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

A dissertation presented

by

Nicholas Genes

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

August 11, 2003

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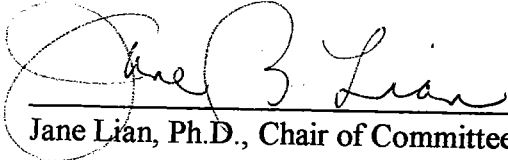
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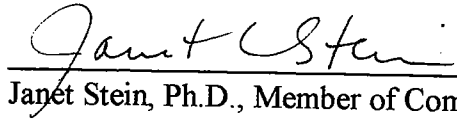
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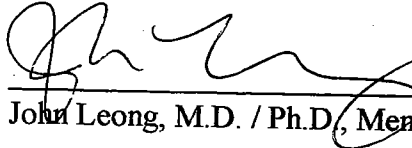
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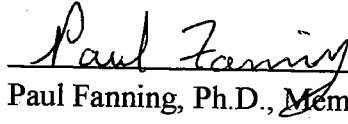
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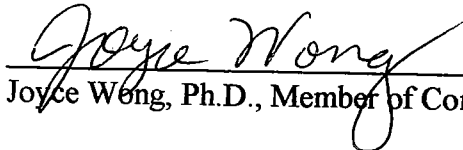
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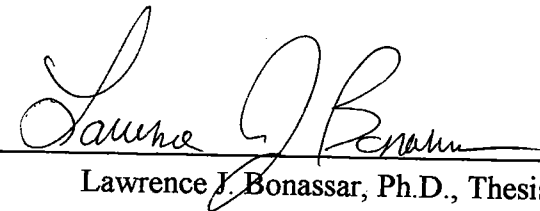

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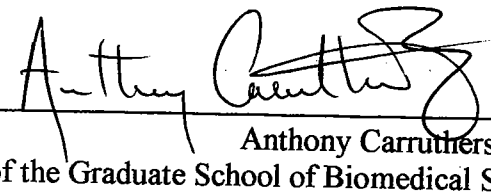

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ABSTRACT

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 mechanotransductive 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 [³⁵S] SO₄ and [³H] 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 $\alpha 1$, $\alpha 5$, αV , $\beta 1$, $\beta 4$ and $\beta 5$. Chondrocytes in osteoarthritic cartilage, in addition, express the $\alpha 2$, $\alpha 4$, and $\beta 2$ 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 , $\alpha 5$, $\beta 1$ and $\beta 5$ [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 α 1,4-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 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

present, under certain circumstances, for chondrocytes to express integrin subunits which can serve as receptors for fibronectin ($\alpha 5\beta 1$), types II and VI collagen ($\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 10\beta 1$), laminin ($\alpha 6\beta 1$), and vitronectin and osteopontin ($\alpha V\beta 3$) [7].

The experiments described in this dissertation coupled established techniques for measuring the chondrocyte response to mechanics, with a hydrogel culture system that promotes cell-matrix interaction. These two tools provided a novel way to study how attachment to a matrix regulates chondrocyte behavior.

Section IV – Summary of Hypothesis & Aims

It is the hypothesis of this research that the RGD-modification of alginate will enable chondrocyte attachment, and this attachment will facilitate sensation of intrinsic mechanics and externally applied compression, via integrins and the cytoskeleton. This signaling will in turn 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. Attachment was characterized by the independent blocking of cell adhesion by soluble RGD, anti-integrin mAb, and cytochalasin. Mechanics were modulated by a novel method of crosslinking a small volume of alginate in the bottom of wells, and a method for varying alginate stiffness by changing crosslink density or species (see figure 1).

The second aim was to examine how chondrocyte attachment to RGD-alginate in three-dimensional culture modulates biosynthesis of ECM molecules. Cell attachment was confirmed with the observation of stiffening of RGD-alginate discs in a cell-dependent and time-dependent manner, and chondrocyte metabolism was observed to decrease with integrin binding site occupancy (figure 2).

The final aim of this research was to determine the metabolic response of chondrocytes sensitized to matrix mechanics. Chondrocytes bound to RGD-alginate adjusted their metabolism with the internal mechanical environment of the hydrogel (figure 2). Static compression affected chondrocyte matrix content to a greater extent in the presence of integrin attachment (figure 3).

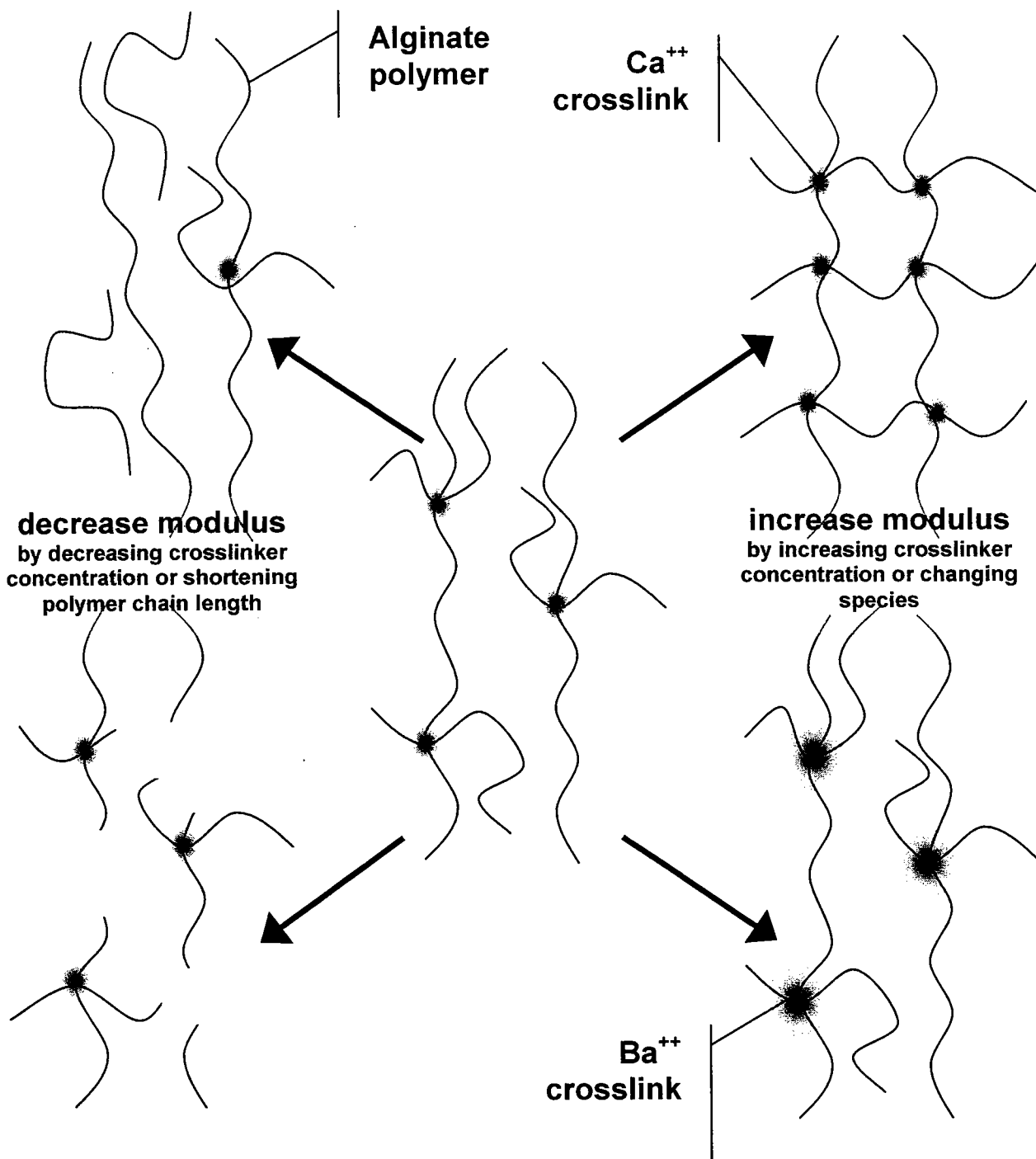


Figure 1. Two methods of changing alginate gel mechanical properties: by changing crosslinker characteristics (which can increase or decrease alginate stiffness) or degradation of the alginate polymer (which can decrease stiffness).

Unmodified
Alginate

RGD-
Alginate

integrin

cell

A

B

mAb

sol. RGD

C

D

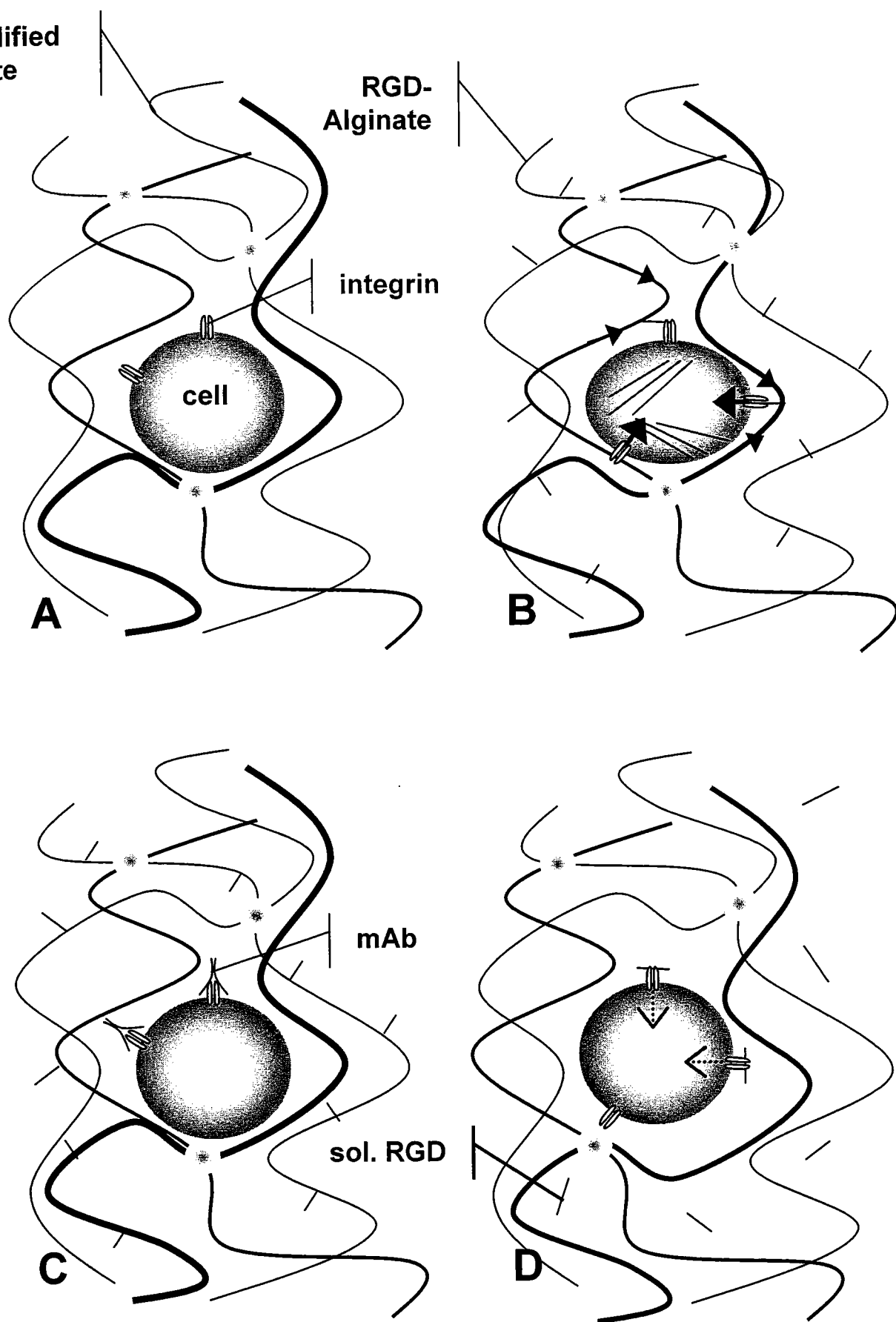


Figure 2. Integrin signaling in alginate and RGD-alginate. **A:** Chondrocytes in unmodified alginate are unbound and unresponsive to integrin signals. **B:** Chondrocyte integrin attachment in RGD-alginate creates metabolic responsiveness to mechanical signals. **C:** Anti-integrin antibodies block antagonize all integrin-mediated signaling. **D:** Soluble RGD peptide agonist abolishes mechanical responsiveness, but activates some integrin signaling.

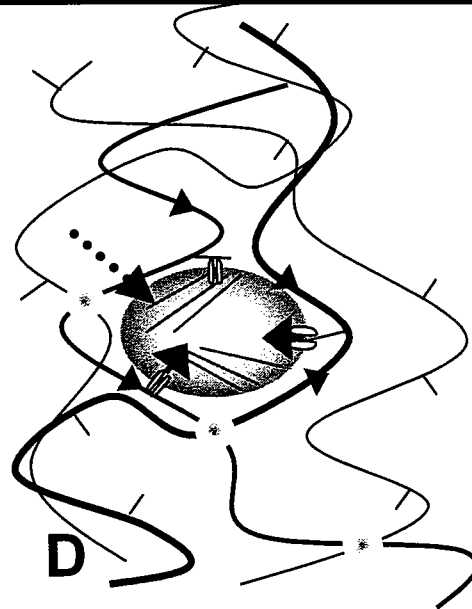
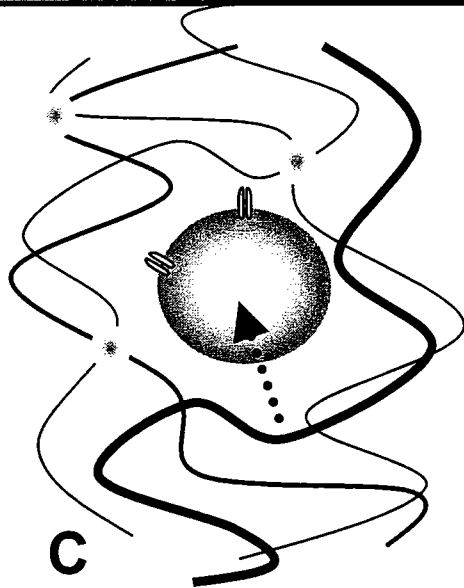
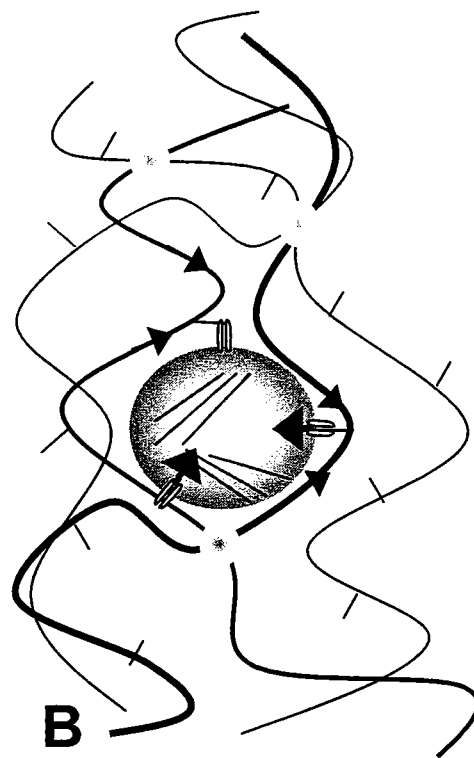
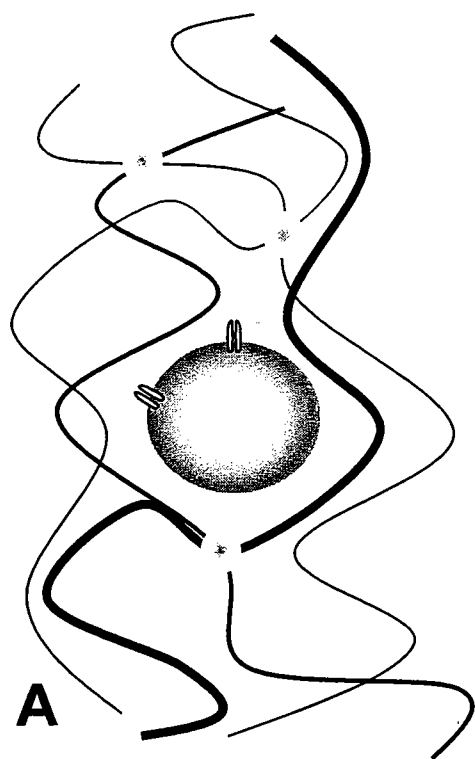


Figure 3. Compression affects attached cells differently than unattached cells. **A:** Chondrocyte in uncompressed, unmodified alginate. **B:** Chondrocyte in uncompressed RGD-alginate. **C:** Chondrocytes in unmodified alginate under compression experience changes in local charge density, and possibly pH and other variables. These non-adhesion-dependent changes can still affect chondrocyte metabolism. **D:** Chondrocytes attached to RGD-alginate will experience the changes noted compressed unmodified cartilage. Furthermore, chondrocytes attached to a scaffold or matrix will experience perturbations secondary to this attachment, either through direct deformation at the linkage sites, or signaling via integrin activation.

CHAPTER I

EFFECT OF SUBSTRATE MECHANICS ON CHONDROCYTE ADHESION TO MODIFIED ALGINATE SURFACES

Abstract

This study characterized the attachment of chondrocytes to RGD-functionalized alginate by examining the effect of substrate stiffness on cell attachment and morphology. Bovine chondrocytes were added to wells coated with 2% alginate or RGD-alginate. The alginate was crosslinked with divalent cations ranging from 1.25-62.5 mmol / g alginate. Attachment to RGD-alginate was 10-20 times higher than attachment to unmodified alginate, and was significantly inhibited by antibodies to integrin subunits α_3 and β_1 , cytochalasin-D, and soluble RGD peptide. The equilibrium level and rate of attachment increased with crosslink density and substrate stiffness. Substrate stiffness also regulated chondrocyte morphology, which changed from a rounded shape with nebulous actin on weaker substrates, to a predominantly flat morphology with actin stress fibers on stiffer substrates. The dependence of attachment on integrins and substrate stiffness suggests that chondrocyte integrins may play a role in sensing the mechanical properties of the matrices to which they are attached.

Introduction

Though articular cartilage is avascular, alymphatic and aneural, the chondrocytes within are nonetheless capable of responding to environment cues. This ability arises largely from the cartilage extracellular matrix (ECM), an abundant network of collagen, proteoglycan, and other molecules. The ECM interacts with chondrocytes through a variety of receptors to modulate chondrocyte metabolism, phenotype, and response to mechanical load [8]. Cartilage responds to compressive loads by adjusting biosynthetic behavior [24, 25], but the mechanism of chondrocyte mechanotransduction is not well-characterized.

Understanding how chondrocytes process and respond to specific, individual ECM signals would provide insight into the pathogenesis of diseases like osteoarthritis, which is known to be precipitated by mechanical factors [98, 99]. Chondrocytes from osteoarthritic cartilage have been shown to feature abnormal cell integrin expression and irregular processing of extracellular matrix signals [22, 100]. The degradation of cartilage ECM seen in osteoarthritis may arise from these aberrant mechanotransduction, during or following abnormal loading conditions [23].

Though worthwhile, isolating and examining mechanotransduction in chondrocytes has proven more difficult than in fibroblasts and other cell types. *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

quickly dedifferentiate to a more fibroblastic phenotype [47-50]. The integrin profile associated with cartilage has also been shown to change in monolayer culture [7, 51, 89, 101].

To better preserve the cartilage phenotype, chondrocytes have been cultured by growing the cells in media suspensions or gels [64, 65]. When cultured in alginate gels, chondrocytes maintained their differentiated phenotype and produced more proteoglycans and type II collagen compared to chondrocytes in monolayer [50, 67, 68]. Alginate is an anionic block copolymer consisting of α 1,4-linked D-mannuronic and L-guluronic acid residues. The extent to which the polysaccharide will gel can be controlled, and reversed, by varying the availability of divalent cations such as Ca^{2+} [70].

One drawback to hydrogels, however, is that cells suspended within the polymer do not directly adhere to the matrix as they would *in vivo*. In articular cartilage, chondrocytes receive ECM signals through many receptors, including the hyaluronic acid receptor CD44 [81], annexin V [10], and integrins [7, 42]. With alginate, however, there is no specific interaction between mammalian cells and the polysaccharide. Furthermore, alginate carries a negative charge balance, such that proteins are not readily adsorbed, due to electrostatic repulsion. [82].

A modified alginate designed to include the integrin receptor ligand RGD has been shown to facilitate attachment of myoblasts [102] presumably through integrins. Integrin attachment has been implicated in transduction of mechanical signals in many cell types, including chondrocytes [29, 53, 103]. It has been shown that these external

mechanical signals are transmitted by integrins to the cytoskeleton and cause changes in cell locomotion [31] and morphology [104].

Because RGD-alginate facilitates integrin adhesion and integrins are putative mechanotransducers, it was hypothesized that RGD-functionalization (modification) would enable chondrocytes to attach to alginate via integrins and thus sense their mechanical environment. Specifically, the objective of this study was to determine the extent to which the mechanical properties of RGD-modified substrates regulated chondrocyte attachment and morphology.

Methods

Chondrocyte Cell Isolation.

Articular cartilage from gleno-humeral and humero-ulnar joints of 1-2 week old calves (Research 87, Boston, MA) was incubated for 12 hours in a solution of 0.3% collagenase Type 2 (Worthington, Lakewood, NJ) in F-12 media, 100 U / ml penicillin, 100 μg / ml streptomycin, and 250 ng / ml amphotericin B (GIBCO, Grand Island, NY) at 37°C. The digested cartilage solution was filtered and resuspended in F-12 / 10% FBS media (GIBCO) and cells were plated in T-175 flasks at 1.4×10^3 cells / cm^2 .

Chondrocytes were maintained in culture for 7-10 days, with media changed every 2-3 days. Near confluence, the cells were rinsed with PBS and incubated for approximately 20 minutes in F-12 media containing 0.05% trypsin, then resuspended in serum-free F-12 media with 1% antibiotics / antimycotics for use in adhesion studies.

Surface Preparation

Using carbodiimide chemistry, the peptide sequence GGGGRRGDY was covalently bonded to medium viscosity alginate (FMC Biopolymer, Drammen, Norway) as previously described [82]. This method for the engraftment of 10.0 mg of peptide per gram of alginate producing a ligand spacing of 23 nm. This density is well in excess of previously reported minimum requirements for focal adhesion complex formation, actin stress fiber formation, and cell spreading [105]. The RGD-alginate was suspended in PBS at 2% (w/v) and sterilized with 0.45 μm filters. Aliquots of 200 μl were pipetted into the wells of a 24-well plate and spread uniformly. Volumes of 1M CaCl_2 or 1M BaCl_2 (Sigma, St. Louis, MO) were added to the surface to crosslink alginate, to a range of concentration of 1.25-62.5 mmoles crosslinker per gram of alginate. After one minute, the crosslinker was removed and the surfaces were rinsed twice with 2 ml of F-12 media before a 1 ml solution of F-12 media containing 50,000 chondrocytes was added to each well.

Adhesion Studies

At multiple timepoints, ten 200X phase contrast images were obtained using a Nikon TE220 microscope, a Spot Jr. CCD camera (MVI, Milford, MA), and NIH Image software. The number of adhered (spread) and non-adhered (rounded) cells was calculated separately for each field and averaged at each time point. The characterization of chondrocyte attachment was based on an experiment in which osteoblasts were plated onto a quartz surface affixed with the peptide CGGNGEPRGDTYRAY [106]. The cell

counting assay was based on approaches described in the literature [104, 107] using morphology as determined by phase-contrast microscopy.

Attachment data was found to follow first-order binding kinetics when fit to the following equation:

$$\% \text{ Attached} = A(1 - e^{-t/\tau}) \quad (1)$$

where A is the equilibrium attachment level, t is time, and τ is the characteristic time constant of attachment.

In parallel studies, attachment of chondrocytes to control and RGD-alginate surfaces crosslinked with 12.5 mmol CaCl₂ was evaluated in the presence of potential adhesion inhibition factors. Cells were suspended in F-12 with 0.1% BSA and 5 μ g / ml blocking antibody for integrin subunits β_1 and $\alpha_1, \alpha_2, \alpha_3, \alpha_4, \alpha_5,$ and α_v (Chemicon, Temeculah, CA); or 160 μ M linear GRGESP or linear GRGDSP peptides (GIBCO).

Further attachment studies were conducted in the presence of varying amounts of soluble RGD peptides. Cell adhesion in these studies was quantified as described above.

Mechanical Analysis

A 6 mm disc cut from the center of each surface was placed in uniaxial confined compression between an impermeable polymethacrylate surface and a porous polyethylene platen in a Dynastat mechanical spectrometer (IMASS, Hingham, MA). A series of ten steps in displacement, up to a maximum of approximately 20% total strain, were imposed on the samples and resultant forces were measured at a rate of 2.5 Hz for 30 sec / relaxation. Displacements and loads were normalized to sample geometry to

yield values for strains and stresses. Stress relaxation curves were fit to a poroelastic model of hydrogel behavior to determine a compressive modulus at mechanical equilibrium. [108]

Morphology Studies

Cell and cytoskeletal morphology was assessed using rhodamine-conjugated phalloidin to visualize filamentous actin. Cells on alginate surfaces were washed twice in 25 mM HEPES buffer (Sigma) supplemented with 100 mM CaCl₂ and 100 mM NaCl, before fixing with 4% paraformaldehyde and permeabilizing with 0.1% triton. Following two more rinses, the cells were exposed to rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) for 20 minutes. The alginate surfaces were placed on coverslips and cells were visualized at 400X upon excitation using a Nikon TE200 microscope with an Chroma epifluorescence attachment equipped with a Spot Jr. digital camera (MVI, Milford MA). The morphology of the chondrocytes was classified according to an established system [104]. In this system, cells are categorized as nearly spherical, rounded with extensions, or flattened with stress fibers (Types I – IV, respectively).

Statistics

The significance of the counting assays results was determined by one-way ANOVA using Dunn's test as post-hoc analysis for pairwise comparison. The r-values of the empirical best-fit lines to equilibrium adhesion vs. modulus data and τ vs. modulus data were determined by root-mean-square calculation.

Results

Adhesion Studies

Chondrocytes attached and spread on RGD-alginate surfaces to a greater extent than on unmodified alginate, as visualized by phase contrast microscopy (figure 4). The equilibrium level of attachment of chondrocytes on RGD-alginate surfaces crosslinked with 12.5 mmol Ca^{2+} per gram alginate was approximately 37%, and the time constant of attachment was about 10 hours (figure 5).

By fitting attachment levels under increasing amounts of blocking peptide to the first-order equations for receptor binding, a dissociation constant between the blocking peptide and the cell receptors of approximately 7.3 μM was determined (figure 6). Maximum inhibition of cell spreading was achieved at concentrations over 100 μM .

Subsequent studies showed that chondrocytes did not adhere to unmodified alginate, even after 48 hours (figure 7A). In contrast, chondrocytes on RGD-alginate demonstrated significantly more attachment. The addition of 160 μM linear RGD peptide significantly inhibited chondrocyte attachment, and the addition of RGE peptide did not block attachment. Cytochalasin D, a fungal toxin that destabilizes the actin cytoskeleton, also significantly inhibited chondrocytes spreading on RGD-alginate.

To further characterize adhesion, attachment studies were carried out in the presence of antibodies to integrin subunits α_1 , α_2 , α_3 , α_4 , α_5 , α_v , and β_1 (figure 7B).

Antibodies to the β_1 subunit blocked chondrocyte adhesion to RGD-alginate ($p < 0.001$), as did α_3 antibodies ($p < 0.05$). Attachment inhibition by β_1 was significant to $p < 0.001$, and for α -three a significance to $p < 0.05$ was obtained for the 48 hour timepoints. Combining α_1 , α_3 and α_5 antibodies failed to enhance the blockade of attachment (not shown).

Effect of Substrate Stiffness on Chondrocyte Adhesion

As calcium crosslinker increased, the fraction of attached chondrocytes also increased (figure 8). The same trend was seen with barium-crosslinked surfaces, but with more attached chondrocytes at each crosslinker concentration. Upon direct measurement of stiffness by stress-relaxation testing, it was found that surfaces crosslinked with 2.5-62.5 mmol Ca^{2+} / g alginate had moduli ranging from 12.3 kPa \pm 6.2 to 127 kPa \pm 16.8 (mean \pm SEM). Surfaces crosslinked with 1.25-25 mmol Ba^{2+} had moduli of approximately 29.8 kPa \pm 14.9 to 147 kPa \pm 20. Equilibrium attachment increased with substrate stiffness (figure 9A) with maximum cell attachment level of approximately 40%, achieved on RGD substrates with moduli greater than 75 kPa. The increase in stiffness did not appreciably affect attachment to control surfaces (not shown). Similarly, the time constant τ was shown to decrease linearly with modulus (figure 9B); increasing the stiffness of the substrate decreased the characteristic time constant for attachment.

Attachment to stiffer substrates was also more rapid, with characteristic attachment times decreasing from 17.5 hours at a modulus of 16 kPa to 2.7 hours at 147 kPa.

Effect of Substrate Stiffness on Chondrocyte Morphology

A variety of cytoskeletal morphologies on the RGD-alginate substrates were seen upon rhodamine-phalloidin staining of actin filaments (figure 10A). Counting the populations of chondrocytes of each type revealed that the substrate stiffness affected not only the number of cells attached, but also the arrangement of the cytoskeleton (figure 10B). Cells on the less-stiff surfaces typically resembled those on control alginate, with approximately spherical cell shapes and nebulous, punctate actin (approximately 80%).

On the stiffest substrates, the predominant cell morphology (approximately 40%) was a large, flattened cell with stress fibers.

Discussion

This study has shown that RGD functionalization enables chondrocytes attachment to alginate through the participation of integrins, specifically α_3 and β_1 , and

the actin cytoskeleton. The dependence of integrins in mediating chondrocyte adhesion to RGD-alginate, coupled with the known role of integrin subunits in mechanotransduction, led to the hypothesis that substrate mechanics regulated chondrocyte attachment. By increasing the crosslinker density to modulate substrate stiffness, it was found that chondrocytes attached more rapidly and to a greater degree on stiffer substrates. The stiffer substrates directed the cells to a flattened, more spread morphology.

Previous studies have illustrated the role of integrins in transmitting mechanical signals to the chondrocytes [21, 53]. The results from the current study are novel in part because they expand the role of integrins to that of relaying additional environmental information to the chondrocytes. The mechanical state of the substrate was sensed by the chondrocytes in the absence of external signaling, which implies the stiffness of the matrix itself is a mechanical signal.

The dissociation constant derived from the blocking peptide dose-response curve (figure 6) is similar to the K_d for endothelial cell integrins on a fixed fibronectin substrate [109]. The fact that dissociation constants derived from these cell-spreading models are several times greater than K_d 's calculated by molecular interaction studies [110, 111] is likely due to assumptions present in the model. Cell spreading requires many integrin binding events, and the initiation of spreading may depend on a threshold number of bound integrins. This threshold would have the effect of skewing the K_d to greater concentrations.

The inhibition assays revealed the specificity of the chondrocyte interaction with RGD peptide, and the role of α_1 , α_3 , and β_1 integrins in mediating adhesion to RGD-

alginate. High levels of α_3 and α_5 subunit expression have been reported for chondrocytes *in vivo*, though *in vitro* reports also increased levels of integrin α_1 (α_2 and α_4 are not normally expressed by chondrocytes) thus it is not surprising they have no involvement in blocking adhesion in this system [7]. It is interesting to note that osteoarthritis can cause increased expression of α_1 and α_5 in chondrocytes, and possibly α_3 , though there is disagreement [7, 92]. Increased expression of these β_1 integrins, two of which cause spreading of chondrocytes on RGD-alginate, may also encourage the spreading and fibroblastic morphology seen in OA histology.

Upon confirming that adhesion was mediated by mechanotransductive integrins, the mechanics of the alginate were altered in an attempt to change chondrocyte behavior. The method chosen to stiffen the alginate by increasing the amount of crosslinker per gram of alginate, though other methods are being developed [112]. The possibility exists, however, that the calcium ions used to crosslink the alginate interfere with integrin adhesion. High calcium ion concentrations have been shown (at 5.5 mM) to inhibit $\alpha_2\beta_1$ adhesion [113, 114] and (at levels over 30 mM) inhibit $\alpha_v\beta_1$ adhesion [115]. Though an effort is made to remove excess calcium chloride solution after casting the alginate, it is likely that concentrations on the order of 5-20 mM exist after the media is added. Furthermore, high extracellular Ca^{2+} may modulate intracellular calcium ion levels, which has been shown to modulate integrin expression and attachment in several cell types [116-118].

To address this concern, alginate surfaces were crosslinked with Ba^{2+} . Barium ions have been shown to bind strongly to alginate [119] and do not appear to be involved

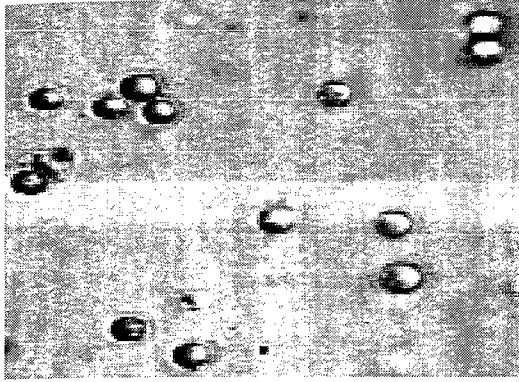
with integrin expression or cell adhesion. For comparison, the equilibrium attachment level for chondrocytes on RGD-alginate crosslinked with 5.0 mmol Ca^{2+} per gram of alginate (modulus = 21.1 kPa, A = 21.6%, figure 9A) was lower than that of cells on surfaces crosslinked with 62.5 mmol of Ca^{2+} per gram (modulus = 127 kPa, A = 37%). In contrast, cells on surfaces crosslinked with 5.0 mmol of Ba^{2+} per gram of alginate (modulus = 147 kPa, A = 37.9%) had equilibrium attachment levels similar to that of higher Ba^{2+} concentrations (modulus = 147 kPa, A = 40.0%). The increased attachment to Ba^{2+} crosslinked surfaces compared to surfaces crosslinked with equivalent amounts of Ca^{2+} also suggests that this phenomenon is not an artifact of charge density or osmotic effects.

Thus, the major factor influencing chondrocyte adhesion and morphology on RGD-alginate appears to be the substrate stiffness. Chondrocytes attached more rapidly, and to a greater degree, on the RGD-alginate substrates with higher modulus. Furthermore, the stiffer substrates directed the cells to a flattened, more spread morphology.

This finding is similar to the durotaxis phenomenon in which fibroblast preferentially migrate to stiffer substrates [120]. The results described in this study have implications for osteoarthritis, a disease in which matrix mechanics are altered and chondrocyte behavior is aberrant. In articular cartilage, the stiffness of the tissue, which is known to change with disease and age [121-123], could influence chondrocyte ECM

synthesis, which would in turn cause further changes in matrix mechanics. More broadly, the phenomenon of substrate signaling has implications in carcinogenesis, embryology, and other fields in which alterations in matrix mechanics coincide with changes in cell behavior and motility.

With these cell adhesion results established, it will be worthwhile to investigate the signaling molecules activated downstream of chondrocyte integrins bound to RGD-alginate, and draw comparisons to other mechanotransductive cell pathways. Also, the role of integrin attachment in mediating changes to chondrocyte behavior can be further explored by examining the response of chondrocyte biosynthesis in 3D RGD-alginate gels under compression.



Control alginate



RGD-alginate

Figure 4. Phase-contrast images at 200X of chondrocytes on unmodified RGD-bonded alginate.

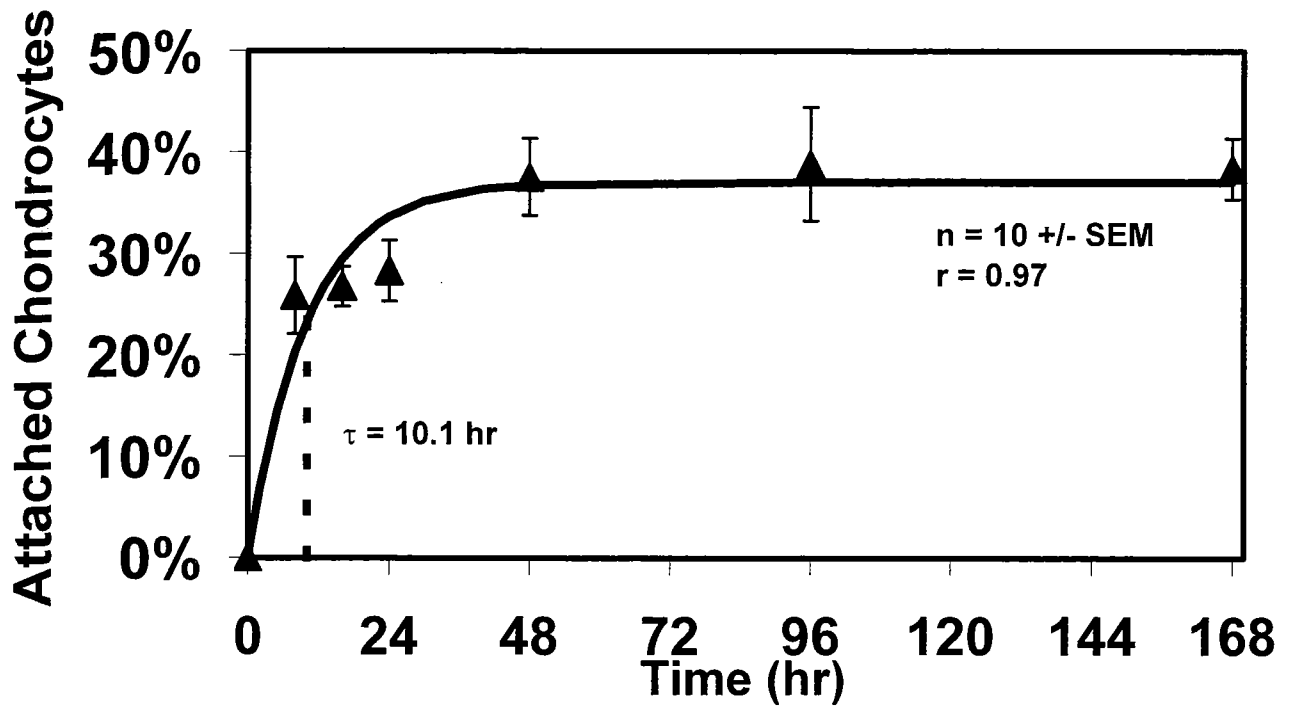


Figure 5. Attachment kinetics fit a first-order model. A surface crosslinked with 12.5 mmol CaCl₂ had a characteristic time constant of attachment of 10.1 hr, and an equilibrium attachment level of 37%.

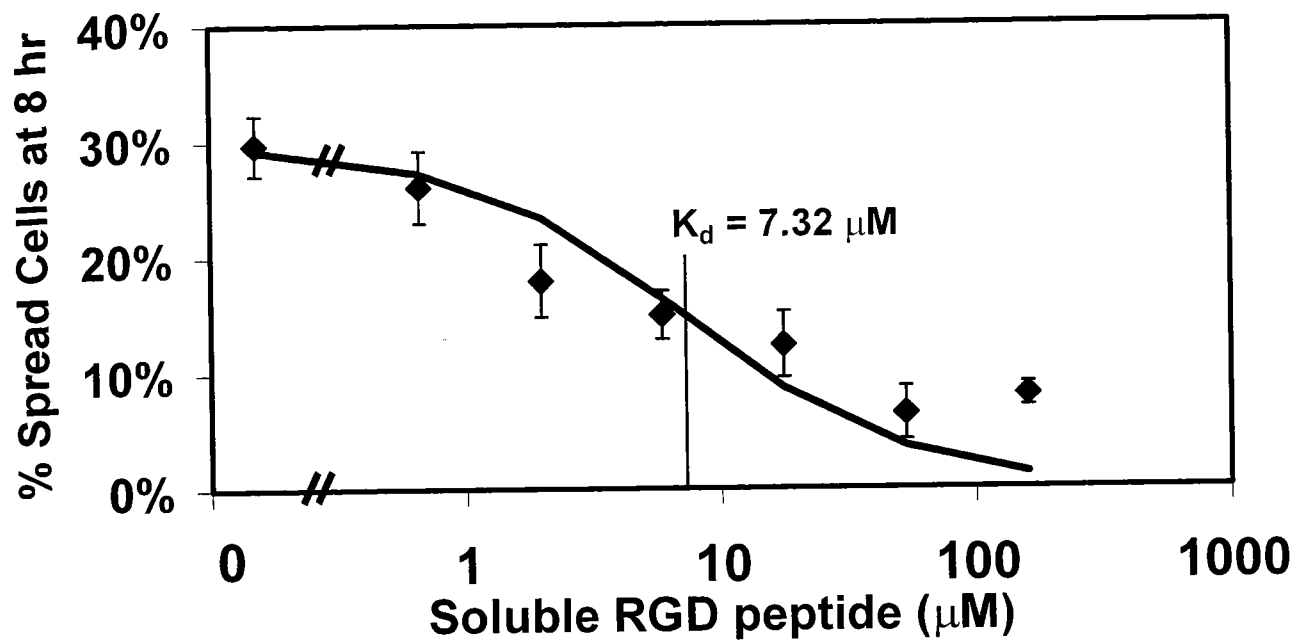


Figure 6. In the presence of increasing amounts of soluble RGD peptide, chondrocyte attachment data was fit to a model for receptor binding.

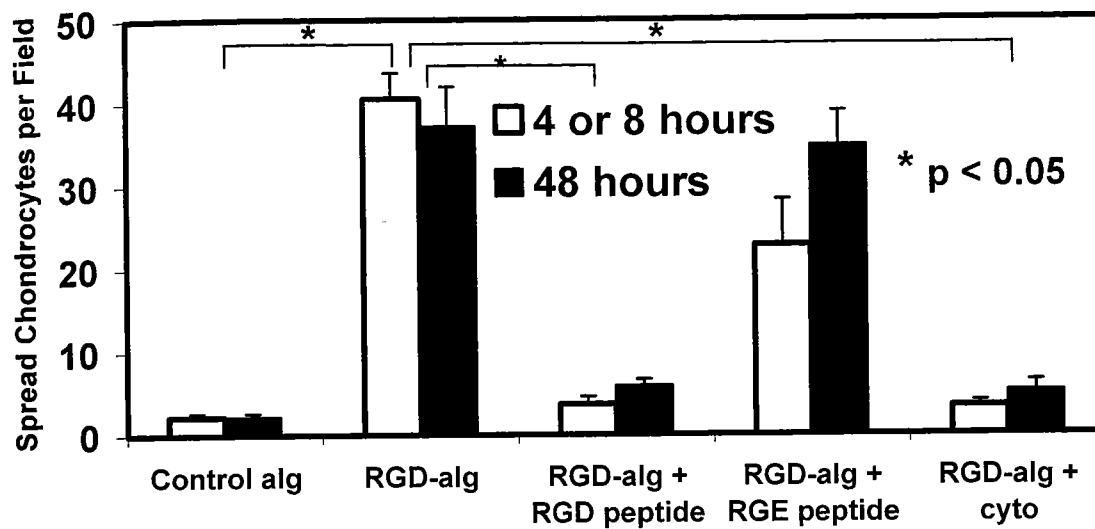
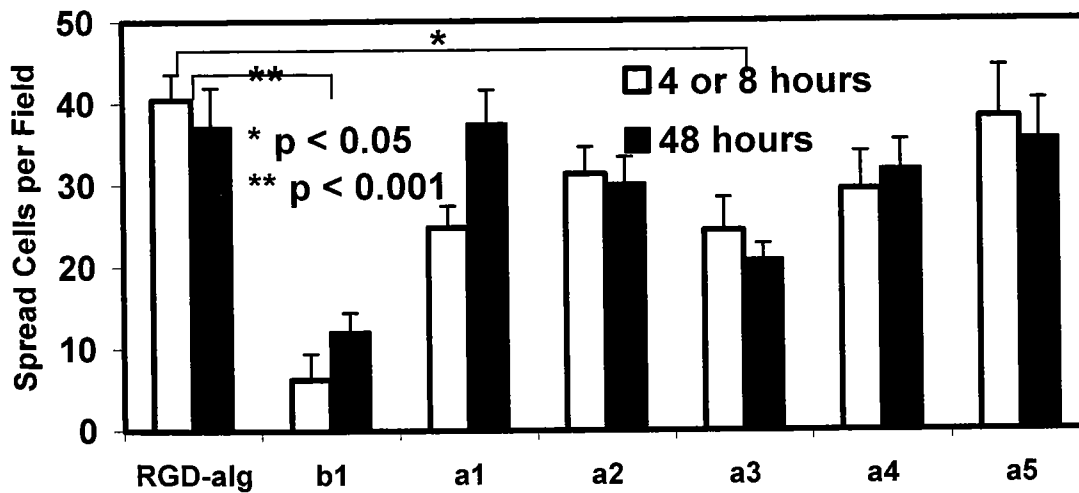
A**B**

Figure 7. Chondrocyte attachment and spreading to RGD-alginate is blocked by agents that interfere with integrins and the cytoskeleton. **A:** Chondrocyte spreading was greater on RGD-alginate surfaces compared to control surfaces. The attachment was blocked by soluble RGD peptide, but not soluble RGE peptide. Cytochalasin-D also blocked attachment. The changes were significant, as shown by One-Way ANOVA using Dunn's Test as post-hoc analysis for pairwise comparison. **B:** Chondrocyte spreading to RGD-alginate was significantly inhibited by the addition of antibodies to integrin subunits β_1 and α_3 . The β_1 blockade was significant to $p < 0.001$, and the α_3 blockade was $p < 0.05$ for the 48 hour timepoints.

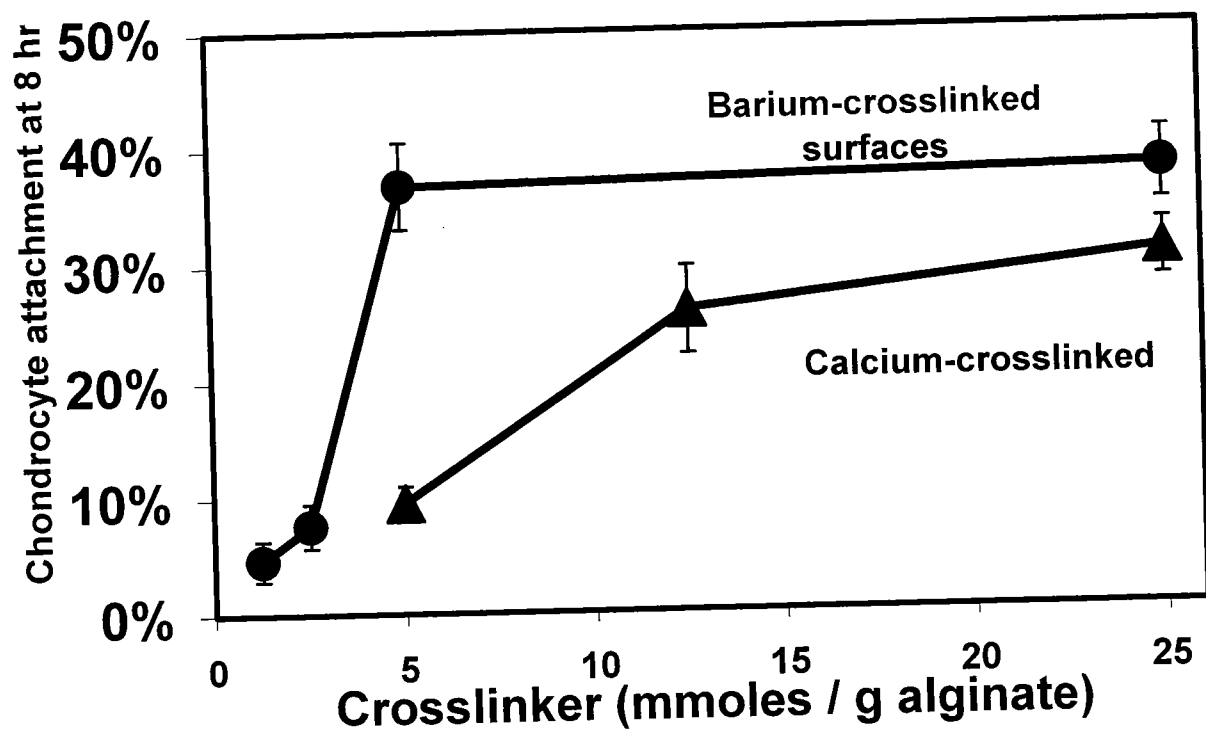


Figure 8. Chondrocyte attachment increased with higher concentrations of crosslinker per gram of alginate.

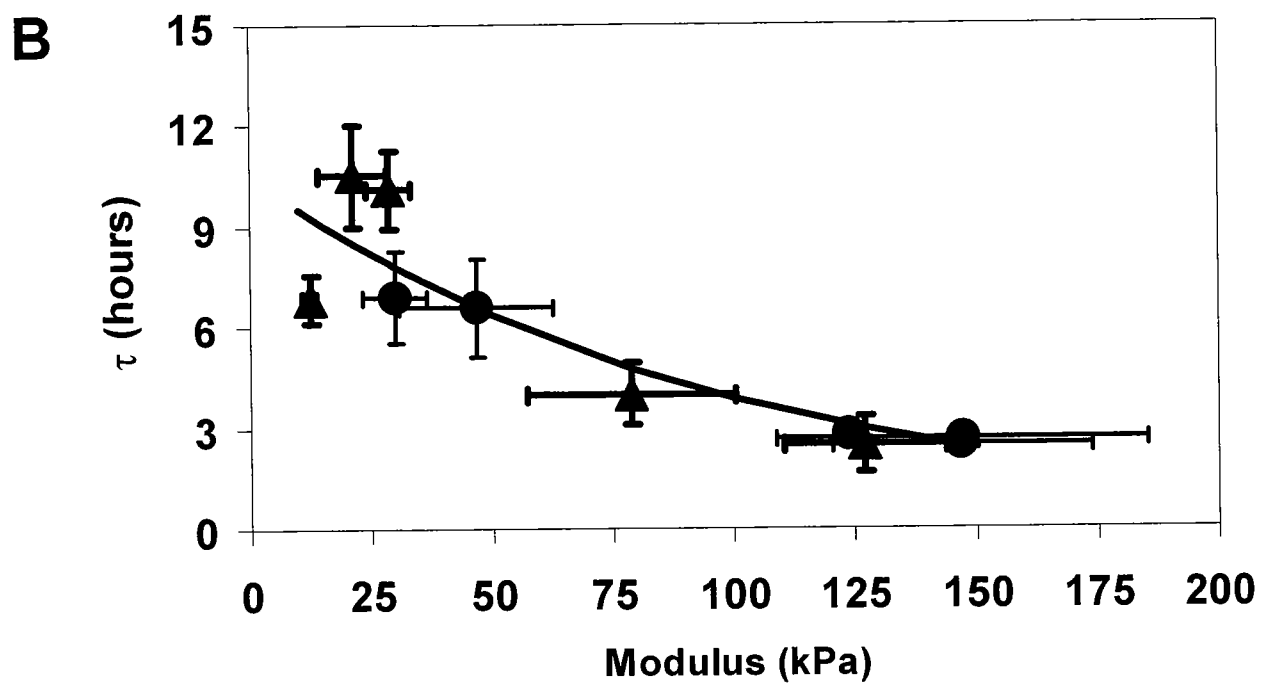
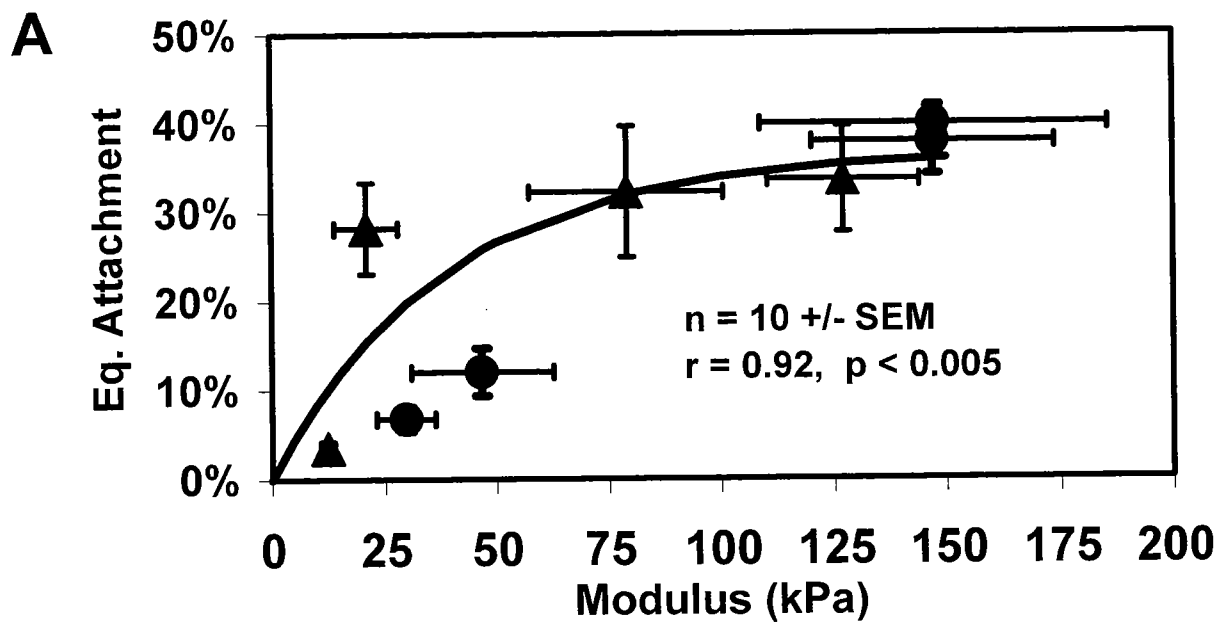


Figure 9. Chondrocyte attachment kinetics depend on matrix stiffness. **A.** The equilibrium level of attachment of chondrocytes depended on substrate stiffness. An empirical fit to a first-order equation gave a r-value of 0.92. Triangles represent Ca^{2+} crosslinked surfaces, and Ba^{2+} is represented with circles. **B:** The characteristic time constant for attachment was dependent on substrate stiffness as well. An empirical fit to a first-order equation had a r-value of 0.94. Triangles represent Ca^{2+} crosslinked surfaces, and Ba^{2+} is represented with circles.

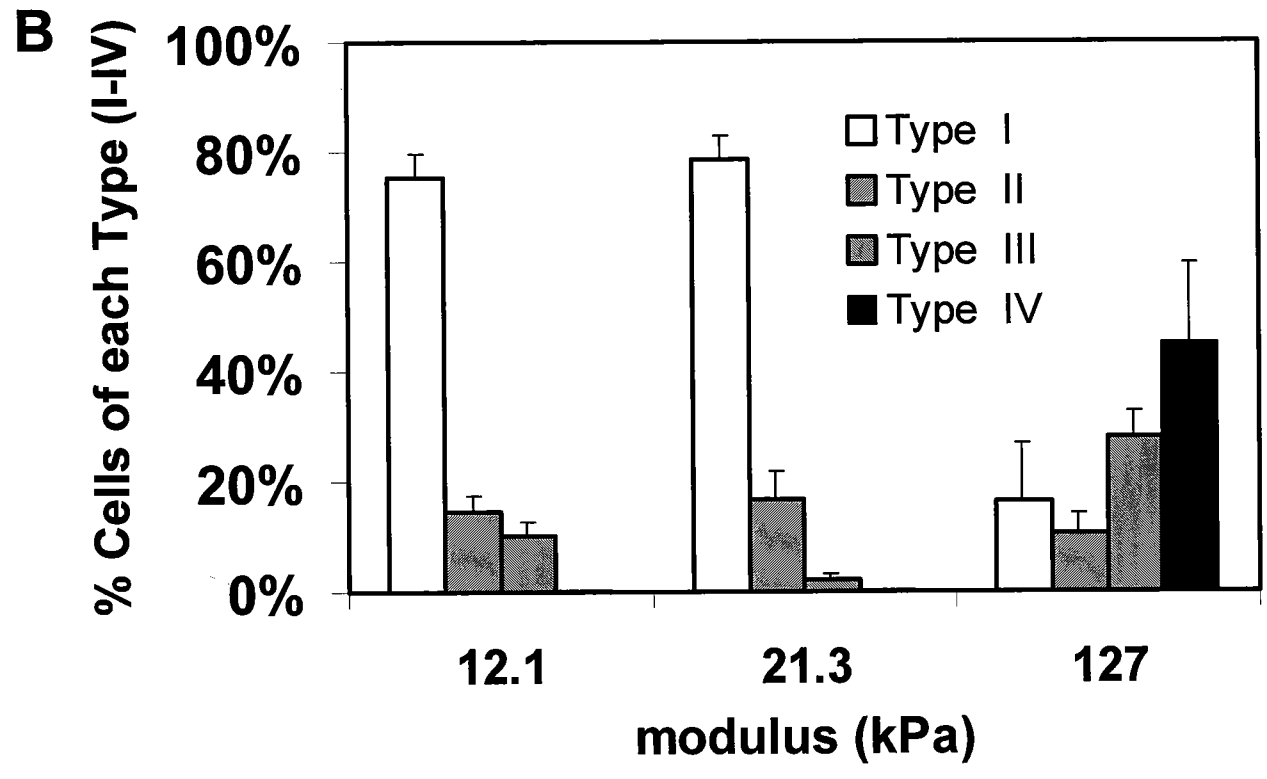
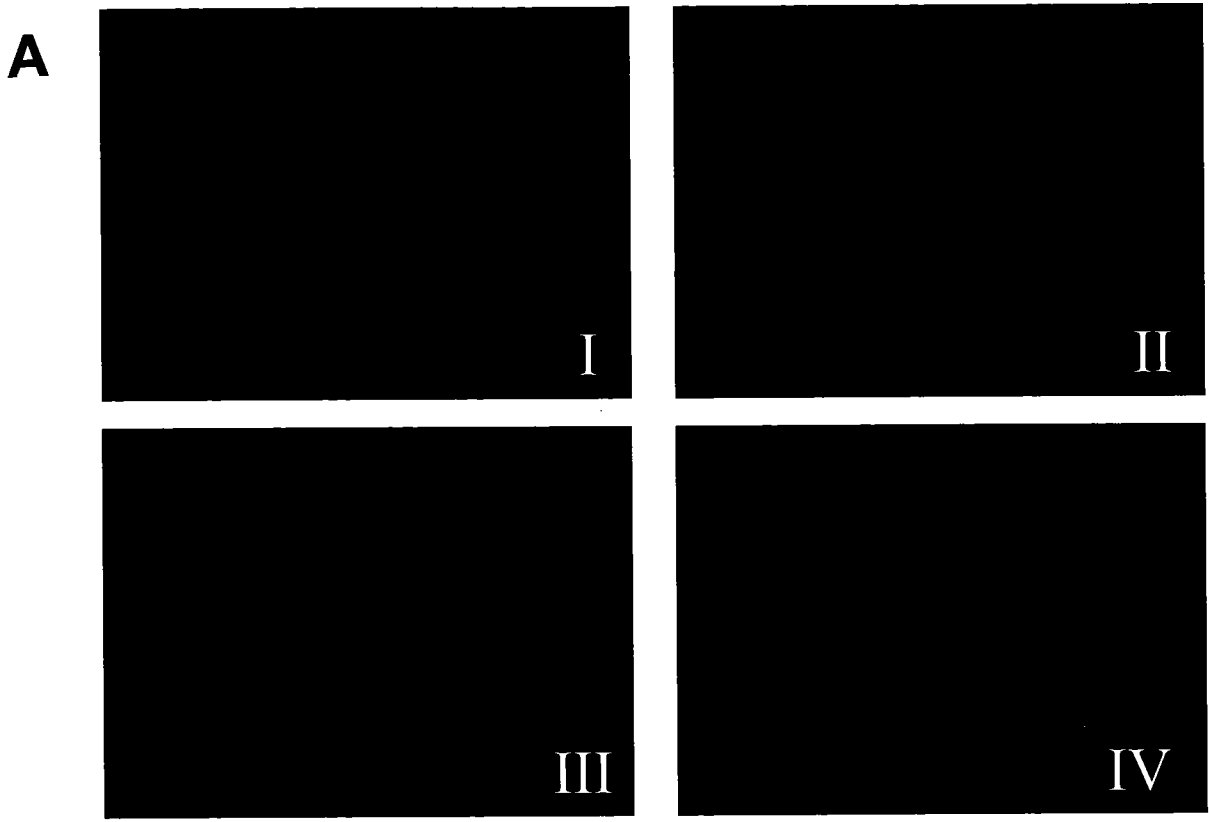


Figure 10. Chondrocyte morphology is regulated by substrate stiffness. **A:** A rhodamine-phalloidin stain of actin filaments allowed for classification of chondrocyte morphologies, based on scanning electron micrographs of fibroblasts on an RGD substrate (Massia and Hubbell, 1991). **B:** Counting the populations of chondrocytes of each type revealed that the substrate stiffness affects the arrangement of the cytoskeleton.

CHAPTER II

CONTROLLING ALGINATE MODULUS: TWO METHODS FOR INTRODUCING MECHANICAL CONSIDERATIONS TO ALGINATE TISSUE CULTURE

Abstract

Alginate is widely used in many fields, including tissue engineering and cell culture applications, due to its regulatable gelation at desirable temperatures. The growing understanding of cell mechanosensation necessitates tissue culture systems with adjustable mechanical properties. In this paper we describe two methods for generating alginate constructs across a range of stiffnesses for these purposes.

The first method involved coating wells of a plate with alginate, then dripping increasing amounts of divalent cation crosslinkers to stiffen the gel. A wide range of moduli (12 kPa to over 150 kPa) were observed with increasing crosslinker concentration (1.5 mmol – 62.5 mmol / gram alginate). The surfaces were inhomogeneous as viewed by freeze-fracture SEM and MRI.

The second method involved casting and shaping gels of decreasing polymer length. Heating the alginate decreased the viscosity, as confirmed by viscometry, and the

molecular weight, as confirmed by size exclusion chromatography. A smaller range of stiffnesses (10-60 kPa) was observed, but inhomogeneity and the confounding effects of increased cation density are avoided.

Investigators can use these methods for introducing variable mechanical environments into future adhesion assays and matrix culture systems.

Introduction

Since its initial characterization and manufacturing refinements many years ago, alginate has found use in a variety of applications, in fields as diverse as baking, dentistry, and drug delivery. Like other hydrogels, alginate has been particularly useful in the tissue culture of cells which depend on a 3D matrix to preserve their *in vivo* phenotype. The use of hydrogels for chondrocyte culture, for instance, has led to discoveries about cell matrix synthesis and mechanotransduction – discoveries that would not have been possible by using cartilage explants or monolayer culture [50, 79].

Alginate has several advantages over agarose and other hydrogels. First, its gelation depends on divalent cation concentration, and not temperature. Thus, alginate can gel and ungel at temperatures hospitable for cell culture. Secondly, the alginate backbone can be modified to include covalently bonded bioactive peptides, which can further the study of cell-matrix interactions [80, 82].

Because of this utility, tissue culture with alginate has led to important advances in the understanding of cell behavior within a matrix. Perhaps because of this success,

few refinements or variations on the original alginate gel procedure have been attempted. Recent discoveries regarding cell mechanosensation, however, compel new directions in tissue culture procedures [37, 39, 120]. With a growing understanding of cellular responsiveness to the mechanical environment, it makes sense to exploit the unique properties of alginate to study this phenomenon.

One conventional way of modulating hydrogel stiffness is to shorten the polymer chain length. Various methods exist for accomplishing this, from radiation to enzymatic degradation [112, 124] [72, 125]. One straightforward method is to thermally degrade the hydrogel [126]. The prolonged exposure to high temperature shortens the polymer chain, thus assuring that the addition of crosslinker will produce a weaker gel.

But alginate offers an additional avenue for controlling stiffness. Though Ca^{2+} has been the preferred cation for alginate gelation in tissue culture, it has long been known that other divalent cations, such as Ba^{2+} , crosslink alginate to a greater extent [127]. Further, the concentration of cation similarly affects the degree of crosslinking. Finally, the method of delivery of the cation has a profound effect on crosslinking – the sparingly soluble ionic solution will gel alginate slower, but ultimately to a greater extent, than a more soluble solution [82]. It has already been shown that changes in the degree of crosslinking, either by changing cation species, concentration, or delivery, leads to changes in the alginate modulus [128]. Thus, among hydrogels, alginate is best suited for precise variation of mechanical properties.

In this paper, we explored the aforementioned methods of modulating alginate mechanics, with an eye toward future applications in tissue culture. The first method used completely soluble Ca^{2+} or Ba^{2+} to crosslink surfaces of alginate lining the bottom of wells in a 24-well plate. This approach will be of use for investigations into cell adhesion on peptide-coated surfaces; the ability to bond different peptides to alginate surfaces allows for similar comparisons. Moreover, the durotaxis phenomenon [120] indicates that substrate stiffness alone can guide cell behavior.

The second method involved the construction of 3D alginate discs with a less soluble crosslinker, CaSO_4 . This procedure is similar to other 3D matrix approaches in the literature, and has been used previously to construct gels into shapes of clinical importance [80, 129]. In these experiments, the mechanics of the alginate gel were adjusted by thermally lowering the molecular weight of the polymer, pre-crosslinking.

The goal of this research was to introduce means of modulating substrate stiffness into existing procedures for alginate tissue culture. The two methods examined here, involving surfaces for cell adhesion assays and 3D matrices for phenotype preservation, are based on methods of broad appeal to current investigations. By incorporating precise control of the substrate modulus, future studies can examine the effect of mechanics on cell behavior across a wide range of cell type and culture conditions.

Methods

CaCl₂ and BaCl₂ discs

Medium-viscosity alginate (FMC Biopolymers, Norway) was suspended in PBS at 2% (w/v) and sterilized through a 0.45 μm syringe filter (Nalgene). Aliquots of 200 μl were pipetted into the wells of a 24-well plate and spread uniformly to coat the surface. Specific volumes of 1M CaCl₂ from 5 - 250 μl (2.5 – 62.5 mmol / g alginate) and 1M BaCl₂ from 1.5 - 100 μl (2.5 – 25 mmol / g alginate) were added to crosslink the alginate. After one minute, the cation solution was removed and the surfaces were rinsed twice with 2 ml of F-12 media before analysis by SEM, MRI, or mechanical spectrometer.

CaSO₄ discs

2% alginate was heated in PBS (GIBCO / Invitrogen, Grand Island, NY) for periods up to 8 hours in a conventional oven. Every two hours, water was added to restore solution volume. The time required for solution to achieve boiling, approximately 15 minutes per 100 mL solution, was not counted towards heating duration.

The 2% alginate was cooled, filter-sterilized as described above, and 2 mL aliquots were mixed with 80 μL of a well-shaken CaSO₄ solution (concentration: 0.266 g / ml PBS) via a 3-way stopcock as described previously [82]. The gel was quickly spread between sterile plates separated by 1 mm spacers. After allowing 15 minutes for the alginate to harden, the plates were separated. Using a biopsy punch, 6 mm diameter discs were carved out of gel, and each disc was placed in 2 ml of F-12 media for analysis. The

alginate used in some discs was heated at 115°C for 1-8 hours before the addition of crosslinker so as to vary the mechanical properties.

Mechanical Testing

CaSO₄ discs were placed in uniaxial confined compression between an impermeable polymethacrylate surface and a porous polyethylene platen in a Dynastat mechanical spectrometer (IMASS, Hingham, MA). In the case of CaCl₂ discs, a 6 mm section was cut from the center of each surface for testing. A series of ten steps in displacement, up to a maximum of approximately 20% total strain, were imposed on the samples and resultant forces were measured at a rate of 2.5 Hz for 30 sec / relaxation. Displacements and loads were normalized to sample geometry to yield values for strains and stresses. Stress relaxation curves were fit to a poroelastic model of hydrogel behavior to determine a compressive modulus at mechanical equilibrium [108].

Cryo-Microscopy Sample Preparation

The hydrated CaSO₄-, CaCl₂-, and BaCl₂- crosslinked alginate specimens, sample stage, and strips of aluminum foil were immersed in separate containers of liquid nitrogen. After approximately 20 minutes of cooling, the stage was placed back into the sample chamber of the SEM. A specimen was removed and fractured (force-split) with a blunt-tipped spatula. The specimen was then mounted on the sample stage with its cross-section (fractured) plane oriented at 90° to the stage surface. The strips of aluminum foil

were utilized to position the specimen on the stage, and to assist in reducing the charging of the specimen surface.

Using the P-SEM standard console (Version 4.02i, 1997), Variable Pressure (VP) mode was selected, and filament saturation began when 0.1 torr was reached in the sample chamber. To obtain secondary electron imaging, which is the most useful for morphological analysis but not available in VP mode, the SEM was switched over to standard High Vacuum mode. The sample chamber pressure then achieved 10^{-6} torr, and images were taken under these conditions.

Scanning Electron Microscopy (SEM)

Morphological characterization was carried out using an Aspex Variable Pressure PSM-75 TorrSEM Scanning Electron Microscope with a tungsten source. The microscope was operated using an accelerating voltage of 20 kV and working distances between 20 – 35 mm. Alginate pore size was approximated by the formula

$$\text{Eq. 1} \quad d = \sqrt{w_{avg} h_{avg}}$$

Magnetic Resonance

MR imaging experiments were performed with a GE CSI-II 2.0T / 45 cm imaging spectrometer (GE NMR Instruments, Fremont, CA) operating at 85.56 MHz for ^1H and equipped with ± 20 G / cm self-shielding gradients. Single slice, T_2 -weighted images of acellular alginate surfaces were acquired using a spin-echo imaging sequence and echo

times (TE) of 18.0, 25.0, 35.0, 55.0, and 75.0 msec. One sagittal 4-mm-thick slice, centered within the gel with FOV = 15.0x15.0 mm and a 256x128 pixel resolution were acquired (TR = 5 s, NEX = 2). In data processing, the phase encoding direction was zero-filled to 256 giving a final matrix of 256x256. The T_2 -relaxation time constant was estimated using custom software (IDL, Research Systems Inc., Boulder CO) on a pixel-by-pixel basis using a linear-least-squares regression and the equation:

$$\text{Eq. 2} \quad M = M_0 e^{(-TE/T_2)}$$

Where M is the pixel signal intensity, and M_0 is the proton density (signal intensity at TE=0).

Viscosity Measurements

The viscosity of alginate hydrolyzed in PBS at 115 °C for up to 8 hours was assessed using an Oswaldt viscometer in a 25°C water bath. Briefly, alginates of concentrations ranging from 0.05 - 0.50 % alginate were suspended in the viscometer and the time required for fluid flow between demarcations was measured. From these relative viscosities, the reduced and inherent viscosities were calculated for decreasing concentrations of alginate. By extrapolating both lines to an infinitesimal concentration of alginate, the intrinsic viscosity was derived. Fitting intrinsic viscosities and respective molecular weights (see below) into the Marc-Houwink equation

Eq. 3
$$\eta_{\text{int}} = K(M_w)^a$$

allowed for calculation of K and a for FMC medium-viscosity alginate.

Size Exclusion Chromatography (SEC) of Alginate Polymers

Size Exclusion Chromatography of the alginate polymers was performed on a Perkin-Elmer Series 200 instrument, with a Perkin-Elmer Series 200 Autosampler, HPLC Pump, Column Oven, and Refractive Index Detector. The column oven was operated at a constant temperature of 30°C. For optimal chromatographic separation, two size exclusion columns were used in series. The SEC columns used were two Polymer Laboratories PL Aquagel-OH mixed 8 micron (300 x 7.5 mm) columns. A Polymer Laboratories PL Aquagel-OH mixed 8 micron (50 x 7.5 mm) Guard column was also used, and placed in series prior to the two SEC columns. The aqueous mobile phase was a mixed buffer of 0.2M NaNO₃ and 0.01M NaH₂PO₄ in deionized water, and was pH adjusted to 7.1 with 1 M sodium hydroxide. Reagents for mobile phase preparation were purchased from Sigma-Aldrich. The system was operated at a flow rate of 1.0 mL/min, and a sample injection volume of 100 microliters was used throughout. To calibrate the system, a series of 10 polysaccharide (pullulan) polymer standards from Polymer Laboratories were used, with a molecular weight range of 788,000 to 180. Data collection was done using Perkin-Elmer Turbochrom software, and data analysis was done with Cirrus software from Polymer Laboratories (Amherst, MA).

Results

CaCl₂ and BaCl₂ discs

Alginate was coated onto the bottoms of wells in a 24-well plate, and various amounts of 1M CaCl₂ and BaCl₂ crosslinker were added (1.25 – 62.5 mmol crosslinker / gram of alginate). As crosslinker concentration increased, the discs became stiffer (figure 11). For Ba²⁺ crosslinked discs, the moduli spanned a range from 29.8 kPa ± 6.58 S.E.M. to 157 kPa ± 38.4 S.E.M., though crosslinker concentrations above 5 mmol / gram did not increase the stiffness. The Ca²⁺ discs (2.50 – 62.5 mmol Ca²⁺ / gram of alginate) ranged from 12.3 kPa ± 1.51 S.E.M. to 127 kPa ± 16.8 S.E.M., and modulus steadily increased with additional concentration. The number of samples at each data point was 4 or greater.

Cryo-treated alginate samples were viewed with variable-pressure SEM. Increasing homogeneity was revealed in cross-sectioned surfaces with increasing crosslinker density (figure 12A-D). Pore size decreased as disc as stiffness increased (figure 12E).

T₂ relaxation times were measured for four concentrations of Ca²⁺ crosslinked alginate surfaces. Relaxation times were found to be higher and more heterogenous on substrates with low crosslinker concentration, and lower and more homogenous as crosslinker concentration rose (figure 13A). Average T₂ values for a 6 mm diameter volume around the center of Ca²⁺ and Ba²⁺ -crosslinked discs were calculated for

comparison to mechanical testing data. T_2 was correlated with substrate modulus, and indicated decreased molecular mobility in stiffer gels (figure 13B).

CaSO₄ discs

Size exclusion chromatography was performed on alginate that had been heated for periods up to 8 hours, showing decreasing elution times as heating duration increased (figure 14A). When calibrated to pullulan standards, M_w was found to range from 969 kDa for unheated alginate to 540 kDa for alginate heated eight hours. M_w declined linearly with heating duration (figure 14B).

Intrinsic viscosity, which related to polymer molecular weight by the Mark-Houwink equation (eq. 3), was also found to vary with heating duration (figure 15). Fitting M_w to the Mark-Houwink equation by minimizing RMS error revealed the value of K to be 6.85×10^{-6} , and the exponent a was equal to 0.979.

Constructing discs by crosslinking heated alginates with CaSO₄ produced discs with with stiffnesses that correlated with molecular weight. For 2% alginates, stiffness ranged from $49.1 \text{ kPa} \pm 11.45 \text{ S.E.M.}$ for 969 kDa alginate to 5.25 kPa for 540 kDa alginate (figure 16). For 4% alginate discs, the range was from $87.0 \text{ kPa} \pm 7.71 \text{ S.E.M.}$ for 969 kDa alginate to $40.4 \text{ kPa} \pm 2.80 \text{ S.E.M.}$ for 540 kDa alginate.

Cryo-SEM of the CaSO₄ discs suggested relative uniformity compared to that of Ca²⁺ and Ba²⁺ crosslinked gels (figure 17A-D). Alginate pore size was observed to decrease with decreasing molecular weight and modulus (figure 17E).

Discussion

This research examined two fabrication techniques for alginate. One method, using varying amounts of fast-acting CaCl_2 and BaCl_2 crosslinkers, involved coating the bottom of a well and making a surface of controllable stiffness. This method is relevant to investigations of cell adhesion on various peptide-coated substrates. The properties of alginate allow covalent addition of peptides to the polymer chain, which by itself makes it an attractive, nonimmunogenic substrate for cell adhesion. The ability to vary the substrate modulus from 12.5 kPa to 125 kPa with increasing CaCl_2 concentration – a tenfold span – permits the consideration of mechanics as a factor in cell behavior in culture.

Existing methods for varying substrate stiffness [39, 130] use a collagen matrix mixed with either polyacrilamide or glycosaminoclycans. Such substrates are harder to construct, cost more, and do not provide as great a range of stiffnesses.

One drawback to the use of CaCl_2 and BaCl_2 substrates for mechanosensory experiments is the apparent inhomogeneity of the alginates with lower crosslinker concentration. Stress-relaxation testing with the Dynastat mechanical spectrometer is incapable of discerning regions of different stiffness, but the inhomogeneity appeared in scanning electron micrographs as a variability in pore size (by histogram, not shown), and in magnetic resonance imaging as a variability in T_2 relaxation times.

Pores have been noted in other alginate SEM studies [131]. Though pore size and shape can be altered by different freezing regimes [132], the presence of pores itself is

not an artifact of the rapid freezing used here – pores appear in crosslinked alginate frozen at far slower rates [133].

Indeed, the size of pores has been shown to vary with crosslink species and mechanical properties [133]. The results described here, however, are the first attempt we know of to correlate alginate crosslinker density or chain length with both pore size and modulus.

Magnetic resonance has also been used to identify alginate inhomogeneities in previous studies. Increases in T_2 relaxation time was found to correlate with decreases in alginate concentrations, as verified by gravimetric analysis [134]. Increasing pore size was also correlated to increasing T_2 relaxation times in the same study. Recently, inhomogeneity in alginate beads was prevented, as verified by 535 nmR, by simultaneous internal and external exposure of the bead to $BaCl_2$ crosslinker [135].

The present study, however, correlates average relaxation times with substrate modulus, demonstrating that substrate stiffness increases as relaxation times drop. The drawbacks to inhomogeneities in pore size and relaxation times can be countered by the second method examined; crosslinking with slower-acting $CaSO_4$.

Gelling alginate with $CaSO_4$ produces 3D scaffolds similar to those used for culture with agarose, PLGA, or collagen, with the added benefit of injectability and moldability [80]. Controlling substrate mechanics in 3D tissue culture is just as compelling as in the 2D case, but the same cell behaviors cannot be observed. Compared to the transparent thin films of alginate made by dripping $CaCl_2$ or $BaCl_2$, visualizing cells in 3D $CaSO_4$ -crosslinked gels by phase contrast microscopy is difficult. Cell

conformation in alginate can be assessed by confocal microscopy, however [136].

Additionally, by growing cells in 3D alginate gels, long-term, high-density cell culture is possible, permitting the analysis of GAG, collagen and MMP synthesis, proliferation, and other variables.

The microstructure within CaSO_4 – crosslinked gels appears more uniform by SEM, echoing the findings that omnidirectional crosslinker exposure reduces inhomogeneity [135]. The observed range of stiffnesses attainable with this crosslinking method was not as great as that of the CaCl_2 surfaces, but starting with a higher concentration of either alginate crosslinker, and heating under different conditions, can likely increase the range of moduli. Heating in the presence of acid for only four hours was found to decrease the M_w of a different alginate from 913 kDa to 274 kDa – almost twice the degradation achieved here [137]. Similarly, using gamma irradiation, an alginate from FMC with M_w of 286 kDa was shortened to 53 kDa [72], with a lower polydispersity than achieved here (1.3 vs. 3.8).

Hydrolyzing the alginate in PBS for eight hours reduced the modulus of crosslinked gels by sixfold, but lowered both the intrinsic viscosity and M_w by a less than a factor of two. This near-linearity is reflected in the coefficient derived for the exponent in the Mark-Houwink equation, $a = 0.979$. Values of a near unity imply minimal interactions between the polymer and the solvent. This value of a agrees with other values published, including that derived from *Macrocystis pyrifera* alginate across a different range of intrinsic viscosities ($a = 0.963$, [138]). The phenomenon of decreased

gel stiffness correlating with decreased viscosity and molecular weight has been described previously [112].

Interestingly, as molecular weight and modulus decreased in CaSO_4 discs, the average pore size was also observed to shrink. This stands in contrast to CaCl_2 and BaCl_2 substrates, in which smaller pores were associated with lower molecular mobility (by T_2 relaxation times) and increased stiffness. Just as other studies have shown pore size relates to crosslink density and alginate concentration, it also relates to polymer chain length and is, by itself, not an indicator of modulus. This finding is supported by a report that heating crosslinked alginate beads causes a decrease in bead size, coupled with water loss and smaller pores [139]. Instead of pore size, another variable, such as pore wall thickness, might correlate better with substrate stiffness.

The two methods examined in this paper have different strengths and drawbacks, but each allows investigators to quickly and easily control substrate mechanics in a variety of tissue culture applications. Substrate signaling has implications in carcinogenesis, embryology, and other fields in which alterations in matrix mechanics coincide with changes in cell behavior and motility. Developing techniques to examine the phenomenon of mechanosensation *in vitro* will prove critical to our understanding of these fields.

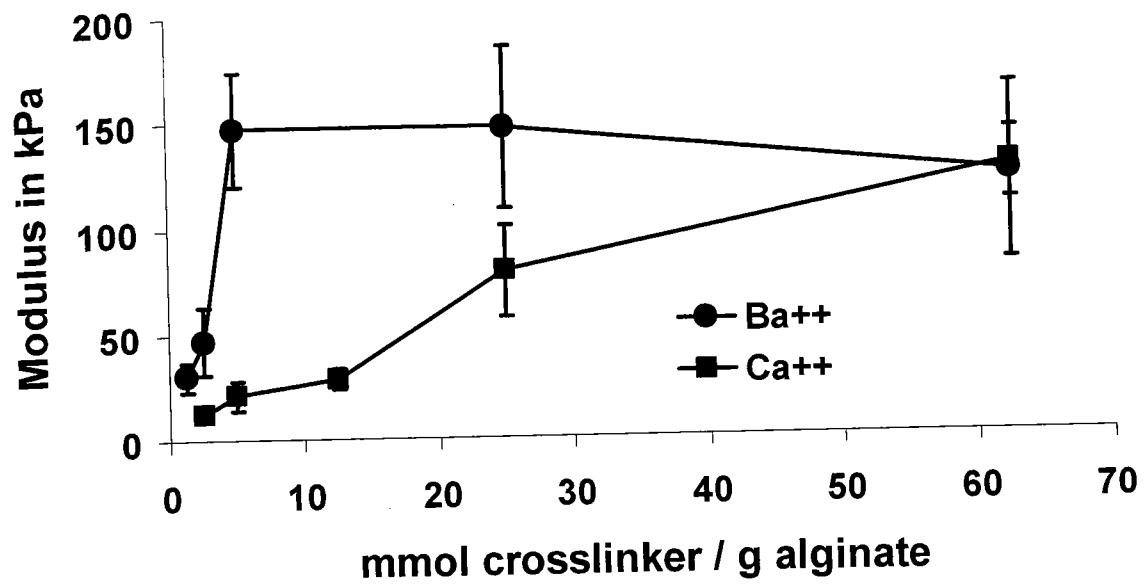


Figure 11. Alginate crosslinked with increasing concentrations of CaCl_2 or BaCl_2 showed increasing stiffness. The modulus of Ba^{++} -crosslinked discs reached a plateau of approximately 150 kPa at concentrations greater than 5 mmol / gram of alginate. Ca^{++} -crosslinked discs required more concentrated cations, consistent with reports that Ca^{++} crosslinks alginate to a lesser degree than Ba^{++} .

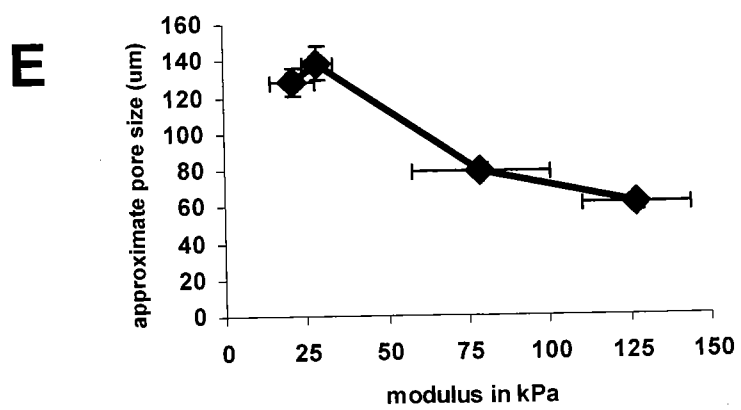
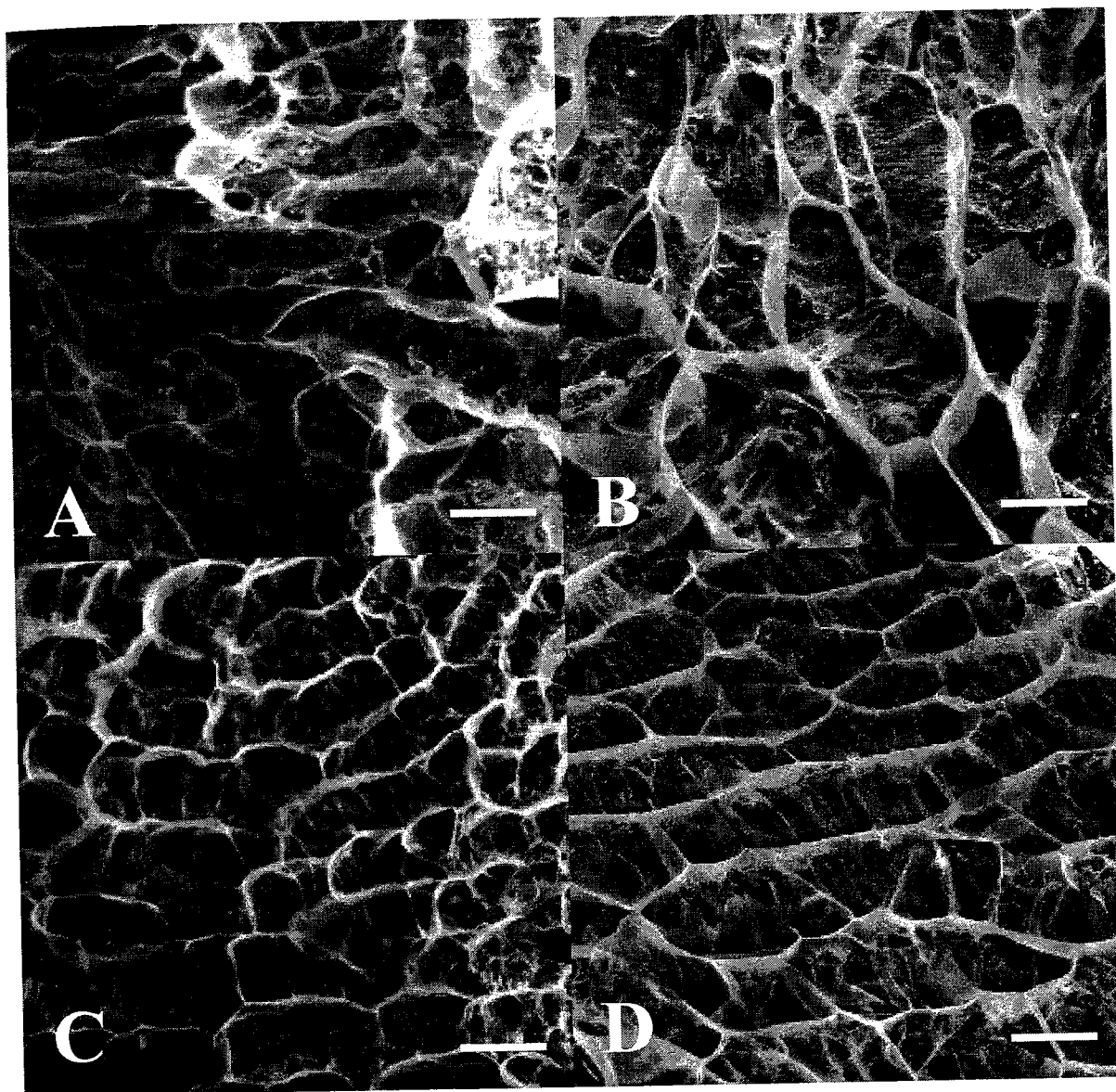


Figure 12. Scanning electron micrographs of CaCl_2 crosslinked alginate. **A:** 5 mmol Ca^{++} / gram of alginate. **B:** 12.5 mmol Ca^{++} / gram of alginate. **C:** 25 mmol Ca^{++} / gram of alginate. **D:** 62.5 mmol Ca^{++} / gram of alginate. **E:** Average pore size correlated with alginate modulus; stiffer substrates had smaller pores.

The bar represents 75 μm .

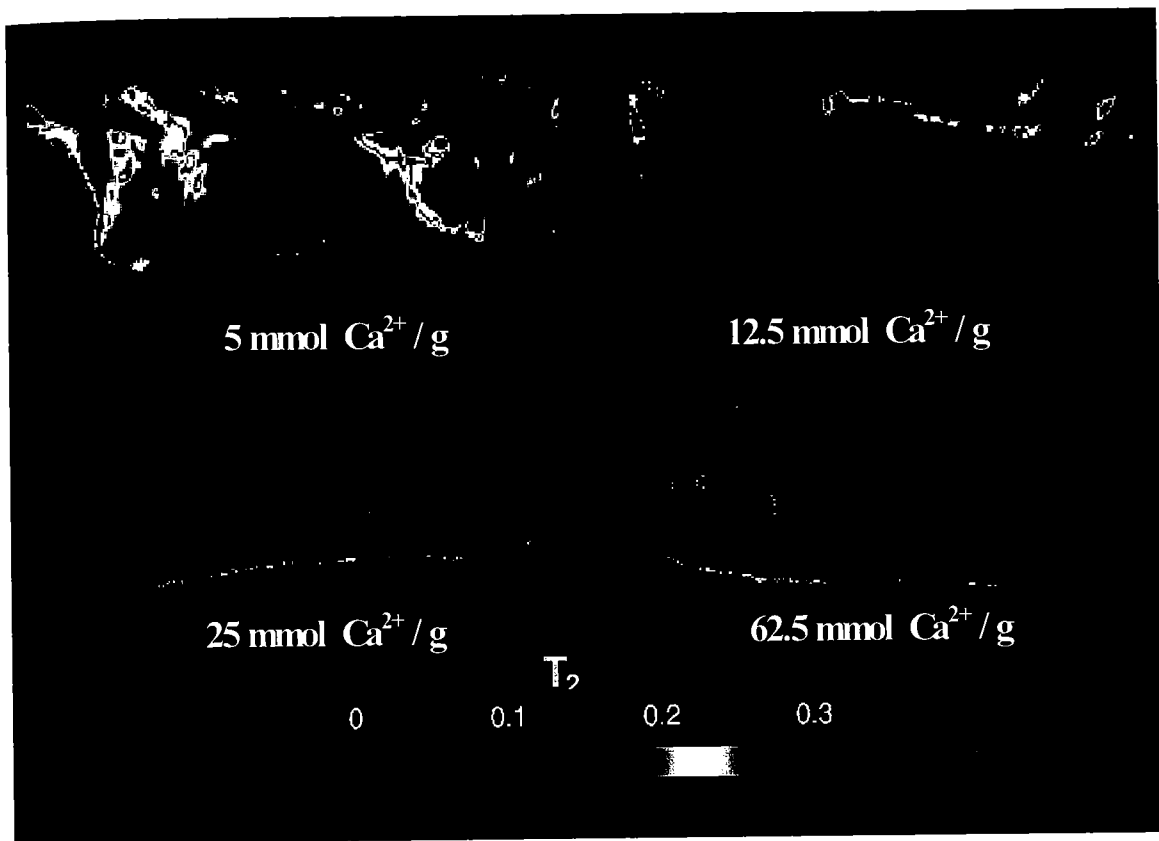
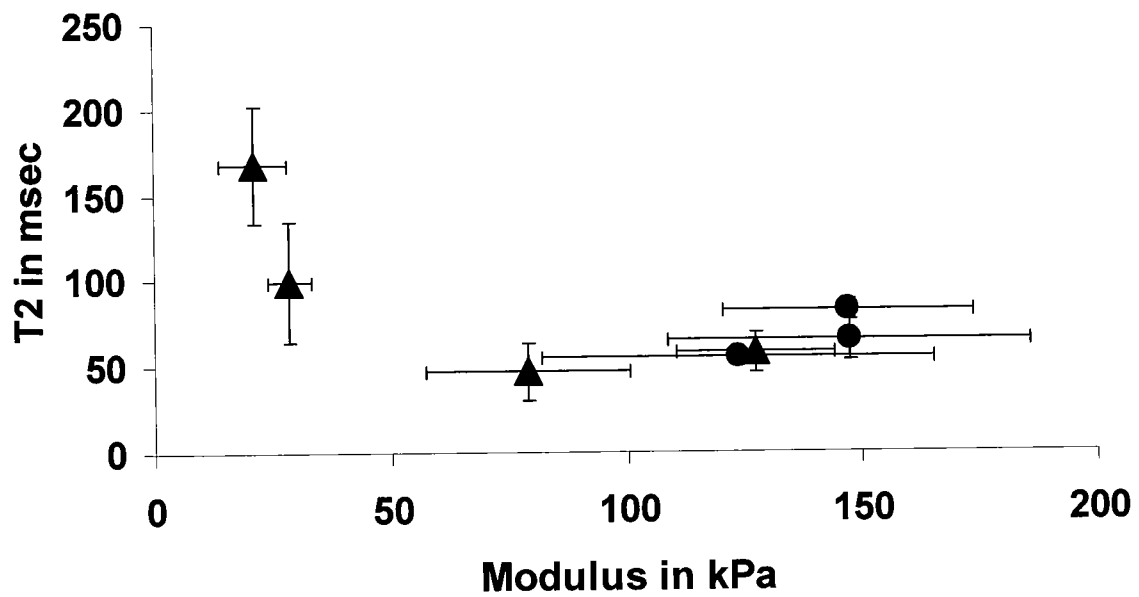
A**B**

Figure 13. T_2 relaxation times decrease in more crosslinked, stiffer gels. **A:** Apparent inhomogeneity and T_2 relaxation times decrease as the concentration of Ca^{++} crosslinker increased. **B:** The stiffest alginate substrates, crosslinked with either Ba^{++} or Ca^{++} , showed the fastest relaxation times.

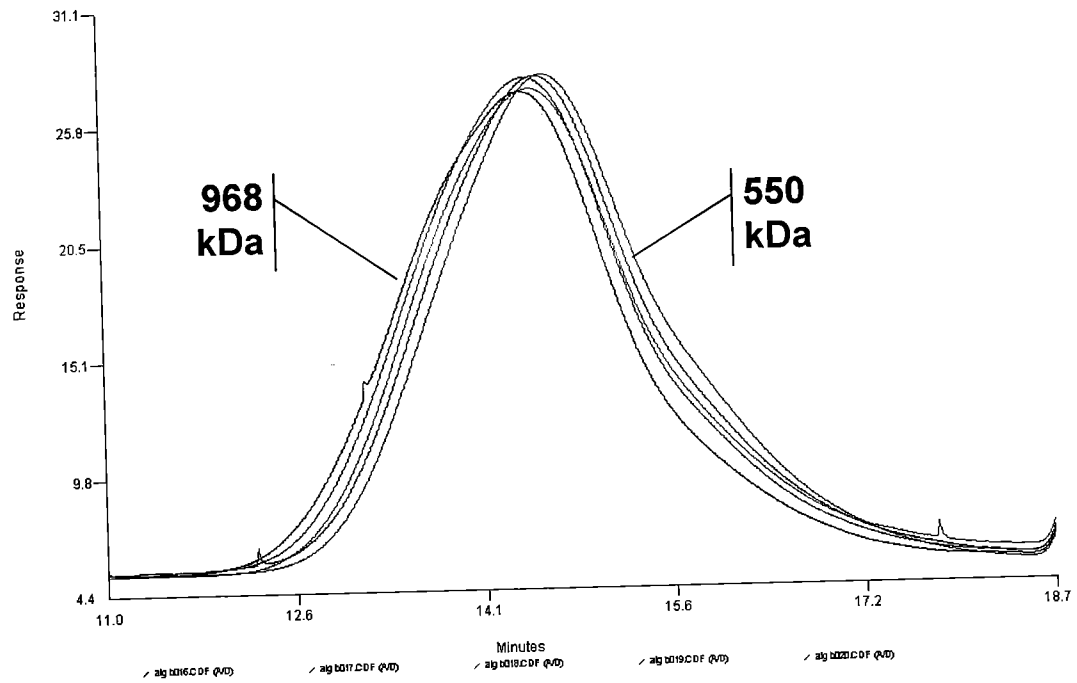
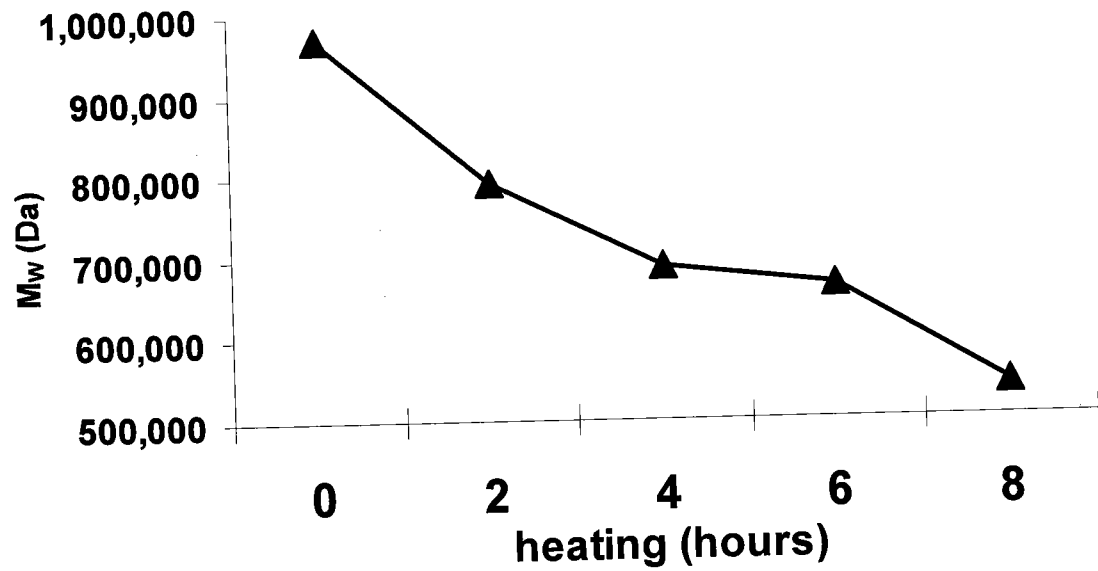
A**B**

Figure 14. Hydrolysis changes alginate molecular weight. **A:** Elution times for size exclusion chromatography of alginate hydrolyzed for varying durations. **B:** M_w decreased as hydrolysis duration increased.

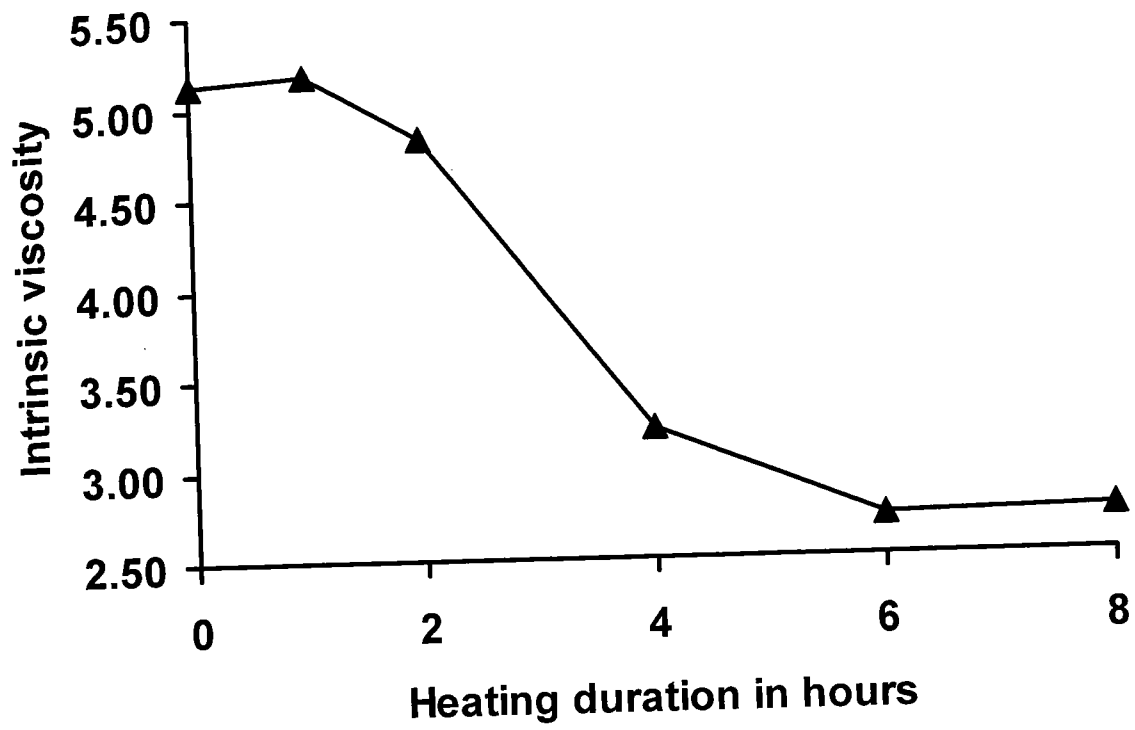


Figure 15. Intrinsic viscosity decreased as heating duration increased. From measurements of relative alginate viscosities, the reduced and inherent viscosities were calculated for decreasing concentrations of alginate. By extrapolating both lines to an infinitesimal concentration of alginate, the intrinsic viscosity was derived.

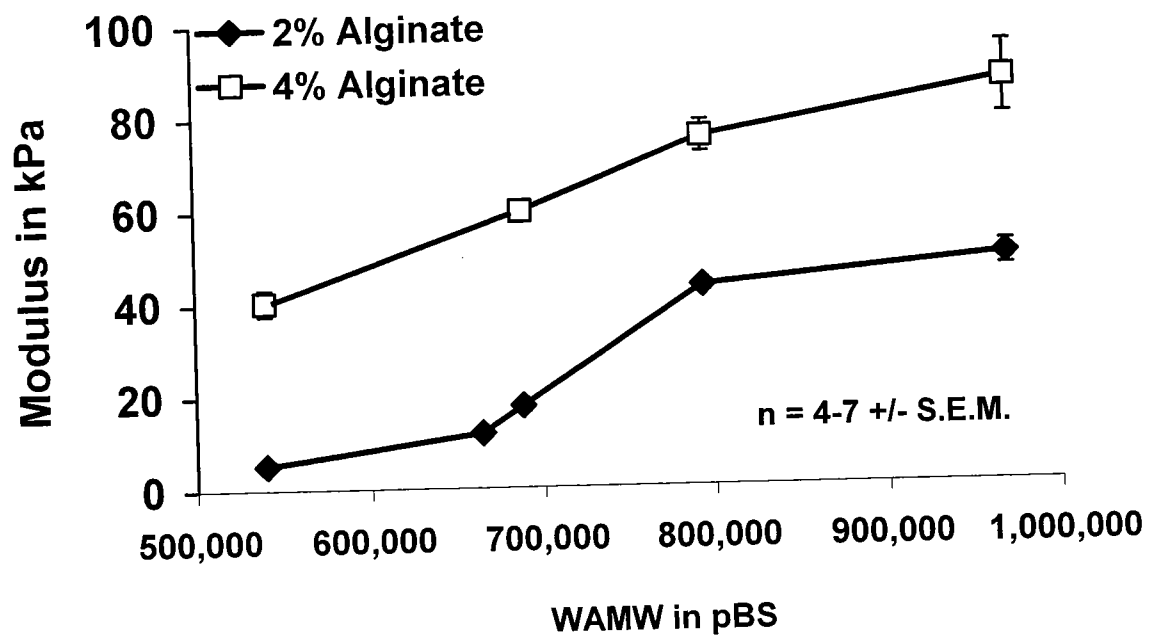


Figure 16. Alginate gelling stiffness depends on polymer length. Alginate hydrogel solutions of 2% and 4%, crosslinked with CaSO_4 , exhibited increasing modulus with increasing molecular weight.

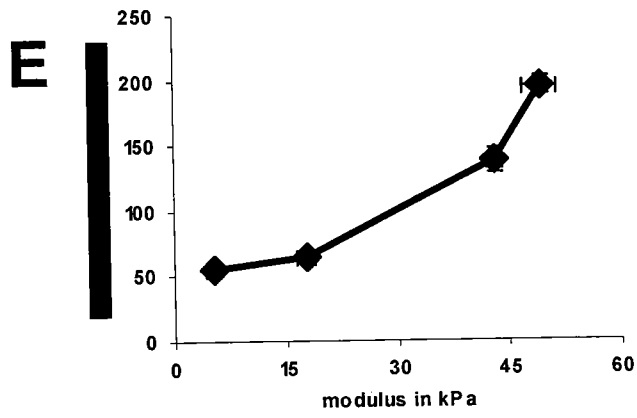
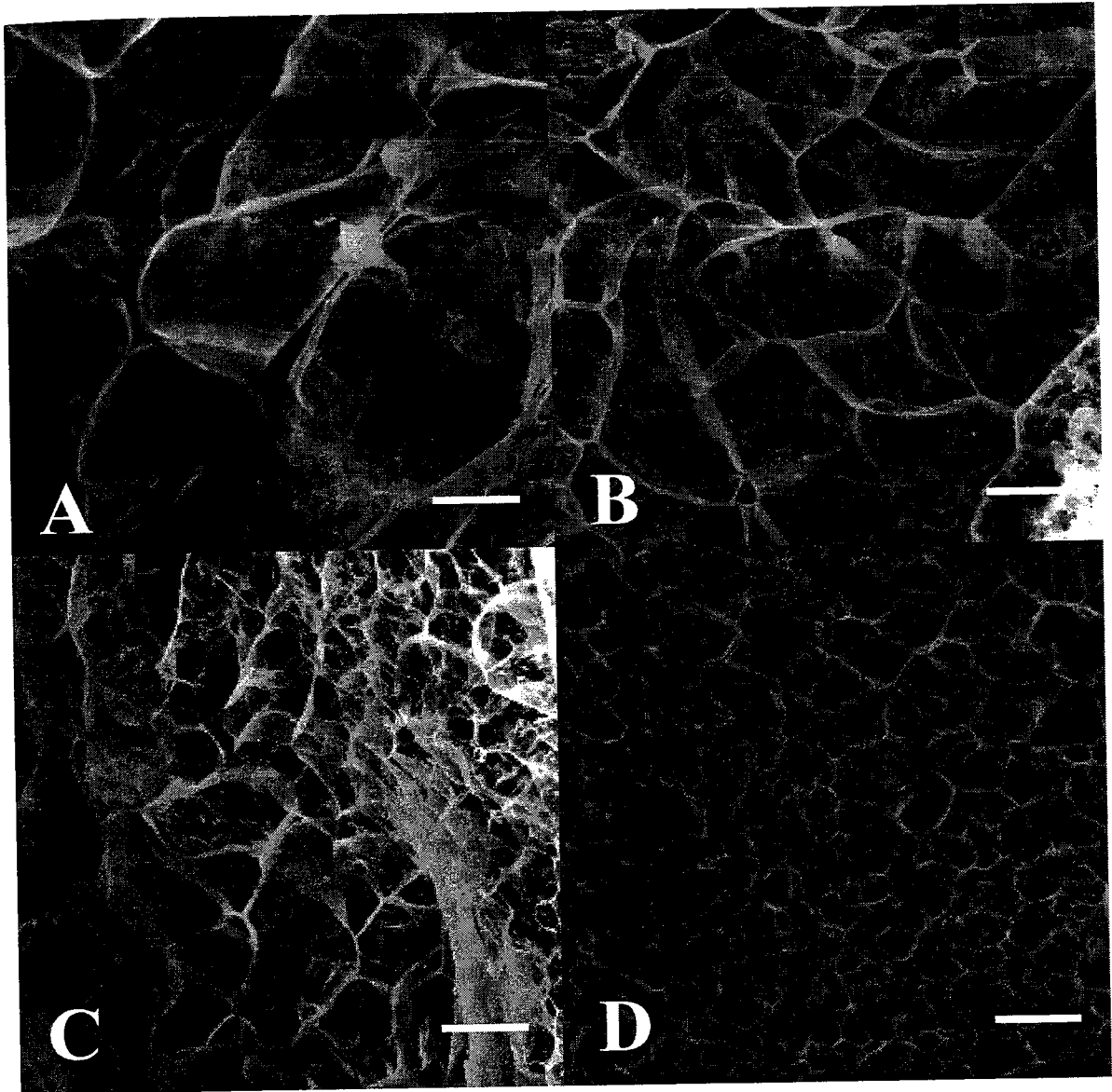


Figure 17. Scanning electron micrographs of hydrolyzed alginates crosslinked with CaSO_4 . **A:** 970 kDa alginate (49 kPa). **B:** 800 kDa alginate (40 kPa). **C:** 700 kDa alginate (18 kPa). **D:** 550 kDa alginate (5 kPa). **E:** Alginate modulus positively correlated with crosslinked alginate pore size.

The bar represents 75 μm .

CHAPTER III

CHONDROCYTE MECHANOTRANSDUCTION OF INTRINSIC MATERIAL PROPERTIES IN RGD-BONDED ALGINATE

Abstract

Culturing chondrocytes in 3D alginate hydrogels preserves the cartilage phenotype and encourages matrix synthesis. When the integrin adhesion ligand RGD is covalently bonded to alginate, chondrocytes form a cytoskeletal attachment that sensitizes the cell to matrix mechanics, at the same time stiffening the disc.

In these studies we constructed unmodified and RGD-alginate discs of variable stiffness and seeded the discs with chondrocytes. Cell proliferation and glycosaminoglycan (GAG) content of the discs were measured, as was radiolabeled sulfate uptake. It was found that the attachment of chondrocytes to RGD alone inhibited GAG synthesis, over both the short term and long term.

Furthermore, stiffer RGD-discs had 40% less GAG content per cell compared to weaker RGD discs. In contrast, in stiff unmodified discs, GAG content was not

significantly higher. The effect of this mechanosensation on matrix metabolism could be blocked with the addition of soluble RGD peptide or anti-integrin antibody to the media.

Introduction

The ability of cells to sense their mechanical environment is important in the controlling processes like embryogenesis and tumor progression. Though some effects of externally applied mechanics have been characterized for a variety of tissue types [31], [32], [33], the response to intrinsic material properties of the extracellular matrix has not been rigorously explored.

Cell motility and metabolism are influenced by substrate rigidity in a number of circumstances. Fibroblasts, for instance, have been shown to migrate towards stiffer substrates [37], [38], and previous studies indicate that chondrocytes modulate attachment kinetics and morphology on stiffer RGD-alginate hydrogels (chapter 1).

Understanding the mechanosensation of the chondrocyte environment has particular relevance for elucidating osteoarthritis (OA). In OA, chondrocyte matrix production is altered as cartilage stiffness declines [122, 123], even as matrix proteoglycan content transiently rises [140] [141]. Though OA is likely initiated by abnormal external mechanical loading [13, 18], the long-term degradation of articular cartilage tissue may depend on the mechanical properties of the matrix.

Investigators attempting to study chondrocyte environmental mechanosensation have been limited by the difficulty of working with cartilage explants. Current techniques make it impossible to change the stiffness of cartilage without disrupting the mechanism by which chondrocytes perceive matrix stiffness.

Culturing chondrocytes in hydrogels, which maintains the cartilage phenotype, holds promise but poses its own limitation to studying mechanotransduction. Chondrocytes seeded in hydrogels are initially insensitive to mechanical compression, and only transduce mechanical signals upon synthesizing matrix of their own [83, 84]. The cell-derived matrix is difficult to penetrate with agents that can help identify and characterize the mechanotransduction pathway.

Altering hydrogel properties in an effort to examine chondrocyte response has been attempted in the past [75]. Polyethylene glycol hydrogels with varying water content, crosslinking density, and polymer degradability were shown to vary collagen II production, though not GAG content, after two weeks in culture [75]. However, these alterations in chondrocyte behavior can be attributed to many factors, not simply matrix interactions. Compression can change permeability, pH, and local charge density, influencing serum growth factor diffusion and other considerations.

Using a hydrogel modified to include the integrin adhesion ligand RGD, chondrocytes have been shown to attach [82] and sense the hydrogel matrix mechanics (chapter 1). A new system of altering matrix mechanics without introducing sources of error from increasing crosslinker concentration has been devised (chapter 2). With this system of variable-stiffness alginate discs, it will be possible to identify and isolate the

putative mechanotransductive element, integrins, and functionalize the matrix so the mechanical environment can be perceived by chondrocytes.

Because integrins are anchored to the cytoskeleton of the cell [103], [40], integrin-matrix attachment of cells has the potential to mechanically alter the matrix itself. Reports indicate fibroblasts have the ability to bend collagen fibrils [142], and cells have measurably contracted their substrates [143, 144]. Just as mechanical signals can modulate chondrocyte behavior, it is logical to expect chondrocyte adhesion to can modulate the mechanics of RGD-alginate.

In light of these considerations, the objective of this research was to examine the dynamic interactions between chondrocytes attached to a matrix via integrins. Through this integrin attachment, it was hypothesized cells will both sense and act on matrix mechanical properties. First, the stiffness of RGD-alginate discs seeded with increasing numbers of chondrocytes was examined. Secondly, RGD-alginate discs of different moduli were assayed with regards to their effect on chondrocyte matrix molecule production. Finally, the role of serum in modulating this matrix mechanosensation was explored.

Methods

Chondrocyte Cell Isolation

Articular cartilage was isolated as described previously [148]. Cartilage from the gleno-humeral and humero-ulnar joints of 1-2 week old calves (Research 87, Boston,

MA) was incubated for 12 hours in a solution of 0.3% collagenase Type 2 (Worthington, Lakewood, NJ) in F-12 media, 100 U / ml penicillin, 100 µg / ml streptomycin, and 250 ng / ml amphotericin B (GIBCO, Grand Island, NY) at 37°C. The digested cartilage solution was filtered in a 100 µm mesh, washed twice in PBS and resuspended in F-12 / 10% FBS media.

RGD Peptide / Alginate Bonding

Peptides containing the sequence GGGGRGDY were be covalently bonded to medium viscosity alginate (FMC) through a condensation reaction with mannuronic or guluronic acid residues as described previously [82]. Briefly, FMC 10/60 alginate was suspended in 100 mM MES (2-[N-Morpholino] ethanesulfonic acid) buffer with 300 mM NaCl at pH 6.5 to make a 1% w/v alginate solution. EDAC (1-ethyl-[dimethylaminopropyl] carbodiimide) was added at 5% molarity to that of the guluronate. Sulfo-NHS (N-hydroxy-sulfosuccinimide) was included at half the molarity of the EDAC. Finally, the octapeptide GGGGRGDY, in 100 mg/ml MES, was added at a ratio of 16.7 mg per gram of alginate. The reaction was allowed to proceed at room temperature for 20 hours before being transferred to 3500 MWCO dialysis tubing and placed in a 4L ddH₂O tank with 7 g NaCl / L. Every 8-12 hours, the salt concentration was lowered 1 g per liter, and the concentration was held at 0 g / L for 24 hours. The contents of the dialysis tube were transferred to a 50 mL conical, frozen, and lyophilized for 48 hours.

Alginate and RGD-alginate was resuspended to form a 2% (w/v) solution in PBS,

and sterilized with 0.45 μm syringe filters.

Thermal Degradation Protocol

2% alginate was hydrolyzed in PBS for periods up to 8 hours in a conventional oven. Every two hours, water was added to restore solution volume. The time required for solution to achieve boiling, approximately 15 minutes per 100 mL solution, was not counted towards heating duration.

Chondrocyte Disc Fabrication and Culture

Refrigerated 2% alginate was warmed and filter-sterilized as described above. Aliquots of 2 mL aliquots were mixed with centrifuged pellets containing 100 million chondrocytes (50 million cells / mL). The suspension was vortexed and mixed with 80 μL of a well-shaken CaSO_4 solution (Sigma, concentration: 0.266 g / ml PBS) via a 3-way stop cock as described previously [82]. The gel was quickly spread between sterile plates separated by 1 mm spacers. After allowing 15 minutes for the alginate to harden, the plates were separated. Using a biopsy punch, 4 mm diameter discs will be carved out of spread culture, and each disc was placed in 300 μL F-12 with 10% FBS, 25 mg/ml ascorbic acid, antibiotics and antimycotics at 37°C, 5% CO_2 .

The media was replaced every 48 hours. Selected discs were incubated in the presence of soluble RGD or RAD peptide (GRGESP or GRADSP) at 640 μM , or 0.1% BSA with blocking mAb to integrin subunit $\beta 1$ (10 μg / mL, Chemicon, Temeculah, CA).

Mechanical Testing

CaSO₄ discs were placed in uniaxial confined compression between an impermeable polymethacrylate surface and a porous polyethylene platen in a Dynastat mechanical spectrometer (IMASS, Hingham, MA). A series of ten steps in displacement, up to a maximum of approximately 20% total strain, were imposed on the samples and resultant forces were measured at a rate of 2.5 Hz for 30 sec / relaxation. Displacements and loads were normalized to sample geometry to yield values for strains and stresses. Stress relaxation curves were fit to a poroelastic model of hydrogel behavior to determine a compressive modulus at mechanical equilibrium [108].

GAG content assay

For assessment of proteoglycan and DNA content, discs were digested with 500 μ L of papain for 24 hours at 60°C. Aliquots of 50 μ L from the papain-digested chondrocyte-alginate discs were added to 950 μ L of 1,9-dimethylene blue (DMB) dye at pH 1.5. At this pH, DMB binds to the sulfate groups on GAG chains but not to the carboxyl groups on alginate [145]. Optical density was measured at 525 nm. GAG concentration was determined by quadratic interpolation with chondroitin-6-sulfate standards.

GAG synthesis assay

Four weeks after casting, 10 $\mu\text{Ci} / \text{mL}$ [^{35}S] SO_4 was added to the culture media for 24 hours to quantify proteoglycan synthesis. The discs were then taken from culture and washed six times with a PBS and Na_2SO_4 solution to remove unincorporated label. The discs were digested in papain and radioactivity was quantified by scintillation counting and normalized to DNA content (see below).

DNA content assay

Aliquots of 5 μL from the papain-digested chondrocyte-alginate discs were added to 195 μL of picoGreen dye solution (Molecular Probes, CA) in a 96-well plate [146]. The contents of the plate were excited at 485 nm and fluorescence was read at 535 nm on a HTS 7000+ spectrophotometer (Perkin-Elmer). DNA concentration was determined by linear interpolation with calf thymus DNA standards.

Results

To confirm cell attachment to the 3D RGD-alginate matrix, mechanical testing was performed. The presence of cells was found to stiffen RGD-alginate discs, compared to controls. This effect was significant at a concentration of 150 million cells / mL, after 24 hours of incubation (figure 18A). The normalized modulus exhibited a near twofold increase over unmodified alginate discs with the same concentration of cells.

With the addition of soluble RGD peptide to the culture media, the stiffening effect of 150×10^6 cell / mL RGD-alginate discs at 24 hours was prevented (figure 18B). Cytochalasin-D also prevented chondrocytes from stiffening the modulus in RGD-alginate.

Second, it was found that GAG accumulation was generally lower in RGD-alginate seeded discs. For every day over a five day period, chondrocytes in RGD-alginate discs had lower total GAG content compared to control alginate, as measured in GAG per mg (figure 19A) and GAG per cell (figure 19B).

This inhibition of synthesis in RGD-alginate discs was observed in long-term cultures as well (figure 19C). Sulfate incorporation rates were lower across initial seeding concentrations ranging from 50 to 150 million cells / mL.

This reduction in GAG accumulation in the presence of RGD-alginate was prevented if the RGD-alginate discs were cultured in the presence of soluble RGD peptide (figure 20A). Furthermore, in the presence of antibodies to the $\beta 1$ integrin subunit in the media, non-RGD discs exhibited a decrease in GAG content after 48 hours of culture (figure 20B).

The third principle finding of this research was a stiffness-dependent increase in GAG accumulation as disc modulus weakened. In contrast, GAG content in non-RGD discs did not significantly increase in 5 kPa discs, as compared to 50 kPa discs. In 5 kPa RGD discs, GAG content rose almost 40% over that of the stiffest, 50 kPa RGD discs. This relative increase in GAG content on weaker RGD discs was reversible in the presence of RGD peptide (figure 21A) or anti- $\beta 1$ antibody (figure 21B).

Fourth, the stiffness-dependent inhibition of GAG synthesis in RGD-alginate discs depended upon the presence of serum in incubation media (figure 22A). GAG content was relatively higher in weaker RGD-alginate discs when serum was present, but not when serum was absent (figure 22B).

Discussion

The principal findings of this research were fourfold: First, that chondrocytes attach to a 3D RGD-alginate matrix; Second, this cell-matrix attachment stiffens RGD-alginate discs and inhibits GAG accumulation; Third, the stiffness of the RGD-alginate hydrogel itself modulates chondrocyte production of GAG; and fourth, this matrix mechanosensation was found to depend on the presence of serum in the media.

An important conclusion in these experiments is that chondrocytes can stiffen their matrix by forming cytoskeletal attachments, mediated by integrins. The increase in modulus on RGD-alginate discs after 24 hours was due to integrin anchorage to the powerful actin cytoskeleton. This is consistent with an earlier report that RGD-alginate stiffens in the presence of cells, while unmodified RGD-alginate weakens with cells [147]. These findings also agree with indications that cells can cause collagen fibril bending [142], can exert forces on their surrounding matrix [143, 144], [148], and suggests that the actin rearrangement observed on RGD-alginate surfaces (chapter 1) may be involved in 3D discs.

Another conclusion of this study was that integrin binding site occupancy, whether from RGD-alginate or soluble RGD peptide, decreased GAG accumulation. The use of RGD peptide as an integrin agonist is with precedent. It has long been recognized that extracellular matrix components in the media can reduce proteoglycan synthesis [28]. RGD-containing fibronectin fragments, and RGD peptide by itself, have been shown to affect matrix metabolism ([149, 150]. Thus, the finding that GAG content per cell declines in RGD-alginate scaffolds, as well as in unmodified alginate discs cultured in media supplemented with soluble RGD peptide, is consistent with previous reports. Thus, it seems that oligopeptides containing RGD are, by themselves, sufficient to trigger integrin signaling.

The Chemicon antibodies to the $\beta 1$ integrin subunit used in these experiments behaved as classic antagonists. At a concentration used previously (chapter 1, [84]), the antibodies blocked cells from attaching to 2D RGD-alginate. Used here to disrupt 3D matrix interaction, GAG content was observed to rise to levels near that of GAG content in unmodified discs.

Previous investigations had led to the development of variable-stiffness alginate through hydrolysis of the polymer backbone (chapter 2). This method did not suffer the confounding effects of different crosslinker concentrations on cell culture (chapter 1), and provided a homogenous environment for assessing long-term chondrocyte behavior. The alginate or RGD-alginate discs developed through the hydrolysis protocol displayed a range of stiffness (5 kPa – 50 kPa) yet did not differentially affect chondrocyte viability or retention (data not shown).

Chondrocytes in RGD-alginate were more sensitive to changes in stiffness than cells in unmodified alginate. In weak 5 kPa discs, GAG accumulation in RGD-alginate discs was greater than in 50 kPa RGD-alginate discs. Furthermore, the increase in GAG content between the weak and stiff RGD-alginate discs was almost 40% – greater than the difference between weak and stiff unmodified alginate discs. In the presence of soluble RGD peptide blockers, matrix attachment was disrupted and sensitivity to mechanics in RGD-alginate discs was lost, resulting in GAG levels similar to those in unmodified alginate.

Matrix rigidity has been shown to modulate cell behavior in the past, both with chondrocytes on RGD-alginate (chapter 1) and in other systems in which fibroblasts have been shown to migrate to stiffer substrates [37, 38]. On less flexible substrates, fibroblasts were seen to exhibit more linearly arrayed focal adhesions [39]. Other reports also note that matrix rigidity organizes focal adhesions, phosphorylation state, and cytoskeletal organization [151, 152]. The data described here suggests cytoskeletal reorganization and matrix modulation occur as a consequence of matrix attachment.

Another possibility is that the increased GAG content of chondrocytes in less-stiff discs was not due to mechanosensation but rather to increased nutrient diffusion, or a more favorable chemical environment. Some reports indicate that decreasing alginate stiffness can increase diffusion of molecules [153]. However, the lack of a significant change in weak unmodified alginate discs reveals the importance of matrix attachment.

Serum components do show dependence on cell-matrix adhesion, according to these results. When chondrocytes in RGD-alginate discs were cultured in serum-free

media, the increase in GAG content as stiffness declined was eliminated. This is consistent with reports suggesting that integrin signal transduction depends on co-stimulation with IGF-1 or other hormones [153-156].

These findings suggest chondrocytes in healthy cartilage exist in equilibrium with the extracellular matrix, both stiffening the matrix via cytoskeletal attachment, and responding to stiffness by modulating GAG content. The degeneration seen in osteoarthritis, then, can be interpreted as a disruption of this steady state.

In the early stages following joint instability or mechanical trauma, articular cartilage has been found to thicken and exhibit increased proteoglycan content [140], [141], before ultimately thinning. The mechanical stiffness of articular cartilage, on the other hand, declines steadily after injury [141], [122], [157]. In light of these clinical findings, the results described in these experiments take on additional relevance: Similarly to native cartilage, chondrocytes in RGD-alginate respond to weaker substrates with increased GAG accumulation. It had been previously proposed that chondrocytes modulate their metabolism in response to pericellular proteoglycan density [158], but this model suggests chondrocytes are responding to matrix stiffness.

The RGD-alginate culture system can demonstrate the nature of the chondrocyte response to the mechanical properties of the surrounding matrix, permitting analyses not possible with unmodified alginate or native cartilage. Furthermore, exploration of the response to mechanical loading is possible, as well.

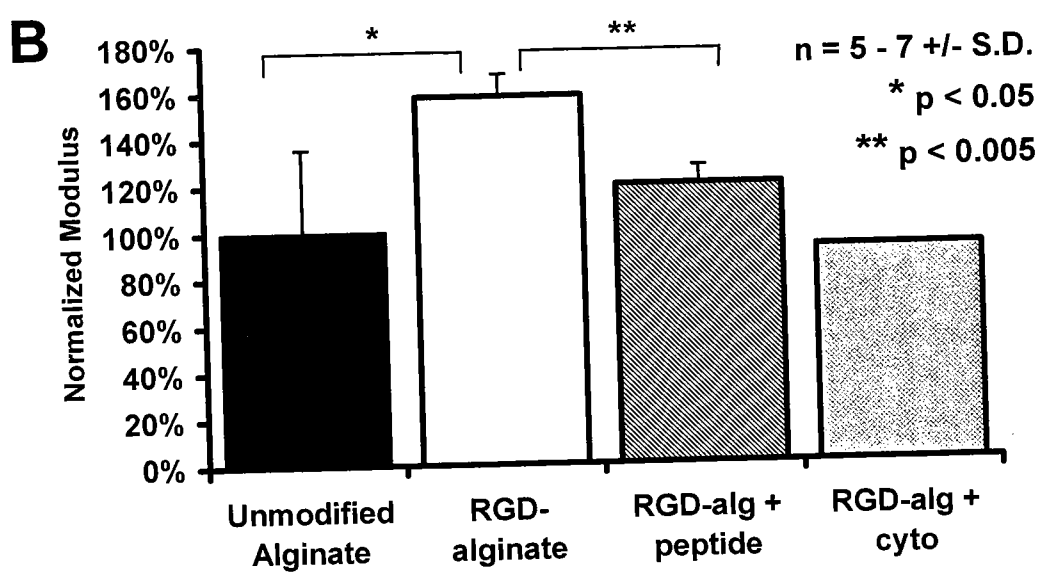
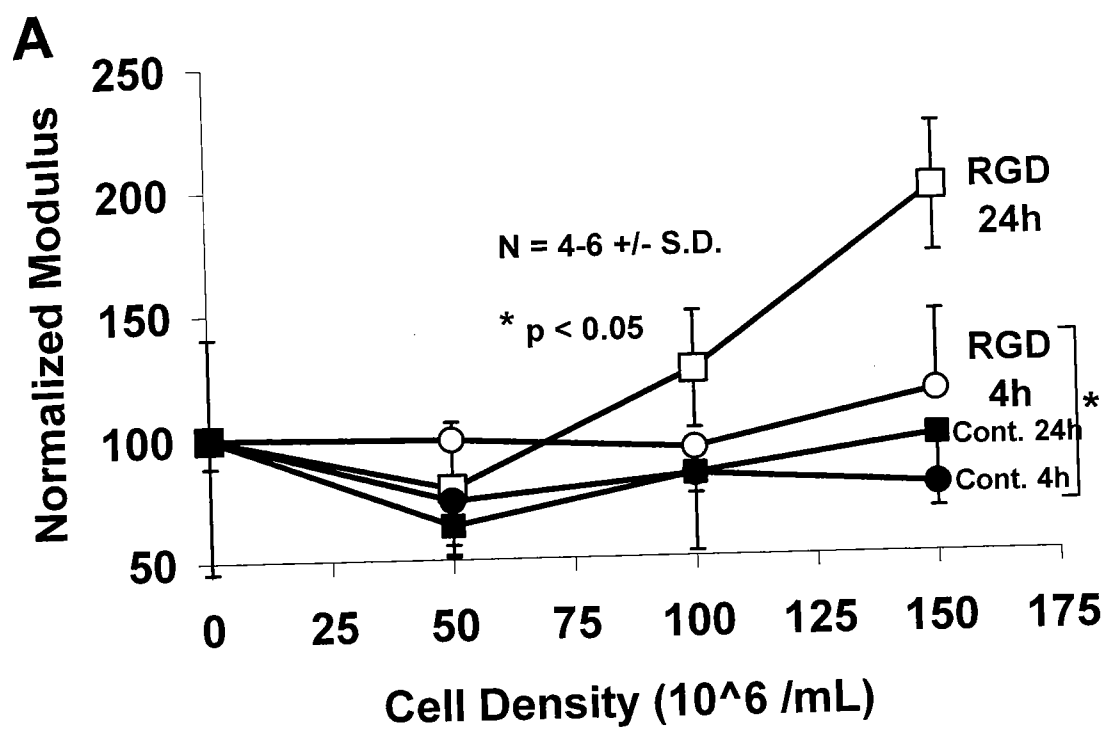


Figure 18. Cells stiffened RGD-alginate via integrin attachment. **A:** As seeding density and time in culture increased, cells stiffened RGD-alginate discs significantly (t-test with Bonferroni correction). **B:** RGD-alginate discs seeded with chondrocytes at a concentration of 150×10^6 cells / mL were significantly stiffer than those in unmodified (control) alginate, or those in RGD-alginate incubated in the presence of $640 \mu\text{M}$ RGD peptide competitor (t-test with Bonferroni correction).

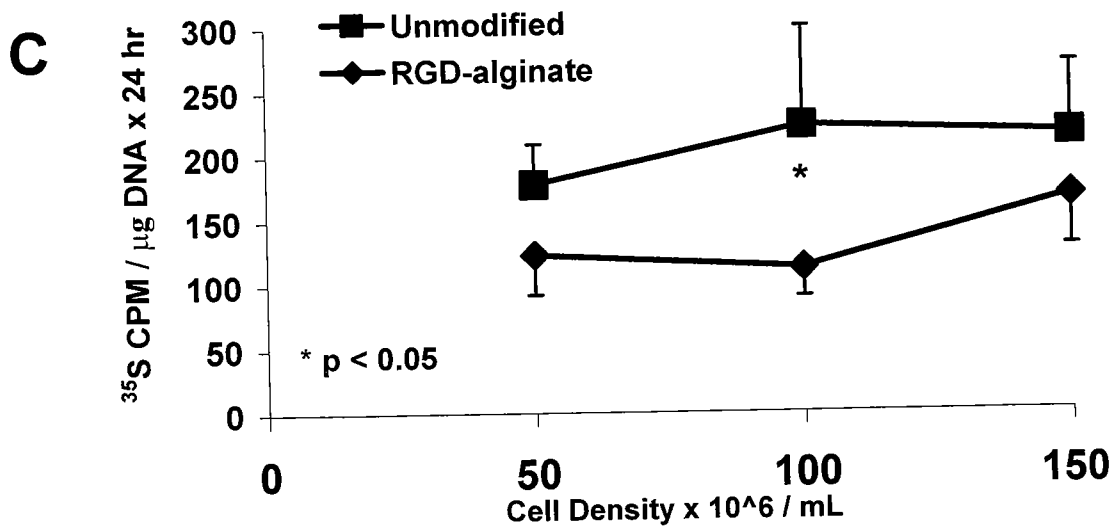
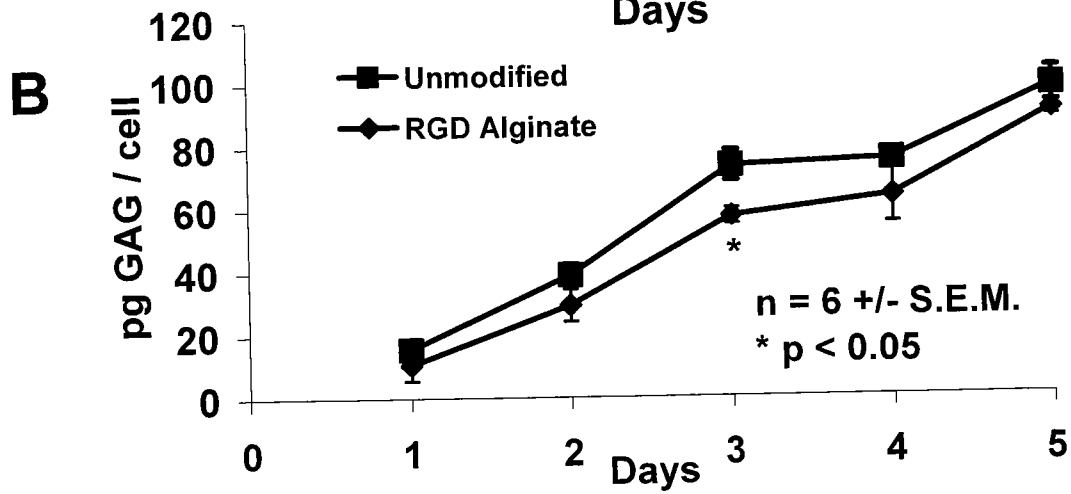
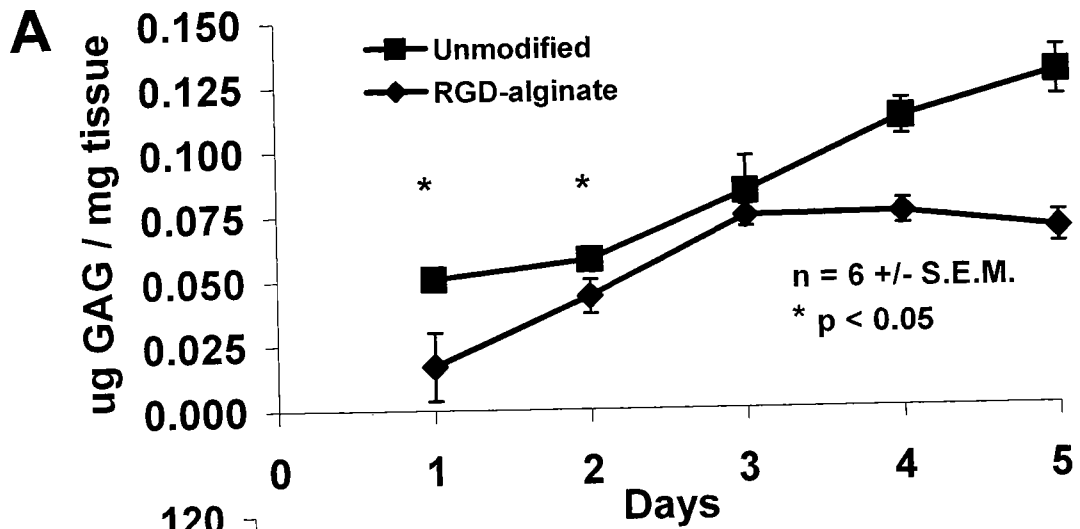


Figure 19. GAG synthesis in chondrocytes in RGD-alginate is inhibited. **A:** Total GAG content per disc over five days of culture in RGD-alginate and unmodified (control) alginate discs. **B:** GAG content per cell over five days of culture. **C:** Radiolabeled sulfate incorporation after four weeks of culture – at a density of 100 million cells / mL, RGD-alginate causes a significant decline in [³⁵S] SO₄ uptake (significance was determined with a two-way ANOVA with Bonferroni's post-hoc pairwise comparison).

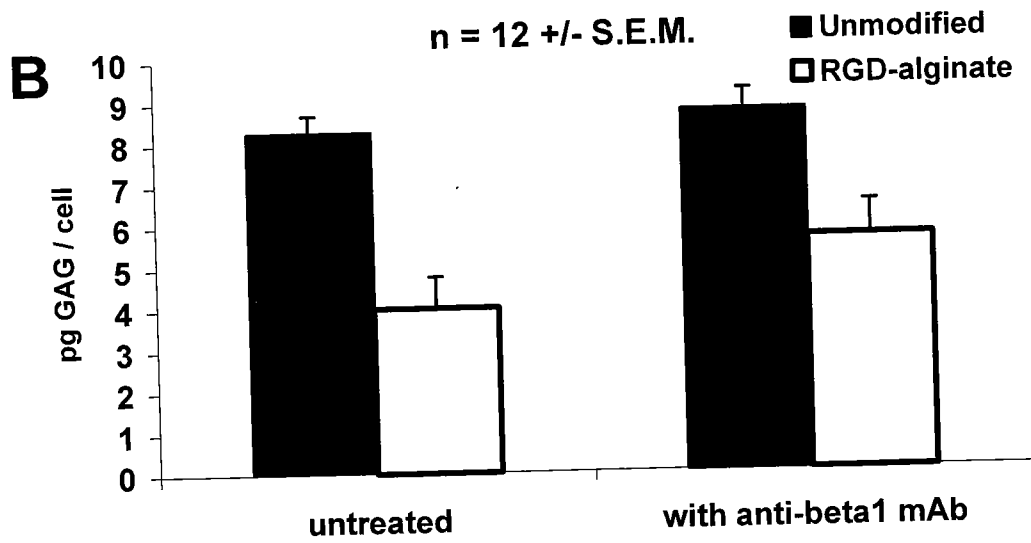
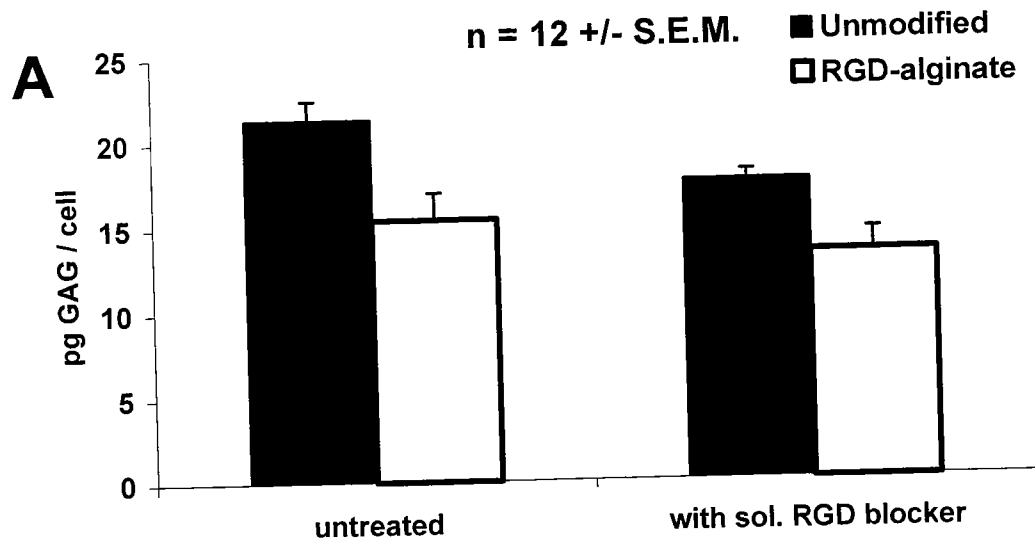


Figure 20: Glycosaminoglycan content per chondrocyte in unmodified and 270 μM RGD-alginate, with and without soluble RGD peptide agonist (640 μM) or anti- $\beta 1$ mAb antagonist (10 $\mu\text{g} / \text{mL}$). **A:** Soluble RGD peptide inhibited GAG accumulation in chondrocytes seeded in unmodified alginate. The soluble RGD acted similarly to RGD-alginate, inhibiting GAG production. **B:** in a separate experiment, mAb against $\beta 1$ integrin reduced the inhibition of GAG synthesis of chondrocytes in RGD-alginate. The anti-integrin antibodies disrupted the inhibitory effect of RGD on GAG accumulation.

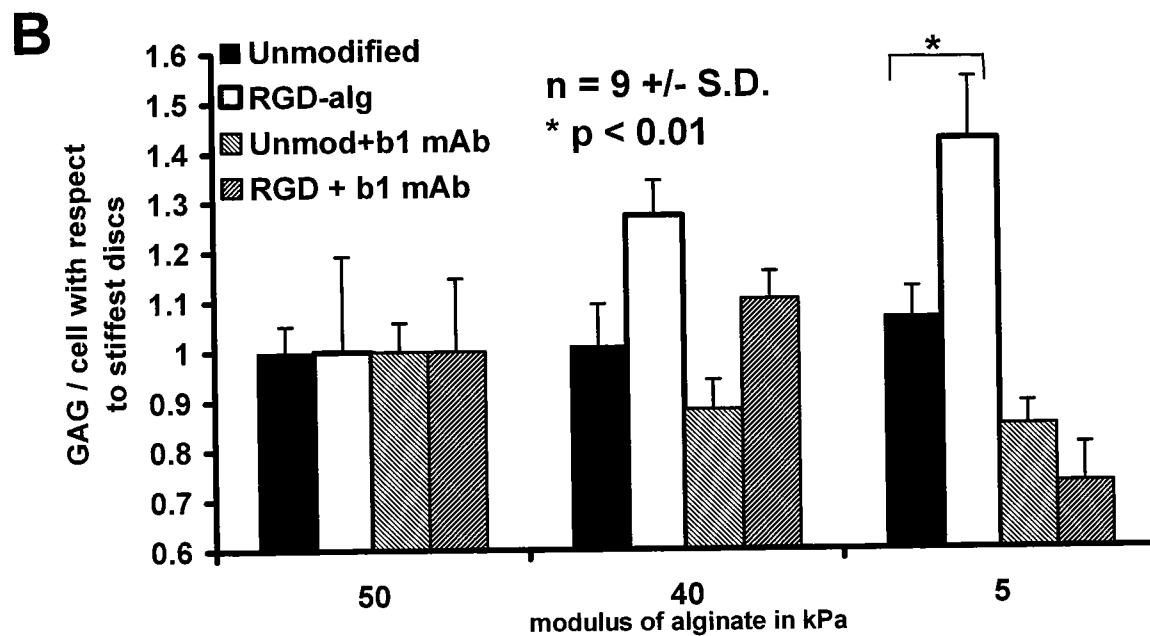
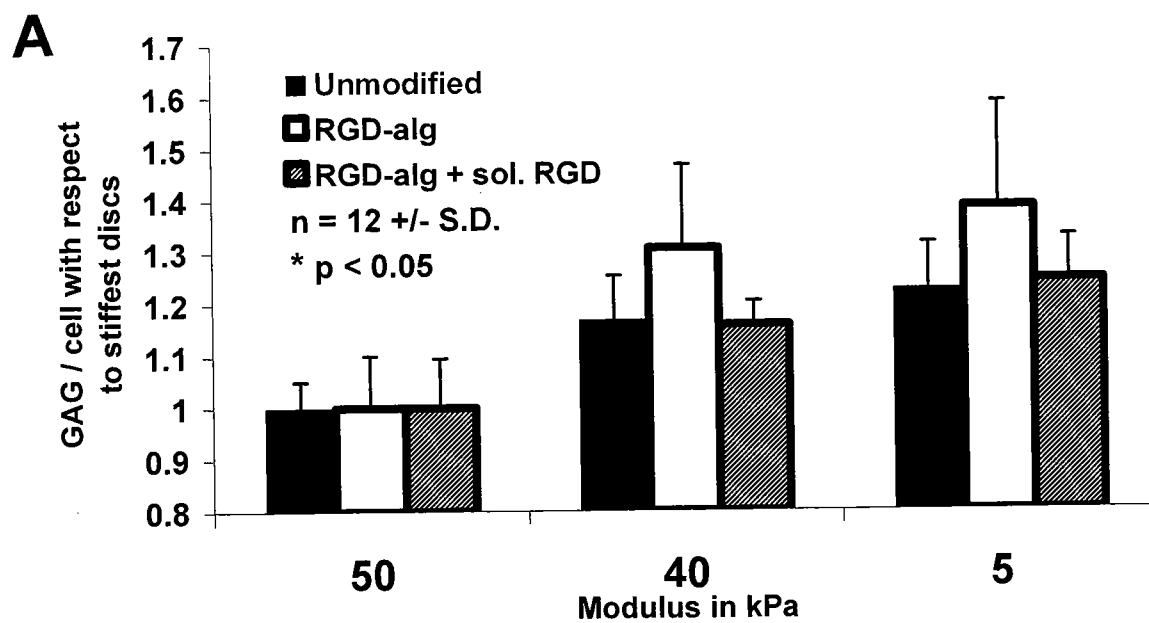


Figure 21: Chondrocytes were seeded in unmodified or RGD-alginate discs of variable stiffness (5-50 kPa). Weaker RGD-alginate discs had the greatest GAG content per chondrocyte, with respect to the stiffer unmodified and RGD-alginate discs. **A:** The increase in GAG content in RGD-alginate discs was reversible in the presence of soluble RGD peptide competitor; disrupting chondrocytes' attachment to RGD-alginate brought GAG accumulation down to that of unmodified alginate (control) levels. **B:** In the presence of the mAb anti- $\beta 1$, the increase in GAG synthesis in weak RGD discs was similarly reversed.

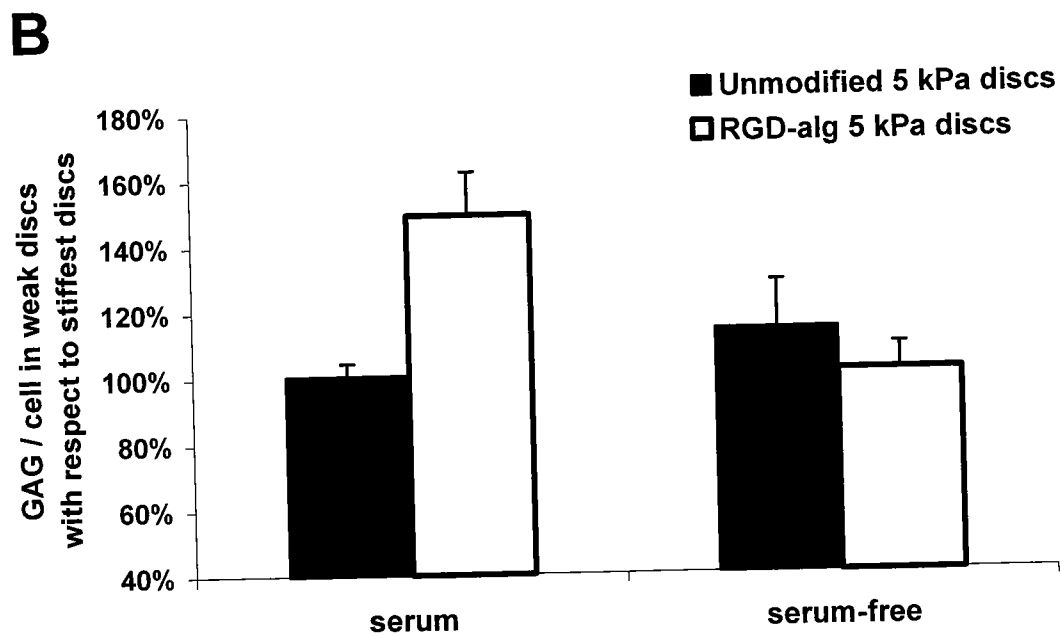
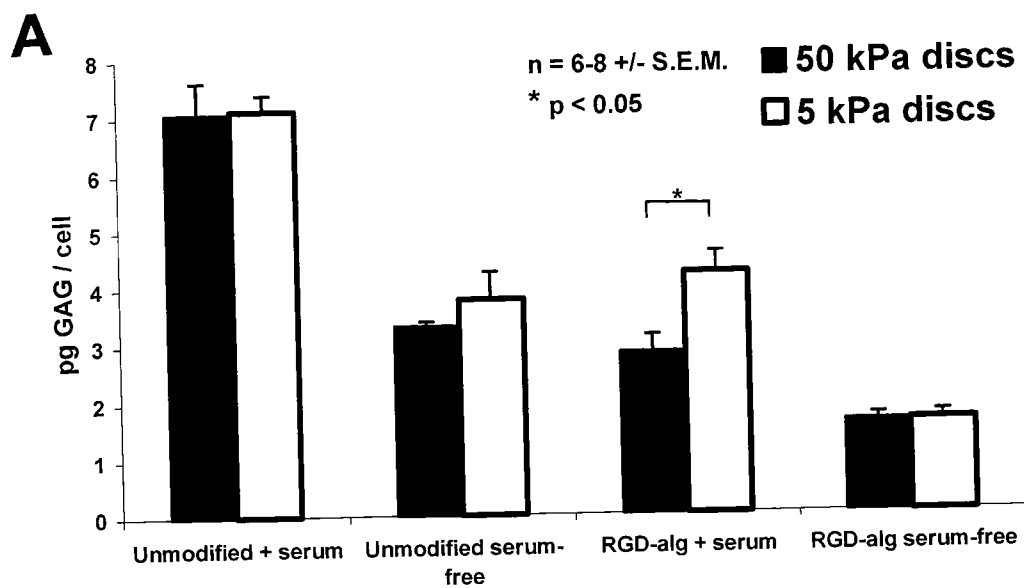


Figure 22: The effect of disc stiffness on GAG content in RGD-alginate was dependent on serum in the culture media. **A:** The presence of serum did not affect the GAG content of cells in unmodified alginate discs, regardless of stiffness. However, GAG content was significantly higher in weak (5 kPa) RGD-alginate discs in the presence of serum, compared to the stiffest (50 kPa) RGD-alginate. Without serum, this increase in GAG content was absent. The data is significant ($p < 0.05$) by three-way ANOVA using Bonferroni's test for post-hoc pairwise comparisons. **B:** The data from panel A was normalized with respect to the stiffest (50 kPa) discs, illustrating the relative increase in GAG content on weak (5 kPa) discs was limited to RGD-alginate discs cultured in the presence of serum.

CHAPTER IV

CHONDROCYTE MECHANOTRANSDUCTION IN RGD-BONDED ALGINATE

Abstract

Cartilage matrix synthesis decreases in response to static compression [24, 25, 155], although the mechanism by which this occurs is unknown. Several studies document that isolated chondrocytes respond differently to compression when cultured in hydrogels such as alginate or agarose [83, 84]. This sensitivity to compression occurs after sufficient time for chondrocytes to produce their own pericellular matrix [83, 84], suggesting that connections to the ECM are involved in the mechanotransduction process. This hypothesis is difficult to test, since native cartilage ECM hinders access of agents that block attachment to cells, and since gels that are commonly used to culture chondrocytes do not enable cell attachment. Recently, the alginate hydrogel, modified to contain the integrin adhesion peptide RGD, has been used as a substrate for myoblasts [82] and chondrocytes (chapter 1). The ability of cells to attach directly to these modified gels allows for the assessment of the role of cell attachment in the response to mechanical stimuli. Therefore, the objectives of this study were to examine the effect of RGD

modification on the response of chondrocytes to compression and to determine the extent to which this effect can be blocked, first by competitive inhibitors, and second by anti-integrin blocking antibodies.

Introduction

Chondrocytes govern matrix metabolism in response to environmental cues. Since cartilage is not vascularized or innervated, signals from the extracellular matrix (ECM) itself are particularly important.

It is believed that abnormal mechanical loading of the ECM signals changes in chondrocyte matrix metabolism that lead to cartilage degeneration and osteoarthritis [18-20] [4]. Clinical observations correlate with studies on cartilage explants, which show that static compression reduces proteoglycan and collagen synthesis [24, 159, 160]. The mechanism of this mechanotransduction, however, is difficult to elucidate, given the density of the ECM and relative scarcity of chondrocytes.

What is known is that early changes in osteoarthritic cartilage include a variety of changes in chondrocyte metabolism. The cell morphology becomes more spread and fibroblastic [4, 63], integrin expression changes [51], proteoglycan synthesis initially rises, then falls [13, 98, 122] and matrix metalloproteinase activity rises [13, 141]. These changes correlate with the declining matrix stiffness and eventual erosion and loss of tissue that is the hallmark of OA.

Several cell culture techniques have been employed to examine chondrocyte mechanotransduction, though none has proved completely suitable. Applying mechanical loads to monolayer cultures [21, 53, 54] allow the use of tools for pathway characterizing the mechanotransductive pathway, but prohibits physiological loading and discourages expression of the cartilage phenotype.

Culturing chondrocytes in hydrogels such as agarose or alginate preserves the expression of cartilage-specific matrix molecules, but has its own limitations. Chondrocytes seeded in hydrogels are initially insensitive to mechanical loads, and only transduce loads upon synthesizing a matrix of their own [83, 84]. The cell-derived matrix is difficult to penetrate with agents that can help identify and characterize the mechanotransduction pathway.

Many studies have, however, implicated integrins as putative mechanotransducers in chondrocytes [53, 54, 84]. Integrins are already known to signal mechanical events in a variety of tissue types [31, 161]. These transmembrane receptors attach not only to matrix molecules but to the actin cytoskeleton as well, triggering changes in cell conformation, metabolism and division [162, 163]. The tripeptide RGD (arginine-glycine-aspartate), present in many ECM molecules, is an integrin receptor ligand for several integrin subunits expressed on chondrocytes [8, 89, 91, 164].

Using alginate modified to include the integrin adhesion ligand RGD, chondrocytes have been shown to attach [102] to alginate and sense the hydrogel matrix mechanics (chapter 1, chapter 3). It was the hypothesis of this research that chondrocytes seeded within RGD-modified alginate will enhance sensitivity to externally applied

strains. This sensitivity to compression is enabled by chondrocyte adhesion to RGD-alginate via integrins.

Compression affects chondrocytes in a matrix in several ways. Changes in cell and nucleus conformation, membrane potential, and physicochemical environment may occur with compression, regardless of matrix adhesion [44, 165]. In RGD-alginate, compression should increase the ligand density perceived by the cell, and introduces the possibility of indirect deformation via cytoskeletally-bound matrix receptors like integrins.

Furthermore, based on evidence that dynamic compression increases fluid flow to chondrocytes in hydrogels, and static compression decreases flow [26, 166], it is logical to suggest that static compression would affect the cell response to serum growth factors in RGD-alginate. Some serum components have been reported to interact with integrin adhesion ligands in a dependant costimulatory fashion [7, 156, 167]. This interaction will be studied in chondrocytes seeded in RGD-alginate discs under compression.

Methods

Chondrocyte Cell Isolation

Articular cartilage was isolated as described previously [148]. Cartilage from the gleno-humeral and humero-ulnar joints of 1-2 week old calves (Research 87, Boston, MA) was incubated for 12 hours in a solution of 0.3% collagenase Type 2 (Worthington,

Lakewood, NJ) in F-12 media, 100 U / ml penicillin, 100 μ g / ml streptomycin, and 250 ng / ml amphotericin B (GIBCO, Grand Island, NY) at 37°C. The digested cartilage solution was filtered in a 100 μ m mesh, washed twice in PBS and resuspended in F-12 / 10% FBS media.

RGD Peptide / Alginate Bonding

Peptides containing the sequence GGGGRGDY were be covalently bonded to medium viscosity alginate (FMC) through a condensation reaction with mannuronic or guluronic acid residues as described previously [82].

Briefly, FMC 10/60 alginate was suspended in 100 mM MES (2-[N-Morpholino]ethanesulfonic acid) buffer with 300 mM NaCl at pH 6.5 to make a 1% w/v alginate solution. EDAC (1-ethyl-[dimethylaminopropyl] carbodiimide) was added at 5% molarity to that of the guluronate. Sulfo-NHS (N-hydroxy-sulfosuccinimide) was included at half the molarity of the EDAC. Finally, the octapeptide GGGGRGDY, in 100 mg/ml MES, was added at a ratio of 16.7 mg per gram of alginate. The reaction was allowed to proceed at room temperature for 20 hours before being transferred to 3500 MWCO dialysis tubing and placed in a 4L ddH₂O tank with 7 g NaCl / L. Every 8-12 hours, the salt concentration was lowered 1 g per liter, and the concentration was held at 0 g / L for 24 hours. The contents of the dialysis tube were transferred to a 50 mL conical, frozen, and lyophilized for 48 hours.

Alginate and RGD-alginate was resuspended to form a 2% (w/v) solution in PBS, and sterilized with 0.45 μ m syringe filters.

Chondrocyte Disc Fabrication

Refrigerated 2% alginate was warmed and filter-sterilized as described above. Aliquots of 2 mL aliquots were mixed with centrifuged pellets containing 100 million chondrocytes (50 million cells / mL). The suspension was vortexed and mixed with 80 μ L of a well-shaken CaSO_4 solution (concentration: 0.266 g / ml PBS) via a 3-way stopcock as described previously [82]. The gel was quickly spread between sterile plates separated by 1 mm spacers. After allowing 15 minutes for the alginate to harden, the plates were separated. Using a biopsy punch, 4 mm diameter discs were carved out of spread culture, and each disc was placed in 300 μ L F-12 with 10% FBS, 25 mg/ml ascorbic acid, antibiotics and antimycotics at 37°C, 5% CO_2 .

Static Compression

Unmodified and RGD-alginate discs seeded with primary chondrocytes were placed in static compression chambers [155]. Discs were placed into wells with 300 μ L of F-12 media with 10% serum and, using spacers, were subjected to 0-0.5 mm uniaxial unconfined displacement with 4 mm-diameter plungers for 48 hours. This displacement compressed the 1 mm-thick discs from 0% to 50% of their original thickness. Some chambers were not equipped with displacement prongs, allowing free swelling of the discs.

Selected discs were incubated in the presence of soluble RGD or RAD peptide (GRGESP or GRADSP) at 640 μM , or 0.1% BSA with blocking mAb to integrin subunit $\beta 1$ 10 $\mu\text{g} / \text{mL}$ (Chemicon, Temeculah, CA).

GAG assay

For assessment of proteoglycan and DNA content, discs were digested with 500 μL of papain for 24 hours at 60°C. Aliquots of 50 μL from the papain-digested chondrocyte-alginate discs were added to 950 μL of 1,9-dimethylene blue (DMB) dye at pH 1.5. At this pH, DMB binds to the sulfate groups on GAG chains but not to the carboxyl groups on alginate [145]. Optical density was measured at 525 nm. GAG concentration was determined by quadratic interpolation with chondroitin-6-sulfate standards.

DNA Assay

Aliquots of 5 μL from the papain-digested chondrocyte-alginate discs were added to 195 μL of picoGreen dye solution (Molecular Probes, CA) in a 96-well plate based on methods described previously [146]. The contents of the plate were excited at 485 nm and fluorescence was read at 535 nm on a HTS 7000+ spectrophotometer (Perkin-Elmer). DNA concentration was determined by linear interpolation with calf thymus DNA standards.

Statistics

The normalized GAG synthesis from chambers with increasing compression thickness was analyzed by a t-test with Bonferroni's correction (figure 23). The individual experiments involving 10% compression and soluble RGD peptide (figure 24), anti-integrin antibodies (figure 25), or serum-free media (figure 26) were analyzed with three-way ANOVA and Bonferroni's post hoc pairwise comparisons.

Results

Chondrocytes in unmodified (control) alginate and in RGD-alginate responded to macroscopic gel compression, although the responses were different (figure 23). Chondrocytes in RGD-alginate decreased total GAG content in response to compression. The change in GAG content was significantly different from that of chondrocytes in unmodified alginate discs at compression thicknesses between 0 mm (in which discs were kept at initial thickness, preventing free swelling) and 0.25 mm (25% nominal strain).

For RGD-alginate seeded discs, a compression thickness of 0 mm (maintaining initial casting thickness) led to decreases of GAG / cell of 29% compared to uncompressed discs. Compression thicknesses of 0.1 mm and 0.25 mm lead to a 39% and 42% decrease in GAG content, respectively, compared to uncompressed discs.

The difference in response to compression was most pronounced at 10% nominal strain, so subsequent studies involving adhesion blockers were conducted at this level.

Analysis of an experiment in which chondrocytes in alginate were exposed to this strain (i.e., 0.1 mm compression thickness, figure 24) revealed that RGD-alginate seeded discs had less GAG content per cell than unmodified alginate discs. This finding was anticipated from previous observations of chondrocyte behavior in RGD-alginate (chapter 3). Additionally, the imposed 10% strain significantly lowered GAG content in RGD-alginate. Chondrocytes in unmodified alginate, however, did not significantly adjust GAG production in response to trials involving 0.1 mm compression.

In the presence of soluble RGD peptide competitor, the metabolic response to 0.1 mm compression thickness (10% nominal strain) was abolished in RGD-alginate discs. It was also noted that free unmodified alginate discs in RGD peptide experienced a significant decrease in GAG content compared to free unmodified alginate discs in normal media, consistent with the observation that RGD acts as an integrin agonist but disrupts mechanical signals from the scaffold matrix (chapter 3).

A similar phenomenon was observed in a separate experiment involving 0.1 mm displacement and anti- β_1 antibodies (figure 25). The presence of β_1 integrin blocking antibody increased GAG synthesis by chondrocytes in RGD-alginate, but did not affect GAG synthesis in control alginate (figure 25). As in previous experiments, in the absence of blocking antibody, the decrease in compression-induced GAG synthesis was ~10% greater in RGD-alginate culture. In the presence of β_1 integrin blocking antibody, there was no difference in the response to compression between control and RGD-alginate.

Furthermore, compared to GAG in unmodified alginate, a compression of 0.1 mm had less of an effect on RGD-alginate discs in the presence of anti- β_1 antibodies. GAG

content per cell in RGD-alginate discs fell from 59% of unmodified discs to 51% under 0.1 mm displacement. However, in the presence of anti- β 1 antibodies, the GAG content in RGD discs remained approximately 73% of that of unmodified discs, regardless of whether compression was applied. In other words, the presence of anti-integrin antibodies abolished the RGD-dependent decrease in GAG content due to compression.

Finally, it was found that the difference in GAG content between two day-old chondrocyte-seeded RGD-alginate and unmodified alginate discs was abolished when 10% fetal bovine serum was excluded from the media (figure 26A). Under compression, however, the difference in GAG content between serum-free RGD-alginate discs and serum-free unmodified discs is more profound than in media with serum (figure 26B). By normalizing GAG content to serum-free levels under compression, it is more apparent that, in the presence of serum, there is relatively more GAG in RGD-alginate discs under compression than in unmodified discs. Thus, the presence of serum in media can be said to increase the GAG content in RGD-alginate discs by 40% under 0.1 mm of compression, with respect to GAG content in RGD-discs in serum-free media. The GAG content in unmodified alginate discs under compression was not affected by the presence of serum.

Discussion

This study demonstrated that the response of chondrocytes to compression is altered by the modification of alginate with an RGD peptide. Chondrocytes seeded into

RGD-modified alginate showed increased sensitivity to anti-anabolic effects of static compression, particularly at low strains, as reflected by significant decreases in GAG content (figure 23). The sensitivity to compression was abolished in the presence of soluble RGD peptide agonist (figure 24) and anti- β_1 integrin antibody antagonist (figure 25). Finally, the response to serum by chondrocytes under compression was found to depend on the presence of adhesion to RGD-alginate (figure 26).

These results suggest a clear role for integrins in mediating the response to chondrocytes in compression. The findings presented here are in agreement with cartilage explant studies that indicate GAG synthesis is decreased under compression [24, 25, 156], and with studies illustrating the role of integrins in mechanotransduction [9, 21, 84]. Culturing chondrocytes in RGD-alginate under compression represents a unique approach for the study of these fields, and has additional versatility for examining other aspects of the chondrocyte response to mechanics (chapter 1, chapter 3).

The fact that the compression-induced effect on metabolism appears to be dependent on serum components suggests there is a combined signaling that requires the engagement of integrins as well. The implication is that integrin-binding and compression-induced integrin signaling can modify the reaction to growth factors contained in serum. This is consistent with other findings that that IGF-1 stimulation and integrin stimulation are linked [154, 168, 169], and echoes reports that mechanotransduction is influenced by IGF-1 or other hormones [154, 156].

It has long been suspected that compression affects the response to growth factors because of limited nutrient diffusion, both in cartilage explants [166, 170, 171] and

hydrogels [26], but these results instead suggest that costimulation with integrins is responsible.

Compression affects many aspects of the chondrocyte-matrix environment, including nutrient diffusion, fluid flow, and local charge density [44, 165]. Thus, it is not surprising that chondrocytes in unmodified alginate were affected by static compression (figure 23). Even in the absence of integrin-mediated matrix attachment, these compression-induced changes in environment – independent of integrin adhesion – contributed to a decrease in GAG production by the cell, most notably at 50% strain.

The largest difference between RGD-alginate discs and unmodified control discs occurred at low compressions – suggesting different mechanisms for metabolic regulation exert influence at different levels of strain. Integrin adhesion regulated the sensitivity to compression for strains of 0% to 25% of cast thickness, and non-adhesion related factors regulated sensitivity to compression at 50% strain. This notion is supported by earlier findings in which unattached chondrocytes nonetheless responded to changes in hydrogel mechanics [74, 75].

The adhesion-dependent effect of 0 mm of compression of GAG content was likely due to disc swelling (at 0 mm, prongs keep the discs at cast thickness and preventing free swelling). Though identical compression was imposed on RGD-alginate discs and unmodified discs, there is the possibility that swelling in the two disc varieties was unequal, causing different perceived strains and different metabolic responses. However, an analysis of the disc weights after culture showed no notable difference, indicating similar water content and swelling (data not shown).

In the past, the differences in proteoglycan metabolism in free swelling discs compared to discs held at cast thickness have been attributed to nutrient transport. The results described here – different GAG content in samples with the same geometry – suggests that nutrient diffusion is not the dominant regulator for GAG synthesis in chondrocytes seeded in RGD-alginate discs.

By examining the effect of compression on net GAG content, the competing influences of matrix synthesis and degradation were summed. There is the possibility, for instance, that GAG content is lower in RGD-alginate discs, not because of any change in proteoglycan synthesis, but because of an increase in MMP activity. If this were the case, digested GAG could be released from the disc to the media and lost. Assaying the culture media for GAG, or assaying MMP activity could further examine this possibility. Studies have shown a compression-dependent increase in MMP activity in monolayer [150] but, as stated above, the elucidating the mechanism of matrix metabolism is beyond the current scope but worthy of future consideration.

Another finding of the current study was that any integrin binding site occupancy, whether from RGD-alginate or soluble RGD peptide, decreased GAG accumulation. The use of RGD peptide as an integrin agonist is with precedent. It has long been recognized that extracellular matrix components in the media can reduce proteoglycan synthesis [28]. Fibronectin fragments, and RGD peptide itself, have by itself been shown to decrease matrix metabolism [149, 150]. Thus, the finding that GAG content per cell declines in RGD-alginate is consistent with these previous reports. Also, GAG content per cell in unmodified RGD-alginate discs declined in media supplemented with soluble RGD

peptide. Thus, it seems that oligopeptides containing RGD are by themselves sufficient to trigger integrin signaling.

RGD integrin signaling alone results in an increase of catabolic enzymes such as MMPs, stromelysin, and others [150, 172], inhibiting matrix accumulation. The further inhibition of GAG accumulation under compression may be due to the increase in RGD ligand density perceived by the cell. Alternatively, compression may cause permanent matrix and cell deformation, triggering changes in gene expression of attached cells [173, 174].

Though the precise mechanism is still not clear, this study demonstrated that the response of chondrocytes to compression is altered by matrix adhesion. Though many characteristics of the matrix environment change with compression, the involvement of integrin attachment has now been shown to significantly modulate cell metabolism.

RGD-alginate represents a unique system to examine the role of chondrocyte attachment via integrins in the response to physiological mechanical compression. Future work should focus on the characterization of individual steps in the integrin signaling and steps regulating matrix synthesis and catabolism. RGD-alginate facilitates such studies and thus is a desirable system for studying chondrocyte mechanotransduction.

Future studies employing this system will employ dynamic compressions regimes to chondrocytes in RGD-alginate. In cartilage explants, dynamic compression has been shown to increase GAG and collagen synthesis [159, 199].

Furthermore, previous studies on cartilage explants were limited because the stresses perceived by the samples could not be calculated due to the confounding effect of

strain. By applying the methods used to construct variable-stiffness RGD-alginate discs (chapter 2), the potential exists for decoupling the effects of stress and strain on the metabolic activity of chondrocytes.

Finally, an examination of how compression changes the availability and efficacy of serum growth factors in regulating cell metabolism should be undertaken. The data in this research suggests serum component(s) are responsible for a co-stimulatory effect with RGD. Adding individual serum components, such as IGF-1, to seeded RGD-alginate discs in serum-free media, will reveal which molecules are acting in conjunction with integrins to activate this mechanotransductive pathway.

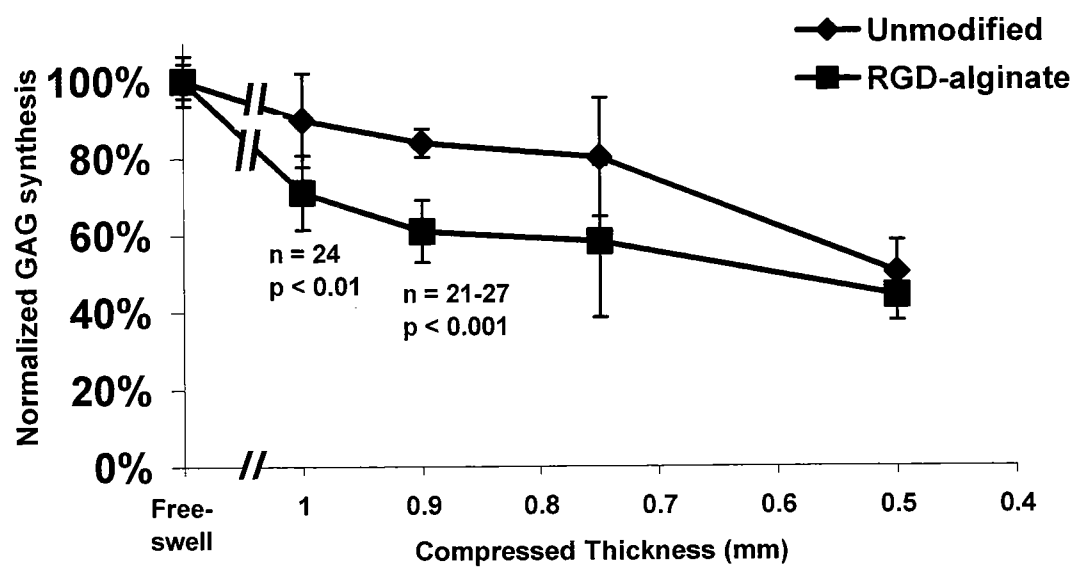


Figure 23. Chondrocytes seeded in control and RGD-alginate discs of 1 mm initial thickness were allowed to swell freely, or compressed to variable final thicknesses (1 mm – 0.5 mm, or 0% to 50% nominal strain). GAG content was found to respond to disc compression, with respect to free-swelling discs. The decrease in GAG content was significant in RGD-alginate discs compressed to a thickness of 1.0 mm and 0.9 mm, compared to unmodified alginate discs, as shown by multiple t-tests with Bonferroni correction. GAG content in unmodified alginate discs under compression showed no significant decrease from free-swelling discs until a compression thickness of 0.5 mm (50% nominal strain) was achieved.

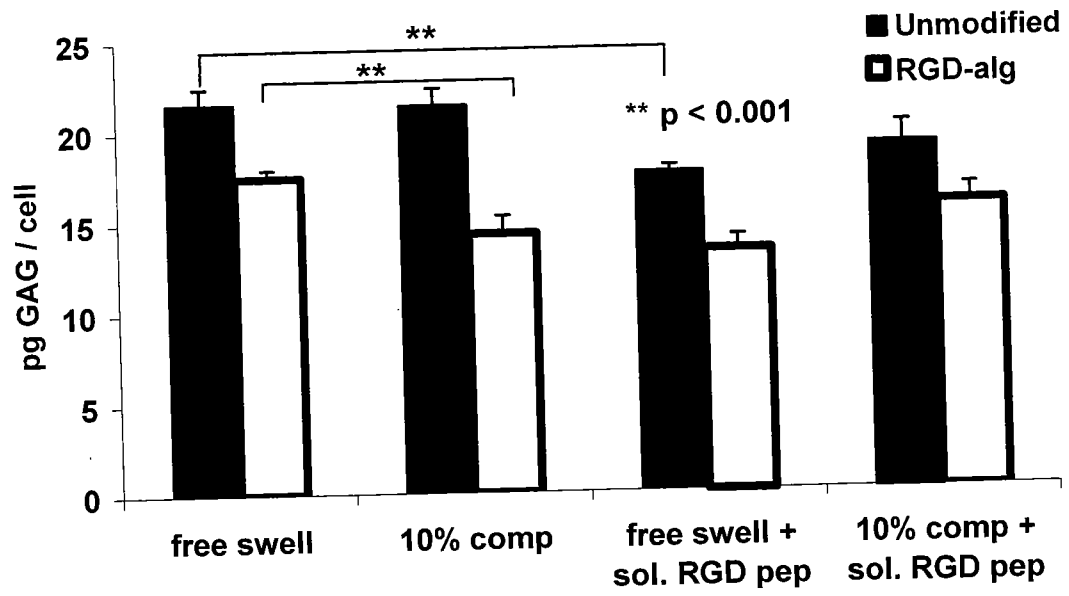


Figure 24. Soluble RGD peptide disrupted mechanical signaling in RGD-alginate discs.

The inhibitory effect of RGD-alginate on GAG content was enhanced when discs are compressed 10% (0.9 mm final thickness), while GAG content in unmodified (control) alginate remained unchanged with compression.

In the presence of 640 μM soluble RGD peptide agonist, GAG production in unmodified alginate was inhibited (similarly to the inhibitory effect of 270 μM RGD-alginate). Compression, however, did not further decrease GAG content in discs exposed to soluble RGD peptide. This was true for both unmodified and RGD-alginate discs. The presence of soluble RGD peptide agonist alone caused some inhibition of GAG synthesis, but disrupted the mechanical signals that would have led to further decreases in RGD-alginate discs.

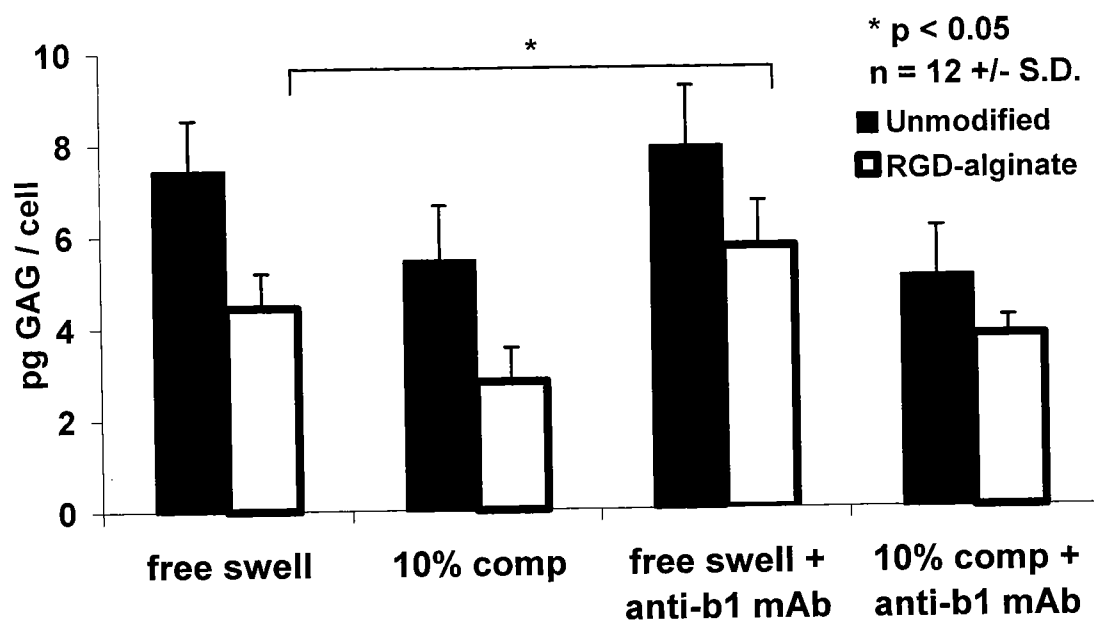


Figure 25. Anti-integrin mAb disrupts mechanical signaling in RGD-alginate discs. The inhibitory effect of RGD-alginate on GAG content was enhanced when discs are compressed 10% (0.9 mm final thickness), while GAG content in unmodified (control) alginate was not significantly decreased with compression.

In the presence of 10 $\mu\text{g} / \text{mL}$ anti-integrin antibodies, GAG production in RGD-alginate was increased (due to the blockade of the inhibitory effect of 270 μM RGD-alginate). Compression did decrease GAG content in RGD-alginate discs cultured with anti-integrin mAb, however, the effect of compression was reduced compared to non-mAb RGD-alginate disc compression.

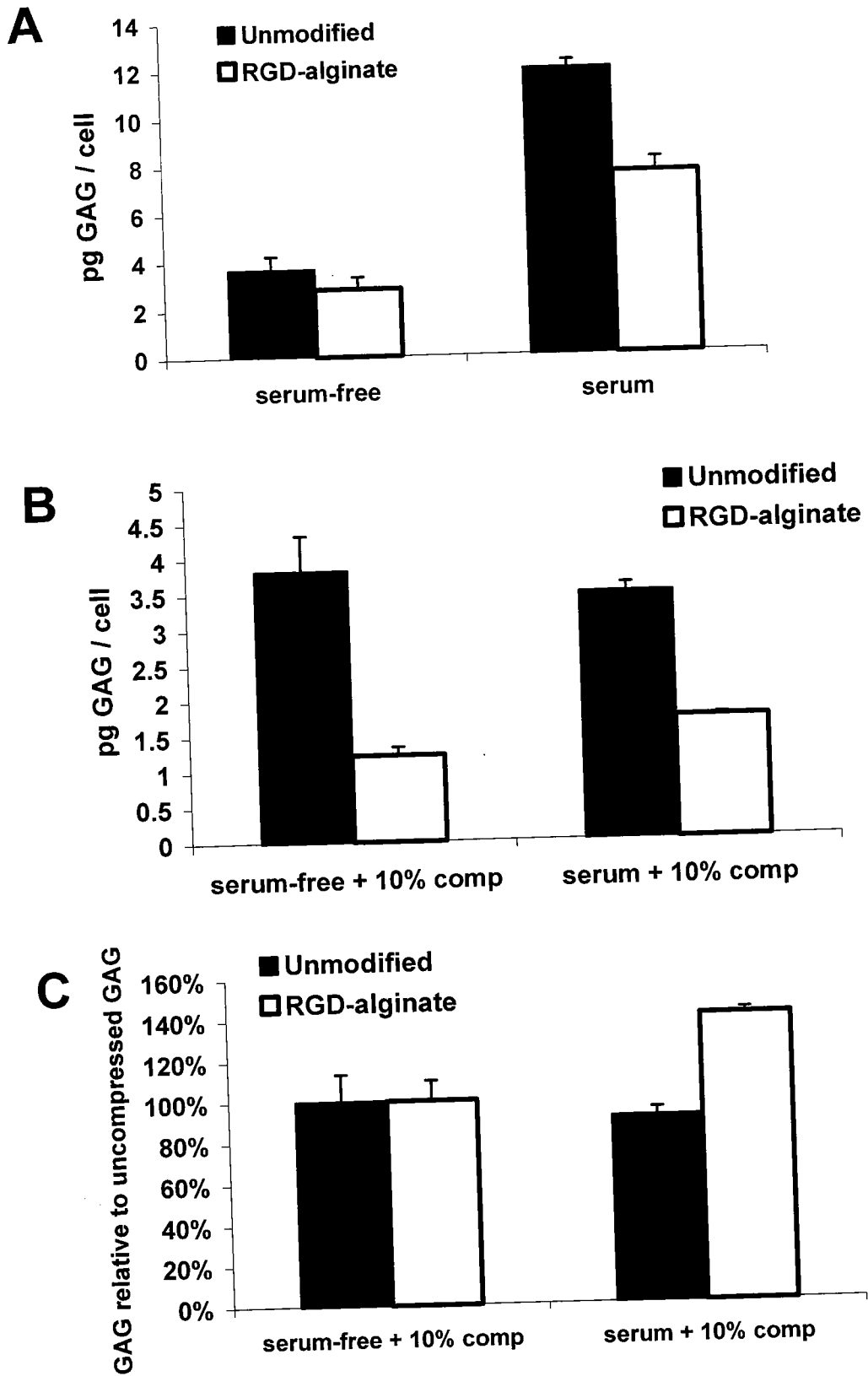


Figure 26. Compression inhibits chondrocytes' response to serum in unmodified (control) alginate, but not in RGD-alginate. **A:** The presence of serum has an anabolic effect on chondrocyte GAG production for chondrocytes in both unmodified and RGD-alginate. **B:** Upon application of 10% compression in media containing serum, chondrocytes in RGD-alginate exhibit a slight increase in GAG / cell, compared to discs compressed in serum-free media. Unmodified alginate discs show no serum-dependent change in GAG content under compression. **C:** Normalizing compressed, serum-free unmodified and RGD-alginate to 100% illustrates that compression abolishes the response to serum in unmodified alginate samples, but not RGD-alginate samples.

DISCUSSION

The multidisciplinary work described in this thesis has significance in the fields of tissue culture, chondrocyte mechanobiology, and for the pathogenesis of osteoarthritis. The principle conclusions of this research are that chondrocytes form an integrin-mediated attachment to RGD-bonded alginate, and that this attachment allows chondrocytes to sense substrate mechanics and externally applied loads. In turn, this mechanosensation regulates chondrocyte adhesion kinetics, morphology, and metabolism of extracellular matrix molecules. The modified hydrogel used in this research, RGD-alginate, can be used further employed to answer additional questions in these fields.

The significance of these findings is discussed below.

Section I – Controlling Alginate Mechanics

The development of the variable-stiffness RGD-alginate culture system is itself a significant outcome from this work. For many years, hydrogels such as agarose, alginate and PEG have been used for the culture of chondrocytes and other anchorage-independent cells [67, 75, 79, 175]. Hydrogels keep chondrocytes in a spherical conformation, which supports the expression of cartilage-specific proteins. With the advent of 3D culturing of chondrocytes, it has become possible to maintain the cartilage

phenotype *in vitro*, and to investigate the effects of many different factors and culture conditions on the synthesis of cartilage matrix molecules.

Hydrogels such as polyethylene glycol and alginate feature regulatable material properties [129, 176]. PEG can be crosslinked with varying amounts of acrylamide to change modulus, pore size and permeability [75]. Though PEG processing is labor-intensive and there is the potential for cytotoxicity, these modifications have been shown to affect some aspects of chondrocyte matrix production. 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, PEG crosslinking agents are potentially cytotoxic, and handling the PEG hydrogel is not as straightforward [76].

Alginate is attractive because its mechanics can be regulated by crosslinking with divalent cations [69, 70, 119]. The experiments described in this research utilized two techniques for fabricating alginates of variable stiffness (figure 2, and chapter 2). One method employed different crosslinker concentrations and species to increase the modulus of the gels. This method allowed for a wide range of moduli (12.5 kPa to 125 kPa) and created a thin transparent surface from which to view cell attachment and spreading (chapter 1). The drawbacks to this method include the inhomogeneities introduced in the rapid gelation, and the confounding effect of increasing cation concentration on cell culture.

The second method involved reducing the polymer chain length by hydrolysis to make gels with weaker moduli. Though the range of stiffness was not as great

(approximately 5 kPa to 50 kPa), the homogeneity of the alginate, the ability to mold desired shapes, and the constancy of ionic concentration across moduli make this method useful for analysis of matrix metabolism.

Together, both methods permitted the straightforward, inexpensive study of many aspects of the chondrocyte response to substrate mechanics, and with one polymer offer many of the advantages found in separate culture systems [39, 72, 177].

Cells, however, do not directly adhere to the alginate polymer, but rather secrete and attach to their own matrix proteins, creating a dense pericellular matrix. Thus, investigations into the mechanisms of chondrocyte adhesion and mechanotransduction have continued to rely on monolayer cultures. Because the spherical cell conformation is lost in 2D culture, chondrocytes have been shown to dedifferentiate, decreasing production of cartilage-specific proteins and altering their characteristic integrin expression profiles. Furthermore, mechanically stimulating 2D cultures is not physiological. This state of affairs has impeded research in chondrocyte-matrix signaling.

Three-dimensional alginate gels modified to contain the RGD adhesion ligand allow chondrocytes to maintain a spherical conformation while attaching to a substrate. This new culture technique permits experiments that decouple cell shape from cell adhesion. In doing so, this system enables investigation of attachment-mediated mechanosensation by chondrocytes in a physiological conformation.

RGD-alginate possesses both the variability in stiffness and the means by which chondrocytes can sense mechanics. Furthermore, RGD-alginate permits the analysis of chondrocyte attachment and matrix metabolism.

*Section II – Chondrocytes Adhere to RGD-Alginate
with Integrins*

Chondrocytes were found to attach and spread on 2D RGD-alginate (chapter 1), as myoblasts had been shown to do previously [82, 102]. This adhesion was disrupted with antibodies against integrin subunits, significantly $\beta 1$ and $\alpha 3$, the actin filament destabilizer cytochalasin D, and by soluble RGD peptide competitor (discussed in the next section). The dissociation constant derived from the blocking peptide dose-response curve is similar to that of integrin binding to a fibronectin substrate [109].

The morphological changes accompanying attachment could not be visually confirmed in 3D RGD-alginate, but the effect was nonetheless noted when RGD-alginate discs stiffened 24 hours after being seeded with chondrocytes at high density (chapter 3). The stiffening effect was blocked with soluble RGD peptide and cytochalasin D, suggesting that integrin adhesion and cytoskeletal contractility mediated the stiffening effect. These findings are consistent with reports that cells can cause collagen fibril bending [142] and exert forces on their surrounding matrix [144, 148, 178]. These findings each support the conclusion that chondrocytes are binding to RGD-alginate with $\beta 1$ integrins.

The antibodies to the $\beta 1$ integrin subunit used in these experiments and others [84, 152] behaved as integrin antagonists. At a concentration of $10 \mu\text{g} / \text{mL}$ [84], the

antibodies blocked cells from attaching to RGD-alginate, as confirmed by phase-contrast microscopy (chapter 1). Furthermore, the use of the antibody antagonists disrupted both the decrease in GAG production typically observed with RGD-binding, and the intrinsic and extrinsic mechanosensitive changes to matrix metabolism (chapters 3 and 4).

Chondrocytes seeded in RGD-alginate discs at high density saw matrix production rise in the presence of anti- $\beta 1$ integrins ($10 \mu\text{g} / \text{mL}$), toward the production levels of chondrocytes in unmodified alginate. The changes due to mechanosensation were similarly attenuated. Significantly, chondrocytes in unmodified alginate exhibited no changes in matrix metabolism, morphology, or attachment kinetics in the presence of anti- $\beta 1$ antibodies. Taken together, these findings suggest integrin binding site occupancy is required for changes in morphology, attachment kinetics, and matrix metabolism.

Section III – Integrin Adhesion to RGD Affects

Chondrocyte Metabolism

The consequences of integrin attachment to the alginate matrix include integrin signaling for cytoskeletal rearrangement and stiffening of the discs (discussed above), and changes to cell metabolism. These metabolic changes can be categorized as either inherent to integrin binding site occupancy, or dependent on matrix mechanics.

The principle observed metabolic effect of chondrocyte binding to RGD – whether bound to alginate or free peptide – is an inhibition of matrix synthesis (chapter 3

and 4). Chondrocytes in unmodified alginate gels exhibited a decrease in GAG per cell in the presence of soluble RGD peptide, and a more profound decrease in RGD-alginate gels.

In contrast to the antagonist actions of antibodies to $\beta 1$ integrins, RGD peptide always behaved like an integrin agonist. This is consistent with reports from other tissues: in rat kidney afferent arteriole, for instance, soluble RGD peptide triggers immediate and dramatic vasoconstriction; RGE elicits no such response [12]. As for cartilage, it has long been recognized that extracellular matrix components (containing RGD) in the media can reduce proteoglycan synthesis [28]. Fibronectin fragments and RGD peptide itself, found on in many matrix molecules, have been shown to affect matrix metabolism [149, 150]. Thus, the result that 160 μM soluble RGD peptide inhibits cell spreading and, at 640 μM , significantly reduces GAG content per cell in unmodified alginate, is consistent with these previous reports (chapter 3). Oligopeptides containing RGD are by itself sufficient to trigger integrin signaling, which in turn reduces net GAG production.

The ligand density of RGD-alginate is equivalent to 270 μM , and the decrease in GAG content in RGD-alginate discs suggests that there is little additional inhibitory affect from 270 μM to 640 μM of RGD peptide. The effect of adding soluble RGD peptide at 640 μM to chondrocytes in unmodified alginate, while causing a decrease in GAG content, did not elicit a decrease of the magnitude seen in RGD-alginate. This finding suggests that RGD fixed to a polymer is a more potent agonist than a soluble, unrestricted agonist.

Chondrocytes binding to either soluble RGD, or RGD on alginate, experience a profound inhibition of GAG synthesis. This inhibition upon integrin binding site occupancy is a greater regulator of metabolism than the mechanosensation observed in other experiments (see below). However, mechanosensation can modulate this adhesion-derived GAG inhibition – intensifying or attenuating it – depending on the signal.

Section IV – Chondrocytes Sense and Respond to the Matrix Mechanical Environment

Previous experiments with fibroblasts suggest that, upon matrix adhesion, the cytoskeleton will strengthen its linkage according to the amount of resistance detected from probing the matrix [38, 152, 179]. In other words, the cytoskeleton generates a level of tension against the matrix, proportional to matrix stiffness [40, 180].

The experiments described in chapter 1 support and extend these observations. Chondrocytes on 2D RGD-alginate surfaces attached to faster and to a greater extent on stiffer surfaces with high crosslink density. These surfaces promoted a more spread chondrocyte morphology and actin stress fibers.

Perhaps cytoskeletal rearrangement and enhanced adhesion kinetics are the most readily observable responses to changing substrate stiffness, but other cell processes are

undoubtedly involved in the response matrix mechanics. In 3D RGD-alginate discs, it was observed that chondrocyte GAG production was more sensitive to changes in stiffness than cells in unmodified alginate. In stiffer RGD-alginate discs, the cells had comparatively less GAG content than in alginate discs weakened with prolonged hydrolysis (chapter 3).

In both 2D and 3D RGD-alginate culture, in the presence of soluble RGD peptide blockers, matrix attachment was disrupted and sensitivity to mechanics in RGD-alginate discs was lost, and the chondrocytes GAG levels were similar to those in unmodified alginate.

These findings, coupled with the observation that cytoskeletal attachment strengthens the hydrogel, suggest a feedback loop exists in which chondrocytes both stiffen the matrix via cytoskeletal attachment, and respond to matrix stiffness by modulating GAG content. In this light, the finding that stiffer substrates cause a decrease in GAG content can be interpreted as necessary to reestablish a steady state.

Furthermore, the observation from 2D surfaces implies stiffer substrates cause chondrocytes to assume a more fibroblastic morphology (chapter 1); it follows that the production of cartilage-specific proteins should diminish as well.

Section V – Chondrocytes Sense External

Mechanical Compression

Chondrocytes attached to the RGD-alginate polymer exhibited greater decreases in GAG content over a range of imposed displacements (chapter 4). These findings agree with data from cartilage explant studies [24, 159] and long-term hydrogel culture studies [83]. The implication of integrins as a mechanotransductive agent, through results on anti- $\beta 1$ subunit antibody and soluble RGD peptide blockade diminishing the mechanotransductive effect under compression, echoes findings previously reported [9, 55, 84]. The particular response to extrinsic mechanical signaling, however, is not the same for all cell types. In contrast with chondrocytes, tendon fibroblasts have been found to increase matrix production under static tension [181-183].

There are many potential changes to the environment perceived by chondrocytes under static compression: increased ligand density, reduced nutrient accessibility [184] changes in pH, charge density, osmolarity, and others [44, 165]. In light of these factors, is not surprising that chondrocytes in unmodified alginate were affected by static compression, though to a lesser degree than RGD-alginate at all but the largest (0.5 mm) imposed displacements.

The basis for these experiments with RGD-alginate was rooted in cartilage explant studies. In calf elbow growth plate explants unconfined static compression for 12 hours, it was found that discs compressed to 75% of their original height (25% strain)

exhibited a decrease in [^{35}S] SO_4 incorporation of 15-36%. Discs compressed 50% showed a 38-63% drop in radiolabeled sulfate incorporation [24].

Confocal laser microscope microscopy studies reveal applying a constant 15% strain on adult canine knee cartilage explants shortened individual chondrocyte heights by approximately 15%, and shrank nuclear heights by about 9%. Upon treatment with cytochalasin D, compression shrank the chondrocyte by approximately 13%, though the nuclear height did not change significantly [185]. The authors concluded that mechanical loads alter the chondrocyte nucleus through the cytoskeleton, and speculated that this modulation of nuclear shape could be responsible for the changes in matrix synthesis seen in cartilage under compression.

Chondrocytes from calves previously cultured in 3% agarose at concentrations of 20 million cells / mL were exposed to static and dynamic compression regimens [83]. It was found that, regardless of the degree of static compression (up to 60%), radiolabeled sulfate incorporation rates were unchanged on the two-day-old discs. In contrast, 41-day-old discs statically compressed to 50% of their original height for 16 hours showed [^{35}S] sulfate incorporation decreased by approximately 20%, and static compression of 60% decreased incorporation by 50%. Similar results were obtained for radiolabeled proline.

Other research on chondrocytes in 3% agarose reveals that, after a short time in culture, cell deformation occurs upon 20% compression [174, 186]. Compression of the hydrogel after the cell has synthesized a pericellular matrix (6 days) reduces the deformation of the cell, though the deformation becomes permanent [173, 186]. This suggests that matrix attachment is not necessary for cells to be perturbed by mechanical

loads, but the decreases in metabolic activity are due to permanent deformations brought on by matrix adhesion.

It's worth considering that static compression of RGD-alginate discs for 48 hours will likely result in new equilibria for a variety of parameters, including as nutrient diffusion [184], cytoskeletal stress and deformation [173], and hydrogel deformation [108, 187]. In cartilage, the static compression translates to an intrinsically stiffer matrix [188, 189]. It follows that, after a new equilibrium is established in RGD-alginate, an attached cell may perceive extrinsic signaling of static compression as intrinsic signaling from a stiffer matrix. The results of static compression studies (chapter 4) are in agreement with intrinsic stiffness studies (chapter 3): attachment to stiffer substrates inhibits GAG synthesis.

Section VI – Influence of Serum on Mechanotransduction

The phenomenon of intrinsic and extrinsic sensation of mechanics influencing chondrocyte metabolism was itself influenced by the presence of 10% fetal bovine serum.

When chondrocytes in RGD-alginate discs were cultured in serum-free media, the increase in GAG content as matrix stiffness declined was eliminated (chapter 3). Furthermore, the presence of serum in culture media increased the GAG content in RGD-alginate discs under 0.1 mm of displacement (10% nominal strain), compared to GAG content in RGD-discs in serum-free media. This suggests a serum component acts to

mitigate the compression-induced decrease in GAG synthesis observed in chondrocytes in RGD-alginate (chapter 4).

These findings are consistent with reports suggesting that integrin signal regulation of metabolism responds to co-stimulation with IGF-1 or other hormones [5, 154-156]. In both experiments performed here, the absence of serum results in selective a decrease in GAG content per cell in RGD-alginate discs. Thus, the findings cannot simply be explained by the anabolic effects of serum hormones on chondrocyte metabolism – those effects would act on chondrocytes in unmodified alginate as well.

Serum-free media was employed exclusively in adhesion studies so as to eliminate the possible confounding factor of exogenous matrix molecules precipitating on alginate and enabling attachment. It is possible, however, based on the results from metabolic studies, that the presence of serum components in the media would have enhanced chondrocyte adhesion to RGD-alginate by other means – through co-stimulation of integrin receptors.

Section VII – Limitations of Methodology

One limitation of this research was the lack of the ideal controls being employed on each experiment. Foremost among these was the use of unmodified alginate as a control to RGD-alginate; a superior control would be RGE-alginate. Since unmodified alginate lacks peptides, , the hydrogel might have different osmolarity or local charge density than RGD-alginate. Cells might perceive these differences in material properties

and modulate their behavior accordingly. Thus, the differences in chondrocyte behavior between RGD-alginate and unmodified alginate, which were attributed to chondrocyte-integrin attachment, may have been due to the absence of an octapeptide in unmodified alginate.

Fortunately, the use of anti- β 1 mAb on chondrocytes in RGD-alginate was found to abolish the effect of integrin adhesion and send chondrocyte metabolic values toward that of unmodified alginate (chapter 3, 4). Furthermore, the use of soluble RGD peptide on unmodified alginate was found to inhibit GAG synthesis toward levels seen in RGD-alginate (chapter 3, 4). Finally, several experiments using RGE-alginate were performed, and chondrocyte response in RGE-alginate was comparable to that of unmodified alginate (appendix C). Thus, unmodified alginate was an acceptable control to RGD-alginate.

The design of many experiments was such that three factors were being examined simultaneously – such as adhesion to RGD, compression, and the presence of RGD blocking peptide – resulting in eight sample sets. Continually including analogues to soluble RGD peptide, such as RAD or RGE peptide, would have complicated the experimental procedure. However, such analogues were used on several occasions and yielded the expected negative results (appendix C).

Though many studies have relied on total GAG content as a measure of chondrocyte metabolism in alginate [79, 80, 175, 190, 191], the use of the DMB dye GAG assay has several limitations. First, total GAG content does not distinguish between fully functional proteoglycans and defective or partially digested molecules. Secondly,

the rates of synthesis and degradation are not assessed; GAG content in RGD-alginate discs could be lower despite increased synthetic rates, so long as MMP or aggrecanases activity is sufficiently increased. For instance, experiments have suggested that the decrease of GAG content of integrin binding is due to upregulation of MMP synthesis [150] as well as a decrease in matrix molecule production [192, 193].

Other established methods for measuring proteoglycan production include aggrecan promoter activity [55], aggrecan mRNA [194], ^{35}S incorporation [77]. Looking at these or other individual steps of the GAG synthetic and degradative pathways, however, is fraught with other limitations.

For instance, methods for Northern blotting of type II collagen and aggrecan have been described for chondrocytes in alginate. Applying these techniques would indicate whether the transcription of these genes was increased for these genes in chondrocytes bound to RGD-alginate. Measuring incorporated [^{35}S] SO_4 and [^3H] proline would reveal whether more matrix molecules were being synthesized and, in conjunction with Northern analyses and GAG and collagen content assays, could shed light on the intracellular processing of these molecules. Further, methods for assessing matrix metalloproteinase (MMP) levels in chondrocyte-alginate beads have been developed [195].

Analysis of some steps in the synthetic or degradative pathways, however, may have counterintuitive results. One study, as an example, indicated increased expression of several IGF binding proteins upon chondrocyte adhesion to fibronectin fragments [149]. Since IGF is a potent anabolic stimulator of chondrocytes, an upregulation of IGFBPs

upon fibronectin binding was interpreted as a catabolic response. Yet one IGFBP was found to decrease its presence in media, and Northern blots showed no correlation between IGFBP levels and mRNA levels [149].

Thus, it can be said that the regulation of synthetic and degradative processes in chondrocytes is complex and not every step is intuitive. The measurement of total GAG content after two days incubation time is both precise enough for the detection of subtle differences between sample sets, and broad enough to relate the complete picture of matrix metabolism. Each of these measures of chondrocyte matrix metabolism may show a dramatic dependence on mechanical signaling [140]. However, each step in matrix production is likely regulated by several factors and may show a negligible or counterintuitive dependence on mechanics.

Elucidating the exact mechanism of how proteoglycan synthesis responds to mechanics is beyond the scope of these studies; rather, the goal was to prove matrix metabolism is modulated by mechanosensitive chondrocytes.

Section VIII – Significance and Future Work

The findings described in this thesis present avenues for future research in several fields. One such avenue of inquiry focuses on the integrin signaling mediating mechanosensation. Future work on this topic should focus on further characterizing the

integrin subunits involved in mediating adhesion. The use of cytochalasin B might be illustrative as well; both cytochalasin B and D disrupt the actin cytoskeleton, but cytochalasin D further blocks tyrosine phosphorylation of pp125 FAK [51]. If integrin signaling affects the cytoskeleton and cell metabolism through separate mechanisms, the use of cytochalasin B would permit the decoupling of cytoskeletal rearrangement from matrix production.

An analysis of the type of adhesions in chondrocyte culture in 3D alginate would be helpful. Focal adhesions are presumably involved in 2D attachment, based on cytoskeletal stains (chapter 1) and previous studies [196, 197]. Determining whether focal adhesions or fibrillar adhesions are involved in chondrocytes in a 3D conformation would be novel, and could shed light on the *in vivo* state of chondrocytes. A third type of connection, dubbed “3D-matrix adhesions” [163], may be involved in mediating organizing integrins and cytoskeletal proteins in spherical chondrocytes attached to the RGD-alginate matrix.

The intracellular consequences of integrin signaling have been characterized in several cell types [29, 198] and but less so in chondrocytes. The RGD-alginate system, which encourages integrin signaling, keeps chondrocytes in a physiological conformation, and permits molecular biology techniques offers promise in this field. Identifying the signaling molecules downstream of integrins, which likely regulate the changes in GAG metabolism observed here, should be a future research goal.

Another goal suggested by these findings is that of isolating the effect of different mechanical signals on chondrocyte metabolism. Simply applying dynamic compressions

regimes to chondrocytes in RGD-alginate would be of value, as dynamic compression has been shown to increase matrix synthesis in cartilage explants [159, 199].

Furthermore, compressing relaxed, seeded hydrogels both stresses and strains on the matrix. By developing methods to construct variable-stiffness RGD-alginate discs, the potential exists for decoupling stress and strain effects on chondrocyte behavior. Applying 10% compression on a weak disc will result in a lower stress than 10% compression on a stiff disc. Until now, although compression has been applied to hydrogels or cartilage explants, the stresses perceived by the samples could not be calculated due to the confounding effect of strain.

How stress and strain change the matrix environment, besides altering the cell-matrix interaction, is also an open question. An avenue of investigation should be to examine how compression changes the availability and efficacy of serum growth factors in regulating cell metabolism. The data in this research suggests serum component(s) are responsible for a co-stimulatory effect with RGD. Testing this by adding IGF-1 to RGD-alginate discs in serum-free media, or adding IGF-1 binding proteins to media containing 10% FBS, is feasible and worthwhile.

This research has indicated a complex interaction between cell and matrix, governing both the metabolism of the cell and the stiffness of the entire construct. Cells adherent to RGD-alginate can sense matrix mechanics, but can also change those mechanics with cytoskeletal forces. Feedback may drive the system to a steady state of proteoglycan synthesis and turnover; the same steady state may exist in healthy cartilage.

Characterizing the consequences of mechanical stimulation on the chondrocyte-matrix attachment will shed light on the pathogenesis of diseases like osteoarthritis, and begin to explain the ECM remodeling that can follow prolonged activity. Furthermore, the identification of components in the mechanotransduction pathway opens up the possibility of pharmaceutical interventions to reverse disease and age-related degenerative processes. All told, the RGD-alginate culture system represents an excellent opportunity for the investigation of integrin adhesion in mechanotransduction.

APPENDIX A

ANALYSIS OF CHONDROCYTE INTEGRIN EXPRESSION WITH FLOW CYTOMETRY

Introduction

It has been observed that chondrocytes cultured from monolayer adhered to and spread on RGD-alginate in a stiffness-dependent manner, mediated by $\beta 1$ integrins (chapter 1). However, primary chondrocytes, digested from cartilage explants in collagenase, did not adhere to RGD-alginate unless first cultured on monolayer. This phenomenon has been noted previously [8].

Another observation was that the time chondrocytes spent *in vitro* before the adhesion assay influenced attachment kinetics – cells cultured longer in monolayer attached faster to RGD-alginate. This is not unexpected, since reports indicate chondrocytes in monolayer will change their integrin profile with time [51, 89, 200]. However, these phenomena taken together suggest a difference between *in vivo* and digested chondrocytes emerges very early after isolation, and is worth studying .

One possible explanation for these finding is that collagenase used to isolate chondrocytes also destroys integrins. This seems unlikely, however, because the attachment inhibition lasts for days, and integrin turnaround time is measured in hours

[8]. Another explanation is that collagenase, or overnight suspension – simply inactivates $\beta 1$ integrins – or that trypsin or monolayer culture activates them.

Examining this hypothesis required an indirect approach, since activation-state specific antibodies were not available for the bovine chondrocyte $\beta 1$ integrin subunit. Fluorescent-activated cell sorting was applied in an attempt to quantize $\beta 1$ integrin expression at different periods of monolayer culture and after enzymatic degradation of cartilage (by collagenase and by other enzymes).

The potential for sortable populations of cells to emerge, with low and high $\beta 1$ expression, was also considered. Studying such populations, and their relative sizes with respect to one another, would aid in optimization of chondrocyte culture in RGD-alginate. Furthermore, by sorting based on $\beta 1$ expression and then culturing on RGD-alginate, it would be possible to demonstrate the dependence of attachment rate and levels on $\beta 1$ expression.

It's worth noting at this time that collagenase-digested chondrocytes will adhere to culture plates and dishes; otherwise, generating the initial monolayer culture from collagenase-digested cells would be impossible. The same mechanism that mediates the attachment of some trypsinized chondrocytes to unmodified alginate (i.e., a non-integrin dependent mechanism) may be responsible for adhesion to plastic plates. Alternatively, the extreme stiffness of the plastic culture surface may reactivate integrins de-activated by collagenase or suspension culture.

Methods

Chondrocyte Cell Isolation

Articular cartilage from gleno-humeral and humero-ulnar joints of 1-2 week old calves (Research 87, Boston, MA) was incubated for 12 hours in a solution of 0.3% collagenase Type 2 (Worthington, Lakewood, NJ) in F-12 media, 100 U / ml penicillin, 100 μ g / ml streptomycin, and 250 ng / ml amphotericin B (GIBCO, Grand Island, NY) at 37°C.

Some primary cells were plated in T-175 flasks at 50×10^3 cells / cm².

Chondrocytes were maintained in culture until confluence days, with media changed every 2-3 days. At various timepoints, the cells were rinsed with PBS and incubated for approximately 20 minutes in F-12 media containing 0.05% trypsin, then prepared for FACS analysis.

Flow Cytometry

Chondrocytes from primary or monolayer culture were transferred to 50 mL conical tubes, spun down at 250g, and resuspended at 1×10^6 cells / mL PBS. One mL was transferred to a 12x75 mm tube (Falcon) and centrifuged. The cells were resuspended in

100 μ L of a PBS / 0.1% Azide / 1% FBS / 1% anti- β 1 (primary) antibody solution at a ratio of 1:1000. The cell suspension was vortexed and incubated on ice.

After 30 minutes, the tube was washed twice with PBS / 0.1% Azide / 5% FBS and resuspended in 2 mL of PBS / Azide / FBS with 2 μ L of IgG : PE secondary antibody (Molecular Probes P-852) at a ratio of 1:2000, vortexed, and incubated on ice.

After 30 minutes, the cells were washed twice and resuspended and pooled to 5 million cells / mL in PBS with 5% FBS and 0.5% paraformaldehyde in PBS / azide. The tubes were kept on ice until FACS analysis by FACScan (BDIS, Mountain View, CA). Negative and positive antibody controls were included.

Results

As time in monolayer culture increased (0, 1, 5, 11 days, figure 27A) the FL-2 median moved rightward, indicating more β 1 expression. This trend is also seen in a second experiment (figure 27B), where chondrocytes passaged twice (day 22 overall) exhibit a biphasic distribution.

Time in culture also led to increases in cell size, as indicated by the FSC-H (y-axis, figure 28). The smallest cells at each timepoint have a wide range of β 1 expression, but the largest cells are always on the high end of β 1 expression.

The cells with the most bound secondary antibody (FL2 channel > 100) were sorted and separated from the least bound cells (FL2 channel < 10, figure 29A). Subsequent efforts at culturing the separated populations on Falcon 6 well-plates revealed

that the most fluorescent cells remained rounded and less likely to spread and attach to the culture dish after eight hours (figure 29B), with the least-fluorescent cells spreading and forming long processes within hours of transfer.

Discussion

The principal finding of this study was that chondrocytes isolated from bovine cartilage by collagenase digest progressively increase their $\beta 1$ integrin expression as they are cultured in monolayer.

The main drawback to this study was the confounding variable of cell size. It was clear that monolayer culture duration increased both chondrocyte size and $\beta 1$ integrin expression, but it was impossible to resolve whether the increase in $\beta 1$ expression was greater than that expected of a larger cell. The actual number of integrin receptors per unit area of cell membrane could not be calculated with available equipment and supplies.

It is interesting that the brightest cell population, when cultured, was unable to spread on a culture surface, even after 12 hours. Since the bright cells $\beta 1$ integrins were bound by primary and secondary antibody, the data suggests that, if the cells are expressing many $\beta 1$ integrins, those $\beta 1$ integrins are necessary for chondrocyte spreading.

Cells not expressing $\beta 1$ integrins (the least-fluorescent population) were able to attach and almost universally spread quickly on the Falcon surface. The absence of $\beta 1$ binding fluorescence in these cells implies another cell surface receptor is responsible for the spreading, or the $\beta 1$ integrins expressed are of a form that does not permit primary antibody binding.

Since the chondrocytes were sorted from the tails of a continuous distribution of $\beta 1$ binding fluorescence, a simple explanation would be that high $\beta 1$ expression crowds out other cell spreading mechanisms, and low $\beta 1$ expression encourages them. This conclusion is supported by a finding that annexin V expression falls as $\beta 1$ integrin expression rises in monolayer [10], though the changes observed take place in the first 24-hours post-culture.

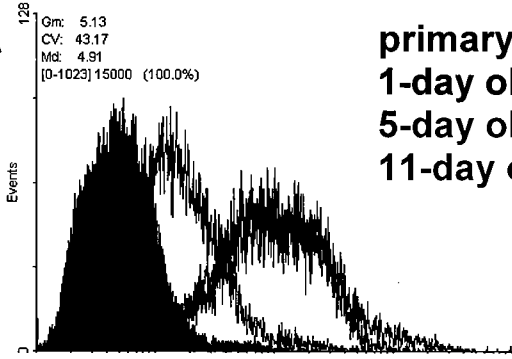
FACS has been used in the past to reveal which chondrocyte surface receptors attach to the pericellular matrix [78], and which integrins are upregulated in response to IL-1 [150]. Others have shown that chondrocytes express relatively high levels of $\alpha 5$ and $\beta 1$ integrin, with low levels of $\alpha 1-3$ and $\beta 3$, after digestion in collagenase and pronase [201].

None of these studies have attempted absolute integrin counts per chondrocyte or per unit surface area, which would be of interest. A size fractionator, in conjunction with an antibody standard (which would compare the intensity of $\beta 1$ staining to the number of illuminated molecules using a known calibration) would further this investigation.

A

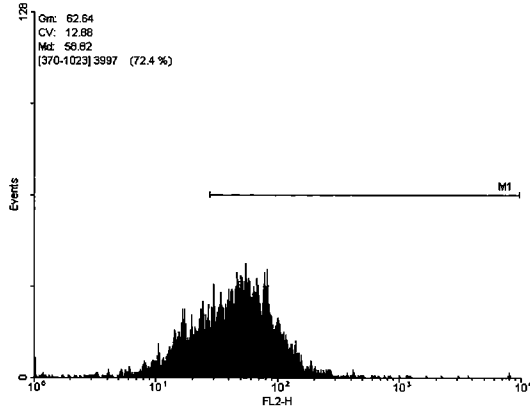
Gm: 5.13
CV: 43.17
Mz: 4.91
[0-1023] 15000 (100.0%)

primary chondrocytes
1-day old monolayer
5-day old monolayer
11-day old monolayer



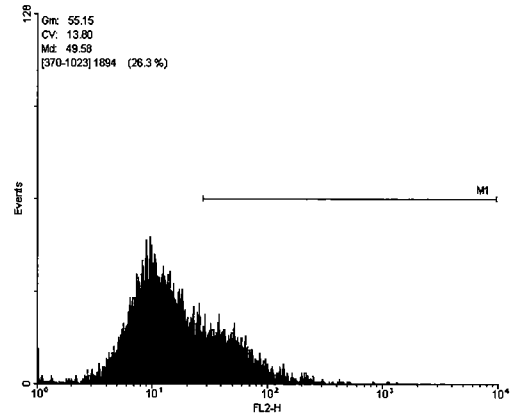
B

Gm: 62.64
CV: 12.88
Mz: 58.82
[370-1023] 3997 (72.4%)



C

Gm: 55.15
CV: 13.80
Mz: 49.58
[370-1023] 1894 (26.3%)



D

Gm: 71.10
CV: 11.51
Mz: 67.93
[370-1023] 7809 (78.1%)

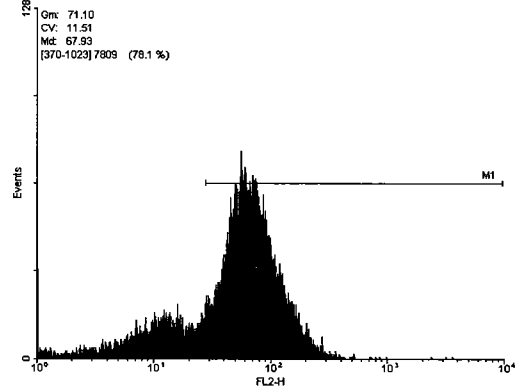


Figure 27A: Increasing time in culture increased overall $\beta 1$ integrin expression, as measured by FITC-conjugated secondary antibody fluorescence. **B:** A separate experiment showing fluorescent intensity on a 4-day old monolayer population; most cells have an intensity below channel 50 (FL2-H). **C:** $\beta 1$ integrin expression on a 7-day old monolayer has shifted so that more than 70% of cells have passed channel 50. **D:** A passaged population of chondrocytes (tertiary cells) after two 11-day incubations, indicating a low-intensity and high-intensity pool of fluorescent chondrocytes.

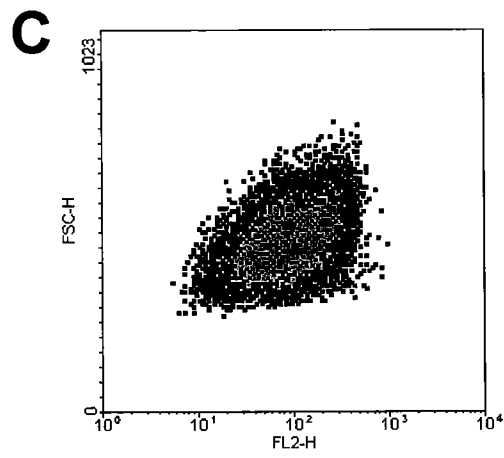
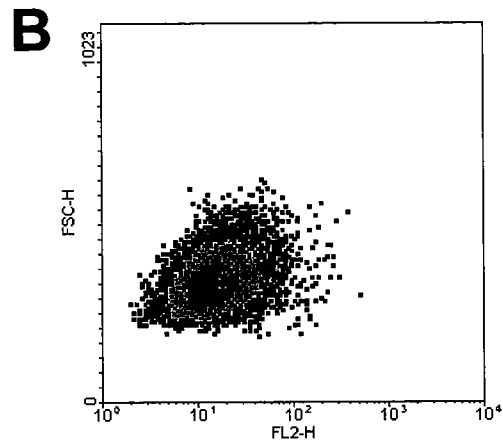
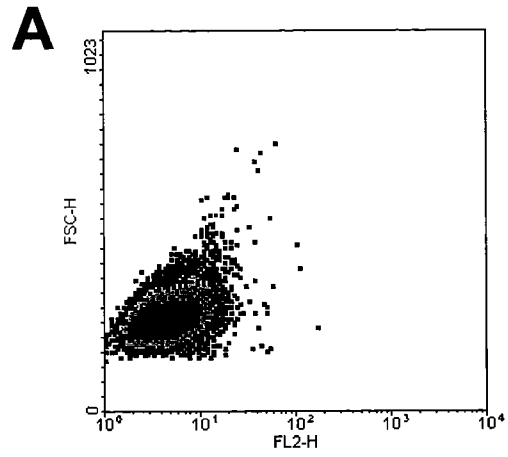


Figure 28: Cell size (Y axis) grows with duration of culture in monolayer, as does β -1 fluorescence (X-axis). **A:** Primary chondrocytes from cartilage digest. **B:** 1 day old monolayer. **C:** By 11 days of monolayer culture, small cells have a wide range of fluorescence; large cells tend to have only high fluorescence.

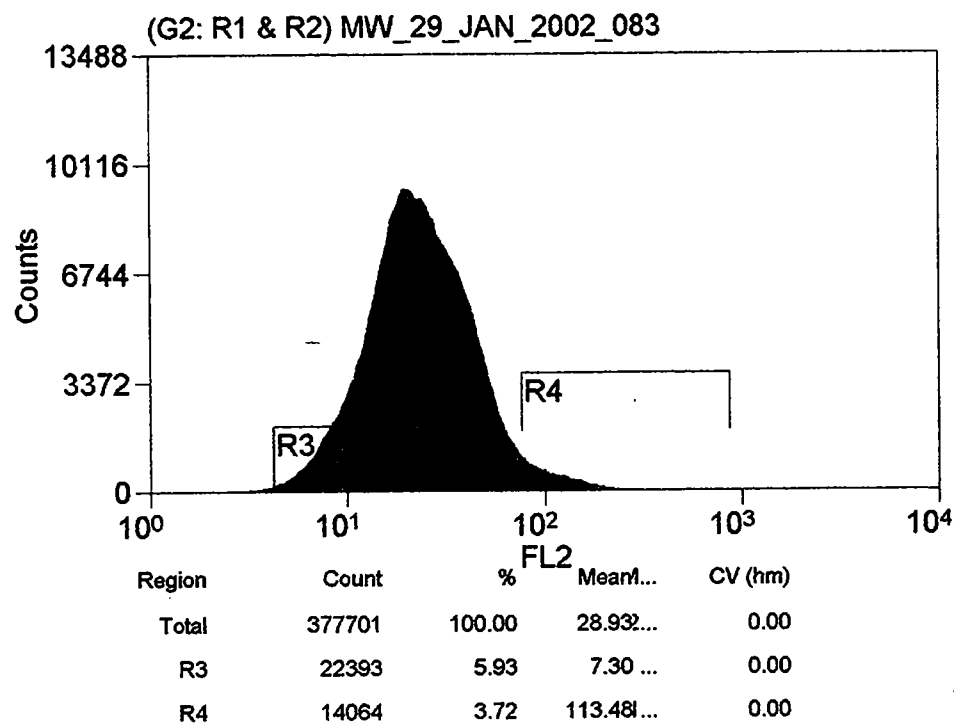
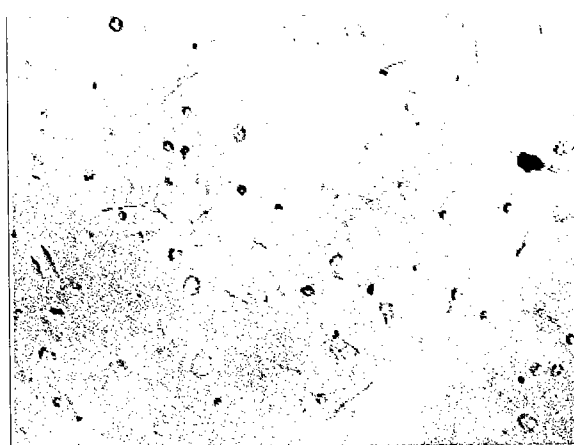
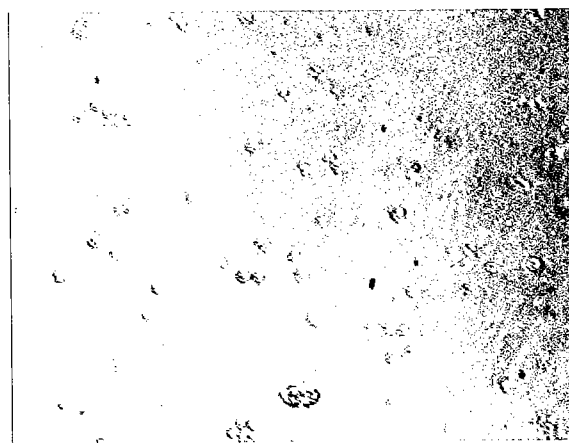
A**B****C**

Figure 29. A: Two cutoffs were selected for sorting chondrocytes from 12-day old monolayer culture: those with fluorescent intensity less than channel 10 and those with intensity greater than channel 100. **B:** The low-intensity population quickly spread and formed attachments to the culture dish. **C:** The high-intensity population remained rounded and unattached to the culture dish.

APPENDIX B

INTRACELLULAR SIGNALING OF INTEGRIN MECHANOTRANSDUCTION

Introduction

Having established GAG content levels are affected by chondrocyte integrin adhesion and mechanotransduction (chapters 3 and 4), preliminary steps were taken in the examination of the integrin signaling pathway.

Based on integrin signaling in fibroblasts and other mechanosensitive cells, it was expected that chondrocyte integrins in RGD-alginate would cluster and form focal adhesions [38, 39]. Focal adhesion kinase (FAK) binds to the cytoplasmic tail of integrin subunits at its N-terminus, and paxillin and the actin cytoskeleton at its C-terminus [103]. FAK has an autophosphorylation site at amino acid 397, which seems to facilitate src-family binding upon integrin adhesion [34].

The organization of the FAK complex after integrin binding is unclear, but may be facilitated by GTP-binding proteins such as Rho [103]. It is known that the src molecule phosphorylates FAK 576 and 577 [36], and the phosphorylation of the FAK925 binding site generates a linkage to the Ras / MAPK pathway [103]. FAK925 phosphorylation triggers a variety of mitogen-activated protein kinases (MAPK) including extracellular signal-related kinase (ERK), p38, and c-Jun NH₂-terminal kinase (JNK) [164, 196].

In cardiomyocytes, ERK, p38 and JNK are activated by mechanical stress [35] and are known to affect proliferation, differentiation and hypertrophy. Chondrocytes binding to a fibronectin fragment exhibited increases in ERK1/2, JNK1 and p38 phosphorylation within 60 minutes [196]. Mechanical stress due to fluid flow has also been shown to stimulate phospho-ERK1/2 expression in chondrocytes, concomitant with a decrease in aggrecan promoter activity [55, 194].

With this in mind, it was logical to hypothesize that chondrocyte adhesion to RGD-alginate and static compression of RGD-alginate discs – both factors which had lowered disc GAG content (chapters 3 and 4) – would also exhibit increased FAK and ERK phosphorylation. These findings would confirm that integrin mechanotransduction occurs via the same pathway in chondrocytes in hydrogel as it does in monolayer and in other cell types, and would further understanding of the mechanism of matrix metabolism in response to ECM signaling.

Methods

Chondrocyte Cell Isolation

Articular cartilage was isolated as described previously [148]. Cartilage from the gleno-humeral and humero-ulnar joints of 1-2 week old calves (Research 87, Boston, MA) was incubated for 12 hours in a solution of 0.3% collagenase Type 2 (Worthington, Lakewood, NJ) in F-12 media, 100 U / ml penicillin, 100 µg / ml streptomycin, and 250 ng / ml amphotericin B (GIBCO, Grand Island, NY) at 37°C. The digested cartilage

solution was filtered in a 100 μm mesh, washed twice in PBS and resuspended in F-12 / 10% FBS media.

RGD Peptide / Alginate Bonding

Peptides containing the sequence GGGGRGDY were be covalently bonded to medium viscosity alginate (FMC) through a condensation reaction with mannuronic or guluronic acid residues as described previously [82].

Briefly, FMC 10/60 alginate was suspended in 100 mM MES (2-[N-Morpholino] ethanesulfonic acid) buffer with 300 mM NaCl at pH 6.5 to make a 1% w/v alginate solution. EDAC (1-ethyl-[dimethylaminopropyl] carbodiimide) was added at 5% molarity to that of the guluronate. Sulfo-NHS (N-hydroxy-sulfosuccinimide) was included at half the molarity of the EDAC. Finally, the octapeptide GGGGRGDY, in 100 mg/ml MES, was added at a ratio of 16.7 mg per gram of alginate. The reaction was allowed to proceed at room temperature for 20 hours before being transferred to 3500 MWCO dialysis tubing and placed in a 4L ddH₂O tank with 7 g NaCl / L. Every 8-12 hours, the salt concentration was lowered 1 g per liter, and the concentration was held at 0 g / L for 24 hours. The contents of the dialysis tube were transferred to a 50 mL conical, frozen, and lyophilized for 48 hours.

Alginate and RGD-alginate was resuspended to form a 2% (w/v) solution in PBS, and sterilized with 0.45 μm syringe filters.

Chondrocyte Disc Fabrication

Refrigerated 2% alginate was warmed and filter-sterilized as described above. Aliquots of 2 mL aliquots were mixed with pellets containing 100 million chondrocytes (50 million cells / mL). The suspension was vortexed and mixed with 80 μ L of a well-shaken CaSO_4 solution (concentration: 0.266 mg / ml PBS) via a 3-way stopcock as described previously [82]. The gel was quickly spread between sterile plates separated by 1 mm spacers. After allowing 15 minutes for the alginate to harden, the plates were separated. Using a biopsy punch, 4 mm diameter discs were carved out of spread culture, and each disc was placed in 300 μ L serum-free F-12 with 1% antibiotics and antimycotics at 37°C, 5% CO_2 .

Static Compression

Chondrocytes were allowed to adhere to RGD-alginate for 8 hours. At that time, discs of both unmodified and RGD-alginate were placed into compression chamber wells with 300 μ L of F-12 media and subjected to 0-0.5 mm uniaxial unconfined displacement with 4 mm-diameter plungers for 1 hour.

SDS-PAGE

Following compression, twelve discs from each condition were pooled, mixed with 500 μ L of homogenization buffer with 55 mM sodium citrate, and homogenized on ice. Proteins were extracted and DNA was assayed to determine lane loading (see below). Normalized quantities of extract were boiled for 10 minutes and loaded in lanes of a 10%

SDS-PAGE gel run at 85V for 60 minutes. The transfer blot was probed with goat anti-mouse pan-ERK antibody (1:1000, Cell Signaling Technology #9102), goat anti-mouse phospho-ERK antibody (1:1000 CST #9102), or goat anti-mouse phospho-FAK-925 antibody (1:1000 Biosource 44-616). The secondary antibody used was FITC-conjugated goat anti-rabbit at a concentration of 1:2000 (CST #7074).

DNA Assay

Aliquots from twelve pooled protein-extracted chondrocyte-alginate discs were added to 195 μ L of picoGreen dye solution (Molecular Probes, CA) in a 96-well plate [146]. The contents of the plate were excited at 485 nm and fluorescence was read at 535 nm on a HTS 7000+ spectrophotometer (Perkin-Elmer). DNA concentration was determined by linear interpolation with calf thymus DNA standards.

Results

Identification of intracellular signaling molecules from chondrocytes in unmodified or RGD-alginate was shown to be possible. Early attempts at ERK phosphorylation blots indicated chondrocytes RGD-alginate discs under 0.5 mm displacement expressed moderately higher levels of p-ERK than in unmodified alginate discs under compression, or RGD-alginate discs allowed to swell freely (figure 30A).

These gels featured distorted bands, likely due to alginate interfering with protein migration. Furthermore, the use of serum in the culture media potentially raised the baseline for phosphorylated ERK, further confounding interpretation.

When the alginate decrosslinker sodium citrate was added to the protein extraction protocol, and when serum was removed from the disc culture media, the band distortion disappeared and baseline ERK phosphorylation fell (figure 30B). No difference was observed in ERK phosphorylation in RGD-alginate or alginate discs, with or without compression. The antibodies detected a several-fold increase in phosphorylated ERK in unmodified alginate discs cultured in media with serum.

Phosphorylation of FAK at the 925 site was most intense in chondrocytes from free swelling, unmodified alginate samples (figure 31). The RGD-alginate bands, free and compressed, were shown to be of approximately equal intensity. The least intense FAK-925 band was chondrocytes from compressed unmodified alginate.

A pair of unknown bands at approximately 30 kDa appear in the lanes of chondrocytes from compressed RGD-alginate and unmodified alginate (right arrow).

Discussion

The principle findings from these experiments is that phosphorylation of ERK is not affected by chondrocyte integrin attachment or compression, and FAK-925 phosphorylation occurs in unanticipated ways.

Early results with this alginate discs were misleading, likely due to the blurring effect of alginate on the migration of bands through the gel and the activation of ERK1/2 by serum in the culture media (figure 30A). The addition of citrate to the homogenization buffer removed the alginate from the protein extract and improved migration. The removal of 10% fetal bovine serum from the culture media lowered the baseline ERK expression and permitted the addition of serum as a positive control for antibody binding.

It was expected that integrin binding to RGD-alginate would lead to higher ERK levels, but no change in phosphorylation was observed (figure 30B). There are several explanations for this, the simplest being that ERK phosphorylation is not involved in this mechanotransduction. It is also possible that the speed of ERK activation rises and falls so quickly that the change is missed by harvesting discs after one hour of compression. Previous reports suggest ERK phosphorylation begins minutes after mechanical loading and shows rapid resolution [55, 196].

The chondrocytes were cultured in RGD-alginate long enough for attachment to occur, and presumably for focal adhesions to form (chapter 1). FAK-925 phosphorylation, in turn, is believed to be a consequence of focal adhesion formation [35, 196], and thus was not expected from chondrocytes in unmodified alginate discs. The decrease in FAK-925 phosphorylation in RGD-alginate (whether compressed or free) was also unexpected, because focal adhesions should be most pronounced in RGD-alginate. This finding may indicate a different kind of focal adhesion forms in spherical chondrocytes attached to RGD-alginate. Besides the well-characterized focal and fibrillar adhesions, a third type of connection, dubbed "3D-matrix adhesions" [163], has been

observed. The 3D-matrix adhesions exhibit different FAK phosphorylation states, different focal adhesion organization, and may be more representative of cell-matrix interactions *in vivo*. Comparing the phosphorylation states of cells in these 3D discs to cells on 2D surfaces would clarify these attachment processes. Thus far, however, it has been difficult to procure adequate amounts of protein from cell – surface experiments.

Further studies are warranted to elucidate the mechanism of how proteoglycan metabolism responds to integrin adhesion and compression. Looking at earlier timepoints might help catch changes in ERK phosphorylation, and later timepoints might reveal changes in FAK. Also, using different antibodies to probe other sites in FAK, and other MAP kinases, is also sensible. Switching from bovine chondrocytes, for which there are relatively few specific antibodies, to another animal or cell type, might be necessary.

However, the basic techniques for identifying intracellular signaling molecules have been established in the course of these experiments. Further characterization of the integrin mechanotransductive pathway is now feasible in the RGD-alginate culture system.

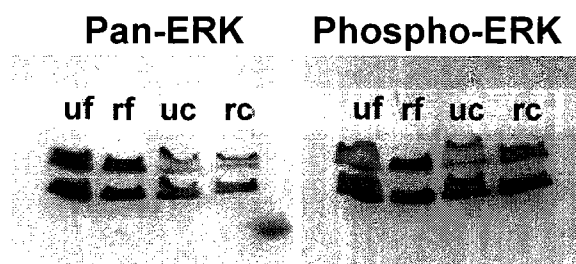
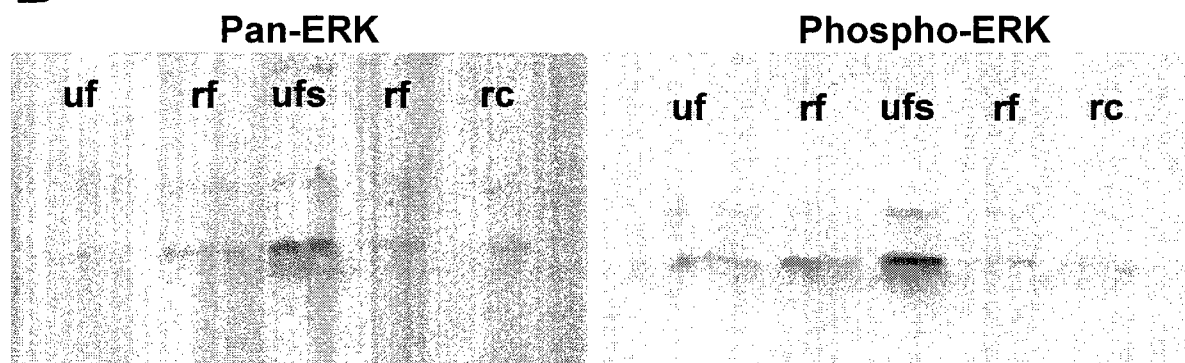
A**B**

Figure 30. ERK phosphorylation in RGD-alginate and unmodified alginate discs, under compression or in a free-swell state. **A:** pan-ERK antibody (left) and phospho-ERK antibody, seemingly indicated a mild increase of phospho-ERK in chondrocytes from RGD-alginate and compressed RGD-alginate samples. Bands were likely contaminated with alginate. **B:** After adding citrate buffer to the protein extraction protocol, alginate was removed and bands cleared, revealing no difference between relative phospho-ERK and pan-ERK staining for unmodified and RGD-alginate, whether compressed or allowed to swelling freely. Serum control increased phospho-ERK intensity severalfold over pan-ERK, as expected.

uf = chondrocytes in nmodified alginate free-swelling samples (uncompressed)

uc = nmodified alginate samples compressed 0.5 mm,

rf = RGD-alginate free-swelling samples

rc = RGD-alginate samples compressed 0.5 mm

ufs = nmodified alginate free-swelling samples in serum.

Total FAK

Phospho-FAK 925

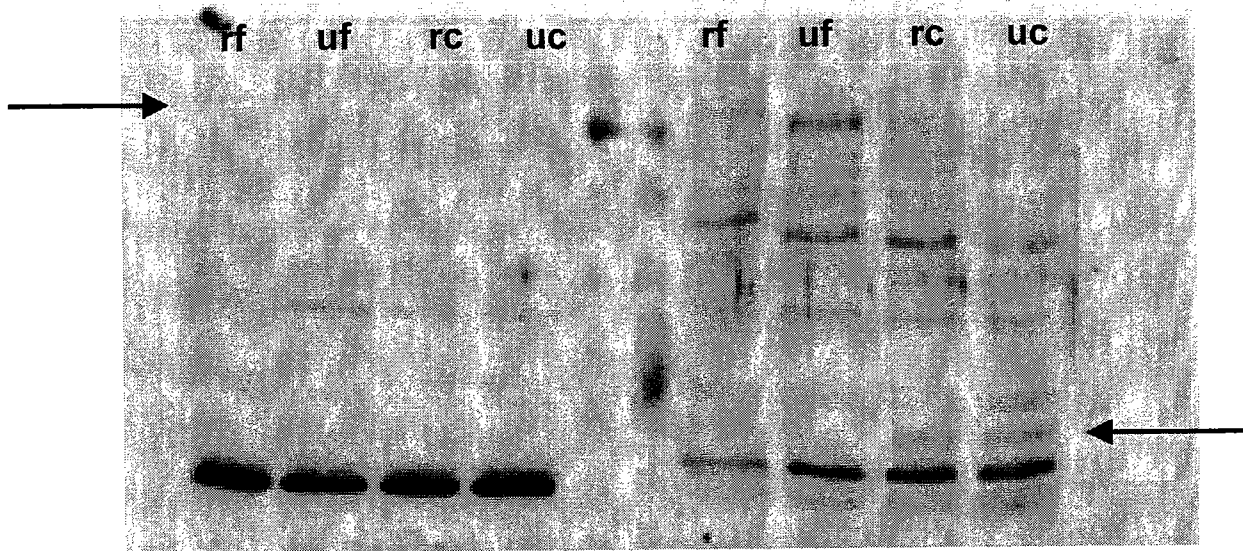


Figure 31. Total FAK blot (left) revealed equal loading based on DNA content, though no discernable FAK at 125 kDa (left arrow). Phospho-FAK 925 staining was strongest in free swelling unmodified alginate; chondrocytes in RGD-alginate had less phospho-FAK925, and compressed unmodified alginate had the weakest staining. Two unknown bands appear at ~30 kDa exclusively in compressed samples (right arrow).

uf = chondrocytes in unmodified alginate free-swelling samples (uncompressed)

uc = unmodified alginate samples compressed to cast thickness

rf = RGD-alginate free-swelling samples

rc = RGD-alginate samples compressed to cast thickness.

APPENDIX C

RGD-ALGINATE FABRICATION AND APPROPRIATE CONTROLS

Introduction

The use of alginate bonded with RGD (Arginine-Glycine-Aspartate) has already been shown to facilitate cell adhesion and regulate phenotype in a ligand-dependent manner [82, 102, 202]. Using techniques previously described, RGD-alginate was fabricated and used to assess the effect of integrin adhesion and mechanics on chondrocyte behavior.

This research faced a potential limitation regarding the use of ideal controls employed for each experiment. Foremost among these was the use of unmodified alginate as a control to RGD-alginate; a better control would be RGE-alginate (Arginine-Glycine-Glutamate). Since unmodified alginate lacks covalently bonded peptides, the unmodified hydrogel might have different osmolarity, pH or local charge density than RGD-alginate. Cells might be sensitive to these differences in material properties and modulate their behavior accordingly. Thus, the differences in chondrocyte behavior between RGD-alginate and unmodified alginate, which were attributed to chondrocyte-integrin attachment, may have been due to the absence of an octapeptide in unmodified alginate.

Furthermore, the design of many experiments in this work was such that three factors were being examined simultaneously – such as adhesion to RGD, compression, and the presence of RGD blocking peptide – resulting in many sample sets. Continually including analogues to soluble RGD peptide, such as RAD or RGE peptide, would have been ideal, but would have complicated the experimental procedure.

The use of such analogues was explored on several occasions in initial studies and yielded expected results. Thus, the ideal controls were not required for subsequent experiments.

Methods

Chondrocyte Cell Isolation

Articular cartilage was isolated as described previously [148]. Cartilage from the gleno-humeral and humero-ulnar joints of 1-2 week old calves (Research 87, Boston, MA) was incubated for 12 hours in a solution of 0.3% collagenase Type 2 (Worthington, Lakewood, NJ) in F-12 media, 100 U / ml penicillin, 100 µg / ml streptomycin, and 250 ng / ml amphotericin B (GIBCO, Grand Island, NY) at 37°C. The digested cartilage solution was filtered in a 100 µm mesh, washed twice in PBS and resuspended in F-12 / 10% FBS media.

RGD Peptide / Alginate Bonding

Peptides containing the sequence GGGGRGDY or GGGGRGEY were be covalently bonded to medium viscosity alginate (FMC) through a condensation reaction with mannuronic or guluronic acid residues as described previously [82].

Briefly, FMC 10/60 alginate was suspended in 100 mM MES (2-[N-Morpholino]ethanesulfonic acid) buffer with 300 mM NaCl at pH 6.5 to make a 1% w/v alginate solution. EDAC (1-ethyl-[dimethylaminopropyl] carbodiimide) was added at 5% molarity to that of the guluronate. Sulfo-NHS (N-hydroxy-sulfosuccinimide) was included at half the molarity of the EDAC. Finally, the octapeptide GGGGRGDY or GGGGRGEY, in 100 mg/ml MES, was added at a ratio of 16.7 mg per gram of alginate. The reaction was allowed to proceed at room temperature for 20 hours before being transferred to 3500 MWCO dialysis tubing and placed in a 4L ddH₂O tank with 7 g NaCl / L. Every 8-12 hours, the salt concentration was lowered 1 g per liter, and the concentration was held at 0 g / L for 24 hours. The contents of the dialysis tube were transferred to a 50 mL conical, frozen, and lyophilized for 48 hours.

Alginate and RGD-alginate was resuspended to form a 2% (w/v) solution in PBS, and sterilized with 0.45 µm syringe filters.

Chondrocyte Disc Fabrication and Culture

Refrigerated 2% alginate was warmed and filter-sterilized as described above. Aliquots of 2 mL aliquots were mixed with centrifuged pellets containing 100 million chondrocytes (50 million cells / mL). The suspension was vortexed and mixed with 80

μL of a well-shaken CaSO_4 solution (Sigma, concentration: 0.266 g / ml PBS) via a 3-way stopcock as described previously [82]. The gel was quickly spread between sterile plates separated by 1 mm spacers. After allowing 15 minutes for the alginate to harden, the plates were separated. Using a biopsy punch, 4 mm diameter discs were carved out of spread culture, and each disc was placed in 300 μL F-12 with 10% FBS, 25 mg/ml ascorbic acid, antibiotics and antimycotics at 37°C, 5% CO_2 .

Selected unmodified or RGD discs were incubated in the presence of soluble RGD or RAD peptide (GRGESP or GRADSP) at 640 μM .

Results

Covalent modification of peptides GGGGRGDY or GGGGRGEY to alginate was accomplished as previously described [80, 82, 102] (figure 32). Cell response to RGE-alginate was similar to the response to unmodified alginate; RGD-alginate elicited an inhibition in GAG synthesis (figure 33).

Chondrocytes responded to culture in 2% RGE-alginate with proteoglycan synthesis comparable to that found in unmodified alginate. Compared to cells in RGD-alginate, GAG content per cell in RGE-alginate was ~25% greater (figure 34).

Discussion

Assuming 60% of the added peptides are incorporated [82], 10.0 mg of peptide is bonded to each gram of alginate, or 135,000 nmol / L of 1% alginate. When the alginate

is concentrated to 2% w/v, the ligand concentration can be expressed as 160,000 ligands per μm^3 , or a ligand spacing of 18 nm. A previous report indicates the minimum ligand spacing for initiation human foreskin fibroblast spreading by identically aligned, immobilized GRGDY peptides was 440 nm, and a spacing of 140 nm was necessary for the appearance of focal contacts and stress fibers [105]. Thus, the RGD spacing in this formulation of alginate is well above the minimum requirements for fibroblast attachment and likely greater than the minimum requirements for chondrocyte spreading. The orientation of peptides on alginate is not identical, but unlike many surface ligand preparations, the orientation is not fixed. It seems likely that many ligands could move into an appropriate orientation for integrin adhesion with time.

The use of RGE-alginate as a control to RGD-alginate is ideal but not always feasible, given the expense and laboriousness of covalently modifying a peptide to alginate. This experiment shows, however, that chondrocyte metabolism in RGE-alginate is similar to that of chondrocytes in unmodified alginate. Thus, the use of unmodified alginate as a control to RGD-alginate is supportable.

Also, the use of soluble RGE or RAD peptide as a control to soluble RGD peptide would be ideal for agonist studies. However, the additional sample sets necessary to accommodate extra controls is prohibitive. The results indicate that RAD peptide does not modulate the metabolic response of chondrocytes seeded in unmodified alginate. Thus, the use of RAD peptide as a control in every experiment is not necessary.

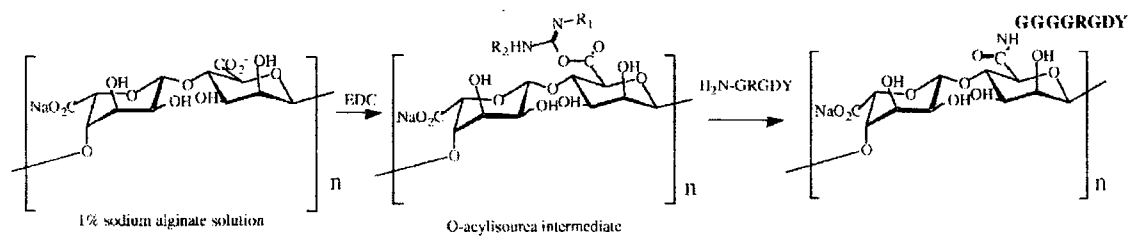


Figure 32. Fabrication of RGD-alginate. EDAC makes an amide linkage to the carboxylic acid moieties on the alginate backbone. Sulfo-NHS (N-hydroxy-sulfosuccinimide) was included at half the molarity of the EDAC, to stabilize EDAC against competing hydrolysis reactions and thereby raise the efficiency of amide bond formation. Finally, the octapeptide GGGGRGDY in 100 mg/ml MES was added at a ratio of 16.7 mg per gram of alginate. The same technique was employed for fabrication of RGE-alginate.

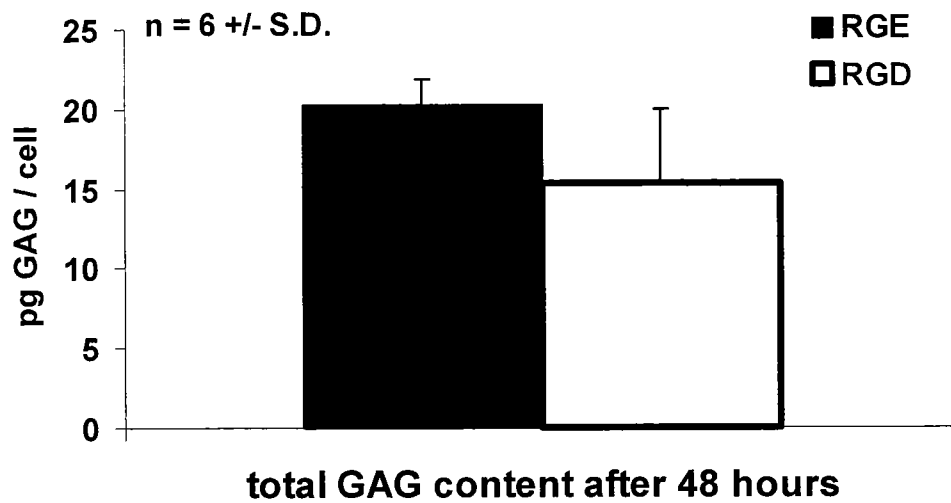


Figure 33. Seeding chondrocytes in RGD-alginate inhibits GAG synthesis. RGE-alginate failed to inhibit GAG synthesis, as was the case with unmodified alginate (chapter 3).

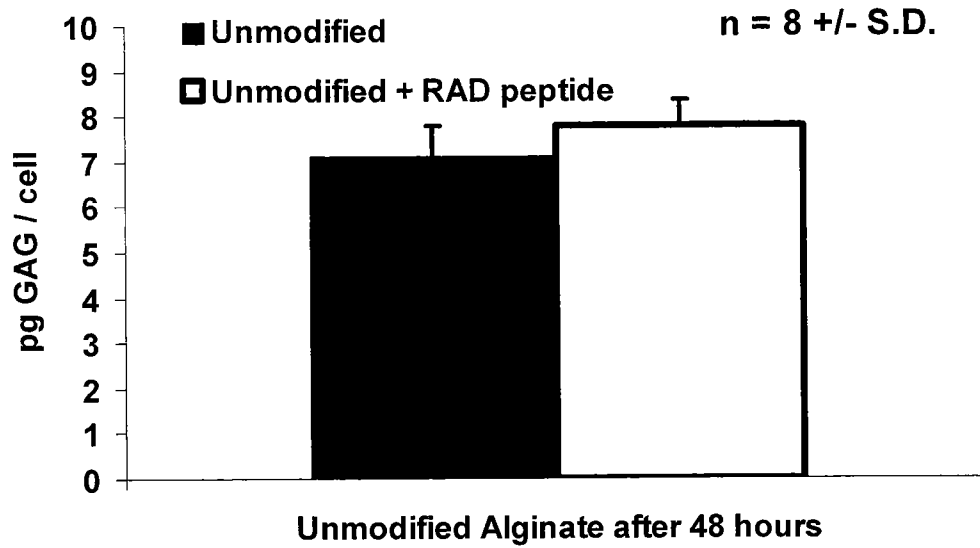


Figure 34. The presence of soluble RAD peptide failed to modulate GAG synthesis in chondrocytes cultured in unmodified alginate discs. Soluble RGD peptide caused an inhibition (chapter 3).

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