Cells and Materials, Volume 8, 1998 (Pages 33-56) 1051-6794/98$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

HYALURONAN-CELL INTERACTIONS DURING CHONDROGENESIS AND MATRIX ASSEMBLY

Cheryl B. Knudson*

Departments of Biochemistry and Pathology, Rush Medical College,
The Rush Arthritis and Orthopedics Institute, Chicago, IL 60612

(Received for publication March 24, 1998 and in revised form April 28, 1998)

Abstract

Coincident with the onset of condensation during chondrogenesis in the embryonic limb is the appearance of specific binding sites for hyaluronan on mesenchymal cells. The use of hyaluronan hexasaccharides can disrupt the interaction of native hyaluronan with potential hyaluronan receptors. Matrix retention during chondrogenesis and by differentiated chondrocytes is mediated via hyaluronan binding to cell surface receptors. We have identified the glycoprotein CD44 as a receptor on articular chondrocytes. The binding of hyaluronan to chondrocytes alters the phosphorylation of CD44 and may regulate actin association.

Key Words: Hyaluronan, CD44, cartilage, chondrocyte, matrix assembly, chondrogenesis.

*Address for correspondence:
Cheryl B. Knudson
Department of Biochemistry
Rush Medical College
1653 West Congress Parkway
Chicago, IL 60612
Telephone number: 312-942-8249
FAX number: 312-942-3053
E-mail: cknudson@rush.edu

Introduction

Hyaluronan and hyaladherins

Hyaluronan (HA) is a ubiquitous component of the extracellular matrix of most animal tissues. A large molecular weight member of the group of polysaccharides termed glycosaminoglycans, HA is a linear macromolecule, composed of a repeating disaccharide unit: β-1,4-glucuronic acid-β-1,3-N-acetyl-D-glucosamine [34, 83]. The simple molecular structure of the molecule belies its functional influence on cell behavior. Its production has been linked to a variety of disease, developmental and physiological processes [70, 84, 85, 140, 142]. Nascent HA is bound to the cell through the membrane synthase complex [114, 158], whilst HA also interacts with cells via specific HA binding proteins, or HA receptors [70, 138, 147]. HA has been shown to be critical for maintaining the structure of the extracellular matrix and to affect such cell behavior as adhesion, migration and differentiation when it is present on cell surfaces. Modulation of these events is also often associated with precise levels of HA in the extracellular matrix, underlying the importance of HA regulation by cells.

The influence of HA on and its association with cells is accomplished by a wide variety of specific binding proteins for HA. These binding proteins can be divided into two categories: those that interact with HA within the extracellular matrix proper (structural matrix HA binding proteins), and those that interact with HA at the plasma membrane of cells (cell surface HA binding proteins). Toole [138] has proposed the use of the term "hyaladherins" for the family of molecules present as membrane proteins or matrix proteins that exhibit high binding affinity for HA (see Table 1). The interaction of HA with cell surface hyaladherins and decoration of HA with matrix hyaladherins can also regulate the effects of HA itself on cell behavior.

Aggrecan [28], the large, aggregating chondroitin sulfate proteoglycan of cartilage interacts with HA and link protein to form a supramolecular ternary complex within the chondrocyte matrix [45, 50]. Aggrecan and link protein are the predominant matrix hyaladherins of cartilage. One filament of HA may serve to anchor more than one hundred aggrecan monomers [16, 49]. These macromolecular

Table 1. Examples of hyaladherins.

Hyaladherins	References
Cell surface	
CD44	[6, 75]
RHAMM	[48]
LEC-receptor	[125, 166]
Matrix	
link protein	[45]
aggrecan	[50]
versican	[169]
PG-M	[67]
neurocan	[99]
brevican	[164]
BEHAP	[63]

aggregates have potent viscoelastic properties which are responsible for controlling the osmotic pressure of the cartilage matrix [100] and their proper assembly in the chondrocyte extracellular matrix is crucial in establishing the important biomechanical properties of normal articular cartilage.

Three cell surface hyaladherins function as HA receptors, namely CD44, RHAMM (**R**eceptor for **HA**-**M**ediated **M**otility) and the liver endothelial cell (LEC) receptor. Endocytosis of HA by the LEC receptor is a primary site for clearance of HA from the circulation [35, 36, 125, 165]. RHAMM, as its acronym foretells, is a regulator of cell motility in normal and transformed cells [48, 145]. Most of the originally described cell surface HA binding proteins on mesenchymal cells, chondrocytes and tumor cells [42, 55, 72, 89, 109, 133, 141, 150, 154] have now been grouped into the CD44 family of hyaladherins; CD44 is the primary receptor for HA [6].

CD44

CD44 is a member of a nonintegrin family of cell surface transmembrane glycoproteins. CD44 exhibits a wide spectrum of biological functions, on a wide variety of cell types. Some of these functions include a central role in cell adhesion [15, 56, 86], tumor cell metastasis [42, 77, 133], endocytosis of HA [20, 23, 58], cell signaling [61] and matrix assembly [68, 74, 75]. How cells regulate the use of this one receptor for all of these functions is the prime research topic of several laboratories. The human CD44 gene consists of 20 exons, twelve of which show variable exon usage [121]. Exons 6-15, also named variant exons v1-v10, are alternatively spliced to a particular site within the extracellular domain of CD44, increasing the length of the exposed receptor and possibly modulating some of its functions. Exon 18 encodes the single-pass transmembrane domain. Either of two exons, exons 19 and 20, representing two variations of the intracellular "tail" portion of the molecule, are also expressed differentially due to alternative splicing. The standard or "generic" form of CD44, sometimes termed CD44s, is an 85-95 kDa glycoprotein, lacking expression of all variant exons (v1-v10) and expressing the "long-tail" highly conserved 70 amino acid cytoplasmic domain (exon 20). Exon 19 contains an early stop codon and thus is represented as 3' untranslated message [121]. The translation of CD44 mRNA containing exon 19 results in a CD44 having a cytoplasmic domain containing only four amino acids encoded in exon 18. It is often called the "short-tailed" form of CD44. The lack of intracellular signaling motifs as well as protein domains necessary for interaction with cytoskeletal components has fueled intense speculation over the role of this CD44 isoform. Our group has also found this isoform to be differentially expressed by chondrocytes [62], hence characterization of its expression and function form an important component of our future research directions. In addition to alternative splicing, variations in glycosylation and phosphorylation have also been shown to contribute to the structural and functional diversity of CD44 [10, 17, 65, 76].

Cell-matrix interactions are crucial in cartilage differentiation and maintenance of cartilage homeostasis. Cell surface matrix "receptors" have been identified and characterized on chondrocytes. One, identical to CD44, specifically interacts with extracellular HA [19, 22, 68, 70, 74, 138]. We have demonstrated that this interaction is responsible for the more than the binding of native HA. Since HA serves as the backbone of aggrecan/HA/link protein aggregates, CD44 is also responsible for proteoglycan (PG)-aggregate retention as well. In fact, the majority of the PG-rich matrix can be displaced by reagents that compete with CD44-HA binding (e.g., anti-CD44 antibodies, HA_6 (HA hexasaccharides) [19, 68, 75]. It should also be noted that, although actively looked for, no aggrecan receptor(s) have ever been documented and, as already stated above, blocking CD44-HA interactions inhibits the majority of proteoglycan binding to the chondrocyte cell surface. Thus, CD44 remains the critical link to the retention of the proteoglycan-rich extracellular matrix to the chondrocyte cell surface. The other receptors characterized on chondrocytes are members of the integrin (e.g., $\alpha_5\beta_1$) and nonintegrin (e.g., annexin V) families [29, 32, 91, 163], and have been shown to interact predominately with collagens and/or fibronectin, the latter present during chondrogenesis [80, 136] and in late osteoarthritis (OA) [94].

Pericellular matrix

The visualization of pericellular matrices on living cells in culture can be facilitated by the particle exclusion assay, first developed by Clarris and Fraser and used to

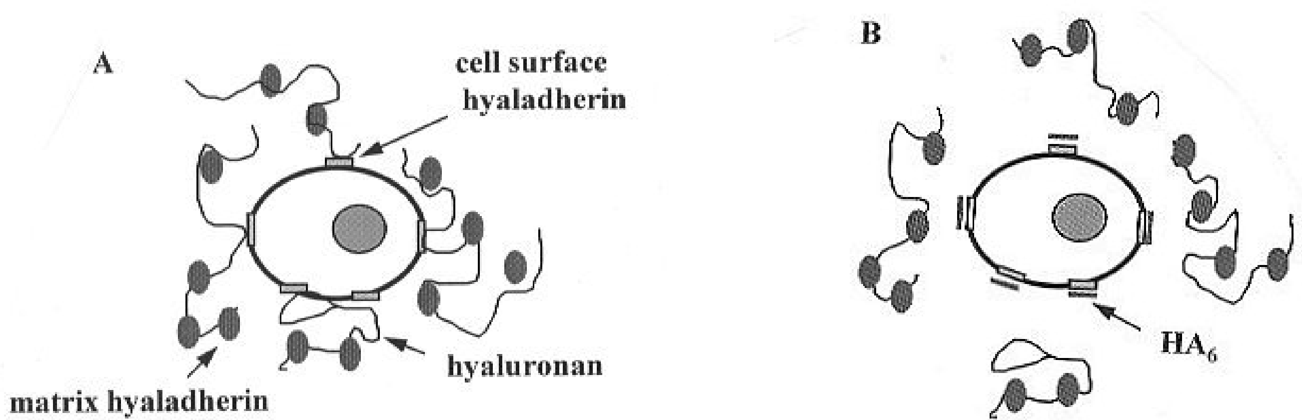

Figure 1. Model for pericellular matrix retention. (**A**). Matrix hyaladherins form supramolecular aggregates with hyaluronan. These aggregates are retained in the pericellular matrix by the interaction of the hyaluronan with cell surface hyaladherins (hyaluronan receptors). (**B**). The presence of hyaluronan hexasaccharides (HA_6) results in the displacement of the hyaluronan-anchored pericellular matrix. The HA_6 represent the smallest oligosaccharide with the capacity to bind to cell surface hyaladherins, resulting in the displacement of the receptor-bound hyaluronan. Thus, HA_6 can be used a tools to disrupt cell-matrix interactions.

distinguish a HA network surrounding synovial fibroblasts [21]. A halo or "cell coat" can be revealed which cannot be penetrated by the particles. The removal of this coat by *Streptomyces* hyaluronidase treatment demonstrates that there is a scaffold of HA within this pericellular matrix. This assay represents the most useful method of visualizing the full extent of the pericellular matrix, since conventional staining of the matrix by histochemical or immunohistochemical techniques often leads to significant collapse of this hydrated structure.

Several cell types in culture, including embryonic and adult chondrocytes, exhibit large pericellular matrices or coats extending from the plasma membrane by as much as one cell diameter. The expression of individual pericellular matrices *in vitro* has been shown to mimic the extracellular volume of cells *in vivo* [71]. On chondrocytes this pericellular matrix is composed predominantly of aggrecan, although its structure depends on both a scaffold of HA and the anchorage of HA to the plasma membrane [68]. Cell surface receptors for HA appear on limb mesenchyme at the onset of condensation and are retained on differentiated chondrocytes [72]. Thus, chondrocyte differentiation is an excellent model for studies on HA-directed matrix assembly. Using the particle exclusion assay, the pericellular matrices of individual chondrocytes in culture were visualized [38, 71, 88]. These matrices are sensitive to degradation by *Streptomyces* hyaluronidase, indicating HA as a scaffold of the chondrocyte pericellular matrix. Endogenous radiolabeled pericellular matrix (including chondroitin sulfate proteoglycan) was displaced by exogenous high molecular weight HA [68, 69, 72]. This suggested that HA is attached to the cell surface by a receptor, which allowed the exchange of exogenous for endogenous HA by competitive binding, and that aggrecan is attached to this receptor-bound HA.

HA hexasaccharides were found as the minimum size of HA oligosaccharide with the capacity for competitive binding to the cell surface for endogenous HA [68, 109]. The endogenous chondrocyte pericellular matrix was displaced following incubation with HA_6 (Fig. 1). As well, "matrix-free" chondrocytes were unable to assemble a pericellular matrix in the presence of HA_6 [68], and newly synthesized matrix components are released to the culture medium [69, 75]. Incubation of cells with HA_6 displaces pre-bound ^{3}H-HA from cell surfaces [109], and competes with binding when added together with ^{3}H-HA to intact cells [150] or isolated cell membranes [150]. This property is helpful in differentiating the specific binding of HA to its cell surface receptor from its binding to extracellular matrix macromolecules. In particular, aggrecan or link protein require a minimum sequence of 10-12 HA monosaccharides for competition of HA binding [47, 50] while link-stabilized aggrecan binding to HA is extremely stable [51]. Binding of cell surface HA receptors by HA_6 competes with binding by large molecular weight HA, but HA_6 are too small to mediate proteoglycan aggregate formation.

Initially, we demonstrated that HA binding sites or "receptors" participate in the assembly and retention of the HA-dependent aggrecan-rich portion of the chondrocyte pericellular matrix [68]. We have recently demonstrated that bovine and human articular chondrocytes do, in fact, express CD44 mRNA as well as CD44 protein [20]. However, the question remains whether CD44 is the primary HA

receptor site involved in assembly and retention of the chondrocyte pericellular matrix. If CD44 is the principal HA binding site on chondrocytes, then spatial organization of CD44 at the cell surface, controlled via cytoskeletal interactions, may function to establish or regulate the structure of the pericellular matrix dependent on an HA scaffolding for aggrecan.

The chondrocyte pericellular matrix within cartilage

The organized extracellular matrix of a chondrocyte is responsible for the specialized physicochemical properties of cartilage. Principal components of this matrix exist in organized supramolecular complexes that function to generate the crucial load bearing properties of cartilage. The cartilage extracellular matrix can be divided into three domains in relationship to the chondrocyte cell surface [60]. The first is the pericellular matrix which is in contact with the plasma membrane of chondrocytes, next the territorial matrix just outside of the pericellular matrix but still associated with a single chondrocyte and third, the interterritorial matrix which is furthest from the chondrocyte cell surface and which links the territorial matrix of individual chondrocytes. Assembly of the chondrocyte extracellular matrix most likely begins with the organization of the pericellular matrix at the cell surface. The chondrocyte pericellular matrix also is a crucial zone for cartilage matrix turnover [53, 106].

The cell-associated matrix can be visualized as a distinct structure within sections of cartilage [60, 119], as well as surrounding individual chondrocytes grown *in vitro* [53, 71, 75, 88]. The composition of the chondrocyte cell-associated matrix is similar to the cartilage extracellular matrix as a whole, however there is some indication of a disproportionate enrichment in HA and aggrecan [57], especially on chondrocytes grown *in vitro*. How this cell-associated matrix is retained, organized and metabolized at the chondrocyte cell surface is not completely understood. Integrin (e.g., $\alpha_1\beta_1$, $\alpha_1\beta_3$, $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$) as well as nonintegrin matrix receptors (e.g., annexin V) have been documented on chondrocytes, and in some cases, investigated for their role in cell-matrix interactions [29, 32, 90, 118, 163]. However, many of these functional assays only test the capacity of a particular receptor to mediate cell attachment to an immobilized substrate. These cell attachment data may fail to describe completely the capacity of soluble, newly synthesized matrix components to attach to cells and then subsequently to undergo self-assembly into pericellular matrix-like structures. Electron micrographs of native as well as composition-defined exogenous pericellular matrices, both preserved via ruthenium hexaminetrichloride fixation procedures, depict strikingly similar networks of HA and proteoglycan extending out from the cell surface [75]. Biochemical as well as morphological

Table 2. Examples of macromolecules upregulated during precartilage condensation.

Molecule	Reference
PG-M	[67]
PNA-binding molecule	[7]
HA-binding protein	[72]
8.5 kDa cadherin	[12]
N-CAM	[161]
N-cadherin	[111]
fibronectin	[25, 80, 136]
syndecan	[41]
tenascin	[95]

analysis of matrix regrowth show that monoclonal antibodies directed against the HA receptor CD44, blocked chondrocyte pericellular matrix assembly [75]. Immunoperoxidase electron microscopy techniques were used to display distinct periodic patterns of HA/proteoglycan assembly similar to the ultrastructural immunolocalization of CD44 at the cell surface [75]. Is a chondrocyte "engaged" with its extracellular matrix via HA/HA receptor interactions? We have used HA_6 to "dis-engage" chondrocytes from their matrix, and to study subsequent changes in matrix accumulation [98], thus allowing us to study the interaction of a matrix assembled *in vivo* with its chondrocyte.

Precartilage condensation in the embryonic limb

A key step in limb development is the event of condensation, during which cells make surface contact with one another and the process of chondrogenesis is triggered through these interactions. Numerous studies on the condensation process have revealed that changes of the extracellular matrix and/or cell surface molecules are chiefly responsible for its occurrence [40, 122, 126]. Table 2 reviews the molecules upregulated during precartilage condensation in the developing limb. Before condensation, limb mesenchyme cells synthesize an extracellular matrix containing type I collagen, fibronectin, large amounts of HA and mesenchyme-characteristic proteoglycan molecules [25, 79, 123, 124]. The onset of condensation [135] coincides with a striking decrease in the amount of extracellular HA [124]. While there is evidence that the early limb mesenchyme is not entirely homogenous, cells taken from every region of the early limb can form cartilage after dissociation and maintenance in high-density cell cultures [128]. During the early stage of limb bud elongation, the cells remain separated from one another (more than one cell-diameter) by large volumes of extracellular matrix [135]. The predominate component of this extracellular space is the glycosaminoglycan HA [79, 124]. HA-enriched extracellular

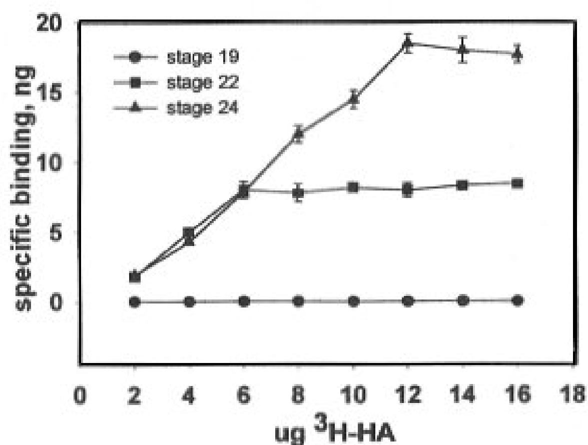

Figure 2. Binding of hyaluronan to limb bud mesenchyme. ^{3}H-Hyaluronan was purified from fibrosarcoma conditioned medium [72] and added to cell suspensions prepared from the mesenchyme of limb buds of stage 19, 22 and 24 chicken embryos. Specific binding was determined by included a 20-fold excess of non-labeled hyaluronan in some tubes, and is represented here by ng bound per 10^5 cells. There is a temporal expression of hyaluronan binding activity on limb mesenchyme.

---

matrices are associated with cells undergoing active migration and proliferation [139]. A transient increase in the accumulation of cell-associated fibronectin [80, 136] and PG-M, a large chondroitin sulfate proteoglycan characteristic of mesenchymal cells [67, 123] within precartilage condensations may reflect mesenchymal cell detachment from the collagen matrix. A newly synthesized molecule which appears during condensation and binds peanut agglutinin (PNA) [7] may also serve to mediate the condensation process. This molecule has been shown to be specific to precartilage condensations and localization of it with PNA lectin has since been used as a useful marker of them [97]. CD44E, epithelial variant form of CD44, may represent one PNA binding molecule with a corresponding temporal and spatial pattern of deposition [59]. Other models suggest that intimate associations between extracellular matrix molecules and adjacent cells or cell-cell associations are responsible for triggering elevated levels of cyclic adenosine monophosphate (cAMP) which may initiate and certainly promote chondrogenesis [87, 130].

Cell surface receptors for HA are first detected at the onset of cell condensation [72] (Fig. 2). Expression of cell surface HA binding ability by limb mesenchyme suggests a function for cell surface HA receptors before matrix assembly. Cell surface HA binding ability begins at stage 22 in the developing chick limb bud [72], while production and assembly of cartilage matrix components begins at stage 26 [78]. The timing of HA cell surface receptor expression coincides with the reduction of intercellular spaces in the areas of prospective cartilage formation [124]. The timely expression of yet other adhesive molecules such as an 8.5 kDa cadherin [12], syndecan [41], N-CAM [161] and N-cadherin [111] has led to several proposals that adhesive interactions are essential to the mechanism of condensation. Some of these have been shown to play major roles in the process of precartilage condensation. Neural cell adhesion molecules (N-CAM) and N-cadherin are both expressed transiently by mesenchyme in precartilage condensations *in vivo* and *in vitro* and antibodies to N-CAM [161] or to N-cadherin [111] inhibit condensation formation in micromass cultures. HA as a cellular cross-linker may represent a first step in bringing cells into close enough proximity for interactions between other extracellular matrix molecules to effect condensation, or it may be one of multiple cooperative mechanisms.

Micromass cultures for *in vitro* chondrogenesis

Significant advances in the knowledge of events in limb chondrogenesis have been made due to the development of an *in vitro* tissue culture model which mimics them [3]. The sequence of events within micromass cultures is similar to the events during *in vivo* chondrogenesis [7, 111, 128]. First is the formation of precartilage condensations, then growth of these condensations, chondrogenic differentiation of the cells, and then synthesis and assembly of cartilage matrix components, forming cartilage nodules. The exact nature of the interactions between cells is unknown, but it has been demonstrated that homotypic cell interactions occur between stages 22-24 in chick limb mesenchyme which eventually lead to cartilage differentiation [127]. Including stage 19 limb bud mesenchyme into stage 22-24 cultures inhibited chondrogenesis in relative proportion to the amount of stage 19 cells used in the culture. Conclusions were then made which support a role for homotypic interactions as a prerequisite for chondrogenesis and, that the ability to interact is acquired after stage 21 [127].

Cell shape and the cytoskeleton

The initiation of chondrogenesis *in vitro* is cell shape dependent [168]. The cell-cell, cell-matrix interactions are of secondary importance compared to the role of cell shape in chondrocyte differentiation [24]. Solitary mesenchymal cells can differentiate into chondrocytes when cultured under conditions that promote a round cell shape [131]. These cells will progress to form cartilage specific matrices and will differentiate to hypertrophic chondrocytes. Treatment of limb mesenchymal cells with cytochalasin promotes the differentiation of the chondrocyte phenotype [167]. The disorganization of microfilaments in cytochalasin-treated mesenchymal cells induces a round cell shape. Cytochalasin treatment also promotes the maintenance of

the chondrocyte phenotype in mature chondrocytes from adult cartilage, without significant changes in cell shape but with a modification of the microfilament organization [14]. The regulation of the chondrocyte phenotype by the actin cytoskeleton and cell shape is influenced by the extracellular matrix microenvironment. Binding of matrix molecules to transmembrane receptors may provide outside-in signaling to regulate the organization of the cytoskeleton. CD44 binding by HA may be one such mechanism.

Studies by other investigators have determined that in cell types such as epithelial cells and lymphocytes, CD44 has the capacity to associate with the underlying cytoskeleton via at least two cytoplasmic binding systems. The ERM family of proteins (ezrin, radixin, moesin) bridge the cytoplasmic tail of CD44 to F-actin [144]. Amieva and Furthmeyer [4] found that, in chondrocytes, moesin was the most highly expressed member of the ERM family. We have also recently identified moesin as a candidate intermediate in the linkage of CD44 to the cytoskeleton in bovine articular chondrocytes. As a second system, Kalomiris and Bourguignon [64] have shown that another intermediate, ankyrin, is responsible for the linkage of the cytoplasmic tail of CD44 to the cytoskeleton in lymphocytes, fibroblasts and endothelial cells. Whether both of these linkage systems occur and operate together or selectively (possibly for different functions) is not known at present and remains a conflict in this field. Nevertheless, we have also recently identified ankyrin and spectrin as proteins that can be co-immunoprecipated with CD44 from bovine articular chondrocytes, and thus represents another candidate intermediate in the linkage of CD44 to actin.

The cytoplasmic tail of CD44 contains threonine and serine residues. In resident macrophages, cell surface CD44 exists both as a nonphosphorylated form in full association with the cytoskeleton and a phosphorylated form, not cytoskeletally associated [17]. In mouse T lymphoma, phosphorylation of serine and threonine residues on CD44 by protein kinase C, enhanced the degree of interaction of CD44 with the actin-based cytoskeleton, via the protein ankyrin [65]. However, except for our study reported herein, there is no information on the phosphorylation state of chondrocyte CD44 or, whether the binding of HA to CD44 changes the state of CD44 phosphorylation. Our recent data indicate that the presence or absence of HA bound to CD44 on chondrocytes, dramatically changes the threonine phosphorylation state of CD44 as well as the association of phosphorylated-CD44 with other co-immunoprecipitating phosphorylated proteins [76]. These "outside-in" experiments clearly demonstrate the potential for "inside-out" regulation of CD44 function via phosphorylation. This opens the door to analysis of how changes in HA and/or PG effect chondrocyte metabolism, an effect that has been observed for decades but never defined mechanistically [103, 110, 129, 162].

Additionally, incorporating models of matrix receptors such as CD44, mechanisms can now be envisioned as to how cytoskeletal changes may control cell-matrix interactions and vice versa. Several agents may be useful in probing the involvement of the chondrocyte cytoskeleton in cell-matrix interactions mediated via CD44. Besides artificial cytoskeletal disrupting agents such as cytochalasin [13, 14, 167], natural agents such as NO have also been shown to inhibit actin polymerization [37]. NO effects are significant because mediators of chondrocyte metabolism such as IL-1, has been shown to induce NO [37, 132]. Thus the effects of IL-1 on CD44 expression and function may, in fact, be driven via NO-mediated cytoskeletal alterations. In summary, it has long been known that in cartilage, the extracellular matrix exerts effects on chondrocyte cell shape, phenotype and metabolism. Conversely, chondrocyte cell shape changes exert effects on matrix organization, assembly and retention. Evidence now suggests that the transmembrane matrix receptor CD44 may be at the heart of many of these cell-matrix interactions.

Materials and Methods

Cells and cell cultures

Limb buds were dissected from chicken embryos of stage 22 Hamburger and Hamilton [43] and treated with 1% trypsin (Type II, Sigma Chem. Co., St. Louis, MO) in Hanks' balanced salt solution for 20 minutes at 37°C. The limbs were then suspended in Hanks' containing 50% horse serum and the epithelial caps dissected away from the mesodermal cores. The mesodermal cores were dissociated in 0.1% trypsin/collagenase (CLS IV, Worthington, Freehold, NJ) for 15 minutes at 37°C to obtain a single cell suspension, with > 90% viability [97]. Micromass cultures [3] were established by plating freshly isolated cells in 10 μl drops containing 4 x 10^7 cells/ml into 24-well culture plates (1 drop/well). After approximately 2 hours of incubation, 0.5 ml of medium was added to each well and attachment continued for 3-4 hours. The experimental conditions were established only after this initial period allowing each micromass culture to attach and recover equally. Cells were grown in supplemented Dulbecco's modified Eagle's medium (S-DMEM) containing 10% fetal bovine serum (FBS), 1% MEM vitamins (Minimum Essential Media vitamin solution, Life Technologies, Inc., Grand Island, NY), L-ascorbate (50 ng/ml), L-glutamine (2 mM) and 1% penicillin/streptomycin.

Tibial explants were established in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS from the distal half of the tibiae from day 12 (stage 38) chick embryos consisting of cartilage zones 1, 2 and 3 [66]. After 48 hours in culture, the cartilage explants were minced and then treated with 0.1% purified collagenase P (Boehringer-

Mannheim, Indianapolis, IN) in DMEM containing 20% FBS for 7 minutes, followed by trituration to liberate chondrocytes.

Chondrocytes were isolated from the full thickness articular surface of bovine (18-24 month steers) metacarpophalangeal joints [8]. Cartilage slices were treated with sequential pronase/collagenase-P digestion to liberate chondrocytes from the tissue as described previously by Aydelotte and Kuettner [8, 53]. Isolated chondrocytes were then cultured in alginate beads as described by Hauselmann *et al.* [53] for 5 days with daily medium changes of Ham's F-12/DMEM supplemented with 10% FBS and 25 μg/ml ascorbic acid (complete medium) during which time matrix production occurs. This method of alginate bead culture, with bovine chondrocytes, maintains the chondrocyte phenotype over an extended time period [54]. To release the chondrocytes into a single cell suspension, the alginate beads were depolymerized using 55 mM citrate in 150 mM sodium chloride, the chondrocytes were pelleted and resuspended in phosphate buffered saline (PBS). These chondrocytes retain a cell-associated matrix and therefore are termed "matrix-intact" chondrocytes. Treatment of these chondrocytes with 3 U/ml *Streptomyces* hyaluronidase in DMEM-F12 supplemented with 10% FBS for 1 hour at 37°C removes the cell-associated matrix [76]. These chondrocyte are rinsed three times with 5 mM ethylenediaminetetraacetate (EDTA) in PBS, resuspended in PBS and are termed "matrix-depleted" chondrocytes.

Particle exclusion assay

Cell-associated matrices are visualized using a particle exclusion assay [21]. In this assay the medium is removed and 0.8 ml of a suspension of formalin-fixed horse erythrocytes {10^8 cells/ml in 0.1% bovine serum albumin (BSA)/PBS} is added to the dishes. Upon settling within 15 minutes, the particles become excluded from a zone or "coat" around the cells that we have termed the pericellular matrix, viewed with phase-contrast microscopy.

Embryonic chondrocytes liberated from the tibial explants or bovine chondrocytes, released from alginate beads with 55 mM citrate, were pelleted and resuspended in PBS. These single cells were transferred to a 6-well culture plate and centrifuged for 10 minutes at 600 x g in a microtiter plate holder. These substrate adherent chondrocytes were then observed within 15 minutes using the particle exclusion assay.

Proteoglycan was extracted from rat chondrosarcoma tumor homogenate [31] and isolated by dissociative cesium chloride equilibrium centrifugation in 4.0 M guanidine HCl with protease inhibitors at a starting density of 1.5 g/ml [52] for 50 hours at 100,000 g at 10°C. The bottom 1/4th of the gradient, density > 1.6 g/ml, was collected (D1 fraction), dialyzed and lyophilized. The D1 proteoglycans were incubated with *Streptomyces* hyaluronidase to degrade small concentrations of HA found within these preparations [68]. The proteoglycans were then recovered by another dissociative equilibrium centrifugation in D1D1 fractions.

We have previously shown that for cells expressing CD44, HA binding is stable to mild fixation [73, 109]. Thus, matrix assembly on chondrocytes fixed at different times following trypsinization was studied. Fixed chondrocytes were "splatted" onto 35 mm culture plates at 600 x g for 20 minutes [98], and then incubated for 4 hours in serum free DMEM containing 12 μg/ml HA and 2 mg/ml D1D1 proteoglycan from the rat chondrosarcoma tumor [68]. The particle exclusion assay was used to detect pericellular matrices; the matrix area: cell area ratio was determined using SigmaScan (Jandel, Corte Madera, CA). Cells with ratios < 1.2 (no detectable matrix) and cells with ratios in the 1.2-2.0 range (positive matrix assembly) were compared; n > 90 for each condition.

Preparation and addition of HA hexasaccharides

Hyaluronan (Sigma, Grade I) was pre-treated with papain (1U/100 mg hyaluronan) and then precipitated in 70% ethanol. This HA was resuspended at 4 mg/ml in PBS to be used as high molecular weight HA, or in testicular hyaluronidase buffer (0.15 M NaCl, 0.1 M sodium acetate, 0.001 M EDTA, pH 5.0). Oligosaccharides were generated by an 18 hours digestion at 37°C with 170 USP/NFU (United States Pharmacopeia/National Formulary Unit) of testicular hyaluronidase (Type 1-S, Sigma) per mg HA and separated on a 2.5 cm x 118 cm column of Bio-Gel P-6 (Sigma) in 0.5 M pyridinium acetate, pH 6.5 [73]. The fractions under the hexasaccharide peak were pooled, lyophilized and resuspended in water three times, and resuspended in serum free-DMEM (SF-DMEM) at 5 mg/ml. Alginate bead chondrocyte cultures were incubated with 150 μg/ml HA_6 (this concentration determined in [68] to be consistently effective to displace chondrocyte coats). HA_6 were added to tibial explant and micromass cultures at 250 μg/ml, with the medium changed every 24 hours [97, 98].

Sulfate labeling of micromass cultures and analysis of proteoglycans

Micromass cultures were incubated for 18 hours in the presence of 30 μCi/ml ^{35}S-sulfate (ICN Radiochemicals, Inc., Irvine, CA). The medium was collected and the cell layer extracted in 4 M guanidine HCl, 50 mM Tris, pH 7.4. Aliquots of medium and cell samples were applied to PD-10 columns (Pharmacia LKB, Piscataway, NJ), eluted with 4 M guanidine HCl buffer, and ^{35}S-proteoglycan detected by scintillation counting [18].

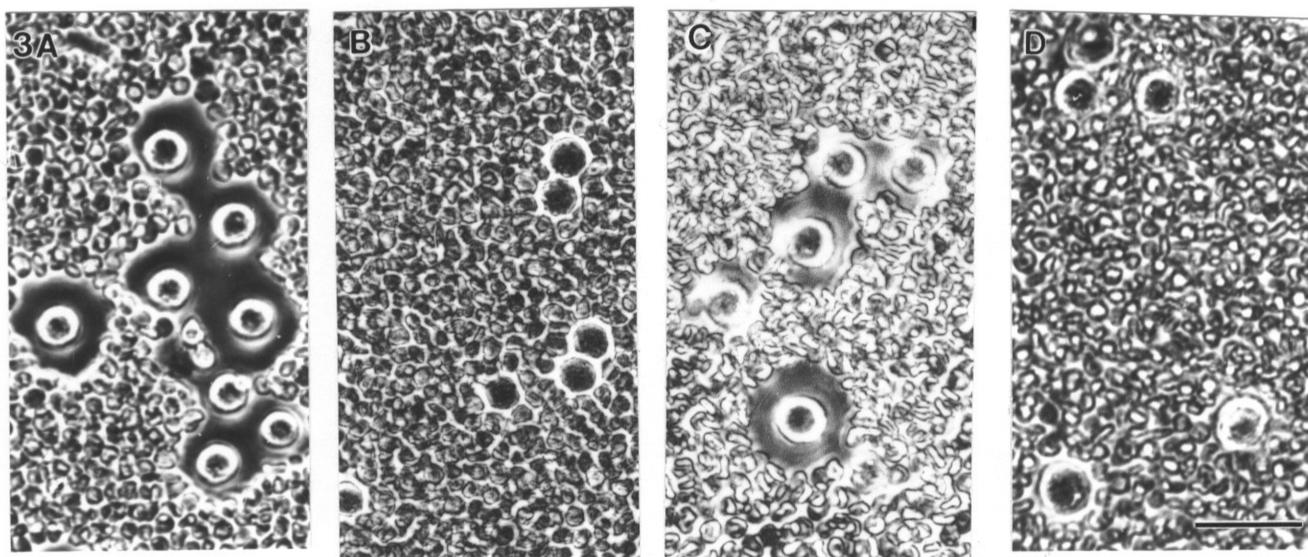

Figure 3. Hyaluronan-dependent chondrocyte matrices. Tibiae removed from day 12 chick embryos were cultured in DMEM + 20% FBS in the absence or presence of HA_6 for 48 hours. Chondrocytes were released with a brief collagenase treatment and the pericellular matrix on living cells revealed by the particle exclusion assay. (**A**). Embryonic chick chondrocytes from control tibiae exhibit prominent pericellular matrices. (**B**). Embryonic chick chondrocytes from tibiae cultured in the presence of HA_6 no longer retain a pericellular matrix. Adult bovine articular chondrocytes were cultured for 5 days in alginate bead cultures. Then they were cultured for an additional 18 hours in absence or presence of HA_6. The alginate was depolymerized and the released chondrocytes were examined with the particle exclusion assay. (**C**). Bovine chondrocytes from control cultures exhibit prominent cell-associated matrices. (**D**). Bovine chondrocytes incubated with HA_6 no longer retain a cell-associated matrix. Bar = 50 µm.

Cell aggregation assay

Freshly isolated cells from stage 22 limb bud mesenchyme were allowed to recover from trypsin/collagenase for 4 hours in S-DMEM in low density suspension cultures in polyhema-coated dishes then passed through a 27-gauge syringe needle to obtain a single cell suspension [97]. Cells were transferred to polyhema-coated 24-well plates at 10^6 cells/ml in serum free-DMEM and incubated at 37°C for 3 hours with constant gentle rocking. This cell number was determined as optimal to obtain cell aggregates by Bee and von der Mark [12]. Experimental cultures were then incubated with 150 µg/ml HA_6. At successive time points, five fields per well were counted for aggregate number, and samples were counted with a hemocytometer for single cell number. Assays were run in triplicate and values reported represent the cells remaining in aggregates in HA_6-treated cultures as a percent of controls, expressed as a mean percentage within the range [12].

Flow cytometry

Adult bovine chondrocytes in alginate bead cultures for 4 days were released into a single cell suspension with 55 mM Na citrate. Cell surface proteins were enzymatically removed by a 30 minutes 0.25% trypsin treatment (t = 0). At various time points between 0.5 and 24 hours, cells were rinsed with PBS and fixed with 0.5% formaldehyde for 10 minutes. To detect CD44 expression, 10^6 chondrocytes were incubated with 1 mg biotin-conjugated rat anti-mouse CD44 IM7.8.1, followed by streptavidin-phycoerythrin. Biotin conjugated rat isotype IgG_{2b} was used as control. Mean fluorescence intensity was measured on a Coulter Epics Profile Analyzer (Coulter Electronics, Miami, FL); n = 10,000 cells counted; data are representative of 3 primary cultures [2].

Detergent extraction, immunoprecipitation, western blotting

Bovine articular chondrocytes were cultured for 4 days in alginate beads, then released with 55 mM Na citrate as "matrix-intact" chondrocytes in suspension. Following digestion with 3 U/ml *Streptomyces* hyaluronidase for 1 hour at 37°C, chondrocytes were "matrix-depleted." Cells were harvested with lysis buffer {Tris buffered saline (TBS) / 1% NP-40 with 5 mM EDTA, 100 mM 6-amino-n-hexanoic acid, 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 ng/ml pepstatin A, 5 mM benzamide, 1 ug/ml sodium orthovanidate, 50 mM KF, and 10 mM sodium pyrophosphate}. For HA add-back experiments, matrix-depleted chondrocytes were incubated with 200 µg/ml HA (Sigma, grade I) for 10 or 20 minutes, followed by 2 ice-cold

PBS rinses then lysis. Samples were incubated overnight with anti-CD44 antibody (1.5 μg IM7.8.1/50 μg lysate protein) and precipitated with goat anti-rat IgG conjugated Sepharose 4B. Following extensive washes, proteins were separated by 6% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and electroblotted. Threonine phosphorylated proteins were detected with anti-P-thr antibodies (Zymed, South San Francisco, CA) or blots were probed with IM7.8.1 (Pharmingen, San Diego, CA) to detect CD44 [76].

Results

Matrix assembly and retention

The integrity of the chondrocyte pericellular matrix was shown in our previous studies to be dependent on a scaffold of HA [68, 71]. Embryonic chick chondrocytes retain a cell-associated matrix after direct isolation from tibial cartilage by a brief treatment with collagenase P in DMEM containing 20% horse serum. Intact tibiae from day 12 embryos were cultured as explants in medium containing 10% FBS ± HA_6 for 48 hours. All chondrocytes released from control tibiae retained a cell-associated matrix (Fig. 3A), sensitive to *Streptomyces* hyaluronidase. However, after 48 hours of culture in the presence of HA_6, chondrocytes were "disengaged" from the matrix and appeared "matrix-free" when released from the explants (Fig. 3B).

Adult bovine articular chondrocytes cultured in suspension in either agarose or alginate gels assemble matrix that can be stained directly within those gels with alcian blue and reflects the capacity of these chondrocytes for matrix assembly when cultured under favorable conditions. Bovine chondrocytes were cultured within alginate beads for 5 days. Upon release of these cells by the depolymerization of the alginate in sodium citrate, the chondrocytes retain prominent cell-associated matrices (Fig. 3C). However, if the chondrocytes within the alginate beads are pre-incubated with HA_6 for 18 hours prior to the release of cells, none of the cells exhibit prominent pericellular matrices (Fig. 3D).

The micromass culture system is a model for chondrogenesis but can also serve to study the maturation of the extracellular matrix. Since HA_6 cannot disrupt HA-aggrecan interactions (this requires minimally a HA decasaccharide; Knudson *et al.*, manuscript in preparation), treatment of cartilage with HA_6 perturbs the native HA-cell interactions and allows analysis of the cell-associated proteoglycan-rich matrix pool. After only 3 days of culture, 50% of the proteoglycan-rich matrix from micromass nodules can be displaced by an overnight incubation with HA_6. However, after 6 days of culture, with more extensive matrix deposition and increase in volume of the micromass nodules, 15% of the proteoglycan-rich matrix can be displaced by an overnight incubation with HA_6 (Fig. 4). The displaced ^{35}S-proteoglycans were recovered in the culture media (data not shown) [96].

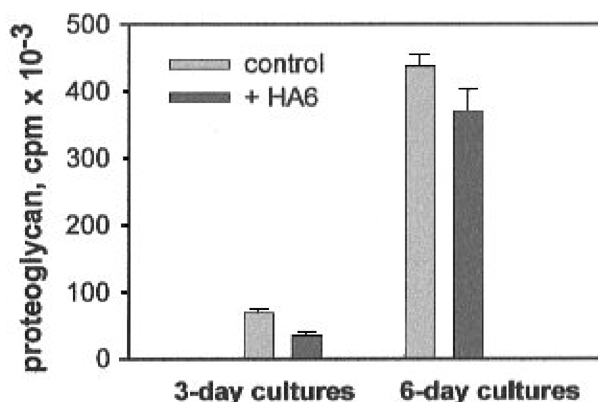

Figure 4. Matrix accumulation during chondrogenesis in micromass cultures. Micromass cultures of stage 22 limb bud mesenchyme were continuously labeled with $^{35}SO_4$ for 3 days or 6 days during which time labeled proteoglycans accumulated within the cultures. To determine the mechanism of retention of proteoglycans in these cultures, HA_6 were added for an 18-hour pulse, and the proteoglycans retained in the micromass cultures were analyzed. The HA_6 treatment resulted in the release of nearly 50% of the ^{35}S-protoeglycans from the 3-day cultures and the release of 15% of the ^{35}S-proteoglycans from the 6-day cultures. The displaced ^{35}S-proteoglycans were recovered in the media (data not shown). Error bars indicate the range from the mean values from ten different cultures; P = 0.001 for 3-day cultures and P = 0.011 for 6-day cultures by t-test analysis.

Formation of cell aggregates

Cell condensations are the initial structures in the formation of proper cartilage and skeletal patterning in the developing vertebrate limb. Coincident with the onset of condensation is the expression by limb mesenchyme of specific HA binding sites (Fig. 2) and the capacity for cell-to-cell adhesion with HA as an extracellular linker molecule. Stage 22 limb bud mesodermal cells in suspension culture form aggregates within 3 hours [97]. These aggregates remain stable over at least a 6 hour incubation period [97]. Aggregates were incubated with HA_6 and the number of cells remaining within aggregates was counted and compared to controls. In controls, nearly 100% of the cells remained within the aggregates during the 5-hour experimental period. However, by 2 hours of incubation with HA_6, ~40% of the cells became loosened from the aggregates and were floating as single cells (or doublets) within the cultures. By 3 hours of incubation with HA_6 (see Fig. 5), less than 30% of the cells remained within the smaller remaining aggregates with a preponderance of single cells

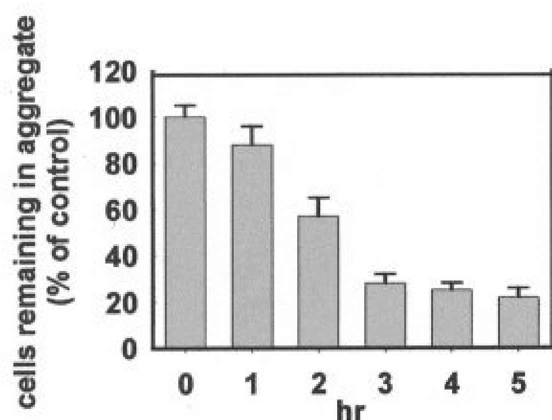

Figure 5. Disruption of cell aggregates by hyaluronan hexasaccharides. Stage 22 limb bud mesenchyme formed aggregates during a three hour pre-incubation in serum free-medium, and these aggregates were stable under these control conditions for 5 hours. The aggregates were also monitored in the presence of exogenous HA_6 for 5 hours, and the bars represent the cells remaining in aggregates as a percent of control. Following 3 hours of HA_6 treatment, nearly 75% of cells had been displaced from the aggregates and seen as single cells within the cultures. Error bars indicate the range from the mean percentage of triplicate determinations.

observed within these cultures.

CD44 expression on bovine articular chondrocytes

Cartilage homeostasis is dependent on the self-assembly of the matrix molecules and their interaction/retention with the cell surface of chondrocytes. CD44 is a HA receptor on chondrocytes that can anchor the HA/aggrecan rich matrix to chondrocytes. The hypothesis tested herein is that the level of cell surface CD44 expression is directly related to the capacity of chondrocytes for matrix assembly. Trypsin treatment eliminated CD44 detection by flow cytometry (Fig. 6). The re-expression observed was biphasic between 0 and 6 hours, suggesting heterogeneity within the articular chondrocytes isolated from the full-thickness of articular cartilage, with a unimodal expression of CD44 by 24 hours (Fig. 6). At 2 hours, 43% of the chondrocytes were positive for CD44, at 4 hours, 66% were positive, and by 24 hours, > 72% of chondrocytes were positive for CD44 (Table 3). The function of CD44 was tested in parallel with CD44 epitope detection. Chondrocytes were analyzed in light of our previous observations that nonviable cells, following brief fixation retain the capacity to bind ^{3}H-HA [39, 109] and can serve as nucleating sites for matrix assembly in the presence of purified exogenous aggrecan plus HA [73]. The percentage of chondrocytes capable of assembling an exogenous matrix increased with time after trypsin treatment (Table 3). There was good correlation between cells positive for CD44 by flow cytometry at a time point and the capacity for exogenous matrix assembly; r = 0.91.

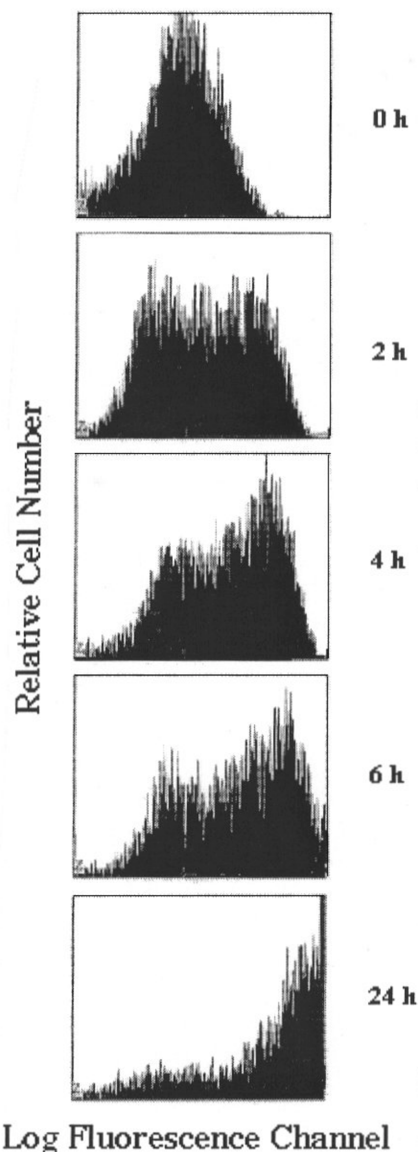

Figure 6. Flow cytometry histograms of CD44 re-expression. Bovine articular chondrocytes were treated with 0.25% trypsin for 30 minutes, which reduced immunoreactivity to the anti-CD44 antibody IM7.8.1 to background; t = 0, top panel. Cells were cultured for 2 to 24 hours in complete media in suspension and then fixed with 1% formaldehyde. Re-expression of cell surface CD44 was measured by flow cytometric analysis and observed as a biphasic response between 2-6 hours, with unimodal positive expression of CD44 by 24 hours; bottom panel.

Table 3. CD44 expression by bovine articular chondrocytes and the capacity for exogenous matrix assembly.

time after trypsin (hours)	cells with exogenous coats (%)	re-expression level of CD44*
0	3	0
2	50	42
4	66	66
6	76	72
24	86	92

*Re-expression level of CD44 values represent the ratio of mean fluorescence value at that timepoint to the pre-trypsin control value.

Bovine chondrocytes cultured in alginate beads for 5 days were isolated, solubilized and total CD44 immunoprecipitated. Chondrocytes from the same cultures were pre-treated with *Streptomyces* hyaluronidase to degrade the HA-dependent pericellular matrix, and these matrix-depleted chondrocytes were then solubilized and total CD44 immunoprecipitated. Some of the matrix-depleted chondrocytes were incubated for 10 or 20 minutes with HA prior to solubilization and immunoprecipitation of CD44. Equivalent protein samples from these cell treatments were separated by 6% SDS-polyacrylamide gel electrophoresis and electroblotted. On lanes cut from the blot and probed with the anti-CD44 antibody
IM7.8.1, a doublet banding pattern was observed for either matrix-intact chondrocytes (Fig. 7; lane 1) or matrix-depleted cells (data not shown). When western blots of the CD44 immunoprecipitates were probed for protein threonine phosphorylation, only the faster migrating ~87 kDa band of the CD44 doublet, not the ~89 kDa band, as well as a co-immunoprecipated ~71 kDa band were positive. The threonine phosphorylation of CD44 on matrix-intact chondrocytes (lane 2; M = matrix intact) was reduced when chondrocytes were pre-treated with *Streptomyces* hyaluronidase (lane 3; +S = *Streptomyces* hyaluronidase treated). When HA was added back to the matrix-depleted chondrocytes, threonine phosphorylation returned to levels detected on matrix-intact chondrocytes (lane 4 and 5; + HA). Immunoprecipitation of chondrocyte lysates with control IgG_{2b} revealed only the background band at ~102 kDa (data not shown).

Discussion

Chondrocyte pericellular matrix

Understanding matrix assembly during chondrogenesis may reveal mechanisms key in cartilage homeostasis.

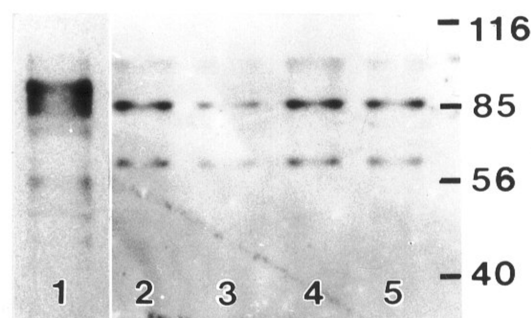

Figure 7. Threonine phosphorylation on bovine articular chondrocyte CD44. Bovine chondrocytes were cultured for 5 days in alginate beads, then released into a single cell suspension. Matrix intact chondrocytes (M) were extracted and total CD44 was immunoprecipitated. Other chondrocytes were treated with *Streptomyces* hyaluronidase for 90 minutes to remove the pericellular matrix (+S). To some of these matrix-depleted chondrocytes, high molecular weight HA was added back for 10 or 20 minutes of incubations (+HA). Chondrocytes from these three experimental conditions were extracted and total CD44 was immunoprecipitated. Lanes from western blots were probed with either an anti-CD44 antibody (lane 1) revealing CD44 in a doublet banding pattern, or an anti-phosphothreonine antibody (lanes 2-5). An 87 kDa band representing one of the doublet CD44 bands from matrix intact chondrocytes showed intense immunoreactivity for phosphothreonine (lane 2; M). This phosphorylation was decreased on CD44 from matrix-depleted chondrocytes (lane 3; +S) but was restored following the addition of HA (lanes 4 and 5; +HA). Positions of molecular weight standards are indicated in kilodaltons at the right.

One strategy we took to approach the elucidation of processes that control matrix assembly was to focus on the developing chondrocyte cell surface as the site of assembly of newly synthesized extracellular matrix macromolecules. Catalysis of cartilage matrix assembly may require specific cell-matrix interactions, which are in turn, mediated via membrane binding proteins or receptors. The interactions of matrix macromolecules with cell surface receptors would allow macromolecules to be clustered in proportions and concentration that would assure optimal assembly in the pericellular compartment. The re-expression of bovine chondrocyte cell surface CD44 following trypsin-treatment indicates that only 25% of normal cell surface CD44 expression on bovine chondrocytes is necessary for the assembly of an HA-anchored, cell-associated matrix. CD44 density may be critical in regulating the amount of HA which can be bound to the cell surface of chondrocytes, suggesting that a critical density of CD44 is required for proper

chondrocyte matrix assembly.

Cells with an intact pericellular matrix were isolated from embryonic chick cartilage by disruption of the collagenous matrix. Thus, the cell-anchored proteoglycan-rich chondrocyte matrix pool apparently is collagen independent. Following incubation of tibial explants in the presence of HA_6, the chondrocytes liberated with collagenase P no longer retain a pericellular matrix. These data suggests that HA/proteoglycan-rich cell-associated matrices do exist as a structural unit within cartilage tissues, and that a collagenous network is not necessary for the initial assembly of the proteoglycan-rich matrix found in close proximity to the chondrocyte cell surface. Later, these cartilages in the presence of HA_6 show decreased safranin O staining, due predominantly to a lack of retention of proteoglycans which are recovered in the medium [96]. Thus, interference by HA_6 with proteoglycan aggregate interactions at the chondrocyte cell surface results not only in disengaging the cell-associated matrix from the cells but disruption of the interterritorial matrix as well. Therefore, the pericellular matrix may serve as a nucleating template or as a matrix anchor regulating assembly or retention of the interterritorial matrix.

Significant progress has been made in the implementation of cell culture conditions for chondrocytes to maintain the expression of the phenotype found in cartilage. For adult bovine articular chondrocytes, we use the alginate microbead culture system in which cells retain their spherical morphology and matrix synthesis/turnover [53, 106]. Bovine and human articular chondrocytes cultured in alginate beads retained their spherical shape and continued synthesis of aggrecan, collagens types II, IX and XI (but not type I) for at least 3 months [54]. Adult human chondrocytes that no longer synthesized type II collagen in monolayer cultures, regained this cartilage phenotype in alginate cultures. Matrix reached an apparent steady state after 2 weeks of culture. At this time, the concentration of aggrecan in the cell-associated matrix was as high as in articular cartilage (60-78 mg/gm). Densely packed collagen fibrils also form within the alginate cultures, which become progressively enriched in mature pyridinium crosslinks [113].

Alginate is a negatively charged unsulfated copolymer of L-guluronan and D-mannuronan, which polymerizes to form a gel in the presence of Ca^{2+} and depolymerizes in physiological buffer containing a calcium chelator [102], releasing individual cells with intact cell-associated matrix. Bovine articular chondrocytes released from alginate beads explants after 6 days of culture, retained cell-associated matrices that had been assembled *in vitro*. These chondrocytes appeared "matrix-free" when released from alginate beads treated with HA_6 from day 5 to 6 of culture. These data suggest that HA/proteoglycan-rich cell-associated matrices also form within the alginate bead cultures and do exist as a structural unit within cartilage tissues and; chondrocytes within those cultures can also become "disengaged" from this cell-associated matrix by the disruption of endogenous HA-chondrocyte binding by exogenous HA_6.

Chondrogenic condensations and cell adhesion

During limb development, cells make surface contact with one another during the condensation event, triggering the process of chondrogenesis [126, 135]. By culturing limb mesenchyme at high density on either side of Nucleopore filters (Poretics Corp., Livermore, CA), the ability of condensations on one side of the filter membrane to induce cells into aggregates on the opposite side of the membrane was examined [120]. Pore sizes were chosen to permit cell-cell or cell-extracellular matrix contact across large pore filters but permit only interactions via diffusible molecules across small pore filters. Examination of these cultures for the alignment of similar patterns of condensation across the membranes suggest that prechondrogenic condensations enlarge in an autocrine manner dependent on direct cell-cell or cell-extracellular matrix contact provided by living cells. The signals involved in the induction can be transmitted across large pore filters but not small pore filters, indicating that the mechanism requires the direct contact permitted by the extension of cell processes across large pore filters.

Prior to the condensation process, an extensive extracellular matrix with HA being the predominant glycosaminoglycan separates the early limb mesenchymal cells. With the initiation of condensation, the HA distribution becomes patterned. The uniform distribution seen at earlier stages persists in the limb periphery, but there is a progressive decline in HA within the prechondrogenic and premyogenic condensations [71, 79, 124]. Coincident with the onset of condensation is the appearance of specific binding sites for HA on limb mesenchyme cells [72].

A significant model was proposed by Toole [137] to explain the dual role of HA in mediating aggregation as well as inhibiting aggregation. In this model a minimal requirement for relatively high molecule weight HA was proposed to facilitate the cross-bridging of cells via the multivalent interaction of HA with receptors on adjacent cells. Residual HA fragments resulting from hyaluronidase treatment would be of such reduced size that these fragments would be unable to cross-bridge cells. Saturation of binding sites on cells by the over-production of endogenous HA (also mimicked by the addition of increasing amounts of exogenous HA) would prevent cell aggregation. This model was based on observations with mouse 3T3 fibroblasts and SV-40 transformed 3T3 cells [147, 149, 152].

Condensation of embryonic limb bud mesenchyme

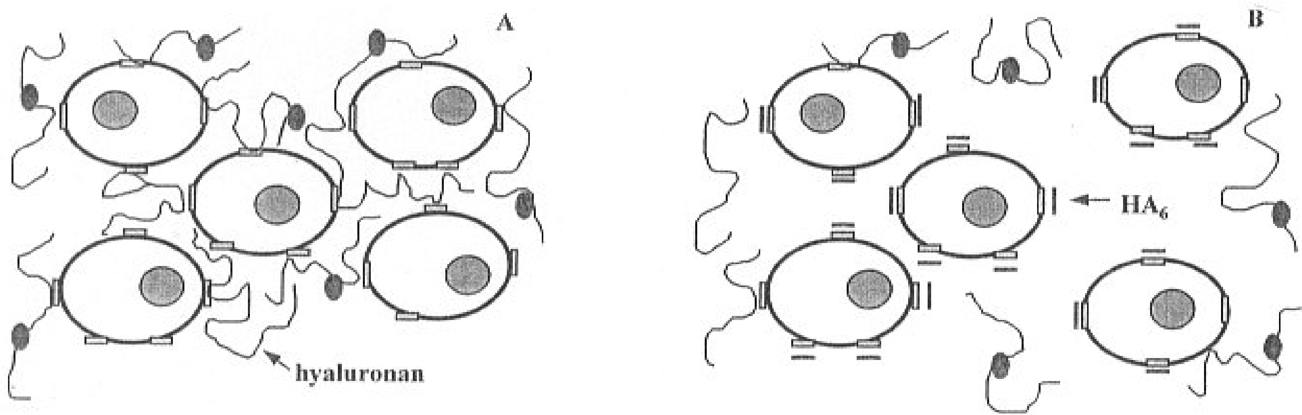

Figure 8. Model for early events in chondrogenic condensation. (**A**). Mesenchymal cells within the embryonic limb bud express hyaluronan binding activity and cell-to-cell aggregation may be mediated by the multivalent interaction of HA with these binding proteins (rectangles) on adjacent cells. (**B**). Addition of HA_6 to cell aggregates displaces endogenous HA, resulting in the release of single cells, and the inhibition of HA-mediated condensation.

can be studied with a variety of *in vitro* assays. Using an intercellular adhesion assay [157], we detected stage specificity of adhesion; earlier stage limb bud cells did not adhere, whereas cells derived from early to late condensation stages show adhesion [97]. This is adhesion apparently mediated by endogenous HA. It should be stressed that this mechanism would be an initial step in the formation of cellular condensations. As a tool, we use small HA oligosaccharides to disrupt the interaction of native HA with cells. Using a cell aggregation assay developed by Bee and von der Mark [12], most cells within single cell suspension of stage 22 mesenchyme incubated in serum-free medium become part of cell aggregates within 3 hours. In the presence of HA_6, the aggregates formed initially disperse into single cells (Fig. 5). Therefore, larger HA molecules must be present for cross-bridging receptor sites on adjacent cells since, in fact, single cells were displaced from the cell aggregates formed in the suspension aggregation assay in the presence of HA_6. The presence of HA_6 apparently can prevent the multiple receptor interactions which occur with native HA macromolecules (Fig. 8). Thus, as receptors become occupied with these small HA oligosaccharides, cells within the aggregate disperse. Competition between HA of different sizes could modulate tissue formation during organogenesis.

Expression of cell surface HA binding ability by limb mesenchyme suggests a function for cell surface HA receptors before matrix assembly. Cell surface HA binding ability begins at stage 22 in the developing chick limb bud [72], while production and assembly of cartilage matrix components begins at stage 26. The timing of HA cell surface receptor expression coincides with the reduction of intercellular spaces in the areas of prospective cartilage formation.

In the model for chondrogenic cellular condensations by Oster *et al*., high concentrations of HA hydrate and inflate the tissue inhibiting intercellular interactions, whereas a decrease in HA in later stages initiates osmotic de-swelling of the tissue, bringing the cells closer together, favoring cellular aggregation [112]. In this model, their hydrated pericellular matrices enriched in HA mechanically isolate early precondensation stage cells. We have observed that stage 19-20 limb bud mesenchyme do exhibit a HA-enriched pericellular matrix as revealed by a particle exclusion assay [71]. The addition of stage 19 limb mesenchyme inhibited cartilage nodule formation by stage 24 mesenchyme [127]. Oster *et al.* [112] discussed the possibility that if the HA-enriched pericellular matrix could be degraded substantially, cells could be brought into closer proximity allowing condensation. In our observations, limb mesenchyme derived from condensation stages (stage 22-25) no longer exhibited pericellular matrices enriched in HA [71] but do exhibit specific cell surface binding sites for HA [72]; in addition, these cells produce hyaluronidase [81].

The initiation of condensation may involve the receptor-mediated removal of extracellular HA coupled with the preliminary formation of cellular aggregates by multivalent HA cross-bridging of adjacent cells. HA binding sites have been found, in other systems, to be involved in the endocytosis of HA en route to its degradation by lysosomal hyaluronidases [58, 104, 105, 125, 155]. Kulyk and Kosher [81] found, by analysis of hyaluronidase activity, that HA turnover in the developing limb bud would have to be regulated by the binding and endocytosis of

HA followed by degradation by lysosomal hyaluronidase. Thus, it seems reasonable to propose that the decrease in the amount of extracellular HA, during the process of precartilage condensation formation, would be receptor mediated.

Speculation remains as to the identity of the HA-binding proteins expressed during precartilage cellular condensation. A very likely possibility is that these HA receptors are part of the CD44 family of hyaladherins. Wheatley *et al.* [160] have immunohistochemical evidence in the mouse that CD44 expression appears in the developing limb at the time of cellular condensation and in the central region that undergoes chondrogenesis. Another possible candidate is the chick cell-surface HA receptor recognized by a monoclonal antibody termed IVd4 described by Banerjee and Toole [9]. Treatment of micromass cultures with this antibody has been shown to decrease cartilage nodule formation as assessed by alcian blue staining and ^{35}S-sulfate incorporation [143]. The identification and further characterization of the receptor protein will allow finer analysis of the role HA and its binding proteins/receptors play in chondrogenesis.

Accumulation of specific extracellular matrix molecules has led to several proposals that adhesive interactions are essential to the mechanism of condensation. The timely expression of new adhesive molecules, such as, an 8.5 kDa cadherin [12] and an as yet unidentified molecule which binds PNA-lectin [7] suggests that interactions between the extracellular matrix and the mesenchymal cell surface could play an important role in the condensation process (see Table 2). HA-mediated adhesion may be the first step in bringing cells into close enough proximity for interactions between other extracellular matrix molecules to effect condensation, or it may be one of multiple mechanisms that cooperate to bring about a crucial developmental end, chondrogenesis.

Matrix stability during cartilage maturation

Micromass cultures were used to obtain cartilage nodules with a relatively immature matrix (after 3 days of chondrogenesis) versus cartilage nodules with a more mature extracellular matrix (after 6 days of chondrogenesis). Thus with the increasing accumulation of matrix in the micromass culture system, there is a decreased sensitivity to proteoglycan displacement by HA_6. The mature cartilage nodules after day 6 of micromass culture had gained properties similar to the cartilaginous anlagen of the embryonic tibiae. Chondrocytes released from these mature cartilages retain a HA/aggrecan-rich cell-associated matrix, and can be "disengaged" from that matrix by HA_6 treatment.

Cell shape and characteristic pericellular matrices can provide evidence to the state of differentiation of these cells from the limb or from micromass cultures. Undifferentiated limb mesenchyme exhibits a flattened, fibroblastic morphology whereas chondrocytes are typically round or polygonal cells which exhibit large pericellular matrices in culture [71]. Individual cells were released from micromass cultures with 0.25% trypsin/ EDTA and examined by phase contrast microscopy [97]. The cells released from an 8-day micromass culture exhibited a typical rounded chondrocyte morphology. In addition, when these cells were assayed with a particle exclusion assay to visualize the presence of a cell-associated matrix, they displayed a matrix characteristic of a differentiated chondrocyte [71]. Individual cells from a parallel HA_6-treated micromass culture exhibited mostly flattened stellate morphology, typical of mesenchymal cells. These cells, when assayed with the particle exclusion assay, produced no detectable pericellular matrix, characteristic of stage 22-26 limb bud mesoderm [71].

Matrix stabilization of the chondrocyte phenotype

It seems likely that connections between the cell-associated matrix and the chondrocyte could participate in transducing signals necessary toward proper turnover and maintenance of the extracellular matrix. Considering the crucial load-bearing properties of cartilage, variations in pressure at the chondrocyte cell surface could be transduced to the cell interior through the HA-cell surface receptor complexes resulting in proper response to these changes in load. The role of the intimate connections between the chondrocyte and the extracellular matrix allows us to view cartilage not solely as structural shock absorbing material, but rather as a tissue with specific and dynamic responses to external forces to effect its functions.

If 9-day HA_6-treated micromass cultures are dissociated into single cells and plated at low density, most cells consistently demonstrate a spread or flattened morphology [97]. These cultures do have accumulated matrix, and it would be expected that a large percentage of cells would demonstrate a chondrocytic morphology. These results also not an effect of selective attachment since there are very few unattached cells in these low-density cultures derived from the micromass cultures. This result has led us to speculate that continued and stable maintenance of chondrocyte phenotype relies on the intimate connection between the cell-associated matrix and the cell surface, namely, the HA receptor. Competitive binding of cell surface HA receptors by hexasaccharides would disengage the accumulated matrix from cells. Entrapped matrix within the micromass could continue to maintain cells in a rounded configuration similar to culturing in collagen or agarose gels but without the crucial cell surface-pericellular matrix connection crucial to a stable phenotype.

Maintenance of the chondrocyte phenotype may also depend on the close association of the cell with its matrix. Chondrocytes have a tendency to dedifferentiate *in vitro* if stripped of their matrix and plated on adhesive substrata [24] or by treatment with specific agents [168].

This dedifferentiation can be evidenced by changes in both cell shape and biosynthetic activity. Typically, dedifferentiation involves a change from a round to a flattened configuration. Dedifferentiation can always be prevented by culturing systems which prevent cell flattening such as collagen gels or agarose [13, 168]. Thus cell shape and the actin cytoskeleton may influence continued expression of the cartilage phenotype as well as initial cartilage differentiation. Components of the chondrocyte extracellular matrix and their association with the cell surface may maintain cell shape and the expression of cartilage phenotype.

A doublet banding pattern in the range of 90-95 kDa is typically seen for bovine chondrocyte CD44 [1, 76]. Although the structural basis for the doublet pattern is unknown, only the faster migrating band is a substrate for protein phosphorylation. Endocytosis of CD44 is apparently upregulated following pericellular matrix depletion and reduced by occupancy with HA [2]. Higher levels of threonine phosphorylation on CD44 were detected on matrix-intact versus matrix-depleted chondrocytes (Fig. 7). The decreased threonine phosphorylation on matrix-depleted chondrocytes was reversed following incubation with HA, suggesting that CD44 occupancy or cross-bridging by HA alters its phosphorylation. The ~71 kDa phosphoprotein that co-precipitated with CD44 but did not react with IM7.8.1 by subsequent western analysis may represent a key component in the pathway of HA signaling through CD44. Thus multiple pools of CD44 may exist that serve varied functions on chondrocytes.

CD44 has been shown to be associated with actin filaments either through interactions with ankyrin [64] or members of the ERM family of proteins [144]. Cell surface binding of HA could affect actin filament alignment and therefore cell shape and/or actin filaments could control the distribution of HA receptors (Fig. 9). Single molecules of HA can bind to more than one receptor at a time resulting in increased affinity [116, 151]. Clustering of cell surface receptors would strengthen the interaction between extracellular HA and the cell surface. Specific distribution of HA receptors may also contribute to the proper assembly and retention of the extracellular matrix.

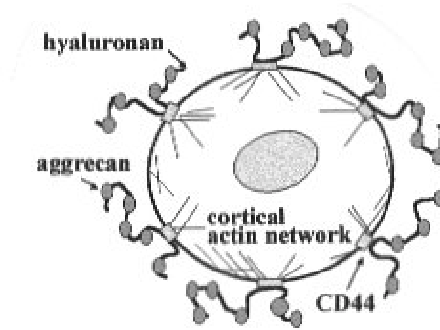

Figure 9. An intact matrix stabilizes chondrocyte cell shape. Binding of HA to chondrocytes enhances the phosphorylation of CD44. Phosphorylated CD44 exhibits increased binding to components of the cortical actin cytoskeleton. Thus, matrix assembly may mediate the stability of the shape of chondrocytes, as maintained by the cortical actin network, critical to the chondrocyte phenotype.

Cartilage matrix homeostasis

Although CD44-HA interactions are likely to play a central mechanistic role in chondrocyte matrix retention, our current long-term goal remains to address the question as to whether or not CD44-HA interactions are actually altered during osteoarthritis or other degenerative musculoskeletal conditions. Disruption of CD44-HA matrix interactions results in a loss of proteoglycan from cartilage tissues in explant culture. Although osteoarthritis is a heterogeneous disease process, one consistent observation, in either experimental animal models of osteoarthritis or cases of human osteoarthritis, is the lack of retention of PG by articular cartilage [11]. This is reflected in the loss of safranin O staining in histologic sections of human articular cartilage, an essential criterion used in the classification of osteoarthritis by both the Mankin or Collins grading scales [115]. Thus, disruption of cell-matrix interactions that are mediated via CD44 results in progressive proteoglycan loss, supporting our overall hypothesis that HA-CD44 interactions are crucial for cartilage homeostasis. Still, we do not have evidence as yet, that CD44 function is altered or disregulated in association with the onset of actual human degenerative disease.

Although active degradation via matrix metalloproteinases (e.g., stromelysin, aggrecanase) are likely responsible for much of the PG loss [82, 101, 117], it is also clear that, in the early stages of the disease, chondrocytes mount a substantial biosynthetic response to counter the PG deficit [11, 27] and stabilize the balance of matrix synthesis and turnover. Nonetheless, this burst of new PG synthesis typically does not lead to successful repair; the PG is not retained by the cartilage, and PG loss continues, as does the disease progression. Is this lack of PG retention due to an altered or inappropriate PG being synthesized or, continued rapid degradation of newly synthesized PG? This is apparently **not** the case as newly synthesized PG extracted from human osteoarthritis cartilage is fully functional and retains its capacity to aggregate and assemble with HA [11]. This would imply that other defects are also present in these diseased tissues. Lack of aggregate retention because of faulty HA receptors could have a dramatic effect on aggrecan retention, even if reparative attempts were made

by chondrocytes to increase proteoglycan and HA synthesis. Tissue remodeling and repair involve interaction of the cell surface with the extracellular matrix. Inappropriate tissue repair can lead to the loss of matrix characteristics and produce severe changes in the mechanical properties of cartilage which occur in degenerative arthritis.

The structure of the cartilage tissue network and the biomechanical properties of its components are essential for the function of articular cartilage; to withstand compressive load forces and undergo reversible deformation. Changes in this unique matrix structure, due to faulty maintenance or repair, trauma, or possibly intrinsic age-related changes, give rise to degenerative disease states such as osteoarthritis [26, 27, 44, 134]. Therefore, considerable attention has been given to understanding the balance between processes of matrix biosynthesis and matrix turnover. The structure of several of the matrix macromolecules has been elucidated [30, 46, 108], the sites of their synthesis determined [92, 114, 158], and regulatory mediators that control their synthesis partially defined [107, 146]. At the other end of the spectrum, enzymes potentially involved in matrix catabolism [5, 33, 82, 101, 117], and the enzyme inhibitors that modulate their function [93, 156, 159] have also been detailed. These studies allow us to understand how stoichiometric levels of appropriate matrix macromolecules are maintained. However, a gap exists in our understanding of processes that control matrix **assembly** and how these macromolecules are immobilized within cartilage once they have been synthesized and secreted by the cells. Such assembly may be critical during the early stages of osteoarthritis. Activated proteases may cleave chondrocyte receptors as well as matrix components. The detection of CD44 neoepitopes generated from proteolytic degradation may in the future serve as a marker of cartilage degeneration. The ability for cell-associated matrix repair may correlate with the re-expression of cell surface receptors.

Acknowledgements

Carita T. Constable performed the experiments to detect phosphothreonine and Dean J. Aguiar, Ph.D. performed the flow cytometry. This work was supported in part by National Institutes of Health grants AR39507 and AR39239.

References

[1] Aguiar DJ, Knudson CB (1995) EM-immunolocalization and immunoprecipitation of bovine chondrocyte CD44. Trans. Ortho. Res. Soc. **20**: 6 (abstract).

[2] Aguiar DJ, Mikecz K, Knudson CB (1996) CD44/HA receptor turnover on "matrix-intact" and "matrix-free" adult bovine articular chondrocytes. Trans. Ortho. Res. Soc. **21**: 86 (abstract).

[3] Ahrens PB, Solursh M, Reiter RS (1977) Stage-related capacity for limb chondrogenesis in cell culture. Develop. Biol. **60**: 69-82.

[4] Amieva MR, Furthmayr H (1995) Subcellular localization of Moesin in dynamic filopodia, retraction fibers, and other structures involved in substrate exploration, attachment, and cell-cell contacts. Exp. Cell Res. **219**: 180-196.

[5] Arner EC, Decicco CP, Cherney R, Tortorella MD (1997) Cleavage of native cartilage aggrecan by neutrophil collagenase (MMP-8) is distinct from endogenous cleavage by aggrecanase. J. Biol. Chem. **272**: 9294-9299.

[6] Aruffo A, Stamenkovic I, Melnick M, Underhill CB, Seed B (1990) CD44 is the principal cell surface receptor for hyaluronate. Cell **61**: 1303-1313.

[7] Aulthouse AL, Solursh M (1987) The detection of a precartilage, blastema-specific marker. Develop. Biol. **120**: 377-384.

[8] Aydelotte MB, Kuettner KE (1988) Differences between subpopulations of cultured bovine articular chondrocytes. Connect. Tiss. Res. **18**: 223-234.

[9] Banerjee SD, Toole BP (1992) Hyaluronan-binding protein in endothelial cell morphogenesis. J. Cell Biol. **119**: 643-652.

[10] Bartolazzi A, Nocks A, Aruffo A, Spring F, Stamenkovic I (1996) Glycosylation of CD44 is implicated in CD44-mediated cell adhesion to hyaluronan. J. Cell Biol. **132**: 1199-1208.

[11] Bayliss MT (1992) Metabolism of animal and human osteoarthritis cartilage. In: Articular cartilage and osteoarthritis. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 487-500.

[12] Bee JA, von der Mark K (1990) An analysis of chick limb bud intercellular adhesion underlying the establishment of cartilage aggregates in suspension culture. J. Cell Sci. **96**: 527-536.

[13] Benya PD, Shaffer JD (1982) Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. Cell **30**: 215-224.

[14] Benya PD, Brown PD, Padilla SR (1988) Microfilament modification by dihydrocytochalasin B causes retinoic acid-modulated chondrocytes to reexpress the differentiated collagen phenotype without change in shape. J. Cell Biol. **106**: 161-170.

[15] Berg EL, Goldstein LA, Jutila MA, Nakache M, Picker LP, Streeter PR, Wu NW, Zhou D, Butcher EC (1989) Homing receptors and vascular addressins: Cell adhesion molecules that direct lymphocyte traffic. Immunol. Rev. **108**: 5-18.

[16] Buckwalter JA, Rosenberg LC (1982) Electron micrographic studies of cartilage proteoglycans. J. Biol.

Chem. **257**: 9830-9839.

[17] Camp RL, Kraus TA, Pure E (1991) Variations in the cytoskeletal interaction and posttranslational modification of the CD44 homing receptor in macrophages. J. Cell Biol. **115**: 1283-1292.

[18] Carrington JL, Chen P, Yanagishita M, Reddi AH (1991) Osteogenin (bone morphogenetic protein-3) stimulates cartilage formation by chick limb bud cells *in vitro*. Develop. Biol. **146**: 406-415.

[19] Chow G, Homandberg G, Knudson W (1995) Effects of interleukin-1α and fibronectin fragments on CD44/hyaluronan receptor expression in bovine articular cartilage. Trans. Orthop. Res. Soc. **20**: 377 (abstract).

[20] Chow G, Knudson CB, Homandberg G, Knudson W (1995) Increased CD44 expression in bovine articular chondrocytes by catabolic cellular mediators. J. Biol. Chem. **270**: 27734-27741.

[21] Clarris BJ, Fraser JRE (1968) On the pericellular zone of some mammalian cells *in vitro*. Exp. Cell Res. **49**: 181-193.

[22] Crossman M, Mason R (1990) Purification and characterization of a hyaluronan binding protein from rat chondrosarcoma. Biochem. J. **266**: 399-406.

[23] Culty M, Nguyen HA, Underhill CB (1992) The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan. J. Cell Biol. **116**: 1055-1062.

[24] Daniels K, Solursh M (1991) Modulation of chondrogenesis by the cytoskeleton and extracellular matrix. J. Cell Sci. **100**: 249-254.

[25] Dessau W, von der Mark H, von der Mark K, Fischer S (1980) Changes in the patterns of collagens and fibronectin during limb- bud chondrogenesis. J. Embryol. Exp. Morphol. **57**: 51-60.

[26] Dieppe P, Cushnaghan J, McAlindon T (1992) Epidemiology, clinical course, and outcome of knee osteoarthritis. In: Articular Cartilage and Osteoarthritis. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 617-628.

[27] Dingle JT (1993) Mechanisms of cartilage destruction and repair:The outlook for therapeutic interventions. Clin. Rheum. **3**: 1-5.

[28] Doege K, Rhodes C, Sasaki M, Hassell JR, Yamada Y (1990) Molecular biology of cartilage proteoglycan (aggrecan) and link protein. In: Extracellular Matrix Genes. Sandell LJ, Boyd CD (eds.). Academic Press, NY. pp. 137-155.

[29] Durr J, Goodman S, Potocnik A, von der Mark H, von der Mark K (1993) Localization of B1-integrins in human cartilage and their role in chondrocyte adhesion to collagen and fibronectin. Exp. Cell Res. **207**: 235-244.

[30] Eyre DR, Wu JJ (1995) Collagen structure and cartilage matrix integrity. J. Rheum. **43S**: 82-85.

[31] Faltz LL, Reddi AH, Hascall GK, Martin D, Pita JC, Hascall VC (1979) Characteristics of proteoglycans extracted from the Swarm rat chondrosarcoma with associative solvents. J. Biol. Chem. **254**: 1375-1380.

[32] Fernandez MP, Selmin O, Martin GR, Yamada Y, Pfaffle M, Deutzmann R, Mollenhauer J, von Der Mark K (1988) The structure of anchorin CII, a collagen binding protein isolated from chondrocyte membrane. J. Biol. Chem. **263**: 5921-5925.

[33] Fosang AJ, Last K, Maciewicz RA (1996) Aggrecan is degraded by matrix metalloproteinases in human arthritis. J. Clin. Invest. **98**: 2292-2299.

[34] Fraser JRE, Laurent TC (1989) Turnover and metabolism of hyaluronan. In: Ciba Foundation Symposium 143, The Biology of Hyaluronan. John Wiley, Chichester, U.K. pp 41-59.

[35] Fraser JRE, Alcorn D, Laurent TC, Robinson AD, Ryan GB (1985) Uptake of circulating hyaluronic acid by the rat liver. Cell Tiss. Res. **242**: 505-510.

[36] Fraser JRE, Kimpton WG, Laurent TC, Cahill RNP, Vakakis N (1988) Uptake and degradation of hyaluronan in lymphatic tissue. Biochem. J. **256**: 153-158.

[37] Frenkel SR, Clancy RM, Ricci JL, Di Cesare P, Rediske JJ, Abramson SB (1996) Effects of nitric oxide on chondrocyte migration, adhesion, and cytoskeletal assembly. Arthritis Rheum. **39**: 1905-1912.

[38] Goldberg RL, Toole BP (1984) Pericellular coat of chick embryo chondrocytes: Structural role of hyaluronate. J. Cell Biol. **99**: 2114-2122.

[39] Goldberg RL, Underhill CB, Toole BP (1982) Affinity chromatography of hyaluronate glutaraldehyde - fixed SV-3T3 cells. Anal. Biochem. **125**: 59-65.

[40] Gould RP, Day A, Wolpert L (1972) Mesenchymal condensation and cell contact in early morphogenesis of the chick limb. Exp. Cell Res. **72**: 325-336.

[41] Gould SE, Upholt WB, Kosher RA (1992) Syndecan 3: A member of the syndecan family of membrane-intercalated proteoglycans that is expressed in high amounts at the onset of chicken limb cartilage differentiation. Proc. Natl. Acad. Sci. USA **89**: 3271-3275.

[42] Gunthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussmann I, Matzku S, Wenzel A, Ponta H, Herrlich P (1991) A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. Cell **65**: 13-24.

[43] Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. J. Morphol. **88**: 49-92.

[44] Hamerman D (1993) Aging and Osteoarthritis: Basic mechanisms. J. Am. Geriatric Soc. **41**: 760-770.

[45] Hardingham TE (1979) The role of link-protein in the structure of cartilage proteoglycan aggregates. Biochem. J. **177**: 237-247.

[46] Hardingham TE, Fosang AJ (1992) Proteo-

glycans: Many forms and many functions. FASEB J. **6**: 861-870.

[47] Hardingham TE, Muir H (1973) Binding of oligosaccharides of hyaluronic acid to proteoglycans. Biochem. J. **135**: 905-908.

[48] Hardwick C, Hoare K, Owens R, Hohn HP, Hook M, Moore D, Cripps V, Austen L, Nance DM, Turley EA (1992) Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. J. Cell Biol. **117**: 1343-1350.

[49] Hascall VC (1977) Interaction of cartilage proteoglycans with hyaluronic acid. J. Supramol. Struct. **7**: 101-120.

[50] Hascall VC, Heinegard D (1974) Aggregation of cartilage proteoglycans. II. Oligosaccharide competitors of the proteoglycan-hyaluronic acid interaction. J. Biol. Chem. **249**: 4242-4249.

[51] Hascall VC, Sajdera SW (1969) Protein polysaccharide complex from bovine nasal cartilage. The function of glycoprotein in the formation of aggregates. J. Biol. Chem. **244**: 2384-2396.

[52] Hascall VC, Oegema TR, Brown M, Caplan AI (1976) Isolation and characterization of proteoglycans from chick limb chondrocytes grown *in vitro*. J. Biol. Chem. **251**: 3511-3519.

[53] Hauselmann HJ, Aydelotte MB, Schumacher BL, Kuettner KE, Gitelis SH, Thonar EJ-MA (1992) Synthesis and turnover of proteoglycans by human and bovine adult articular chondrocytes cultured in alginate beads. Matrix **12**: 130-136.

[54] Hauselmann HJ, Fernandes RJ, Mok SS, Schmid TM, Block JA, Aydelotte MB, Kuettner KE, Thonar EJ-MA (1994) Phenotypic stability of bovine articular chondrocytes after long- term culture in alginate beads. J. Cell Sci. **107**: 17-27.

[55] Haynes BF, Hua-Xin L, Patton KL (1991) The transmembrane hyaluronate receptor (CD44): Multiple functions, multiple forms. Cancer Cells **3**: 347-350.

[56] Haynes BF, Telen MJ, Hale LP, Denning SM (1989) CD44 - A molecule involved in leukocyte adherence and T-cell activation. Immunol. Today **10**: 423-428.

[57] Heinegard D, Lorenzo P, Sommarin Y (1995) Articular cartilage matrix proteins. In: Osteoarthritic Disorders. Kuettner KE, Goldberg VM (eds.). American Academy of Orthopaedic Surgeons, Rosemont, IL. pp. 239-237.

[58] Hua Q, Knudson CB, Knudson W (1993) Internalization of hyaluronan by chondrocytes occurs via receptor-mediated endocytosis. J. Cell Sci. **106**: 365-375.

[59] Hudson DL, Sleeman J, Watt FM (1995) CD44 is the major peanut lectin-binding glycoprotein in human epidermal keratinocytes and plays a role in intercellular adhesion. J. Cell Sci. **108**: 1959-1970.

[60] Hunziker EB (1992) Articular cartilage structure in humans and experimental animals. In: Articular Cartilage and Osteoarthritis. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Reaven Press. pp. 183-199.

[61] Isacke CM (1994) The role of the cytoplasmic domain in regulating CD44 function. J. Cell Sci. **107**: 2353-2359.

[62] Jiang H, Knudson CB, Koepp H, Eger W, Knudson W (1998) Alternative expression of cytoplasmic tail-less isoform of CD44 (CD44exon 19) by human articular chondrocytes. Trans. Ortho. Res. Soc. **23**: 913 (abstract).

[63] Jaworski DM, Kelly GM, Hockfield S (1995) BEHAB, a new member of the proteoglycan tandem repeat family of hyaluronan-binding proteins that is restricted to the brain. J. Cell Biol. **125**: 495-509.

[64] Kalomiris EL, Bourguignon LYW (1988) Mouse T lymphoma cells contain a transmembrane glycoprotein (GP85) that binds ankyrin. J. Cell Biol. **106**: 319-327.

[65] Kalomiris EL, Bourguignon LYW (1989) Lymphoma protein kinase C is associated with the transmembrane glycoprotein, GP85, and may function in GP85-ankyrin binding. J. Biol. Chem. **264**: 8113-8119.

[66] Kim JJ, Conrad HE (1977) Properties of cultured chondrocytes obtained from histologically distinct zones of the chick embryo tibiotarsus. J. Biol. Chem. **252**: 8292-8299.

[67] Kimata K, Yasuteru O, Katsuko T, Shinomura T, Yamagata M, Uritani M, Suzuki S (1986) A large chondroitin sulfate proteoglycan (PG-M) synthesized before chondrogenesis in the limb bud of chick embryo. J. Biol. Chem. **261**: 13517-13525.

[68] Knudson CB (1993) Hyaluronan receptor-directed assembly of chondrocyte pericellular matrix. J. Cell Biol. **120**: 825-834.

[69] Knudson CB (1997) Hyaluronan in embryonic cell adhesion and matrix assembly. In: The Chemistry, Biology and Medical Applications of Hyaluronan and its Derivatives. Laurent TC (ed.). Portland Press, London, U.K. Wenner-Gren Internatl. Series, **72**: 161-168.

[70] Knudson CB, Knudson W (1993) Hyaluronan-binding proteins in development, tissue homeostasis and disease. FASEB J. **7**: 1233-1241.

[71] Knudson CB, Toole BP (1985) Changes in the pericellular matrix during differentiation of limb bud mesoderm. Develop. Biol. **112**: 308-318.

[72] Knudson CB, Toole BP (1987) Hyaluronate-cell interactions during differentiation of chick embryo limb mesoderm. Develop. Biol. **124**: 82-90.

[73] Knudson W, Knudson CB (1991) Assembly of a chondrocyte-like pericellular matrix on non-chondrogenic cells. J. Cell Sci. **99**: 227-235.

[74] Knudson W, Bartnik E, Knudson CB (1993) Assembly of pericellular matrices by COS-7 cells transfected with CD44 homing receptor genes. Proc. Natl. Acad.

Sci.USA, **90**: 4003-4007.

[75] Knudson W, Aguiar DJ, Hua Q, Knudson CB (1996) CD44-anchored hyaluronan-rich pericellular matrices: An ultrastructural and biochemical analysis. Exp. Cell Res. **228**: 216-228.

[76] Knudson CB, Constable CT, Nofal GA (1997) Changes in phosphorylation and actin-association of CD44 with receptor occupancy on articular chondrocytes. Trans. Orthop. Res. Soc. **22**: 34 (abstract).

[77] Knutson JR, Iida J, Fields GB, McCarthy JB (1996) CD44/Chondroitin sulfate proteoglycan and alpha 2 beta 1 integrin mediate human melanoma cell migration on type IV collagen and invasion of basement membrane. Mol. Biol. Cell, **7**: 383-396.

[78] Kosher RA, Kulyk WM, Gay SW (1986) Collagen gene expression during limb cartilage differentiation. J. Cell Biol. **102**: 1151-1156.

[79] Kosher RA, Savage MP, Walker KH (1981) A gradation of hyaluronate accumulation along the proximodistal axis of the embryonic chick limb bud. J. Embryol. Exp. Morphol. **63**: 85-98.

[80] Kosher RA, Walker KH, Ledger PW (1982) Temporal and spatial distribution of fibronectin during development of the embryonic chick limb bud. Cell Differentiation **11**: 217-228.

[81] Kulyk WM, Kosher RA (1987) Temporal and spatial analysis of hyaluronidase activity during development of the embryonic chick limb bud. Develop. Biol. **120**: 535-541.

[82] Lark MW, Gordy JT, Weidner JR, Ayala J, Kimura JH, Williams HR, Mumford RA, Flannery CR, Carlson SS, Iwata M, Sandy JD (1995) Cell-mediated catabolism of aggrecan. J. Biol. Chem. **270**: 2550-2556.

[83] Laurent TC, Fraser JRE (1986) The properties and turnover of hyaluronan. In: Functions of the Proteoglycans, Ciba Foundation Symposium 124. Evered D, Whelan J (eds.). John Wiley. pp. 9-29.

[84] Laurent TC, Fraser RE (1992) Hyaluronan. FASEB J. **6**: 2397-2404.

[85] Laurent TC, Laurent UB, Fraser JRE (1995) Functions of hyaluronan. Ann. Rheum. Dis. **54**: 429-432.

[86] Lazaar AL, Pure E (1995) CD44: A model for regulated adhesion function. The Immunologist **3**: 19-25.

[87] Lee Y, Chuong C (1997) Activation of protein kinase A is a pivotal step involved in both BMP-2 and cyclic AMP-induced chondrogenesis. J. Cell. Physiol. **179**: 153-165.

[88] Lee GM, Johnstone B, Jacobson K, Caterson B (1993) The dynamic structure of the pericellular matrix on living cells. J. Cell Biol. **123**: 1899-1907.

[89] Lesley J, Schulte R, Hyman R (1990) Binding of hyaluronic acid to lymphoid cell lines is inhibited by monoclonal antibodies against Pgp-1. Exp. Cell Res. **187**: 224-233.

[90] Loeser RF (1993) Integrin-mediated attachment of articular chondrocytes to extracellular matrix proteins. Arthritis Rheum. **36**: 1103-1109.

[91] Loeser RF (1994) Modulation of integrin-mediated attachment of chondrocytes to extracellular matrix proteins by cations, retinoic acid, and transforming growth factor B. Exp. Cell Res. **211**: 17-23.

[92] Lohmander LS, Kimura JH (1986) Biosynthesis of cartilage proteoglycan. In: Articular Cartilage and Osteoarthritis. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 93-111.

[93] Lohmander LS, Hoerrner LA, Lark MW (1993) Metalloproteinases, tissue inhibitor, and proteoglycan fragments in knee synovial fluid in human osteoarthritis. Arthritis Rheum. **36**: 181-189.

[94] Lust G, Burton-Wurster N (1992) Fibronectin in osteoarthritis. Comparison of animal and human diseases. In: Articular Cartilage and Osteoarthritis. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 447-454.

[95] Mackie EJ, Thesleff I, Chiquet-Ehrismann R (1987) Tenascin is associated with chondrogenic and osteogenic differentiation *in vivo* and promotes chondrogenesis *in vitro*. J. Cell Biol. **105**: 2569-2579.

[96] Maleski MP, Knudson CB (1992) Matrix assembly during chondrogenesis occurs via hyaluronan -cell surface interactions. Trans. Orthop. Res. Soc. **17**: 105 (abstract).

[97] Maleski MP, Knudson CB (1996) Hyaluronan meditated aggregation of limb bud mesoderm and mesenchymal condensation during chondrogenesis. Exp. Cell Res. **225**: 55-66.

[98] Maleski MP, Knudson CB (1996) Matrix accumulation and retention in embryonic cartilage and *in vitro* chondrogenesis. Connect. Tiss. Res. **34**: 75-86.

[99] Margolis RU, Margolis RK (1994) Aggrecan-versican-neurocan family of proteoglycans. Meth. Enzymol. **245**: 105-126.

[100] Maroudas A (1979) Physicochemical properties of articular cartilage. In: Adult Articular Cartilage. Freeman MAR (ed.). Pitman, London. pp. 215-290.

[101] Martel-Pelletier J, McCollum R, Fujimoto N, Obata K, Cloutier JM, Pelletier JP (1994) Excess metalloproteinases over tissue inhibitor of metalloproteinases may contribute to cartilage degradation in osteoarthritis and rheumatoid arthritis. Lab. Investigation **70**: 807-815.

[102] Martinsen A, Skjak-Braek G, Smidsrod O (1989) Alginate as immobilization material: I. Correlation between chemical and physical properties of alginate gel beads. Biotechnol. Bioeng. **33**: 79-89.

[103] Mason RM, Crossman MV, Sweeney C (1989) Hyaluronan and hyaluronan-binding proteins in

cartilaginous tissues. In: The Biology of Hyaluronan, Ciba Foundation Symposium 143. Evered D, Whelan J (eds.). John Wiley. pp. 107-120.

[104] McGary CT, Raja RH, Weigel PH (1989) Endocytosis of hyaluronic acid by rat liver endothelial cells, evidence for receptor recycling. Biochem. J. **257**: 875-884.

[105] McGuire PG, Castellot Jr. JJ, Orkin RW (1992) Size dependent hyaluronate degradation by cultured cells. J. Cell. Physiol. **133**: 267-276.

[106] Mok SS, Masuda K, Hauselmann HJ, Aydelotte MB, Thonar EJ-MA (1994) Aggrecan synthesized by mature bovine chondrocytes suspended in alginate. J. Biol. Chem. **269**: 33021-33027.

[107] Morales TI (1992) Polypeptide regulators of matrix homeostasis in articular cartilage. In: Articular Cartilage and Osteoarthritis. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 265-280.

[108] Muir H (1995) The chondrocyte, architech of cartilage: Biomechanics, structure, function and molecular biology of cartilage matrix macromolecules. BioEssays **17**: 1039-1048.

[109] Nemec RE, Toole BP, Knudson W (1987) The cell surface hyaluronate binding sites of invasive human bladder carcinoma cells. Biochem. Biophys. Res. Comm. **149**: 249-257.

[110] Ng CK, Handley CJ, Preston BN, Robinson HC, Bolis S, Parker G (1995) Effect of exogenous hyaluronan and hyaluronan oligosaccharides on hyaluronan and aggrecan synthesis and catabolism in adult articular cartilage explants. Arch. Biochem. Biophys. **316**: 596-606.

[111] Oberlender SA, Tuan RS (1994) Expression and functional involvement of N-cadherin in embryonic limb chondrogenesis. Development **120**: 177-187.

[112] Oster GF, Murray JD, Maini PK (1985) A model for chondrogenic condensations in the developing limb: The role of extracellular matrix and cell tractions. J. Embryol. Exp. Morphol. **89**: 93-112.

[113] Petit B, Masuda K, D'Souza A, Otten L, Pietryla D, Hartmann D, Morris N, Uebelhart D, Schmid TM, Thonar EJ-MA (1996) Characterization of cross-linked collagens sythesized by mature articular chondrocytes cultured in alginate beads: Comparison of two distinct matrix compartments. Exp. Cell Res. **225**: 151-161.

[114] Prehm P (1984) Hyaluronate is synthesized at plasma membranes. Biochem. J. **220**: 597-600.

[115] Pritzker KPH (1992) Cartilage histopathology in human and rhesus macaque osteoarthritis. In: Articular Cartilage and Osteoarthritis. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 473-485.

[116] Raja RH, McGary CT, Weigel PH (1988) Affinity and distribution of surface and intracellular hyaluronic acid receptors in isolated rat liver endothelial cells. J. Biol. Chem. **263**: 16661-16668.

[117] Roughley PJ, Nguyen Q, Mort JS, Hughes CE, Caterson B (1993) Proteolytic degradation of human articular cartilage: Its relationship to stromelysin. Agents Actions **39S**: 149-159.

[118] Salter DM, Hughes DE, Simpson R, Gardner DL (1992) Integrin expression by human articular chondrocytes. British J. Rheumatology **31**: 231-234.

[119] Schenk RK, Eggli PS, Hunziker EB (1986) Articular cartilage morphology. In: Articular Cartilage Biochemistry. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 3-22.

[120] Schramm CA, Reiter RS, Solursh M (1994) Role for short-range interactions in the formation of cartilage and muscle masses in transfilter micromass cultures. Develop. Biol. **163**: 467-479.

[121] Screaton GR, Bell MV, Jackson DG, Cornelis FB, Gerth U, Bell JI (1992) Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. Proc. Natl. Acad. Sci. USA **89**: 12160-12164.

[122] Shinomura T, Kimata K (1990) Precartilage condensation during skeletal pattern formation. Develop. Growth Differentiation **32**: 243-248.

[123] Shinomura T, Nishida Y, Kimata K (1992) A large chondroitin sulfate proteoglycan (PG-M) and cartilage differentiation. In: Articular Cartilage and Disease. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 35-44.

[124] Singley CT, Solursh M (1981) The spatial distribution of hyaluronic acid and mesenchymal condensation in the embryonic chick wing. Develop. Biol. **84**: 102-120.

[125] Smedsrod B, Pertoft H, Eriksson S, Fraser JRE, Laurent TC (1984) Studies *in vitro* on the uptake and degradation of sodium hyaluronate in rat liver endothelial cells. Biochem. J. **223**: 617-626.

[126] Solursh M (1984) Cell and matrix interactions during limb chondrogenesis *in vitro*. In: The Role of Extracellular Matrix in Development. Trelstad RL (ed.). A.R. Liss, New York. pp. 277-303.

[127] Solursh M, Reiter S (1980) Evidence for histogenic interactions during *in vitro* limb chondrogenesis. Develop. Biol. **78**: 141-150.

[128] Solursh M, Ahrens PB, Reiter RS (1978) A tissue culture analysis of the steps in limb chondrogenesis. In Vitro **14**: 51-61.

[129] Solursh M, Hardingham TE, Hascall VC, Kimura JH (1980) Separate effects of exogenous hyaluronic acid on proteoglycan synthesis and deposition in pericellular matrix by cultured chick embryo limb chondrocytes. Develop. Biol. **75**: 121-129.

[130] Solursh M, Reiter R, Ahrens PB, Pratt RM (1979) Increase in levels of cyclic AMP during avian limb

chondrogenesis *in vitro*. Differentiation **15**: 183-186.

[131] Solursh M, Linsenmayer TF, Jensen KL (1982) Chondrogenesis from single limb mesenchymal cells. Develop. Biol. **94**: 259-264.

[132] Stefanovic-Racic M, Stadler J, Evans CH (1993) Nitric oxide and arthritis. Arthritis Rheum. **36**: 1-10.

[133] Sy MS, Guo YJ, Stamenkovic I (1991) Distinct effects of two CD44 isoforms on tumor growth *in vivo*. J. Exp. Med. **174**: 859-866.

[134] Thonar EJ-MA, Bjornsson S, Kuettner KE (1986) Age-related changes in cartilage proteoglycans. In: Articular Cartilage Biochemistry. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 273-291.

[135] Thorogood PV, Hinchliffe JR (1975) An analysis of the condensation process during chondrogenesis in the embryonic chick hind limb. J. Embryol. Exp. Morphol. **33**: 581-606.

[136] Tomasek JJ, Mazurkiewicz JE, Newman SA (1982) Nonuniform distribution of fibronectin during avian limb development. Develop. Biol. **90**: 118-126.

[137] Toole BP (1981) Glycosaminoglycans in morphogenesis. In: Cell Biology of the Extracellular Matrix. Hay ED (ed.). Plenum Press, NY. pp. 259-294.

[138] Toole BP (1990) Hyaluronan and its binding proteins, the hyaladherins. Curr. Opin. Cell Biol. **2**: 839-844.

[139] Toole BP (1991) Proteoglycans and hyaluronan in morphogenesis and differentiation. In: Cell Biology of Extracellular Matrix. Hay ED (ed.). Plenum. pp. 305-339.

[140] Toole BP, Banerjee SD, Turner R, Munaim S, Knudson CB (1992) Hyaluronan-cell interactions in limb development. In: Developmental Patterning of the Vertebrate Limb. Hinchliffe J, Hurle J, Summerbell D (eds.). Plenum. pp. 1-9.

[141] Toole BP, Goldberg RL, Chi-Rosso G, Underhill CB, Orkin RW (1984) Hyaluronate-cell interactions. In: The Role of the Extracellular Matrix in Development. Trelstad RL (ed.). A.R. Liss. pp. 43-46.

[142] Toole BP, Munian SI, Wells S, Knudson CB (1989) Hyaluronate-cell interactions and growth factor regulation of hyaluronate synthesis during limb development. In: The Biology of Hyaluronan, Ciba Foundation Symposium 143. Evered D, Whelan J (eds.). John Wiley. pp. 138-149.

[143] Toole BP, Turner RE, Banerjee SD (1993) Hyaluronan-binding protein in chondrogenesis and angiogenesis in the developing limb. In: Limb development and regeneration. Part B. Fallon JF, Goetinck PF, Kelley RO, Stocum DL (eds.). Wiley-Liss, NY. pp. 437-444.

[144] Tsukita S, Oishi K, Sato N, Sagara J, Kawai A (1994) ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. J. Cell Biol. **126**: 391-401.

[145] Turley EA, Belch AJ, Poppema S, Pilarski LM (1993) Expression and function of a receptor for hyaluronan-mediated motility on normal and malignant B lymphocytes. Blood **81**: 446-453.

[146] Tyler JA, Bolis S, Dingle JT, Middleson JFS (1992) Mediators of matrix catabolism. In: Articular Cartilage and Osteoarthritis. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 251-264.

[147] Underhill CB (1982) Interaction of hyaluronate with the surface of simian virus 40- transformed 3T3 cells: Aggregation and binding studies. J. Cell Sci. **56**: 177-189.

[148] Underhill CB (1992) CD44: The hyaluronan receptor. J. Cell Sci. **103**: 293-298.

[149] Underhill CB, Dorfman A (1978) The role of hyaluronic acid in intercellular adhesion of cultured mouse cells. Exp. Cell Res. **117**: 155-164.

[150] Underhill CB, Toole BP (1979) Binding of hyaluronate to the surface of cultured cells. J. Cell Biol. **82**: 475-484.

[151] Underhill CB, Toole BP (1980) Physical characteristics of hyaluronate binding to the surface of simian virus 40 - transformed 3T3 cells. J. Biol. Chem. **255**: 4544-4549.

[152] Underhill CB, Toole BP (1981) Receptors for hyaluronate on the surface of parent and virus-transformed cell lines. Exp. Cell Res. **131**: 419-423.

[153] Underhill CB, Chi-Rosso G, Toole BP (1983) Effect of detergent solubilization on the hyaluronanate-binding protein from membranes of Simian virus 40-transformed 3T3 cells. J. Biol. Chem. **258**: 8086-8091.

[154] Underhill CB, Green SJ, Cologlio PM, Tarone G (1987) The hyaluronate receptor is identical to a glycoprotein of 85, 000 Mr (gp 85) as shown by a monoclonal antibody that interferes with binding activity. J. Biol. Chem. **262**: 13142-13146.

[155] Underhill CB, Nguyen HA, Shizari M, Culty M (1993) CD44 positive macrophages take up hyaluronan during lung development. Develop. Biol. **155**: 324-336.

[156] Vincenti MP, Clark IM, Brinckerhoff CE (1994) Using inhibitors of metalloproteinases to treat arthritis. Arthritis Rheum. **37**: 1115-1126.

[157] Walther BT, Ohman R, Roseman S (1973) A quantitative assay for intercellular adhesion. Proc. Natl. Acad. Sci. USA **70**: 1569-1573.

[158] Weigel PH, Hascall VC, Tammi M (1997) Hyaluronan synthases. J. Biol. Chem. **272**: 13997-14000.

[159] Werb Z (1992) The biologic role of metalloproteinases and their inhibitors. In: Articular Cartilage and Osteoarthritis. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 295-304.

[160] Wheatley SC, Isacke CM, Crossley PH (1993) Restricted expression of the hyaluronan receptor, CD44, during postimplantation mouse embryogenesis suggests key roles in tissue formation and patterning. Development

119: 295-306.

[161] Widelitz RB, Jiang T, Murray BA, Chuong C (1993) Adhesion Molecules in skeletogenesis: II. Neural cell adhesion molecules mediate precartilaginous mesenchymal condensations and enhance chondrogenesis. J. Cell. Physiol. **156**: 399-411.

[162] Wiebkin OW, Hardingham TE, Muir H (1975) Hyaluronic acid-proteoglycan interactions and the influence of hyaluronic acid on proteoglycan synthesis by chondrocytes from adult cartilage. In: Extracellular Matrix Influences on Gene Expression. Slavkin HC, Greulich RC (eds.). Academic Press, NY. pp. 209-223.

[163] Woods VL, Schreck PJ, Gesink DS, Pacheco HO, Amiel D, Akeson WH, Lotz M (1994) Integrin expression by human articular chondrocytes. Arthritis Rheum. **37**: 537-544.

[164] Yamada H, Watanabe K, Shimonaka M, Yamaguchi Y (1994) Molecular cloning of brevican, a novel brain proteoglycan of the aggrecan/versican family. J. Biol. Chem. **269**: 10119-10126.

[165] Yannariello-Brown J, Weigel PH (1992) Detergent solubilization of the endocytic Ca^{2+}-independent hyaluronan receptor from rat liver endothelial cells and separation from a Ca^{2+}-dependent hyaluronan-binding activity. Biochem. **31**: 576-584.

[166] Yannariello-Brown J, Frost SJ, Weigel PH (1992) Identification of the Ca^{2+}-independent endocytic hyaluronan receptor in rat liver sinusoidal endothelial cells using a photoaffinity cross-linking reagents. J. Biol. Chem. **267**: 20451-20456.

[167] Zanetti NC, Solursh M (1984) Induction of chondrogenesis in limb mesenchymal cultures by disruption of the actin cytoskeleton. J. Cell Biol. **99**: 115-123.

[168] Zanetti NC, Solursh M (1989) Effect of cell shape on cartilage differentiation. In: Cell Shape: Determinants, Regulation, and Regulatory Role. Stein WD, Bronner F (eds.). Academic Press. pp. 291-327.

[169] Zimmermann DR, Ruoslahti E (1989) Multiple domains of the large fibroblast proteoglycan, versican. EMBO J. **8**: 2975-2981.

Discussion with Reviewers

V. Hascall: The description of CD44 could benefit from a figure illustrating the exon structure and a model of the standard and "epican" forms.

Author: Figure A illustrates the human CD44 gene, indicating the exons numbered 1 through 20, based on the work of Screaton *et al*. [121]. Exons 1 through 17 encode the extracellular domain, exon 18 encodes the transmembrane (TM) domain, while exon 20 encodes the standard cytoplasmic domain. Exon 19 is used alternatively, and would generate a short cytoplasmic tail. The mRNA for the standard form of CD44 would consist of exons 1-5, 16-18 and 20. Variant forms of CD44 use alternative splicing of exons 6 through 15, which are also called v1-v10 (variant 1-10). The epithelial variant form, CD44E or "epican" contains the v8-10 exons and therefore the mRNA would consist of exons 1-5, 13-18 and 20.

P. Prehm: How was it shown that the hexasaccharide preparation did not contain higher oligosaccharides that could disrupt hyaluronan-aggrecan interactions?

Author: The profile of the P-6 column shows that there is fairly good separation of the HA oligosaccharides. We have tested several column fractions, including the higher numbered oligosaccharides, for their capacity to compete for aggrecan-HA binding using an ELISA for hyaluronan [170]. In this assay, the aggrecan-HA binding is not stabilized by link protein, whereas in cartilage the majority of aggrecan bound to HA is link stabilized. HA and HA_{10} or higher numbered oligosaccharides when pre-incubated with aggrecan, would inhibit aggrecan binding to target HA in a dose-dependent manner (Knudson *et al*., manuscript in preparation) HA_4, HA_6 and HA_8 fractions were not effective inhibitors of aggrecan binding to high molecular weight HA in the ELISA.

P. Prehm: The concentrations of the hexasaccharides used in the experiments are extremely high: 150-250 μg/ml. Are conditions known that such high concentrations occur *in vivo* so that the observed effects are relevant for physiological processes in cartilage?

Author: The molecular size of HA isolated from cartilage decreases with age [11], suggesting that there may be HA oligosaccharides generated. The amount of HA oligosaccharides in cartilage has not been determined. Yes, the high concentrations used in our experiments were determined empirically [68], and are effective in displacing the chondrocyte cell-associated matrix in a relatively short time frame, which we are currently using as a model system. Although lower concentrations (< 100 μg/ml) do not result in a noticeable change in cartilage explants cultured for up to two weeks, we do not know whether or not low concentrations effect changes in a slow progressive manner, similar to the slow degenerative changes seen in human disease.

W.M. Kulyk: Micromass cultures prepared from whole limbs of early-stage chick embryo contain considerable population of myogenic as well as chondrogenic progenitor cells. Do you know whether both cell types express hyaluronan receptors such as CD44? To what extent might the presence of premyogenic cells in your embryonic limb mesenchyme cell cultures complicate interpretation of your experimental data regarding the role of hyaluronan/HA

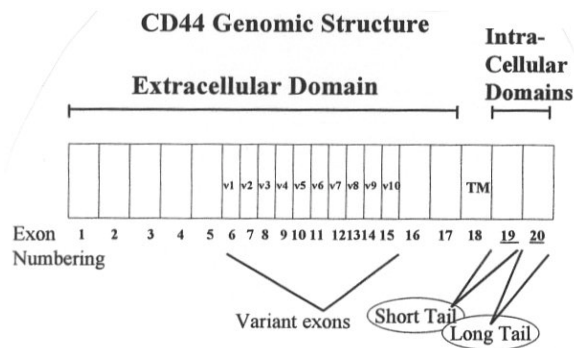

Figure A. The human CD44 gene, indicating the exons numbered 1 through 20, based on the work of Screaton *et al.* [121].

---

receptor interaction in chondrogenic cell aggregation/ differentiation?

Author: We have not focused on myogenesis, but in our earlier work we studied myoblasts prepared from stage 38 embryonic chick limbs and documented hyaluronan binding capacity of those cells [72]. But yes, certainly the presence of premyogenic cells confounds the interpretation of experiments using limb bud mesenchyme. Clearly however, the presence of hyaluronan hexasaccharides inhibited the formation of chondrogenic condensations and chondrocyte differentiation in micromass cultures. Nonetheless, we did not examine these cultures for the presence of myoblasts or myocytes.

V. Hascall: In **Discussion**, the comparison with the work of Oster *et al.* [112] with that of the author is unclear. Please comment.

Author: The work of Oster *et al.* [112] complements ours in that we also found that very high concentrations of hyaluronan do inhibit cell aggregation, and that in later stages when hyaluronan levels are reduced (but not absent), precartilage condensation begins.

W.M. Kulyk: The phosphorylated form of CD44 is cytoskeleton-associated in T lymphoma cells, but phosphorylation hinders CD44 interaction with the cytoskeleton in macrophages. Given that HA-occupancy elevates CD44 phosphorylation in chondrogenic cells, and that a rounded cell shape favors the chondrogenic phenotype, can you predict at this time the specific effect that CD44 phosphorylation is likely to have on its association with the **chondrocyte** cytoskeleton? Is this relationship likely to be identical in embryonic cartilage progenitor cells (limb bud mesenchyme) and fully differentiated (e.g., articular) chondrocytes?

Author: Our preliminary results suggest that HA-occupancy increases CD44 association with the chondrocyte cytoskeleton [76], so we would therefore predict that CD44 phosphorylation would enhance cytoskeletal interactions. Since the cortical actin network of articular chondrocytes is so different from the cytoskeleton of chondrogenic mesenchymal cells, it is possible that different actin binding proteins mediate CD44-cytoskeletal associations in these cells and therefore the role of CD44 phosphorylation in modulating the binding to these proteins may differ.

R.S. Tuan: The threonine phosphorylation data in Figure 7 are certainly very interesting, in that a possible outside-in signal transduction step upon hyaluronan binding is suggested. Does the author have data on serine phosphorylation? Any hint as to which threonine (or serine) residues on the cytoplasmic tail of CD44 are phosphorylation targets?

Author: Our preliminary experiments using the anti-phosphoserine antibodies have given parallel findings to those presented herein on threonine phosphorylation. In regard to which threonine or serine residues might be crucial, please refer to the work of Isacke [61] and her future publications.

R.S. Tuan: Also, it is generally assumed that the slower migrating band of a candidate phosphorylated molecules is the phosphorylated species. It is therefore interesting to learn that the 87 kDa, but not the 89 kDa, band is the P-Thr reactive protein. What is known about the difference between the 87 kDa and 89 kDa species of CD44? Other post-translational modifications?

Author: Based on nucleic acid sequences, the predicted molecular weight of CD44 due to amino acids is 39 kDa [121]. Thus, glycosylation with both N- and O-linked oligosaccharides moieties accounts for the increased molecular weights. Immunoprecipitation from bovine articular chondrocytes or from cells derived from bovine synovium typically yields a doublet banding pattern for CD44 whereas other cell types show a primary band at 90 kDa with higher molecular weight species, such as CD44E. We are still curious as to the identity of the two species.

W.M. Kulyk: Endocytosis of CD44 is apparently upregulated following pericellular matrix depletion and reduced by occupancy with HA. In addition, you have observed a decline in CD44 phosphorylation following matrix depletion that is reversed by incubation with HA. Does a dephosphorylation of CD44 represent a signal for endocytosis? Have you examine the relative distribution of phosphorylated versus dephosphorylated forms of CD44 in intracellular versus cell-surface compartments?

Author: It is an interesting hypothesis that dephosphor-

ylation may serve as a signal for CD44 endocytosis. The cytoplasmic tail of CD44 does not contain any of the consensus sequences identified so far that mediate the formation of clathrin coats on forming endocytotic vesicles. We have evidence from cell surface biotinylation studies that CD44 that is on the plasma membrane can be internalized, but have not yet examined the relative phosphorylation of CD44 in these compartments.

W.M. Kulyk: Is there any information available to date on the extents to which levels of either hyaluronan or its receptors (e.g., CD44) are altered in osteoarthritic cartilage?
Author: Studies of human osteoarthritic cartilage show great variability of cellularity as well as matrix composition. Evidence for an imbalance in HA turnover has been observed in experimental models of osteoarthritis. For example, in the canine anterior cruciate ligament model of osteoarthritis, HA content decreases in weight-bearing areas of femoral cartilage in the early stages prior to detectable loss of matrix proteoglycans [171]. However, hyaluronan synthesis in actually increased in osteoarthritic cartilage explants, implying that the newly synthesized hyaluronan is either being rapidly removed or not properly retained and assembled into the matrix. Work is currently in progress to correlate CD44 expression in human articular cartilage with age and disease progression.

W.M. Kulyk: Does CD44 appear to be an exclusive receptor for hyaluronan? Is there any evidence for a direct interaction of CD44 with other cell-surface or extracellular matrix components?
Author: Most of the reported interactions of CD44 with other extracellular matrix components are low affinity interactions. It is difficult to appreciate the significance of these within tissues. CD44-hyaluronan interactions are of relatively low affinity if considered as monovalent interactions; it is the multivalent interaction of hyaluronan with several receptors that contributes to the high avidity binding on chondrocytes and other cells. The role of CD44 in lymphocyte homing to high endothelial venules of lymph nodes (as characterized by the Hermes antibodies) does not involve hyaluronan, but the identity of the binding partner for lymphocyte CD44 in lymph nodes has not yet been resolved.

Additional References

[170] Li XQ, Thonar EJMA, Knudson W (1989) Accumulation of hyaluronate in human lung carcinoma as measured by a new hyaluronate ELISA. Connect. Tissue Res. **19**: 243-253.

[171] Pita JC, Muller FJ, Manicourt DH, Buckwalter JA, Ratcliffe A (1992) Early matrix changes in experimental osteoarthritis and joint disuse atrophy. In: Articular Cartilage and Osteoarthritis. Kuettner KE, Schleyerbach R, Peyron JG, Hascall VC (eds.). Raven Press, NY. pp. 455-470.