CHONDROCYTE ADHESION TO RGD-BONDED ALGINATE: EFFECT ON MECHANOTRANSDUCTION AND MATRIX METABOLISM

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ABSTRACT

The mechanism of mechanotransduction in chondrocyte matrix metabolism is not well understood, in part because of the density of cartilage and in part because of limitations in *in vitro* culture systems. Using alginate covalently modified to include the integrin adhesion ligand R-G-D (arginine-glycine-aspartate) represents a unique approach to studying mechanotransduction in that it allows for exploration of the role of integrin adhesion in mediating changes to chondrocyte behavior.

The hypothesis of this research was that chondrocytes will form a cytoskeletal adhesion to RGD-alginate mediated integrins, that this attachment will enable chondrocyte sensation of mechanical signals, and this signaling will alter chondrocyte matrix metabolism. The first aim of this research was to characterize chondrocyte attachment to RGD-alginate, and assess the role of substrate mechanics on chondrocyte attachment kinetics and morphology. Secondly, the effect of chondrocyte attachment to RGD-alginate in 3D culture on matrix biosynthesis was assessed, as were changes in substrate mechanics. Finally, this research aimed to determine the metabolic response of chondrocytes to changes in intrinsic and extrinsic mechanics.

It was found that the RGD ligand functionalized the alginate scaffold, enabling chondrocytes to sense the mechanical environment. Attachment kinetics, morphology, and proteoglycan metabolism were found to adapt to hydrogel matrix stiffness when an integrin adhesion was present. Externally applied compression was transmitted through this integrin attachment, causing changes in proteoglycan synthesis. Components of media serum were found to modulate the effects of integrin mechanotransduction.

These results were obtained by analyzing a novel approach with established techniques, such as the DMB dye assay for sulfated GAG content. The conclusions conform to diverse data from cartilage explant loading and monolayer culture studies, yet were accomplished using one versatile system in a straightforward manner. The potential of this system extends further, into identification of intracellular signaling pathways and extracellular modulation of matrix components. Seeded RGD-alginate is well suited for studying consequences of integrin attachment.

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INTRODUCTION

Section I – Overview of Cartilage and Osteoarthritis

Articular cartilage is an unusual tissue in that it receives no blood supply, no innervation, and has no lymphatic drainage. Such features are apparently not necessary in fulfilling the primary physiological functions of articular cartilage: to distribute and bear loads, and to lubricate joint movement. These functions are fulfilled by the mechanical properties of the cartilage extracellular matrix (ECM), which is a complex network of collagen, proteoglycan, and other molecules.

The ECM makes up 90% of the dry weight of articular cartilage [2]. More than half of that ECM dry weight is made up of proteoglycans (PG) [1]. Proteoglycans like aggrecan are composed of large core protein, and covalently bound, sulfated glycosaminoglycan (GAG) chains. In cartilage, a typical large PG has 100 long chondroitin sulfate chains and 130 much shorter keratin sulfate chains, and accounts for approximately 90% of the molecular weight of the PG [1].

When hydrated, the sulfated GAG chains exhibit electrostatic repulsion and swell. In cartilage, the swelling is checked by a collagen fibril network, which restricts PG to as little as 20% of their potential size [1]. The collagen, which is the second major constituent of the cartilage ECM, is primarily type II collagen. The type II collagen fibrils

feature fibromodulin, decorin and by type IX collagen bound at specific intervals along the fiber, aiding in cross-linking and forming a network [3, 4]. The hydrated proteoglycans account for the ability of articular cartilage to resist compressive loads. The collagen mesh, in turn, allows the tissue to resist tension and shear forces [2].

Chondrocytes maintain the mechanical properties of articular cartilage by adjusting the synthesis and degradation rates of matrix components. Though collagen turnover is very slow (the half life is approximately 100 years), PG turnover is faster and regulatable [1]. The GAG content and chain length is variable [3], and the activity of degradative matrix metalloproteinases (MMP) and aggrecanases is variable as well [5].

Chondrocytes regulate matrix metabolism by responding to a variety of signals. Besides synovial cytokines and growth factors, a major source of chondrocyte signals is the extracellular matrix itself [6, 7]. The ECM interacts with chondrocytes through a variety of receptors to modulate chondrocyte metabolism, phenotype, and response to mechanical load [8].

Proteoglycans noncovalently bind, via link proteins, to hyaluronic acid (HA) chains – approximately fifty PGs per HA molecule in articular cartilage. Chondrocytes do not bind directly to aggrecan and PG core proteins, but via the CD44 receptor to HA [3]. Chondrocytes bind to collagen fibrils via anchorin II and integrins [9, 10]. Chondrocytes also adhere to other matrix molecules, such as fibronectin, vitronectin and tenascin, via integrins [4] [8]. Integrins have been implicated as mechanotranductive elements in a variety of tissue types, such as skeletal muscle and arterial walls [11, 12]. Thus it is

possible chondrocytes are regulating cartilage matrix metabolism from mechanical signals originating or transmitted through the ECM.

Understanding how these cells process and respond to specific, individual ECM signals would be useful, given how cartilage matrix tends to deteriorate. The characteristics of matrix proteoglycans changes with advancing age, exhibiting decreasing core protein size and shortening GAG chains [13]. Mitotic and synthetic activity of chondrocytes decrease, as does response to the anabolic growth factor, IGF-1 [14]. This leads to the deterioration of mechanical properties. These age-related changes predispose cartilage to developing osteoarthritis.

Osteoarthritis (OA) is the second leading cause of long-term disability in the US [15-17]. Half of all people 65 and over have at least some features of OA in some joints, and by age 75 features of the disease are almost universally prevalent. The incidence of OA correlates with increasing age, increasing weight, joint trauma and repetitive mechanical loading [4]. Animals studied with de-stabilized or impact-loaded joints also develop features of OA, further suggesting that the pathogenesis of OA is abnormal loading [18].

Osteoarthritis is not simply senescent cartilage. OA is a degenerative disease that leads to fissures in the cartilage surface, ulcerations and eventual loss of tissue. Matrix metalloproteinases, including collagenases and aggrecanases, exhibit increased activity in OA and catabolize matrix components. Synthesis of new matrix components does not keep pace with degradation, and the new components have decreased functionality [4, 19, 20].

Cells isolated from OA cartilage feature abnormal cell integrin expression and irregular processing of extracellular matrix signals [21, 22]; others have suggested that these differences may even be the underlying cause of osteoarthritis [23]. However, studying the pathogenesis of OA in native cartilage has proven difficult. The density of the ECM and scarcity of cells has prohibited physiologic analysis of signaling between chondrocytes and their matrix.

Section II – Overview of Chondrocyte Mechanotransduction

Part A: Cartilage Explant Studies

Due to these constraints, many studies have focused on the process of explanted cartilage metabolism under various compression regimes. It has long been known, for instance, that static compression of cartilage explants reduces PG synthesis [24]. The decrease in GAG synthesis was found to be strain-dependent, as was collagen synthesis. Dynamic compression of explants between 1-5% strain at frequencies between 0.1 Hz and 1 Hz was found to increase GAG and collagen synthesis [25]. Matrix synthesis rates also have been shown to be regulated by compression-dependent properties such as hydraulic permeability, pH, osmolarity, and charge density. [5, 26, 27]

These estimations of PG and collagen synthesis under loading regimes were based on uptake of [^{35}S] SO_4 and [^{3}H] proline, respectively. Radiolabeled sulfates are incorporated into chondroitin- and keratin-sulfate as the glycosaminoglycans are added to PG core proteins in the Golgi [28]. The radiolabeled PG or collagen remains in the

cartilage matrix or is degraded by MMP and lost to the media. Thus, radiolabel incorporation methods can indicate comparative levels of net matrix molecule synthesis at a given timepoint.

These investigations focused on how changes to the biophysical environment affect chondrocyte metabolism, and not how the interaction between cell and matrix regulates behavior. The mechanism responsible for sensing and reacting to mechanical signals in chondrocytes is likely to involve integrins [4, 29, 30]. Integrins, which are membrane-spanning dimers common to all tissue types, have been implicated in a variety of mechanotransduction processes, such as those in cardiac and smooth muscle [31] [32] [33]. The "extrinsic" forces encountered by tissues are transmitted to the cells through integrins, which trigger an array of signaling events [34]. Such extrinsic signaling influences cell morphology, division, and protein synthesis [34] [35, 36].

More recently, focal adhesions (complexes which include clusters of integrins) have been implicated in the durotaxis phenomenon, in which fibroblasts migrate to stiffer substrates [37],[38]. On these stiff substrates, fibroblasts were seen to exhibit more focal adhesions in a linear arrangement [39]. Because integrins are linked to the actin cytoskeleton, tension can be generated by the cell against integrin attachment [40]. Using this tension, cells can probe their mechanical environment, even in the absence of explicit, externally applied stimulation. This "intrinsic" substrate stimulation may provide important cues to cells in conditions where the matrix stiffness changes, such as in embryology, lung disease, or osteoarthritis.

The interest in integrin signaling has led to examinations of integrin expression in cartilage. Investigators have found variability in integrin expression based on cartilage layer, the age of the specimen, the presence of OA, and in chondrosarcoma cells. *In situ* cross-sections of human knee and femoral articular cartilage stained with monoclonal antibodies revealed α1, α5, αV, β1, β4 and β5. Chondrocytes in osteoarthritic cartilage, in addition, express the α2, α4, and β2 subunits [41] [22].

Further work has identified which integrins are responsible for chondrocyte adhesion to cartilage. Using trypsinized chondrocytes from monolayer culture, attachment assays have shown that integrins are necessary and sufficient for chondrocyte adhesion to cartilage [42] and the integrins subunits involved are αV, α5, β1 and β5 [43].

Though some progress has occurred, studies using cartilage explants cannot by themselves confirm the role of integrins in mechanotransduction. Explant studies can look at sulfate incorporation or total GAG content under a variety of conditions, but receptor antagonists cannot be used in explants; the matrix is too dense. The cells are too few, and extraction takes too long, to obtain mRNA levels, promoter activity, or phosphorylation states. Furthermore, the material properties of cartilage cannot be changed without profoundly affecting the mechanotransductive pathway. Thus, identification of the mechanotransductive pathway requires other culture systems.

Section II – Overview of Chondrocyte Mechanotransduction

Part B: Monolayer Culture of Chondrocytes

In an attempt to identify mechanotransductive elements, chondrocyte culture in monolayer has obvious advantages and drawbacks. The advantage is established agents and methods can be employed to identify elements of the mechanotransduction pathway. There are several disadvantages, however. Stripped of ECM and cultured at high density, chondrocytes do not behave as they do *in vivo.* In addition to biological concerns, cells cannot be stimulated as they are *in vivo* – chondrocytes do not physically deform under hydrostatic pressure as they do under compression, for instance [6, 44, 45]. Other techniques to mechanically stimulate monolayer culture, such as stretching chondrocytes on a flexible membrane, are also nonphysiological [21, 46].

In vitro chondrocyte culture in monolayer has been shown to decrease gene expression and production of cartilage-specific proteins such as type II collagen and aggrecan, and within days "de-differentiate" to a more fibroblastic phenotype [47-50]. The integrin profile associated with cartilage has also been shown to change in monolayer culture [7, 51].

Furthermore, the effects of mechanical loads perceived by chondrocytes in monolayer can differ substantially from those transmitted through the ECM. For instance, cyclical mechanical stretching of chondrocyte monolayer culture has been found to activate hyperpolarize chondrocytes via $\alpha 5\beta 1$ signaling [52]. This hyperpolarization, triggered by K^+ channels, is further dependent on IL-4 release and paracrine stimulation

[53], [54]. Though intriguing, there is no indication that chondrocyte membrane hyperpolarization or IL-4 signaling play a role in cartilage matrix metabolism *in vivo*.

Similarly, a study involving supraphysiological fluid flow over chondrocytes in monolayer was found to trigger a 3-fold increase in ERK1/2 phosphorylation and increase aggrecan promoter activity by 40% [55]. The involvement of MAP kinases such as ERK1/2 or p38 is a common feature of integrin mechanotransduction in many tissue types [29, 56, 57], but it is not clear whether the same signal transduction observed in fluid flow over a monolayer occurs in compressed cartilage of the synovial joint.

Still, there has been useful information learned from monolayer culture of chondrocytes. Attachment assays have demonstrated the involvement of integrins or RGD-binding receptors for chondrocytes to adhere to surfaces coated with collagen, fibronectin, or other molecules [58], [59], [60]. The modulation of chondrocyte attachment by TGF-beta or other cytokines may illustrate important phenomena in cartilage [61].

Increases in $\alpha 1$ integrin expression on monolayer chondrocytes with a spread, fibroblastic appearance, compared to round or oval cells, has been noted [62]. This $\alpha 1$ expression increases with duration in culture [51] and may correlate with the behavior of osteoarthritic chondrocytes, which also can be fibroblastic in appearance [63].

Section II – Overview of Chondrocyte Mechanotransduction

Part C: Hydrogel Culture of Chondrocytes

To better preserve the cartilage phenotype, investigators have cultured growing the chondrocytes in media suspensions or gels [64, 65]. When cultured in agarose gels, for instance, chondrocytes exist in the spherical conformation seen *in vivo*. The differentiated cartilage phenotype is maintained, and cells produce more proteoglycans and type II collagen compared to chondrocytes spread in monolayer [65, 66].

Like agarose, alginate has been shown to keep chondrocytes in a spherical conformation, allowing expression of the cartilage-specific proteins [50, 67, 68]. Alginate is an anionic block copolymer consisting of α1,4-linked D-mannuronic and L-guluronic acid residues. Liquid alginate forms a gel in the presence of divalent cations [69, 70]. Unlike agarose, alginate gelation is largely temperature independent [71], so cells can be introduced to and extracted (with cation chelators like citrate or EDTA) from the gel with ease. Alginate makes small-scale and large-scale culture applications are more feasible, and simplifies the process of harvesting cells for biochemical and molecular biology assays.

Another attractive feature of alginate is that the polysaccharide gelling can be controlled, and reversed, by varying the availability of divalent cations like Ca^{2+} [70]. Stiffening the alginate can be accomplished by multiple mechanisms, including raising the cation concentration used for gelation, or using cations that bind to alginate to a

greater degree. Other methods that alter hydrogel mechanics, such as shortening the polymer chain with heat or radiation, are possible with alginate as well [72].

Scaffold mechanics can be adjusted in hydrogels like polyethylene glycol (PEG), which has also been used in the culture of chondrocytes [73-75]. Like alginate, PEG characteristics like stiffness and permeability can be altered using different crosslinking techniques; these methods have been shown to affect chondrocyte metabolism and behavior [74, 75]. Unlike alginate, however, PEG crosslinking agents are potentially cytotoxic, and handling the PEG hydrogel is not as straightforward [76].

Chondrocytes cultured for extended durations in hydrogels produce a matrix with similar properties to cartilage. Using articular chondrocytes from several species grown in alginate beads based on Guo's technique, investigators consistently find the two matrix compartments – a pericellular / territorial matrix and a "further-removed matrix" – seen *in vivo* [77-79]. After thirty days, human articular chondrocytes in alginate cultured *in vitro* show cell densities and matrix volumes per cell statistically identical to human cartilage [78]. After thirty weeks, bovine articular chondrocytes cultured in alginate molds implanted within nude mice had an elastic modulus equal to 60% of that of human nasal cartilage [80].

Integrin expression can be easily studied in hydrogel culture, as well [51]. Thus, as a method for maintaining a differentiated phenotype and generating cartilage *in vitro*, chondrocyte culture in alginate has demonstrated some success. One drawback to the hydrogel, however, is that cells suspended within the alginate polymer do not directly adhere to the matrix as they would *in vivo.* In articular cartilage, chondrocytes attach to

the ECM signals through many receptors, including the HA receptor CD44 [81], the collagen II receptor annexin V [10], and the aforementioned integrins (receptors for collagen, fibronectin, and other ECM molecules, [7].

In alginate, however, mammalian cells do not specifically interact with the polysaccharide and, due to the hydrophobic nature of alginate, secreted proteins are not readily adsorbed [82]. Chondrocytes in agarose, too, do not immediately adhere to the polymer scaffold. As a consequence, chondrocyte sulfate incorporation rates do not change in response to static compression for several days after seeding [83]. After several weeks, however, chondrocytes in alginate decrease radiolabeled sulfate uptake by 20% in response to 50% static compression, with similar results for collagen synthesis [83]. These findings are similar to results with chondrocyte synthesis of cartilage oligomeric matrix protein (COMP) in alginate, which is also modulated by compression (in this case, dynamic compression) [84].

In each case, the presence of a fully developed pericellular matrix, synthesized by the chondrocytes, was shown to be a prerequisite for mechanical modulation of matrix synthesis. In the period immediately after seeding in the hydrogel, the chondrocytes have no matrix attachment and thus cannot respond to mechanical signals. Yet once the chondrocytes secrete enough matrix molecules to become mechanosensitive, the ability to study mechanotransduction is impaired, for the reasons discussed earlier.

Section III – Modifying Alginate to Facilitate Chondrocyte Adhesion

Thus, there has been motivation to modify an alginate to include a matrix attachment ligand for chondrocytes. With this modified alginate, seeded chondrocytes would attach to the hydrogel matrix immediately, instead of synthesizing and adhering to their own ECM molecules. An engineered attachment to the alginate matrix would offer many advantages for investigations into chondrocyte behavior, besides the advantages of alginate over explants, monolayer and other hydrogels. The chondrocyte synthetic response to ligand species and density could be assessed by changing the ligand molecule and concentration. The ligands could be blocked by adding antibodies to the ligand receptor or soluble ligand analogues.

Most importantly, by simplifying chondrocyte matrix attachment to a single type of molecule, before chondrocytes make their own matrix, ligand-modified alginate would allow for the study of controlled matrix perturbations on chondrocyte metabolism. In theory, the chondrocyte seeded in RGD-alginate has only one mechanotransductive link to the environment – the integrin attachment.

The ligand that was chosen for addition to alginate was RGD, a tripeptide identified in integrin adhesion to molecules including fibronectin, vitronectin, osteopontin, collagen, fibrinogen, von Willebrand factor, and thrombospondin [85]. The hydrophilic region RGDSP (arginine-glycine-aspartate-serine-proline), believed to

exposed in a beta-turn on fibronectin, was discovered to be sufficient, in itself, for stimulating cell attachment [86, 87].

Previous studies along these lines led to the realization that the amino acid immediately following aspartate was responsible for some of the molecular specificity of attachment, and that cyclic RGD could inhibit cell adhesion to collagen [88]. It is now known RGD peptides encourage a variety of molecular attachments based on the arrangement of the peptides themselves, flanking sequences, and other secondary factors. Some integrins that recognize the RGD sequence include $\alpha 3\beta 1$ (collagen type II and fibronectin), $\alpha 5\beta 1$ (fibronectin), $\alpha v\beta 3$ [89]. Chondrocytes are known to express a variety of RGD-binding ligands, including $\alpha 3\beta 1$ and $\alpha 5\beta 1$ [90-92].

With appropriate flanking sequences, the RGD peptide has since been used in a variety of adhesion assays [93], [94] and is employed as a coating to enhance cell interactions with proposed clinical wound repair devices [95, 96].

RGD was first bonded to polyethylene glycol [97]. Covalently bonding the GGGGRGDY peptide to alginate was first performed at the University of Michigan [82]. The process of bonding RGD to alginate was optimized and the efficiency of the reaction was found to be 60%. Murine C2C12 skeletal myoblasts from existing cultures were found begin attaching and spreading on RGD-alginate within 4 hours, but not on control surfaces.

Predicting which chondrocyte integrins will mediation adhesion to RGD-alginate is nontrivial, since integrin expression varies with different layers of cartilage [41], different disease states [22], and different culture conditions [7, 62]. The potential is