UNIVERSITY OF CALIFORNIA, SAN DIEGO

CARTILAGE GROWTH AND REMODELING: MODULATION OF GROWTH PHENOTYPE AND TENSILE INTEGRITY

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Bioengineering

by

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2006

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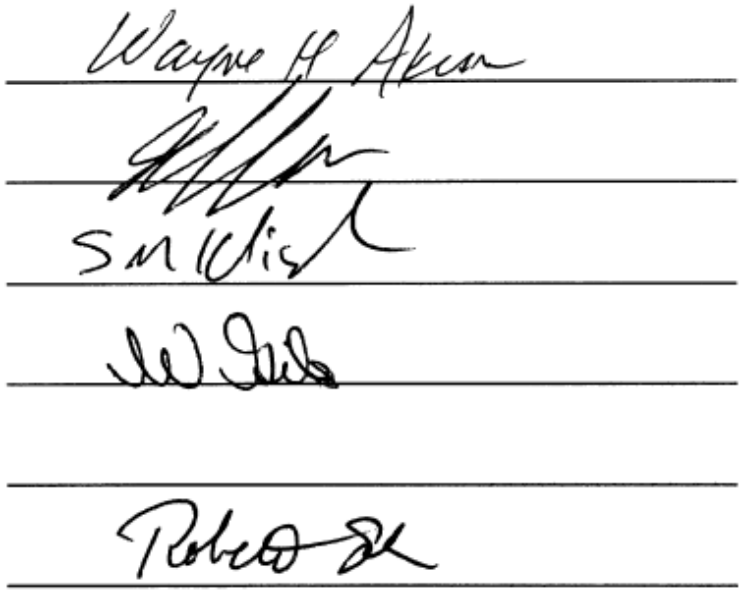

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Journal Articles

Asanbaeva A, Masuda K, Thonar EJ-MA, Klisch SM, Sah RL: Regulation of Immature Cartilage Growth by IGF-I, TGF-β1, BMP-7, and PDGF-AB: Role of Metabolic Balance Between Fixed Charge and Collagen Network *Biomechanics and Modeling in Mechanobiology* (accepted), 2006.

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Klisch SM, **Asanbaeva A**, Masuda K, Thonar EJ-MA, Sah RL: Modeling Articular Cartilage Growth: Polyconvex Strain Energy Functions for the Proteoglycan-Collagen Solid Matrix, Collagen Remodeling, and Validation Protocols. *ASME Journal of Biomechanical Engineering* (in preparation), 2006.

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Klisch SM, **Asanbaeva A**, Sah RL, Davol A: Cartilage Growth Mixture Model: Finite Strain Theory, Constitutive Equations, and Boundary Value Problem Solutions. *11th International Symposium on Plasticity*, 2005.

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ABSTRACT OF THE DISSERTATION

CARTILAGE GROWTH AND REMODELING:
MODULATION OF GROWTH PHENOTYPE
AND TENSILE INTEGRITY

by

Anna Asanbaeva

Doctor of Philosophy in Bioengineering
University of California, San Diego, 2004
Professor Robert L. Sah, Chair

Articular cartilage function as a low friction, wear-resistant, load-bearing material in joints depends on the molecular composition and structure of the extracellular tissue matrix. The proteoglycan component provides the tissue with a fixed negative charge, which imparts a swelling pressure. The crosslinked collagen network resists the swelling tendency of the proteoglycans, and provides the tissue with tensile integrity. During growth of articular cartilage *in vivo*, composition and function evolve dramatically and both chemical and mechanical stimuli have profound regulatory effects. However, it is unknown how immature tissue evolves into its adult form, and if and how various types of chemical stimuli modulate this process. The

overall hypothesis proposed here is that cartilage growth results from a chemically regulated dynamic imbalance between the swelling pressure of glycosaminoglycans (GAG) and the restraining function of the collagen network.

Manipulation of matrix content, metabolism, and assembly distinctly altered the growth phenotype of immature articular cartilage, as assessed by culture-associated variations in tissue geometry, composition, and function. *In vitro* growth of cartilage explants was regulated differentially by growth factors. Overall, cartilage explants grew in volume, as GAG content, an indicator of swelling pressure, increased and tensile modulus and strength, indicators of collagen network integrity, decreased. The propensity of cartilage tissue to grow was additionally enhanced during growth in presence of an inhibitor of collagen crosslink formation, and reduced during growth of explants which were depleted of GAG prior to culture. Thus, factors that lead to growth of cartilage explants *in vitro* involve a shift in the balance between the swelling pressure of the proteoglycans and the restraining ability of the collagen network, toward an overall expansive effect resulting from the swelling pressure.

The tensile integrity of articular cartilage was modulated through depletion of certain extracellular matrix components; however the extent of this modulation was dependent on the maturity of the source tissue. Certain resident matrix components of articular cartilage were implicated in the development of tensile integrity.

An understanding of cartilage growth mechanisms may ultimately allow the development of methods to guide appropriate growth of cartilage tissue grafts for emerging tissue engineering and cartilage repair therapies.

CHAPTER 1

INTRODUCTION

1.1 General Introduction to the Dissertation

Articular cartilage normally functions as a low friction, wear-resistant, load-bearing material for many decades; however it is susceptible to injury- and age-associated degeneration. One approach to repairing articular defects is to regenerate cartilage by mimicking fetal and postnatal growth [132]. It is important to determine the physical and chemical regulators of cartilage growth, and molecular mechanisms underlying such growth, if tissue engineering is to be successful in fabricating cartilage with properties approaching those of normal adult cartilage.

The growth of articular cartilage from fetus, through newborn, to the adult involves a dramatic increase in the size of articular cartilage as well as changes in its composition and mechanical integrity. Transformation of articular cartilage from a lax immature type tissue that is able to expand and sustain growth to a stable mature state whose primary function is to withstand the mechanical demands of adulthood involves precisely orchestrated deposition of the extracellular matrix (ECM) components and tissue expansion. *The overall motivation of this dissertation was to contribute to the understanding of the nature of processes that drive the transformation of cartilage tissue from immature to a mature state by examining how cartilage growth can be*

modulated in vitro through manipulations of content, synthesis, and assembly of matrix components, as well as examining how this transformation may be accomplished through remodeling of the collagen network.

Growth and remodeling are biological processes that, together, transform cartilage tissue *in vivo* from an immature to a mature state. Tissue growth is generally defined as an increase in tissue size due to accretion of one or more solid tissue components similar to those already present, while tissue remodeling is defined as a change in tissue composition and/or structure of tissue components [78, 154]. Many tissues, including articular cartilage, can expand due to accretion of fluid; however this process is not generally considered growth, but rather tissue swelling. Two distinct mechanisms of tissue growth have been recognized: appositional growth, or growth at a tissue surface, and interstitial growth, or growth within tissue volume [32]. While it is possible that tissues can grow appositionally in the absence of remodeling, interstitial tissue growth must involve both growth and remodeling since accretion of a single tissue component will change the overall tissue structure and mechanical properties. Tissues can exhibit growth and remodeling in the form of hyperplasia (increase in the number of cells), hypertrophy (addition of cellular components), deposition of the extracellular matrix components or any number of these processes occurring concomitantly. In articular cartilage, the incidence of cell division is low and matrix deposition is the major contributor to the increase in size (i.e. growth) and changes in biochemical composition (i.e. remodeling) of this tissue *in vivo* [74, 171, 172]. Since articular cartilage tissue may undergo both appositional and interstitial growth and the major contributor to cartilage growth is matrix deposition, the term *growth* is used subsequently in this dissertation to refer, collectively, to both growth and remodeling, which can occur in presence or absence of cellular proliferation.

Chapter 1 begins with a description of composition, structure and function of articular cartilage and how it is accomplished through synthesis, assembly and remodeling of cartilage matrix componenets. Then, various scenarios of cartilage growth, such as normal and pathological growth/tissue expansion *in vivo*, as well as growth *in vitro* during serum-supplemented culture are described. Next, the regulation of cartilage growth by chondrocyte metabolism in response to chemical, mechanical, and genetic stimuli is reviewed. Finally, analytical models of cartilage metabolism and growth are described briefly.

Chapter 2, which has been accepted for publication in *Biomechanics and Modeling in Mechanobiology*, examines the effects of IGF-I, TGF-β1, BMP-7, and PDGF-AB on *in vitro* growth of immature bovine articular cartilage explants. Culture-associated variation in tissue geometry, accumulation of proteoglycan and collagen network components, and changes in tensile mechanical properties were assessed. Additionally, whether tissue size is related to the accumulation of GAG in the tissue and the swelling-resistant properties of the collagen network and whether tensile mechanical properties are related to the biochemical composition of the tissue was assessed by correlative analysis.

Chapter 3, which has been submitted to the *Osteoarthritis and Cartilage*, examines the effects of reduction of the restraining function of the collagen network, by disrupting collagen network assembly during growth with ß-aminopropionitrile (BAPN), an inhibitor of collagen crosslinking. Immature bovine articular cartilage explants were cultured in presence of serum with or without supplementation with BAPN. Culture-associated variation in tissue geometry, accumulation of proteoglycan and collagen network components, and changes in tensile mechanical properties were assessed, similar to the methods in Chapter 2.

Chapter 4, which has been accepted for publication in *Arthritis and Rheumatism*, examines the effects of reduction of cartilage swelling pressure, by diminishing GAG content prior to culture by enzymatic treatment with chondroitinase-ABC (C-ABC), on *in vitro* growth of immature bovine articular cartilage explants. Culture-associated variation in tissue geometry, accumulation of proteoglycan and collagen network components, and changes in tensile mechanical properties were assessed, similar to the methods in Chapter 2 and 3.

While Chapters 2-4 concentrated on the effects of manipulations of matrix content, synthesis and assembly on cartilage growth, in Chapter 5 the focus is shifted towards examining the mechanisms of collagen network remodeling. Chapter 5 determines the effects of increasingly selective ECM depletion on tensile mechanical properties of immature and mature articular cartilage, and thereby establishes a framework for identifying molecules that regulate cartilage network remodeling. The effects of ECM depletion using guanidine HCl (Gnd) on tensile mechanical properties were examined and whether these effects are dependent on the stages of maturity of the source tissue was investigated. Immature and mature articular cartilage from bovines and healthy human donors was analyzed. To further examine the nature of the effect of ECM depletion on tensile integrity, the chapter examines the effect of ECM depletion using C-ABC, chondroitinase-AC (C-AC), and *Streptomyces* hyaluronidase (sHAase) on tensile mechanical properties of immature articular cartilage, where the effect of matrix depletion with Gnd was the greatest. Treatment-associated variations in tissue geometry, proteoglycan and collagen contents, and changes in tensile mechanical properties were assessed.

Chapter 6 summarizes the major findings and discusses future directions of this work.

1.2 Structure, Composition, and Function of Mature Articular Cartilage

Articular cartilage is a connective tissue that covers the ends of long bones [21] and normally functions as a low friction, wear-resistant, load-bearing material that facilitates joint movement [92, 105]. Adult cartilage is composed of a relatively small fraction of cells, within a fluid-filled ECM (Fig. 1.1). The solid component of cartilage matrix consists of collagen (60% of the dry weight) [21] and proteoglycan (25-35% of the dry weight) which appear to be predominantly responsible for the functional mechanical properties of the tissue [55, 92, 105].

Collagen type II forms the primary component of the collagen fibers with minor contribution (on weight basis) from collagens type IX and XI [41]. Because collagen type IX may have a chondroitin-sulfate chain, it is also considered a proteoglycan [129]. One of the domains of collagen IX projects away from the surface of the fibril and may be serve to anchor the molecule onto another collagen II or IX molecule or interact with the proteoglycan component of the matrix [37, 178]. The collagen II:IX:XI heterofibers are organized into a tight network that is crosslinked by reaction based on lysyl oxidase [43], providing tensile and shear stiffness and strength to articular cartilage [105, 160, 175].

Small leucine-rich proteoglycans decorin and fibromodulin, have a similar horseshoe-shaped three dimensional structure [141, 168] which allows them to bind to the surface of the collagen fibers. The amino terminus of decorin core protein is substituted with a single dermatan sulfate chain and fibromodulin has multiple keratan sulfate chains, however, binding of these proteoglycans to collagen depends on their core protein. These molecules may have a role in organizing and stabilizing the

collagen network, although their direct function in the cartilage matrix is still not clear [64, 129].

Aggrecan, the major proteoglycan present in cartilage, non-covalently binds to hyaluronic acid with the help of link protein that strengthens the attachment, to form a large proteoglycan aggregates of ~$2.5*10^6$ Da [58]. Numerous sulfate and carboxylate groups on aggrecan GAG chains provide the tissue with a fixed negative charge that increases the tissue's propensity to swell (due to a Donnan effect) and to resist compressive loading [13, 81]. The balance between the swelling propensity of proteoglycan molecules and the restraining function of the collagen network governs the load-bearing biomechanical function of cartilage [91].

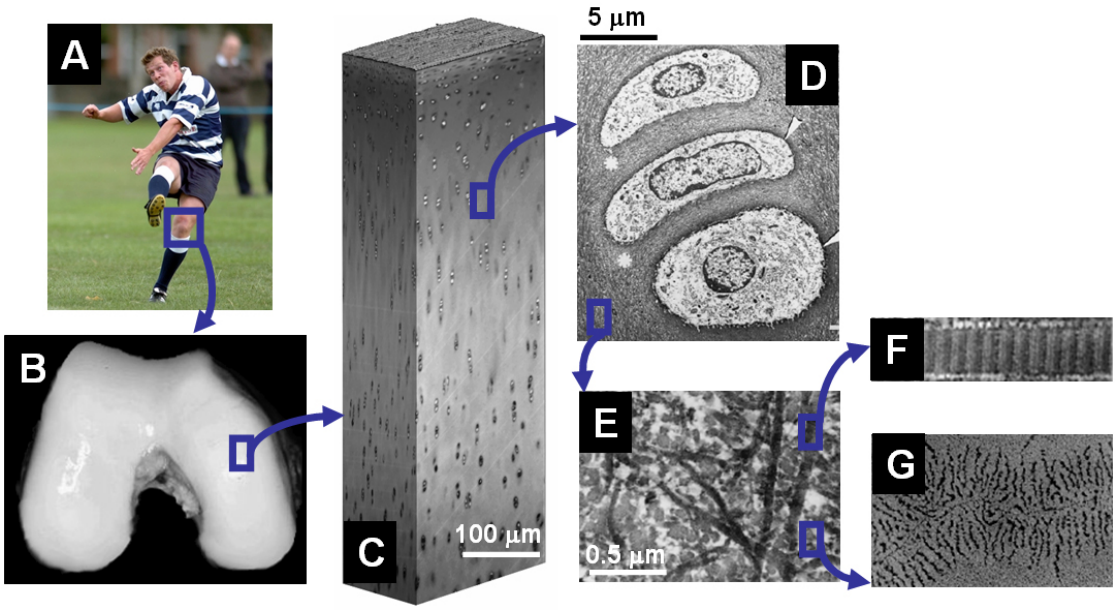

Figure 1.1: Articular cartilage structure and composition. (**A**) Human knee joints are (**B, C**) covered with articular cartilage, which is comprised of (**D, E**) chondrocytes sparsely embedded in extracellular matrix, largely consisting of collagen (**F**) proteoglycans (**G**). Cartilage in the body is hydrated in the synovial fluid. (Photo in panel B is provided by Dr. Michele M. Temple, in C is provided by Dr. Kyle D. Jadin. Micrographs were adapted from [21]).

1.3 Synthesis, Assembly, and Degradation of Articular Cartilage Matrix Components

1.3.a Collagen and Collagen Network Components

The fibril-forming collagens or articular cartilage (i.e. collagen types II and XI) are initially synthesized as procollagen molecules, comprised of three polypeptide chains wound into a tight triple helix. Collagen is formed from procollagen by cleavage of the amino and carboxy terminus propeptides by the procollagen metalloproteinases [85]. It is thought that the long collagen fibrils found in mature connective tissues are assembled from the short fibril segments. The mean fibril segment length has been shown to increase slowly between 12 and 16 days of embryonic tendon development and to increases rapidly at 17 days [16]. From 19 days of development lateral fibril growth predominates and the average diameter of the collagen fibrils increases [46].

Collagen fibrillogenesis, i.e. the formation of mature fibrils from smaller fibril segments, can occur via linear (end-to-end) and/or lateral (side-to-side) associations and collagen fibril-binding proteoglycans, which may include small leucine-rich proteoglycans and collagen type IX, appear to regulate this process [53]. Enzymatic treatment of collagen fibers with trypsin, which may remove at least some of the fibril-coating proteoglycans, appears to enhance the lateral associations of collagen fibers *in vitro* [53]. The rapid increase in mean fibril length at 17 days of development occurs contemporary with a significant decrease in fibril-associated decorin [16].

Crosslinking is important in stabilizing the collagen fibrils and contributes to the tensile strength of articular cartilage. During synthesis and assembly of procollagen into collagen fibril segments and into collagen fibrils, collagen crosslinks are formed as a result of the action of the enzyme lysyl oxidase. Lysyl oxidase

mediates covalent intermolecular and intramolecular crosslinking of collagen II, IX and XI fibrils [42, 44] by oxidizing hydroxylysine residues to hydroxylysyl aldehydes which then, through several reactions, lead first to immature dehydroxylysinorleucine (DHLNL) crosslinks, then to stable pyridinoline crosslinks [126]. Lysyl oxidase-mediated collagen crosslink formation can be inhibited with a lathyrogen such as β-aminopropionitrile (BAPN), which binds covalently and irreversibly to the active site of the enzyme [4, 5, 72]. BAPN has been found to cause a 50% inhibition of lysyl oxidase at a concentration of 3–5 μM [71], and at even higher concentrations (e.g., 0.1–0.25 mM), BAPN has little or no discernible effect on matrix synthesis by chondrocytes [5, 39]. Specific inhibition of collagen crosslink formation without an effect on general biosynthesis makes BAPN a useful tool to study the effect of inhibition of functional assembly of the collagen network on the integrity of the collagen network and on cartilage growth.

During the long life of collagen molecules, several non-enzymatic crosslinks can form. Maillard crosslinks formed through non-enzymatic glycation accumulate slowly after skeletal maturity [120, 158], but not during growth [10]. These crosslinks can be produced *in vitro* through the addition of sugars such as ribose or threrose [34] and have been used to study the effects of such crosslinks on biochemical composition [34, 35] and biomechanical properties [28, 161] of cartilage tissue. Also, reactions catalyzed by transglutaminase can result in collagen crosslinking and collagen network bonding [2, 95], but they are generally associated with cartilage regions undergoing terminal differentiation [3].

Collagen molecules are susceptible to degradation by various proteinases, including serine proteases, such as leukocyte elastase [49], and matrix metalloproteinases (MMP), which include collagenases (MMP-1, 8, 13), gelatinases

(MMP-2, 9) and stromelysin (MMP-3). Collagen fibril degradation into classic ¾ and ¼ fragments is attributed to cleavage by collagenases (MMP-1, 8, 13) and has been implicated in collagen breakdown associated with osteoarthritis [14, 101]. The rates of collagen hydrolysis differ between various MMPs. MMP-13 appears to be 5-6 times more efficient with collagen type II as a substrate than with collagen type I [79].

While MMP activity is generally associated with catabolic processes, certain actions of MMPs are important in remodeling of collagen during development and growth and are part of normal tissue remodeling. A number of MMPs appear to play important roles in regulation of development through the embryonic stages, as indicated by their specific temporal and spatial patterns of expression in the developing joint [75, 111, 137]. MMP-2, 3, 8, 9 and 13 show a peak of expression in the stages of early growth, with levels dropping off during maturation and aging [51]. Production of MMPs may also mediate the rapid fusion of collagen segments [16] and consequent tissue strengthening [117] during development [26]. MMP-3 can decouple collagens type II and IX, thus potentially initializing collagen fibril depolymerization. Such cleavage within the collagen network may allow reorganization and remodeling of the collagen network that may be important during active growth [41, 179].

1.3.b Aggregating Proteoglycan

Aggrecan is synthesized as core protein, which is glycosylated, and then assembled with link protein and hyaluronic acid to form proteoglycan aggregates. At the amino terminus of the aggrecan core protein reside two globular domains, termed G1 and G2. The G1 domain provides aggrecan core protein the ability to interact with hyaluronic acid. Another globular domain, termed G3, is present at the carboxy terminus of the aggrecan protein. The GAG chains are attached to the long span

between the G2 and G3 domains, where over many consensus sites for their attachment are present. Those for keratan sulfate reside closer to the G2 domain, whereas those for chondroitin sulfate are closer to the G3 domain.

In the cartilage matrix most aggrecan molecules exist in the form of proteoglycan aggregates, with many aggrecan molecules bound to hyaluronic acid. The interaction between the aggrecan molecules and hyaluronic acid is non-covalent, and this interaction can be disrupted, among other things, by high ionic strength of 4M guanidine HCl [66]. In cartilage tissue the interaction between the aggrecan molecules and hyaluronic acid is stabilized through non-covalent interactions by link protein [57].

GAG molecules are synthesized as polymers of repeating disaccharides in the Golgi apparatus and require a protein acceptor to initiate their synthesis. β-D-xyloside can act as artificial acceptor of a newly synthesized sulfated GAG chain and, thus, can inhibit the deposition of newly-synthesized GAG in the cartilage tissue [60]. β-D-xyloside can be used to manipulate the extent of GAG deposition in cartilage as well as decouple the effects of core protein and GAG synthesis on various biochemical and metabolic parameters [99, 100]. In contrast to the sulfated GAG, hyaluronic acid is synthesized at the plasma membrane of the cell and the growing GAG chain is extruded directly into the ECM [123].

GAGs of cartilage are susceptible to enzymatic cleavage by commercially available enzymes [22]. Such enzymes allow the manipulation of the content of various matrix molecules within cartilage tissue. Chondroitinase ABC (C-ABC) depolymerizes chondroitin sulfate (CS) and dermatan sulfate (DS) and, to some degree, hyaluronic acid (HA) [180], thus potentially affecting CS and DS-containing molecules such as aggrecan, decorin, and collagen type IX as well as HA. Depending

on the extent of HA cleavage with specific treatment conditions, C-ABC may also affect proteoglycan aggregates, as some of the aggregates which are attached to small HA fragments, may be able to diffuse out of the tissue. Collagen and collagen network arrangement are generally unaffected by C-ABC treatment [31, 90, 107]. Chondroitinase AC II (C-AC) is more specific than C-ABC in that it catalytically cleaves CS and HA but not DS [180]. Thus, C-AC may affect similar molecules as C-ABC, except DS-containing molecules like decorin. *Streptomyces* hyaluronidase (sHAase) splits HA [112] and, thus, may remove some proteoglycan aggregates, similar to C-ABC and C-AC.

Like collagen molecules, aggrecan molecules are susceptible to degradation by various proteinases, including MMPs, serine proteases (plasmin and leukocyte elastase), cystein proteases (cathepsin B) as well as the recently discovered aggrecanases (ADAM-TS-4, and 5, disintegrin and metalloproteinase-thrombospondin type) [12, 59, 104, 149]. Both MMPs and aggrecanases cleave aggrecan core protein at multiple sites, generating proteoglycan fragments which are rapidly lost form the cartilage matrix [25, 67, 136]. The span between G1 and G2 domains, termed IGD (for the interglobular domain), is particularly sensitive to the proteolytic attack, potentially due to its relatively inflexible structure [118]. MMP-3 is also known to degrade link protein [110]. Although many aspects of aggrecanolysis are now understood, the relative importance of MMPs and aggrecanases in aggrecan degeneration is still a topic of heated debate [135].

1.4 Articular Cartilage Metabolism and Growth

The balance between the rate of matrix synthesis and degradation is important in physiologic (such as that during growth) and pathologic matrix remodeling. Cartilage tissue growth is generally dominated by an increase in the rate of matrix synthesis by the chondrocytes and a decrease in the rate of degradation and subsequent loss of matrix constituents from the tissue.

1.4.a Articular Cartilage Growth and Maturation *in vivo*

During *in vivo* embryonic development and postnatal growth into skeletal maturity, cartilage undergoes significant volumetric growth accompanied by matrix remodeling/maturation of the extracellular matrix (Fig. 1.2). Immature cartilage is characterized by the high density of cells and their homogeneous distribution, while in the adult tissue high cellularity is confined to the most superficial region, with few cells in the deep region [68]. This is accompanied by matrix remodeling/maturation such that the net deposition of collagen network components exceeds that of proteoglycans Fetal and postnatal growth of the articular cartilage normally involves a net deposition of collagen that is greater than that of proteoglycan, as well as an increase in mechanical integrity. Throughout the maturation of bovine articular cartilage, from the fetal stage, through newborn calf, and to skeletally mature adult, there is an increase in the collagen content and pyridinoline crosslink density, but little or no change in the GAG content [115, 151, 155]. The overall composition of cartilage GAGs also change with age: the proportion of chondroitin sulfate falls with increasing age, whereas that of keratin sulfate and hyaluronate rises [128]. These biochemical changes are accompanied by an increase in the tensile modulus and strength and each

of these biomechanical properties is positively correlated with collagen content and the pyridinoline crosslink density [172].

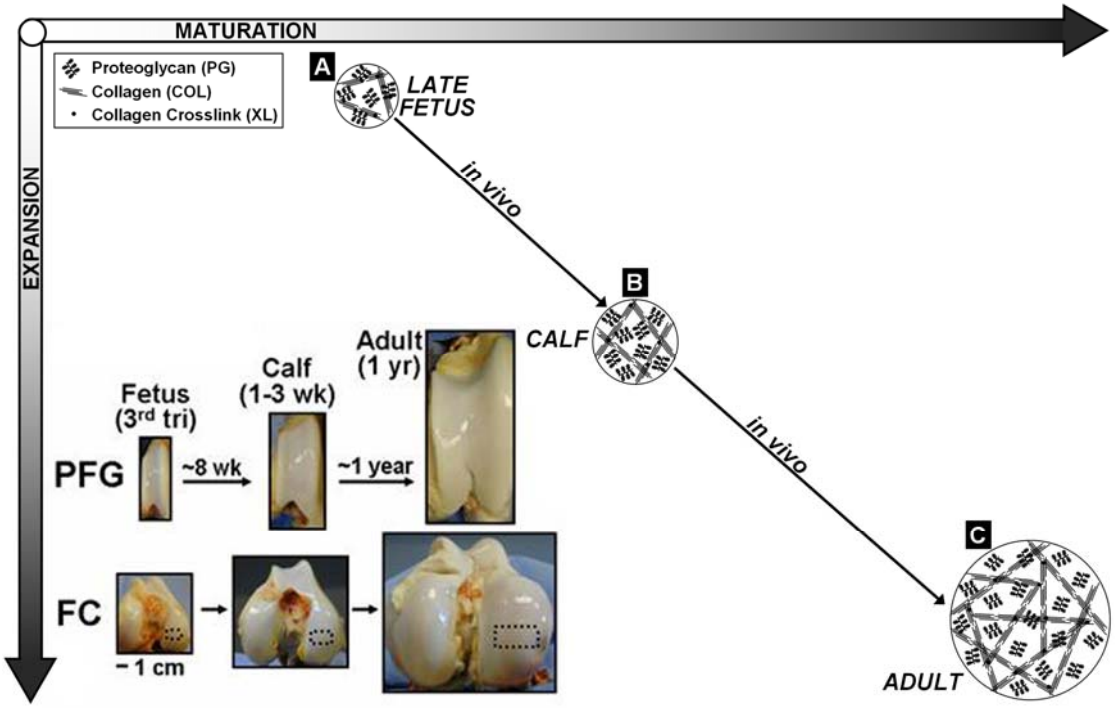

Figure 1.2: During *in vivo* growth of articular cartilage from fetus (**A**) through calf (**B**) and to the adult state (**C**) significant expansion (vertical axis) occurs. This is accompanied by matrix remodeling/maturation (horizontal axis) such that the net deposition of collagen network components exceeds that of proteoglycans (joint images of bovine patellofemoral groove (PFG) and femoral condyle (FC) are from Dr. Amanda K. Williamson).

1.4.b Articular Cartilage Expansion/Swelling in Disease

Certain pathological processes *in vivo* induce biochemical and biomechanical changes in articular cartilage that maybe classified as types of growth/swelling. Osteoarthritis (OA) is a degenerative joint disease that affects over 60 million Americans [7] and has large economic impact, over $50 billion [122], annually. Characteristics of OA include roughening of the surface, articular cartilage softening, and eventual erosion down to the subchondral bone. In OA, changes in the composition and structure of cartilage matrix components lead to changes in biomechanical properties of the tissue and to tissue swelling [11, 93]. Increased proteolytic activity results in a release of proteoglycan fragments into the joint fluid and weakening of the collagen network [121, 124]. These changes lead to a decrease of the proteoglycan content in the tissue matrix and a reduction of collagen network stiffness and strength [6, 95]. Thus, even though the concentration and swelling pressure of GAG decreases, the weakened collagen network has lost its ability to restrain the swelling pressure effectively. The net result is an increased hydration or swelling of the tissue with a concomitant reduction of mechanical integrity.

Hypertropy, or increase in thickness/volume, of articular cartilage tissue is another pathological process that can occur under conditions of altered mechanical environment. In the case of experimental transection of the anterior cruciate ligament in a dog, an increase in proteoglycan concentration resulted in cartilage thickening [2, 17]. Thus, the supranormal fixed charge provides sufficient swelling pressure to expand the tissue leading to cartilage hypertrophy.

1.4.c Articular Cartilage Growth and Maturation *in vitro* during serum-supplemented culture

In contrast to *in vivo* growth and maturation of articular cartilage, growth of cartilage explants *in vitro* in serum-supplemented medium is characterized by extensive volumetric tissue growth accompanied by remodeling that leads to a reversion towards a more immature tissue state (Fig. 1.3). Cartilage growth in serum-supplemented medium results in a net deposition of proteoglycan that is greater than that of collagen and maintenance or a decrease in mechanical integrity. For adult bovine cartilage explants, addition of fetal bovine serum to the culture medium results in a stimulation of proteoglycan synthesis, an inhibition of proteoglycan degradation, a maintenance of proteoglycan concentration at steady-state levels [23, 60, 133]. For cartilage explants from bovine fetus and calf, and neonatal rat, incubation in serum-supplemented medium results in overall growth, maintenance of proteoglycan concentration and a decrease in collagen and crosslink concentrations [50, 130, 131]. These changes in composition are associated with a decrease in tensile modulus and strength [173]. The reason for the discrepancy between *in vitro* growth and *in vivo* growth and maturation may involve a different balance in metabolism of proteoglycans and collagen.

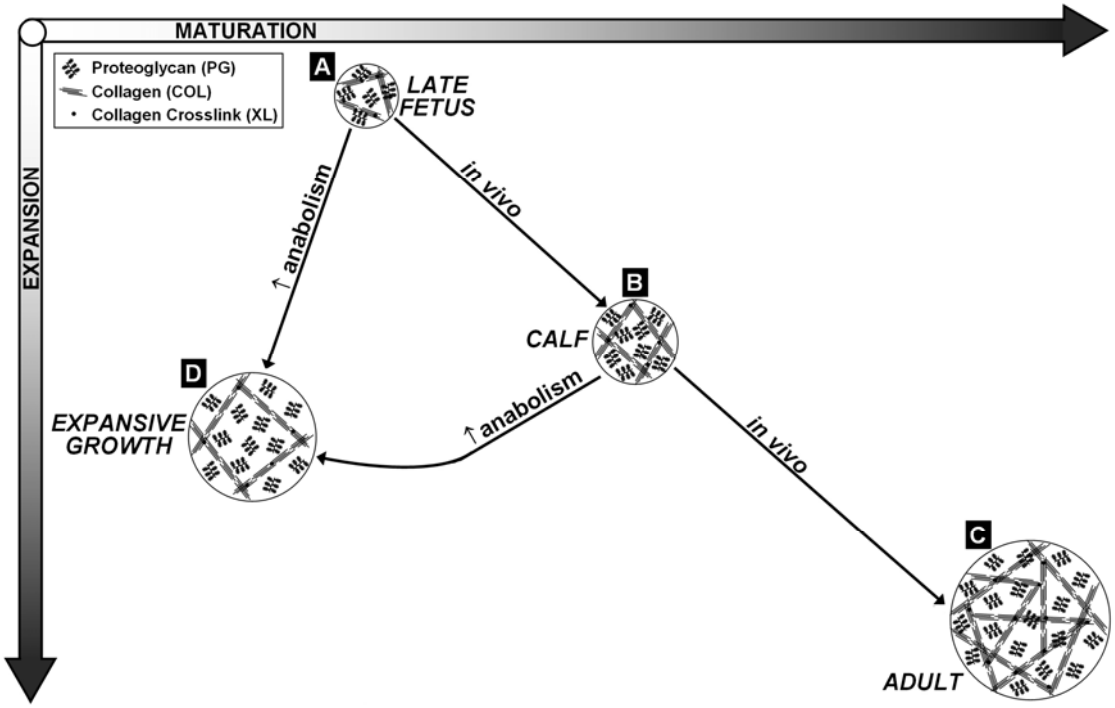

Figure 1.3: Growth of immature articular cartilage *in vitro* during serum-supplemented culture results in *expansive growth* phenotype (**D**), characterized by significant tissue expansion (vertical axis), deposition of proteoglycans that exceeds that of collagen network components and overall reversion towards a more immature state (horizontal axis).

1.5 Regulation of Articular Cartilage Growth

The metabolic processes underlying growth and remodeling of cartilage from an immature to a mature state are regulated by mechanical and chemical stimuli (Fig.1.4). These stimuli govern matrix metabolism, which can lead to changes in the composition, structure, and mechanical properties of the tissue. Genetic alterations in the ECM components or molecules involved in the assembly of the ECM can also alter the normal course of cartilage growth [96].

1.5.a Biochemical regulation of articular chondrocytes and articular cartilage growth

With development through the embryonic stages, and pre-natal and post-natal growth to reach the adult form, various growth factors assume a different tempo-spatial profile of expression. Concentrations of growth factors continue to change with maturation or with injury or disease. Insulin-like growth factor 1 (IGF-1), transforming growth factor- β1 (TGF-β1) and bone morphogenic protein-7 (BMP-7) are present during early stages of limb development as well as in immature and mature articular cartilages [47, 48, 56, 65, 156]. Their concentration in joint fluid and cartilage decline with maturation, and increase after traumatic insult or in disease [29, 30, 170]. Platelet derived growth factor-AB (PDGF-AB) may also play a role in maintenance and repair of cartilage as it is abundant in serum and synovial fluid and is released from platelets during injury. These patterns of expression suggest that growth factors alone and in combination are associated with certain events during growth of articular cartilage [9, 54, 162].

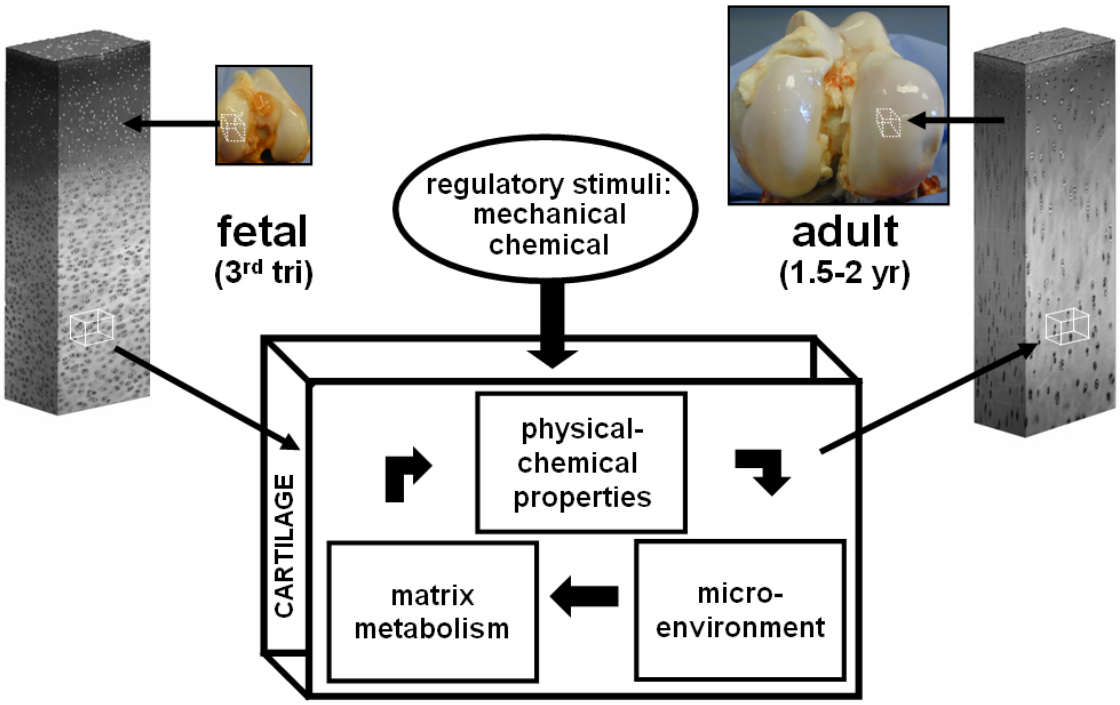

Figure 1.4 The metabolic processes underlying the growth and remodeling of cartilage from the immature to mature state are regulated by mechanical and chemical stimuli. Images of cartilage blocks are adapted from [68] and images of bovine joints are from Dr. Amanda K. Williamson.

The complexity of the effects of various growth factors on cartilage development and growth is further accentuated by disruption of normal skeletal development in natural or targeted mutations of genes encoding for certain growth factors. IGF-1 is a major regulator of cartilage growth and deficiencies in either *IGF-I* or *IGF-I receptor* cause intrauterine growth retardation and postnatal growth failure in mice and humans [1, 89, 138, 177]. Lack of growth differentiation factor-5 (GDF-5) alters the length and the number of bones in the limbs of mice with brachypodism mutation [150], while activation of GDF-5 results in cartilage expansion [157]. The role of fibroblast growth factor receptor-3 (FGFR-3) in the growth plate cartilage appears to be one of negative regulation of intrinsic growth rates. Achondroplasia, dwarfism with reduced size of long bones, is caused by point mutations in the FGFR-3 gene, overactivating the receptor and restricting growth [146]. Contrarily, mice that are homozygous for FGFR-3 null alleles show overgrowth of long bones with enlargement of growth plate cartilage [36].

1.5.b Biomechanical regulation of articular chondrocytes and articular cartilage growth

Several experimental and clinical observations as well as computer models suggest that changes in the in normal pattern of joint loading influence the development and maintenance of articular cartilage [8, 24, 145]. Alteration of mechanical loading during *in utero* development of a human fetus, in conditions such oligohydramnios or breech position, can predispose to developmental hip dysplasia [27]. Experimental embryonic paralysis or restriction of fetal movement leads to joint malformations [62, 98] and alterations in biochemical content and mechanical properties [97]. Limb immobilization in young and mature joints induced a reduction

of tissue' proteoglycan content [69], collagen crosslinking and equilibrium and dynamic shear moduli [84]. Furthermore, joint unloading in presence of join motion, such as that after spinal cord injury or paw transection models, demonstrated biochemical changes similar to those that occurred during immobilization [116, 159]. Continuous joint loading was also destructive of cartilage tissue in the areas of contact [134]. These findings suggest that an optimal regime of joint loading and not joint motion alone is responsible for cartilage development and maintenance. Indeed, adaptive changes in biochemical and mechanical properties were noted in animals subjected to exercise routines. In addition, alteration of mechanical stimuli in certain disease conditions such as hip dysplasia, anterior cruciate ligament and meniscal insufficiency can lead to premature osteoarthritis of the affected joint [73, 169]. These studies indicate that mechanical forces have a profound effect on cartilage during development, growth, and at skeletal maturity, and that abnormal movement and joint loading can interfere with the normal course of joint maturation and maintenance.

1.5.c Regulation of articular cartilage development and growth by genetic alterations in the ECM components

Alterations in the content and structure of proteoglycans and collagens, the major structural components of the ECM, can change the normal course of development of cartilaginous tissues, as demonstrated by natural mutations and gene knockout models [88, 106, 139]. Nanomelia (*nm*) in chickens [82] and cartilage matrix deficiency (*cmd*) in mice [127] result in a truncated aggrecan core protein [86, 165], which is incapable of binding hyaluronic acid. Cartilage of *nm/nm* chickens and *cmd/cmd* mice demonstrate the absence of aggrecan in the cartilage matrix although the levels of collagen are normal [76, 94]. This type of aggrecan deficiency results in

shorter cartilage anlagens and a decrease in epiphysial cartilage volume, leading to shorter bones and dwarfism [82, 119, 127, 167]. Similar reduction in cartilage volume resulted in mice null for link protein (*LP−/−*) [166]. ECM of *LP−/−* mice had a significantly reduced levels of aggrecan, while the amount of type II collagen was similar to that in wild-type mice [167].

Brachymorphism (*bm*) is another disorder in mice characterized by normal size at birth and but a 50% reduction, as compared to normal, in limb length and cartilage ECM volume during postnatal growth [19, 83]. The defect of the *bm*/*bm* mice is the undersulfation of GAG due to a mutation of a sulfate donor 3`-phosphoadenyl-5`-phosphosulfate (PAPS) [80]. *Bm*/*bm* cartilage matrix contains normal collagen fibrils, but stains poorly for GAG [113]. Undersulfation of proteoglycans in the ECM probably alters the normal growth process through reduction in the content of fixed negative charge associated with the sulfate groups. Overall, it appears that low levels of negative charge in the cartilage matrix that can result from either a reduction in the content of sulfate groups (*bm*/*bm*) or aggrecan (*nm*/*nm*, *cmd*/*cmd*, *LP−/−*) maybe decreasing the tissues propensity to expand during growth and consequently resulting in reduced cartilage volume.

Mutations in genes coding for fibrillar collagens also lead to alterations in growth and mechanical integrity of cartilage. Disproportionate micromelia (*dmm*) [18], a severe form of dwarfism, is caused by a mutation in procollagen *Col2a1* [114] which interferes with the assembly of procollagen II homotrimers. As a result the amount of type II collagen in the matrix is greatly reduced. Low levels of type II collagen in cartilage matrix may contribute to the fragile or “liquid” appearance of cartilage in these animals [19]. Another mutations in *Col2a1* gene in mice, spondyloepiphyseal dysplasia congenita (*sedc*), results in mice with thicker articular

cartilage than that of wild-type mice [40]. Mutation in *COL11a1* gene in murine chondrodysplasia (*cho*) [143], is characterized by shortened limbs and absence of COL11 from the ECM of cartilage, while collagen II is present at normal levels [87]. The collagen fibers are thicker than usual suggesting that COL11 may play a role in the lateral assembly of the collagen fibers [103, 144]. The resulting phenotype of (*cho*/*cho*) mice is an increase in the epiphysial cartilage volume and an unusually soft cartilage matrix [87, 143]. *COL9a1* knockout mice develop mild chondrodysplasia with early-onset osteoarthritis [45, 109]. It appears that alterations in the restraining function of the collagen network can lead to differences in growth both in terms of cartilage tissue volume and its mechanical integrity. Reduction in the collagen network integrity may enhance tissue's propensity to expand during growth, resulting in thicker cartilage layer.

The regulation of collagen fibril synthesis, assembly, and remodeling is also important for cartilage growth. Various studies suggest that small proteoglycans that bind to collagen fibers may have a role in regulation of collagen fibril length and diameter. Animal models employing genetic mutations lacking decorin demonstrate collagen fibrils with irregular diameters and decreased skin strength [33]. Down regulation of decorin has been shown to lead to development of collagen fibrils with larger diameters and higher ultimate tensile strengths in ligament scar [108]. Similarly, knockout mice for fibromodulin, tend to have fibers of smaller diameter with irregular outlines [152] accompanied by a tendency towards ligament rupture and early degenerative changes in articular cartilage [52]. Studies *in vitro* indicate that decorin [163, 164] and fibromodulin [63] retard the formation of type II collagen fibrils, and that in embryonic tendon growth, a dramatic decrease in fibril-associated decorin occurs contemporary with lateral collagen fibril fusion to form more continuous fibrils

[15]. In addition, enzymatic treatment of cartilage with trypsin, which may remove at least some of the small proteoglycans, appears to enhance cartilage repair *in vivo* [102] and the association of collagen fibers from tendon *in vitro* [53]. These findings suggest that a decrease in decorin or other collagen-binding molecules is necessary to permit the lateral association of collagen segments. In corollary, binding of collagen-binding molecules to the collagen fibers competitively inhibits the accretion of additional collagen segments and, in this way, regulates the growth of collagen fibrils during development [53, 70, 140]. A similar mechanism may be responsible for controlling the restraining function of the collagen network during growth through mediating the adherence and sliding between fibrils to modulate tissue shape [20, 142, 176]. While the exact nature of interactions between collagen and small proteoglycans to form a functional collagen network remains to be established, collagen-binding molecules have been proposed as functional non-covalent linkages [125, 153] in the ECM.

1.6 Models of Cartilage Metabolism and Growth

Dynamics of cartilage growth, in terms of evolution of tissue size, biochemical composition and mechanical properties can be described by mathematical models. Both compartmental and continuum models to describe the composition of cartilage explants and tissue engineered cartilage have been developed [38, 61, 77, 174]. However these models are designed to only describe the metabolism of a single or multiple matrix components. If cartilage growth models are to be successfully used for applications in tissue engineering, such as for design of growth conditions to generate

tissue with desired geometry, composition and function, they have to be able to relate matrix metabolism to the evolution of tissue shape and mechanical function.

Tissue growth can be described as a displacement field, where points within and on the tissue surface move relative to a fixed reference point, and relative to each other [147, 148]. Cell proliferation and matrix deposition throughout the tissue volume can be regarded as a spatial distribution of mass sources. Conversely, cell death and matrix resorption are considered a spatial distribution of mass sinks. In this analysis, growth is represented by a tensor, transforming a tissue region R_1 at time t_1 to the grown configuration occupying R_2 at time t_2, by the relative motion of points that constitute the tissue. The time rate of change of concentration of tissue constituents can be described by introducing the growth rate (the local biosynthesis or transport rate) into the equation of continuity.

Recently, a continuum mechanics model of cartilage growth that is able to predict biomechanical parameters and the changes in matrix composition has been developed [78]. This cartilage growth model describes growth as deposition of components of a mixture, including proteoglycan and collagen network components, which lead to deformations of the tissue and alteration of tissue composition and mechanical properties. Variables of this model include tissue deformation, contents of proteoglycan and collagen network components, and mechanical properties of the collagen network. These variables can be obtained experimentally and used to further refine the cartilage growth mixture model.

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CHAPTER 2

REGULATION OF IMMATURE CARTILAGE GROWTH BY IGF-I, TGF-β1, BMP-7, AND PDGF-AB: ROLE OF METABOLIC BALANCE BETWEEN FIXED CHARGE AND COLLAGEN NETWORK

2.1 Abstract

Cartilage growth may involve alterations in the balance between the swelling tendency of proteoglycans and the restraining function of the collagen network. Growth factors, including IGF-I, TGF-β1, BMP-7, and PDGF-AB, regulate chondrocyte metabolism and, consequently, may regulate cartilage growth. Immature bovine articular cartilage explants from the superficial and middle zones were incubated for 13 days in basal medium or medium supplemented with serum, IGF-I, TGF-β1, BMP-7, or PDGF-AB. Variations in tissue size, accumulation of proteoglycan and collagen, and tensile properties were assessed. The inclusion of serum, IGF-I, or BMP-7 resulted in expansive tissue growth, stimulation of proteoglycan deposition but not of collagen, and a diminution of tensile integrity. The

regulation of cartilage metabolism by TGF-β1 resulted in tissue homeostasis, with maintenance of size, composition, and function. Incubation in basal medium or with PDGF-AB resulted in small volumetric and compositional changes, but a marked decrease in tensile integrity. These results demonstrate that the phenotype of cartilage growth, and the associated balance between proteoglycan content and integrity of the collagen network, is regulated differentially by certain growth factors.

2.2 Introduction

Articular cartilage is a layer of connective tissue located on the ends of long bones [12] that normally functions as a low friction, wear-resistant, load-bearing material, facilitating joint motion [61, 70]. The ability of cartilage to withstand compressive, tensile, and shear forces depends on the composition and structure of the extracellular matrix [35, 61, 70]. The proteoglycan constituent of the extracellular matrix provides the tissue with a fixed negative charge that increases the tissue's propensity to swell and to resist compressive loading [8, 52]. The crosslinked collagen network resists the swelling tendency of the proteoglycan molecules and provides the tissue with tensile and shear stiffness and strength [70, 95, 103]. Chondrocytes in cartilage normally maintain a functional matrix by modulating synthesis and degradation of the matrix components.

Growth and remodeling are biological processes that, together, transform cartilage tissue *in vivo* from an immature to a mature state. Growth is generally defined as an increase in size due to accretion of material similar to that already present, while remodeling is defined as a change in the endogenous or the overall material, and, concomitantly, a change in mechanical properties [50, 91]. Biological tissues can be viewed as composite materials that grow and remodel due to changes in the quantity and/or structure of tissue components such as cells and constituents of the hydrated extracellular matrix. Two distinct mechanisms of tissue growth have been recognized: appositional growth, or growth at a surface of tissue, and interstitial growth, or growth within a volume of tissue [23]. While it is possible that tissues can

grow appositionally in the absence of remodeling, interstitial tissue growth must involve both growth and remodeling since accretion of a single tissue component will change the overall tissue structure and mechanical properties. Since articular cartilage tissue may undergo both appositional and interstitial growth, the term *growth* is used subsequently in this paper to refer, collectively, to both growth and remodeling.

Alterations of cartilage function, structure, and composition during growth *in vivo* and during serum-supplemented culture *in vitro* appear to depend on the metabolic balance between proteoglycan molecules and the components of the collagen network. Fetal and postnatal growth of articular cartilage normally involves a net deposition of collagen that is greater than that of proteoglycan, as well as an increase in mechanical integrity. During maturation of bovine articular cartilage, from the fetal stage, through the newborn calf, and to the skeletally mature adult, there is an increase in the collagen and pyridinoline crosslink densities, but little or no change in the content of glycosaminoglycan (GAG) [75, 93, 98, 102]. These biochemical changes are accompanied by an increase in the tensile modulus and strength, and each of these biomechanical properties is positively correlated with the collagen and pyridinoline crosslink densities [97]. In contrast to this type of *in vivo* growth, growth of immature cartilage tissue *in vitro* in serum-supplemented medium results in a net deposition of proteoglycan that is greater than that of collagen and a decrease in mechanical integrity. For cartilage explants from bovine fetus and calf, and neonatal rat, incubation in serum-supplemented medium results in an increase in tissue size, maintenance of proteoglycan concentration and a decrease in the concentrations of

collagen and pyridinoline crosslinks [33, 80, 97]. These changes in composition are associated with a decrease in tensile modulus and strength [99].

A number of growth factors appear to play important roles in regulation of development through the embryonic stages, as well as during pre-natal and post-natal growth. Insulin-like growth factor-I (IGF-I), transforming growth factor-β1 (TGF-β1), bone morphogenic protein-7 (BMP-7, also known as osteogenic protein-1 (OP-1)), and platelet derived growth factor (PDGF) are localized to certain regions of a developing limb in a specific temporal pattern [4, 31, 40, 53, 74, 76, 78]. Deficiencies in either IGF-I or IGF-I receptor cause intrauterine growth retardation and postnatal growth failure [1, 55, 104], while deficiencies in BMP-7 result in skeletal abnormalities and death at birth [57]. The levels of IGF-I in serum and of TGF-β1 in cartilage are low in a neonate and rise during post-natal growth [18, 30, 69]. IGF-I, TGF-β1, and BMP-7 continue to be expressed in immature and adult articular cartilages [3, 17, 26, 32, 47, 58, 67, 71, 86] and reach concentrations of 1-50 ng/g cartilage tissue [58, 86], 0.02-0.2 ng/ml synovial fluid [96], 1-20 ng/g cartilage tissue [16], respectively. Pathological cartilage/bone growth, such as that seen during the formation of osteophytes, also involves activities of growth factors such as TGF-β and PDGF [43, 44].

In vitro studies on articular cartilage explants have delineated the ability of certain growth factors to regulate cartilage metabolism and mechanical integrity. Various investigations point to IGF-I as the major factor in fetal bovine serum (FBS) [7, 58, 59, 65, 80, 92] that stimulates proteoglycan and collagen synthesis in immature and adult cartilage [80], and results in a decreased rate of proteoglycan loss in adult

tissue [80]. Overall, IGF-I results in the net deposition of proteoglycan [80] and maintenance of tissue mechanical function in confined compression [82]. In explant culture of calf cartilage, TGF-β1 has similar effects on proteoglycan metabolism as IGF-I. TGF-β1 increased proteoglycan synthesis and decreased the rate of proteoglycan catabolism, but did not affect the content of collagen [68]. While addition of BMP-7 to explant culture of immature cartilage enhanced the net accumulation of proteoglycan through stimulation of proteoglycan synthesis and diminution of proteoglycan release [54, 73], inhibition of autocrine BMP-7 decreased the accumulation of proteoglycan in cartilage matrix [87]. In immature and adult bovine cartilage explants, PDGF-AB stimulated proteoglycan synthesis and decreased the rate of proteoglycan catabolism [83].

Cartilage hydration and the load-bearing biomechanical function are influenced by the balance between the swelling propensity of proteoglycan molecules and the restraining function of the collagen network. This idea was proposed by Maroudas [60] and has been supported by theoretical models [8, 13, 25, 52]. The increased hydration and loss of mechanical integrity of osteoarthritic cartilage, compared with normal cartilage, is due to a weakening of the collagen network and an associated swelling of the tissue [2, 6, 62]. Analogously, in the context of growth, a low or reduced restraining function of the collagen network, due to either variations in network composition or structure or due to excessive swelling pressure imposed by the newly synthesized proteoglycan, is predicted to allow tissue swelling and growth [50]. Thus, we hypothesized that cartilage growth results from a dynamic imbalance

between the swelling pressure of endogenous and newly synthesized GAG and the restraining function of the collagen network.

Since growth factors, such as IGF-I, TGF-β1, BMP-7, and PDGF-AB, regulate chondrocyte metabolism, they may consequently regulate cartilage growth. Thus, the objectives of this study were to examine the effects of IGF-I, TGF-β1, BMP-7, and PDGF-AB on *in vitro* growth of immature bovine articular cartilage explants in terms of culture-associated variations in tissue size, accumulation of GAG and collagen, and tensile mechanical properties. Additional objectives were to assess, by correlative analysis, whether tissue size is related to the accumulation of GAG in the tissue and the swelling-resistant properties of the collagen network and whether tensile mechanical properties are related to the biochemical composition of the tissue.

2.3 Materials and Methods

Sample Preparation and Culture

Articular cartilage was harvested from the patellofemoral groove of 3 newborn (1-3 weeks old) bovine calves. Blocks, 9 x 3 x ~0.4 mm^3 (length x width x thickness), were prepared using a sledge microtome to either include the intact articular surface (S) or to include the middle zone, starting at a distance of ~0.6 mm from the articular surface (M). The long axis of the blocks was in the anterior-posterior direction and, thus, approximately perpendicular to the split line direction. Blocks were weighed wet (WW_i) under sterile conditions.

Some blocks were **(a)** analyzed immediately. Other blocks were incubated in medium (DMEM supplemented with 100 μg/mL of ascorbate, 0.1 mM nonessential amino acids, 0.4 mM L-proline, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B) [24] at 37°C in a humidified 5% CO_2 - 95% air incubator with modifications as noted: **(b)** 0.01% cell culture tested bovine serum albumin (BSA, basal medium) (Sigma, St. Louis, MO), **(c)** 20% FBS, or 0.01% BSA with either **(d)** 50 ng/mL recombinant human (rh) IGF-I, **(e)** 10 ng/mL rhTGF-β1, **(f)** 50 ng/mL rhBMP-7, or **(g)** 50 ng/mL rhPDGF-AB. All growth factors were from PeproTech, Inc. (Rocky Hill, NJ), except rhBMP-7 that was from R&D Systems Inc. (Minneapolis, MN). Non-tissue culture treated plates were used for incubation to minimize cell adhesion to the plate and cell outgrowth from the explants. Medium (0.5 mL/block) was changed every other day, and, during the first 12 days of culture, supplemented with 10 μCi/mL [^{3}H]proline and

2 μCi/mL [^{35}S]sulfate. To remove unincorporated isotopes, blocks were then washed (6 times over 1.5 hr., 37°C), transferred to a new culture plate, and incubated for an additional day in medium without radiolabel. At termination, blocks were weighed wet (WW_f) and punched to form a tapered tensile test strip and residual cartilage.

Biochemical Analysis

Residual cartilage and the failed portions of the corresponding tensile strip were analyzed together to quantify the biochemical composition of the fresh and cultured samples. Samples were lyophilized, weighed dry, and solubilized with proteinase K [84]. Portions of the tissue digest were analyzed to quantify the content of DNA [64], GAG [27], and hydroxyproline [101]. DNA was converted to cell number by using a conversion constant of 7.7 pg of DNA per cell [49]. Hydroxyproline content was converted to collagen content by assuming a mass ratio of collagen to hydroxyproline equal to 7.25 [41, 75]. A portion of spent medium collected over 13 days of culture was analyzed for the content of GAG [27] to asses the loss of this matrix component from the tissue. The net change in GAG during culture was calculated for each explant as the difference between the sum of GAG content in the tissue on day 13 and GAG released into the medium over 13 days and GAG content in fresh cartilage of the same layer. This represented GAG synthesis as measured by the DMB dye-binding assay [27]. Total GAG content was calculated as the sum of GAG content in the tissue and GAG content released into the medium over 13 days. Biochemical parameters were normalized to initial wet weight of the tissue

(WW_i) to represent constituent content and to final wet weight of the tissue (WW_f) to represent constituent concentration.

Analysis of Matrix Metabolism

Other portions of the solubilized tissue and portions of the medium were analyzed for the incorporated radioactivity to assess matrix metabolism. [^{35}S] and [^{3}H] radioactivity was determined as indices of sulfated GAG and protein synthesis respectively. To more specifically assess collagen synthesis, portions of tissue digests were pooled for each layer/experimental condition and analyzed for [^{3}H]hydroxyproline residues [81, 89]. A portion of spent medium was analyzed for the content of [^{35}S]GAG using the Alcian Blue precipitation method [63]. The rates of incorporation of [^{35}S]sulfate and [^{3}H]proline were used to estimate the absolute rates of sulfate and proline incorporation based on the specific activity of [^{35}S]sulfate and [^{3}H]proline in the medium and an assumed equivalence between the specific activity of the intracellular biosynthetic precursor pools and of the medium. The sulfate concentration in the medium was about 0.86 mM, assuming the sulfate concentration in serum of 0.32 mM [20] and proline concentration in the medium was about 0.42mM, with serum proline concentration of 0.16mM (HyClone, Logan, UT). Total sulfate incorporation was calculated as the sum of the content of sulfate incorporation in the tissue and medium and represented GAG synthesis as measured by [^{35}S]sulfate incorporation.

Biomechanical Analysis

Tapered tensile strips were analyzed to determine mechanical properties as described previously [15]. From each cartilage block, a tapered strip [48] with a gage region of 4 mm x 0.80 mm was prepared using a punch. The thickness of each tensile strip was measured at three locations in the gage region, using a contact-sensing micrometer, and the average was used for cross-sectional area calculations. Tapered specimens were then secured in clamps (4.0 mm apart) of a mechanical tester and elongated at a constant extension rate (5 mm/min) until failure. Structural tensile parameters were obtained from the load-displacement curves. Structural tensile strength was determined as the maximum load sustained at failure. Ramp stiffness was calculated as the slope of the linear regression of the load-displacement curve from 25-75% of the maximum load. Load and displacement were converted to stress (defined as load normalized to the cross-sectional area of the gage region) and strain (defined as the elongation distance normalized to the initial clamp-to-clamp distance) to obtain material tensile parameters of tensile strength, strain at failure, and ramp modulus. The strain at failure was the strain at which maximum stress was attained. The failed portions of each tensile strip, resulting from the tensile test, were saved for biochemical analysis (described above) in addition to the adjacent cartilage samples obtained during preparation of the tensile strips.

Statistical Analysis

For each layer (S and M), the effects of experimental conditions **(a-g)** were assessed by analysis of variance (ANOVA) with experimental condition as fixed

factor and donor animal as a random factor. Where significant ($p<0.05$) differences were detected, Tukey post-hoc testing was performed. To analyze the effect of 13 days of incubation on wet weight, repeated measures ANOVA was performed for each layer and experimental condition with wet weight (WW_i and WW_f) as a repeated factor. All correlative relationships were analyzed by univariate linear regression. A significance criterion of 0.05 was used when assessing regression slopes. To determine whether the level of correlative relationships in the S and M layers were similar, the regression slopes for these groups of samples were compared by *t* test. Data are expressed as mean ± SEM. Statistical analysis was performed using Systat 10.2 (Systat Software, Inc., Richmond, CA).

2.4 Results

Assessment of Volumetric Tissue Growth

The extent of *in vitro* volumetric growth of articular cartilage blocks was markedly affected by experimental conditions as assessed by changes in thickness ($p<0.05$ in each layer) (Fig. 2.1A), wet weight ($p<0.001$ in each layer) (Fig. 2.1B), and water content (Fig. 2.1C). The thickness increased during culture with FBS (104%, $p<0.001$ in S, 45%, $p<0.05$ in M) and IGF-I (78%, $p<0.001$ in S) and, in the S layer, was larger than the thickness of explants cultured in basal medium (66%, $p<0.001$ for FBS; 44%, $p<0.05$ for IGF-I). Similarly, large extents of wet weight change occurred during culture with FBS (82% in S, 54% in M), IGF-I (82% in S, 37% in M), and BMP-7 (46% in S, 24% in M) (all $p<0.001$) and exceeded the extent of wet weight change during culture in basal medium (62% in S, 44% in M for FBS; 61% in S, 27% in M for IGF-I, $p<0.001$; 26% in S, 14% in M for BMP-7, $p<0.05$). In contrast, incubation in basal medium or with TGF-β1 or PDGF-AB did not affect the thickness (all $p>0.38$); consistent with this, the change in wet weight of these explants was small (21%, $p<0.05$ in S and 10%, $p<0.001$ in M for basal; 12%, $p<0.05$ in S and 9%, $p<0.001$ in M for TGF-β1; 29%, $p<0.01$ in S and 17%, $p<0.01$ in M for PDGF-AB). The volumetric growth of all explants appeared to be predominantly axial, as changes in wet weight can mostly be accounted for by changes in thickness alone. The content of water varied slightly in the S layer ($p<0.001$), increasing during basal culture or culture with IGF-I, BMP-7, or PDGF-AB (3%, $p<0.01$ each). Since water content varied slightly, changes in wet weight were largely due to changes in tissue

volume and not density and, thus, along with changes in thickness, represent volumetric growth of tissue samples.

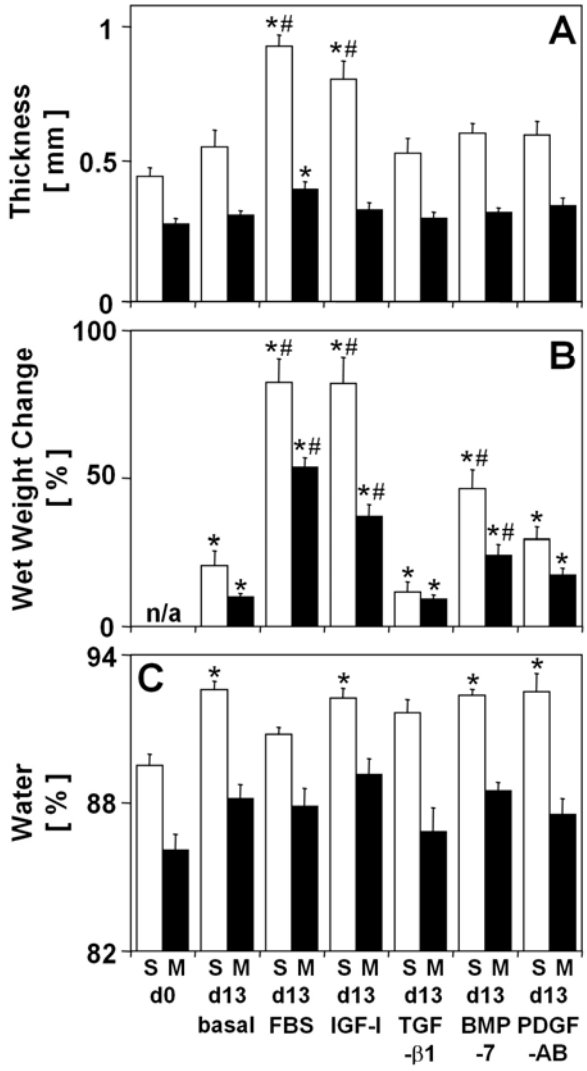

Figure 2.1: Effect of experimental conditions on general indices of *in vitro* growth of calf articular cartilage explants from the superficial (S) and middle (M) layers. Blocks were analyzed on day 0 (d0), or incubated for 13 days (d13) in basal medium (0.01% BSA) or medium supplemented with either 20% FBS, 50 ng/ml IGF-I, 10 ng/ml TGF-β1, 50 ng/ml BMP-7, or 50 ng/ml PDGF-AB. (**A**) Thickness, (**B**) change in wet weight, and (**C**) percent water of cartilage blocks. Data are mean ± SEM, n = 8-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. basal condition.

Biochemical Analysis

The content of cells (Cells/WW_i, Fig. 2.2A) did not vary with experimental conditions and, when normalized to WW_f to give a measure of concentration of cells (Cells/WW_f, Fig. 2.2D), reflected the changes in wet weight ($p<0.001$ in each layer). During culture, Cells/WW_f decreased in explants cultured in basal medium (-24% in S, -22% in M), with FBS (-57% in S, -34% in M), IGF-I (-45% in S, -26% in M), BMP-7 (-31% in S, -19% in M), and PDGF-AB (-31% in S, -22% in M) (all $p<0.01$). In contrast, during culture with TGF-β1 Cells/WW_f did not change ($p=0.44$ in S) or decreased slightly (-19%, $p<0.01$ in M).

The extent of volumetric growth was generally paralleled by variations in the tissue content of GAG but not collagen (COL). GAG content (GAG/WW_i, Fig. 2.2B) varied with experimental conditions ($p<0.001$ in each layer), while the content of COL (COL/WW_i, Fig 2C) did not vary. GAG/WW_i increased during culture with FBS (36%, $p<0.01$ in S, 43%, $p<0.001$ in M), IGF-I (43%, $p<0.001$ in S, 31%, $p<0.001$ in M), and BMP-7 (41%, $p<0.01$ in S, 19%, $p=0.09$ in M) and exceeded GAG/WW_i in explants cultured in basal medium (28%, $p<0.05$ in S, 58%, $p<0.001$ in M for FBS; 34%, $p<0.01$ in S, 45%, $p<0.001$ in M for IGF-I; 32%, $p<0.05$ in S, 31%, $p<0.01$ in M for BMP-7). In contrast, GAG/WW_i did not change during basal culture or culture with TGF-β1 or PDGF-AB (all $p>0.24$).

When normalized to WW_f to give an index of concentration in the tissue, the concentrations of extracellular matrix components reflected changes in the content of the components and in the wet weight of the tissue during culture. The concentrations of GAG (GAG/WW_f, Fig. 2.2E) and COL (COL/WW_f, Fig. 2.2F) varied with

experimental conditions in the S layer ($p<0.01$) but did not change during culture of explants from the M layer. In the S layer, GAG/WW_f did not change ($p>0.51$ for basal, TGF-β1, BMP-7, and PDGF-AB) or decreased slightly (-21-25%, $p<0.01$ for FBS and IGF-I), while COL/WW_f decreased markedly during culture with FBS (-43%, $p<0.001$), IGF-I (-45%, $p<0.001$), BMP-7 (-40%, $p<0.001$), and PDGF-AB (-31%, $p<0.05$), but not with TGF-β1 or during basal culture ($p>0.27$).

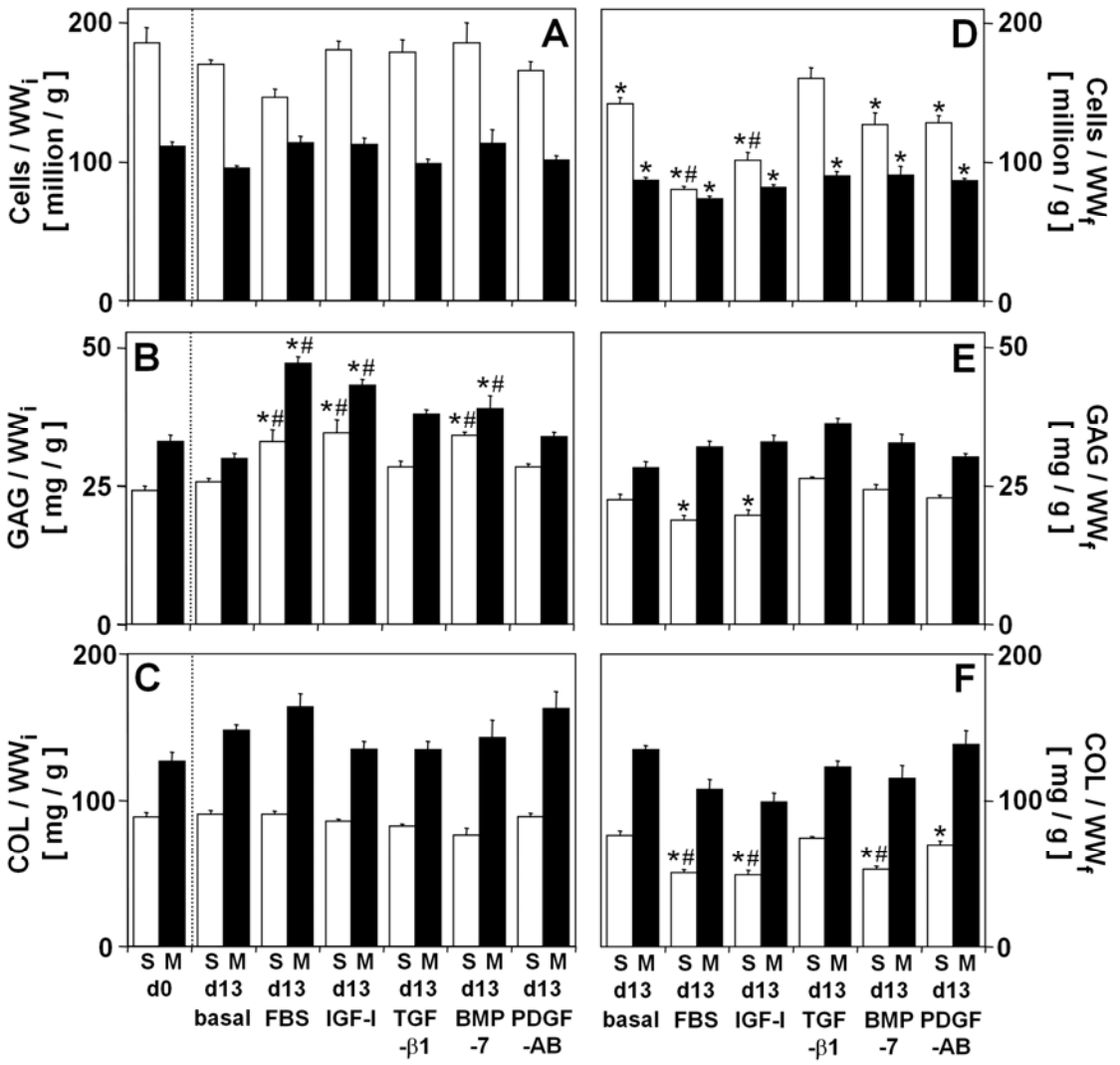

Figure 2.2: Effect of experimental conditions on biochemical composition of calf articular cartilage explants from the S and M layers (**A**, **D**) Cells, (**B**, **E**) glycosaminoglycan (GAG), and (**C**, **F**) collagen (COL) normalized to initial wet weight (WW_i) to represent constituent content (**A**-**C**) and final wet weight (WW_f) to represent constituent concentration (**D**-**F**). The dotted line separates explants analyzed on day 0, where $WW_f = WW_i$. Data are mean ± SEM, n = 8-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. basal condition.

Analysis of Matrix Metabolism

Changes in GAG content (Fig. 2.3B) were generally paralleled by similar variations in the incorporation of sulfate (Fig. 2.3A). The experimental conditions had only a minor effect on GAG released into the medium, with greater release during incubation with IGF-I than during incubation in basal medium (27%, p=0.08 in S; 26%, p<0.05 in M) or with FBS (31%, p<0.05 in S; 39%, p<0.001 in M). While the total GAG content increased in all cultures (44-57% for basal; 85-91% for FBS; 98-107% for IGF-I; 71-77% for TGF-β1; 78-96% for BMP-7; 61-69% for PDGF-AB, all p<0.001), only incubation with FBS, IGF-I, and BMP-7 resulted in a higher total GAG content than that in explants incubated in basal medium (18%, p=0.08 in S and 33%, p<0.001 in M for FBS; 32-38%, p<0.001 for IGF-I; 24-25%, p<0.01 for BMP-7). Consistent with this, the net GAG change during culture with FBS, IGF-I, and BMP-7 was higher than that during incubation in basal medium (50%, p=0.08 in S and 107%, p<0.001 in M for FBS; 90-123%, p<0.01 for IGF-I; 70-77%, p<0.05 for BMP-7). Similarly, incubation with FBS, IGF-I, and BMP-7 stimulated the total sulfate incorporation and incorporation of sulfate into the tissue and medium above the levels in explants incubated in basal medium (187-255%, p<0.001 for FBS; 135-142%, p<0.01 for IGF-I; 89-95%, p<0.05 for BMP-7). A correlation analysis indicated a positive correlation ($p<0.01$, $R^2 = 0.47$ in S; $p<0.001$, $R^2 = 0.51$ in M) between the total sulfate incorporation, an indicator of GAG synthesis measured by [^{35}S]sulfate incorporation, and the net GAG change during culture, an indicator of GAG synthesis measured by the DMB dye-binding assay (Fig. 2.3D). Assuming one mole of sulfate per disaccharide, the regression slopes should yield the molecular

weight of GAG and, indeed, approach 502.4 g/mol in both the S (490 g/mol) and M (600 g/mol) layers. Thus, the metabolism of [^{35}S] can be used to quantitatively assess the amount of sulfated GAG deposition.

Variations in the incorporation of sulfate (Fig. 2.3A) were paralleled by variations in proline incorporation (not shown) (all $p<0.05$) and hydroxyproline formation (not shown). Incubation with FBS, IGF-I, and BMP-7 enhanced incorporation of proline into the tissue above the levels in explants incubated in basal medium (247% in S, 461% in M for FBS; 124% in S, 155% in M for IGF-I; 75% in S, 95% in M for BMP-7, all $p<0.01$). In the absence of FBS, the percent of hydroxyproline residues did not vary and reached 27-31%, while in explants incubated with FBS, the percent of hydroxyproline residues reached 36-38%. The resultant variations in hydroxyproline formation paralleled variations in proline incorporation.

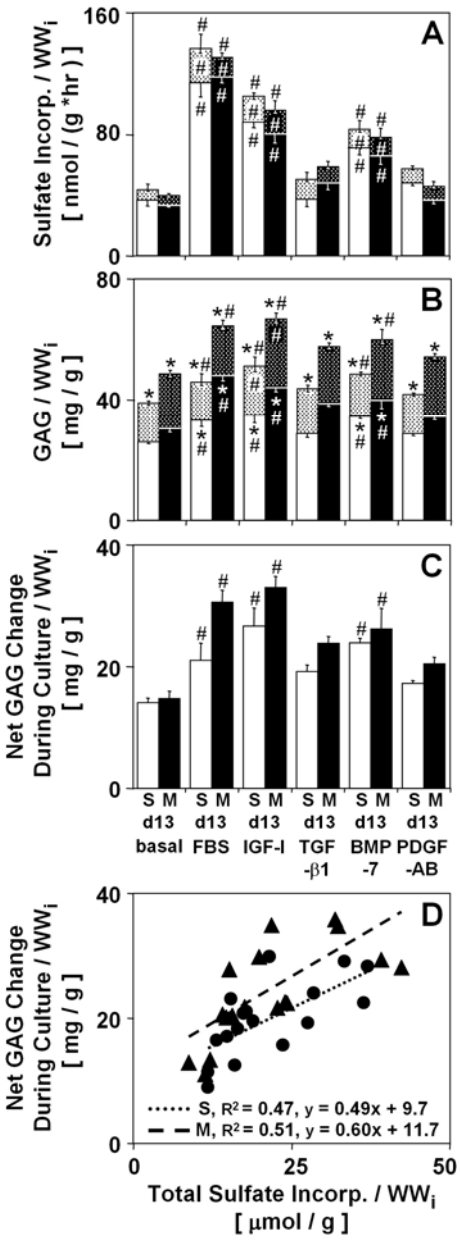

Figure 2.3: Effect of experimental conditions on GAG balance of calf articular cartilage explants from the S and M layers. The dotted regions (above) represent (**A**) sulfate and (**B**) GAG released into the culture medium, while the solid color regions (below) represent sulfate incorporation (**A**) and GAG in the tissue (**B**). The overall bar height represents the total (**A**) incorporated sulfate and (**B**) GAG content. (**C**) The net change in GAG during culture, and (**D**) relationship between the net change in GAG during culture and total incorporated sulfate. Regression lines, corresponding R^2 values and equations are shown for S (●) and M (▲) layers, where the statistical significance $p<0.05$. Data are mean ± SEM, n = 8-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. basal condition.

Biomechanical Analysis

Structural biomechanical properties of tensile ramp stiffness (Fig. 2.4A) and strength (Fig. 2.4B) varied with experimental conditions (all $p<0.001$) and the variations were consistent with the variations in the material biomechanical properties of tensile ramp modulus (Fig. 2.4C) and strength (Fig. 2.4D), which are described below. Ramp modulus decreased during culture in basal medium (-55%, $p<0.001$ in S), with FBS (-73%, $p<0.001$ in S, -75%, $p<0.001$ in M), IGF-I (-80%, $p<0.001$ in S, -59% $p<0.01$ in M), BMP-7 (-74%, $p<0.001$ in S, -63%, $p<0.001$ in M), and PDGF-AB (-66%, $p<0.001$ in S, -40% $p=0.08$ in M). Similar to variations in ramp modulus, strength decreased during culture in basal medium (-53%, $p<0.001$ in S), with FBS (-64%, $p<0.001$ in S, -62%, $p<0.001$ in M), IGF-I (-76%, $p<0.001$ in S, -46% $p<0.01$ in M), BMP-7 (-75%, $p<0.001$ in S, -36%, $p<0.05$ in M), and PDGF-AB (-59%, $p<0.001$ in S). In contrast, after culture with TGF-β1 both ramp modulus and strength remained unchanged (all $p>0.24$). Strain at failure (Fig. 2.5C) did not vary with experimental conditions in the S layer but varied slightly in the M layer ($p<0.001$). Strain at failure increased during culture with BMP-7 (51%, $p<0.01$) and, although not significantly, increased (12-33%) after incubation in basal medium, with FBS, IGF-I, and PDGF-AB. In explants incubated with TGF-β1, strain at failure tended to decrease (-12%) and was lower than that of explants incubated in basal medium (-34%, $p<0.05$).

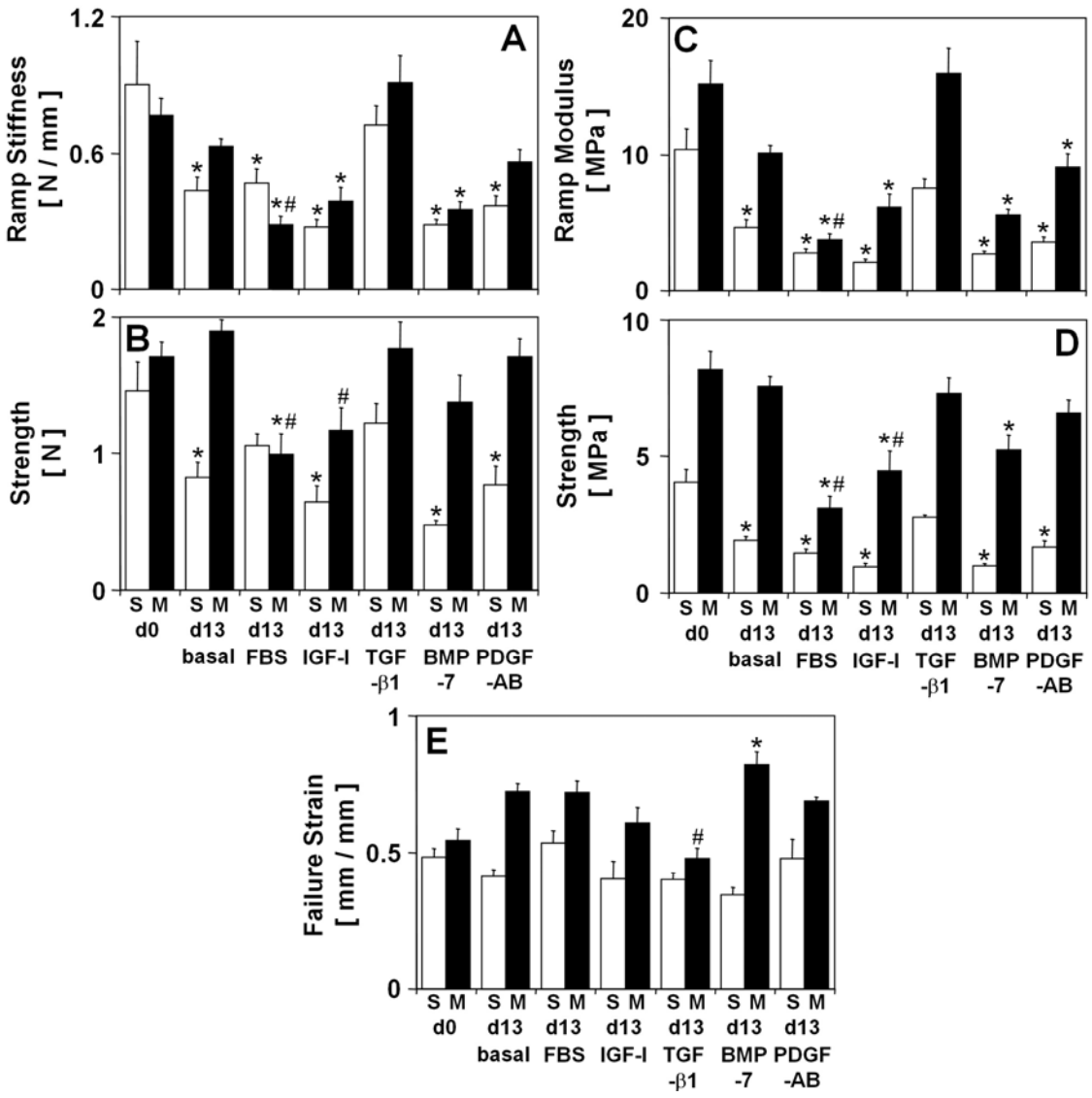

Figure 2.4: Effect of experimental conditions on structural and material tensile mechanical properties of calf articular cartilage explants from the S and M layers. Structural properties: (**A**) Ramp modulus, and (**B**) strength. Material properties: (**C**) ramp modulus, (**D**) strength, and (**E**) strain at failure. Data are mean ± SEM, n = 8-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. basal condition.

Correlative Analysis

The correlations between certain parameters were examined to assess factors involved in volumetric growth (Fig. 2.5). The change in wet weight correlated positively with GAG/WW_i in the tissue (p<0.001 for each layer), reaching R^2 values of 0.55 (Fig. 2.5B), while it did not correlate to COL/WW_i (p>0.35, R^2 <0.06) (Fig. 2.5A). The tensile mechanical properties were used in the correlative analysis as they reflect the swelling-resistant properties of the collagen network. Both ramp modulus and strength showed a negative correlation with the change in wet weight (all p<0.01) with R^2 values of 0.31-0.46 (Fig. 2.5C-D). The level of dependency of the change in wet weight on GAG/WW_i and mechanical properties was more pronounced in the S layer (3-5 fold, all p<0.05).

Tensile mechanical properties were correlated with the concentration of several matrix components (Fig. 2.6). In each layer, ramp modulus and strength showed a negative correlation with water content (each p<0.05, Fig 6A-B), and a strong positive correlation with COL/WW_f (each p<0.01, Fig. 2.6C-D). The correlation coefficients were highest for strength, reaching R^2 values of 0.56-0.68 for COL/WW_f (Fig. 2.6D) and 0.22-0.41 for water (Fig. 2.6B). The level of dependency of mechanical properties on COL/WW_f were similar in each layer, while the level of dependency of mechanical properties on water was more pronounced in the S layer (3-4 fold, each p<0.05). The tensile mechanical properties did not correlate with GAG/WW_f (p>0.16, R^2 <0.27) (Fig. 2.6E-F). Strain at failure did not correlate with the biochemical constituents measured (p>0.16, R^2 <0.18).

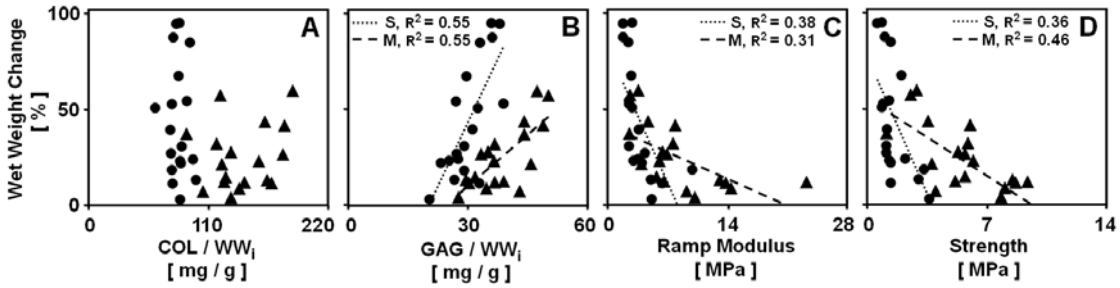

Figure 2.5: Relationships between the change in wet weight during culture and constituent content and tensile mechanical properties. Data are for cartilage from the S (●) and M (▲) layers for blocks analyzed on day 0 and incubated in medium. Regression lines, and corresponding R^2 values are shown where the statistical significance $p<0.05$.

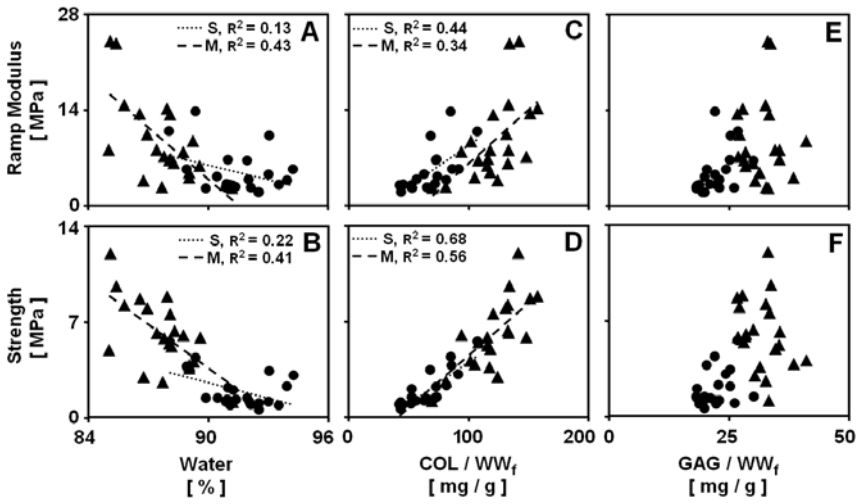

Figure 2.6: Relationships between cartilage tensile mechanical properties and constituent concentrations. Data are for cartilage from the S (●) and M (▲) layers for blocks analyzed on day 0 and incubated in medium. Regression lines, and corresponding R^2 values are shown where the statistical significance $p<0.05$.

2.5 Discussion

The data presented here demonstrate that *in vitro* growth of immature articular cartilage explants, as indicated by changes in tissue size, content of matrix components, and the integrity of the collagen network, is regulated differentially by certain growth factors. Incubation with FBS, IGF-I, or BMP-7 resulted in *expansive cartilage growth*, characterized by an increase in tissue size (Fig. 2.1A-B), stimulation of GAG synthesis and deposition (Fig. 2.3A-C), and loss in the concentration of matrix components (Fig. 2.2D-F) and the tensile mechanical integrity (Fig. 2.4A-D). Many of the effects of serum on cartilage growth were similar to those reported previously [99] and appear attributable to IGF-I. Incubation in basal medium or with PDGF-AB also resulted in growth with some, but not all, of the characteristics of expansive growth. Under these conditions, tissue volume and composition changed slightly (Fig. 2.1-2), but were accompanied by a marked decrease in mechanical integrity (Fig. 2.4A-D). In contrast, the regulation of cartilage metabolism by TGF-β1 induced a state of *homeostasis*. Volumetric growth and biosynthetic rates were minimal under these conditions (Fig. 2.1A-B, Fig. 2.3A) and content and concentration of GAG and collagen were maintained (Fig. 2.2). Notably, these changes were accompanied by maintenance of the tensile mechanical integrity (Fig. 2.4A-D). These findings suggest that remodeling and reorganization of the tissue matrix that takes place during culture in basal medium or culture with serum, IGF-I, BMP-7, and PDGF-AB facilitates a relatively loose and weak collagen network that

allows tissue expansion, while TGF-β1-supplemented culture induces conditions that restrict volumetric growth while maintaining the integrity of the collagen network.

The assessment of cartilage growth in response to growth factors required consideration of certain experimental and theoretical issues. The overall culture duration was chosen based on pilot and previous studies [99] which indicated that changes in tissue size, biochemical content and mechanical integrity become apparent after ~2 weeks. Further analysis could address the time course of these changes. The selection of growth factors and their concentrations was based on their ability to stimulate chondrocytes in explant culture and the intent of testing physiologic concentrations. Although the dose response studies were not performed, it is likely that the concentrations of growth factors used here achieved maximal or near maximal stimulation. Analysis of articular cartilage explants incubated with 30 or 300 ng/ml of IGF-I indicated that these doses of IGF-I produced relatively similar effects on the contents of proteoglycan and collagen, and mechanical properties in compression [80, 82]. Stimulation of proteoglycan synthesis in cartilage explants with TGF-β1 saturated at around 10 ng/ml [68]. Incubation with 30 or 100 ng/ml of BMP-7 showed no detectable dose-dependence of sulfate incorporation [54]. As a result, incubation with IGF-I or with TGF-β1, as well as incubation with serum, have served as control conditions for interpreting the stimulatory effects of other biochemical factors [42, 59, 77, 80, 92]. Other growth factors [42, 59, 80], such as basic fibroblast growth factor (bFGF), BMP-2, 3, 4, 9, and 13, and growth factor combinations [56, 94], such as IGF-I and BMP-7, which stimulate proteoglycan synthesis of calf articular cartilage explants, may also regulate cartilage growth and could be examined in future studies.

The regulatory effects of growth factors on net cell proliferation, and synthesis and deposition of matrix components within the cartilage tissue, were generally consistent with those reported in previous studies. Maintenance of the content of cells and collagen in explant cultures with serum, IGF-I, TGF-β1, or PDGF-AB is generally consistent with previous studies that investigated the effect of these factors on proliferation [68, 83] and collagen deposition [59, 68, 83]. While the effects of serum, IGF-I, and BMP-7 on proteoglycan synthesis and deposition also agree with other studies [7, 54, 59, 73, 80, 83], the effects of TGF-β1 and PDGF-AB on proteoglycan synthesis are somewhat different. In contrast to results obtained here, in cartilage derived from 1-6 month old bovines, TGF-β1 stimulated proteoglycan synthesis [66, 68]. This difference may be due to the age of calf animals that were used, as the effects of TGF-β1 on proteoglycan synthesis appear to be dependent on the stage of development of the source tissue. In cultures of articular cartilage explants from horses aged 3 months to 14 years, stimulation of sulfate incorporation by TGF-β1 was enhanced markedly in the older but not in the immature animals [47]. It also does not appear that the absence of stimulation of proteoglycan synthesis was due to inactivity of TGF-β1, as it did markedly stimulate the secretion of proteoglycan 4 (PRG4) (data not shown) in the explants from the S layer, consistent with the specific effect of TGF-β1 on PRG4 secretion [85]. Results of the present study are also different than those of Schafer and coworkers, where 50 ng/ml of PDGF-AB enhanced proteoglycan synthesis ~2.6 fold over basal cultures in bovine explants derived from full thickness metacarpophalangeal cartilage [83]. This difference may be due to site-associated and

depth-dependent differences in response of articular cartilage to PDGF-AB. Full-thickness tissue, with high content of chondrocytes derived from deep zones of cartilage, may have a stronger response to PDGF-AB than chondrocytes in the top ~1 mm of tissue. Indeed, although insignificant, the percent increase in the net change of GAG in explants during incubation with PDGF-AB over that during incubation in basal medium was 40% in the M layer and only 23% in the S layer (Fig. 2.3C). In future studies, it maybe possible to determine if the effects of a certain growth factor involve modulation of other growth factors using neutralizing antibodies [36].

The growth rate of cartilage explants *in vitro* was generally greater than that which occurs naturally *in vivo*. To estimate the rate of cartilage growth *in vivo*, measurements of the radius of the femoral condyle of a bovine fetus at a gestational age of 207 days (based on femur and tibia length and an overall gestational duration of 282 days [75]) (11 mm), and of a 2 weeks of age bovine calf (16 mm) were used. Growth rate in terms of radial growth (proportional to radius) and volumetric growth (proportional to radius cubed) was calculated as ~54 μm/day and ~2.2 %/day, respectively and is comparable to the radial growth of articular cartilage of the femoral head in neonatal rat (~47 μm/day) [38], and the volumetric growth of cartilage from the femoral condyle in immature marsupial *Monodelphis domestica* (~0.18 %/day) [39]. In contrast, growth rate during culture supplemented with serum or IGF-I was ~2.7 %/day or ~180 μm/day in terms of thickness (calculated based on the rate of change in thickness of the S layer (31 μm/day, Fig. 2.1A) and by extrapolating the rate of change in thickness of the M layer (6.7 μm/day, Fig. 2.1A) for the tissue below the first 400 μm and assuming cartilage thickness of the donor joints of ~6 mm) and ~3.7

%/day in terms of wet weight (Fig. 2.1B). Similarly, during a long-term serum-supplemented culture, immature bovine articular cartilage from the most superficial 400 μm grew ~2.6 %/day in thickness and ~6.4 %/day in wet weight [99] and cartilage from the mandibular condyle of neonatal rat extended ~8.2 %/day along the longitudinal axis [33]. Even higher rates of growth were observed during a short-term serum-free culture of embryonic chick tibiae, where the wet weight of the cartilaginous portion increased by ~25 %/day [11, 28]. In addition, a direct comparison of the rate of cartilage lengthening during *in vitro* incubation and normal *in vivo* development of mandibular condyles from age-matched neonatal rats demonstrated rates of extension of up to ~3.7 %/day *in vitro* and a decrease in the amount of cartilage *in vivo* (~-2.9 %/day) [21].

The indices of metabolism of certain matrix molecules were consistent with their role in determining the volumetric growth of cartilage tissue. Cartilage explants grew in volume as the content of GAG in the tissue, an indicator of swelling pressure, increased (Fig. 2.5B) and as the tensile modulus and strength, indicators of the integrity of the collagen network, decreased (Fig. 2.5C-D). Inhibition of biosynthesis with cycloheximide or at 4°C halted cartilage growth (changes in tissue size and composition) and remodeling (changes in the mechanical properties) for at least 13 days in serum-supplemented culture (data not shown), suggesting that volumetric expansion of cartilage tissue during culture is indicative of active growth and remodeling mediated by chondrocytes and is not a passive swelling process. Thus, it appears that factors that lead to growth of immature cartilage explants *in vitro* involve a shift in the balance between the swelling pressure of the proteoglycan molecules and

the restraining ability of the collagen network, toward an overall expansive effect resulting from the swelling pressure. Studies to investigate the spatial distribution of newly deposited GAG molecules and other matrix components may provide additional insights into the mechanisms of cartilage growth.

Large extents of volumetric cartilage growth *in vitro* appeared to be due to deposition of proteoglycans that exceeded the deposition of collagen. Normal rate of volumetric growth of bovine cartilage from fetus to calf *in vivo*, involves deposition of collagen (~1.7 %/day) that exceeds that of GAG (which exhibits a ~steady content) and an increase in the tensile mechanical integrity (~5.7 %/day) [97]. A notably higher rate of volumetric growth in the S layer during serum or IGF-I-supplemented growth *in vitro* was associated with GAG deposition (~-1.8 %/day on concentration basis, Fig. 2.2E) that exceeded that of collagen (~-3.4 %/day on concentration basis, Fig. 2.2F) and with a decrease in the tensile mechanical integrity of the tissue (~-5.6 %/day, Fig. 2.4C-D). Similarly, in comparison with articular cartilage, a higher tendency to grow is expected in cartilage of the growth plate where the proportion of GAG is higher and tensile strength and modulus are lower than those of articular cartilage [19, 72]. Indeed, a relatively high rate of axial growth is observed in the growth plate *in vivo* (up to ~400 μm/day) [100], as well as *in vitro* where larger increases in length were observed in cartilages containing one or more osteogenic zones as compared to that of entirely cartilaginous explants [22].

An additional determinant of volumetric growth of cartilage tissue appeared to be the structural organization of the collagen network and its resultant restraining function. Different extents of volumetric growth in the S and M layers for a given

increase in GAG content and a given decrease in mechanical integrity, as indicated by steeper regression slopes for the S layer (Fig. 2.5), may be associated with the depth-associated variations in the structural organization of the collagen network. It is possible that the parallel orientation of collagen fibrils that exists in the superficial zone of cartilage [10] allows the swelling pressure of the newly synthesized GAG to be dissipated between the collagen fibers, resulting in, for the S layer, growth with minimal remodeling of the collagen network. In contrast, randomly oriented collagen fibrils in the middle zone of cartilage are necessitated to bear the swelling pressure, leading to, in the M layer, growth that is accompanied by major remodeling of the collagen network and, concomitantly, a marked tensile softening and weakening.

The relationship between tensile mechanical properties and collagen concentration was generally maintained for cartilage explants from both the S and M layers at explant, as well as after incubation *in vitro*. During growth, the tensile material properties of ramp modulus and strength, diminished as did the concentration of collagen. The dependence of properties was similar to that observed previously [97, 99] and agrees with the general notion that tensile properties of cartilage are primarily dependent on the collagen network. However, these relationships may be nonlinear, in that the decrease in tensile material properties was somewhat more than the decrease in concentration of collagen. The residual variation in tensile properties may be due to a variety of factors, including components of the cartilage tissue and the structural organization of tissue components. One of these major factors may be the remodeling of the collagen network that changes its stress-free state. Similar determinants can also

contribute to the nonlinearity in the relationship between the change in wet weight and tissue GAG content.

Expansive cartilage growth shows many attributes of a distinct immature and growing phenotype, rather than signs of progression to a more mature state. The presence of serum, IGF-I or BMP-7 resulted in deposition of GAG that exceeded the deposition of collagen, creating swelling pressures that were excessive relative to the restraining ability of the collagen network. This metabolic imbalance facilitated a relatively loose and weak collagen network that allowed volumetric expansion. While expansive growth is also observed during incubation in basal medium and with PDGF-AB, it occurs in the absence of changes in biochemical composition. It appears that growth under these conditions may be driven by alterations in the structural organization of the collagen network itself that render it weak and malleable. Such remodeling may involve induction of various proteinases such as collagenase, which can be stimulated by PDGF [9].

Remodeling and reorganization of cartilage tissue that occurs during incubation with TGF-β1 allows maintenance of tissue composition and function resulting in a state of *homeostasis*. Preservation of collagen network integrity may result from low swelling pressures, as a result of ~steady GAG content, as well as from modulation of remodeling through suppression of proteinase synthesis and stimulation of production of protease inhibitors. TGF-β has the ability to decrease the activity of various extracellular proteinases found in connective tissues, including collagenase, stromelysin, and plasminogen activator [5, 45, 79]. In addition, TGF-β1

can induce secretion of protease inhibitors such as tissue inhibitors of metalloproteinase (TIMPs) [37, 45, 90].

These findings may facilitate the development of growth factor-based strategies for treatment and prevention of articular cartilage pathology associated with aging, injury, and disease. A decline in the concentrations of anabolic growth factors with increasing age [16, 18, 29, 47, 96, 105] may contribute to aging-related changes in articular cartilage, while the apparent increase in the expression of certain anabolic growth factors in states of injury or disease [14, 34, 51, 71, 86, 88, 96] may stimulate chondrocytes to repair the damaged tissue matrix. Thus, specific alteration of the growth factor environment that can shift chondrocyte metabolism towards matrix deposition (as with serum, IGF-I, or BMP-7) and stabilization (as with TGF-β1) may be used to treat or prevent conditions associated with aging, disease, and injury.

These findings may also have practical utility for tissue engineering of cartilaginous tissue for the purposes of repairing articular cartilage defects. Specific *in vitro* conditions may aid in designing tissue engineered constructs that attain the geometry of an *in vivo* defect and the structural and functional properties of native cartilage surrounding the defect. For example, the expansive growth phenotype may be useful to produce a tissue construct quickly but with characteristics typical of immature cartilage. Subsequently, methods, such as mechanical stimulation, may be used to enhance maturation of the immature cartilaginous tissue [46]. The homeostasis phenotype may then be used to maintain a tissue construct or graft until implantation with a steady-state maintenance of biochemical and biomechanical characteristics.

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CHAPTER 3

CARTILAGE GROWTH AND REMODELING: MODULATION OF BALANCE BETWEEN PROTEOGLYCAN AND COLLAGEN NETWORK *IN VITRO* WITH β-AMINOPROPIONITRILE

3.1 Abstract

Objective: To examine the effect of β-aminopropionitrile (BAPN), an inhibitor of lysyl oxidase, on growth and remodeling of immature articular cartilage *in vitro*. *Design:* Immature bovine articular cartilage explants from the superficial and middle zones were cultured for 13 days in serum-containing medium with or without BAPN. Variations in tissue size, accumulation of proteoglycan and collagen, and tensile mechanical properties were assessed. *Results:* The inclusion of serum resulted in expansive tissue growth, stimulation of proteoglycan and collagen deposition, and a diminution of tensile integrity. Supplementation of medium with BAPN accentuated this phenotype in terms of a further increase in tissue size and diminution of tensile integrity, without affecting the contents of proteoglycan and collagen. *Conclusion:* Collagen crosslinking is a major factor in modulating the phenotype of cartilage

growth and the associated balance between proteoglycan content and integrity of the collagen network.

3.2 Introduction

Articular cartilage is a layer of connective tissue located on the ends of long bones [9] that normally functions as a low friction, wear-resistant, load-bearing material, facilitating joint motion [33, 39]. The ability of cartilage to withstand compressive, tensile, and shear forces depends on the composition and structure of the extracellular matrix [21, 33, 39]. The proteoglycan constituent of the extracellular matrix provides the tissue with a fixed negative charge that increases the tissue's propensity to swell and to resist compressive loading [7, 31]. The crosslinked collagen network resists the swelling tendency of the proteoglycan molecules and provides the tissue with tensile and shear stiffness and strength [39, 53, 60]. Chondrocytes in cartilage normally maintain a functional matrix by modulating synthesis and degradation of the matrix components.

Growth and remodeling are biological processes that, together, transform cartilage tissue *in vivo* from an immature to a mature state. Tissue growth is generally defined as an increase in tissue size due to accretion of one or more solid tissue components similar to those already present, while tissue remodeling is defined as a change in tissue composition and/or structure of tissue components [30, 49]. Many tissues, including articular cartilage, can expand due to accretion of fluid; however this process is not generally considered growth, but rather tissue swelling. Two distinct mechanisms of tissue growth have been recognized: appositional growth, or growth at a tissue surface, and interstitial growth, or growth within tissue volume [12]. While it is possible that tissues can grow appositionally in the absence of remodeling,

interstitial tissue growth must involve both growth and remodeling since accretion of a single tissue component will change the overall tissue structure and mechanical properties. Tissues can exhibit growth and remodeling in the form of hyperplasia (increase in the number of cells), hypertrophy (addition of cellular components), deposition of the extracellular matrix components or any number of these processes occurring concomitantly. In articular cartilage, the incidence of cell division is low and matrix deposition is the major contributor to the increase in size (i.e. growth) and changes in biochemical composition (i.e. remodeling) of this tissue *in vivo* [27, 54, 56]. Since articular cartilage tissue may undergo both appositional and interstitial growth and the major contributor to cartilage growth is matrix deposition, the term *growth* is used subsequently in this paper to refer, collectively, to both growth and remodeling, which can occur in presence or absence of cellular proliferation.

Alterations of cartilage function, structure, and composition during growth *in vivo* and during serum-supplemented culture *in vitro* appear to depend on the metabolic balance between proteoglycan molecules and the components of the collagen network. Fetal and postnatal growth of articular cartilage normally involves a net deposition of collagen that is greater than that of proteoglycan, as well as an increase in mechanical integrity. During maturation of bovine articular cartilage, from the fetal stage, through the newborn calf, and to the skeletally mature adult, there is an increase in the collagen and pyridinoline (PYR) crosslink densities, but little or no change in the content of glycosaminoglycan (GAG) [41, 50, 56, 59]. These biochemical changes are accompanied by an increase in the tensile modulus and strength, and each of these biomechanical properties is positively correlated with the

collagen and PYR crosslink densities [55]. In contrast to this type of *in vivo* growth, growth of immature cartilage tissue *in vitro* in serum-supplemented medium results in a net deposition of proteoglycan that is greater than that of collagen and a decrease in mechanical integrity. For cartilage explants from bovine fetus and calf, and neonatal rat, incubation in serum-supplemented medium results in an increase in tissue size, maintenance of proteoglycan concentration and a decrease in the concentrations of collagen and PYR crosslinks [20, 45, 55]. These changes in composition are associated with a decrease in tensile modulus and strength [57].

Cartilage hydration and the load-bearing biomechanical function are influenced by the balance between the swelling propensity of proteoglycan molecules and the restraining function of the collagen network. This idea was proposed by Maroudas [32] and has been supported by theoretical models [7, 10, 16, 31]. The increased hydration and loss of mechanical integrity of osteoarthritic cartilage, compared with normal cartilage, is due to a weakening of the collagen network and an associated swelling of the tissue [4, 6, 34]. Analogously, in the context of growth, a low or reduced restraining function of the collagen network, due to either variations in network composition or structure or due to excessive swelling pressure imposed by the newly synthesized proteoglycan, is predicted to allow tissue swelling and growth [30]. Thus, we hypothesize that cartilage growth results from a dynamic imbalance between the swelling pressure of endogenous (that present at the time of explant) and newly synthesized GAG and the restraining function of the collagen network.

One way to test this hypothesis is to examine the effect of blocking the metabolic pathway for the formation of enzyme-mediated collagen crosslinks on

growth of cartilage explants. Collagen crosslinks are a major element in stabilizing the collagen network, hence explants where crosslinking is blocked should have an enhanced propensity to grow. Lysyl oxidase mediates covalent crosslinking among collagen II, IX and XI fibrils [17, 18] by oxidizing hydroxylysine residues to hydroxylysyl aldehydes which then, through several reactions, lead first to immature dehydroxylysinorleucine (DHLNL) crosslinks, then to stable PYR crosslinks [44]. Lysyl oxidase-mediated collagen crosslink formation can be inhibited with a lathyrogen such as β-aminopropionitrile (BAPN), which binds covalently and irreversibly to the active site of the enzyme [2, 3, 26]. BAPN has been found to cause a 50% inhibition of lysyl oxidase at a concentration of 3–5 μM [25], and at even higher concentrations (e.g., 0.1–0.25 mM), BAPN has little or no discernible effect on matrix synthesis by chondrocytes [3, 15]. Specific inhibition of collagen crosslink formation without an effect on general biosynthesis makes BAPN a useful tool to study the effect of inhibition of functional assembly of the collagen network on the integrity of the collagen network and on cartilage growth. Thus, to address the hypothesis of this study, the objectives were to examine the effect of BAPN on culture-associated variations in (i) tissue size, (ii) accumulation of GAG and collagen network components, and (iii) tensile mechanical properties.

3.3 Materials and Methods

Sample Preparation and Culture

Articular cartilage was harvested from the patellofemoral groove of 5 newborn (1-3 weeks) bovine calves, as described previously [57]. Blocks, 9 x 3 mm^2 in area, were prepared using a sledge microtome to either include the intact articular surface (superficial layer, ~0.4 mm thick, S) or to include the middle zone, starting at a distance of ~0.6 mm from the articular surface (middle layer, 0.25 mm thick, M). The term layer rather than zone is used to avoid associations with classical zonal classification of articular cartilage. The long axes of the blocks were in the anterior-posterior direction and, thus, approximately perpendicular to the split line direction. Blocks were weighed wet (WW_i) under sterile condition.

Some blocks were **(a)** analyzed immediately. Other blocks were incubated in **(b)** medium (DMEM supplemented with 20% fetal bovine serum (FBS) and 100 μg/mL of ascorbate) or **(c)** medium with 0.1mM β-aminopropionitrile (BAPN) (Sigma, St. Louis, MO). Medium was changed every other day, and during the first 12 days of culture supplemented with 4.5 μCi/mL [^{3}H]proline and 1.8 μCi/mL [^{35}S]sulfate. To remove unincorporated isotopes, blocks were washed, transferred to a new culture plate, and incubated for an additional day in medium without radiolabel. Spent medium was collected throughout the culture duration. At termination, blocks were weighed wet (WW_f) and punched to form a tapered tensile test specimen and residual cartilage.

Biochemical Analysis

Residual cartilage and the failed portions of the corresponding tensile strip (see below) were analyzed together to quantify the biochemical composition of the fresh and cultured samples. Samples were lyophilized, weighed dry, and solubilized with proteinase K [57]. Portions of the tissue digest were analyzed to quantify the content of DNA [37], GAG [19], and hydroxyproline [58]. Digest portions from each animal/layer/experimental condition were pooled for analysis of PYR [52]. DNA was converted to cell number by using a conversion constant of 7.7 pg of DNA per cell [29]. Hydroxyproline content was converted to COL content by assuming a mass ratio of collagen to hydroxyproline equal to 7.25 [23, 41]. The molar ratio of PYR per collagen molecule was calculated, assuming the molecular weight of collagen triple helix of 300,000. Because initial tissue wet weight (WW_i) varied among samples on day 0, mass of each biochemical constituents on day 13 was normalized to WW_i, to provide information about the content of biochemical constituents in the tissue. To represent biochemical constituent concentration, mass of each biochemical constituents on day 13 was normalized to the final wet weight of the tissue (WW_f).

Analysis of Matrix Metabolism

Other portions of the solubilized tissue and portions of the medium were analyzed for the incorporated radioactivity to assess matrix metabolism. The contents of [^{35}S] and [^{3}H] radioactivity were determined in the tissue digest and used to estimate the absolute rates of sulfate and proline incorporation as indices of 12-day average sulfated-GAG and protein synthesis, respectively. To more specifically assess

collagen synthesis, portions of tissue digests and medium were pooled for each animal/layer/experimental condition and analyzed for [^{3}H]hydroxyproline residues using Dowex columns [45, 48]. The content of [^{3}H]hydroxyproline was used to estimate the absolute rate of HYPRO formation. To estimate the absolute rate of sulfate release into the medium, a portion of spent medium was analyzed for the content of [^{35}S]GAG using the Alcian Blue precipitation method [35].

Biomechanical Analysis

Tapered tensile specimens were analyzed to determine mechanical properties as described previously [11]. From each cartilage block, a tapered strip [28] with a gage region of 4 mm x 0.80 mm was prepared using a punch. The thickness of each tensile strip was measured at three locations in the gage region, using a contact-sensing micrometer, and the average was used for cross-sectional area calculations. Tapered specimens were then secured in clamps (4.0 mm apart) of a mechanical tester and elongated at a constant extension rate (5 mm/min) until failure. Structural tensile parameters were obtained from the load-displacement curves. Structural tensile strength was determined as the maximum load sustained at failure. Ramp stiffness was calculated as the slope of the linear regression of the load-displacement curve from 25-75% of the maximum load. Load and displacement were converted to stress (defined as load normalized to the cross-sectional area of the gage region) and strain (defined as the elongation distance normalized to the initial clamp to clamp distance) to obtain material tensile parameters of tensile strength, strain at failure (the strain at which maximum stress was attained), and ramp modulus. The failed portions of each

tensile strip, resulting from the tensile test, were saved for biochemical analysis (described above) in addition to the adjacent cartilage samples obtained during preparation of the tensile strips.

Statistical Analysis

For each layer (S and M), the effects of experimental conditions (d0, d13, d13+BAPN) were assessed by analysis of variance (ANOVA) with experimental condition as fixed factor and donor animal as a random factor. For dependent variables that involved PYR and HYPRO, which were determined from a pool of samples, the effects of experimental conditions were assessed by ANOVA with experimental condition and layer as fixed factors and donor animal as a random factor. Tukey post-hoc testing was performed to compare groups. To analyze the effect of day in culture on wet weight, repeated measures ANOVA was performed for each layer and experimental condition with wet weight (WW_i, WW_f) as a repeated factor. Data are expressed as mean ± SEM, and significance level was set to 0.05. Statistical analysis was performed using Systat 10.2 (Systat Software, Inc., Richmond, CA).

3.4 Results

The extent of *in vitro* volumetric growth of articular cartilage blocks was markedly affected by experimental conditions as assessed by changes in thickness ($p<0.05$) (Fig. 3.1A), wet weight ($p<0.001$) (Fig. 3.1B), and water content (Fig. 3.1C). Both the thickness and wet weight increased during culture in medium (42-56%, 45-73%, respectively; $p<0.05$) and medium with BAPN (44-80%, 44-88%, respectively; $p<0.05$). The extent of change in wet weight during culture with BAPN exceeded that of explants cultured without BAPN, with the largest difference detected in the S layer (34%, $p<0.05$). The volumetric growth of all explants appeared to be predominantly in the direction perpendicular to the articular surface, as changes in wet weight can mostly be accounted for by changes in thickness alone. The content of water did not vary with experimental conditions in the S layer ($p=0.30$) but varied in the M layer ($p<0.01$), decreasing slightly during culture in medium without BAPN (~4%, $p<0.01$). Since the content of water did not change during culture with BAPN ($p>0.44$), it was higher than that of explants cultured in medium (~3%, $p<0.05$). Since water content varied only slightly on an absolute basis, changes in wet weight were largely due to changes in tissue volume and not density and, thus, along with changes in thickness, represent volumetric growth of tissue samples.

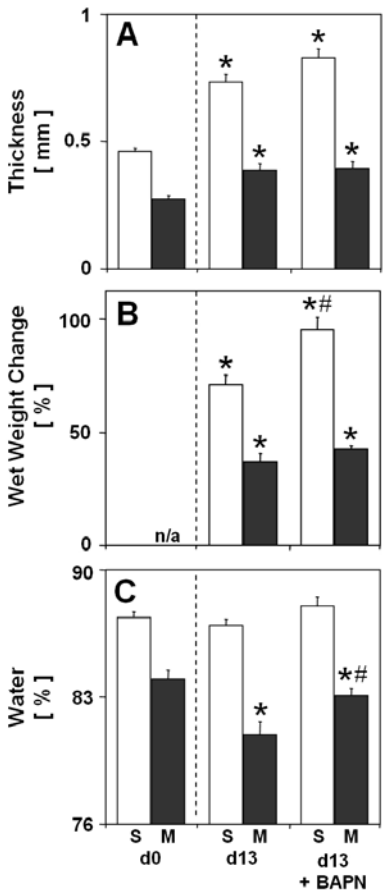

Figure 3.1: Effect of experimental conditions on general indices of *in vitro* growth of calf articular cartilage explants from the superficial (S) and middle (M) layers. Blocks were analyzed on day 0 (d0), or incubated for 13 days (d13) in medium (20% FBS) or medium supplemented with 0.1mM β-aminopropionitrile (BAPN). (**A**) Thickness, (**B**) change in wet weight, and (**C**) percent water of cartilage blocks. The dotted line separates explants analyzed on day 0. * indicates $p<0.05$ vs. d0, # indicates $p<0.05$ vs. d13 for a corresponding layer. Data are mean ± SEM, n = 10-20 blocks from 5 animals.

The content of cells (Cells/WW_i, Fig. 3.2A) did not vary with experimental conditions ($p>0.25$), and, when normalized to WW_f to give a measure of concentration of cells (Cells/WW_f, Fig. 3.2E), reflected the changes in wet weight ($p<0.001$). Cells/WW_f decreased in explants cultured in medium (~43%, $p<0.001$), and medium with BAPN (~50%, $p<0.001$).

The extent of volumetric growth was generally paralleled by variations in the tissue content of GAG, collagen (COL) and PYR. GAG content (GAG/WW_i, Fig. 3.2B) varied with experimental conditions ($p<0.001$), while the content of COL (COL/WW_i Fig 2C) varied only in the S ($p<0.05$) layer. While GAG/WW_i increased markedly during culture in medium (~53%, $p<0.001$) and medium with BAPN (~46%, $p<0.001$), COL/WW_i remained unchanged (during culture with BAPN and in the M layer during culture in medium $p>0.87$), or increased slightly (~14% during culture in medium, $p<0.05$ in S). The variations in COL/WW_i were small, so that the extents of deposition of both GAG/WW_i and COL/WW_i were similar in explants incubated with and without BAPN ($p>0.15$). The content of PYR (PYR/WW_i, Fig. 3.2D) varied with experimental conditions ($p<0.05$), such that PYR/WW_i increased during culture in medium (37%, $p=0.07$), but remained unchanged during incubation in medium with BAPN ($p=0.83$). As a result PYR/WW_i was higher in explants incubated in medium than in explants incubated in medium with BAPN (33%, $p<0.05$).

When normalized to WW_f to give an index of concentration in the tissue, the concentrations of extracellular matrix components reflected changes in the content of the components and in the wet weight of the tissue during culture. The concentration of GAG (GAG/WW_f, Fig. 3.2F) varied with experimental conditions in the S layer

(p<0.001) but did not change during culture of explants from the M layer (p>0.20), while the concentration of COL (COL/WW_f, Fig. 3.2G) varied in both layers (p<0.001). GAG/WW_f decreased slightly during incubation in medium with BAPN (11%, p<0.001), but not during incubation in medium (p=0.33), while COL/WW_f decreased during incubation in medium (~24%, p<0.05) and medium with BAPN (~37%, p<0.001). As a result, both GAG/WW_f and COL/WW_f were similar among explants incubated with and without BAPN, except explants from the S layer where GAG/WW_f and COL/WW_f were slightly lower after incubation with BAPN than after incubation without BAPN (14% and 23%, respectively; p<0.05). The concentration of PYR (PYR/WW_f, Fig. 3.2H) varied with experimental conditions (p<0.01). PYR/WW_f did not change during incubation in medium (p=0.78), and decreased (~44%, p<0.01) during incubation in medium with BAPN, so that it was lower than PYR/WW_f in explants cultured without BAPN (~37%, p<0.05). As a result of variations in PYR/WW_f the ratio of PYR to COL (Fig. 3.2I) varied with experimental conditions (p<0.05). While the ratio did not change during culture with and without BAPN (p>0.20), it was lower in explants incubated with BAPN than of those incubated without BAPN (~24%, p<0.05).

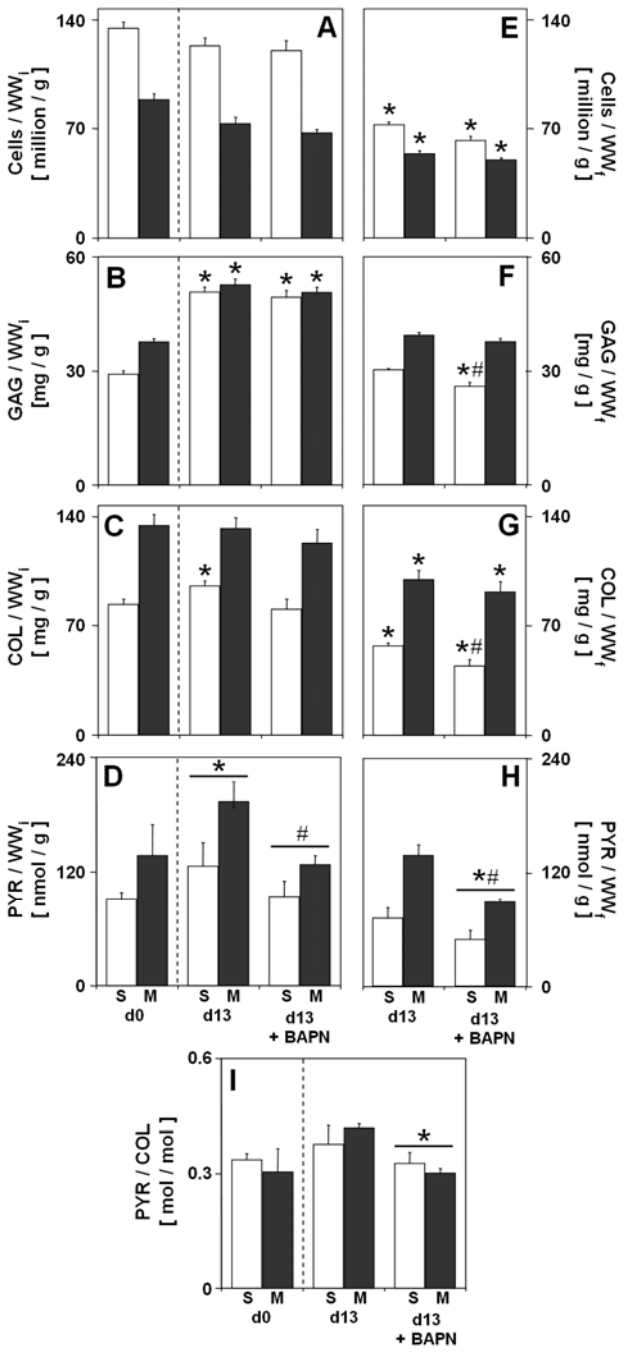

Figure 3.2: Effect of experimental conditions on general indices of *in vitro* growth of calf articular cartilage explants from the S and M layers. (**A**, **E**) Cells, (**B**, **F**) glycosaminoglycan (GAG), (**C**, **G**) collagen (COL), and pyridinoline crosslink (PYR) (**D**, **H**) normalized to initial wet weight (WW_i) to represent constituent content (**A-D**) and final wet weight (WW_f) to represent constituent concentration (**E-H**). (**I**) Molar ratio of PYR to COL. The dotted line separates explants analyzed on day 0, where $WW_f = WW_i$. * indicates $p<0.05$ vs. d0, # indicates $p<0.05$ vs. d13 for a corresponding layer, while ____ indicates where both layers were analyzed together. Data are mean ± SEM, n = 10-20 blocks from 5 animals.

The changes in GAG content (Fig. 3.2B) during culture were generally paralleled by similar variations in sulfate incorporation (Fig. 3.3A), as well as proline incorporation (Fig. 3.3B) and hydroxyproline (HYPRO) formation (Fig. 3.3C). The addition of BAPN to culture medium did not affect sulfate incorporation into the tissue ($p>0.28$), while the incorporation of proline into the tissue decreased slightly (~20%, $p\leq0.05$). The percent of HYPRO residues in the tissue did not vary and reached 36%. As a result, the content of HYPRO in the tissue paralleled variations in proline incorporation with a slightly lower HYPRO content (~20%, $p<0.05$) in explants incubated with BAPN. While the release of both sulfate and HYPRO into the medium was not affected by BAPN ($p>0.32$, $p=0.24$, respectively), the release of HYPRO into the medium tended to be slightly higher (~15% in M) in explants that were cultured with BAPN. BAPN did not affect the overall collagen synthesis, as the total HYPRO formation (in tissue and medium) was similar ($p=0.64$) for explants incubated with and without BAPN.

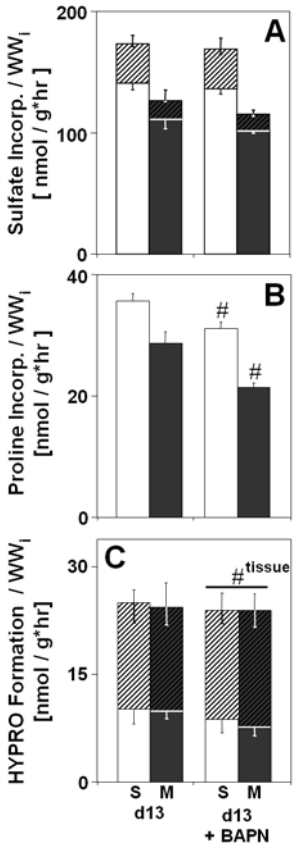

Figure 3.3: Effect of experimental conditions on sulfate and proline incorporation and hydroxyproline (HYPRO) formation of calf articular cartilage explants from the S and M layers. Content of (**A**) sulfate and (**B**) proline incorporation, and (**C**) HYPRO formation in the tissue (solid) and released into the medium, where applicable, (striped). Total sulfate incorporation and HYPRO formation is represented by the overall bar height and upward error bar. # indicates $p<0.05$ vs. d13 for a corresponding layer, while ____ indicates where both layers were analyzed together. Data are mean ± SEM, n = 10-20 blocks from 5 animals.

Experimental condition also affected the tensile biomechanical behavior of the cartilage explants as demonstrated by the average load-displacement and stress-strain curves and the mechanical properties derived from these curves (Fig. 3.4-3.5). Incubation of cartilage explants in medium and medium with BAPN reduced the strength at failure and the slopes of the average load-displacement and stress-strain curves (Fig. 3.4) with a greater reduction of tensile parameters in explants incubated with BAPN than of those incubated without BAPN.

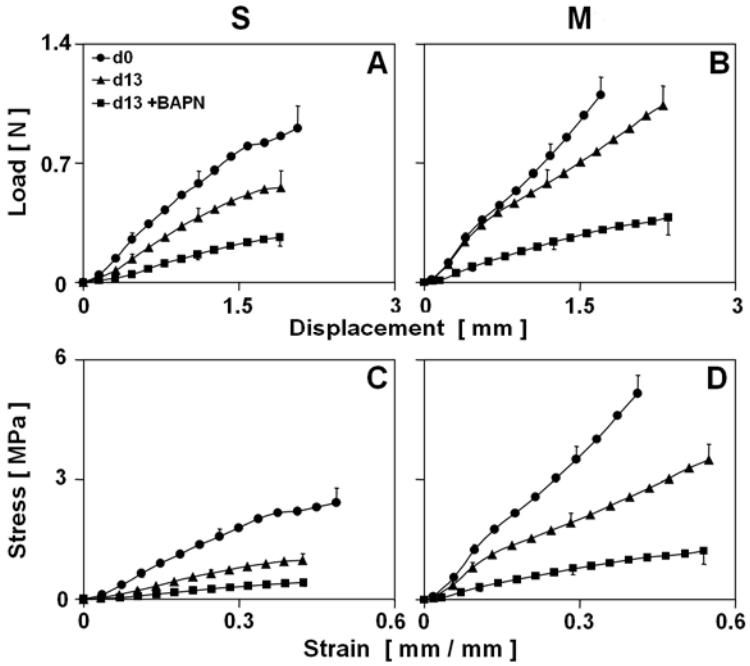

Figure 3.4: Effect of experimental conditions on growth associated changes in structural and material tensile mechanical behavior of calf articular cartilage explants from the S (**A** and **C**) and M (**B** and **D**) layers. Average load-displacement (**A**-**B**), and stress-strain (**C**-**D**), profiles preceding the failure stress of the weakest sample within a group are displayed for blocks that were analyzed on day 0 (d0) (●) and on day 13 (d13) after incubation in medium (▲) or medium supplemented with 0.1mM β-aminopropionitrile (BAPN) (■). Values are the mean ± SEM at selected points, n = 10-20 blocks from 5 animals.

Certain structural biomechanical properties of cartilage explants varied with experimental condition ($p<0.001$). Both ramp stiffness (Fig. 3.5A) and strength (Fig. 3.5B) decreased during culture in medium (~36%, $p<0.01$; -31% in S, $p<0.05$, ~17% in M, respectively) and sharply decreased during incubation in medium with BAPN (~65%, ~68%, respectively; $p<0.001$). As a result, the decrease in ramp stiffness and strength of explants incubated in medium with BAPN was more marked than that of explants incubated in medium without BAPN (~49%, ~59%, respectively; $p\leq0.05$).

The variations in the material biomechanical properties of cartilage explants paralleled the statistical variations and accentuated the trends in the structural biomechanical properties. Both ramp modulus (Fig. 3.5C) and strength (Fig. 3.5D) decreased during incubation in medium (~53%, ~47%, respectively; $p<0.01$) and sharply decreased during incubation in medium with BAPN (~76%, ~79%, respectively; $p<0.001$). As a result, the decrease in ramp modulus and strength of explants incubated in medium with BAPN was more marked than those of explants incubated in medium without BAPN (~46%, ~60%, respectively, $p<0.05$). Failure strain (Fig. 3.5E) did not vary with experimental conditions in the S layer ($p>0.22$) but varied in the M layer ($p<0.01$). Failure strain increased slightly (17%, $p<0.01$) in explants incubated in medium so that it exceeded the failure strain of explants incubated in medium with BAPN (~10%, $p<0.01$).

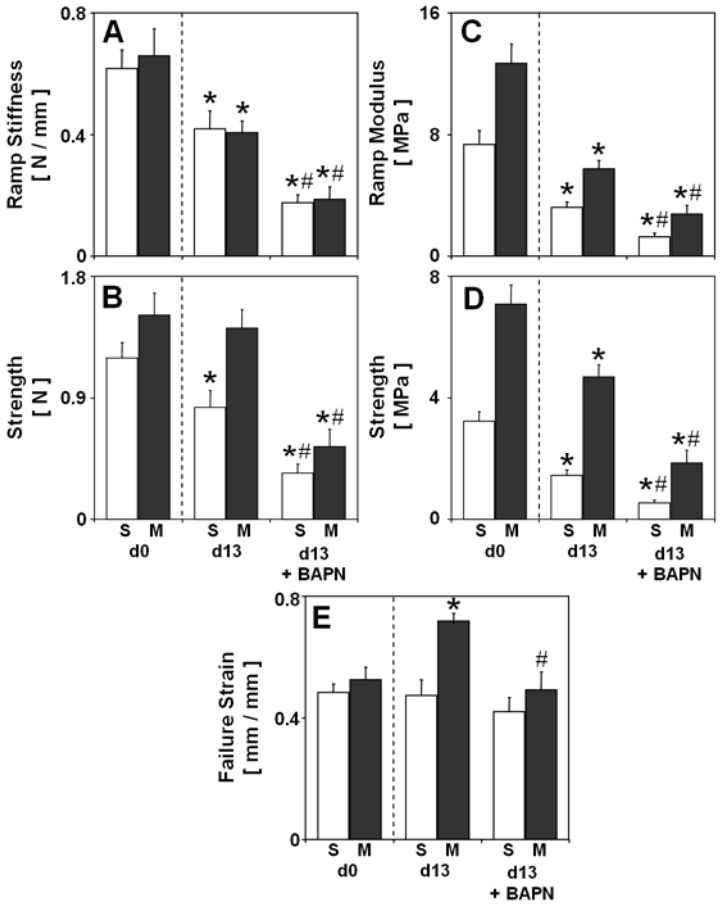

Figure 3.5: Effect of experimental conditions on structural and material tensile mechanical properties of calf articular cartilage explants from the S and M layers. Structural properties: (**A**) Ramp modulus, and (**B**) strength. Material properties: (**C**) ramp modulus, (**D)** strength, and (**E**) failure strain. The dotted line separates explants analyzed on day 0. * indicates $p<0.05$ vs. d0, # indicates $p<0.05$ vs. d13 for a corresponding layer. Data are mean ± SEM, n = 10-20 blocks from 5 animals.

3.5 Discussion

The data presented here demonstrate that manipulation of collagen network assembly can distinctly alter the growth phenotype in explants of immature bovine articular cartilage, as indicated by changes in tissue size, content of matrix components, and the integrity of the collagen network. **(I)** Incubation with 20% FBS resulted in *expansive cartilage growth* (Fig. 3.6B) characterized by a marked increase in tissue volume (Fig. 3.1A-B) and the content of GAG (Fig. 3.2B), and a slight increases in the contents of COL and PYR (Fig. 3.2C-D). The result was a maintenance of the concentration of GAG and PYR (Fig. 3.2F,H), reduction in the concentration of COL (Fig. 3.2G), and a concomitant diminution in tensile mechanical integrity (Fig. 3.4-5). **(II)** Addition of BAPN induced *accelerated cartilage growth* (Fig. 3.6C) by accentuating the *expansive cartilage growth* phenotype in terms of a further increase in tissue volume (Fig. 3.1A-B) and diminution of mechanical integrity (Fig. 3.4-3.5), without affecting the contents of GAG and COL on day 13 (Fig. 3.2B-C). Consistent with the specific effect of BAPN on collagen crosslinking, the increase in PYR content during culture was blocked by inclusion of BAPN (Fig. 3.2D).

The use of immature cartilage explants for studying mechanisms of articular cartilage growth required consideration of a number of issues. The tissue was harvested in layers, using the top ~1mm of articular cartilage (Fig. 3.1A). The superficial 0.4 mm layer included the articular surface and was prepared to include the cells that are situated to become those present in mature articular cartilage. Because the various

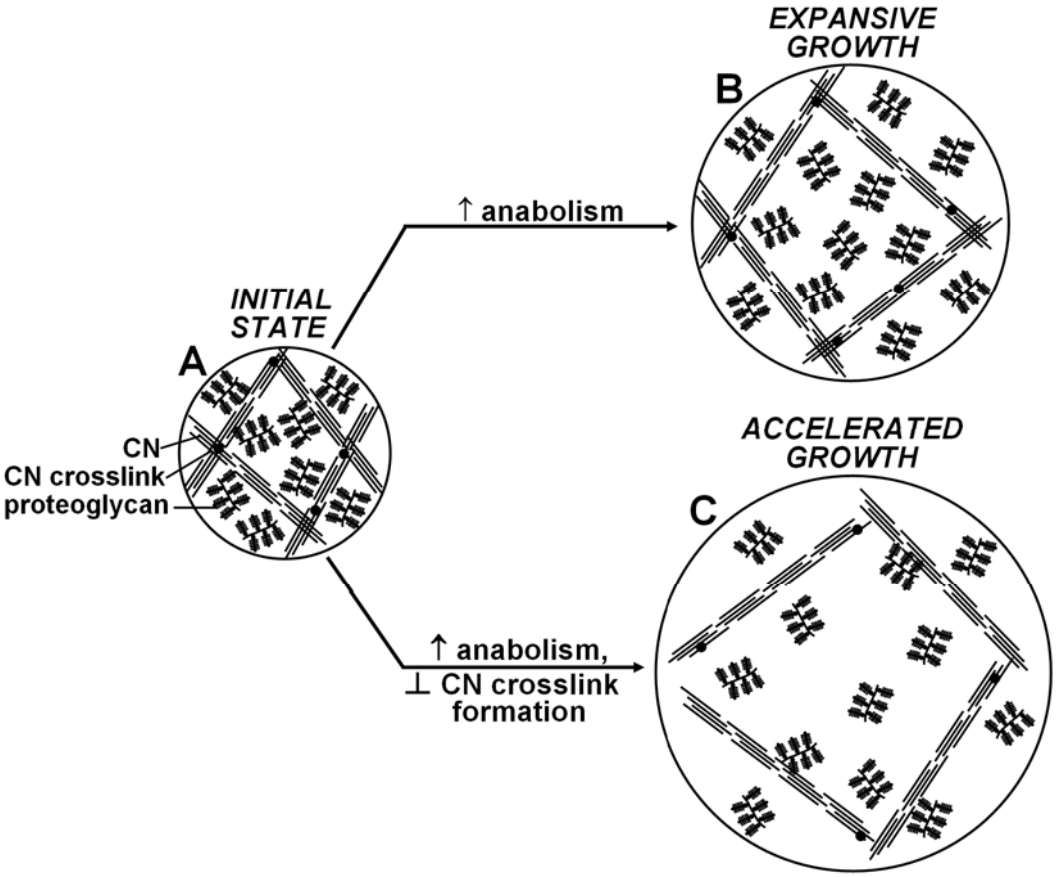

Figure 3.6: Schematic of metabolism of proteoglycan and collagen network components, leading to different types of cartilage growth. Initial state (**A**) with the major components of the solid extracellular matrix. Stimulation of anabolism with 20%FBS leads to (**B**) *expansive growth* or (**C**) *accelerated growth* when collagen crosslink formation is inhibited with BAPN.

zones of normal cartilage exhibit differences in biochemical composition and mechanical properties, a middle layer was also analyzed, and displayed an initial state different than that of the superficial layer. The patellofemoral groove was used as the source of tissue, similar to tissue used in previous studies [45, 55, 57]. Consequently, the biochemical and biomechanical properties of tissue samples at the time of explant were similar to those reported previously [55, 57].

While BAPN at doses effective at blocking crosslink formation may not effect matrix synthesis in adult bovine cartilage, with levels being similar to samples incubated in medium without BAPN [3, 15], the slight inhibitory effect observed here is consistent with previous studies on calf cartilage [15]. The inhibitory effect on protein synthesis (Fig. 3.3B) is combined with a slight increase in secretion of HYPRO into the culture medium (Fig. 3.3C) [15], suggesting that BAPN does not affect the overall synthesis of collagen, as is also indicated by similar rates of total HYPRO formation (in tissue and medium) (Fig. 3.3C), but reduces the retention of newly synthesized molecules in the tissue. The increased extractability of newly synthesized collagen after culture with BAPN [3, 15] may facilitate the release of a small number of these molecules into the medium. While the retention of the newly synthesized collagen may be lower in explants incubated with BAPN than of those without BAPN, it is not likely to be the major factor in determining the phenotype of growth, as the overall collagen deposition is not affected (Fig. 3.2C).

It appears that free-swelling growth of immature cartilage explants *in vitro* in presence of serum involves a shift in the balance between the swelling pressure of the proteoglycan molecules and the restraining ability of the collagen network, in favor of

the swelling pressure. Factors that contribute to this imbalance, and consequent volumetric tissue growth, involve both the additional swelling pressure associated with the newly synthesized GAG (Fig. 3.2B) as well as remodeling and reorganization of the collagen network that render it loose and weak (Fig. 3.4-5). During *expansive cartilage growth*, the swelling pressure of intrinsic and newly synthesized GAG was sufficient to not only expand the weakened endogenous collagen network, but also prevent its functional reinforcement with the newly deposited collagen. This was accentuated further during *accelerated cartilage growth*, when the formation of collagen crosslinks, a major element in stabilizing the collagen network, was inhibited. Similar levels of the swelling pressure resulted in even further volumetric expansion of the tissue. Such types of tissue growth appear to result from active metabolic processes that are mediated by chondrocytes and not from a passive process of tissue swelling since inhibition of biosynthesis at 4°C (data not shown) resulted in maintenance of cartilage explant geometry (±2% of initial wet weight) for up to 13 days in serum-supplemented culture. Thus, collagen crosslinking is a major element in modulating the phenotype of cartilage growth and the associated balance between proteoglycan content and integrity of the collagen network.

The effect of BAPN on the development of a distinct cartilage growth phenotype is consistent with the specific inhibitory effect of BAPN on collagen crosslink formation [26] and kinetics of collagen crosslinking in articular cartilage [2]. During culture of adult bovine articular cartilage, PYR crosslinks increase gradually, with a characteristic time constant of formation of 7-30 days [2], and thus can form during a 13 day incubation. While other forms of crosslinks that are not blocked by

BAPN can also form during culture, they are not likely to contribute to the growth of immature cartilage tissue during a relatively short, 13 day incubation *in vitro*. Maillard crosslinks formed through non-enzymatic glycation accumulate slowly after skeletal maturity [43, 51], but not during growth [5], while collagen network crosslinking mediated by transglutaminase is generally associated with cartilage regions undergoing terminal differentiation [1]. The conversion of endogenous DHLNL into mature PYR crosslinks can still occur during culture with BAPN since lysyl oxidase acts upstream of DHLNL formation. However, this conversion is estimated to be <4% of DHLNL that mature into PYR in the absence of BAPN, based on the kinetics of maturation of the newly formed DHLNL in bovine cartilage during culture with and without BAPN [2, 36].

The assembly of a functional collagen network through synthesis of collagen fibrils and the formation of collagen crosslinks is critical to the integrity and tensile properties of articular cartilage as well as other connective tissues. In animals, where enzyme-mediated collagen crosslinking has been inhibited with administration of BAPN, the mechanical properties [40, 47] and wound healing response [13, 14, 42] of various tissues greatly diminished. *In vitro*, incubation of cartilage explants with BAPN blocked the development of strength between apposing cartilage surfaces in a model of integrative cartilage repair [15].

Growth of all explants appeared to be inhomogeneous with respect to the depth from the articular surface of the source tissue and also anisotropic. Inhomogeneity of growth, as demonstrated by a large extent of volumetric growth in the superficial layer (~80%, Fig. 3.1B) and only modest volumetric growth in the middle layer (~45%, Fig.

3.1B), could be due to depth-associated variations in the content and structural organization of matrix components and the associated mechanical integrity of cartilage tissue (Fig. 3.2B-D, 3.4-3.5) [8, 33, 46]. It is possible that the parallel orientation of collagen fibrils of the superficial zone of cartilage [8] has a larger propensity to expand upon application of the swelling pressure associated with the newly synthesized GAG than the collagen network in the middle layer where collagen fibrils do not exhibit a preferred orientation. Alternatively, such inhomogeneous growth could be due to a number of resident chondrocytes, which also varies with depth from the articular surface (Fig. 3.2A) [24]. Although different extents of volumetric growth occurred in the superficial and middle layers, in each layer the volumetric growth appeared to be mostly axial, as >80% of the change in wet weight can be accounted for by the change in thickness. While it is possible that tissue dimensions may determine the preferential axis of growth, these patterns suggest that cartilage tissue may grow appositionally or that appositional growth mechanisms are dominant [22].

While the relationship between manipulations *in vitro* and growth and maturation control mechanisms *in vivo* remain to be defined, the findings of the current study may have practical utility for tissue engineering and cartilage repair. The *expansive growth* phenotypes (**I**) may be beneficial for volumetric tissue growth and used to create cartilage tissue constructs *in vitro* or fill cartilage defects directly *in vivo*. The *accelerated growth* phenotypes (**II**) may be useful for promoting large extents of volumetric tissue growth and, after generating a cartilage tissue construct of a desired volume, used to fill cartilage defects *in vivo*. In addition to being able to fill

large defect spaces, cartilage tissue constructs formed in presence of BAPN can potentially accelerate and enhance integration of this tissue with the host tissue [38] by providing a source of the non-crosslinked collagens that build up during culture with BAPN [15].

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CHAPTER 4

MECHANISMS OF CARTILAGE GROWTH: MODULATION OF BALANCE BETWEEN PROTEOGLYCAN AND COLLAGEN *IN VITRO* USING CHONDROITINASE ABC

4.1 Abstract

Objective: To examine the cartilage growth-associated effects of a disruption in the balance between the swelling pressure of glycosaminoglycans (GAG) and the restraining function of the collagen network by diminishing GAG content prior to culture using enzymatic treatment with chondroitinase-ABC (C-ABC). *Methods:* Immature bovine articular cartilage explants from the superficial and middle layers were analyzed immediately or incubated in medium for 13 days in the presence of serum. Other explants were treated with C-ABC to deplete tissue GAG and also either analyzed immediately or incubated in medium for 13 days in the presence of serum. Treatment- and incubation-associated variations in tissue volume, contents of proteoglycan and collagen network components, and tensile mechanical properties were assessed. *Results:* Incubation in serum-supplemented medium resulted in *expansive growth* with a marked increase in tissue volume that was associated with a

diminution of tensile integrity. In contrast, C-ABC treatment on day 0 led to a marked reduction of GAG content and enhancement of tensile integrity and subsequent incubation led to *maturational growth* with minimal changes in tissue volume and maintenance of tensile integrity at the enhanced levels. *Conclusions:* The data demonstrate that a manipulation of GAG content in articular cartilage explants can distinctly alter the growth phenotype of cartilage. This may have practical utility for tissue engineering and cartilage repair. For example, the *expansive growth* phenotype may be useful to fill cartilage defects, while the *maturational growth* phenotype may be useful to induce matrix stabilization after filling defect spaces.

4.2 Introduction

Articular cartilage normally functions as a low friction, wear-resistant, load-bearing material that facilitates joint motion [3]. The functional mechanical properties of this connective tissue are attributed to two of the molecular components of its extracellular matrix, proteoglycan and collagen [11, 24]. The proteoglycan constituent imparts a fixed negative charge to the tissue that increases the propensity of the tissue to swell and to resist compressive loading [2, 20]. The crosslinked collagen network resists the swelling tendency of the proteoglycans and provides the tissue with tensile and shear stiffness and strength [28, 47]. The balance between the swelling propensity of proteoglycan molecules and the restraining function of the collagen network governs cartilage hydration and its load-bearing biomechanical function [23]. For example, in osteoarthritic cartilage, the increased hydration and loss of mechanical integrity is due to a weakened collagen network that can no longer resist normal levels of swelling pressure [1].

Growth and remodeling are biological processes that, together, transform cartilage tissue *in vivo* from an immature to a mature state. Tissue growth is generally defined as an increase in tissue size due to accretion of one or more solid tissue components similar to those already present, while tissue remodeling is defined as a change in tissue composition and/or structure of tissue components [19, 38]. Many tissues, including articular cartilage, can expand due to accretion of fluid; however this process is not generally considered growth, but rather tissue swelling. Two distinct mechanisms of tissue growth have been recognized: appositional growth, or

growth at a tissue surface, and interstitial growth, or growth within tissue volume [6]. While it is possible that tissues can grow appositionally in the absence of remodeling, interstitial tissue growth must involve both growth and remodeling since accretion of a single tissue component will change the overall tissue structure and mechanical properties. Tissues can exhibit growth and remodeling in the form of hyperplasia (increase in the number of cells), hypertrophy (addition of cellular components), deposition of the extracellular matrix components or any number of these processes occurring concomitantly. In articular cartilage, the incidence of cell division is low and matrix deposition is the major contributor to the increase in size (i.e. growth) and changes in biochemical composition (i.e. remodeling) of this tissue *in vivo* [15, 41, 43]. Since articular cartilage tissue may undergo both appositional and interstitial growth and the major contributor to cartilage growth is matrix deposition, the term *growth* is used subsequently in this paper to refer, collectively, to both growth and remodeling, which can occur in presence or absence of cellular proliferation.

The metabolic balance between proteoglycan molecules and the components of the collagen network may be responsible for the evolution of cartilage function, structure, and composition during growth *in vivo* and during growth stimulated by serum *in vitro* [19]. Fetal and postnatal growth of the articular cartilage normally involves a net deposition of collagen that is greater than that of proteoglycan, as well as an increase in mechanical integrity. During maturation of articular cartilage from fetus to skeletal maturity, there is an increase in collagen and pyridinoline crosslink densities but little or no change in the content of glycosaminoglycans (GAG) [30, 39, 43, 46]. These biochemical changes are accompanied by an increase in tensile

modulus and strength [42]. In contrast to this type of *in vivo* growth and maturation, growth of cartilage explants *in vitro* in serum-supplemented medium results in a net deposition of proteoglycan that is greater than that of collagen and a decrease in mechanical integrity. For explants of immature cartilage, incubation in serum-supplemented medium results in an increase of tissue volume, maintenance of proteoglycan concentration and a decrease in the concentrations of collagen and pyridinoline crosslinks [9, 34, 44]. These changes in composition are associated with a decrease in tensile modulus and strength [44].

Based on the known phenotypes of *in vivo* growth and maturation and *in vitro* growth during serum-supplemented culture of immature cartilage, we hypothesized that cartilage growth can result from a dynamic imbalance between the swelling pressure of endogenous (that present at the time of explant) and newly synthesized GAG and the restraining function of the collagen network. One way to test this hypothesis is to compare the growth of cartilage explants, some of which are depleted of proteoglycan before subsequent culture, and hence should have a reduced propensity to grow. A number of studies have used chondroitinase ABC (C-ABC) to deplete cartilage of GAG and to investigate the immediate effects on both mechanical properties and biological responses to such perturbation of the tissue. C-ABC specifically depolymerizes chondroitin and dermatan sulfate [48], while collagen, collagen network arrangement, keratan sulfate, link protein, and virtually all the hyaluronic acid remain unaffected by the enzyme [5, 22, 29]. In response to C-ABC treatment, altered mechanical properties include a reduction of the initial slope of the stress-strain curve [16, 17] and an increase in the overall tensile modulus [21, 35]. The

biological response of chondrocytes to C-ABC treatment appears to be minimal: no changes were detected in cell density and volume [32] as well as in gene expression of certain collagens, aggrecan, and decorin [14].

To address the hypothesis of this study, the objectives were to examine the effect of depletion of GAG content in immature bovine articular cartilage by enzymatic treatment using C-ABC on culture-associated variations in (i) tissue size, (ii) accumulation of GAG and collagen network components, and (iii) tensile mechanical properties.

4.3 Materials and Methods

Sample Preparation and Culture

Articular cartilage was harvested from the patellofemoral groove of three newborn (1-3 week old) bovine calves as described previously [44]. Blocks, 9 x 3 mm^2 in area, were prepared using a sledge microtome to either include the intact articular surface (superficial layer, ~0.4 mm thick, S) or to include the middle zone, starting at a distance of ~0.6 mm from the articular surface (middle layer, 0.25 mm thick, M). The term layer rather than zone is used to avoid associations with classical zonal classification of articular cartilage. Blocks were weighed wet (initial wet weight: WW_i) under sterile condition.

Some blocks were either **(a)** analyzed immediately or **(b)** incubated in medium (DMEM supplemented with 20% FBS and 100 μg/mL of ascorbate) for 13 days. Other blocks were treated in medium with 2 U/ml protease-free C-ABC (Seikagaku America, East Falmouth, MA) (4 hr, 37°C) to deplete GAG by >90%. After treatment, the samples were washed in medium, and then either **(c)** analyzed or **(d)** incubated in medium for 13 days. During the first 12 days of culture, medium included 4.5 μCi/mL [^{3}H]proline and 1.8 μCi/mL [^{35}S]sulfate. To remove unincorporated isotopes, blocks were washed, transferred to a new culture plate, and incubated for an additional day in medium without radiolabel. Spent medium was collected throughout the culture duration. At termination, blocks were weighed wet (final wet weight: WW_f) and punched to form a tapered tensile test specimen and residual cartilage.

Biochemical Analysis

The residual cartilage and failed portions of the corresponding tensile strip (see below) were analyzed together to quantify the biochemical composition of the explants. Samples were lyophilized, weighed dry, and solubilized with proteinase K [44]. Portions of the tissue digest were analyzed to quantify the content of DNA [26], GAG [7], and hydroxyproline [45]. Digest portions from each animal/layer/experimental condition were pooled for analysis of pyridinoline (PYR) [40]. DNA was converted to cell number by using a conversion constant of 7.7 pg of DNA per cell [18]. Hydroxyproline was converted to collagen (COL) by assuming a mass ratio of collagen to hydroxyproline equal to 7.25 [13, 30]. Portions of spent medium were analyzed for GAG [7]. Biochemical parameters were normalized to initial wet weight of the tissue (WW_i) to represent constituent content and to final wet weight of the tissue (WW_f) to represent constituent concentration.

Histological Analysis

Histological analysis of GAG was performed on tissue cryosections using Alcian Blue dye. Tissue was embedded in OCT compound, snap frozen by immersion in isopentane cooled with liquid nitrogen. Tissue was then sectioned normal to articular surface at 5 μm, stained with AlcianBlue (0.002% Alcian Blue in 0.4 M $MgCl_2$, 0.025 M sodium acetate, 2.5% glutaraldehyde, pH 5.6) and imaged at 20x magnification with an inverted Nikon Eclipse TE 300 microscope equipped with SPOT RT camera (Diagnostic Instruments, Burlingame, CA).

Analysis of Matrix Metabolism

To assess matrix metabolism, other portions of the tissue digest and portions of the medium were analyzed for the incorporated radioactivity. The contents of [^{35}S] and [^{3}H] radioactivity were determined in the tissue digest and the medium and used to estimate the absolute rates of sulfate incorporation and hydroxyproline formation as indices of sulfated-GAG and collagen synthesis. A portion of spent medium was analyzed for the content of [^{35}S]GAG using the Alcian Blue precipitation method [25]. For [^{3}H]hydroxyproline analysis using Dowex columns [34, 36], portions of tissue digests and medium were pooled for each animal/layer/experimental condition.

Biomechanical Analysis

Tapered tensile specimens were analyzed to determine mechanical properties as described previously [4]. The thickness of 0.8 mm wide gage region of each tensile strip was measured using a contact-sensing micrometer. Tensile specimens were secured in clamps (4.0 mm apart) of a mechanical tester and elongated at a constant rate (5 mm/min) until failure. Structural tensile parameters were obtained from the load-displacement curves. Structural tensile strength was determined as the maximum load sustained at failure. The displacement at failure was the displacement at which maximum load was attained. Ramp stiffness was calculated as the slope of the linear regression of the load-displacement curve from 25-75% of the maximum load. Load and displacement were converted to stress (load normalized to the cross-sectional area of the gage region) and strain (elongation distance normalized to the initial gage

length) to obtain material tensile parameters of tensile strength, strain at failure, and ramp modulus.

Statistical Analysis

For each layer (S and M), the effects of C-ABC treatment (untreated, C-ABC-treated) and culture duration (0, 13) were assessed by two-way analysis of variance (ANOVA) with donor animal as a random factor. For dependent variables where data for a certain grouping factors were not applicable (e.g., sulfate incorporation), one-way ANOVA was used to examine the effect of experimental conditions. Tukey post-hoc testing was performed to compare groups. To analyze the effect of culture duration on wet weight, repeated measures ANOVA was performed for each layer and experimental condition with wet weight (WW_i, WW_f) as a repeated factor. Data are expressed as mean ± SEM, and the significance level was set to 0.05.

4.4 Results

The extent of *in vitro* growth of articular cartilage blocks was markedly affected by C-ABC treatment as assessed by changes in thickness and wet weight (Fig. 4.1A-B). In each layer, thickness and wet weight increased during culture ($p<0.001$). While C-ABC treatment did not have an independent effect on thickness in the S layer ($p=0.16$), and only minor effects in the M layer ($p<0.05$), an interactive effect existed between C-ABC treatment and culture duration in each layer ($p<0.01$). Treatment of cartilage with C-ABC on day 0 did not affect tissue thickness ($p>0.63$), and, consistent with this, the change in wet weight of these explants was near 0% ($p>0.19$). Untreated explants grew during culture, both in thickness (96% in S, 51% in M, $p<0.001$) and in wet weight (109% in S, 57% in M, $p<0.001$). In contrast, during culture after C-ABC treatment, explants from the M layer did not grow ($p=0.70$ for thickness and $p=0.11$ for wet weight) and explants from the S layer grew (51%, $p=0.09$ in thickness and 44%, $p<0.001$ in wet weight), but the extent of this growth was less than that of the untreated explants (23%, $p<0.05$ in thickness and 65%, $p<0.001$ in wet weight).

The content of water (Fig. 4.1C) did not vary with C-ABC treatment and culture duration in the M layer, but an interactive effect existed in the S layer ($p<0.01$). Treatment with C-ABC did not affect water content on day 0 ($p>0.73$), but during subsequent incubation of these explants it decreased slightly (2%, $p<0.05$). The content of water did not change during culture of the untreated explants ($p>0.42$) and on day 13 was slightly higher (2%, $p<0.05$) than that of C-ABC-treated explants.

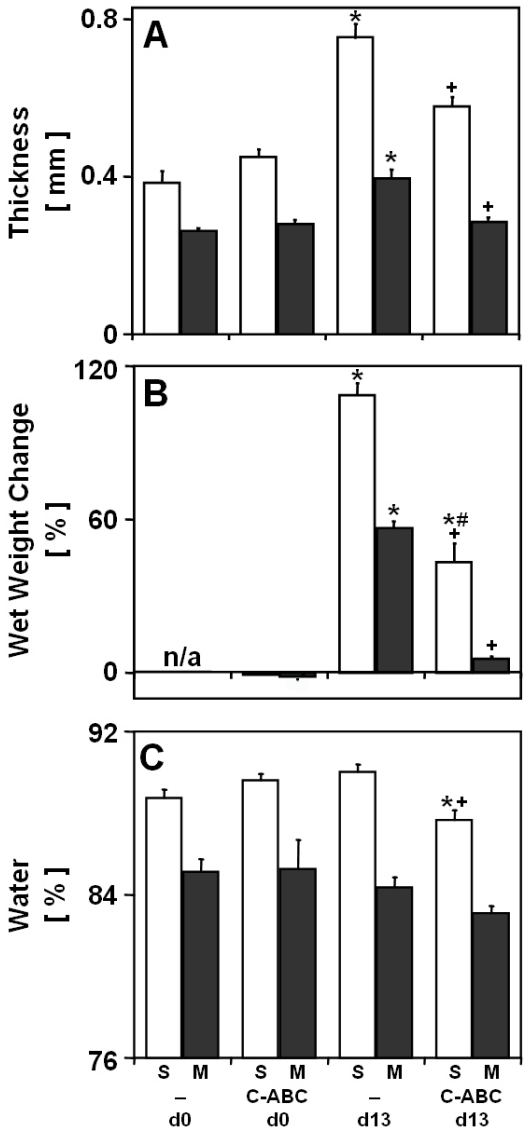

Figure 4.1: Effect of chondroitinase ABC (C-ABC) pre-treatment on general indices of *in vitro* growth of calf articular cartilage explants from the superficial (S) and middle (M) layers. On the day of explantation some blocks were treated with C-ABC. Blocks were either analyzed on day 0 (d0), or incubated for 13 days in medium (d13). (**A**) Thickness, (**B**) change in wet weight, and (**C**) percent water of cartilage blocks. Data are mean ± SEM, n = 6-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. d0 C-ABC, + = $p<0.05$ vs. d13.

The content of cells (Cells/$WW_{i,}$ Fig 2A) did not vary with C-ABC treatment or culture duration ($p>0.14$), and when normalized to WW_f to give a measure of concentration of cells (Cells/WW_f, Fig. 4.2A) reflected the culture-associated changes in wet weight. The Cells/WW_f decreased during culture ($p<0.01$). While C-ABC treatment had only a minor independent effect ($p<0.05$), an interactive effect existed between C-ABC treatment and culture duration ($p=0.09$ in S, $p<0.01$ in M). At day 0, treatment with C-ABC did not affect Cells/WW_f ($p>0.86$). During culture, Cells/WW_f decreased in explants that exhibited volumetric growth (30-45%, $p<0.001$ in the untreated explants and 26%, $p<0.01$ in the S layer of C-ABC-treated explants), and did not change in explants that did not grow ($p>0.37$ in the M layer of C-ABC-treated explants).

The extent of volumetric growth was generally paralleled by variations in the tissue contents of GAG and COL, but not PYR. In each layer, GAG content (GAG/$WW_{i,}$ Fig. 4.2B) was reduced by C-ABC treatment and increased during culture, with an interactive effect ($p<0.001$). At day 0, treatment with C-ABC reduced GAG/WW_i by 93-95% ($p<0.001$). During culture of the untreated explants, GAG/WW_i increased markedly (28-59%, $p<0.01$). Similarly, during culture of C-ABC-treated explants, GAG/WW_i increased from the very low levels to levels higher than (64%, $p<0.001$ in S) or similar to ($p=1.0$ in M) those at explant, and similar to ($p>0.48$ in S) or lower than (26%, $p<0.001$ in M) those of the untreated explants on day 13. The content of collagen (COL/WW_i, Fig. 4.2C) increased during culture ($p<0.001$ in each layer) and decreased with C-ABC treatment in the S layer ($p<0.01$), without interactive effects ($p>0.25$). Treatment with C-ABC did not affect COL/WW_i

on day 0 ($p>0.28$). During culture of both the untreated and C-ABC-treated explants, COL/WW_i increased (31-43% and 25-40%, respectively, $p<0.01$). On day 13, the result was that C-ABC-treated and subsequently cultured explants had a similar COL/WW_i ($p>0.11$) as the untreated explants. The cartilage PYR content (PYR/WW_i, Fig. 4.2D) did not vary with C-ABC treatment or culture duration ($p>0.26$).

When the content of extracellular matrix components was normalized to WW_f to give an index of concentration in the tissue, the concentrations of GAG, COL, and PYR reflected the changes in wet weight during culture. In each layer, the concentration of GAG (GAG/WW_f, Fig. 4.2B) was reduced by C-ABC treatment, and increased during culture, with an interactive effect ($p<0.001$). During culture of the untreated explants, GAG/WW_f decreased slightly (13-25%, $p<0.05$). During culture of C-ABC-treated explants, GAG/WW_f increased to levels similar to those in freshly explanted cartilage ($p>0.61$) and the untreated explants ($p=0.39$ in M) or higher than those of the untreated explants (43% in S, $p<0.001$). Histochemical staining confirmed the quantitative changes in GAG/WW_f, with uniform initial depletion by C-ABC and subsequent restoration by day 13 (Fig. 4.3). The concentration of collagen (COL/WW_f, Fig. 4.2C) decreased during culture in the S layer ($p<0.001$), and increased with C-ABC treatment in the M layer ($p<0.001$), with an interactive effect in each layer ($p<0.05$). During culture of the untreated explants, COL/WW_f either decreased (32%, $p<0.001$ in S) or did not change ($p=0.75$ in M). In contrast, during culture of C-ABC-treated explants, COL/WW_f remained unchanged ($p>0.86$ in S) or even increased (17%, $p=0.05$ in M). As a result, on day 13, C-ABC-treated explants had a higher COL/WW_f (19%, $p=0.12$ in S, 50%, $p<0.01$ in M) than the untreated

explants. The cartilage PYR concentration (PYR/WW_f, Fig. 4.2D) did not vary with C-ABC treatment ($p>0.41$) or culture duration in the M layer ($p=0.24$), but there was a slight effect of culture duration in the S layer ($p=0.06$). During culture of both the untreated and C-ABC-treated explants, PYR/WW_f decreased (39% and 12%, respectively).

Variations in the content of GAG in tissue and medium and COL content in the tissue were generally paralleled by similar variations in the total incorporation of sulfate (sum of sulfate incorporation in tissue and medium) and hydroxyproline formation, respectively. C-ABC treatment stimulated the total sulfate incorporation, as well as individual incorporations in tissue and medium (Fig. 4.4A) during subsequent culture in the S (25-36%, $p\leq0.08$), but not the M layer ($p>0.22$). Also, C-ABC treatment led to a decrease in GAG release to the culture medium (23%, $p=0.12$ in S, 71% in M, $p<0.001$). The formation of hydroxyproline (Fig. 4.4B) did not vary with C-ABC treatment ($p>0.34$).

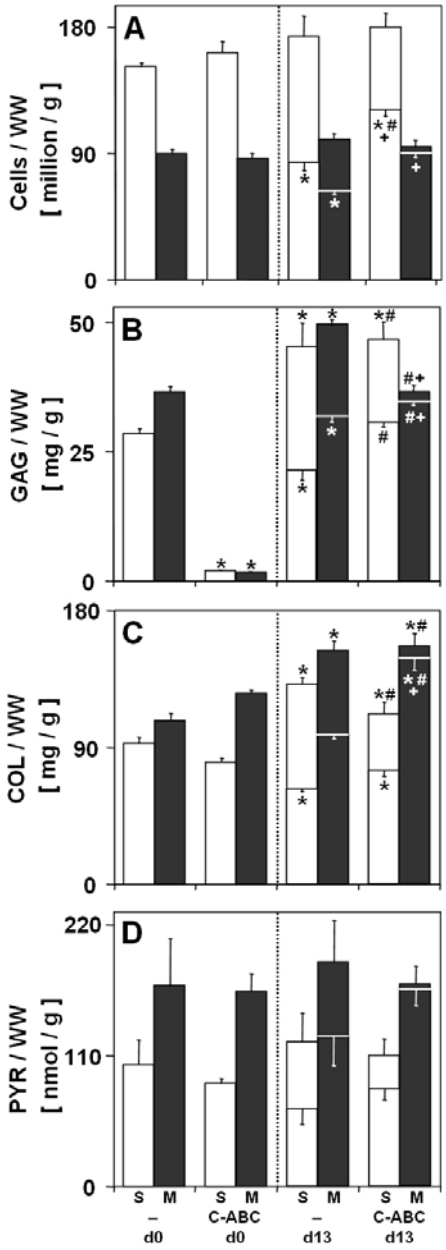

Figure 4.2: Effect of C-ABC pre-treatment on changes in the biochemical composition of calf articular cartilage explants from S and M layers. (**A**) Cells, (**B**) glycosaminoglycan (GAG), (**C**) collagen (COL), and (**D**) pyridinoline cross-link (PYR). To the left of the dotted line data are expressed per initial wet weight (WW_i) since for these explants final wet weight (WW_f) equals or is similar to WW_i. To the right of the dotted line data are expressed per WW_i (bar with an upward error bar) to represent constituent content and WW_f (bar with a downward error bar) to represent constituent concentration. Data are mean ± SEM, n = 6-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. d0 C-ABC, + = $p<0.05$ vs. d13.

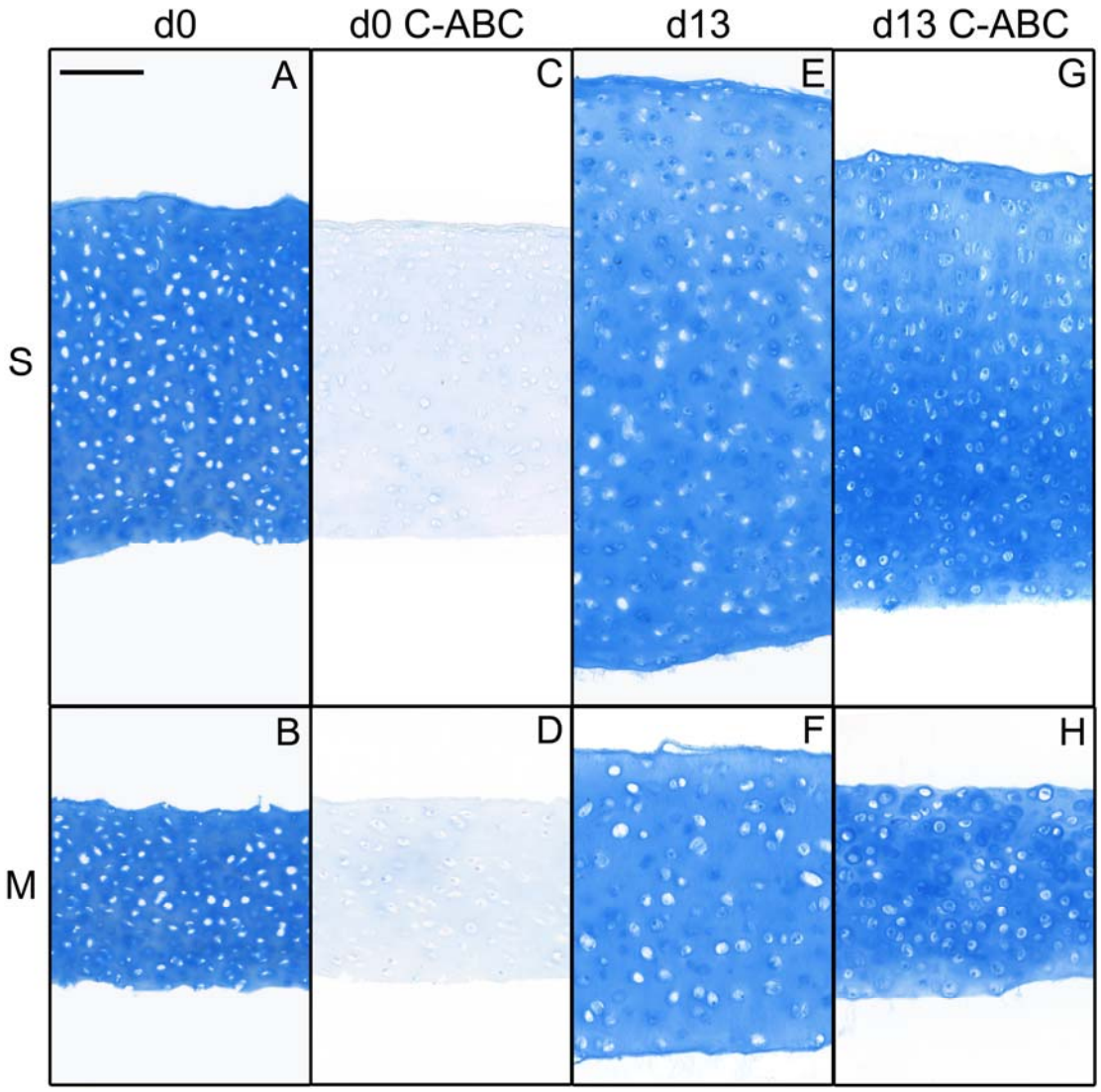

Figure 4.3: Effect of C-ABC pre-treatment on localization of GAG in calf articular cartilage explants from S and M layers. Tissue sections were stained with Alcian Blue for the untreated (**A**-**B**, **E**-**F**) and C-ABC treated (**C**-**D**, **G**-**H**) explants on day 0 (**A**-**D**) and day 13 (**E**-**H**). Bar = 100 μm.

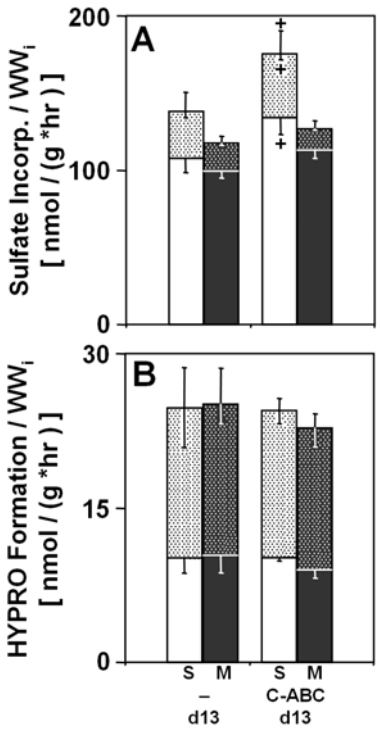

Figure 4.4: Effect of C-ABC pre-treatment on the content of sulfate incorporation and hydroxyproline (HYPRO) formation of calf articular cartilage explants from S and M layers. The dotted regions (above) represent (**A**) sulfate and (**B**) HYPRO released from tissue into the culture medium, while the solid color regions (below) represent sulfate incorporation (**A**) and HYPRO formation in the tissue (**B**). The overall bar height represents the total (**A**) incorporated sulfate and (**B**) formed HYPRO. Data are mean ± SEM, n = 6-9 blocks from 3 animals. + = $p<0.05$ vs. d13.

C-ABC treatment also affected the tensile biomechanical behavior of cartilage explants as demonstrated by the average load-displacement and stress-strain curves and the mechanical properties derived from these curves for individual samples (Fig. 4.5-4.6). Ramp stiffness and strength increased with treatment ($p<0.01$) and decreased with culture duration in the M ($p<0.05$), but not the S layer ($p>0.97$), with an interactive effect ($p<0.01$) in each layer. On day 0, C-ABC treatment lead to a marked increase in ramp stiffness (110-190%, $p<0.001$) in each layer and strength in the S (78%, $p<0.001$), but not the M layer ($p>0.48$), while displacement at failure decreased (32-46%, $p<0.001$) in each layer. During subsequent culture of these explants, the mechanical properties were maintained ($p>0.24$), so that on day 13 ramp stiffness and strength were higher (88-190%, $p<0.01$, 29-91%, $p<0.05$, respectively) and displacement at failure was lower (29-35%, $p<0.05$) than those at the time of explant. During culture of the untreated explants, mechanical properties were maintained ($p>0.62$) in the S layer, but in the M layer ramp stiffness and strength decreased (33-59%, $p<0.05$). As a result, on day 13, ramp stiffness and strength were higher (216-390% and 124-155%, respectively, $p<0.01$) and displacement at failure was lower (18-37%, $p<0.05$) in C-ABC-treated than in the untreated explants. The average stress-strain curves and the calculated material mechanical properties (Fig. 4.6), accentuated the above-described trends in the structural mechanical properties. In addition, in the untreated explants of the S layer both ramp modulus and strength decreased during culture (58-65%, $p<0.05$), similar to variations in ramp modulus and strength in the M layer.

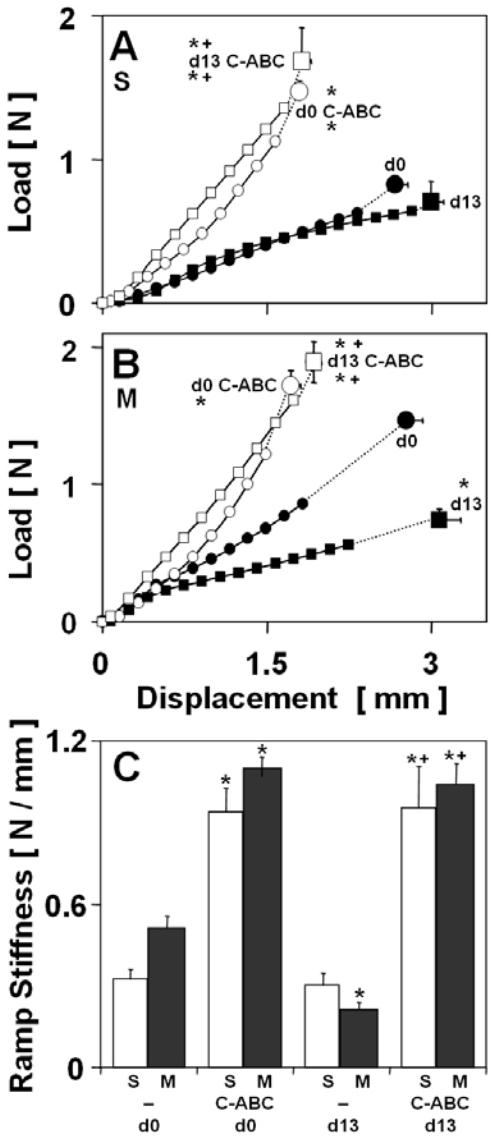

Figure 4.5: Effect of C-ABC pre-treatment on changes in structural tensile mechanical behavior and properties of calf articular cartilage explants from S (**A**, **C**) and M (**B**, **C**) layers. (**C**) Ramp stiffness, (**A**-**B**) strength and (**A**-**B**) displacement at failure of cartilage blocks. Average load-displacement profiles preceding the failure strength of the weakest sample within a group are displayed (**A**-**B**) for C-ABC-treated (white) and untreated (black) explants, which were analyzed on day 0 (circles) or on day 13 (squares). The load-displacement profiles are then extended (dotted line) until the average maximum strength and failure displacement for each group (larger symbol). Data are mean ± SEM, n = 6-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. d0 C-ABC, + = $p<0.05$ vs. d13 for strength (above name) and displacement at failure (below name).

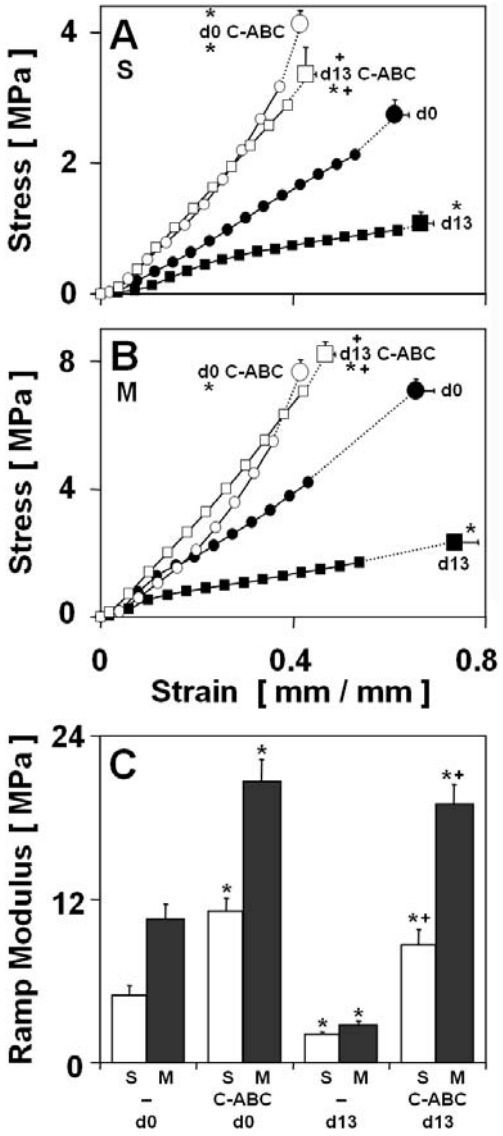

Figure 4.6 Effect of C-ABC pre-treatment on changes in material tensile mechanical behavior and properties of calf articular cartilage explants from S (**A**, **C**) and M (**B**, **C**) layers. (**C**) Ramp modulus, (**A**-**B**) strength and (**A**-**B**) strain at failure of cartilage blocks. Average stress-strain profiles preceding the failure stress of the weakest sample within a group are displayed (**A**-**B**) for C-ABC-treated (white) and untreated (black) explants, which were analyzed on day 0 (circles) or on day 13 (squares). The stress-strain profiles are then extended (dotted line) until the average maximum strength and failure strain for each group (larger symbol). For each experimental group, data are mean ± SEM, n = 6-9 blocks from 3 animals. Data are mean ± SEM, n = 6-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. d0 C-ABC, + = $p<0.05$ vs. d13 for strength (above name) and strain at failure (below name).

4.5 Discussion

The data presented here demonstrate two distinct *in vitro* growth phenotypes (**I** and **II**) in explants of immature bovine articular cartilage, as indicated by changes in tissue size, content of matrix components, and the integrity of the collagen network. (**I**) Incubation with 20% FBS resulted in *expansive cartilage growth* (Fig. 4.7B) characterized by a marked increase in tissue volume (51-109%) (Fig. 4.1A-B) and the contents of GAG (28-59%) and COL (31-43%) (Fig. 4.2B-C). The result was a reduction in the concentrations of GAG, COL, and PYR (Fig. 4.2B-D), and a concomitant diminution in tensile mechanical integrity (Fig. 4.5-4.6), similar to previous reports on growth of immature cartilage in serum-supplemented medium [9, 34, 44]. These findings suggest that free-swelling, serum-supplemented culture environment induces remodeling and reorganization of the tissue matrix that facilitates a relatively loose and weak collagen network and allows tissue expansion. (**II**) In contrast, treatment with C-ABC and subsequent incubation with 20% FBS induced *maturational cartilage growth* (47D). C-ABC treatment on day 0 induced a depletion of matrix GAG, leaving the collagen network intact, and resulted in a marked increase in tensile mechanical integrity (Fig. 4.5-4.6). During subsequent culture, the volumetric cartilage growth was slight (Fig. 4.1A-B), the content of GAG restored, and COL content increased slightly (Fig. 4.2B-C). Consequently, the concentration of PYR decreased slightly, and the concentrations of GAG and COL remained unchanged (Fig. 4.2B-D). Notably, these changes were accompanied by maintenance of the tensile mechanical integrity at enhanced levels attained after treatment with C-

ABC (Fig. 4.5-4.6). These findings suggest that C-ABC treatment and subsequent free-swelling, serum-supplemented culture induce conditions that restrict volumetric tissue growth while enhancing the integrity of the collagen network. Thus, during *in vitro* growth, phenotype (**I**) shows many hallmarks of a distinct immature and growing tissue, while phenotype (**II**) exhibits signs of progression to a more mature tissue state.

The use of immature cartilage explants for studying mechanisms of articular cartilage growth required consideration of a number of issues. The tissue was harvested in layers, using the top ~1mm of articular cartilage. The S layer included the articular surface and was prepared to include cells that are situated to become those present in mature articular cartilage. Because the various zones of normal cartilage exhibit differences in biochemical composition and mechanical properties, the M layer were also analyzed, and displayed an initial state different than that of the S layer. The patellofemoral groove was used as the source of tissue, similar to tissue used in previous studies [34, 42, 44]. Consequently, the biochemical and biomechanical properties of tissue samples at the time of explant were similar to those reported previously [42, 44].

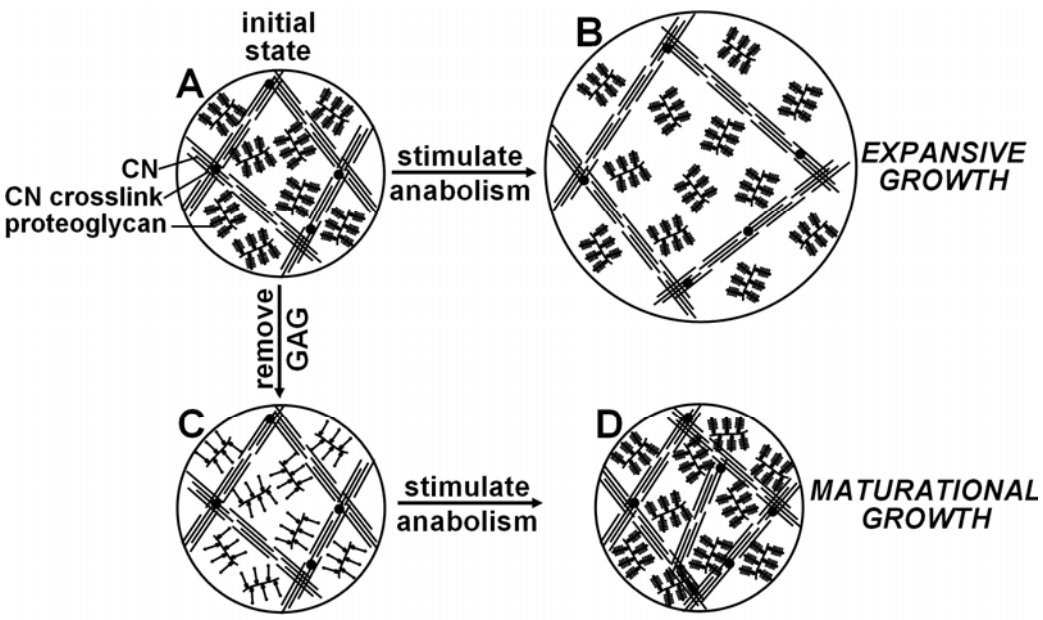

Figure 4.7: Schematic of the manipulation of GAG content and metabolism of GAG and collagen network (CN) components leading to different types of cartilage growth. Initial state (**A**) with the major components of the solid extracellular matrix. The *expansive growth* phenotype (**B**) is characterized by deposition of proteoglycan and collagen and an increase in tissue size. Enzymatic treatment to remove GAG (**C**), and subsequent growth lead to *maturational growth* phenotype (**D**), with deposition of proteoglycan and collagen but a maintenance of tissue size.

The individual direct contributions of GAG and the collagen network to the tensile properties of cartilage may explain some, but not all, of the increase in the overall tensile integrity after C-ABC treatment demonstrated in this study and observed by others [21, 35]. In normal cartilage, proteoglycans act to inflate the collagen network and establish a level of pre-stress [23], while in GAG-depleted cartilage, in the absence of pre-stress, the collagen network would be expected to relax and straighten. Although C-ABC treatment had little effect on thickness and wet weight (Fig. 4.1A-B), the small decrease in wet weight due to treatment may indicate a contraction of the collagen network. The absence of pre-stress in the collagen network of treated cartilage may contribute to a higher strength, while collagen network straightening can account for some of the decrease in failure strain and, consequently, an increase in the ramp stiffness/modulus. The residual variation in tensile properties between freshly explanted and C-ABC-treated explants may involve different interactions between collagen fibers within the collagen network. Although the nature of such interactions in normal cartilage is not well established, collagen-binding molecules (such as collagen IX and decorin) can regulate collagen fibril length and diameter during development [10, 49], and may also mediate the adherence and sliding between collagen fibrils and act as functional non-covalent linkages within the extracellular matrix [33, 37]. While aggrecan contains most of tissue's C-ABC-susceptible material, depletion of GAG with C-ABC may alter or displace some of these other chondroitin sulfate- or dermatan sulfate-containing molecules and allow new interactions between collagen fibrils that enhance the tensile integrity of the collagen network. Thus, although the tensile behavior of cartilage in the high strain

region is generally attributed to the tensile response of the collagen network [16, 42], these results demonstrate an indirect, but dramatic, contribution of the GAG component.

It appears that free-swelling growth of immature cartilage explants *in vitro* in presence of serum involves a shift in the balance between the swelling pressure of the proteoglycan molecules and the restraining ability of the collagen network, in favor of the swelling pressure. Factors that contribute to this imbalance, and the consequent *expansive cartilage growth*, involve both the additional swelling pressure associated with the newly synthesized GAG (Fig. 4.2B) and remodeling of the collagen network that render it soft and weak (Fig. 4.5B-C and 4.6B-C). Pilot studies (data not shown) indicate that such growth was mediated by the metabolic activities of chondrocytes and was not a passive swelling process, as inhibition of biosynthesis by addition of cycloheximide to block protein synthesis or incubation at 4°C resulted in maintenance of cartilage explant geometry within 6% of initial wet weight up to 2 weeks in serum-supplemented culture, much less than the observed increase in tissue volume during expansive growth (Fig. 4.1B). Thus, the swelling pressure of intrinsic and newly synthesized GAG may be sufficient to not only expand the weakened endogenous collagen network, but also prevent its functional reinforcement with the newly deposited collagen. Studies to investigate the spatial distribution of newly deposited GAG molecules and other matrix components may provide additional insights into the mechanisms of cartilage growth.

While remodeling of the collagen network during growth is an important determinant of volumetric growth of cartilage tissue, the structural organization of the

collagen network and its resultant restraining function at the initial state also play a role. Although the S and M layers were not compared directly, differences in statistical variations of certain parameters in the two layers were evident. During growth of the S layer, the collagen network remained functional, as indicated by the maintenance of structural mechanical integrity (Fig. 4.5A, C), despite large extents of volumetric growth (Fig. 4.1A-B). In contrast, during growth of the M layer, even lower extents of volumetric growth induced a weakened collagen network (Fig. 4.5B-C). These trends suggest that cartilage from the S layer is more resistant to free-swelling *in vitro* growth-associated collagen network weakening than cartilage from the M layer and potentially may be better suited to accommodate expansion associated with growth *in vivo*. Depth-associated variations in matrix components and their structural organization, such as the orientation of the collagen fibrils, may be responsible for these differences.

C-ABC-treated and subsequently cultured explants, remarkably, were able to restore GAG content to native levels (Fig. 4.2B, 4.3), similar to previous reports that demonstrated the ability of cartilage to restore proteoglycan content and organization within 4-10 days after enzymatic digestion by increasing the rate of proteoglycan synthesis [8, 12, 27, 31]. Although C-ABC treatment stimulated sulfate incorporation in the S layer, the level of sulfate incorporation in the M layer was similar to that of the untreated explants (Fig. 4.3A), despite marked GAG deposition (Fig. 4.2B). This could be largely explained by a higher rate of GAG turnover in the untreated explants, as indicated by the elevated GAG release to the culture medium, combined with preferential turnover of GAG in the matrix further removed from the cells, as

indicated by a lower specific activity of GAG in the medium. Thus, while in C-ABC-treated explants, high rates of synthesis are needed to restore GAG content, in the untreated explants, similar rates of GAG synthesis are required to replenish non-radiolabeled GAG that is being turned over.

Remodeling and reorganization of cartilage tissue that occurs during C-ABC treatment and subsequent incubation with serum allows enhancement of tissue function and results in *maturational cartilage growth*. While the overall content of the newly synthesized and deposited GAG on day 13 was high, the majority of newly synthesized collagen was probably deposited during the time when tissue GAG content and the associated swelling pressure were low. This temporal mismatch in the contents of GAG and collagen may have permitted the development of a stiff collagen network that was able to resist the swelling pressure and tendency towards expansion when GAG content increased. Factors other than the dynamic imbalance between the swelling pressure of GAG and the restraining function of the collagen network may also contribute to the observed cartilage growth phenotypes. Alterations in matrix content induced by C-ABC may change how certain chemical (medium components) or mechanical (e.g. osmotic) signals are perceived by the cells. This, in turn, may perturb the cell-mediated matrix metabolism in a way that can lead to the observed changes in the composition and the mechanical properties of the tissue.

While the relationship between manipulations *in vitro* and growth and maturation control mechanisms *in vivo* remain to be defined, the findings of the current study may have practical utility for tissue engineering and cartilage repair. The *expansive growth* phenotype (**I**) may be beneficial for volumetric tissue growth and

used to create cartilage tissue constructs *in vitro* or fill cartilage defects directly *in vivo*. The *maturation growth* phenotype (**II**) may be beneficial for promoting tissue integrity and used to induce matrix stabilization after generating a cartilage tissue construct of a desired volume or after filling defect spaces.

4.6 Acknowledgments

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CHAPTER 5

ARTICULAR CARTILAGE TENSILE INTEGRITY: MODULATION BY MATRIX DEPLETION IS MATURATION-DEPENDENT

5.1 Abstract

Articular cartilage function depends on the molecular composition and structure of its extracellular matrix (ECM). The collagen network (CN) provides cartilage with tensile stiffness and strength, but must also remodel during growth. Such remodeling may depend on matrix molecules, such as proteoglycans, interacting with the CN to modulate the tensile behavior of cartilage. The objective of this study was to determine the effects of increasingly selective matrix depletion on tensile properties of immature and mature articular cartilage, and thereby establish a framework for identifying molecules involved in CN remodeling. Articular cartilage blocks from bovine (immature, bCALF; young adult, bYOUNG) and human (teen aged, hTEEN; skeletally mature, hADULT) tissue were prepared. Blocks were treated, rinsed, tested in tension and analyzed for biochemical composition. Treatment with Gnd released ~80% of GAG, with little effect on collagen network components.

Depletion of matrix with Gnd and rinse/test in PBS increased tensile integrity markedly in bCALF, and moderately in bYOUNG, and, in contrast, did not affect the integrity in hTEEN or hADULT. Treatment with Gnd and rinse/test in Gnd, decreased tensile integrity markedly in bCALF, bYOUNG, and hTEEN, indicating that the effects of Gnd were dependent on rinse with PBS, while the integrity of hADULT remained unaltered. For bCALF, the effects of C-ABC, C-AC and sHAase were similar to the effects of Gnd. For Gnd-treated bCALF, the effects of PBS rinse were reversible, since subsequent testing in Gnd decreased tensile properties, suggesting that modulated CN interactions are non-covalent. The enhanced tensile integrity of immature cartilage after matrix depletion suggests that certain ECM components of immature matrix serve to inhibit CN interactions; such molecules are candidates for modulating physiological alterations of cartilage geometry and tensile properties during growth and maturation and can be tested through depletion/repletion studies.

5.2 Introduction

Articular cartilage is a layer of connective tissue located on the ends of long bones [5] that normally functions as a low friction, wear-resistant, load-bearing material, facilitating joint motion [34, 36]. The ability of cartilage to withstand compressive, tensile, and shear forces depends on the composition and structure of the extracellular matrix (ECM) [14, 34, 36]. The proteoglycan constituent of the extracellular matrix provides the tissue with a fixed negative charge that increases the tissue's propensity to swell and to resist compressive loading [2, 30]. The crosslinked collagen network (CN) resists the swelling tendency of the proteoglycan molecules and provides the tissue with tensile and shear stiffness and strength [36, 56, 63]. In adult cartilage, the compressive modulus has been correlated strongly with glycosaminoglycan (GAG) content [14, 36] and the tensile modulus and strength have been attributed primarily to the CN [26, 36]. An indirect, but dramatic contribution of the proteoglycan component to the tensile properties or cartilage has also been demonstrated. Chondroitinase ABC (C-ABC) treatment, which specifically depolymerizes chondroitin and dermatan sulfate GAG chains [66], resulted in a reduction of the initial slope of the stress-strain curve [28, 29] and an increase in the overall tensile modulus [31, 49]. In addition, collagen to proteoglycan ratio correlated strongly with equilibrium tensile modulus of cartilage [1]. Thus, the load-bearing function of cartilage may also depend on the ability of ECM components to transfer stress through intermolecular interactions.

The nature of the interactions among collagen fibrils and between fibrils and other members of the extracellular matrix to form a functional CN remains to be established. Sites for covalent pyridinoline crosslinks, catalyzed by lysyl oxidase, are located both within and between fibrils, linking collagens type II and IX (i.e., II-II, II-IX, and IX-IX) [10]. Such sites on collagen type IX are a source of covalent interfibrillar bonds [9, 11]. Other type of bonds may exist between collagen type IX and proteoglycans, providing a link between the CN and the extrafibrillar matrix components of cartilage [43, 55]. Collagen-binding molecules, including the small leucine-rich proteoglycans, such as decorin and fibromodulin, associate with distinct and specific bands at the surface of the collagen fibers through noncovalent binding of their core proteins [18] and therefore can be considered as constituents of the CN. These molecules have been proposed as functional non-covalent linkages within the ECM [18, 45, 46, 52]. Aggrecan, the major proteoglycan in cartilage, while distinct from the CN, may also form interactions with the CN through hyaluronic acid, or its KS and CS-rich regions [19, 24, 39, 44]. Together, extrafibrillar matrix components may mediate the adherence and sliding between collagen fibrils to modulate cartilage tissue shape [4, 51, 64].

The regulation of collagen fibril synthesis, assembly, and remodeling also points out the existence of non-covalent linkages in the ECM. Various studies suggest that small proteoglycans that bind to collagen fibers may have a role in regulation of collagen fibril length and diameter. Studies *in vitro* indicate that decorin [58, 59] and fibromodulin [17] retard the formation of type II collagen fibrils, and that the dermatan sulfate chain is not required for the interaction of decorin with collagen [57].

In embryonic tendon growth, a dramatic decrease in fibril-associated decorin occurs contemporary with lateral collagen fibril fusion to form more continuous fibrils [3]. In addition, enzymatic treatment of cartilage with trypsin, which may remove at least some of the small proteoglycans, appears to enhance cartilage repair *in vivo* [35] and the association of collagen fibers from tendon *in vitro* [13]. These findings suggest that a decrease in decorin or other collagen-binding molecules is necessary to permit the lateral association of collagen segments. In corollary, binding of collagen-binding molecules to the collagen fibers competitively inhibits the accretion of additional collagen segments and, in this way, regulates the growth of collagen fibrils during development [13, 25, 50]. A similar mechanism may be responsible for controlling the restraining function of the collagen network during cartilage growth and maturation.

Articular cartilage has distinct needs for remodeling at different stages of maturation. During prenatal or early postnatal growth periods, expansion of articular cartilage tissue occurs to accommodate the rapidly increasing size of the underlying bones and to meet the loading demands *ex utero*. Articular cartilage grows radially at a rate of ~47 μm/day in the femoral head of a neonatal rat [15], and volumetrically at ~0.18 %/day in the femoral condyle of an immature marsupial *Monodelphis domestica* [16]. Based on the radius of the femoral condyle of a bovine fetus at a gestational age of 207 days [41] (11 mm), and of a 2 weeks of age bovine calf (16 mm), radial growth rate from fetus to calf is ~54 μm/day and volumetric growth rate reaches ~2.2 %/day. It is possible that relatively weak interactions among matrix components could be more desirable in a tissue matrix that needs to grow and remodel rapidly. As the tissue increases in size, these weak interactions then can be gradually replaced by stronger

ones, fit to withstand the loading environment of the adult joint. At skeletal maturity, after cessation of growth, articular cartilage homeostasis both in terms of composition and function is desirable.

While the exact nature of interactions between collagen fibrils and extrafibrillar matrix components to form a functional collagen network remains to be established, cartilage growth and maturation may depend critically on the modulation of the CN and cartilage tensile properties. A detailed understanding of the tensile properties of cartilage may provide insight into molecular mechanisms that regulate interactions within the CN and the associated changes in the CN function during growth as well as in cartilage homeostasis and disease. Thus, the objective of this study was to determine the effects of increasingly selective matrix depletion on tensile mechanical properties of immature and mature articular cartilage, and thereby establish a framework for identifying molecules that regulate remodeling of the CN.

5.3 Materials and Methods

Sample Preparation and Culture

Articular cartilage blocks, 9 x 3 mm^2 in area, were harvested from the patellofemoral groove of bovines that were immature (1-3 week calf, bCALF, n=3) or were young adults (1-2 years, bYOUNG, n=3) and of humans that were teen aged (15-16 years, hTEEN, n=2) or were skeletally mature adults (37-39 years, hADULT, n=3). Bovine joints were obtained from an abattoir and harvested as described previously [60]. Human donor tissue was obtained from San Diego Life Sharing (San Diego, CA) within 72 hours after death and stored at -70°C. Only normal human articular cartilage (grade 1A [38]) was used in the study. Cartilage blocks were prepared using a sledge microtome to either include the intact articular surface (superficial layer, ~0.45 mm thick, S) or to include the middle zone, starting at a distance of ~0.6 mm from the articular surface (middle layer, 0.25 mm thick, M). The long axes of the blocks were in the anterior-posterior direction and, thus, approximately perpendicular to the split line direction.

Experimental Design

Blocks were weighed wet (WW_i), treated, weighed wet again (WW_f), rinsed, tested in tension, and analyzed for biochemical composition as described below. All solutions included protease inhibitors (2 mM Na_2-EDTA, 1 mM PMSF, 5 mM Benz-HCl, 10 mM NEM). Two series of experiments were carried out.

Experiment 1

To investigate the effect of non-covalent intermolecular bonds among the extracellular matrix molecules on the tensile integrity of articular cartilage, the specimens were extracted with 4M guanidine HCl (Gnd), a strong chaotrophic agent [20]. The maximum amount of GAG, as measured by hexuronate content, that can be extract with Gnd is ~85% of total, while the residual ~15% remains behind with the insoluble matrix [48]. To investigate the effects of Gnd on tensile properties apart from the effect on Gnd-induced matrix depletion alone, Gnd extracted tissue was mechanically tested under associative conditions (in PBS) and under dissociative conditions (in Gnd). Blocks of bCALF and bYOUNG, as well as hTEEN and hADULT cartilage were treated (600 μl/block, 48 hr, 4°C, pH 7.0), rinsed (200 μl/block, 30 min, 22°C, pH 7.0), and mechanically tested in treat/rinse/test combinations: **(1a)** PBS/PBS/PBS (sham control), **(1b)** Gnd/PBS/PBS, or **(1c)** Gnd/Gnd/Gnd.

Experiment 2

While Gnd is a relatively non-specific depletion agent, more specific enzymatic tissue digestions [6] were used with bCALF specimens. Chondroitinase ABC (C-ABC) depolymerizes chondroitin sulfate (CS) and dermatan sulfate (DS) and, to some degree, hyaluronic acid (HA) [66], thus potentially affecting CS and DS-containing molecules such as aggrecan, decorin, and collagen type IX as well as HA. Depending on the extent of HA cleavage with specific treatment conditions, C-ABC may also affect proteoglycan aggregates, as some of the aggregates which are attached

to small HA fragments, may be able to diffuse out of the tissue. Collagen and collagen network arrangement are generally unaffected by C-ABC treatment [7, 32, 37]. Chondroitinase AC II (C-AC) is more specific than C-ABC in that it catalytically cleaves CS and HA but not DS [66]. Thus, C-AC may affect similar molecules as C-ABC, except DS-containing molecules like decorin. *Streptomyces* hyaluronidase (sHAase) splits HA [40] and, thus, may remove some proteoglycan aggregates, similar to C-ABC and C-AC.

Explants from bCALF were subjected to either digestion with protease-free enzyme preparations or the corresponding sham solution (buffer solution that did not contain the enzyme) treatment (0.1 ml/block, 24hr at 37°C): **(2a)** 0.2 U/ml C-ABC in a buffer solution containing 0.05 M Tris buffer, pH 8.0, 0.01 M sodium acetate, and 0.02% BSA, **(2b)** 0.1 U/ml C-AC in a buffer solution containing 0.033 M acetic acid/sodium acetate buffer, pH 6.0, and 0.01% BSA, **(2c)** 10 TRU/ml sHAase in a buffer solution containing 0.02 M acetic acid/sodium acetate buffer, pH 6.0 and 0.15M NaCl. After treatment, the samples were washed and mechanically tested in PBS. C-ABC, C-AC, and sHAase were from Seikagaku America (East Falmouth, MA).

The effects of Gnd on bCALF tissue were also examined further. To investigate whether the effects of PBS wash of Gnd treated blocks (as in **1b**) are reversible (i.e. if interactions that form in PBS, under associative conditions, are covalent) the blocks were transferred to Gnd (dissociative conditions) and tested as such: **(2d)** Gnd/PBS/Gnd. To investigate if the effects of Gnd on tensile properties depend on extraction of matrix components, bCALF tissue was incubated in Gnd saturated with cartilage extract (Gnd+), solution that allows incubation under

dissociative conditions without extracting matrix components: **(2e)** (Gnd+)/PBS/PBS. To prepare Gnd+, articular cartilage from bCALF stifle joints was finely minced (1-3 mm^3 pieces), incubated in Gnd (48 hr, 4°C, pH 7.0) in a ratio of cartilage to Gnd of 2:1 and filtered through gauze.

Biochemical Analysis

Residual cartilage and the failed portions of the corresponding tensile strip (see below) were analyzed together to quantify the biochemical composition of the samples. Samples were solubilized with proteinase K [61] and analyzed to quantify the content of sulfated GAG [12] and hydroxyproline [62]. Digest portions from each animal or donor/layer/experimental condition were pooled for analysis of HA (Corgenix Inc., Denver, CO) and pyridinoline (PYR) [54]. Hydroxyproline content was converted to COL content by assuming a mass ratio of collagen to hydroxyproline equal to 7.25 for bovine cartilage [21, 41] and 7.1 for human cartilage [23]. The molar ratio of PYR per collagen molecule was calculated, assuming the molecular weight of collagen triple helix of 300,000. Biochemical parameters were normalized to initial wet weight of the tissue (WW_i) to represent constituent content.

Biomechanical Analysis

Tapered tensile specimens were analyzed to determine mechanical properties as described previously [61]. From each cartilage block, a tapered strip [27] with a gage region of 4 mm x 0.80 mm was prepared using a punch. The thickness of each tensile strip was measured at three locations in the gage region and the average was

used for cross-sectional area calculations. Tapered specimens were then secured in clamps (4.0 mm apart) of a mechanical tester. The test sequence consisted of applying a positive displacement at 0.5 mm/min until a tare load of 0.05 N (equivalent to a stress of ~0.2 MPa) was attained, then elongating the specimen to 10% strain (relative to the length at the tare load) at a constant strain rate of 0.25%/s, allowing stress relaxation to equilibrium over 900 seconds, elongating the specimen to 20% strain, allowing stress relaxation to equilibrium over 900 seconds, and then elongating the specimen at a constant rate of 5 mm/min until failure.

The measured load and displacement were converted to stress (defined as load normalized to the cross-sectional area of the gage region) and strain (defined as the elongation distance normalized to the initial clamp to clamp distance). The equilibrium tensile modulus was calculated as the slope of the equilibrium stress-strain data by linear regression. Tensile ramp modulus was calculated from the last part of the mechanical test (elongation to failure) as the slope of the linear regression of the stress-strain curve between 25% and 75% of the maximum stress. Tensile strength was determined as the maximum stress sustained at failure, and strain at failure was determined as the strain at which maximum stress was attained. Blocks that were tested in Gnd fractured during the tare phase of the test. Tensile ramp modulus was calculated as the slope of the linear regression of the stress-strain curve for tare phase of the test and equilibrium modulus was calculated by multiplying the calculated ramp modulus by the average ratio of equilibrium to ramp modulus for blocks tested in PBS of the same layer. The failed portions of each tensile strip, resulting from the tensile

test, were saved for biochemical analysis (described above) in addition to the adjacent cartilage samples obtained during preparation of the tensile strips.

Statistical Analysis

For each maturation stage/species (bCALF, bYOUNG, hTEEN, and hADULT) and each layer (S and M), the effects of experimental conditions were assessed by analysis of variance (ANOVA) with experimental condition as fixed factor and animal/human donor as a random factor. Tukey post-hoc testing was performed to compare groups. Data for equilibrium modulus of bCALF blocks in Figures 1 and 3 were log-transformed to improve normality. To analyze the effect of experimental conditions on wet weight, repeated measures ANOVA was performed for each layer and experimental condition with wet weight (WW_i, WW_f) as a repeated factor. Data are expressed as mean ± SEM or mean ±5% CI, where applicable, and significance level was set to 0.05. Statistical analysis was performed using Systat 10.2 (Systat Software, Inc., Richmond, CA).

5.4 Results

Experiment 1

Treatment of bovine and human cartilage with Gnd did not affect thickness of cartilage blocks ($p>0.16$) and, consistent with this, changes in tissue wet weight were minimal (Fig. 5.1). In bCALF and bYOUNG cartilage incubation in PBS did not change tissue wet weight ($p>0.12$) but during incubation in Gnd tissue wet weight decreased slightly (−7.4-10.2%, $p<0.001$). In hTEEN and hADULT cartilage both incubation in PBS and Gnd decreased tissue wet weight (−1.3-3.2% in PBS and −2.8-8.2% in Gnd; $p<0.01$), but such decrease was generally larger after incubation with Gnd than in PBS.

Gnd treatment affected biochemical composition of cartilage blocks from bCALF, bYOUNG, hTEEN, and hADULT. Gnd treatment released 70-85% ($p<0.001$) of GAG (Fig. 5.2) and up to 20% of HA, with little effect on collagen content (<5% release, $p>0.13$) or content of PYR crosslinks.

Experimental conditions affected the tensile biomechanical properties of cartilage explants from bCALF, bYOUNG, and hTEEN, but not hADULT (Fig. 5.2). Depletion of matrix with Gnd and then rinse/test in PBS markedly affected bCALF cartilage, with increased equilibrium modulus (+775-1176%, $p<0.001$), ramp modulus (+189%-199%, $p<0.001$) and strength (+44-148%, $p<0.001$) and a decrease in strain at failure (−28-43%, $p<0.05$), and moderately affected bYOUNG cartilage with a slightly increased equilibrium modulus (+105-120%, $p<0.05$) and ramp modulus (+37% in S, $p<0.05$; +33% in M, $p=0.18$), but not strength ($p>0.14$) and strain at

failure (p>0.28). In contrast, such treatment did not affect tensile integrity of hTEEN (p>0.25) or hADULT (p>0.16) cartilage.

Depletion of matrix with Gnd and then rinse/test in Gnd markedly affected bCALF cartilage, with decreased equilibrium modulus (−77-93%, p<0.001), ramp modulus (−82-92%, p<0.001), strength (~ −94%, p<0.001), and strain at failure (−49-58%, p<0.05), as well as bYOUNG cartilage with decreased equilibrium modulus (−86-92%, p<0.05), ramp modulus (−88-93%, p<0.001), and strength (−74%-89%, p<0.001) but not strain at failure (p>0.30). Such treatment affected hTEEN cartilage, but only moderately, with decreased equilibrium modulus (~ −57%, p<0.01), ramp modulus (−45-53%, p<0.05), but not strength (p>0.28) and a slight increase in strain at failure (+32-52%, p<0.05). In contrast, such treatment did not affect tensile integrity of hADULT cartilage (p>0.13).

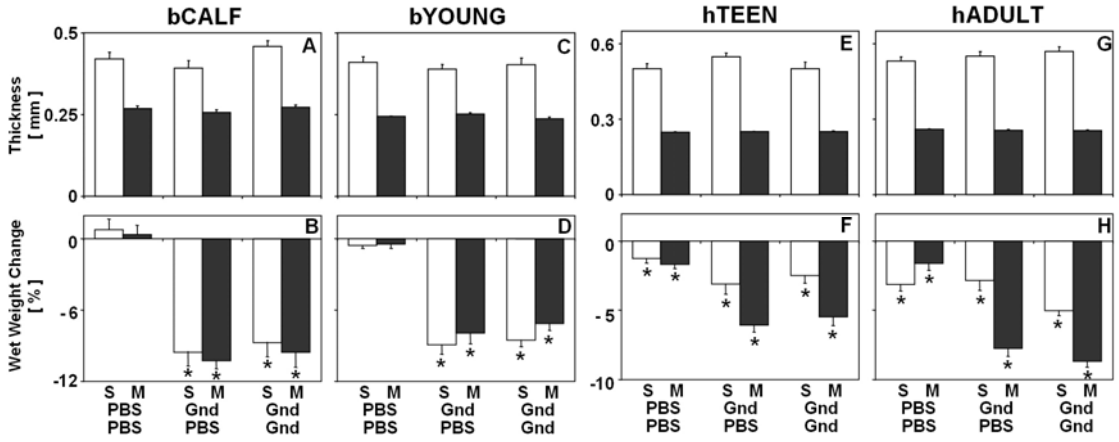

Figure 5.1: Effect of experimental conditions on geometry of bCALF (**A-B**), bYOUNG (**C-D**), hTEEN (**E-F**), and hADULT (**G-H**) articular cartilage explants from S and M layers. Blocks were incubated (48hr at 4°C), rinsed and mechanically tested in combinations of PBS and Gnd. * indicates $p<0.05$ between WW_i and WW_f. Data are mean ± SEM, n = 12-16 blocks from 3 bCALF animals, n=12-15 block from 3 bYOUNG animals, n=10-12 blocks from 2 hTEEN donors, and n=14-18 blocks from 3 hADULT donors.

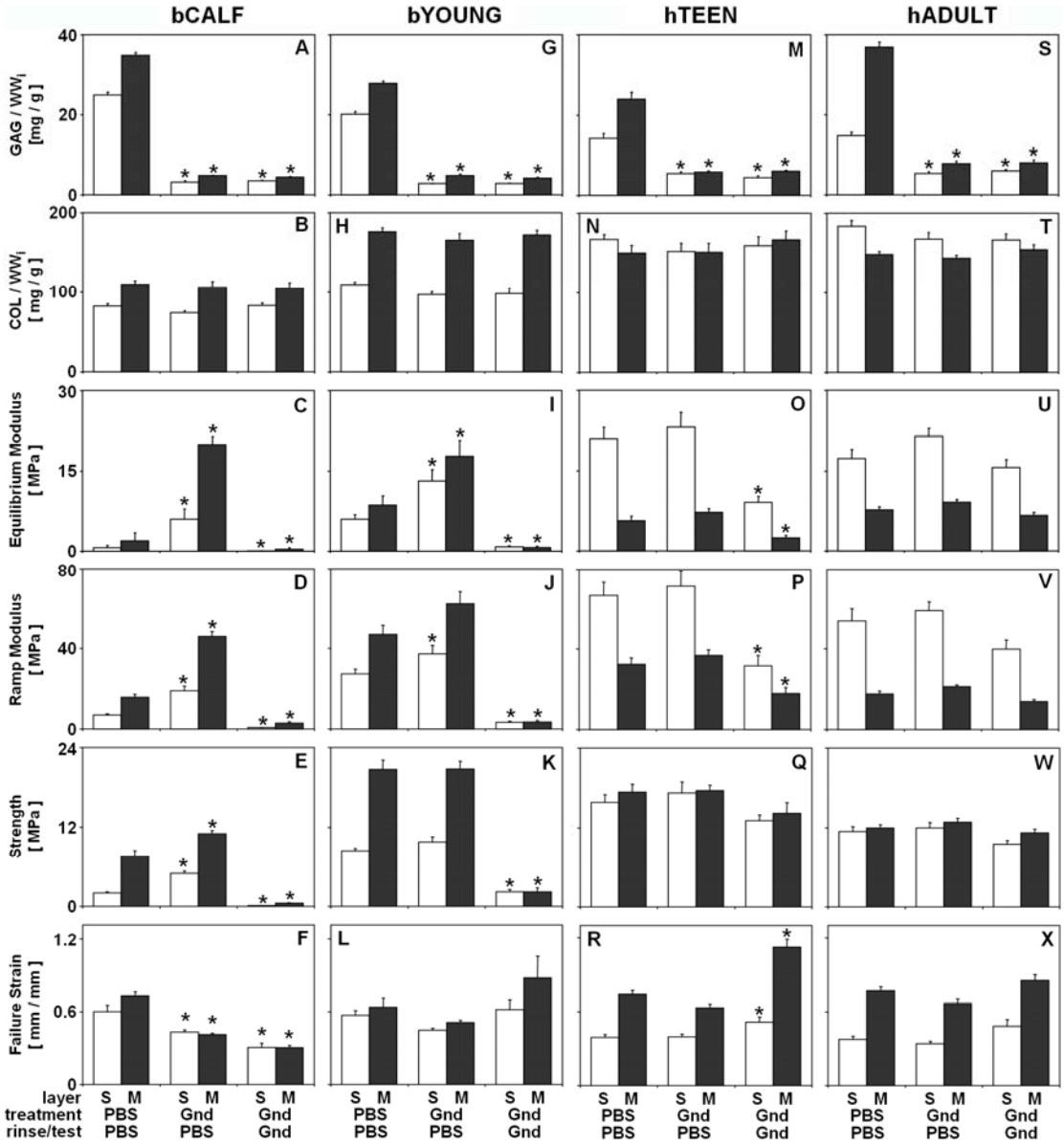

Figure 5.2: Effect of experimental conditions on composition and material tensile mechanical properties of bCALF (**A-F**), bYOUNG (**G-L**), hTEEN (**M-R**), and hADULT (**S-X**) articular cartilage explants from S and M layers. Blocks were incubated (48hr at 4°C), rinsed and mechanically tested in combinations of PBS and Gnd. * indicates $p<0.05$ vs. PBS/PBS/PBS control for a corresponding layer. Data are mean ± SEM or ± 5% CI, n = 12-16 blocks from 3 bCALF animals, n=12-15 block from 3 bYOUNG animals, n=10-12 blocks from 2 hTEEN donors, and n=14-18 blocks from 3 hADULT donors.

Experiment 2

Enzymatic treatment of bCALF cartilage did not affect thickness of cartilage blocks ($p>0.26$) and, consistent with this, changes in tissue wet weight were minimal (Fig. 5.3). Incubation in sham solutions did not change tissue wet weight in the S layer ($p>0.13$) and increased wet weight slightly in the M layer (up to +3%, $p<0.001$) while during incubation in solutions containing C-ABC, C-AC and sHAase tissue wet weight decreased (−7.5-10.5%, $p<0.001$). Similar to effects of Gnd on tissue wet weight in experiment 1, incubation in Gnd decreased tissue wet weight slightly (−7.4-10.2%, $p<0.001$), while during incubation in PBS and Gnd+, tissue wet weight remained unchanged ($p>0.08$) (Fig. 5.5).

The effects of treatments with C-ABC, C-AC and sHAase of bCALF blocks were similar to the effect of Gnd, in terms of both biochemical and biomechanical parameters. C-ABC, C-AC and sHAase treatments released ~95%, ~95% and ~63% of GAG ($p<0.001$) (Fig. 5.4), and ~64%, ~69% and ~86% of HA, respectively, with little effect on collagen content (<5% release, $p>0.17$) or content of PYR crosslinks. Depletion of tissue matrix with C-ABC, C-AC and sHAase and subsequent rinse/test in PBS resulted in a marked increase in equilibrium modulus (+246-782%, $p<0.01$), ramp modulus (+149-373%, $p<0.01$) and strength (+80-300%, $p<0.05$). The strain at failure did not vary or decreased after enzymatic treatment (up to -45%, $p>0.05$).

Further investigation of the effect of Gnd on bCALF tissue agreed with findings of experiment 1 on both biochemical and biomechanical parameters and yielded more information about the nature of the effects of ECM depletion. Gnd treatment released ~85% ($p<0.001$) of GAG (Fig. 5.4) and up to 20% of HA, with

little effect on collagen content (<5% release, $p>0.30$) or content of PYR crosslinks (Fig. 5.6). In contrast, incubation in Gnd+ did not affect biochemical parameters, with GAG, COL, HA and PYR crosslinks being similar to those in tissue incubated in PBS ($p>0.99$). Similar to effects of Gnd on biomechanical properties in experiment 1, depletion of matrix with Gnd and then rinse/test in PBS resulted in a marked increase in equilibrium modulus (+530-736%, $p<0.001$), ramp modulus (+246-295, $p<0.001$) and strength (+63-178%, $p<0.05$) and a decrease in strain at failure (−38% in S, $p=0.12$; −48% in M, $p<0.001$), and depletion of matrix with Gnd and then rinse/test in Gnd resulted in a decrease in equilibrium modulus (−77-90%, $p<0.05$), ramp modulus (−78-90%, $p<0.05$), strength (~ −95%, $p<0.05$), and strain at failure (~ −60%, $p<0.05$). Incubation in Gnd, with a PBS rinse, and subsequent test in Gnd, resulted in a decreased equilibrium modulus (−59-86%, $p<0.05$), ramp modulus (−68-86%, $p<0.05$), strength (~ −93% $p<0.05$), and strain at failure (−48% in S, $p=0.13$; −60% in M, $p<0.001$) and these mechanical properties approximated those of Gnd-treated tissue tested in Gnd but not exposed to PBS ($p>0.39$). Finally, incubation in Gnd+ restored mechanical properties to native levels, with equilibrium and ramp moduli, strength, and strain at failure similar to those of tissue incubated and tested in PBS ($p>0.93$).

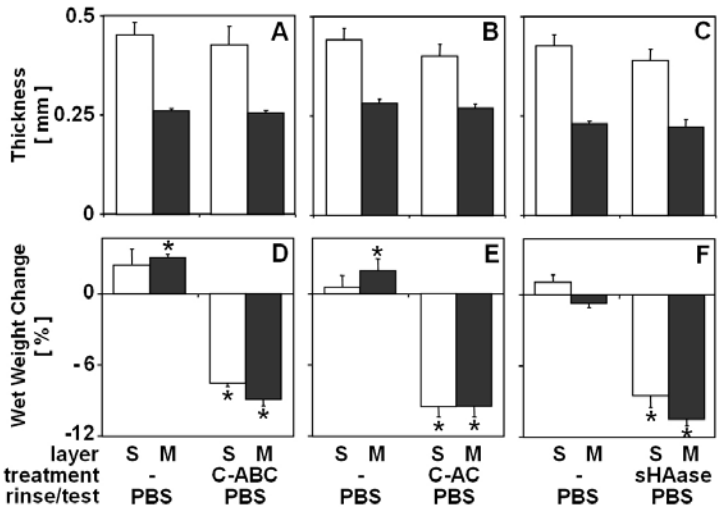

Figure 5.3: Effect of experimental conditions on geometry of bCALF articular cartilage explants from S and M layers. Blocks were incubated (24hr at 37°C) in either C-ABC (**A, D**), C-AC (**B, E**), sHAase (**C, F**) or a corresponding sham buffer (-) and mechanically tested in PBS. * indicates $p<0.05$ between WW_i and WW_f. Data are mean ± SEM, n = 4-6 blocks.

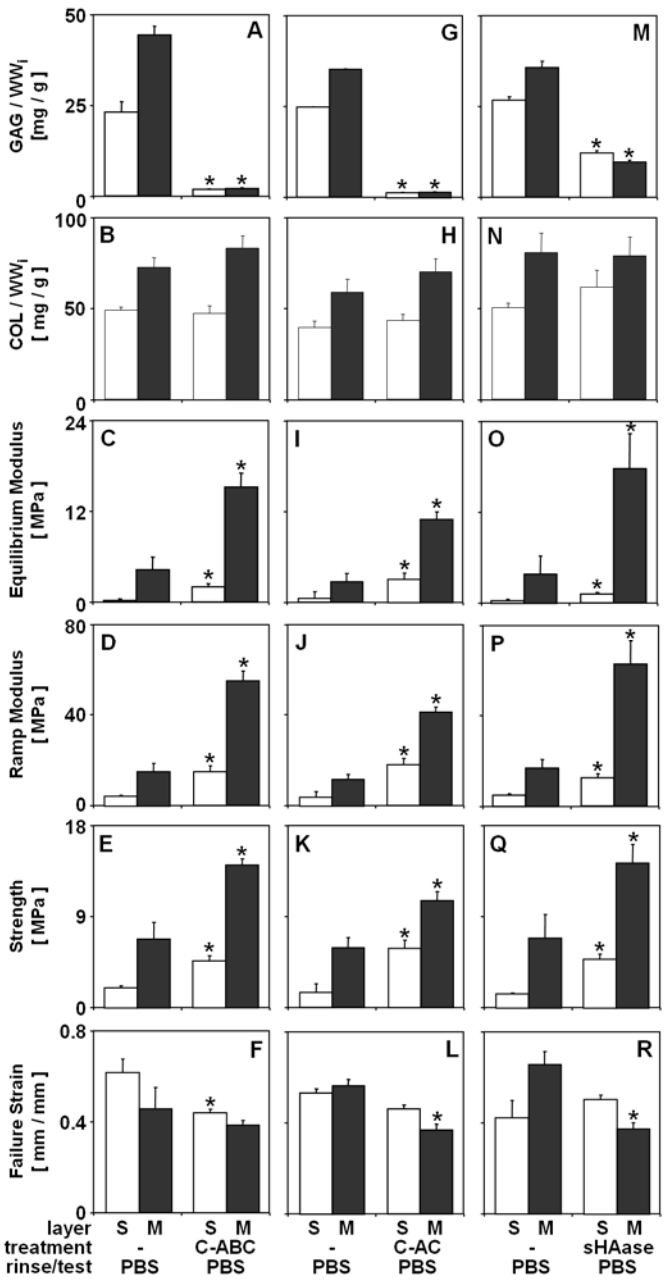

Figure 5.4: Effect of experimental conditions on composition and material tensile mechanical properties of bCALF articular cartilage explants from S and M layers. Blocks were incubated (24hr at 37°C) in either C-ABC (**A-F**), C-AC (**G-L**), sHAase (**M-R**) or a corresponding sham buffer (-) and mechanically tested in PBS. * indicates $p<0.05$ vs. sham control for a corresponding layer. Data are mean ± SEM, n = 4-6 blocks.

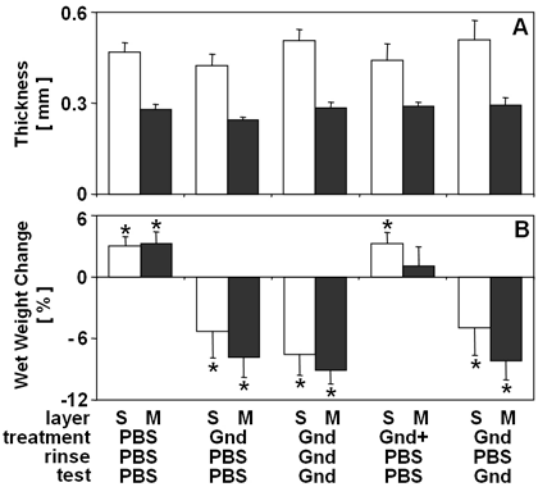

Figure 5.5: Effect of experimental conditions on geometry of bCALF articular cartilage explants from S and M layers. Blocks were incubated (48hr at 4°C), rinsed and mechanically tested in combinations of PBS, Gnd, and Gnd saturated with cartilage extract (Gnd+). * indicates $p<0.05$ between WW_i and WW_f. Data are mean ± SEM or ± 5% CI, n = 4-6 blocks.

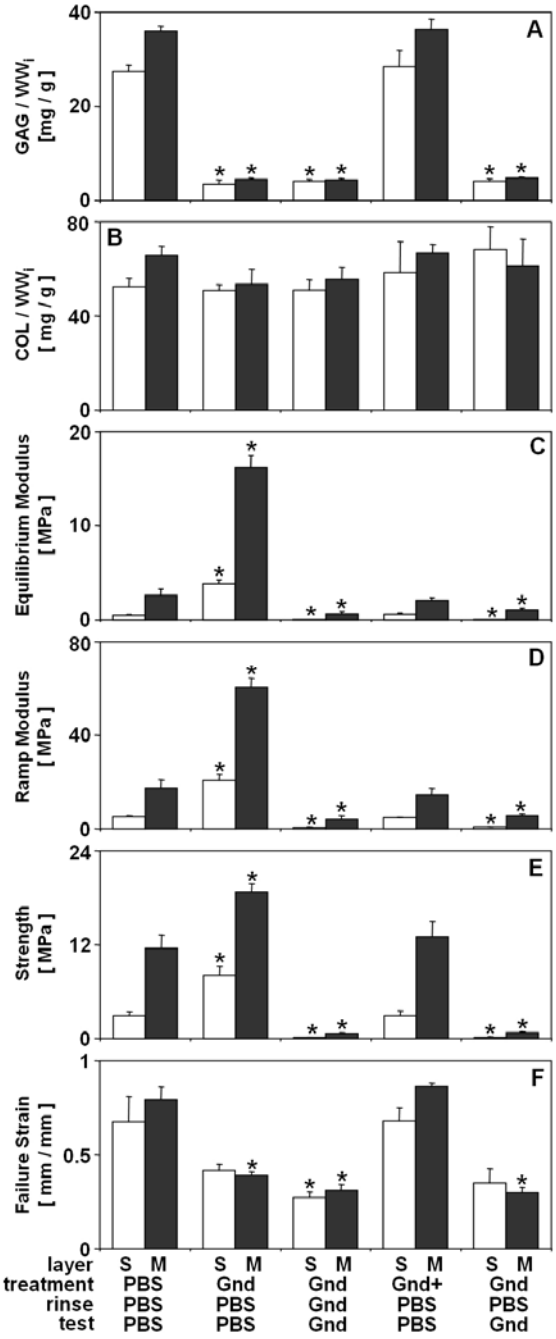

Figure 5.6: Effect of experimental conditions on composition and material tensile mechanical properties of bCALF articular cartilage explants from S and M layers. Blocks were incubated (48hr at 4°C), rinsed and mechanically tested in combinations of PBS, Gnd, and Gnd saturated with cartilage extract (Gnd+). * indicates $p<0.05$ vs. PBS/PBS/PBS control for a corresponding layer. Data are mean ± SEM or ± 5% CI, n = 4-6 blocks.

5.5 Discussion

The data presented here demonstrate that tensile mechanical properties of articular cartilage can be modulated through depletion of certain extracellular matrix components; however the extent of this modulation is dependent on the maturity of the source tissue. Matrix depletions with Gnd resulted in a marked enhancement of tensile integrity in the immature tissue (bCALF) when mechanically tested under associative conditions and a marked decrease in tensile integrity when mechanically tested under dissociative conditions. The enhanced tensile integrity of immature cartilage after matrix depletion suggests that certain ECM components of immature matrix serve to inhibit CN interactions, while reduction of tensile integrity to very low levels after depletion and test in Gnd indicates that matrix components within immature tissue are weakly bound to each other. As the tissue maturity increases (from bCALF to bYOUNG to hTEEN to hADULT), a progressively more moderate enhancement after matrix depletion and testing under associative conditions and less marked decrease after matrix depletion and testing under dissociative conditions is observed, until hADULT tissue that is not affected by Gnd. The lack of effect of Gnd on hADULT cartilage on tensile properties suggests the CN attains a state in which it is not disrupted by even by the strong denaturing conditions of 4M Gnd.

For bCALF, the effects of C-ABC, C-AC and sHAase were similar to the effects of Gnd both in terms of biochemical and biomechanical parameters narrowing down the list of candidate ECM components that may serve to inhibit tensile integrity-promoting CN interactions within the immature tissue matrix. For Gnd-treated

bCALF, the effects of PBS rinse were reversible, since subsequent testing in Gnd decreased tensile properties, suggesting that modulated CN interactions are non-covalent. In addition, lack of effects on biochemical composition and tensile integrity of incubation in Gnd+, indicates that the formation of modulated CN interactions is dependent on matrix depletion.

The use of bovine and human cartilage explants for studying the modulation of CN and cartilage tensile properties required consideration of a number of issues. The tissue was harvested in layers, using the top ~1mm of articular cartilage. The superficial 0.4 mm layer of the immature cartilage included the articular surface and was prepared to include the cells that are situated to become those present in mature articular cartilage. Because the various zones of normal cartilage exhibit differences in biochemical composition and mechanical properties, the middle layers were also analyzed, and displayed an initial state different than that of the superficial layer. The patellofemoral groove of bovines was used as the source of tissue, similar to tissue used in previous studies [47, 60, 61]. Consequently, the biochemical and biomechanical properties of bCALF and bYOUNG tissue samples incubated and tested in PBS were similar to those reported previously [60, 61]. The tensile specimens of human cartilage were taken from patellofemoral groove based on preliminary studies that showed little variation among the specimens from this location for an individual joint. Biochemical and biomechanical parameters of hTEEN and hADULT were generally similar to those reported for human cartilage [1, 53].

The stage of maturity of the source tissue, as indicated by tissue age, also agrees with measures of content of collagen and PYR crosslinks and of tensile moduli

and strength, parameters that are sensitive to the degree of tissue maturity [60]. The content of collagen and PYR crosslinks in bCALF tissue was lower than that in the bYOUNG tissue. This variation was accompanied by an increase in tensile moduli and strength (Fig. 5.2). Similar differences between these age groups were reported previously [60]. The contents of collagen and PYR crosslinks in both hTEEN and hADULT were even higher than that of bYOUNG, accompanied by higher tensile moduli and strength (Fig. 5.2). Older bovine tissue could not be examined because of difficulty finding joints that were macroscopically normal, while younger human tissue could not be examined because of supply limitations.

Immature cartilage can be rendered stiff or compliant experimentally by alterations in the tissue matrix through enzymatic or chemical treatments, suggesting that the restraining function of the collagen network can be regulated through alterable interactions of components of the extracellular matrix. These components of the extracellular matrix may be responsible for controlling the stiffness and the restraining function of the collagen network through mediating the adherence and sliding between fibrils. It has been proposed that the collagen fibrils, or groups of fibrils, slide past one another mediating the response of cartilaginous tissues to mechanical loading and modulating tissue length/shape. The deflection patterns of lines photobleached or fluorescently stained into the extracellular matrix suggest that collagen sliding occurs in rat tendon and ligament and bovine annulus fibrosis [4, 64, 65]. The presence of gaps within transversely sectioned fibril bundles in electron micrographs of experimentally elongated sea urchin ligament, is also indicative of fibril sliding [22]. It is possible that if molecules that mediate sliding are removed, collagen fibers can

associate laterally to increase the fibril diameter and consequently increase tensile stiffness and strength of the tissue overall [8, 13, 42].

While the enhanced tensile integrity of immature cartilage after matrix depletion suggests the involvement of different interactions between collagen fibers within the collagen network, some of the increase in the overall tensile integrity after matrix depletion demonstrated in this study and observed by others [31, 49] can be explained by direct individual contributions of GAG and the collagen network components to the tensile properties of cartilage. In normal cartilage, proteoglycans act to inflate the collagen network and establish a level of pre-stress [33], while in matrix-depleted cartilage, in the absence of pre-stress, the collagen network would be expected to relax and straighten. Although matrix depletion did not affect thickness and had little effects tissue wet weight, the small decrease in wet weight due to treatment may indicate a contraction of the collagen network. The absence of pre-stress in the collagen network of treated cartilage may contribute to a higher strength, while collagen network straightening can account for some of the decrease in failure strain and, consequently, an increase in the ramp stiffness/modulus. Thus, although the tensile behavior of cartilage in the high strain region is generally attributed to the tensile response of the collagen network [28, 60], these results demonstrate an indirect, but dramatic, contribution of the proteoglycan component.

It is possible that a relatively weak interactions within the collagen network and collagen fibril sliding could be more desirable in an immature tissue matrix that may need to remodel as it rapidly increases in size in the process of tissue growth. Thus, such extensible tissue state and collagen sliding may be a part of a normal

response to the growth stresses. Inhibition of fiber sliding mechanism and a resultant stiff tissue state may be desirable in adult tissue that mainly functions to withstand the rigors introduced by various loading regimes throughout life.

While the exact mechanism of collagen sliding and interaction of matrix components within cartilage tissue remains to be established, interactions between the collagen network and proteoglycans component of the tissue matrix have been proposed to mediate the adherence and sliding of the collagen fibers [45, 52]. In the case when these molecules are present, such as in the immature tissue, the may play a role in modulating physiological alterations of cartilage geometry and tensile properties during growth and maturation, while the absence these matrix molecules, such as in the chemically or enzymatically depleted immature tissue or a mature tissue, results in a stiff tissue state contributes to maintenance of cartilage tissue homeostasis. Modulation of the content and organization of such components may be important in collagen network remodeling during growth and maturation.

The finding that modulated CN interactions are dependent on matrix depletion, as indicated by lack of effects of incubation in Gnd+, and are non-covalent, since effects of PBS rinse were reversible, suggest that candidate molecules, such as those binding collagen, can be tested individually and in combination through depletion/repletion studies. Increasingly selective treatments with C-ABC, C-AC, and sHAase, provide insight into the identity of such inhibitory molecules. While aggrecan contains most of tissue's C-ABC-susceptible material, depletion of GAG with C-ABC may alter or displace some of these other chondroitin sulfate- or dermatan sulfate-containing molecules such as collagen type IX and decorin. The involvement of

decorin, however, is excluded as treatment with C-AC does not remove this molecule, but resulted in enhancement of tensile integrity similar to that produced by C-ABC treatment. Treatment with sHAase was most selective as sHAase susceptible material includes HA and, consequently, may also involve proteoglycan aggregates, suggesting that these molecules are primary candidates as the "inhibitory" components in the immature tissue.

Since the tensile integrity-promoting interactions in immature tissue are non-covalent, it may be possible to deconstruct and reconstruct the constituents to modulate CN function. Identification of methods to reconstruct constituents, may allow the assembly of desired components that would enhance functional propertied of cartilage tissue, thus producing a mature tissue construct in a short time period.

5.6 Acknowledgments

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CHAPTER 6

CONCLUSIONS

6.1 Summary of Findings

The overall motivation of this dissertation work was to contribute to the understanding of the nature of processes that drive the transformation of cartilage tissue from immature to a mature state by examining how cartilage growth can be modulated in vitro through manipulations of content, synthesis, and assembly of matrix components, as well as examining how this transformation may be accomplished through remodeling of the collagen network. In summary, the major findings were:

1. *In vitro* growth of immature articular cartilage explants, as indicated by changes in tissue size, content of matrix components, and the integrity of the collagen network, is regulated differentially by certain growth factors.
   a. Incubation with FBS, IGF-I, or BMP-7 resulted in *expansive cartilage growth*, characterized by an increase in tissue size, stimulation of GAG synthesis and deposition, and loss in the concentration of matrix components and the tensile mechanical integrity.
   b. Incubation in basal medium or with PDGF-AB also resulted in growth with some, but not all, of the characteristics of expansive growth. Under these

conditions, tissue volume and composition changed slightly, but were accompanied by a marked decrease in mechanical integrity.

c. In contrast, the regulation of cartilage metabolism by TGF-β1 induced a state of *homeostasis*. Volumetric growth and biosynthetic rates were minimal under these conditions and content and concentration of GAG and collagen were maintained. Notably, these changes were accompanied by maintenance of the tensile mechanical integrity.
d. Correlative analysis indicated that cartilage explants grew in volume as the content of GAG in the tissue, an indicator of swelling pressure, increased and as the tensile modulus and strength, indicators of the integrity of the collagen network, decreased.
e. Tensile mechanical properties of modulus and strength were correlated with the concentration collagen, but not concentration of GAG.

2. Manipulation of collagen network assembly can distinctly alter the growth phenotype in explants of immature bovine articular cartilage, as indicated by changes in tissue size, content of matrix components, and the integrity of the collagen network.
   a. Addition of BAPN, an inhibitor of collagen crosslinking, to culture medium containing 20% FBS, induced *accelerated cartilage growth* by accentuating the *expansive cartilage growth* phenotype in terms of a further increase in tissue volume and diminution of mechanical integrity, without affecting the contents of GAG and COL.
   b. Consistent with the specific effect of BAPN on collagen crosslinking, the increase in PYR content during culture was blocked by inclusion of BAPN.

c. Collagen crosslinking is a major factor in modulating the phenotype of cartilage growth and the associated balance between proteoglycan content and integrity of the collagen network.

3. Manipulation of GAG content by depletion of GAG content prior to culture can distinctly alter the growth phenotype in explants of immature bovine articular cartilage.
   a. C-ABC treatment on day 0 induced a depletion of matrix GAG, leaving cell density and collagen network intact and resulted in a marked increase in tensile mechanical integrity.
   b. Treatment with C-ABC on day 0 and subsequent incubation with 20% FBS induced *maturational cartilage growth*. This growth phenotype was characterized by the slight volumetric cartilage growth, increase in the contents of GAG and COL and maintenance of GAG and COL concentrations. Notably, these changes were accompanied by maintenance of the tensile mechanical integrity at enhanced levels attained after treatment with C-ABC.

4. The tensile integrity of articular cartilage can be modulated through depletion of certain extracellular matrix components; however the extent of this modulation is dependent on the maturity of the source tissue.
   a. Matrix depletions with Gnd resulted in a marked enhancement of tensile integrity in the immature tissue (bCALF) when mechanically tested under associative conditions and a marked decrease in tensile integrity when mechanically tested under dissociative conditions.

b. As the tissue maturity increases (from bCALF to bYOUNG to hTEEN to hADULT), a progressively more moderate enhancement and less marked decrease in mechanical integrity is observed, until hADULT tissue that is not affected by Gnd. The lack of effect of Gnd on hADULT cartilage on tensile properties suggests the CN attains a state in which it is not disrupted by Gnd.
c. The enhanced tensile integrity of immature cartilage after matrix depletion suggests that certain ECM components of immature matrix serve to inhibit collagen network interactions.
d. The modulated collagen network interactions appear non-covalent, since effects of PBS rinse after extraction with Gnd were reversible.
e. The finding that enhancement of tensile integrity is depended on the extraction of certain molecules suggests that candidate molecules, such as those binding collagen, can be tested individually and in combination through depletion/repletion studies.

6.2 Discussion

The results presented here demonstrate that manipulation of matrix content, metabolism, and assembly can distinctly alter the growth phenotype of cartilage. **(I)** Incubation with 20% FBS, IGF-I, and BMP-7 results in *expansive cartilage growth*, increase in the contents of matrix components, and a concomitant diminution in tissue tensile mechanical integrity (tensile ramp modulus and strength), consistent with previous studies of *in vitro* growth in presence of serum [18]. **(II)** Incubation with BAPN results in *accelerated cartilage growth*, by accentuating expansive cartilage growth phenotype in terms of even further increase in tissue size and diminution of

tensile mechanical integrity, consistent with the specific inhibitory effect of BAPN on collagen crosslink formation [9], without affecting the contents of matrix molecules. **(III)** Treatment with C-ABC and subsequent incubation with 20% FBS, results in *maturational cartilage growth* characterized by an increase in tissue tensile integrity and geometrical homeostasis. **(IV)** Remodeling and reorganization of cartilage tissue that occurs during incubation with TGF-β1 allows maintenance of tissue size, composition, and function resulting in a state of *homeostasis*.

These findings suggest that free-swelling, serum, IGF-I, or BMP-7-supplemented culture environment induces remodeling and reorganization of the tissue matrix that facilitates a relatively loose and weak collagen network and allows tissue expansion. Such a dynamic growth state is further accentuated by the addition of BAPN. In contrast, C-ABC treatment and subsequent free-swelling, serum-supplemented culture induces conditions that restrict volumetric tissue growth while enhancing the integrity of the collagen network. Thus, during *in vitro* growth, phenotype (**I**) and (**II**) show many hallmarks of a distinct immature and growing tissue, while phenotype (**III**) exhibits signs of progression to a more mature tissue state (Fig. 6.1).

Thus, it appears that factors that lead to growth of immature cartilage explants *in vitro* involve regulation of metabolism of proteoglycan and collagen network components by chondrocytes. Specifically, the shift in the balance between the swelling pressure of the proteoglycan molecules and the restraining ability of the collagen network, toward an overall expansive effect resulting from the swelling pressure. Cartilage explants grew in volume, as the content of GAG in the tissue, an indicator of swelling pressure, increased and as the tensile modulus and strength, indicators of the integrity of the collagen network, decreased. *Expansive cartilage*

growth appeared to be due to deposition of proteoglycans that exceeded the deposition of collagen. Normal rate of volumetric growth of bovine cartilage from fetus to calf *in vivo* (~2.2 %/day in terms of wet weight), involves deposition of collagen (~1.7 %/day) that exceeds that of GAG (which exhibits a ~steady content) and an increase in the tensile mechanical integrity (~5.7 %/day) [17]. A notably higher rate of volumetric growth for the most superficial 400 μm of tissue during serum or IGF-I-supplemented growth *in vitro* (~6.3 %/day in terms of wet weight) was associated with GAG deposition (~-1.8 %/day on concentration basis) that exceeded that of collagen (~-3.4 %/day on concentration basis) and with a decrease in the tensile mechanical integrity of the tissue (~-5.6 %/day).

The results of Chapter 5 provide insights into the nature of interactions among matrix components in cartilage tissue that may be important in collagen network remodeling during growth and maturation. Immature cartilage can be rendered stiff or compliant experimentally by alterations in the tissue matrix through enzymatic or chemical treatments, suggesting that the restraining function of the collagen network can be regulated through alterable interactions of components of the extracellular matrix. It is possible that a relatively weak interactions within the collagen network could be more desirable in an immature tissue matrix that may need to remodel as it rapidly increases in size in the process of tissue growth. Thus, such extensible tissue state in immature cartilage may be a part of a normal response to the growth stresses. Modulation of matrix components and the resultant stiff tissue state may be desirable in adult tissue that mainly functions to withstand the rigors introduced by various loading regimes throughout life. While the nature of interactions among matrix components within cartilage tissue remains to be established, interactions between the collagen network and proteoglycans of the tissue matrix have been proposed to

mediate the adherence and sliding of the collagen fibers to modulate tissue shape [13, 16]. Modulation of the content and organization of such regulatory components may be important in collagen network remodeling during growth and maturation.

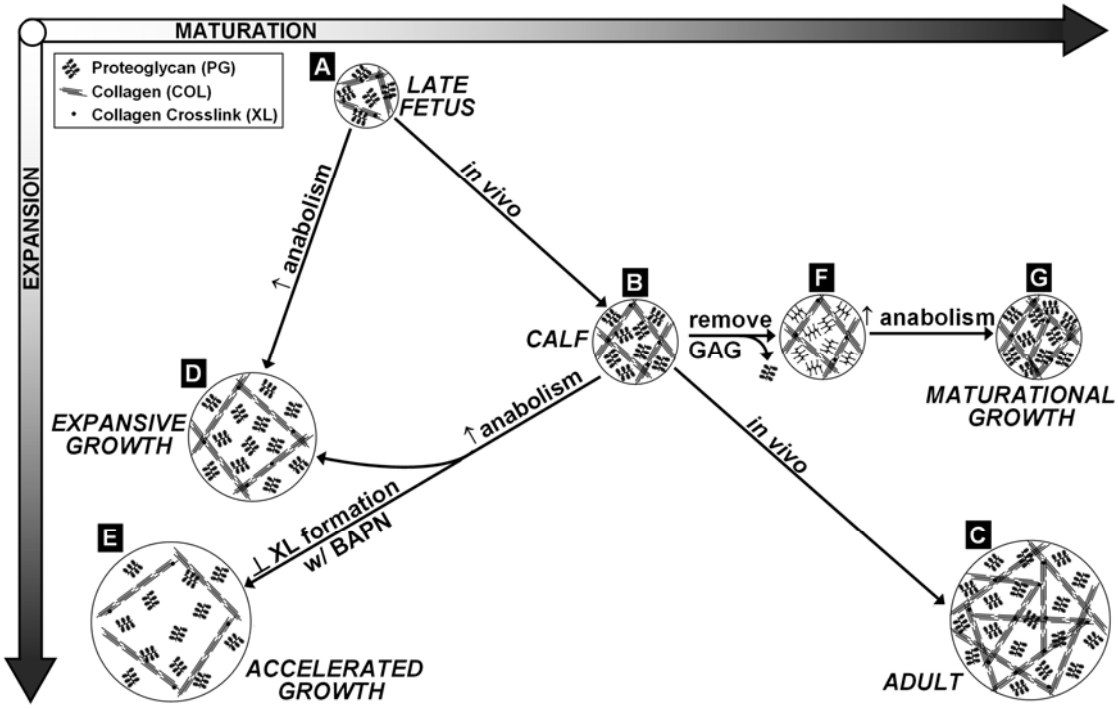

Figure 6.1: Phenotypes of immature articular cartilage growth in vivo (**A**-**C**) *in vitro* during serum-supplemented culture (**D**), culture supplemented with β-aminopropionitrile (BAPN) (**E**), and that after pre-treatment with chondroitinase ABC (C-ABC) (**F**-**G**).

6.3 Future Work

The work presented in this dissertation can be expanded in a number of ways. Some of the major directions include investigating the effects of mechanical stimulation on immature cartilage growth and identifying some of the molecular mechanisms involved in the weakening of the collagen network during serum-supplemented culture as well as applying some of the principles described here to the generation of cartilage grafts to improve on current tissue engineering and cartilage repair strategies.

Growth of immature cartilage tissue *in vitro* in serum-supplemented medium resulted in a net deposition of proteoglycan that was greater than that of collagen and a decrease in mechanical integrity, in contrast to fetal and postnatal growth *in vivo*, which involves a net deposition of collagen that is greater than that of proteoglycan, as well as an increase in mechanical integrity. In addition, the growth rate of cartilage explants *in vitro* was generally greater than that which occurs naturally *in vivo*. These differences suggest that factors other than those present *in vitro* during free-swelling, serum-supplemented growth are necessary for remodeling and maturation of cartilage that could resemble what occurs *in vivo*. The presence of biochemical factors other than those present in serum, or chemically defined medium containing IGF-I, BMP-7, or PDGF-AB, may be necessary for biomechanical maturation. For example, chemically defined medium containing TGF-β1 allows maintenance of mechanical function *in vitro* and biomechanical stimuli modulate cartilage matrix metabolism and remodeling *in vitro* and *in vivo*. Application of load during culture *in vitro* may result in improved mechanical function compared to a free-swelling condition.

Current work suggests that the mechanisms for cartilage growth include the swelling pressure that exceeds the restraining function of the collagen network to

result in an increase in tissue size. Application of external load may not only approximate the environment in which cartilage grows *in vivo*, but also direct the swelling pressure and consequently growth in a predictable way. For example, axial loading of a cartilage disc may decrease the rate of axial growth and increase the rate of radial growth. Such directional manipulation of the swelling pressure and of growth, may allow functional maturation of the collagen network, as well as further understanding of how cartilage tissue can be altered in size and state of maturity.

It may be of interest to investigate whether remodeling of the collagen network that leads to maintenance or weakening of tensile integrity results from modulation of the activity of MMPs. While some of the tensile weakening can be induced by a physical disruption of the collagen network by the damaging forces of the swelling pressure associated with the endogenous and newly synthesized GAG, remodeling that involves induction of various proteinases such as collagenases may also be involved [2]. Certain sites of cleavage in the collagen network (such as that of MMP-3) can potentially initialize collagen fibril depolymerization which may allow reorganization and remodeling of the collagen network that may be important during active growth [5]. On the other hand, maintenance of collagen network integrity may result from modulation of remodeling through suppression of proteinase synthesis [1, 7, 14] and stimulation of production of protease inhibitors such as tissue inhibitors of metalloproteinases (TIMPs) [6, 7, 15]. MMP-induced matrix remodeling can be inhibited by broad spectrum MMP inhibitors such as hydroxamates [3] or coumarins [4, 12] which have activity against both the MMPs and the ADAM-TS.

Some of the principles of growth *in vitro* can be utilized in the engineering of cartilage tissue grafts for the purposes of repairing articular cartilage defects. Specific *in vitro* conditions may aid in designing tissue engineered grafts that attain the

geometry of an *in vivo* defect and the structural and functional properties of native cartilage surrounding the defect. For example, the space-filling phenotypes (**I**, **II**) may be useful to produce a tissue graft quickly but with characteristics typical of immature cartilage. Subsequently, methods, such as mechanical stimulation, may be used to enhance maturation of the immature cartilaginous tissue [8]. Alternatively, the integrity-promoting phenotype (**III**) may be useful to induce matrix stabilization after filling defect spaces. The homeostasis phenotype **(IV)** may then be used to maintain a tissue graft or graft until implantation with steady-state maintenance of biochemical and biomechanical characteristics.

The data on biochemical and biomechanical parameters during various *in vitro* growth protocols can be used as an experimental validation for analytical models of cartilage growth [10, 11]. The solutions to such models have possible implications for tissue engineering experiments. They may be able to predict a specific concentration of biochemical factors and/or intensities of mechanical stimuli for a specific outcome (i.e. desired geometry, biochemical composition and mechanical function of a cartilage graft).

To further identify the molecular mechanisms responsible for the enhancement of tensile properties after GAG depletion, candidate molecules, such as those binding collagen, can be tested individually and in combination through depletion/repletion studies. While some of this work was attempted (see Appendix A), experimental methods for functional cartilage repletion remain to be identified. Identification of such methods, preferably under associative conditions to maintain cell viability, may allow the assembly of desired components that would enhance functional propertied of cartilage tissue, thus producing a more mature tissue graft in a shorter time period.

6.4 References

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APPENDIX A

ARTICULAR CARTILAGE TENSILE INTEGRITY: MODULATION BY MATRIX DEPLETION AND REPLETION WITH CHONDROITIN SULFATE

A.1 Introduction

The enhanced tensile integrity of immature cartilage after matrix depletion, as was demonstrated in Chapter 5, suggests that certain ECM components in immature cartilage serve to inhibit collagen network interactions that promote tensile tissue integrity. The findings that the modulated collagen network interactions arise after extraction of ECM components from the tissue matrix and that the interactions appear to be non-covalent, suggest that candidate molecules, such as those binding collagen, can be tested individually and/or in combination through depletion/repletion studies.

Thus, the objective of this study was to determine the effects of selective repletion of matrix molecules following matrix depletion with 4M guanidine HCl, on tensile mechanical properties of immature articular cartilage, and thereby identify molecules that regulate collagen network remodeling.

A.2 Materials and Methods

Detailed description of materials and methods can be found in Chapter 5. The following describes deviations from the description in Chapter 5, as well as additional methodology. For mechanical testing, cartilage blocks were subjected to the treat, rinse test combination as described in Chapter 5 and indicated in Figures 2 and 3. All solutions included protease inhibitors (2 mM Na_2-EDTA, 1 mM PMSF, 5 mM Benz-HCl, 10 mM NEM).

Preparation of Gnd Containing Cartilage Extract

Gnd containing cartilage extract was prepared from articular cartilage bovine calf stifle joints and included cartilage lining the femur, tibia, and patella. Tissue was finely minced (1-3 mm^3 pieces), incubated in Gnd (48 hr, 4°C, pH 7.0) in a ratio of cartilage to Gnd of 2:1 to yield Gnd saturated with cartilage extract (Gnd+) or in a ratio of 1:1 to yield $Gnd^{(1:1)}$. Gnd+ containing [^{35}S]sulfate-labeled GAG was prepared from cartilage tissue that was incubated in medium with 20% FBS and 100 μg/ml of ascorbate, supplemented with 15 μCi/mL [^{35}S]sulfate for 4 days.

Purification of Aggrecan Monomer

The method of Rosenberg [5] was followed with minor modifications. Articular cartilage was harvested from PFG, patella and condyles of immature (1-3 weeks) bovine calf, minced and extracted with 5 volumes of 4M Gnd in 0.05 M NaAc with PIs (pH 5.8) for 48 hr at 4°C. The mixture was then filtered through glass wool and dialyzed through a membrane (MWCO 6-8000) against 9 volumes of 0.05 M

NaAc containing protease inhibitors, pH 5.8 for 24 hr at 4 °C. The density of the dialysate was adjusted to 1.62-1.64 g/ml by addition of solid CsC1 and centrifuged at 37000 rev./min for 48 h and at 10°C. The bottom one third (A1) was collected and recycled into another associative gradient. Two volumes of 5.5 M Gnd, 5 mM EDTA, 0.15 M sodium acetate, pH 5.8, containing protease inhibitors were added to one volume of fraction Al. The solution was stirred for 4 hr, then dialyzed overnight against 20 volumes of 0.15 M sodium acetate, 5 mM EDTA, pH 5.8, containing protease inhibitors. Equilibrium density gradient centrifugation under associative conditions was carried out in 3.5 M CsCl at 10°C for 40 h at 37,000 rpm. The bottom one third (A1A1) was collected and subjected to equilibrium density gradient centrifugation under dissociative conditions in 4 M Gnd, 0.05 M NaAc, 5 mM EDTA, pH 5.8 at 10°C for 40 h at 37,000 rpm, with starting CsCl density of 1.45 g/ml. The gradient was cut into six equal fractions, the bottom one sixth (A1A1D1) was collected, dialyzed through a membrane (MWCO 6-8000) against 9 volumes of 0.05 M NaAc containing protease inhibitors, pH 5.8 for 24 hr at 4°C. The then content of GAG in this fraction was assed by DMMB assay [3]. From 25g of cartilage, 162g of GAG was obtained which, considering there is 30mg GAG per g cartilage tissue and 90% of aggrecan monomer is GAG, corresponds to about 22% yield. The fraction was then lyophilized and reconstituted in either PBS or 4 M Gnd, at concentration of aggrecan monomer equivalent to 30 mg/ml of GAG.

Cartilage Repletion

Articular cartilage blocks were treated with Gnd to released ~85% of GAG, as described in Chapter 5, the tissue was then repleted (24 hr, 4°C, pH 7.0) with either **(a)** Gnd+, **(b)** PBS with 30 mg/ml of shark chondroitin sulfate (CS) (Sigma, St. Louis, MO), **(c)** Gnd with 30 mg/ml of CS, **(d)** PBS with aggrecan monomer (AGG) at a concentration equivalent to 30 mg/ml of GAG or **(e)** Gnd with AGG at a concentration equivalent to 30 mg/ml. The concentration of 30 mg/ml for both CS and AGG was chosen to match GAG content of bovine calf tissue. Following repletion, the tissue was rinsed quickly (2min) in PBS. GAG content of the final PBS rinse, as well as that in the tissue was quantified.

A.3 Results

Treatment of cartilage with Gnd released ~85% of GAG (Fig. A.1), while treatment of cartilage with Gnd saturated with cartilage extract (Gnd+) did not affect tissue GAG content. Depletion of cartilage matrix with Gnd, and subsequent incubation with Gnd+ did not result in repletion of GAG, which remained at levels similar to those after depletion with Gnd. Low levels in GAG in the rinse indicate that GAG from Gnd+ did not enter the tissue matrix. Repletion of cartilage tissue occurred in PBS with CS to levels similar to those of native tissue, however, a portion of repleted GAG rapidly exited the tissue during the brief rinse in PBS, resulting in tissue GAG content at ~45% of that in native tissue. A smaller extent of repletion (to ~55% of GAG in native tissue) occurred in Gnd with CS, which exited the tissue completely during the rinse in PBS. As a result, tissue GAG content was similar to that after depletion with Gnd. While, incubation with AGG in either PBS or Gnd did not result in repletion of GAG content, as levels of combined GAG in tissue and PBS rinse were similar to those after depletion with Gnd, it appeared as more GAG bearing AGG may be entering the tissue in presence of PBS.

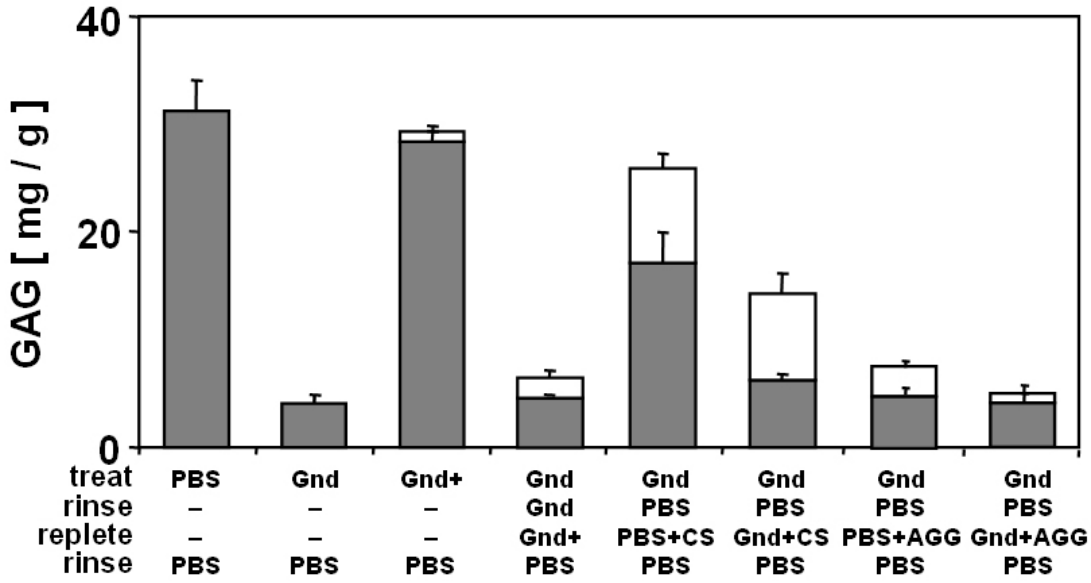

Figure A.1: Effect of depletion of calf articular cartilage explants from M layer with 4M Guanidine HCl (Gnd) or Gnd saturated with cartilage extract (Gnd+) and its subsequent repletion with Gnd+, PBS/Gnd with 30mg/ml of chondroitin sulfate (CS) or PBS/Gnd with aggrecan (AGG), at concentration equivalent to 30mg/ml of GAG. Tissue GAG content (grey) and GAG content in the 2nd rinse (white) are shown. Data are mean ± SEM, n = 3-6 blocks.

The nature of the effect of repletion with PBS containing CS was investigated further in term of effects on tensile integrity (Fig. A.2). While the repletion was only partial (to ~50% of GAG in native tissue), such treatment had similar effects in terms of biomechanical parameters as did depletion with Gnd without repletion. Depletion of matrix with Gnd and then rinse/test in PBS markedly affected cartilage, with increased equilibrium modulus (+894%), ramp modulus (+200%) and strength (+110%). Similarly, depletion of tissue matrix with Gnd, and subsequent repletion with PBS containing CS increased equilibrium modulus (+1070%), ramp modulus (+250%), and strength (+120%).

To investigate whether, a ~50% reduction in GAG content is sufficient to produce the enhancement of tensile integrity seen with a near complete GAG depletion (such as that as with Gnd), the effect of partial depletion of GAG content on tensile mechanical properties was investigated (Fig. A.3). Incubation of tissue with $Gnd^{(1:1)}$, released 50% of GAG. Following a rinse in PBS, such tissue did not exhibit an enhancement in tensile integrity, which remained at levels of native tissue. This was in contrast to the ~85% GAG depletion with Gnd, and subsequent test in PBS, which resulted in increased equilibrium modulus (+340%), ramp modulus (+260%) and strength (+130%).

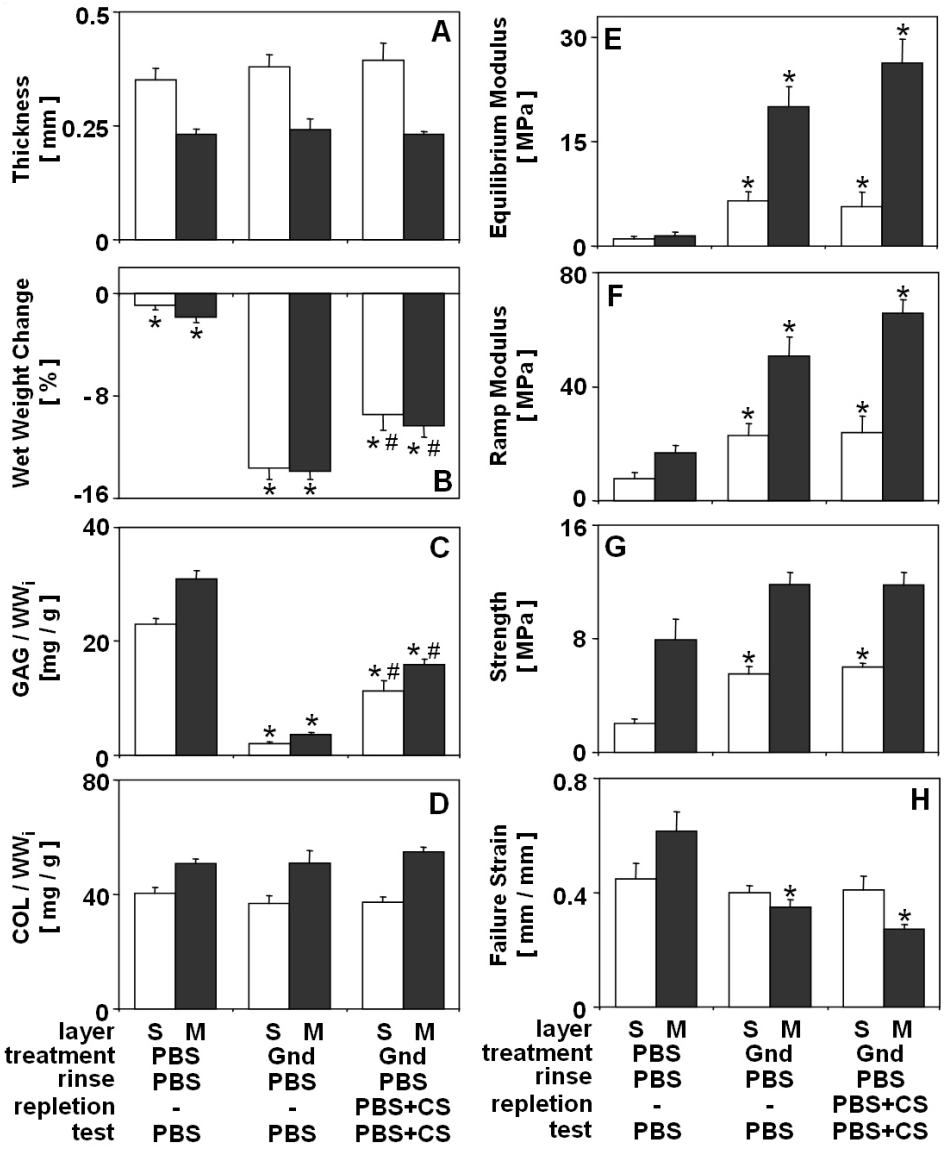

Figure A.2: Effect of repletion with chondroitin sulfate on tissue geometry, composition and tensile mechanical properties of calf articular cartilage explants from S and M layers. Tissue thickness (**A**) and change in wet weight (**B**) content of GAG (**C**) and collagen (**D)**, equilibrium modulus (**E**), ramp modulus (**F)** strength (**G**), and failure strain (**H)**. * indicates $p<0.05$ vs. PBS/PBS/PBS control, # indicates $p<0.05$ vs. Gnd/PBS/PBS for a corresponding layer. Data are mean ± SEM, n = 6.

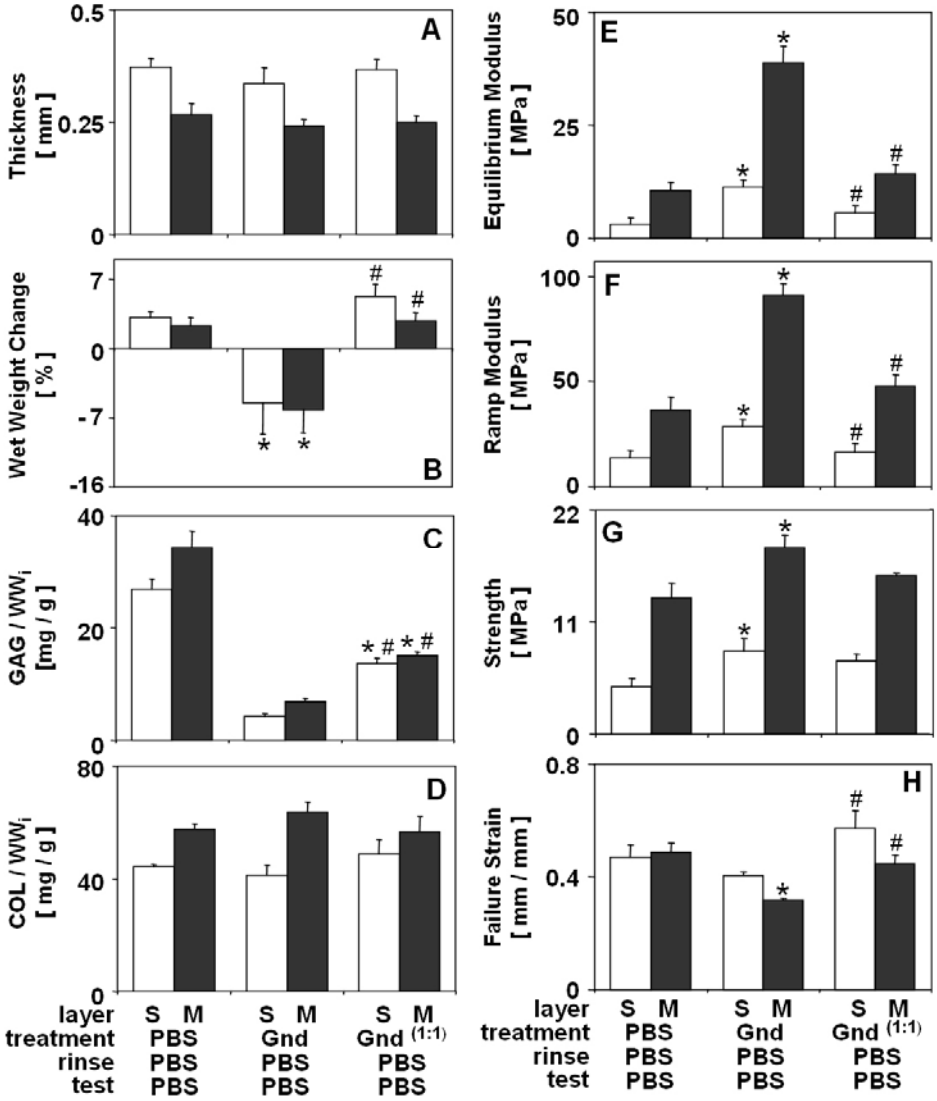

Figure A.3: Effect of partial depletion in guanidine on tissue geometry, composition and tensile mechanical properties of calf articular cartilage explants from the S and M layers. Tissue thickness (**A**) and change in wet weight (**B**) content of GAG (**C**) and collagen (**D)**, equilibrium modulus (**E)**, ramp modulus (**F)** strength (**G**), and failure strain (**H)**. * indicates $p<0.05$ vs. PBS/PBS/PBS control, # indicates $p<0.05$ vs. Gnd/PBS/PBS for a corresponding layer. Data are mean ± SEM, n = 4.

A.4 Discussion

Repletion of cartilage tissue with CS and AGG indicate that certain molecules may be able to enter the cartilage matrix after Gnd depletion. The ability of a molecule to enter such tissue may depend on its molecular size, as larger extents of repletion were obtained with CS, a 60 kDa molecule as compared to AGG, which is ~$2.5*10^6$ Da [4]. While proteoglycan content is an important determinant of transport properties of articular cartilage, as its enzymatic removal greatly increases the diffusivity of low molecular weight molecules within cartilage explants [6], removal of the proteoglycan component may act to effectively shrink the spacing between neighboring collagen fibers, potentially hindering the diffusion of large molecules back into the tissue. Enzymatic removal of proteoglycan in rabbit synovium reduced the extrafibrillar volume fraction within the synovial collagen bundles, accompanied by a reduction in collagen fiber diameter and the intrafibrillar center to center spacing [1]. Similar treatment of cornea, also resulted in a decrease in spacing between collagen molecules [2]. The extent of repletion appeared to depend on the solvent as is demonstrated by larger extents of repletion in PBS than in Gnd. This may be due to changes in the structure of the residual tissue matrix or the in the molecule being repleted in presence of Gnd.

The inability of Gnd+ to replete cartilage tissue matrix taken together with its capacity to prevent cartilage tissue depletion suggests that Gnd may saturate with dissociated proteoglycans at relatively low levels. Indeed, the concentration of GAG in Gnd+ was 6 mg/ml, similar to that in the tissue after it has been extracted with

excess amounts of Gnd (Fig. 1). Incubation in Gnd solution that is saturated with dissociated proteoglycan molecules (i.e. Gnd+) may prevent further dissociation of GAG within the tissue and its subsequent extraction into the surrounding Gnd. Incubation of tissue with Gnd+ containing [^{35}S]sulfate-labeled GAG, prevented cartilage tissue depletion, however scintillation counting analysis did not detect the presence of [^{35}S]sulfate-labeled GAG molecules in the tissue, suggesting that there is not exchange of GAG between tissue and Gnd+. While this indicates that in the presence of Gnd+ GAG molecules are not diffusing out of the tissue, whether tissue GAG undergoes dissociation in such conditions remains to be determined.

The enhanced tensile integrity of immature cartilage after matrix depletion suggests that certain ECM components of immature matrix serve to inhibit collagen network interactions. Partial matrix depletion (~50% of GAG remains in the tissue) with Gnd$^{(1:1)}$, suggests that the molecules that serve to inhibit such interaction are either not depleted or that they are present in excess and are effective at inhibiting the interactions at reduced concentrations. The inability of CS to result in functional repletion of cartilage, as indicated by an enhancement of tensile integrity similar to that produced by matrix depletion with Gnd, suggests that CS alone is ineffective at inhibiting the integrity promoting interactions within the collagen network and that a more complex molecular structure may be required. Unfortunately, repletion with a molecule that may lend such a molecular structure (e.g. aggrecan monomer) did not occur under the conditions described here and thus its effects on mechanical properties could not be investigated at present time in a repletion experiment.

A.5 Acknowledgments

The dissertation author is the primary investigator and thanks Barbara Schumacher for help with preparation of aggrecan monomer, as well as Drs. Koichi Masuda and Robert Sah for their contribution. This work was supported by the Howard Hughes Medical Institute, Musculoskeletal Transplant Foundation, National Institute of Health and National Science Foundation.

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APPENDIX B

CARTILAGE GROWTH AND REMODELING: THE ROLE OF CHONDROCYTE METABOLISM

B.1 Abstract

Objective: To examine the effect of cycloheximide (CH), an inhibitor of cellular biosynthesis, and culture at 4°C, on growth and remodeling of immature articular cartilage *in vitro*. *Design:* Immature bovine articular cartilage explants from the superficial and middle zones were cultured for 13 days in serum-containing medium with or without CH or at 4°C. Variations in tissue size, accumulation of proteoglycan and collagen, and tensile mechanical properties were assessed. *Results:* The inclusion of serum resulted in expansive tissue growth, deposition of glycosaminoglycan (GAG) and collagen, and a diminution of tensile integrity. Supplementation of medium with CH or incubation at 4°C reduced the metabolic activity to a very low level and resulted in maintenance of tissue size, content of GAG and collagen, and a slight enhancement of tensile mechanical integrity. *Conclusion:* Growth and remodeling of cartilage tissue in serum-supplemented culture results from active metabolic processes that are mediated by chondrocytes and not from a passive process of tissue swelling.

B.2 Introduction

The mechanical function of cartilage depends on the molecular composition and structure of the extracellular matrix, particularly the swelling pressure of glycosaminoglycan (GAG) and the tensile stiffness of the collagen network [6]. Physiologic alterations of cartilage function, structure, and composition during growth and maturation depend on the balance between synthesis/assembly and degradation/release of matrix components. Previously, culture of immature cartilage *in vitro* in serum-supplemented medium induced growth, i.e., increases in tissue size and the overall amount of matrix in a tissue sample [12]. However it is unclear whether such tissue expansion occurs as a result of active growth or passive tissue swelling (i.e. addition of solid matrix components rather than water). Cycloheximide (CH) inhibits protein biosynthesis by inhibition of binding and release of transfer RNA from ribosomes [10]. In chondrocytes, CH inhibits protein synthesis, including that of aggrecan core protein, but may allow the addition of chondroitin sulfate chains onto the aggrecan core protein that was synthesized prior to addition of CH [5, 9]. Thus, the present study examined the effect of reduction of cellular metabolism with CH and at 4°C in immature bovine articular cartilage on culture-associated variations in tissue size, deposition of matrix components, and tensile mechanical properties.

B.3 Materials and Methods

Sample Preparation and Culture

Articular cartilage was harvested from the patellofemoral groove of 4 newborn (1-3 weeks) bovine calves, as described previously [12]. Blocks, 9 x 3 mm^2 in area, were prepared using a sledge microtome from three successive layers of cartilage to either include the intact articular surface (superficial layer, ~0.4 mm thick, S) or to include the middle zone (middle layers, 0.25 mm thick, M1 and M2). The long axes of the blocks were in the anterior-posterior direction and, thus, approximately perpendicular to the split line direction. Blocks were weighed wet (WW_i) under sterile condition.

Some blocks were **(a)** analyzed immediately. Other blocks were incubated at 37°C in **(b)** medium (DMEM supplemented with 20% fetal bovine serum (FBS) and 100 μg/mL of ascorbate) or **(c)** medium with 0.5mM cycloheximide (CH) (Sigma, St. Louis, MO) or **(c)** medium at 4°C. Medium was changed every other day, and during the first 12 days of culture supplemented with 4.5 μCi/mL [^{3}H]proline and 1.8 μCi/mL [^{35}S]sulfate. To remove unincorporated isotopes, blocks were washed, transferred to a new culture plate, and incubated for an additional day in medium without radiolabel. Spent medium was collected throughout the culture duration. At termination, blocks were weighed wet (WW_f) and punched to form a tapered tensile test specimen and residual cartilage.

Biochemical Analysis

Residual cartilage and the failed portions of the corresponding tensile strip (see below) were analyzed together to quantify the biochemical composition of the fresh and cultured samples. Samples were lyophilized, weighed dry, and solubilized with proteinase K [12]. Portions of the tissue digest were analyzed to quantify the content of DNA [8], GAG [2], and hydroxyproline [13]. DNA was converted to cell number by using a conversion constant of 7.7 pg of DNA per cell [4]. Hydroxyproline content was converted to COL content by assuming a mass ratio of collagen to hydroxyproline equal to 7.25 [3, 11].

Analysis of Matrix Metabolism

Other portions of the solubilized tissue and portions of the medium were analyzed for the incorporated radioactivity to assess matrix metabolism. The contents of [^{35}S] and [^{3}H] radioactivity were determined in the tissue digest and used to estimate the absolute rates of sulfate and proline incorporation as indices of 12-day average sulfated-GAG and protein synthesis, respectively. To estimate the absolute rate of sulfate release into the medium, a portion of spent medium was analyzed for the content of [^{35}S]GAG using the Alcian Blue precipitation method [7].

Biomechanical Analysis

Biomechanical analysis is described in detail in Chapter 2.

Statistical Analysis

For each layer (S, M1, and M2), the effects of experimental conditions (d0, d13, d13+CH, d13@4°C) were assessed by analysis of variance (ANOVA) with experimental condition as fixed factor and donor animal as a random factor. Tukey post-hoc testing was performed to compare groups. To analyze the effect of day in culture on wet weight, repeated measures ANOVA was performed for each layer and experimental condition with wet weight (WW_i, WW_f) as a repeated factor. Data are expressed as mean ± SEM, and significance level was set to 0.05. Statistical analysis was performed using Systat 10.2 (Systat Software, Inc., Richmond, CA).

B.4 Results

The extent of *in vitro* volumetric growth of articular cartilage blocks was markedly affected by experimental conditions as assessed by changes in thickness ($p<0.001$) (Fig. B.1A), wet weight ($p<0.001$) (Fig. B.1B), and water content (Fig. B.1C). Both the thickness and wet weight increased during culture in medium (42-59%, 38-71%, respectively; $p<0.001$). In contrast, explants incubated with CH or at 4°C did not grow in thickness ($p>0.30$), and consistent with this the change in wet weight of these explants was near zero. The change in wet weight of explants incubated with CH decreased slightly during culture (4-7%, $p<0.01$), while that of explants incubated at 4°C did not change ($p>0.10$). The content of water did not vary with experimental conditions in the S layer ($p=0.60$) but varied in the middle layers ($p<0.05$), decreasing slightly during culture in medium (~2-4%, $p<0.01$), but not during culture with CH or at 4°C ($p>0.22$). Since water content varied only slightly on an absolute basis, changes in wet weight were largely due to changes in tissue volume and not density and, thus, along with changes in thickness, represent volumetric growth of tissue samples.

The content of cells (Cells/WW_i, Fig. B.2A) varied with experimental conditions ($p<0.01$), decreasing slightly during culture with CH (38-45%, $p<0.01$), and, when normalized to WW_f to give a measure of concentration of cells (Cells/WW_f, Fig. B.2D), reflected the changes in Cells/WW_i and in wet weight ($p<0.001$). Cells/WW_f decreased in explants cultured in medium (~42%, $p<0.001$), and medium with CH (~35%, $p<0.01$), but not during culture at 4°C ($p>0.26$).

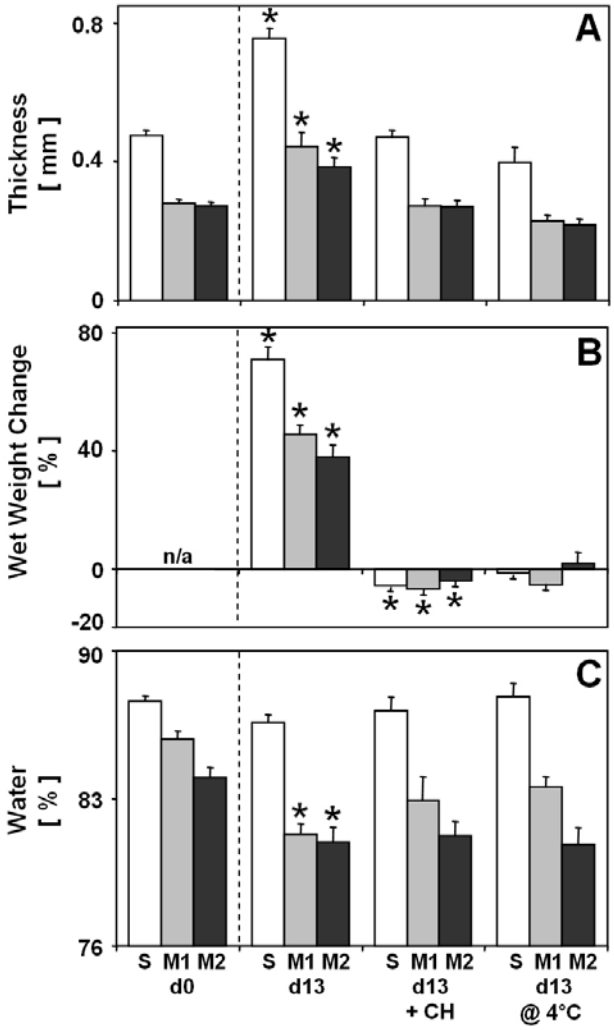

Figure B.1: Effect of experimental conditions on general indices of *in vitro* growth of calf articular cartilage explants from the superficial (S), middle 1 (M1) and middle 2 (M2) layers. Blocks were analyzed on day 0 (d0), or incubated for 13 days (d13) in medium (20% FBS) or medium supplemented with 0.5 mM cycloheximide (CH). (**A**) Thickness, (**B**) change in wet weight, and (**C**) percent water of cartilage blocks. The dotted line separates explants analyzed on day 0. * indicates $p<0.05$ vs. d0 for a corresponding layer. Data are mean ± SEM, n = 8-12 blocks from 4 animals.

The extent of volumetric growth was generally paralleled by variations in the tissue content of GAG and collagen (COL). GAG content (GAG/WW_i, Fig. B.2B) varied with experimental conditions ($p<0.001$), while the content of COL (COL/WW_i Fig 2C) varied only in the S layer ($p<0.001$) and slightly in the M1 layer ($p=0.07$). While GAG/WW_i and COL/WW_i increased during culture in medium (40-69%, and ~15%, respectively; $p<0.09$), they remained unchanged during culture with CH and at 4°C ($p>0.11$).

When normalized to WW_f to give an index of concentration in the tissue, the concentrations of extracellular matrix components reflected changes in the content of the components and in the wet weight of the tissue during culture. The concentration of GAG (GAG/WW_f, Fig. B.2E) did not vary with experimental conditions ($p>0.30$), while the concentration of COL (COL/WW_f, Fig. B.2F) varied in all three layers ($p<0.01$). COL/WW_f decreased during incubation in medium (15-30%, $p<0.05$) but did not change during culture with CH and at 4°C ($p>0.25$).

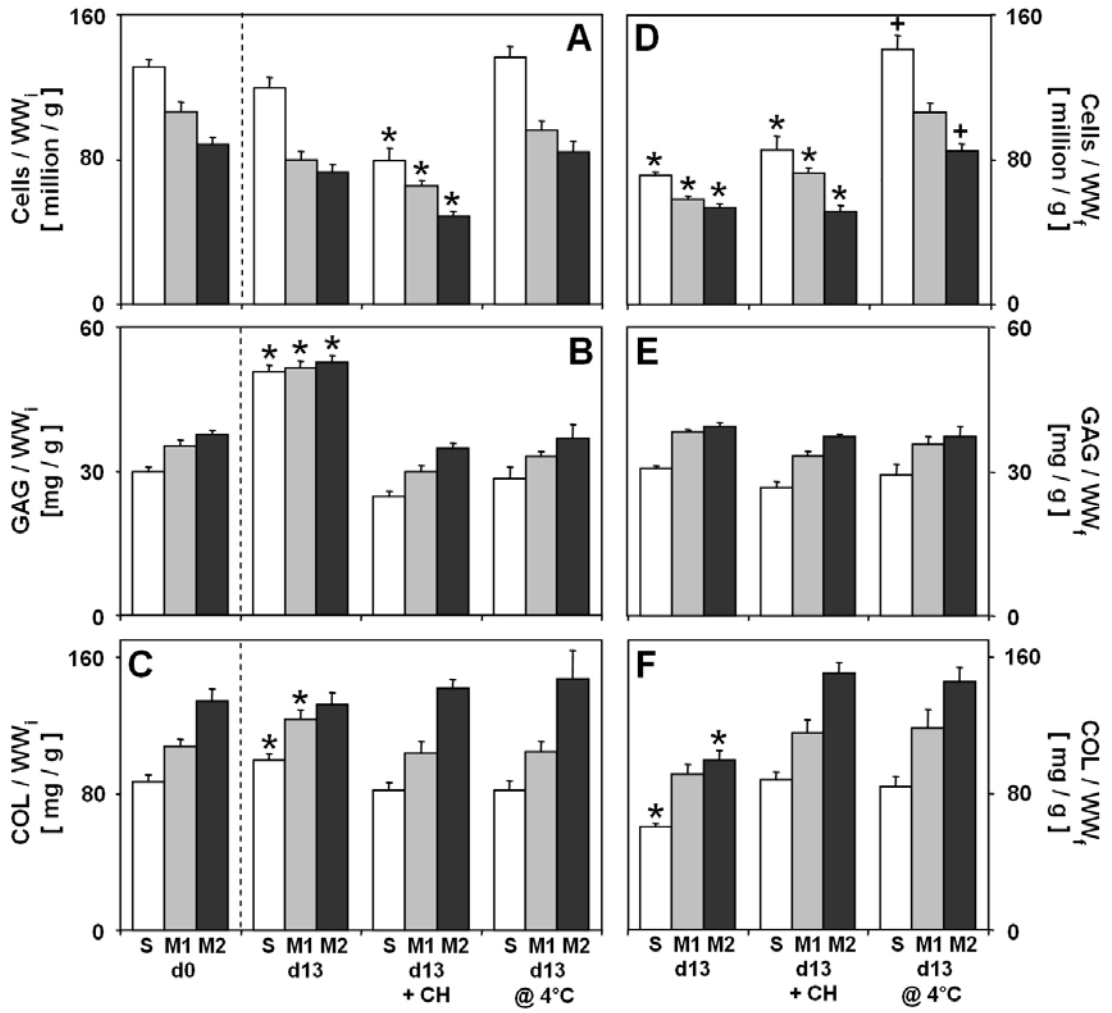

Figure B.2: Effect of experimental conditions on general indices of *in vitro* growth of calf articular cartilage explants from the S, M1, and M2 layers. (**A**, **D**) Cells, (**B**, **E**) glycosaminoglycan (GAG), (**C**, **F**), and collagen (COL) normalized to initial wet weight (WW_i) to represent constituent content (**A**-**C**) and final wet weight (WW_f) to represent constituent concentration (**D**-**F**) The dotted line separates explants analyzed on day 0, where $WW_f = WW_i$. * indicates $p<0.05$ vs. d0 and + indicates $p<0.05$ vs. d13+CH for a corresponding layer. Data are mean ± SEM, n = 8-12 blocks from 4 animals.

The changes in GAG and COL content (Fig. B.2B-C) during culture were generally paralleled by similar variations in sulfate (Fig. B.3A) and proline incorporation (Fig. B.3B), respectively. Both sulfate and proline incorporation into tissue and sulfate loss into medium varied with experimental conditions ($p<0.001$). While serum stimulated incorporation of both sulfate and proline, the addition of CH to culture medium or culture at 4°C reduced incorporation to very low levels (99-100%, $p<0.001$). The release of sulfate into the culture medium was also reduced significantly by the addition of CH to culture medium or culture at 4°C (96-99%, $p<0.001$).

The variations in the material biomechanical properties of cartilage explants paralleled the statistical variations and accentuated the trends in the structural biomechanical properties. Both ramp modulus (Fig. B.5C) and strength (Fig. B.5D) decreased during incubation in medium (40-57%, 48-57%, respectively; $p<0.05$). During culture with CH both ramp modulus and strength increased in the S and M1 layers (65%, 38%, respectively; $p<0.05$), but remained unaltered in the M2 layer ($p>0.36$). During culture at 4°C, ramp modulus increased (60-91%, $p<0.05$) in all three layers and strength increased in only in M1 layer (45%, $p<0.05$). Generally, both ramp modulus and strength were similar for explant culture with CH and those culture at 4°C ($p>0.18$). Failure strain (Fig. B.5E) did not vary with experimental conditions in the S and M1 layers ($p>0.75$) but varied in the M2 layer ($p<0.001$). Failure strain increased slightly (27%, $p<0.01$) in explants incubated in medium.

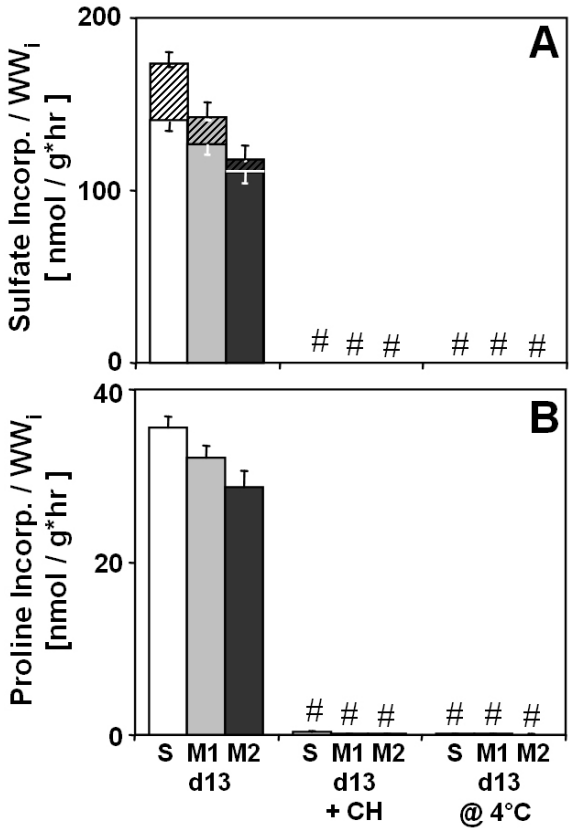

Figure B.3: Effect of experimental conditions on sulfate and proline incorporation of calf articular cartilage explants from the S, M1, and M2 layers. Content of (**A**) sulfate and (**B**) proline incorporation in the tissue (solid) and released into the medium (striped). Total sulfate incorporation is represented by the overall bar height and upward error bar. # indicates $p<0.05$ vs. d13 for a corresponding layer. Data are mean ± SEM, n = 8-12 blocks from 4 animals.

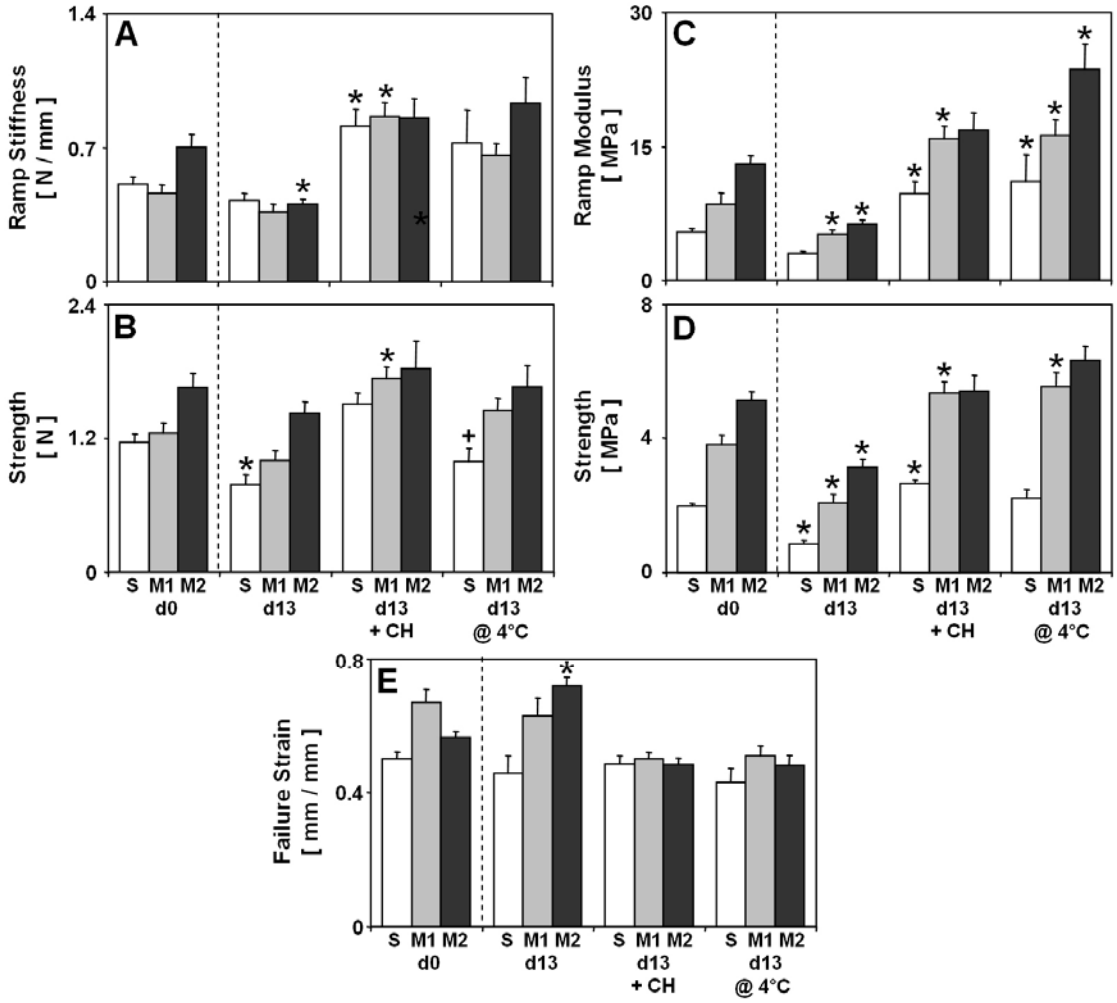

Figure B.4: Effect of experimental conditions on structural and material tensile mechanical properties of calf articular cartilage explants from the S, M1, and M2 layers. Structural properties: (**A**) Ramp modulus, and (**B**) strength. Material properties: (**C**) ramp modulus, (**D)** strength, and (**E**) failure strain. The dotted line separates explants analyzed on day 0. * indicates $p<0.05$ vs. d0 and + indicates $p<0.05$ vs. d13+CH for a corresponding layer. Data are mean ± SEM, n = 8-12 blocks from 4 animals.

B.5 Discussion

The data presented here demonstrate that inhibition of protein synthesis can abolish volumetric growth in explants of immature bovine articular cartilage, as indicated by changes in tissue size, but does not fully inhibit matrix remodeling, as indicated by the change in the integrity of the collagen network. Incubation with 20% FBS resulted in *expansive cartilage growth* characterized by a marked increase in tissue volume (Fig. B.1A-B) and the content of GAG (Fig. B.2B) and a slight increase in the contents of COL (Fig. B.2C). The result was a maintenance of the concentration of GAG (Fig. B.2E), reduction in the concentration of COL (Fig. B.2F), and a concomitant diminution in tensile mechanical integrity (Fig. B.4). Addition of CH or culture at 4°C reduced the rates of GAG and collagen synthesis to very low levels, resulting in maintenance of tissue size (Fig. B.1A-B) and biochemical composition (Fig. B.2), but a slight enhancement of tensile mechanical integrity (Fig. B.4).

The indices of metabolism of GAG and collagen were consistent with their role in determining the volumetric growth of cartilage tissue. Cartilage explants grew in volume as the content of GAG in the tissue, an indicator of swelling pressure, increased and as the tensile modulus and strength, indicators of the integrity of the collagen network, decreased, and did not grow when metabolism of these components was inhibited. Thus, cartilage tissue growth appears to result from active metabolic processes that are mediated by chondrocytes and not from a passive process of tissue swelling. Consequently, deposition of matrix components is a major element in modulating the volumetric growth of cartilage tissue.

Although the conditions of incubation with CH are slightly different from those at 4°C, the size, composition and biomechanical function of both types of explants on day 13 day were generally similar. CH inhibits the synthesis of proteins but since the culture is carried out at 37°C, the enzymes present in the cartilage matrix prior to the addition of CH are, presumably, still active. In contrast, incubation at 4°C slows down both the cellular metabolism as well as the activity of various enzymes of the extracellular matrix. The major difference between the CH and 4°C conditions was a reduced cellularity after incubation with CH, but not at 4°C. This could be due to chondrocyte death and destruction by matrix proteases during prolonged state of inhibited biosynthesis and active matrix enzymes.

The extents of matrix remodeling were similar during incubation with CH and at 4°C, as indicated by the slight enhancement of mechanical properties, and could be due to activity of certain matrix enzymes that reside in the tissue matrix (i.e. matrix metabolism). Certain sites of cleavage in the collagen network (such as that of MMP-3) can potentially initialize collagen fibril depolymerization which may allow reorganization and remodeling of the collagen network [1]. While such enzymes are less active at 37°C than at 4°C, 13 days of incubation at 4°C may be long enough for some remodeling events to occur. Shorter culture durations may be more sensitive for detection of matrix remodeling mediated by matrix metabolism. Thus, cartilage tissue remodeling can result from processes mediated by matrix metabolism in the absence of chondrocyte metabolism.

B.6 Acknowledgments

The dissertation author is the primary investigator and thanks co-author, Dr. Robert Sah, for his contribution. This work was supported by National Institute of Health and National Science Foundation.

B.7 References

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APPENDIX C

SUPPLEMENTAL MATERIAL FOR CHAPTER 2

C.1 Materials and Methods

Detailed description of materials and methods can be found in Chapter 2. The following describes additional methodology not included in the chapter.

Proteoglycan 4 (PRG4) analysis

PRG4 expression was quantified by enzyme-linked immunosorbent assay (ELISA) of spent medium. Spent medium from the cultures was quantitatively analyzed in duplicate for PRG4 by indirect ELISA, as previously described [1], using mouse monoclonal antibody (mAb) 3A4 (a generous gift from Dr. Bruce Caterson, University of Wales, Cardiff, UK) [3]. Briefly, samples were diluted serially, adsorbed, and then reacted with mAb 3A4, horseradish peroxidase–conjugated secondary antibody, and ABTS substrate, with 3 washes with PBS–0.1% Tween (Bio-

Rad, Hercules, CA) between each step. PRG4 levels were calculated using a PRG4 standard that was purified by isopyknic CsCl density-gradient ultracentrifugation and ion-exchange chromatography on DEAE-Sephacel [2], verified for purity by Western blot analysis, and quantified by bicinchoninic acid protein assay (Pierce, Rockford, IL). An appropriate diluent was used so that the slopes of the control and sample absorbance curves were equivalent in the linear range of the sigmoidal curve. Secretion rates were normalized to the surface area of the superficial surface on day 0 as calculated by dividing WWi by the average thickness of d0 group.

C.2 Results

The results are reported in Chapter 2, data in the form of figures is provided here as a supplemental material.

C.3 Acknowledgments

The dissertation author is the primary investigator and thanks Dr. Tannin Schmidt and Jennifer Antonacci for PRG4 secretion analysis, as well as co-authors, Drs. Koichi Masuda, Eugene Thonar, Stephen Klisch, and Robert Sah for their contribution. This work was supported by National Institute of Health, National Science Foundation, and National Aeronautics and Space Administration.

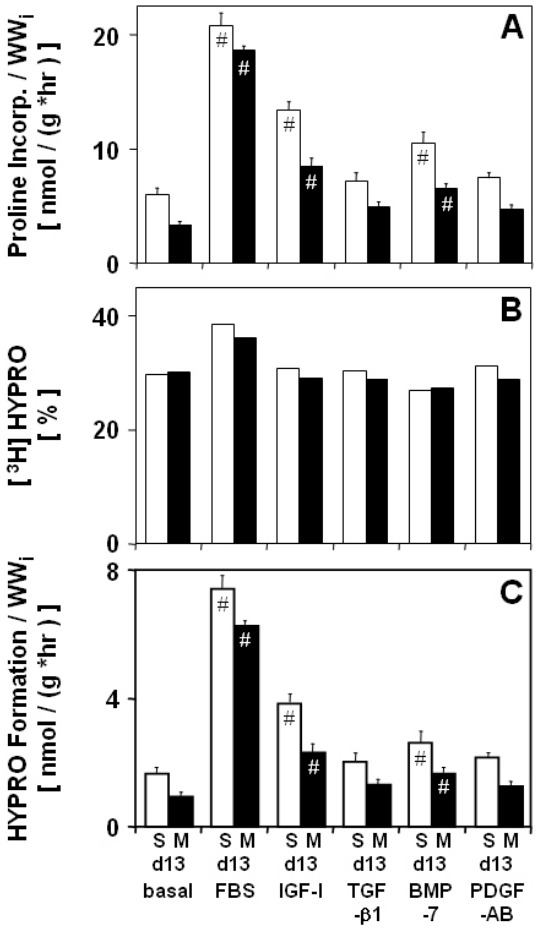

Figure C.1: Effect of experimental conditions on proline incorporation and hydroxyproline (HYPRO) formation of calf articular cartilage explants from the S and M layers. Content of (**A**) proline incorporation and (**C**) HYPRO formation in the tissue. (**B**) Percent of [^{3}H]HYPRO in the tissue. # indicates $p<0.05$ vs. basal condition for a corresponding layer. Data are mean ± SEM, n = 8-9 blocks from 3 animals.

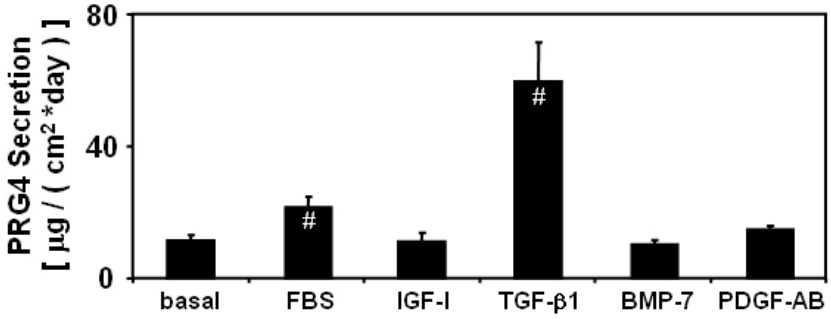

Figure C.2: Effect of experimental conditions on secretion rate of PRG4 by calf articular cartilage explants from the S layer. # indicates $p<0.05$ vs. basal condition. Data are mean ± SEM, n = 8-9 blocks from 3 animals.

C.4 References

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APPENDIX D

SUPPLEMENTAL MATERIAL FOR CHAPTER 3

D.1 Introduction

Because various zones of normal articular cartilage exhibit differences in biochemical composition and mechanical properties, three successive ~0.4 mm layers of cartilage, with the first being superficial (S) and including the intact articular surface and the next two being from the middle layers (M1, M2) were studied in Chapter 3. However, for brevity and clarity, only data from the S and M2 (defined as M in Chapter 3) layers was shown. The data for M1 layer, along that for S and M2 layers to allow comparison, is now presented as supplemental information.

D.2 Materials and Methods

Detailed description of materials and methods can be found in Chapter 3. The following describes deviations from the description in Chapter 3.

Sample Preparation and Culture

Articular cartilage blocks were prepared using a sledge microtome from three successive ~0.4 mm layers of cartilage, with the first being superficial (S) and including the intact articular surface and the next two being from the middle layers (M1, M2).

D.3 Results

In the M1 layer, the data paralleled those observed in the M2 layer, defined as M in Chapter 3.

D.4 Acknowledgments

The dissertation author is the primary investigator and thanks co-authors, Drs. Koichi Masuda, Eugene Thonar, Stephen Klisch, and Robert Sah for their contribution. This work was supported by National Institute of Health and National Science Foundation.

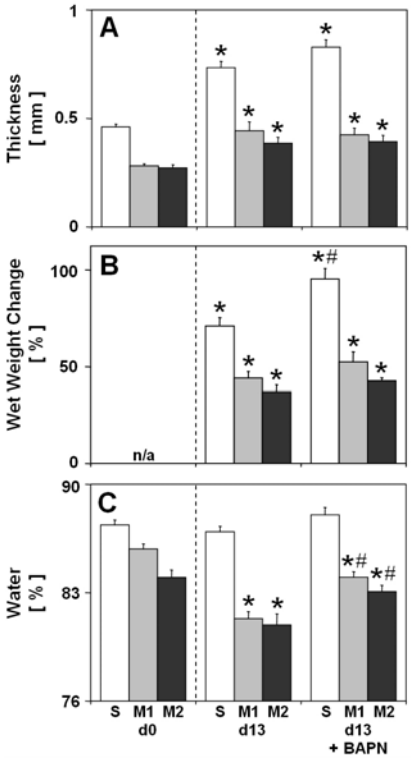

Figure D.1: Effect of experimental conditions on general indices of *in vitro* growth of calf articular cartilage explants from the superficial (S), middle 1 (M1), and middle 2 (M2) layers. Blocks were analyzed on day 0 (d0), or incubated for 13 days (d13) in medium (20% FBS) or medium supplemented with 0.1mM β-aminopropionitrile (BAPN). (**A**) Thickness, (**B**) change in wet weight, and (**C**) percent water of cartilage blocks. The dotted line separates explants analyzed on day 0. * indicates $p<0.05$ vs. d0, # indicates $p<0.05$ vs. d13 for a corresponding layer. Data are mean ± SEM, n = 10-20 blocks from 5 animals.

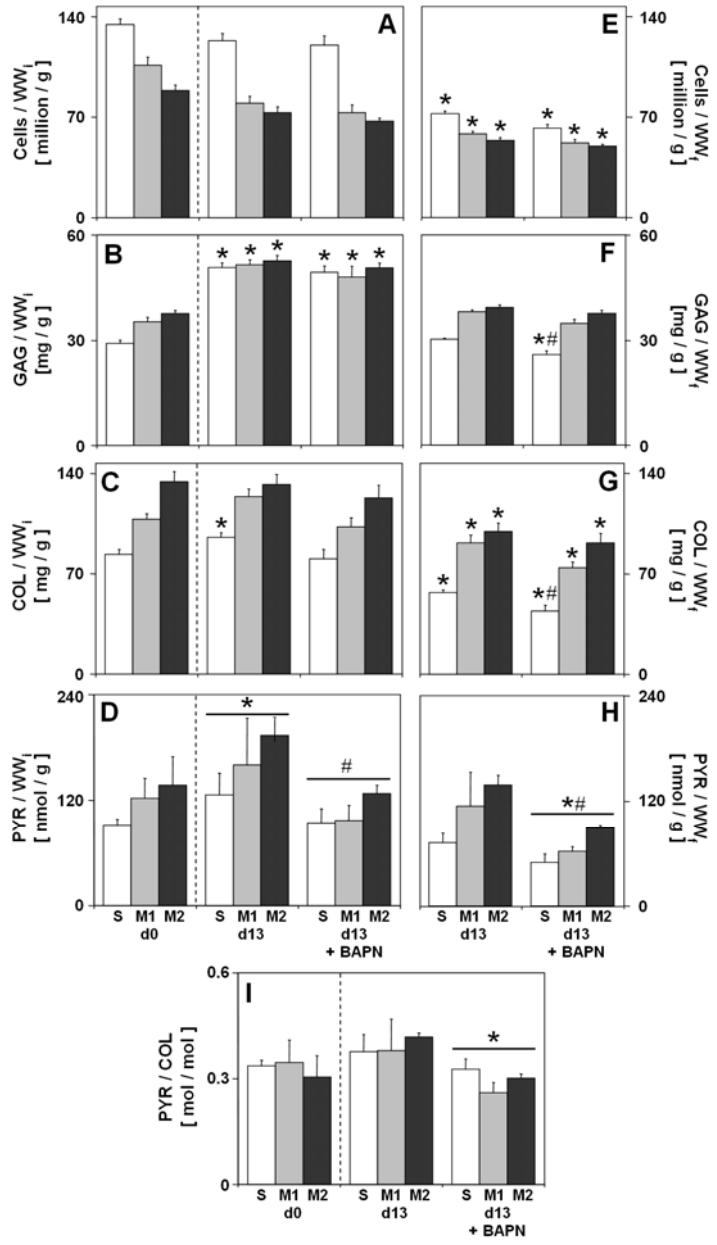

Figure D.2: Effect of experimental conditions on biochemical composition of calf articular cartilage explants from the S, M1, and M2 layers. (**A**, **E**) Cells, (**B**, **F**) glycosaminoglycan (GAG), (**C**, **G**) collagen (COL), and pyridinoline crosslink (PYR) (**D**, **H**) normalized to initial wet weight (WW_i) to represent constituent content (**A**-**D**) and final wet weight (WW_f) to represent constituent concentration (**E**-**H**). (**I**) Molar ratio of PYR to COL. The dotted line separates explants analyzed on day 0, where $WW_f = WW_i$. * indicates $p<0.05$ vs. d0, # indicates $p<0.05$ vs. d13 for a corresponding layer, while ____ indicates where all three layers were analyzed together. Data are mean ± SEM, n = 10-20 blocks from 5 animals.

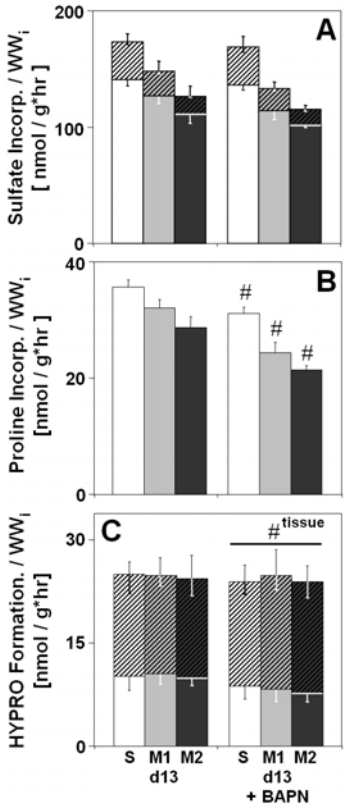

Figure D.3: Effect of experimental conditions on sulfate and proline incorporation and hydroxyproline (HYPRO) formation of calf articular cartilage explants from the S, M1, and M2 layers. Content of (**A**) sulfate and (**B**) proline incorporation, and (**C**) HYPRO formation in the tissue (blank) and released into the medium, where applicable, (striped). Total sulfate incorporation and HYPRO formation is represented by the overall bar height and upward error bar. . # indicates $p<0.05$ vs. d13 for a corresponding layer, while ____ indicates where all three layers were analyzed together. Data are mean ± SEM, n = 10-20 blocks from 5 animals.

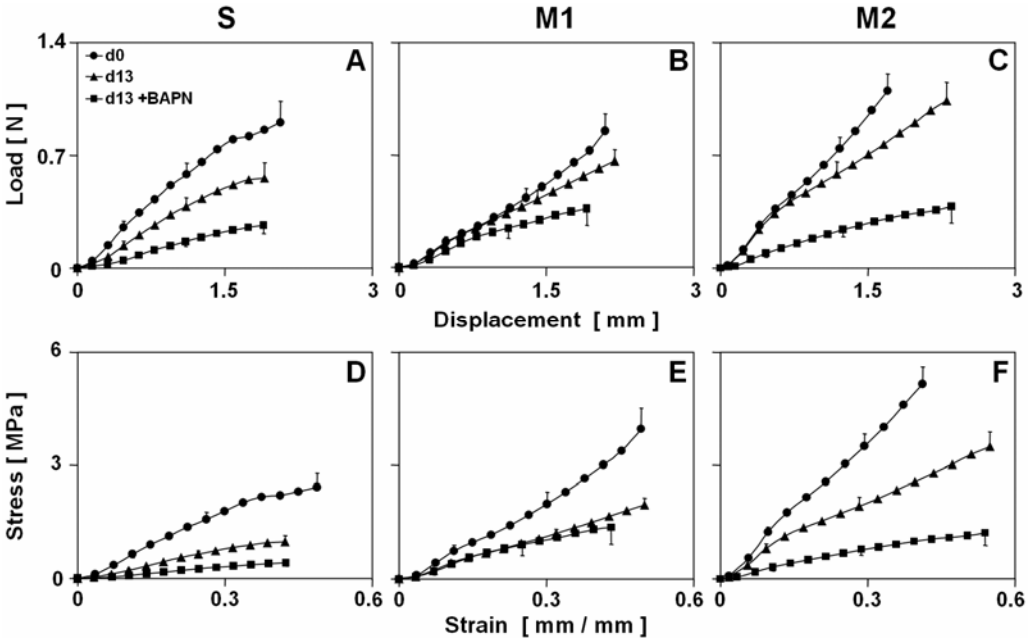

Figure D.4: Effect of experimental conditions on growth associated changes in structural and material tensile mechanical behavior of calf articular cartilage explants from S (**A** and **D**), M1 (**B** and **E**), and M2 (**C** and **F**) layers. Average load-displacement (**A**-**C**), and stress-strain (**D**-**F**), profiles preceding the failure stress of the weakest sample within a group are displayed for blocks that were analyzed on day 0 (d0) (●) and on day 13 (d13) after incubation in medium (▲) or medium supplemented with 0.1mM β-aminopropionitrile (BAPN) (■). Values are the mean ± SEM at selected points, n = 10-20 blocks from 5 animals.

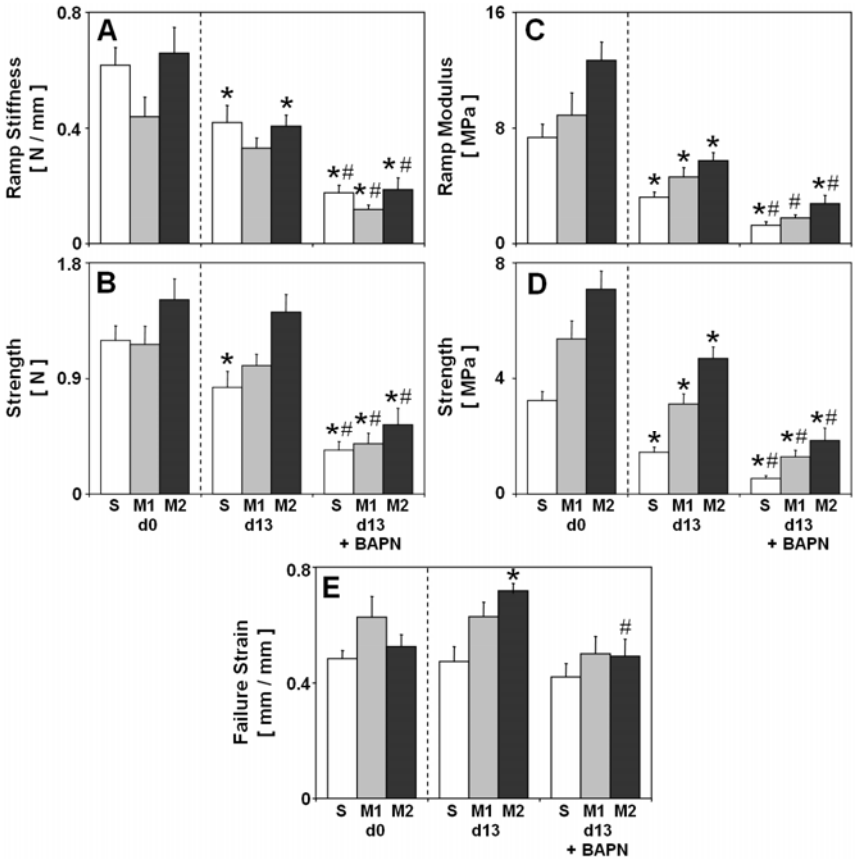

Figure D.5: Effect of experimental conditions on structural and material tensile mechanical properties of calf articular cartilage explants from the S, M1, and M2 layers. Structural properties: (**A**) Ramp modulus, and (**B**) strength. Material properties: (**C**) ramp modulus, (**D)** strength, and (**E**) failure strain. The dotted line separates explants analyzed on day 0. * indicates $p<0.05$ vs. d0, # indicates $p<0.05$ vs. d13 for a corresponding layer. Data are mean ± SEM, n = 10-20 blocks from 5 animals.

APPENDIX E

SUPPLEMENTAL MATERIAL FOR CHAPTER 4

E.1 Introduction

Because various zones of normal articular cartilage exhibit differences in biochemical composition and mechanical properties, three successive ~0.4 mm layers of cartilage, with the first being superficial (S) and including the intact articular surface and the next two being from the middle layers (M1, M2) were studied in Chapter 3. However, for brevity and clarity, only data from the S and M2 (defined as M in Chapter 3) layers was shown. The data for M1 layer, along that for S and M2 layers to allow comparison, is now presented as supplemental information.

E.2 Materials and Methods

Detailed description of materials and methods can be found in Chapter 4. The following describes deviations from the description in Chapter 4.

Sample Preparation and Culture

Articular cartilage blocks were prepared using a sledge microtome from three successive ~0.4 mm layers of cartilage, with the first being superficial (S) and including the intact articular surface and the next two being from the middle layers (M1, M2).

E.3 Results

In the M1 layer, the data paralleled those observed in the M2 layer, defined as M in Chapter 4.

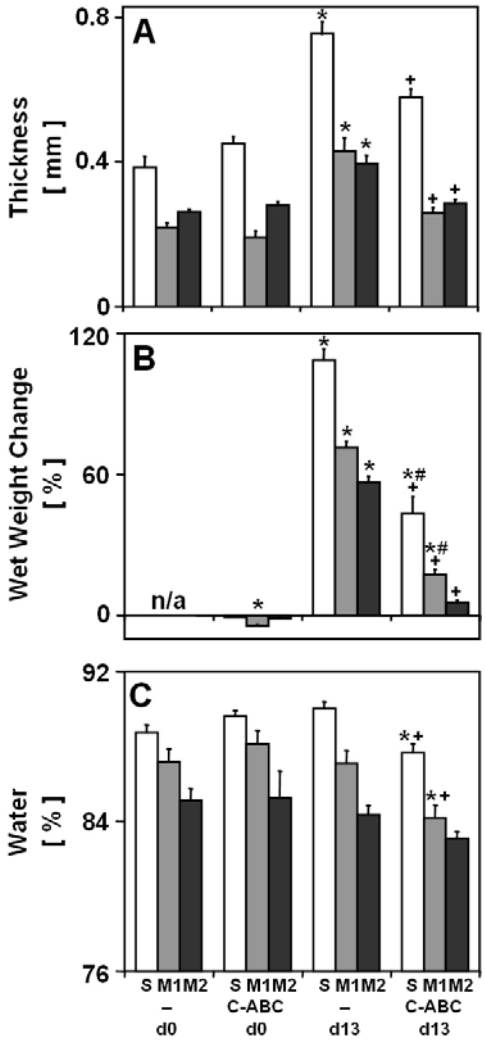

Figure E.1: Effect of chondroitinase ABC (C-ABC) pre-treatment on general indices of *in vitro* growth of calf articular cartilage explants from the superficial (S), middle 1 (M1), and middle 2 (M2) layers. On the day of explantation some blocks were treated with C-ABC. Blocks were either analyzed on day 0 (d0), or incubated for 13 days in medium (d13). (**A**) Thickness, (**B**) change in wet weight, and (**C**) percent water of cartilage blocks. Data are mean ± SEM, n = 6-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. d0 C-ABC, + = $p<0.05$ vs. d13.

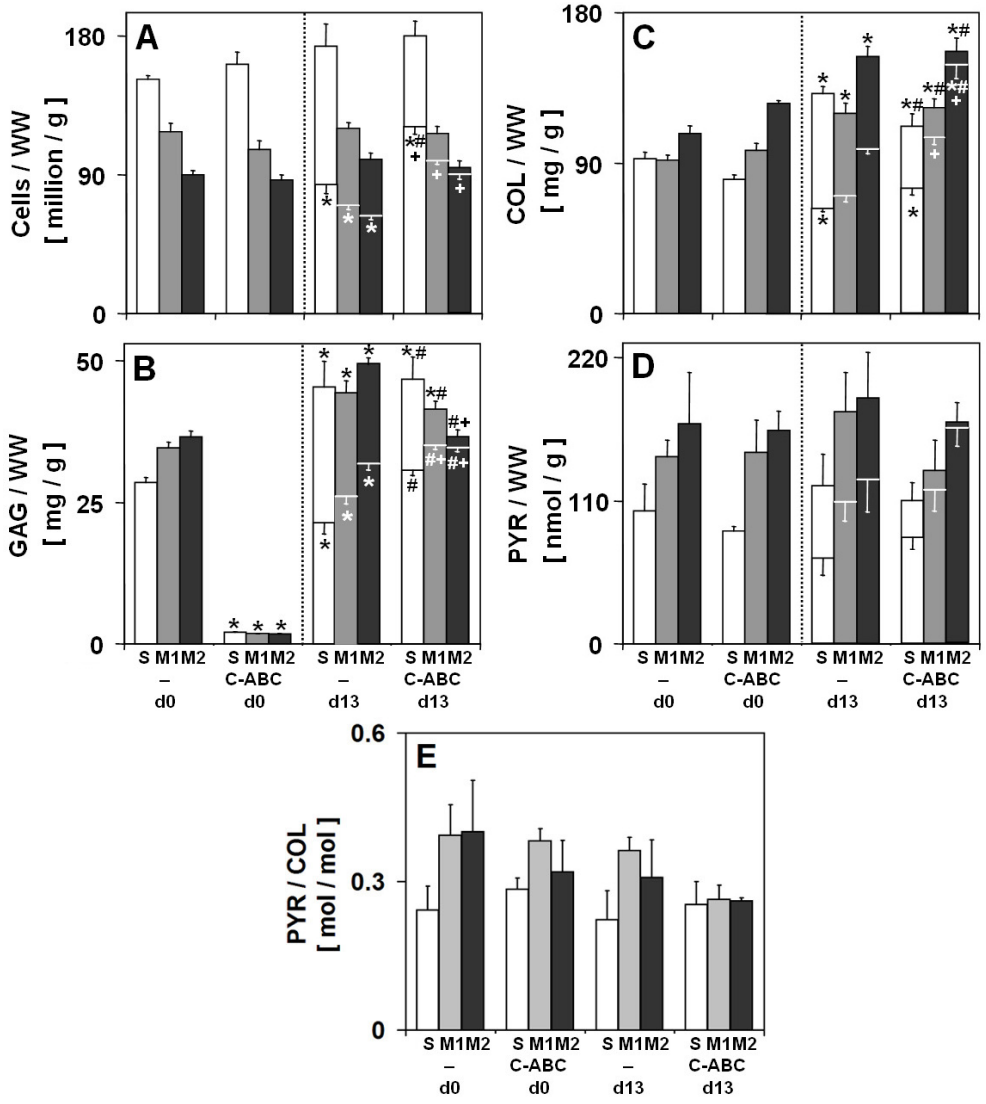

Figure E.2: Effect of C-ABC pre-treatment on changes in the biochemical composition of calf articular cartilage explants from S, M1, and M2 layers. (**A**) Cells, (**B**) glycosaminoglycan (GAG), (**C**) collagen (COL), and (**D**) pyridinoline cross-link (PYR). To the left of the dotted line data are expressed per initial wet weight (WW_i) since for these explants final wet weight (WW_f) equals or is similar to WW_i. To the right of the dotted line data are expressed per WW_i (bar with an upward error bar) to represent constituent content and WW_f (bar with a downward error bar) to represent constituent concentration. (**E**) Molar ratio of PYR to COL. Data are mean ± SEM, n = 6-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. d0 C-ABC, + = $p<0.05$ vs. d13.

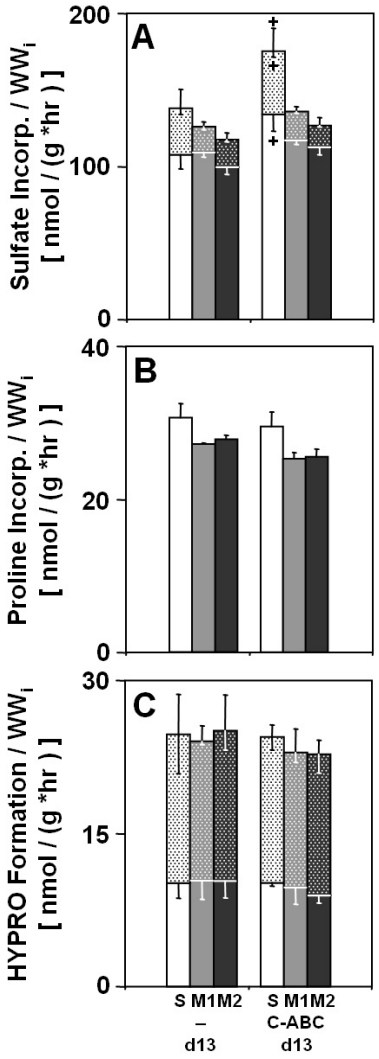

Figure E.3: Effect of C-ABC pre-treatment on sulfate and proline incorporation and hydroxyproline (HYPRO) formation of calf articular cartilage explants from the S, M1, and M2 layers. Content of (**A**) sulfate and (**B**) proline incorporation, and (**C**) HYPRO formation in the tissue (blank) and released into the medium, where applicable, (dotted). Total sulfate incorporation and HYPRO formation is represented by the overall bar height and upward error bar. Data are mean ± SEM, n = 6-9 blocks from 3 animals. + = $p<0.05$ vs. d13.

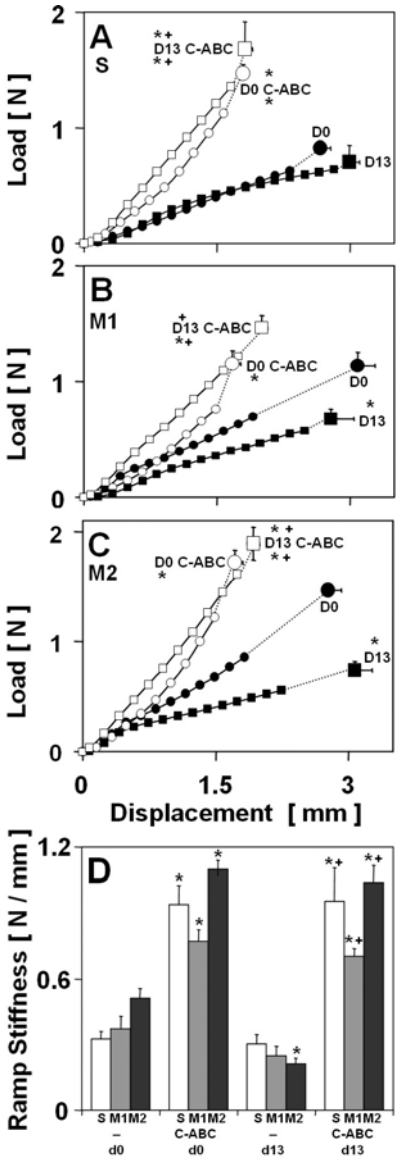

Figure E.4: Effect of C-ABC pre-treatment on changes in structural tensile mechanical behavior and properties of calf articular cartilage explants from S (**A**, **D**), M1 (**B**, **D**), and M2 (**C**, **D**) layers. (**D**) Ramp stiffness, (**A**-**C**) strength and (**A**-**C**) displacement at failure of cartilage blocks. Average load-displacement profiles preceding the failure strength of the weakest sample within a group are displayed (**A**-**C**) for C-ABC-treated (white) and untreated (black) explants, which were analyzed on day 0 (circles) or on day 13 (squares). The load-displacement profiles are then extended (dotted line) until the average maximum strength and failure displacement for each group (larger symbol). Data are mean ± SEM, n = 6-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. d0 C-ABC, + = $p<0.05$ vs. d13 for strength (above name) and displacement at failure (below name).

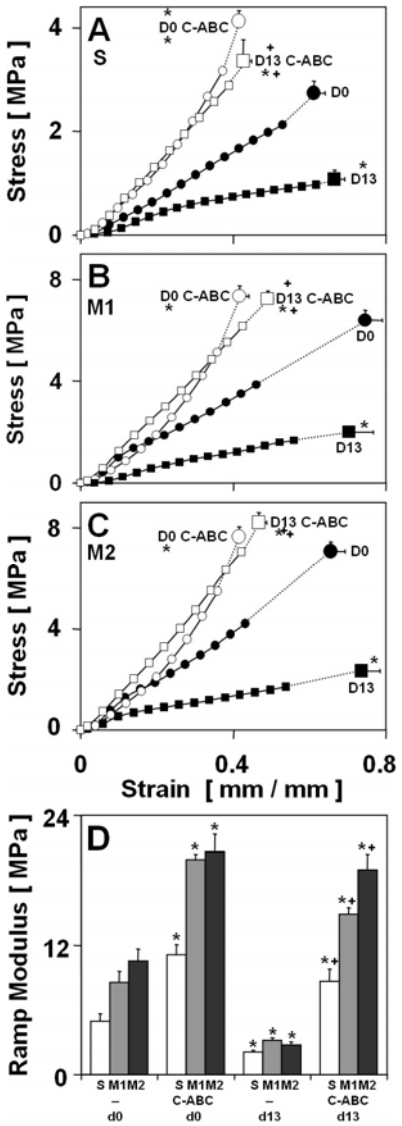

Figure E.5 Effect of C-ABC pre-treatment on changes in material tensile mechanical behavior and properties of calf articular cartilage explants from S (**A**, **D**), M1 (**B**, **D**), and M2 (**C**, **D**) layers. (**D**) Ramp modulus, (**A**-**C**) strength and (**A**-**C**) strain at failure of cartilage blocks. Average stress-strain profiles preceding the failure stress of the weakest sample within a group are displayed (**A**-**C**) for C-ABC-treated (white) and untreated (black) explants, which were analyzed on day 0 (circles) or on day 13 (squares). The stress-strain profiles are then extended (dotted line) until the average maximum strength and failure strain for each group (larger symbol). For each experimental group, data are mean ± SEM, n = 6-9 blocks from 3 animals. Data are mean ± SEM, n = 6-9 blocks from 3 animals. * = $p<0.05$ vs. d0, # = $p<0.05$ vs. d0 C-ABC, + = $p<0.05$ vs. d13 for strength (above name) and strain at failure (below name).

E.4 Discussion

Details of the tensile load-displacement response and the deduced stress-strain response of cartilage reveal the complexities of the interactions between matrix components in the region of small tensile strain [1, 4, 6]. Removal of the proteoglycan component from the cartilage matrix increases tissue permeability [5] and reduces the resistance to collagen fiber movement. These processes appear to accelerate collagen fiber alignment along the direction of principal stress [7] and may be responsible for the apparent tissue softening of GAG-depleted cartilage in the region of small strain in both equilibrium and kinetic response to tension [2, 3, 8]. Indeed, such softening, as demonstrated by a reduction of the initial slope of the stress-strain curve after enzymatic treatment, is accentuated in specimens where larger extents of collagen fiber alignment will occur, such as that in samples tested perpendicular to the collagen fiber orientation as compared to the parallel orientation [2], or in the middle layers, where the orientation of collagen fibers is more random, as compared to the superficial layer (Fig. E.5B-C).

E.5 Acknowledgments

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