, affects integrin function in terms of activation and localization (Kim and Yamada, 1997). Integrins can also be activated from outside the cell by ligand binding and by divalent cations: Mg^{2+} and Mn^{2+} are stimulatory while Ca^{2+} tends to inhibit adhesiveness (Sanchez-Mateos *et al*., 1996). Inside out signaling regulates the affinity of the integrins for their ligands by modifications to the cytoplasmic domains (Ginsberg, 1995; Sanchez-Mateos *et al*., 1996) and involves conformational change of the integrin molecule (Kim and Yamada, 1997). One function of inside-out signaling is to allow rapid change in adhesive properties (Ginsberg, 1995). The α subunit mediates this signaling process, and the intracellular molecules involved are highly dependent on cell type (Ginsberg, 1995).

When chondrons are plated in monolayer, adhesion of the chondrocyte to the substratum precedes the exodus of the chondrocyte from its pericellular matrix. Thus, it appears that signaling initiated through cell contact with the substratum has an inhibitory affect on the binding affinity for the pericellular matrix. This could occur by a mechanism that has been described in other cell types where the modulation of one specific integrin can inhibit the function of a different integrin through inside-out signaling (Blystone *et al*., 1994; Diaz-Gonzalez *et al*., 1996; Huhtala *et al*., 1995). Diaz-Gonzalez *et al*. (1996) studied Chinese hamster ovary (CHO) cells transfected to express αIIbβ3 as well as their endogenous α5β1 integrins. An inhibitor specific for the αIIbβ3 blocked adhesion of the α5β1 integrin to fibronectin. The inhibitor had no effect on adhesion to fibronectin by non-transfected cells. Thus, inhibition of the αIIbβ3 integrin indirectly blocked α5β1 activity. Recent work by the same laboratory showed that the signal transduction pathway involved a GTP-binding protein, H-Ras, its effector kinase, Raf-1 and the ERK MAP kinase pathway {extracellular signal-regulated protein kinase (ERK) mitogen-activated protein (MAP)} (Hughes *et al*., 1997). As more is learned about integrin activity and intracellular signaling molecules in chondrocytes, the integrins involved in maintaining matrix integrity can be identified.

Degradation in the pericellular area

In arthritic cartilage, degraded molecules are most evident in the pericellular region, and some degradation of the pericellular matrix is necessary for the chondrocytes to exit the chondron *in vitro*. For a variety of cell types, enhancement of proteolytic activity in the pericellular matrix frequently involves the plasma membrane. Matrix metalloproteinases can either be associated with the plasma membrane as in membrane type metalloproteinases (MT-MMP's) (Takino *et al*., 1995) or can be activated by transmembrane serine proteinases (Pei and Weiss, 1995; for review, see, Basbaum and Werb, 1996). MT1-MMP, which activates pro-MMP2, is found in osteoarthritic cartilage and is synthesized by the chondrocytes (Imai *et al*., 1997). The exodus of the chondrocyte from the chondron probably requires the activity of serine proteases since the pericellular matrix in adult articular cartilage is composed to a large extent of type VI collagen, which is resistant to digestion by matrix metalloproteinases but is degraded by serine proteinases (Kielty *et al*., 1993). Serine proteases are involved in the degradation of the extracellular matrix by invadopodia associated with transformed cells, macrophages and osteoclasts (Kelly *et al*., 1994). Invadopodia have not been described in chondrocytes, but thin cell projections can be seen in the electron micrograph shown in Figure 4 and serine proteases have been isolated from cartilage (Rodgers *et al*., 1995) and shown to be synthesized by chondrocytes (Kikuchi *et al*., 1996).

Conclusion

This work demonstrates that chondrocytes use a variety of methods to actively alter their surrounding pericellular matrix. These alterations are dependent on environmental cues and are mediated through receptor molecules and intracellular signaling pathways. The elucidation of these mechanisms in chondrocytes as well as other cell types will facilitate our understanding of chondrocyte physiology in health and disease.

Acknowledgements

We thank Dr. Scott Kelley for provision of surgical waste tissue, Mr. Greg Faris and Ms. Eleanor Hilliard for technical assistance, Dr. C. William Davis for use of his confocal microscope, and Ms. Vicoria madden for Fig. 4 and the cover micrograph. The work was funded by an Arthritis Investigator Award from the Arthritis Foundation (GML) and NIH grants, AR 43883 (GML) and AR 41656 (RFL).

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Discussion with Reviewers

Q. Chen: The authors stated in Results that "Unlike the monolayer cultures,...the pericellular matrix was retained with a similar structure as when isolated..." Judging from Figure 3, there is a dynamic change of pericellular matrix morphology during incubation. This is particularly striking if one compares panels B, C and D. Does the pericellular matrix structure change or remain similar in three-dimensional culture?
Authors: There is considerable variability in chondron morphology at any given time point and this is reflected in the figure. The general structure is retained over time in alginate bead culture. However, synthesis of new matrix proteins, such as fibronectin, can produce rearrangements in the matrix molecules that were present at the time of chondron isolation.

Q. Chen: The authors review integrins extensively in the discussion, although there is no data on integrins at all in the results. Can the authors either add more data on integrins or emphasize pericellular matrix molecules examined in this manuscript, to achieve a balance between Results and Discussion?
Authors: A manuscript titled "Cell Surface Receptors Transmit Sufficient Force to Bend Collagen Fibrils" has been submitted for publication. This manuscript presents the data showing that $\beta 1$ integrin subunits are required for collagen fibril bending. We do not wish to jeopardize publication of the full manuscript by presenting the same data in this review paper. The review is on interactions of the chondrocyte with its pericellular matrix. The review is not on pericellular matrix molecules per se. C.A. Poole has just published such a review (C.A. Poole, 1997).

A. Plaas: How were the cells released from the beads, was this done by solubilization in a citrate solution? How does the DTAF-prelabeling influence the metabolic activity of the cells? Have the authors performed any viability tests on the chondrons?
Authors: Yes, the alginate beads were dissolved with 50 mM NaCl 55 mM Na citrate after DTAf labeling. The DTAF-prelabeling appeared to have no effect on the cells. Actual measures of viability in DTAF-labeled cells have not been done; however, Hoechst stain (a good indicator of viability, Lee *et al*., 1997b) is used routinely when preparing DTAF labeled cells for microscopy. There are abundant DTAF-labeled chondrons with viable cells after 18 days of culture.

A. Plaas: Is the "exit" of the cells from the chondron (a) the result of matrix proteolysis that took place during the isolation procedure (i.e., from collagenase and dispase), or (b) the results of proteolysis by enzymes produced by the chondrocytes during culture? Can this process be observed with chondrons prepared by mechanical disruption of cartilage? Can it be induced by treatment of cells with a cytokine (such as IL-1, oncostatin) known to induce proteolysis of the ECM?
Authors: There is precedence for other cell types locally secreting enzymes during migration through the ECM. Those studies have not yet been done with the chondrocytes but are planned. These studies have not been done with mechanically isolated chondrons. Contact with a substratum or matrix supporting adhesion seems to be the triggering mechanism. This occurs with untreated cells. Whether IL-1 would enhance or speed the process is not known.

A. Plaas: In Figure 5, is this morphological appearance uniform throughout the culture? To what extent does the added type II collagen compete with the collagen molecules synthesized by the cells? The authors' description of the process of collagen fibril organization by the cell suggests that it occurs in several stages. In addition to the role of integrins, have the authors considered the possible involvement of collagen-binding small proteoglycans (decorin, biglycan, fibromodulin) or minor collagens (Type IX and XI), in this process? Can these ECM components be immunolocalized to both endogenous and exogenous collagens?
Authors: The amount of collagen added is considerably greater than the amount synthesized by the cells. In addition, culture with and without ascorbic acid had no effect on the cells ability to acquire and arrange collagen fibrils. We do not know of any references in the literature suggesting a specific interaction of the small proteoglycans or minor collagens (types IX and XI) with cell surface receptors. Type IX collagen immunoreactivity was found on the exogenous collagen fibrils.

R. Sah: The abstract further implies that the remodeling of PCM is adhesion-dependent, and that this illustrates inside-out and outside-in signaling. However, in the absence of specific experimental data that specifically modulates intracellular signals and observes extracellular alterations, it is difficult to understand (how) the experiments specifically demonstrate inside-out signaling. With regard to outside-in signaling, it would be useful to explicitly state when the described events are indicative of this.
Authors: These experiments do not "specifically" demonstrate but they are highly suggestive of inside-out signaling mechanisms. In Discussion, we state that the details of the stimulation and signaling which result in the release of the matrix from the cell surface, and the selective degradation of the pericellular matrix have not been elucidated. The review of signaling mechanisms in chondrocytes and other

cell types is presented as a means of exploring the possible mechanisms that are involved.

R. Sah: It is stated that the exodus of the chondrocyte from its PCM would likely require changes in integrin affinity. However, is it unlikely that this could occur just after PCM degradation.
Authors: Matrix degradation and changes in integrin affinity could occur simultaneously but not necessarily in the same location, i.e., the cell could be releasing attachments around the cell body while digesting matrix around the lamellipodia.

R. Sah: The abundance of CD44 on the surface of chondrocytes is critical for interpretation of the results. It would be useful to provide an appropriate reference to the statement that it is abundant and uniformly distributed (e.g., to C. Knudson and co-worker's EM studies).
Authors: The abundance of CD44 is not relevant to the results except that there is enough on the cell surface that it serves as a useful marker for the plasma membrane. The EM studies were based on immunolabeling just as our immunofluorescence is.

C. McDevitt: The detection of cell lamellipodia by staining for CD44 is very unconvincing. Could the authors not have used some of the lipid stains to establish the boundaries of the cell and thus confirm the CD44 staining?
Authors: CD44 labeling is on the plasma membrane as can be seen in the confocal micrographs in Figures 2 and 3. The absence of intracellular labeling in Figures 3a, 3c and 3e, and the clean circular profile of the cells without extensions clearly shows that CD44 labeling is confined to the plasma membrane. In addition, the absence of label in the pericellular matrix at Day 1 further supports the specificity of the antibody. Lipid stains did not enter the chondrons except when a cell had extended a lamellipodium out to the surface of the chondron and then the plasma membrane of the whole cell was labeled.

C. McDevitt: The reason why the cells should even attempt to escape from their chondrons, their native environment, receives little attention.
Authors: One can only wonder if the chondron is a jail or a cozy little home for the chondrocyte.

C. McDevitt: The investigators used 2% serum in their experiments. Would depleting the serum of fibronectin influence the escape process?
Authors: Cell motility responses are activated by LPA and by insulin; both are found in serum. The chondrocytes synthesize a lot of fibronectin, and fibronectin produced by the cells is added to the pericellular matrix during culture. The chondrons will still adhere, and the chondrocytes will spread when plated in serum-free medium that contains insulin.

C. McDevitt: What is the *in vivo* relevance of this exodus from the chondron?
Authors: It is our speculation that this process could be involved in the formation of fibrocartilage, but we have no data to support this. It is also possible that this occurs in osteoarthritic cartilage as part of a process, along with cell proliferation, which results in the formation of chondrocyte clusters or "clones."

Editor: Please provide details of the submitted reference mentioned in the text.
Authors: Please see below:

Larson CM, Kelley SS, Blackwood AD, Banes AJ, Lee GM (submitted) Retention of the native chondrocyte pericellular matrix results in significantly improved matrix production. Submitted to Osteoarthritis Cartilage. (A reviewed 1998 abstract by the same title and authors was published in Trans. Orthop. Res. Soc. 23: 448).

Note added in proof: A new integrin, $\alpha 10\beta 1$, which binds type II callagen and is found in chondrocytes has recently been described [Camper L, Hellman U, Lundgren-Åkerlund E (1998) Isolation, cloning and sequence analysis of the integrin subunit $\alpha 10$, a $\beta 1$-associated collagen binding integrin expressed on chondrocytes. J. Biol. Chem. 273: 20383-20389].