Development of a Collagen-Glycosaminoglycan Analog of Extracellular Matrix to Facilitate Articular Cartilage Regeneration

by

Howard Alan Breinan

B.S. Materials Science and Engineering
Stanford University, 1990

M.S. Materials Science and Engineering
Stanford University, 1991

Submitted to the Division of Health Sciences and Technology in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Medical Engineering at the Massachusetts Institute of Technology

June 1998

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Signature of Author: Division of Health Sciences and Technology

May 29, 1998

Certified by: Myron Spector, Ph.D.
Lecturer in Health Sciences and Technology
Thesis Supervisor

Accepted by: Martha L. Gray, Ph.D.
J. W. Kieckhefer Associate Professor of Electrical Engineering
Co-Director, Division of Health Sciences and Technology

JUL 08 1998
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ABSTRACT

An investigation was performed in vitro and in an animal model toward the development of a collagen-glycosaminoglycan analog of extracellular matrix to facilitate articular cartilage regeneration in vivo.

Highly porous, sponge-like matrices were fabricated via a freeze-drying process from 1) type I collagen and chondroitin 6-sulfate, and 2) reconstituted porcine hyaline cartilage extracellular matrix (containing predominantly type II collagen). Specimens of varying pore diameter, degree of cross-linking, and collagen type were seeded with adult canine chondrocytes and cultured for up to six weeks. The phenotype and proliferative and biosynthetic activity of the cells were assessed by histology, immunohistochemistry, DNA and glycosaminoglycan analyses, and radiolabeled sulfate incorporation. Certain characteristics of the matrix were found to affect the chondrocyte distribution in the cell-seeded construct or the behavior of the chondrocytes.

A canine model of cartilage injury was developed and the baseline healing of untreated full-thickness chondral defects was established. Distinct phases of healing in this model were identified and described. The model was used to test the regenerative effects of relevant clinical treatments (including microfracture and autologous chondrocyte transplantation) and a specific porous type II collagen matrix. A quantitative histomorphometric method of evaluating the contribution to healing of distinct tissue types was developed and employed. The various treatments yielded different amounts of reparative tissue and percentages of specific tissue types after 15 weeks. Autologous chondrocyte transplantation resulted in the most hyaline cartilage, but only approximately 50% filling of the defect; by one year, this reparative tissue displayed degenerative changes. Treatments with collagen-
glycosaminoglycan matrices resulted in greater filling at 15 weeks (up to approximately 90%), but predominantly with fibrocartilage. Long-term follow-up will be required to assess the fate of this tissue.

Thesis Supervisor: Myron Spector

Title: Lecturer in Health Sciences and Technology,
       Professor of Orthopedic Surgery (Biomaterials), Harvard Medical School
Acknowledgments

I would like to apologize in advance for not acknowledging all of the details of the contributions that made this work possible. I hope that this reflects the incredible amount of help I received in completing this work.

I must first thank Dr. Spector, for leading me through my thesis work from beginning to end. Without his guidance and support through times of doubt I could not have completed this experience and learned so much.

Equally important has been my fiancee, Beth, who patiently agreed to support me through the most difficult times.

I sincerely thank my committee, Professors Yannas, Grodzinsky, Griffith, and Thomas, for their regular advice and guidance.

I cannot do justice to the many valuable contributions were made by members of the Orthopedic Research Laboratory at Brigham and Women’s Hospital where the bulk of this work was performed. I must first thank Sonya Shortkroff for being a constant resource in learning techniques, designing experiments, and handling laboratory issues, and Dr. Hsu for working so diligently on the animal studies. To Tom, Arun, Stefan, and Stefan, the students and fellows who worked closely with me on cartilage projects, thank you for providing company during many long hours in the lab, helping develop ideas and laboratory techniques, and sharing in the labor of surgery, culture, and analysis. For their help with histology and other assays, I thank Sandra, Gretchen, Christina, George, and Pam-- I couldn’t have done it without you. I also extend gratitude to Drs. Glowacki and Mizuno, Wei, Qi, Thomas, and Mosi for taking time from their research to make contributions to this work, to Cassandra for helping with grading scale work, and to Aamir and Brady for their work as undergraduate researchers in the lab. Finally, thanks to Karen, Atha, Henry, Patricia, Jean, Michelle, Pam, and Manisha, for keeping the lab going and helping with all of the peripheral but necessary aspects of the scientific work.

In the Brigham department of Orthopedic Surgery, I must thank Dr.’s Sledge and Minas for their contributions to work with the animal model and expanding my knowledge of the clinical aspects of this work. To Dr. Scott Martin, I owe these same thanks and more for his support of other academic endeavors and personal development.

To everyone at the Fibers and Polymers lab (Libby, Lila, Diane, Mark, Bernie, Donna, Toby, Debbie, and Gema), thank you for the help with work with the collagen matrices and for providing plenty of smiles during my regular trips to building 3.
I would like to further acknowledge those from other laboratories and institutions for their participation in this work: the animal research staffs at the West Roxbury and Jamaica Plain VA Hospitals; Genzyme Tissue Repair for financial support and helping design a significant portion of the animal work; Ron Bosch, Bob Lew, Betsy Meyer and Sara Wilson for providing support with statistics; and Dr. Andrew Rosenberg for providing advice on cartilage grading.

And finally, I would like to thank the Division of Health Sciences and Technology, the Department of Defense, the Veterans Administration, the Brigham Orthopedic Foundation, and Asa Davis with the Biokinetix Foundation for their additional financial support of this work.
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1 Introduction

1.1 Statement of the Problem

When injured, articular cartilage has limited capacity for self repair. A method of regenerating articular cartilage to resurface damaged joints would be of great clinical value.

1.2 Clinical Significance

Articular cartilage is a specialized connective tissue covering the ends of bones in synovial joints. The articular surface is responsible for transmitting loads across joints and for providing a very low-friction bearing surface, both of which are critical to normal joint function. Failure of the articular surface may lead directly to pain or dysfunction or to inflammation of the joint and damage to the underlying bone that will eventually necessitate joint replacement.

Articular cartilage is susceptible to both traumatic and degenerative injury, and to disease. These processes may be closely related, with incidents of trauma or degeneration inciting or accelerating a disease process. Traumatic injuries such as tearing of the cartilage commonly occur in sports injuries, accidents, and other situations where joints are subjected to unusual or severe loading. Traumatic injuries may affect only the cartilage (chondral fractures or lesions), or both the cartilage and underlying subchondral bone (osteochondral fractures). Such lesions can vary significantly in size, location, and complexity, including whether or not the opposing load-bearing surface of the joint is also involved.

The most common primary disease of articular cartilage is osteoarthrosis (also called degenerative joint disease or, improperly, osteoarthritis). This disease, which can also occur idiopathically or secondary to traumatic injury or other abnormalities, may begin with subtle changes in the biochemical composition of the cartilage, but eventually leads to mechanical breakdown of cartilage, damage to the underlying bone, and loss of the entire articular surface. In other situations, osteoarthrosis may cause isolated lesions. In another disorder of the cartilage, osteochondritis dissecans, the cartilage separates cleanly from the underlying bone, which is often damaged. Cartilage may also be damaged by diseases affecting other parts of the joint. For example, in rheumatoid arthritis, inflammation in the synovium of the joint can impair cartilage function, while avascular necrosis may destroy cartilage due to pathology arising from the underlying bone.

Regardless of the cause, damage to the cartilage is likely to cause pain and dysfunction to the patient. Damage to the upper extremity can result in inability to perform routine daily tasks, while damage to the lower extremity
can severely hamper mobility or activity level. Pain, then dysfunction, are the basis for intervention.

If successfully developed, a collagen-GAG matrix, possibly seeded with cells, might be used for the following types of lesions, all of which are localized to some extent within a joint:

- isolated, small chondral lesions (< 2 cm diameter)
- more extensive chondral lesion (>2 cm diameter)
- isolated osteochondral lesions
- osteochondritis dissecans

It is possible that the performance of such a matrix could vary with the type of lesion, as well as other variables involved in specific clinical cases. The construct would not be designed to treat generalized destruction of cartilage, as in advance osteoarthrosis.

The prevalence of these types of lesions is generally unknown because not all cases are symptomatic or diagnosed. Only recently has there been an attempt to understand the patient populations which would benefit from the latest cartilage repair techniques including chondrocyte transplantation or resurfacing techniques using porous matrices, both of which are studied in this work.

It is generally accepted that the treatments to be discussed would be most appropriate for the population of young patients (40 years of age or younger) with isolated chondral lesions, erosion of the cartilage to the calcified layer, and no associated joint pathologies (including osteoarthrosis, meniscal tears, ligament insufficiencies, or malalignment of the joint). A recent study investigating over 30,000 arthroscopies has suggested that the prevalence of this very specific situation taken from all arthroscopic examinations is approximately 1.5% [40]. Given approximately 1.4 million arthroscopic procedures performed on the knee annually in the United States, this would represent approximately 20,000 patients. In practice, cartilage repair techniques are being used clinically in a much wider range of patients including older patients and those with damaged bone, mild osteoarthrosis, multiple lesions or insufficiency of other tissues. The prevalence of this defect type in these more complicated cases totals approximately 20% of arthroscopies [40]. As this work is in its infancy, many years of clinical and basic research are needed to elicit the proper criteria for patient selection and treatment.

1.3 Current clinical treatments for cartilage defects

Clinical treatments for articular cartilage injury will depend on the extent of the injury, patient age, severity of symptoms, the desired result for the patient, and willingness of the patient and surgeon to undertake risky or experimental procedures. The primary goals are to relieve pain and to replace lost function
to a level satisfactory to the patient. It is generally desired to achieve an optimum combination of long-lasting relief from a minimally invasive procedure, although normally it is not possible to achieve both simultaneously. Depending on the situation, treatments currently implemented clinically may focus on one or more of the following: correcting conditions causing the damage, relieving symptoms, or resurfacing the articular cartilage.

If acceptable to the patient, a first option is altering the patient's lifestyle in an effort to relieve the symptoms. However, restriction of activity may be impossible or undesirable, and invasive treatment may be indicated. In cases where loose pieces or fibrillated cartilage cause swelling, inflammation, or locking/catching of the joint, arthroscopy may bring relief. In arthroscopic surgery, small instruments for removing loose pieces of tissue or shaving the articular surface are introduced through tiny incisions (~1 cm). Such minimally invasive surgery is preferable to any procedure which requires opening of the joint through larger incisions (arthrotomy), typically 4 cm or more. This method does not repair cartilage defects, but if the injury to the cartilage is not serious, it may prevent the damage from progressing further. If there is no damage to the bone, the surgeon will first attempt to bring relief while keeping the bone intact.

Further intervention may be required due to excessive damage to the joint or failure of arthroscopy. The primary options in treatment are resurfacing the damaged area or altering joint loading. When damage is caused by excessive forces stemming from abnormal joint anatomy, several methods may be used to alter joint loading, including osteotomy, release of muscles or tendons pulling abnormally on the joint, or other realignment surgery. These procedures may either correct the underlying abnormality which has caused the damage, and/or provide relief to the patient by shifting the load to a non-damaged area. While this method leaves the joint intact, it may not correct certain underlying disease processes. Success may be only temporary if the new joint anatomy is also susceptible to degeneration. Realignment surgery is often combined with methods of resurfacing.

Methods focused on resurfacing damaged articular cartilage seek either to regenerate the damaged tissue or to replace it with a different tissue that will carry enough load to allow the joint to function near normally, at least temporarily. One set of alternatives deliberately penetrate the subchondral bone to allow blood and marrow cells to fill the defect and form a reparative tissue. In drilling, abrasion arthroplasty, and microfracture, only the extent and nature of the penetration vary. All of these methods may be applied arthroscopically. Because penetration of the subchondral bone has been found to increase the incidence of pain and may introduce complications in the bone [85], the consensus is to use the least invasive procedure. Currently, that treatment is microfracture, which uses very sharp picks to puncture the
surface, but leaves most of the bone intact. In microfracture, the defect is filled through punctate bleeding and infiltration of marrow from the small pick holes. The result of any of these procedures is a fibrocartilaginous repair tissue that often provides at least temporary relief to the patient. However, the long term success is variable and generally not considered satisfactory. Break down of this tissue may cause more serious joint damage.

Alternatively, autografts or allografts may be used as a source of cells for cartilage resurfacing. While allografts, especially if frozen, could be made widely available, they have significant drawbacks due to the possibility of immunologic rejection and disease transmission. The main drawbacks of autografts are the need for additional surgical procedures and the limited supply of donor tissue.

The simplest autograft materials are those in which a bulk piece of tissue containing chondrogenic cells can be fixed to the defect site. Autologous perichondrial grafts, isolated from the rib [74, 75, 171], and autologous periosteal grafts, isolated from the tibia [73, 95, 133], have been used clinically. Preliminary results with periosteal grafts have been mixed and require more follow-up [121], while perichondrial grafts have shown a tendency to calcify or delaminate [21, 121].

One recent technique uses a combination autograft to improve repair: cultured autologous chondrocytes held in place by an autologous periosteum cover. In this technique, chondrocytes are obtained from the patient via a cartilage biopsy, isolated and proliferated in culture, and then returned to the patient. This procedure has generated a great deal of interest and controversy since publication in 1994 [22]. While preliminary results have been encouraging, there has been no controlled trial including treatments of periosteum alone. Thus, the efficacy of the cell transplantation has been questioned. An additional question is raised by possible abnormal behavior of chondrocytes due to possible alterations in the phenotype caused by culturing.

Each of the aforementioned autografts offer the advantage of being useful in chondral defects or osteochondral defects. These methods can be applied without causing additional damage to the bone. However, in defects where there is already significant damage to the bone, the surgeon is more likely to choose from more invasive techniques.

One choice has been that of osteochondral autografts, or in some cases allografts [37, 61, 119]. Because autograft tissue can only be taken from the non-articulating margins of cartilage surface, the size of a lesion to be treated with an autograft is limited. However, a new technique seeks to extend the use of osteochondral allograft procedures. In mosaicplasty, instead of one large graft, small osteochondral cores are spread throughout the defect area, with the gaps allowed to fill with clot from the underlying bone. Thus, a larger area
can be resurfaced, and the smaller donor sites can be selected from a greater range of the cartilage margins.

Ultimately, if all else fails, total joint replacement using metallic and polymeric implants are performed. This procedure is undesirable due to the invasive nature of the surgery and the limited lifespan of the implants and difficulty of revision of the procedure.

1.4 Scope of the present research

The present laboratory and animal research seeks to evaluate the capability of porous collagen analogs of extracellular matrix to facilitate regeneration of articular cartilage in vivo. It was not intended to grow cartilage in vitro, but rather to provide a construct to support in vivo regeneration of the tissue. The in vivo strategy may have advantages over the in vitro approach because: 1) the ability of natural biomechanical forces in the joint to influence and guide regeneration, and 2) integration of the reparative tissue with adjacent structures is likely to be facilitated and more complete when occurring in parallel with new tissue synthesis.

In vitro work was used to study cell-matrix interactions in the process of developing a cell-seeded construct for implantation in an animal model. Due to the time and expense associated with experimentation with an animal model, the in vitro work provided rationale for the choice of constructs to be used in vivo. Certain matrix characteristics were varied in vitro to determine how certain parameters in the design of the matrix might affect cell behavior. The results in culture could be correlated with behavior in the animal model and used to further engineer the material.

The in vivo work, performed in a canine model, was designed to evaluate the effects of the matrices engineered in vitro on the healing of a specific type of defect in articular cartilage. The matrix was studied alone and in conjunction with or comparison to other clinically relevant treatments for cartilage repair. An important component of the work was to assess the potential for spontaneous regeneration in this type of lesion, and to ensure that the defect was beyond the critical size at which healing could be expected to occur without intervention. The defect chosen was limited to the articular layer (chondral defect), and therefore resembles only a subset of clinical defects. The performance of matrices in this type of defect may not reflect their performance in other types of defects or related applications.

1.4.1. Specific aims

The specific aims of this thesis were to:
1) Synthesize and characterize analogs of extracellular matrix comprised of both type I and type II collagen
2) Evaluate *in vitro* the interaction of matrices and chondrocytes including:
- Developing methods of seeding matrices with chondrocytes
- Evaluating chondrocyte proliferation in matrices
- Evaluating biochemical activity of chondrocytes in matrices
- Engineering implantable chondrocyte-AECM composites

3) Develop an animal model for studying cartilage regeneration including:
- Establishing a well-defined surgical lesion
- Developing methods for quantitative analysis and characterization of healing in the model
- Characterizing spontaneous healing
- Characterizing healing due to autologous cultured chondrocytes alone

4) Evaluate engineered matrices from (2) in the animal model.

1.4.2 Hypotheses

The following are hypotheses that were synthesized to direct the research in the process of the achieving the specific aims:

- The chemical composition of a matrix, primarily the predominant collagen type, will influence cell morphology and synthesis of DNA and glycosaminoglycan *in vitro*.
- The pore structure of a matrix, primarily the pore diameter, will influence cell morphology and synthesis of DNA and glycosaminoglycan *in vitro*.
- The application of cultured autologous chondrocytes under a periosteal flap to a chondral defect of articular cartilage will increase the amounts of reparative tissue and hyaline cartilage found filling the defect relative to an untreated control.
- The application of a cultured autologous chondrocyte-seeded engineered type II collagen matrix to a chondral defect of articular cartilage will increase the amounts of reparative tissue and hyaline cartilage found filling the defect relative to a defect treated with cultured autologous chondrocyte transplantation.
- Chondrocyte-seeded collagen matrices implanted in chondral defects will form more reparative tissue and hyaline cartilage than identical treatments using unseeded matrices.
- The implantation of a porous collagen matrix in a microfracture-treated chondral defect will increase the amounts of reparative tissue and hyaline cartilage filling the defect.
2 Background

2.1 Articular cartilage

2.1.1 Articular cartilage structure and function

Articular cartilage is a load-bearing connective tissue covering the ends of bones in synovial joints. It has a unique structure which gives rise to its equally unique mechanical and functional properties. Its main functions include transmitting loads across the joint and providing a smooth, low friction surface to facilitate articulation. No other material, natural or synthetic has been found to reproduce these specialized functions.

Composed predominantly of water (65-80 percent by weight), articular cartilage is basically avascular, aneural, and alymphatic. Articular cartilage is also relatively acellular, but the few chondrocytes synthesize and maintain a complex extracellular matrix. Structural materials in the matrix include collagens (10-30 wt%) and proteoglycans (5-10 wt%). Chondrocytes account for less than 5% of the dry weight, and occupy 2 to 10 percent of the tissue volume. The balance of the tissue consists of numerous non-collagenous proteins and lipids such as fibronectin, anchorin, cartilage oligomeric matrix protein (COMP), and chondrocalcin.

Collagens provide the primary three dimensional structure of articular cartilage, giving the tissue tensile strength. Normal articular cartilage contains predominantly type II collagen (90%), but also includes small amounts of types VI, IX, X, and XI collagen. Type II collagen is normally found only in specialized tissues such as hyaline cartilage and the nucleus pulposis of the intervertebral disc. The collagens are arranged in fibrils and are cross-linked to each other and entrap the network of proteoglycans. Type IX collagen is thought to be an important component of cross-linking the type II network. Type X is found in calcifying regions, type XI within type II fibrils, and type VI surrounding chondrocytes. The majority of the large proteoglycan molecules (aggrecan) attach to hyaluronic acid molecules to form proteoglycan aggregates. A special protein specific to articular cartilage, link protein, stabilizes this attachment. Each aggregcan molecule consists of a core protein which is divided into a hyaluronic acid binding region and glycosaminoglycan (GAG) rich regions. GAGs are linear, negatively charged polysaccharides. The high density of hydrophilic negative charges on GAGs creates a large swelling pressure within the cartilage and provides compressive strength. The predominant GAGs in human articular cartilage are chondroitin 6-sulfate, keratan 6-sulfate, and chondroitin 4-sulfate.

In the light microscope, chondrocytes appear surrounded by distinct spherical matrix structures known as lacunae. The extracellular matrix structure varies with proximity to chondrocytes. In the pericellular region, surrounding the
chondrocytes, the matrix consists predominantly of proteoglycans, non-collagenous proteins, and glycoproteins. This matrix provides a means of cellular attachment to the extracellular matrix as a whole. A territorial matrix surrounds the pericellular matrix. It consists of thin fibrils and serves to link the chondrocyte to the main interterritorial matrix. The interterritorial matrix, as described above, occupies most of the volume in cartilage and is responsible for the bulk of the mechanical properties of the tissue as a whole.

Articular cartilage is divided into zones based on the horizontal plane level relative to the smooth surface. Many structural and biochemical characteristics change from zone to zone including collagen structure, chondrocyte appearance and metabolism, proteoglycan content, etc. It has been shown that collagen forms arching leaf-like structures which emanate from the subchondral bone and bend over when they reach the articular surface. Thus, the leaves display a different orientation in each zone. The thin superficial zone found at the articular surface of the cartilage lies furthest from the bone. In this zone, the collagen leaves are closely packed, oriented basically parallel to the surface, and consist of a fine mesh. The most superficial layer is bare of chondrocytes. Deeper in the layer, elongated, relatively inactive chondrocytes lie parallel to the surface. Since there is very little hyaluronic acid here, proteoglycan monomers may exist and may react directly with collagen fibrils. Below the superficial layer lies the intermediate or transitional zone. This zone provides a bridge to the deep zone and exhibits larger collagen fibrils and more rounded chondrocytes.

The large region of cartilage adjacent to the subchondral bone includes the deep zone or radiate zone, the calcified zone, and the tide mark, a distinct line separating the two zones. The calcified zone consists of a layer of calcified cartilage attached to the subchondral bone and may contain blood vessels. Collagen leaves emanate from the calcified zone normal to the joint surface and stretch vertically through the deep zone. The fibrils are largest in the deep zone and increased proteoglycan content is also found. In addition, chondrocytes in the deep zone are typically rounded and arranged in vertical columns.

As mentioned above, cartilage function depends on this specialized structure. This pattern may be altered either by abnormal changes to the grossly intact articular structure, or, in the case of removal of large pieces of cartilage, complete replacement by a very different reparative tissue (see below, 2.1.3). The resulting tissues can be distinguished from articular cartilage based on changes in the matrix and cells populating the tissue. Other tissue types (characteristic of damaged cartilage or reparative responses) include fibrous tissue, fibrocartilage, or hyaline cartilage. Fibrous tissue consists of fibrous bundles of type I collagen easily distinguishable through low power light microscopy and elongated, fibroblastic cells. A fibrocartilaginous tissue would have characteristics intermediate between fibrous and hyaline tissue. Often
this appearance includes macroscopically visible collagen fibers interspersed with chondrocyte-like cells in lacunae. Typically, the end result of cartilage injury will be described as fibrocartilage, making it the articular cartilage equivalent of scar tissue in skin. The term hyaline refers to the ground-glass appearance of cartilage matrix and is generally used to refer to a material that grossly resembles articular cartilage. However, hyaline tissue may lack certain elements of the highly specialized articular structure described above, and therefore must be distinguished.

The relationship between articular cartilage structure and function by measuring mechanical or electromechanical properties of modified articular cartilage has been well-studied and reviewed [128]. Experiments have included studies of cartilage in which damage occurs naturally (as in osteoarthrosis) or is induced by experimental enzyme treatment or joint destabilization [6, 17, 31, 49, 71, 153, 165], as well as studies of reparative and surrounding tissues in healing of cartilage defects [7, 92, 117, 131, 154, 188, 195, 196]. It is clear from this work that altering the collagen and or proteoglycan structure of normal cartilage will alter its mechanical function.

2.1.2 Mechanisms of injury and disease in articular cartilage

As introduced in section 1.2, articular cartilage can fail when it is subject to disease or injury. Under normal conditions, chondrocytes are responsible for maintaining the cartilage structure needed for proper function. Thus, all injury can be described as the inability of the chondrocytes to maintain the normal articular cartilage extracellular matrix structure. This may be due to abnormalities in the chondrocytes themselves (such as genetic abnormalities or direct damage or death of chondrocytes), or simply the inability of normal chondrocytes to repair damage to the matrix caused by external factors (such as repeated mechanical injury or excesses of destructive enzymes). For example, traumatic injuries may cause irreparable disruption of the matrix or directly kill chondrocytes. In other cases, the problem may stem from a subtle imbalance in chemical regulators of the chondrocytes.

2.1.3 Spontaneous healing of articular cartilage

Articular cartilage self-repair depends on the nature of the damage. This discussion will be limited to the general type of defects studied in this thesis: one in which a bulk quantity of cartilage tissue is removed, i.e. excisional injury. The most relevant types include chondral (defects limited to the thickness of the articular cartilage) and osteochondral defects (those penetrating the subchondral bone). For these injuries, there is no critical sized defect, defined as a defect that will spontaneously heal with normal articular cartilage. This is the consensus of years of clinical observations [27, 111] and animal experimentation. The majority of scientific work in this area, as outlined below, has focused on the animal models of repair.
In a chondral defect, repair is limited due to the avascularity and relative acellularity in the tissue. Some authors have observed that chondral defects display minimal healing (i.e. there is little filling with reparative tissue)[33, 42, 57, 59, 107, 125, 152]. In other cases investigators have described some fibrous tissue or fibrocartilage as the end result of healing [13, 28, 74, 97, 155, 167]. Responses to these defects included cloning of chondrocytes and increased synthesis of proteoglycans, but chondrocytes appeared trapped by their extracellular matrix and did not migrate to adjacent regions to enact repair. While the specific responses observed in chondral defects have varied due to differences in animal models, surgical procedures, and the extent of the lesion, there has been a consensus that reparative responses were incapable of fully regenerating any significant portion of the damaged. There is some evidence that synovial cells can migrate into the wound to participate in repair, but the spontaneous effect was minimal [78]. While these lesions do not heal by regeneration, there remains uncertainty about the clinical consequences, for example the likelihood of progression to osteoarthrosis [13, 24, 28, 57, 59, 64, 89, 97, 109, 115, 125, 176].

Osteochondral defects, which violate the subchondral bone, introduce a second repair mechanism. Cells and soluble factors from the bone marrow and subchondral vessels may infiltrate the defect area and participate in a reparative process. Unlike chondral defects, defect filling is always observed. Numerous studies have reported the formation of a fibrous repair tissue during early stages of healing [3, 4, 26, 38, 58, 60, 79, 82, 88, 89, 116, 124, 131, 146, 152, 161, 168]. Over time, the cells may undergo metaplasia into chondrocytes, forming a fibrocartilage, and in some cases even a hyaline-like tissue [38, 125, 152, 167]. However, this hyaline tissue was either a minor component of healing, or at least could still be distinguished histologically from normal articular cartilage. In addition, long term results in animal models have suggested that fibrocartilage is the final product of healing, and that this tissue may be susceptible to degeneration [125, 168]. The repair response may vary significantly from animal to animal in a given experiment, and especially across different models. Injury response may also depend on defect size [38, 79]. Biochemical and mechanical studies have shown that the fibrocartilage reparative tissue contains type I collagen [3, 58] and is less stiff and more permeable than normal cartilage [195].

2.1.4 Summary

The structure and function of articular cartilage are intimately related. Changes in the specialized structure, from injury or disease, will invariably affect the mechanical properties of cartilage. The response of articular cartilage to injury depends on many factors, most notably the type of defect. For the excisional type defects studied in this thesis, the best controlled observations of spontaneous healing have been performed in animal models. It has not been shown that any cartilage defect can fully regenerate. In
essence, there is no critical sized defect, although the reparative response is much more effective in defects which penetrate the subchondral plate. Even so, the reparative response results in a tissue which is easily distinguishable from normal cartilage. This “scar” of articular cartilage is normally referred to as fibrocartilage. Any effort to regenerate articular cartilage will have to improve on the natural response to injury.

2.2 Chondrocyte-matrix interactions

2.2.1 Gross effects of extracellular matrix

It is clear from the literature that the environment of the chondrocyte, specifically the extracellular matrix, can affect chondrocyte function and phenotypic expression. Much work has focused on finding environments which support expression of the chondrocytic phenotype in culture.

When placed in 2-dimensional culture, chondrocytes spread on their plastic substrate, adopting a stellate or elongated morphology. This shape change had been correlated with loss of expression of cartilage-specific molecules and is therefore taken as a sign of loss of chondrocyte phenotype. However, it is well established that chondrocytes cultured under special conditions regain their rounded morphology or retain the ability to synthesize chondrocyte-specific macromolecules. In 2-dimensional culture, conditions of high seeding density [192, 203] or hydrogel coating of the culture surface [151] have been found more favorable to chondrocytic expression.

A wide range of 3-dimensional systems have been found to support the chondrocytic phenotype, including agarose [16, 25, 39, 169] collagen gels [90, 203], alginate [19], many porous or fibrous matrices, both natural and synthetic [50, 105, 123], and microcarrier beads [56]. The ability of chondrocytes to express their native phenotype in these and other matrices has been the basis for current research focused on efforts to induce cartilage regeneration.

One study of chondrocyte behavior in porous sponge-like matrices has shown different chondrocyte expression in constructs made of collagen versus a synthetic polymer [63]. This work was not able to determine the specific factors which influence the chondrocyte behavior (i.e. chemical vs. physical).

2.2.2 Molecular level interactions

The extracellular matrix likely regulates cell function through two means: 1) direct cell-matrix interaction, and 2) regulation of other (soluble) factors which can affect the cells.
On one hand, the ability of the extracellular matrix to modulate chondrocyte phenotype and activity can be due to effects of chondrocyte-matrix interactions through integrins. Recent studies have found at least three different specific integrins (α1β1, α2β1, and α3β1) are involved in chondrocyte attachment to extracellular matrix [2, 46, 182]. The importance of these integrins is suggested by a study showing integrin dependence of chondrocyte shape and proliferation [47]. Alternatively, the extracellular matrix may affect cells indirectly, through interaction with growth factors. The ability of the matrix to bind and modulate growth factors has been studied in many systems such as wound healing [113] and angiogenesis [81], however little work has been performed to determine growth factor binding effects in chondrocyte culture. One recent study showed that the collagen type of the extracellular matrix was able to modulate chondrocyte response to one growth factor, TGF-β1 [149]. Thus, while more work is needed to extend this knowledge to the many other chondrocyte-matrix-growth factor systems of interest, it is clear that the growth factor-binding properties of the matrix can exert considerable influence over chondrocyte behavior.

2.2.3 Matrix contraction

One phenomena that is reflective of cell-matrix interactions is matrix contraction. Several investigators have studied the ability of the extracellular matrix to regulate cell contraction of that matrix [44, 66, 162]. If it occurs in vivo, chondrocyte contraction of the matrix in which it is seeded has the potential to affect the performance of the construct as an implant: 1) by reducing the pore diameter and thus impairing cell proliferation and migration; 2) by reducing the volume that the implant occupies in the defect; and 3) by separating the implant from the adjacent tissue, thus impairing integration of the newly forming reparative tissue.

2.2.4 Summary

Natural or artificial extracellular matrix can affect cellular function of most cell types, including chondrocytes. Matrix components have been found to regulate chondrocyte interactions with growth factors, integrin expression, biosynthetic activity, morphology, and contractile properties. Attempts to regenerate articular cartilage need consider the influence of a synthesized or engineered matrix on this behavior.

2.3 Collagen-GAG analogs of extracellular matrix

2.3.1 Introduction

Collagen is one of the most versatile materials that can be used as a biodegradable, implantable matrix material. It can be used in several forms, including gelatin, gels or lattices, and sponges or scaffolds. Gelatin refers to the
naturally occurring form of collagen consisting of randomly coiled individual chains. Gels or lattices are hydrated networks of small fibrils (banded bundles of collagen 50-500 nm in diameter) with interfiber spaces of "pores" of 1-10 μm. Sponges or scaffolds are made of large sheets or fibers of collagen with pores from 5 to over 500 μm. The walls may be of varying thickness (on the order of 10 μm, and may display either banded or unbanded collagen structure. A porous collagen-GAG cross-linked copolymer has not yet been used in articular cartilage experimentation. Because it has been studied in depth for other regenerative purposes, its effects in an articular cartilage model would be of great interest. In addition, the above findings of differential effects of collagen type on cell behavior suggest that culture of chondrocytes in collagen-GAG matrices composed predominantly of different collagen types would be of interest in searching for an optimal method of facilitating regeneration.

2.3.2 Method of fabrication

In general, porous materials can be produced by a variety of methods including woven or non-woven structures formed from fibers, polymerization in the presence of sacrificial particles or other insoluble phase of solution, or through a freeze-drying process. The method of freeze-drying, which involves forming of a slurry of a predominantly hydrous solution, freezing to form ice crystals, and lyophilization of the ice phase under vacuum has been used with collagen materials. The structure of the pores of the collagen sponge are negative replicas of the ice crystals formed in the freezing process. Thus, the principles of heat transfer may be applied to alter the formation of the solid phase, and thus, indirectly, the pore structure achieved.

2.3.3 Cross-linking of collagen matrices

Several methods are available for cross-linking collagen. The effect of cross-linking can be to improve mechanical properties, decrease the rate of degradation, or even to reduce antigenicity [187, 194]. Techniques include dehydrothermal treatment, ultraviolet and gamma radiation, and chemical cross-linking. All of these methods can offer the additional advantage of sterilization of the collagen material during the course of treatment. Each method introduces interchain bonds into the collagen structure through a different mechanism. Dehydrothermal treatment removes water form the environment of the matrix, thereby inducing condensation reactions between neighboring amino acid residues-- likely carboxyl and amino groups [193, 198]. There will be a theoretical limit to the cross-linking induced by this method based on the chemical makeup of the amino acid residues. Ultraviolet radiation creates free radicals on adjacent collagen fibers (most likely aromatic amino acid residues such as tyrosine and phenylalanine [193], which then combine to form a covalent crosslink. There are several chemical methods of cross-linking collagen, usually based on bifunctional compounds which can
react with amino acid side chains on neighboring collagen fibers [68, 194]. Some commonly used chemical cross-linking agents include glutaraldehyde (or other aldehydes), diamines, diisocyanates, imidoesters, and carbodiimides [68, 87, 194]. A potential disadvantage to this method is hydrolysis of the chemical into toxic byproducts. Finally, ethanol, which is often used as a tissue fixative, was used as a pre-wetting and sterilization agent in this work. Previous work has noted less shrinkage in collagen matrices treated with ethanol versus aqueous solutions, suggesting a slight cross-linking effect [41]. However, the effect of ethanol as a cross-linking agent on the collagen biomaterials used in this work is unknown.

For this work, cross-linking was used primarily as a means to control the rate of degradation of the matrix and mechanical properties after periods in culture. It was desired that the matrices remain intact throughout in vitro experiments to allow proper assays. In addition, it was desired that constructs seeded in vitro and transplanted to an animal model be easily handled by the surgeon.

2.3.4 Previous use in tissue engineering

The use of collagen-GAG copolymer matrices to regenerate articular cartilage follows logically from previous work using engineered collagen-based materials in hard and soft tissue regeneration or repair. Collagen-GAG matrices were first used by Yannas in 1977 to facilitate regeneration of skin in guinea pigs and subsequently humans [199]. These matrices were formed from a homogenized acetic acid solution of reconstituted bovine type I collagen and chondroitin 6-sulfate (isolated from shark) by various freeze-drying methods. The GAGs and collagen were cross-linked by a combination of vacuum drying treatment and soaking in acidic glutaraldehyde solution to provide mechanical strength and to prevent the elution of GAGs in vivo.

Attention was paid to engineering the copolymers for optimum regenerative capabilities. Variation of cross-linking technique controlled the degradation rate of the material in vivo [200, 201]. Pore characteristics (percent porosity, pore size, and pore orientation) were controlled by varying the freeze-drying techniques [41]. These effects were studied in depth, and it was found that a limited range of pore characteristics and degradation rates provided optimum activity in delaying wound contraction of skin lesions [202]. It was later found that seeding of the matrices with cultured cells could improve the performance of the construct by arresting contraction and supporting the regeneration of dermis instead of formation of scar [199]. Finally, it was more recently suggested that the chemical composition of the matrix, specifically the nature of the GAG component, may affect the biological activity of the construct [166].

In subsequent work, similar materials were used to treat peripheral nerve defects. These experiments found that different parameters were required in
engineering matrices appropriate for nerve regeneration [29, 30]. Optimally regenerated nerve resulted from implants with smaller pore sizes (5-10μ versus 20-125μ for skin) and quicker degradation rates (180 assay units versus less than 140 assay units for skin). In addition, it was found that nerve grafts performed better with the pores oriented axially along the length of the severed nerve. Presently, collagen-GAG copolymers are being investigated in treating spinal cord, tendon, and ligament defects.

Stone has investigated the use of collagen-GAG copolymers in meniscus regeneration in pig and dog models [173, 174]. Using the same basic sources of material and synthesis procedures as Yannas, he included dermatan sulfate and hyaluronic acid in the copolymer. In vitro experiments indicated that the matrix could be seeded with chemotactic factors, specifically fibronectin, to improve cell migration through the matrix in culture. Stone reported that the matrix did not impair meniscal healing in the pig, and may have improved healing in the dog. Meniscus differs from articular cartilage in that it is a fibrocartilage and contains areas of vasculature, thus improving its self regenerative capabilities. Although Stone mentions the possibility of optimizing his matrix through control of pore characteristics, degradation rate, he reports no experiments where he actually changes matrix characteristics. Recently, this technique has been introduced into the clinic in ongoing human trials [175].

2.3.5 Summary

The history of the use of collagen-GAG copolymers has demonstrated success in tissue engineering of several different tissue types. The chemical and physical properties of the matrix have proven to be important variables in optimizing regeneration. Because the nature of cell-matrix interactions are cell- and tissue- specific, a construct designed to facilitate articular cartilage regeneration will likely have different properties from those used in other tissues. Specifically, based on results in skin and nerve, pore size is expected to be an important variable. In addition, because the natural collagen type of articular cartilage differs from that in skin and nerve, the nature of the collagen component of the matrix is likely to be important. Finally, because of the limited reparative response inherent in cartilage, it is anticipated that addition of an exogenous cell source to the matrix may improve its activity as it does in skin.

2.4 Animal models of cartilage injury

2.4.1 Animal species

Studies of healing of cartilage defects have been carried out in many animal models. Specifically, work in regeneration has utilized rooster, rabbit, and dog
models as well as clinical trials on humans. The most common animal model, the rabbit, however, has several problems. First, the rabbit knee experiences considerably different loading and stresses on the articulating surface compared to a human knee. In addition the defect depth is limited by the thickness of the articular cartilage in the rabbit knee joint (generally 0.5 mm or less). The thicker articular cartilage layer (approximately 0.5 to 1.0 mm) in the dog improves this condition, and the activity level and loading more closely resemble human conditions.

2.4.2 Animal age

Healing may be affected by animal age, as studies performed in adolescent species have reported very good healing compared to other studies [26, 89]. While it has been known for many years that chondrocyte response to injury may be age dependent [108] only recently has a study conclusively shown age dependent differences in repair in the rabbit [195]. Because a treatment modality that would work only in young patients (prior to skeletal maturity) would be very limited in applications, adult models are generally preferred to provide more clinically applicable tests of efficacy.

2.4.3 Defect depth

Although there is no critical defect size in articular cartilage for regeneration (section 2.4.4), the depth of a defect determines the process of repair. In intact cartilage, the calcified layer prevents direct transfer of large molecules between bone and cartilage. Thus, it is convenient to classify defects as either limited to the non-calcified articular layers (chondral defects) or penetrating to the subchondral bone (osteochondral defects). It is unclear in the adult whether defects extending into, but not through the calcified cartilage should be considered chondral or osteochondral. The vascular supply to the calcified layer is extremely limited or absent in the adult, thus penetration into the calcified cartilage may bring no visible response. However, if vessels are penetrated, communication extends to the subchondral bone, albeit on a much smaller scale than occurs with fracture or intentional drilling into bone. The terminology used for defects in this work is depicted in Figure 2-1.

The filling of osteochondral defects was discussed in section 2.1.3. In fact, this filling by reparative tissue is the aim of clinical treatments designed to stimulate repair by access to the subchondral bone (section 1.3). It is important to note that the violation of the bone requires the additional step of bone reformation for full resolution of this healing process.

In contrast, it is generally agreed that in chondral defects, i.e. those limited to the articular layers, the reparative response is weak in that little or no reparative tissue fills the defect (section 2.1.3). In the context of the present work, it is instructive to consider differences among chondral defects. For
example, a single laceration will heal differently than an injury where a bulk amount of cartilage is removed (such as shaving). Some articles suggest differences in responses to chondral lesions of different depths. This should not be surprising due to the depth-dependent nature of chondrocyte and matrix properties in the cartilage. Each type of defect may have different clinical significance.

The use of a “full-thickness” chondral defect, one made through all non-calcified layers of articular cartilage, reaching to, but not through the tidemark, is a new development [22, 23, 64]. No study has reported on the initial appearance of these defects (immediately after creation), and the natural course healing of these defects is not well established. Grande reported on only 5 defects at 6 weeks, and Brittberg reported on only 6 defects at 12 weeks, both in a rabbit model. However, there are several important differences between this type of defect and more superficial defects, most notably the exposed basal surface to which reparative tissue must bind (the calcified cartilage vs. articular cartilage), and the mechanical force environment to which reparative tissue will be exposed, which will depend on defect geometry, location in the joint, the nature of joint loading, and the hardness of underlying surface. It is also possible that the proximity to the subchondral bone may also expose full-thickness chondral defects to a different chemical environment when compared to more superficial defects, however little is known about chemical communication through the calcified cartilage.
2.4.4 Critical size defect

It is generally accepted that for any adult species, there is no critical size chondral defect in articular cartilage, i.e. that even a slight defect (e.g. an incision) will not heal if the defect is wholly contained within the articular cartilage layers. Studies have shown that even a scalpel incision, in which no material is moved will not fully heal [27, 33, 115]. As discussed above, the response to deeper defects will include formation of reparative tissue usually described as fibrocartilaginous in nature. Because this material is clearly not articular cartilage, it is considered reparative or scar-like tissue, not regenerated cartilage. Thus, the absence of a critical size defect also applies to osteochondral defects (at least in adults).

2.4.5 Methods of evaluation

There are three primary methods for evaluation of the outcome of healing cartilage in an animal model: histology, biochemistry, and mechanical properties. Interestingly, none of these match the criteria used to evaluate success clinically: pain relief, then function. Other clinical assessments which can be made through arthroscopic procedure include viewing or probing the surface, however, these results do not always correlate with patient symptoms, and thus are not used as reliable indicators of the success of healing.

In experimental work, most authors use histological methods of evaluating cartilage repair. This allows evaluation of many important factors in the reparative process: the types of tissues filling the defect (including both cell and extracellular matrix characteristics), attachment to adjacent structures (cartilage, calcified cartilage, or bone), and the health of the adjacent tissues. The method of staining may also be useful in eliciting biochemical information. For example several staining methods are specific to sulfated glycosaminoglycans (safranin O, alcian blue), while immunohistochemical stains can be used to demonstrate collagen type and cartilage specific proteins. Finally, histology can reveal structural information, primarily collagen organization, which may give a general idea of the functionality of the reparative tissue.

A semi-quantitative schema for assessing the degree of degradation of articular cartilage- the Mankin scale [112]- has recently been adapted to provide quantitative assessment of the success of cartilage healing in reparative procedures [12, 140, 146, 189]. However, this approach must be exercised with caution. The use of an ordinal semi-quantitative scale precludes accurate use of parametric statistics. Still, in many studies the ordinal data are used as parametric input for the reporting of statistical comparisons [12, 54]. The meaning of these statistics should be considered only approximate. Furthermore, often a composite score of many categories is reported and used for comparison between groups. This comparison is therefore made under the
assumption that the highest score possible in each category accurately reflects its relative importance in healing. This is most certainly not the case, as the “importance” in healing is at this time at best a subjective judgment of the experimenter.

Other methods of analysis are more specialized and may complement histological analysis. The measurement of mechanical properties on reparative tissue may indicate the degree to which the tissue functionally replaces normal cartilage. The major variables examined include modulus and permeability. Biochemical analysis is normally focused on synthesis of the major components of the cartilage extracellular matrix: collagen and proteoglycans. Several studies have measured the relative amounts of collagen types I and II formed in reparative tissue. Both of these methods have been limited in use in part due to the destructive nature of the typical *ex vivo* testing procedures which prevents histological analysis of the same tissue.

It would be desirable to have information from all of these outcomes, however, practical limitations of the research area, including the limited size of defects and expense of animal models, often makes this impossible. For a preliminary investigation of healing, histology provides the widest range of information and is widely accepted. Mechanical and biochemical evaluations are more appropriate for more specialized follow-up studies. Improvements of technology in mechanical testing, including recently developed non-destructive probes which may be used *in situ* [18, 132] promise to expand the use of mechanical testing in analysis of cartilage repair.

2.4.6 Summary

Many types of defects are available for studying cartilage healing. In this work we chose a dog model for reasons of cartilage thickness, accessibility of the surgical site, reproducibility of the lesion, animal activity, and loading of the joint. An adult animal was used to reflect the challenge of repair most often seen clinically. A specific lesion was chosen extending to, but not through the calcified cartilage. This type of lesion mimics situations that are often the direct result of injury or logical steps in minimally invasive repair procedures. While this defect is the subject of recent research and clinical procedures, no comprehensive studies have described its initial histological appearance or the natural course of healing. Based on extensive work with both more superficial and deeper defects, there is critical sized defect in articular cartilage. In this work we sought to employ a quantitative method of evaluating the tissue types comprising the reparative tissue based on histological and immunohistochemical criteria.
2.5 Experimental methods of facilitating articular cartilage regeneration

In general, efforts to regenerate tissues focus on manipulation of the three pillars of tissue engineering: cells, matrices, and soluble regulators. While this work focuses only on cells and matrices, the use of growth factors will be discussed briefly. In addition, as this work attempts to regenerate cartilage in vivo (as opposed to in vitro), this review will concentrate on in vivo techniques.

Several novel methods are under development in an attempt to regenerate articular cartilage, some of which have reached clinical trials and have been discussed in section 1.3. The majority of these approaches may include one or more of the following techniques:

1) Inducing existing chondrocytes to more actively repair the defect through enhanced proliferation, migration, and synthesis, often through application of soluble regulators (i.e. cytokines or growth factors).

2) Providing a new population of cells to express chondrocytic phenotype to synthesize new cartilage. This source may be an autogenous or allogeneic tissue graft, or cells manipulated in culture.

3) Providing a matrix, carrier, or support for cells to facilitate or direct synthesis of new cartilage.

A final factor to consider in treating a defect in articular cartilage is the post-procedural motion and loading to which the joint will be subjected. Before or after injury, there is clearly an acceptable range of loading and motion outside of which cartilage begins to degrade. No loading at all can lead to joint degeneration [160] (a sort of atrophy), while excessive loading can create pathology in the cartilage and underlying bone [150]. A series of studies in animal models have demonstrated the ability of controlled motion to improve the reparative process in certain defect types subjected to certain treatments [127, 136, 138, 140, 141, 161]. While not the subject of this work, the postoperative loading should be investigated in any model of cartilage repair.

2.5.1 Cells with chondrogenic potential/autologous tissue

Several different types of adult cells maintain chondrogenic potential. The discussion of clinical repair techniques revealed that sources may include bone marrow, perichondrium, periosteum, or articular cartilage. Although these cells may normally express different phenotypes in situ, they share a common mesenchymal lineage and under the appropriate conditions may express the chondrogenic phenotype. Since there have been few clinical trials with techniques using several of these sources and there is limited opportunity for follow-up evaluation in humans, research has proved useful in evaluating the efficacy of such procedures. In general, these cells may be used as part of a tissue implant (as with periosteum and perichondrium) or the cells may be
isolated and transplanted by a variety of methods. Cells which have been successfully isolated and transplanted into cartilage defects include articular chondrocytes, mesenchymal stem cells from bone marrow, and perichondrocytes.

In early work in cartilage repair, several investigators attempted to transplant cells, predominantly chondrocytes, directly into cartilage lesions. These studies included both allografts and autografts [14, 15, 32, 65]. This technique could only be applied to deep defects, as there was no method of fixing the cells in a shallow defect without clot forming from the subchondral bone. Results were in general poor. One study suggested that healing was more successful when the cells were not completely separated from their matrix [14]. Thus the presence of matrix may be important in providing support for the cells.

The method of autologous cell transplantation under periosteum is of special significance to this thesis. Two animal experiments have been performed using this technique, both in the rabbit [23, 64]. In these studies, the cell treatment was judged to have a positive effect on healing of chondral defects including increased amount of reparative tissue and improved quality and durability of this tissue.

The use of periosteal or perichondrial grafts has been recently extended in animal models to investigate the use of techniques such as postoperative treatment (such as continuous passive motion) [127, 141, 157, 159] or in combination with growth factors [77, 137]. In some studies in osteochondral defects, these techniques have successfully induced more hyaline-like tissue than controls, although this repair tissue does not duplicate normal articular cartilage. Thus, the long term behavior of this repair tissue is in question. In addition, this technique has the drawback of either requiring an additional operation on the patient for an autograft, or overcoming immunological reactions when performed with donor tissue.

2.5.2 Use of matrices for articular cartilage repair

Matrices may function either as insoluble regulators of cell function (section 2.2) or simply as delivery vehicles or a supporting structure for cell migration or synthesis. Numerous matrices made of either natural (Table 2-1) or synthetic (Table 2-2) components have been investigated for use in cartilage repair. Many experimenters have included exogenous cells or growth factors to further facilitate the reparative process. Natural matrices are made from processed or reconstituted tissue components (such as collagens and GAGs). Because they mimic the structures ordinarily responsible for the reciprocal interaction between cells and their environment, they may serve as regulators with minimal modification. They are generally biocompatible and, because they are ordinarily remodeled in the body, also biodegradable. The ability to
<table>
<thead>
<tr>
<th>Matrix</th>
<th>cells</th>
<th>animal</th>
<th>defect type (s)</th>
<th>author</th>
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<tbody>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>ECM, fibrin</td>
<td>chondrocytes</td>
<td>rooster</td>
<td>osteochondral</td>
<td>Itay 87 [83]</td>
</tr>
<tr>
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<td>rooster</td>
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</tr>
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<td>rabbit</td>
<td>osteochondral</td>
<td>Speer 79 [172]</td>
</tr>
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<td>rabbit</td>
<td>osteochondral</td>
<td>Grande 93 [62]</td>
</tr>
<tr>
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<td>chondrocytes</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Frenkel 97 [54]</td>
</tr>
<tr>
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<td>chondrocytes</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Ben-Yishay 95 [12]</td>
</tr>
<tr>
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<td>chondrocytes</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Wakitani 89 [190]</td>
</tr>
<tr>
<td>collagen gel</td>
<td>mesench. stem</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Wakitani 94 [189]</td>
</tr>
<tr>
<td>collagen gel</td>
<td>mesench. stem</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Mochizuki 96 [126]</td>
</tr>
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<td>collagen gel</td>
<td>chondrocytes</td>
<td>rat</td>
<td>osteochondral</td>
<td>Noguchi 94 [135]</td>
</tr>
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<td>rabbit</td>
<td>osteochondral</td>
<td>Hogervorst 92 [72]</td>
</tr>
<tr>
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<td>chondrocytes</td>
<td>horse</td>
<td>osteochondral</td>
<td>Hendrickson 94 [70]</td>
</tr>
<tr>
<td>fibrin</td>
<td>none</td>
<td>dog</td>
<td>osteochondral</td>
<td>Paletta 92 [145]</td>
</tr>
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</table>

Table 2-1. Summary of natural matrices used to facilitate articular cartilage repair in vivo

<table>
<thead>
<tr>
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<th></th>
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<th></th>
<th></th>
</tr>
</thead>
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<td>non-resorbable</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>poly vinyl alcohol</td>
<td>none</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Speer 79 [172]</td>
</tr>
<tr>
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<td>none</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Engkvist 82 [45]</td>
</tr>
<tr>
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<td>none</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Hanff 90 [67]</td>
</tr>
<tr>
<td>PTFE (polyurethane)</td>
<td>none</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Messner 93, 94 [117, 118]</td>
</tr>
<tr>
<td>Dacron</td>
<td>none</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Messner 93, 94 [117, 118]</td>
</tr>
<tr>
<td>carbon fiber</td>
<td>none</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Minns 82 [123]</td>
</tr>
<tr>
<td>carbon fiber</td>
<td>none</td>
<td>human</td>
<td>osteochondral</td>
<td>Minns 90 [122]</td>
</tr>
<tr>
<td>carbon fiber</td>
<td>chondrocytes</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Hemmen 91 [69]</td>
</tr>
<tr>
<td>carbon fiber</td>
<td>chondrocytes</td>
<td>rabbit</td>
<td>chondral</td>
<td>Brittberg 96 [23]</td>
</tr>
<tr>
<td>carbon fiber</td>
<td>mesench. stem</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Robinson 93 [154]</td>
</tr>
<tr>
<td>resorbable</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Freed 94 [52]</td>
</tr>
<tr>
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<td>rabbit</td>
<td>osteochondral</td>
<td>Vacanti 94 [184]</td>
</tr>
<tr>
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<td>none</td>
<td>dog/rabbit</td>
<td>osteochondral</td>
<td>Klompmaker 92 [93]</td>
</tr>
<tr>
<td>poly-lactide-caprolactone</td>
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<td>dog</td>
<td>osteochondral</td>
<td>Klompmaker 92 [93]</td>
</tr>
<tr>
<td>poly-lactic acid (PLA)</td>
<td>perichondrial</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Chu 95 [35]</td>
</tr>
<tr>
<td>poly-lactic acid (PLA)</td>
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<td>rabbit</td>
<td>osteochondral</td>
<td>von Schroeder 91 [188]</td>
</tr>
<tr>
<td>poly HEMA</td>
<td>none</td>
<td>rabbit</td>
<td>osteochondral</td>
<td>Kon 81 [94]</td>
</tr>
</tbody>
</table>

Table 2-2. Summary of synthetic matrices used to facilitate articular cartilage repair in vivo
remodel an implanted material is a prerequisite for regeneration. An important subset of materials in this category are those made predominantly from collagen, the main structural component in cartilage.

Synthetic matrices are made predominantly of polymeric materials, and offer the advantage of a virtually unlimited range of carefully defined chemical compositions and structural arrangements. It is important to distinguish the subset of synthetic matrices that are not degradable. While the non-degradable subset may aid in repair, they will never be replaced by remodeling and therefore cannot be used to fully regenerate articular cartilage. It is also undesirable to leave foreign materials permanently in a joint due to the problems associated with generation of wear particles, thus only degradable materials would be suggested for work in regeneration. Degradable synthetic matrices can be engineered to control the rate of degradation. However, because synthetic implants are made from non-native materials, it must be shown that they degrade safely, without the evolution of toxic byproducts.

It is important to note that only one of these studies has examined the use of a matrix in a chondral defect [23], and no degradable matrices have been used in this system. The use of a carbon fiber matrix to transplant cultured autologous chondrocytes was compared to the clinical method of implanting the cells under periosteum. In this study of healing of defects in the rabbit, the matrix was found to slightly improve the amount of reparative tissue and its histologic score at 12 weeks when compared to control treatments without matrix. These trends held for the use of periosteum alone, and for periosteum in combination with CAC. However, at one year, the carbon fiber group had the same amount of reparative tissue with slightly inferior histological appearance.

In work with deeper defects, matrices including porous sponges and gels, have had varying effects on healing. Cell-seeded matrices had much greater effects than unseeded matrices. Virtually every study including cell-seeded matrices (sponges or gels) with unseeded matrix controls found some positive effect of the cell seeding with regard to histological, biochemical, or mechanical properties of reparative tissue. However, the magnitude of the effects varied significantly from study to study. The use of chondrocytes from younger animals was very effective [12, 83, 145, 154]. Due to the differences in the models and techniques used, it is difficult to compare the effect of various matrices and cell types among these studies.

In general, unseeded matrices or gels were less successful than seeded matrices. Some studies found that porous matrices (some natural, some synthetic) increased either the amount or maturity of cartilaginous reparative tissue [52, 54, 67, 93, 145, 172]. Two studies of collagen implants reached different conclusions about the ability of such a material to improve the healing of bone underlying the defect [164, 172]. Other experiments, however, found no effect
from matrix or gel implantation [12, 72, 190], or they were deemed unsuccessful [94, 135]. In general, gels have only been used as delivery vehicles for cells, thus there is limited reporting of "gels alone," reflecting controls for cell-seeded experiments.

Even fewer studies have investigated the effects of matrix parameters such as chemical composition or pore structure by direct comparison in vivo. Speer found a collagen implant preferable to one of polyvinyl alcohol [172], while Messner found a porous Dacron implant slightly preferable to one of PTFE [117, 118]. In dogs and rabbits, Klompmaker found no effect of synthetic polymer composition or pore size [93]. All of these studies used osteochondral defects.

In summary, it is clear that a wide range of materials are appropriate for implantation due to the positive effects on healing, however, much more work needs to be done. While the chemical and physical properties of the matrix are recognized as important determinants in cell-matrix interactions, the literature indicates very limited work aimed at optimizing the physical and chemical properties of collagen-based materials for articular cartilage repair. Several of the studies with collagen and non-collagenous materials have addressed the idea that the implant structure may be optimized by changes in physical or chemical properties, however, no study has yet conclusively demonstrated optimized healing in articular cartilage. While using cell grafts in conjunction with matrices raises such issues as immunogenicity (for allogeneic sources) and donor site morbidity (for autologous sources), cell-seeded matrices have been widely studied and appear to hold great promise. In addition, there is especially a need to perform experiments in models other than osteochondral, as this has not been addressed in the literature.

2.5.3 Growth factors

Recently, many authors have investigated the use of growth factors, cytokines, and other small molecules to enhance the reparative response in the healing of cartilage defects. Although not the subject of this thesis, this relatively new approach holds great potential for improving cartilage regeneration. Growth factors are well-known to affect basic cell processes such as proliferation, attachment, migration, and synthesis. The effects on cartilage have been extensively reviewed [106, 110, 179]. Originally, work with growth factors focused primarily on the ability to affect chondrocytes or cartilage explants in culture [10, 20, 34, 55, 76, 99, 100, 104, 142, 144, 156, 158, 178, 185]. More recently, the effect of growth factors on healing of cartilage lesions in animal models has been studied. Growth factors have been delivered by collagen sponge [12, 164], fibrinogen [78], and by injection and periosteum [77]. These experiments concluded that the growth factors used, namely bone morphogenetic protein-2, transforming growth factor-beta, and fibroblast growth factor, produced significant effects in healing. As the cell-matrix-growth factor line of experimentation is likely to be pursued further, the ability of
matrices, such as the one developed in this thesis, to act as delivery vehicles will become an important focus of research.

2.5.4 Summary

Where it was once thought that articular cartilage could heal only with fibrous repair tissue, recent evidence has suggested that methods of tissue engineering, including cells, matrices, and/or growth factors, may be capable of facilitating regeneration, or at least improving healing. Several different cell sources (marrow and periosteal stem cells, perichondrocytes, and chondrocytes) have been shown to express cartilage-like phenotypes in culture or in treatment of animals or humans. Controlling the expression and behavior of these cells is the key to regenerating articular cartilage. For tissue grafts, the primary means of influencing cell behaviors are mechanical forces and soluble factors. For isolated or cultured cells, however, an additional important component of controlling cell behavior is the matrix in which it resides.

Several attempts have been made to use porous matrices to study articular repair. Some porous implants have been shown to improve repair relative to untreated defects. However, over a wide range of experimental systems, much better results were achieved by adding a source of cells to the matrix. Despite the known importance of matrices as cell regulators, little attention has been paid to optimizing and controlling the physical and chemical characteristics of the matrices. Results of studies which did alter the engineering of matrices are inconclusive. Furthermore, no work has been reported in using the novel chemistry of a collagen-GAG copolymer to treat articular cartilage defects. Thus, there is a need to study the use of collagen-GAG porous copolymer implants for use in regenerating articular cartilage.
3. Experimental Design

3.1 In vitro experiments

This set of experiments was designed to meet specific aims #1 and #2 of the thesis: to determine if the chemical composition or pore size of the matrix would affect chondrocyte behavior in vitro. First, analogs of extracellular matrix composed predominantly of either type I or type II collagen were synthesized and characterized. Subsequently the interaction of chondrocytes with these matrices was studied under varying conditions of matrix chemical composition, cross-linking treatment, seeding density, and pore size. The outcome variables included chondrocyte morphology, proliferation, biosynthetic activity, and the ability of the chondrocytes to contract the matrix. The results of these experiments were used to guide and interpret in vivo experiments.

3.1.1 Effects of seeding parameters

Numerous pilot experiments were performed to assess the efficacy of various methods for seeding matrices with chondrocytes, as measured by the retention of chondrocytes in the matrix after the seeding process. The predominant outcome variable was the amount of DNA in the seeded constructs after 2-3 hours, but before adding medium. As it was anticipated that a percentage of cells would be lost through initial medium addition, one experiment investigated this effect. Unseeded constructs were measured and subtracted from totals so the effect of only the cells added was measured. In most groups, n = 4 matrices were used. The specific designs of each component experiment is found in section 4.2.1.3.2.

3.1.2 Culture of chondrocytes in matrices of different chemical composition

In this experiment two matrices with similar pore characteristics but made of different chemical components were used as substrates for chondrocyte culture. The objective was to determine the effect of the chemical composition of the matrix on chondrocyte morphology, proliferation, and the amount of glycosaminoglycans synthesized and retained in the matrix. Matrices made predominantly of type I collagen (from now on referred to as “Type I” matrices) and type II collagen (from now on referred to as “Type II” matrices) were obtained or produced as described in section 4.1. Type I matrices for this experiment were made of bovine tendon collagen and were dehydrothermally cross-linked, while type II matrices were UV cross-linked for 8 hours (section 4.1.1.3).

Forty-two samples, 4 mm in diameter by 2 mm thick (cored from sheets) were used, including 18 of type I collagen and 18 of type II collagen, for simultaneous
cell seeding, and 3 sponges of each material as unseeded controls (Table 3-1). Six type I and six type II matrices were terminated at each of the three time points: 3 hours (before addition to medium), 7 days and 14 days. Three of the seeded matrices at each time point were allocated for histology and three seeded specimens for biochemical assays. Histological analysis included histology, immunohistochemistry for types I and II collagen, and morphological analysis of cell shape (section 4.2.3). Biochemical analysis included measurement of dry weight, and GAG and DNA analysis (section 4.2.2). One unseeded matrix was assayed at each time point: three hours, 1 week, and 2 weeks.

<table>
<thead>
<tr>
<th>time</th>
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<th>unseeded biochemistry</th>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>type II</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>7 days</td>
<td>type I</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>type II</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>14 days</td>
<td>type I</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>type II</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 3-1. Number of matrices evaluated in comparison of matrices of varying chemical composition

Cells were also seeded in agarose (Seaplaque GTG, FMC Bioproducts, Rockland, ME) and in 25 cm²-flasks to show expression of phenotype in an inert hydrogel and in monolayer culture.

3.1.3 Effect of cross-linking treatment and cell seeding density

To produce matrices of varying cross-linking, matrices made predominantly of type II collagen were dehydrothermally cross-linked from 0 to 4 days (section 4.1.1.3). In addition, several samples were UV cross-linked for 12 hours. A pilot study was first run to 2 weeks, then a long term study was performed.

Pilot study. It was desired to see if the length of dehydrothermal treatment altered the ability to handle constructs or the proliferation of chondrocytes in type II matrices in culture up to two weeks. Five groups of matrices, dehydrothermally cross-linked 0, 1, 2, 3, or 4 days, were used. For each of three time points (3 hours, 1 week, and 2 weeks), 4 seeded and 4 unseeded samples were prepared for each cross-linking treatment, making a total of 120 9-mm cores. Seeding was carried out simultaneously on all groups. At the termination times (3 hours, 1 week, and 2 weeks), the 4 seeded and 4 unseeded samples were bisected with a scalpel and the halves processed separately for histology and DNA assay.
Long-term study. This study was designed to determine, if 1) the degree of cross-linking, or 2) the seeding density of chondrocyte-seeded type II matrices would affect the ability to handle the constructs or chondrocyte proliferative activity or the amount of GAG retained in the matrix up to 6 weeks in culture. Three groups of Chondrocell type II matrices were used for this study: dehydrothermally cross-linked for 1 day and 4 days, and UV cross-linked for 12 hours. 9-mm discs were cored from larger pieces. A total of 72 cores were used. Samples were allocated to each of three time points: 1 day, 4 weeks, and 6 weeks. Samples were grouped into one of three seeding categories: unseeded, high seeding density (3.2 million cells/core), or low seeding density (1.6 million cells/core). Only 12 UV-cross-linked samples were available, and therefore were used only for the 6 week time period. Samples were allocated for biochemical analysis (GAG and DNA assays, Table 3-2) or for histology. For each cross-linking treatment, seeding condition, and time for which biochemistry was performed, one additional sample was obtained for histology.

<table>
<thead>
<tr>
<th>time</th>
<th>cell seeding</th>
<th>1 day DHT</th>
<th>4 day DHT</th>
<th>UV</th>
</tr>
</thead>
<tbody>
<tr>
<td>high dens.</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day low dens.</td>
<td>2</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unseeded</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>high dens.</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 weeks low dens.</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>unseeded</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>high dens.</td>
<td>3</td>
<td>3</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>6 weeks low dens.</td>
<td>3</td>
<td>3</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>unseeded</td>
<td>3</td>
<td>3</td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

Table 3-2. Number of seeded samples allocated for biochemistry in culture of matrices with varying degree of cross-linking and seeding density. Also, 1 sample was allocated for histology in each group.

3.1.4 Effect of matrix pore diameter

Pore diameter effects were studied in two different types of matrices: type I collagen GAG matrices and type II matrices, both produced in our laboratory (section 4.1.1). The goal was to determine if the pore diameter of the matrix affected the seeding of the matrix, the phenotypic behavior of chondrocyte, and the resultant construct after various times in culture.

3.1.4.1 Effect of pore diameter on chondrocytes in type I matrices

This study sought to determine whether the pore diameter of cell-seeded matrices comprised of primarily type I collagen would affect chondrocyte morphology or proliferation, or the amount of glycosaminoglycan synthesized.
and retained in the matrix. 4-mm cores of small- and large- pore type I matrices were manufactured and used in this study (section 4.1.1.2.1). This experiment was run in parallel with an experiment of the effect of collagen type, thus the “large pore” type I matrices used here are the same samples used in section 3.1.2. In addition, the conditions of seeding were identical.

29 sponges were used, including 12 small-pore and 12 large-pore collagen matrices for cell seeding and 3 large-pore and 2 small-pore sponges as unseeded controls. Six chondrocyte-seeded type I and six chondrocyte-seeded type II sponges were terminated at each of the two time points: 3 hours and 7 days. Three of the seeded sponges at each time point were dedicated for histology and morphological analysis of cell shape (section 4.2.3), and three seeded specimens for biochemical assays for GAG and DNA (sections 4.2.2).

3.1.4.2 Effect of pore diameter on chondrocytes in type II matrices

This study quantified the ability of the pore diameter of chondrocyte-seeded matrices comprised predominantly of type II collagen to affect 1) cell number, 2) cell morphology, 3) rate of proteoglycan synthesis, 4) accumulation of glycosaminoglycan, and 5) matrix contraction. The matrices were manufactured under different conditions to yield three different pore diameters, designated small, medium, and large (section 4.1.1.2.2).

The experiment was performed in two parts from a common cell source. In the first part, samples were allocated for histological processing, primarily for assessment of cell morphology (Table 3-3).

<table>
<thead>
<tr>
<th>time</th>
<th>description</th>
<th>pore size</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>small</td>
<td>medium</td>
<td>large</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>histo:biochem</td>
<td>histo:biochem</td>
<td>histo:biochem</td>
<td>histo:biochem</td>
</tr>
<tr>
<td>2 hour</td>
<td>seeded</td>
<td>1 : 0</td>
<td>1 : 0</td>
<td>1 : 0</td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>seeded</td>
<td>3 : 4</td>
<td>3 : 4</td>
<td>3 : 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>unseeded</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>seeded</td>
<td>3 : 4</td>
<td>3 : 4</td>
<td>3 : 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>unseeded</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td></td>
</tr>
<tr>
<td>2 week</td>
<td>seeded</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>unseeded</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td></td>
</tr>
<tr>
<td>4 week</td>
<td>seeded</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>unseeded</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td>1 : 4</td>
<td></td>
</tr>
<tr>
<td>* 6 week</td>
<td>seeded</td>
<td>1 : 0</td>
<td>1 : 0</td>
<td>3 : 0</td>
<td></td>
</tr>
</tbody>
</table>

* Extra samples cultured 2 weeks further for histology.

Table 3-3. Number of samples of seeded type II matrices of varying pore size. Table entries given as, n for histology : n for biochemistry.
Most samples were grouped at 1 day and 1 week, as pilot work in the type I material indicated that pore size effects were lost rapidly. Other times were included to provide histological evidence for the second part of the experiment and to aid in evaluation of the suitability of the in vitro cultured constructs for in vivo implantation.

In the second part of the experiment, samples were allocated for biochemical analysis to assay cell proliferation and synthetic activity (Table 3-3). Methods of evaluation included radiolabeled sulfate incorporation, DNA, and GAG analyses. For the biochemical experiment, a total of 96 matrices were used: 4 seeded and 4 unseeded matrices for each pore size at each of 4 time points (1 day, 8 days, 15 days, and 29 days).

3.2 *In vivo* experiments

These experiments were designed to meet specific aims #3 and #4 of the thesis: to determine if transplanted autologous cultured chondrocytes (with or without an engineered matrix), or if engineered matrices alone could increase the amount of total reparative tissue or hyaline cartilage in healing articular cartilage defects in a canine model. An initial study was performed in an effort to develop the animal model, including methods of creation of the defect and quantification of results, and to determine the baseline healing of the lesion created in the model. A series of experiments was then performed to establish methods of implanting matrices, culminating in a final experiment designed to test the effect of an engineered matrix in the model. These results were compared to those using similar matrices in culture to interpret the *in vivo* vs. *in vitro* effects.

3.2.1 Effects of autologous transplanted cells under a periosteal flap

In this experiment, an animal model for a clinical treatment for articular cartilage repair in defects, limited to articular layers, was developed and the course of natural repair studied in untreated defects. Healing was evaluated primarily by quantitative determination of the area occupied by selected types of reparative tissue found in the defect. In examining the use of cultured autologous chondrocytes, implanted under a periosteal flap, comparisons were made between the treatment group and two control groups: one receiving the identical treatment without cells, and that of untreated defects. Finally, as the experimental design included sacrifice times from 6 weeks to 18 months, an evaluation of the time course of healing in this defect type was performed. The distribution of defects among the treatment group (cultured autologous chondrocytes, or CAC) and the two control groups (periosteum, or P, and empty controls, or EC) are shown in Table 3-4. Note that in order to limit the scope of the experiment, periosteum controls were omitted at 3 and 6 months.
Table 3-4 Number of defects evaluated in autologous chondrocyte transplantation study. The time given is the time for the right knee receiving treatment and external fixation. Times for the left knees with untreated defects was three weeks longer. * 6/18 defects were in the right knee for 6 weeks with external fixation, 12/18 defects in left knee for 9 weeks.

All defects were evaluated by histological, immunohistochemical, and a quantitative histomorphometric method.

3.2.2 Effect of matrix composition and cell seeding

This series of studies examined effects on healing in the animal model of a) the microfracture technique, b) the chemical composition of a matrix, and c) two methods of introducing cells into the matrix within the defect. The constructs used were: unseeded type I matrices, type I matrices seeded with chondrocytes, and type II matrices seeded with chondrocytes. Treatment groups in these studies were compared to each other and to untreated controls from the study in 3.2.1 to determine the effect of the treatments. The number of defects studied and conditions of implantation is shown in Table 3-5. In the final phase of the study, a matrix was engineered with a specific pore structure and studied in conjunction with two clinical treatments for cartilage defects. The 15-week time point was chosen based on the findings of the initial animal study showing this to be within the phase of remodeling and in the range where the maximum value of hyaline cartilage was obtained.

Table 3-5. Summary of experimental groups in initial experiments with collagen-GAG matrix implants in a canine model.
All defects in these studies were evaluated by histological, immunohistochemical, and a quantitative histomorphometric method.

3.2.2.1 Studies with type I matrices

Two studies were run using type I collagen matrix made from a bovine hide source (section 4.1.1): one using an unseeded matrix, and the other using an autologous chondrocyte-seeded sponge. 4-mm diameter cores, approximately 1-1.5 mm thick, were cut from a large sheet. In the first study, unseeded matrices were implanted into 24 defects in 6 mongrel dogs and covered with an autologous fascia cover. Both knees of the dog were operated, but 11 weeks apart. Each knee received one defect with only fascia, and one defect with a fascia-covered matrix. Animals were sacrificed 4 weeks after the second procedure, providing time periods of 4 and 15 weeks for the respective knees. Thus, for each time period (4 and 15 weeks) and each treatment (fascia only and matrix with fascia), 6 defects from six different knees were studied.

In the second experiment, 8 defects in 4 mongrel dogs were used. Each dog underwent a harvest procedure in the left knee approximately 2 weeks prior to the actual implantation. Cells were recovered and cultured as described in section 4.2.1. Matrices, identical to those just described, were seeded with autologous cultured cells and implanted into all 8 defects. Autologous fascia was used as a cover for the matrix.

3.2.2.2 Studies with seeded type II matrices

A second cell-seeded study was performed with matrices containing predominantly type II collagen. The matrices used in this experiment are Chondrocell matrices, batch 915905, identical to those used in culture experiments of the nature of the chemical composition of the matrix. These matrices, approximately 2 mm thick, were cored with a 5-mm dermal punch and UV cross-linked for 4 hours on each surface. 8 defects were prepared in 4 mongrel dogs. Each dog underwent a harvest procedure in the left knee approximately 2 weeks prior to the actual implantation. Cells were recovered and cultured as described in section 4.3. Type II matrices seeded with approximately 1 million autologous cultured cells were implanted into all 8 defects. A 6-mm diameter type II collagen sponge cover, sutured to the surrounding articular cartilage, was used to contain the implant within the defect.

3.2.2.3 Engineered type II matrix and microfracture

The objective of this experiment was to determine the behavior of an engineered type II matrix in defects to which endogenous chondrogenic cells were given access through holes perforating the subchondral plate (microfracture). In addition, the use of the endogenous cells from
Microfracture was compared to autologous chondrocyte transplantation with an intact subchondral plate. The following groups were used to make the following assessments:

Effect of matrix: Microfracture group vs. microfracture plus matrix group.
Effect of cell source for healing: Microfracture group (subchondral cells) vs. cell-seeded matrix group (autologous cultured cells).
Effect of microfracture: Microfracture group vs. untreated defects (from 3.2.1).
Effect of specific cell transplantation method: Cell-seeded matrix group vs. autologous cultured chondrocyte treatment group (from 3.2.1).

The type II matrix for this experiment was manufactured under conditions identical to those used to produce the medium-pore size matrix described in section 4.1.1.2.2. Twelve adult hound dogs were used for this study. Four underwent a harvest procedure on the left knee for autologous chondrocyte transplantation. Two defects were made in the right knee of each animal, and both defects received the same treatment. The three experimental groups were:

I. Microfracture alone,
II. Microfracture plus unseeded type II matrix, and
III. Seeded type II matrix.

A new cover, consisting of a thin film made from type II collagen slurry was introduced in this work. The distribution of defects is shown in Table 3-6.

The effect of the treatments was studied by comparison of the quantified amounts of different tissue types filling the defect among these three experimental groups and historical groups (section 3.2.1).

<table>
<thead>
<tr>
<th>group</th>
<th>dogs</th>
<th>defects</th>
<th>cover</th>
<th>CAC</th>
<th>time (wks.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>microfracture</td>
<td>4</td>
<td>8</td>
<td>none</td>
<td>no</td>
<td>15</td>
</tr>
<tr>
<td>microfracture + matrix</td>
<td>4</td>
<td>8</td>
<td>type II film</td>
<td>no</td>
<td>15</td>
</tr>
<tr>
<td>cell-seeded matrix</td>
<td>4</td>
<td>8</td>
<td>type II film</td>
<td>yes</td>
<td>15</td>
</tr>
</tbody>
</table>

Table 3-6. Distribution of defects in study of cell-seeded matrices and microfracture in a canine model.
4. Materials and Methods

4.1 Collagen-GAG analogs of extracellular matrix

4.1.1 Fabrication

Fabrication of matrices involved three steps. First, a slurry was made predominantly of collagen digested from a specific connective tissue. The slurry was then placed in a mold, frozen, and the resulting ice crystals lyophilized to create a porous structure. The third step was cross-linking of the resulting matrices.

4.1.1.1 Collagen slurries

Type I and type II collagen slurries were obtained from the USDA and two companies. The type I slurry was made from type I collagen described by one company as approximately 99% pure. The type II slurry was described as predominantly type II collagen, but no detailed chemical analysis was provided with the solution.

Different types of slurries were used to produce matrices comprised predominantly of type I or type II collagen. The type I slurries were made with pure collagen and glycosaminoglycan, while the type II slurry was reconstituted from animal tissue.

4.1.1.1.1 Type I collagen slurries

Two different sources of type I collagen were used in these experiments: 1) a purified USDA bovine hide source (type I hide collagen) was used to produce type I collagen matrices for the initial studies in vivo; and 2) a purified bovine tendon source from Integra life sciences (type I tendon collagen) was used to produce matrices for in vitro assessment of chondrocyte-matrix interactions.

Type I hide collagen slurry: Before the present work was begun, the following steps were carried out [180]: type I collagen, purified from bovine hide, was obtained in the form of a ground slurry from the H. I. Sinnamon (USDA, Philadelphia, PA) circa 1980. The collagen slurry was lyophilized and ground to small size using a 20 mesh screen in a Wiley mill (Thomas-Wiley Intermediate Mill 3383-L10 series, Arthur H. Thomas Co., Philadelphia, PA), then stored at 4°C until 1988. The ground collagen was then stored at -20°C until use in 1994.

For the present work, two blenders fitted with cooling jackets were equilibrated to 4°C (Eberbach Waring restaurant model blender, Ann Arbor, MI) then filled with 1.65g wet collagen in 600 ml 0.05M acetic acid solution. The solution was blended at high speed (approximately 23,000 rpm) for 1 hour. A solution of
1.1 g GAG (chondroitin-6-sulfate from shark cartilage, Cat. # C-4384, Sigma Chemical, St. Louis, MO) in 1 L 0.05M acetic acid was prepared. While still blending at high speed, 120 ml GAG solution was added to the collagen solution in the blender over 15 minutes by a peristaltic pump (Manostat cassette pump, Manostat, New York, NY). The complete collagen-GAG slurry was blended an additional 15 minutes. The complete slurry was decanted into a separate plastic bottle for each blender and centrifuged at 2200 rpm for 60 minutes at low temperature (swinging cup centrifuge, IEC CRU-5000, International Equipment Co., Needham, MA). 420 ml (out of 720 ml) was decanted and discarded. The 300 ml of concentrated solution from each bottle was combined, rebled at low speed (approximately 14,000 rpm), and placed into a vacuum flask for degassing under vacuum. The slurry was used immediately of stored for future use.

**Type I tendon collagen slurry:** Type I collagen, purified from bovine tendon, was obtained from Integra Life Sciences Corp., Plainsboro, NJ. The complete specific protocol for producing the slurry is found in Appendix A. 13.69g wet collagen was dissolved in a 0.05M acetic acid solution and blended in at 40°C (Eberbach Waring restaurant model blender, Ann Arbor, MI) at high speed (approximately 23,000 rpm). GAG in the form of chondroitin-6-sulfate from shark cartilage (Cat. # C-4384, Sigma Chemical, St. Louis, MO) was slowly added to the blender by a peristaltic pump (Manostat cassette pump, Manostat, New York, NY) to complete the slurry. The slurry was degassed and stored for use. The slurry pH was approximately 3.5, as measured by pH papers.

**4.1.1.1.2 Type II collagen slurry**

Type II collagen slurry was provided by Geistlich Biomaterials, Wolhusen, Switzerland (Chondrocell slurry). The exact composition of the slurry was not given, however, it is known that the material was reconstituted from porcine cartilage and contains predominantly type II collagen. The same batch of slurry, numbered 901037, was used for all experiments.

**4.1.1.2 Freeze-drying**

Freeze-drying consisted of two distinct steps: freezing and lyophilization (sublimating the ice crystal under vacuum). In this work, all slurries were frozen flat on trays. For all experiments, the freezing took place in the same machine in which the vacuum apparatus was found. However, two different machines were used in different parts of the research.

**4.1.1.2.1 Freeze-drying bovine hide collagen**

Freeze-drying of USDA hide collagen was performed in a single batch. 190.5 ml slurry was poured into each half of stainless steel tray at room temperature.
The approximate dimensions of each half of the tray were 21 cm x 29 cm. An FTS freeze-drying unit was used (triphilizer model, FTS Systems, Albany, NY). The lower shelf was precooled to -45°C, then the tray with slurry was placed on the shelf and allowed approximately one hour to freeze before turning on the vacuum pump in the unit. When the pressure in the chamber dropped below 200 mtorr (approximately 1 hour), the temperature was raised to 0°C and the system left overnight. This procedure produced a matrix varying from 1 to 4 mm thick across the sample.

Small pore type I matrices were manufactured by lowering 4-mm diameter tubes into a freezing bath as described by Louie [102].

4.1.1.2.2 Freeze-drying of bovine type I tendon collagen and of type II collagen

Freeze-drying of bovine type I tendon collagen and Chondrocell type II collagen slurry was performed in a VirTis Genesis freeze-drying unit (VirTis, Gardiner, NY). Freeze-drying conditions varied from batch to batch in efforts to control pore characteristics. The slurries were prepared and degassed as described above. Stainless steel freeze-drying pans were cleaned and prepared with appropriate molds. In some cases the pans were pre-cooled to a low temperature, but in others used at room temperature. This yielded different pore structures. Approximately 3 ml of slurry per 8 cm² of pan area was used to yield a matrix of approximately 3 mm thickness.

Type I matrices: Type I tendon sheet matrix was produced in a single batch (HB95-1). One stainless steel tray, taken at room temperature, was used as a mold. The mold was divided into three separate sections, each measuring 16 cm x 25 cm. Slightly different volumes of slurry were used to produce matrix of different thickness. In one section of the mold 160 ml of slurry was poured. In a second section of the mold 125 ml of slurry was poured. The third section was left empty. The shelf temperature in the freeze drier was set to -45°C. After freeze-drying overnight (see Appendix A), the thickness of the respective sections were 3 to 4.25 mm and 1.5 to 4 mm.

Type II Chondrocell matrices. All matrices designated Chondrocell were provided in solid form (already freeze dried) as approximately 2 cm x 2 cm square samples, 2-4.5 mm thick, by Geistlich Biomaterials. These materials were manufactured by the company freeze-drying a slurry reconstituted from porcine collagen. Most samples were provided not cross-linked. Certain batches provided for experiments designed to study the effects of the degree of cross-linking were provided in various states of cross-linking (Table 4-1).

Pore size controlled type II matrices: All matrices for pore size experiments in type II collagen were manufactured in the Fibers and Polymers Laboratory at MIT. The starting material was a collagen slurry reconstituted from porcine
batch | cross-linking
--- | ---
914106 | not cross-linked
914206 | DHT cross-linked* 1 day
914306 | DHT cross-linked* 2 day
914406 | DHT cross-linked* 3 day
914506 | DHT cross-linked* 4 day

Table 4-1. Cross-linking conditions for 914 series of matrices. *DHT conditions: 105°C, under vacuum

cartilage, Chondrocell Slurry, batch #901037, Geistlich Biomaterials, Wolhusen, Switzerland (section 4.1.1.1.2). The slurry was stored at 4°C throughout the experiments. Initial experiments were performed to estimate pore sizes formed at different freezing temperatures and conditions. The temperature of the slurry, the shelf temperature of the freeze dryer, and whether or not the freezing tray was pre-cooled were all found affect the final pore size and architecture. Molds were made by sanding the bottom from an 88-mm diameter plastic petri dish (Falcon #3003, Fisher Scientific, Cat. #08-772-E, Springfield, NJ), leaving a plastic ring of area approximately 60 cm². This ring was secured to a freezing tray by four pieces of tape. A standard temperature of 109°F was used to melt the slurry. The slurry was poured/scraped from the storage bottle to a beaker, which was in turn placed into a water bath maintained at 109°F. Several minutes were required to meld the matrix after attaining this temperature.

Two conditions, based on the trial experiments, were selected to produce pores of different size. To insure reproducibility of pore structure, the same trays were used in repeating the synthesis procedure multiple times, as the resulting pore structure was found to vary somewhat from tray to tray. These trays were chosen such that they were flat-bottomed in the areas used, assuring good contact between the freezing shelf and the tray.

Condition 1: The freeze drier was pre-cooled and shelves equilibrated to -20°C. A full size stainless steel tray (25 cm x 49 cm) was fitted with two 88 mm petri dish molds on opposite ends, and maintained at room temperature. The slurry was melted at 109°F. A 25-ml serological pipet warmed in an oven to 55°C was used to transfer 24 ml of slurry into each mold on the tray. The slurry was allowed to cool at room temperature for at least 30 minutes, with the tray placed flat on a benchtop. The tray was then placed on the bottom freeze drier shelf. Freezing of the slurry was complete within 40 minutes and lyophilization was begun, and continued overnight. Samples cored from the outer 9 mm of the diameter of the final freeze-dried product were considered “small” pore size. Samples cored from the center 5-6 cm diameter were considered “medium” pore size (Figure 4-1).
Figure 4-1. Schematic of coring of small (S) and medium (M) pore size matrices from an 88-mm diameter sheet of matrix. Individual cores are 9 mm in diameter.

Figure 4-2. Trimming of small pore matrices from edge of sheet.
Condition 2: A freezing tray, fitted with one 88 mm petri dish mold, was pre-cooled in the freezedryer with the shelf temperature set to -25°C. The slurry was melted at 109°F. A second freezing tray was placed on the bench immediately adjacent to the water bath. 24 ml of slurry was drawn into a 25 ml serological pipet warmed in an oven to 55°C and rested across the water bath. Immediately, the following steps were performed as rapidly as possible, taking a total of approximately 20 seconds: 1) The tray with a mold was removed from the shelf at -25°C (freezedryer door was shut briefly) and placed on the benchtop in a tilted position. One edge was elevated by the tray already on the bench top, the other edge resting on the benchtop itself. 2) The 24 ml of slurry was pipetted into the 88-mm petri dish mold, specifically into the lowest point of mold, as determined by it's tilted position. 3) Immediately after the slurry was transferred, the tray was leveled and shaken to evenly disperse the slurry. This took place while stepping toward the freezedryer. 4) The freezedryer was reopened, the tray placed on the bottom shelf, and the door closed. Note, that because of the urgency in carrying out these steps rapidly and reproducibly, only one mold at a time could be filled, at least until the previous mold was frozen and the freezedryer re-equilibrated. The sample was completely frozen within 35 minutes. Samples were cored from the center 6 cm of the sample and considered "large" pore size.

At least ten pieces of matrix in each of conditions 1 and 2 (each an 88 mm diameter sheet of thickness approximately 3 mm) were made. For coring of matrices for the actual experiment, three pieces from condition 1 and four pieces from condition 2 were selected. Approximately 100 large-, 66 medium-, and 62 small-pore 9-mm cores were punched with a brass cork borer and hammer from these samples. Samples for characterization and experimentation were selected randomly from these stores. It was necessary to trim a small outcropping of matrix from the small samples that had formed from the meniscus of the slurry up against the plastic mold at the outer edge of the sample (Figure 4-2). These samples were cross-linked for 12 hours total (6 hours on each side) under UV light (section 4.1.1.3).

4.1.1.3 Cross-linking

Dehydrothermal (DHT) cross-linking was performed in a vacuum oven (Fisher Isotemp vacuum oven, Fisher Scientific, Medford, MA) at 105°C and approximately 1 to 3 mmHg for 24 hours.

Ultraviolet cross-linking was performed at a standard distance (30 cm) from an ultraviolet lamp (Philips Sterilamp #G10T5 1/2 L, λ = 253.5 nm) in a sterile tissue culture hood. The UV lamp was rated at 5.3 W total output, 55 μW/cm² at 1 m. Cross-linking time was varied from 8 to 16 hours, with the material being flipped half way through to expose both surfaces to approximately the same radiation.
4.1.2 Characterization

Pores were observed by scanning electron microscopy (to provide images with excellent depth of field) and by light microscopy for the purpose of quantitative pore characterization.

4.1.2.1 Scanning electron microscopy

Scanning electron microscopy was performed with a JEOL model #6320 field emission gun scanning electron microscope (Tokyo, Japan). Samples were coated with carbon with a Varian vacuum thermo-evaporator.

4.1.2.2 Quantitative pore characterization

Quantitative pore characterization involved several key steps: 1) embedding, 2) sectioning, 3) capturing and preparing a digital image, and 4) stereological analysis of the image. The first two steps were critical for two reasons. First, the pore structure of the original matrix must be maintained. Second, the success of these two steps, as reflected in the quality of the sections prepared, was critical in determining the ease of image preparation in step 3. It was desired to provide the method of embedding and sectioning which would allow for the least time spent on image analysis while still capturing a true image.

In order to determine the best method of embedding and sectioning the matrices, several variables in the embedding process were varied including fixation of matrices (to use formalin or not), method of dehydration (including the order of solutions, and the use of a machine or not), and embedding medium (paraffin vs. plastic). Experiments included measuring the gross dimensions of the matrices before and after these treatments, and embedding and comparing some combinations for ease of sectioning and quality of sections. One semiquantitative experiment was performed to determine whether cross-linking or formalin fixation affected the quality of matrix sections obtained from plastic embedding. Twelve 7-mm matrix cores were chosen at random from a single source of type II matrix produced in our laboratory. The matrices were divided equally into a cross-linked group, (6 matrices, UV cross-linked for 12 hours), and a not cross-linked group (6 matrices). From each group, 3 matrices were placed in formalin for 16 hours, then dehydrated by hand to 100% ethanol, while the other 3 matrices were placed directly in 100% ethanol. The matrices were then embedded in paraffin, and sectioned blindly without knowledge to treatment group. The ease and quality of sectioning was rank ordered from 1 to 12, then the treatments revealed. Two analyses were performed using the Mann-Whitney ranked order test: one with the matrices grouped by cross-linking treatment, and a second with the matrices grouped by formalin treatment.
Qualitatively, the response of the matrices to the various treatments varied slightly with matrix stiffness, collagen type, and pore size, but overall the following observations were made:

1) Placing the matrices directly into alcohol created the least change in pore size due to solution. Formalin or water sometimes caused significant (though sometimes reversible) changes in matrix dimensions.

2) Most matrices were damaged significantly by the automatic dehydrating machine (Tissue Tek VIP 1000), possibly due to the vacuum suction and pressure used to change solutions. It is therefore necessary to dehydrate all matrices by hand.

3) Paraffin sections did not preserve the pore structure as well as plastic

4) Paraffin section provided the advantage of much less background in staining.

5) The blinded experiment suggested that improved sections were obtained from type II matrices if: 1) the matrices were NOT UV cross-linked (p=0.05, and 2) the matrices were NOT placed in formalin before dehydration (0.05<p<0.10).

Conclusion: Although embedding in plastic created more background staining (and thus more difficulty in the pore analysis), it was preferred as more consistent and accurate means of preserving pore structure during embedding and sectioning. Still some problems persisted, including crumbling or tearing out of some of the matrix material during sectioning. It was necessary to manually delete certain background elements and to replace matrix material which was torn away in the final digitized image. Thus, official pore analysis was done following the following protocol with embedding in plastic medium.

Matrix pore characteristics, including average pore diameter, percent porosity, and matrix wall thickness were evaluated from digital images. The detailed protocols are presented in Appendix B. Briefly, matrices selected for evaluation were embed in glycol methacrylate (JB-4 embedding system, Polysciences, Inc., Warrington, PA) and 5 μm thick histological sections were prepared. Digital images were captured via videocamera (Hamamatsu CCD Camera Control #C2400 with camera), edited to eliminate background and provide a complete image of pore walls (Scion Image 1.60c), and analyzed by an automated image analysis computer program (Digit) developed for our laboratory. This program uses the method of parallel secants to assess pore diameter, as described previously [102].

The methods of sampling matrices for analysis were as follows:
Multiple pieces of each type of matrix to be characterized were removed at random from the same supply from which samples were taken at random experiments. The samples used were:

**Geistlich type II matrices:** Two groups were examined:

1) Three 9-mm cores were selected from batch 914106 (This batch was one of five used in the experiment on the effect of cross-linking, and was used for the majority of additional experiments with this material).

2) Three 9-mm cores were selected from batch 914306, one of four made in the same manner as 914106, but subjected to varying degrees of cross-linking for the experiment to determine effects of cross-linking.

**Type II pore size experiment matrices:** Three types of matrices, designated Small, Medium, and Large, were produced in this laboratory and were used in *in vitro* experiments (see section 4.1.1.2.2). Three 9-mm cores from each group were analyzed.

**Canine implant type II matrices:** These matrices were produced in our laboratory in the same manner as the medium-pore group of pore diameter experiments. Three matrices were chosen randomly from the extra samples taken to the operating room.

For each of the individual samples, at least three images representative of the overall pore structure were captured. These images were taken from across the breadth of the sample, in some cases from different serial sections. On each image, two circular regions were chosen for analysis. The circles were constructed as large as possible to include as many pores as possible. Due to the size of the images, the circles overlapped significantly, and therefore contained many of the same pores. Thus, the values for the pore diameters from the two regions were averaged to provide a single value for each image.

The values for the three images from a given sample were then averaged and reported.

**Additional pore diameter analysis:** Analyses on several samples were performed using the same embedding and image analysis techniques, as reported previously [102, 130]. The samples analyzed included:

- A sample of Geistlich type II collagen from previous batches used for initial *in vivo* studies and *in vitro* comparison of type I and type II materials.
- A sample of type I collagen used for *in vitro* comparison of type I and type II materials.
- small pore size type I material made in tubular form by Libby Louie [102], used for \textit{in vitro} comparison on matrix pore size of type I collagen materials.

4.1.2.3 \textit{Chemical characterization}

The glycosaminoglycan content of freeze-dried matrices was measured and expressed as a weight percent. Three pieces of matrix (9 mm cores or 1 cm x 1 cm squares, approximately 1.5-4 mm thick) were selected from each of the following matrix groups:

1) Type I hide
2) Type I tendon
3) Type II Geistlich (batch 914306)
4) Type II small-pore
5) Type II medium-pore
6) Type II large-pore

The matrices were vacuum lyophilized and immediately weighed on a microgram balance. The matrices were then digested with papain and assayed for GAG content by the dimethyl-methylene blue method (see section 4.2.2). The weight percent of GAG for each individual piece was calculated.

In addition, for types I hide and tendon collagens, the expected value, based on the dry weights of the purified raw materials, was calculated.

4.1.3 Preparation of matrices for experiments

Matrices for experimental use were cored from larger sheets with either a dermal punch (4 mm or 5 mm in diameter) or brass cork borers (for diameters over 5 mm). All coring was performed under sterile conditions in a laminar flow tissue culture hood on a sterile teflon cutting board. Matrices were stored in sterile tubes or petri dishes at room temperature until use.

4.2 \textit{In vitro} experiments

4.2.1 Methods of seeding matrices with chondrocytes

Seeding of chondrocytes in matrices involved several steps. First, articular cartilage was harvested from canine knee joints. The chondrocytes were then isolated from the harvested cartilage, expanded in culture, in some cases frozen, recovered from culture, and actually seeded into the matrices. These procedures are reviewed in this section.

4.2.1.1 \textit{Chondrocyte isolation from cartilage}

Cartilage was harvested in sterile procedures in the operating room either from dogs undergoing survival surgery or as soon as possible after sacrifice or animals
undergoing other studies. Shavings were taken with a scalpel, with care being taken not to take bone, synovium, or other surrounding tissues. Cartilage was temporarily stored in PBS supplemented with 1% Penicillin/Streptomycin and 0.5% Fungizone kept on ice. Digestion was performed within 4 hours of harvest. The cartilage shavings were diced to less than 1 mm squared pieces and placed in a spinner flask in sterile filtered collagenase solution. Slightly varying collagenase concentrations were used based on the desired digestion time and collagenase activity. The most often used and recommended concentration was 0.15% collagenase in DMEM/F12 supplemented by 2% penicillin/streptomycin (20,000 U / 20,000 μg per 100 ml) and 1% fungizone (collagenase activities ranged from 212 to 370 U/mg). The solution was placed in a tissue culture incubator (37°C, 5% CO₂) on a magnetic spinner plate (Thermolyne cellgro stirrer type 45600, Dubuque, IA) for 4 hours, with the spinner set for the slowest speed consistent with keeping the cartilage pieces suspended. Digested cartilage was filtered through 70μm nylon cell strainers (Falcon #2350, Fisher #08-771-2) and spun down (1500 rpm X 10 min.) in 50 ml centrifuge tubes. The digest was rinsed 2-3 times by resuspension in tissue culture medium followed by similar centrifugation. The cells were counted in a hemacytometer (including trypan blue viability assay), resuspended one final time, and plated into tissue culture flasks for expansion. Plating density was approximately 200,000 to 2 million cells/75 cm² flask.

4.2.1.2 Culturing of chondrocytes in monolayer culture

Cells were cultured in standard 75 cm² polystyrene tissue culture flasks (Falcon #3084, Fisher #08-772-1). Complete culture medium was made as follows (end values in parentheses indicate amount to make 500 ml total):

- Base medium: Dulbecco's modified Eagle Medium/Nutrient Mixture F12 (HAM), 1:1 mixture, (DMEM/F12), Gibco #11320-033. (420 ml)
- 10% Fetal Bovine Serum (FBS), Hyclone #SV 3002.01. (50 ml)
- 2% Penicillin (5,000 U/ml) /Streptomycin (5,000 μg/ml) Gibco #15070-063. (10 ml)
- 1% L-glutamine (2.5 mM), Gibco #25030-081. (5 ml)
- 2% Ascorbic acid (solution of 0.125 mg, Gibco #13080-023 in 10 ml DMEM/F12). (10 ml)
- 1% Fungizone, Gibco #15295-017. (5 ml)

All Falcon supplies obtained through Fisher Scientific, Springfield, NJ.
All Gibco items obtained from Life Technologies, Grand Island, NY.

Culture medium was changed as indicated by yellowing of the pH indicator in the medium, approximately every two days. Cells were recovered by trypsinization for seeding, passaging, or other uses. The cells were released by 5 ml 0.05% trypsin, 0.53 mM EDTA-4Na, 1x liquid (Trypsin/EDTA, Gibco #25300-
062), and rinsed 3 times in complete medium before being concentrated and allocated as needed.

4.2.1.3 Seeding of matrices

Seeding of matrices occurred in three main steps: 1) pre-wetting/sterilization, 2) cell recovery from 2-dimensional culture in which the cells were proliferated, and 3) the introduction of the cells into the matrices. The specifics of these procedures are contained in Appendix C. Below, the experiments designed to study seeding conditions are first described, then the conditions of seeding for all other experiments.

4.2.1.3.1 General methods used in cell seeding

Matrices were either pre-wet or used dry. Most matrices were pre-wet. These matrices were first sterilized for 10 minutes in 70% ethanol. Ethanol was rinsed by 5 x 10 minutes in PBS, and 2 x 10 minutes in culture medium. The matrices were compressed lightly with rounded-end forceps at each step to ensure thorough rinsing. Pre-wet matrices were stored in medium while cells were being recovered. Matrices used dry were kept in sterile packaging until needed.

Cells were then collected by trypsinization of cell culture flasks and stored temporarily in complete culture medium while matrices were prepared in their wells for seeding.

In preparation for seeding, matrices were placed either in empty plastic wells (12, 24, and 96-well plates, Falcon #’s 3043, 3047, and 3072, Fisher #’s 08-772-29, 08-772-1 and 08-772-2C), or wells lined with agarose or polyhydroxyethylmethacrylate (polyHEMA). Agarose-lined tissue culture plates wells were prepared at least several hours prior to the experiment. 2% agarose (Bio-Rad #062-0100, Cambridge, MA) was autoclaved and aliquotted to cover the bottom of the wells being used (the amount depending on the size of the wells). PolyHEMA-coated wells were prepared at least 16 hours in advance. PolyHEMA was coated onto wells in a thin film by filling wells with a solution of 0.3 gm polyHEMA in 100 ml 95% ethanol and letting the alcohol evaporate overnight in a sterile hood.

Matrices were moved to their wells in one of two conditions: 1) “wet”: pre-wet with medium and transferred by forceps directly to a well for seeding, or 2) “filter-dried”: placed on filter paper to partially dry the matrices before being placed in their wells. Matrices were let dry as much as possible without drying so much as they would collapse irreversibly, as estimated from trial and error. All matrices were placed in the center of the tissue culture plate wells in which they would be seeded.
Cells were resuspended at the desired concentration (typically at 25 million cells/ml) and placed on the matrices by drops from a micropipettor. When many matrices were seeded, the order of seeding matrices was chosen to ensure that matrices in each experimental group received aliquots spaced relatively evenly throughout the seeding process. Matrices in some experiments were seeded on both surfaces. In these instances, after one surface of all matrices were seeded, the matrices were flipped over in their wells and the second surface then seeded. The seeding of the second side was performed in reverse order of the first side. Seeded matrices were placed in the tissue culture incubator for 1-3 hours before they were transferred to tissue culture 24-well plates into wells with 1.5 to 2 ml complete tissue culture medium.

4.2.1.3.2 Pilot studies to determine optimal seeding conditions

In these studies, matrices were seeded with chondrocytes and examined for DNA content within 24 hours. These samples were prepared for DNA analysis by protease digestion. To terminate each experiment, samples were removed from the wells, placed in microcentrifuge tubes, and put directly in a -70°C freezer. The chondrocytes used for seeding were all second passage canine chondrocytes, cultured as described earlier in section 4.2.1. Six separate experiments were performed, as described below.

Effect of medium addition: Forty 9-mm diameter cores of type II Chondrocell matrix (not cross-linked batch 914106) were used and prepared for seeding by filter paper drying and centered in agarose lined wells in 24-well plates. Approximately 940,000 cells were seeded onto one surface of each matrix in a 40 µl aliquot of cell-medium suspension. Four matrices were left without cells. The matrices were placed in the incubator. One group of four matrices was terminated at each of the following times: 20 minutes, 1 hour, 3 hours, and 6 hours. One extra sample was processed for histology at each of these time points. At each of these same times, an additional group of four matrices was transferred to a well containing 1.5 ml of culture medium and placed back in the incubator for termination at 24 hours. Finally, at the one hour time point, a third group of 4 matrices was added to wells containing medium. At 24 hours, the medium was changed, then the samples terminated as just described.

Effect of cell number and flipping matrices: Three groups were used in this experiment to determine the effect of the number of cells delivered to the matrix, and of seeding on one or both sides. Most matrices were found to have one surface more porous than the other (section 5.1):

1) 1 million cells delivered to more porous surface only
2) 2 million cells delivered to more porous surface only
3) 2 million cells delivered: 1 million to one surface, flipping the matrix after a few minutes, then 1 million to the opposite surface.
Four 9-mm cores of type I bovine tendon matrices were used in each group. Each million cells were delivered in 40 μl of medium. The 2 million cell aliquot was delivered in 60 μl medium. Samples were terminated after 3 hours.

**Effect of experimenter and matrix type:** To determine the effect of the experimenter in seeding cells, the number of cells contained in 9-mm diameter cores of two different types of matrices was compared: Chondrocell type II matrices, and type I bovine tendon matrices. One million cells in 40 μl were delivered to the porous side only, with the cores placed in agarose-coated wells. Samples were terminated after 3 hours. The number of samples reported is shown in Table 4-2:

<table>
<thead>
<tr>
<th>Experimenter</th>
<th>Type I</th>
<th>Type II</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>SM</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 4-2. Matrices used in seeding experiment of experimenter and type of matrix (number of matrices seeded)

**Effect of coating of culture wells:** This experiment examined the effect of coating of culture wells on the number of cells retained in matrices. Three groups were examined: uncoated wells, polyHEMA-coated wells, and agarose-coated wells. Agarose coatings were achieved by allowing 0.5 ml 2% Bio-Rad agarose and 120 μl medium to solidify in each 24-well plate. Three to four drops of this mixture were used in 96-well plates.

Two conditions were used to test these groups: 9-mm cores of type I bovine tendon matrix in 24-well plates (an unconfined condition), and 5-mm cores of type I bovine tendon matrix in 96-well plates (a partially confined condition). 4 cores of each size for each coating were filter dried and seeded with 1 million cells in 40 μl. After 2 hours in an incubator, the samples were terminated and the cells counted by DNA assay.

**Effect of filter-drying:** Matrices were seeded in one of two conditions: 1) "wet" or 2) "filter-dried". Two sub-experiments were run on separate days. In the first experiment, cores of bovine tendon matrix of 5 mm, 7 mm, and 9 mm were used in uncoated 24-well plates, each with n=4 wet matrices and n=4 dried matrices. 1.3 million cells in 40 μl were seeded onto each matrix. Matrices were incubated for 2 hours, then terminated for DNA count.

In the second experiment, 5-mm cores of bovine tendon matrix were confined in 96-well plates coated with polyHEMA, coated with agarose, or uncoated, each with n=4 wet matrices and n=4 dried matrices. 1 million cells in 40 μl were
seeded onto each matrix. Matrices were incubated for 2 hours, then terminated for DNA count.

**Effect of confinement of matrices:** This experiment was designed to test whether preventing the cell suspension from escaping from the periphery of the cores to be seeded would increase the number of cells in the matrix. Five-mm diameter cores of type I bovine tendon matrix were used. In the unconfined group, the cores were seeded in the center of 24-well plates (well diameter approximately 16 mm). In the confined group, the cores were seeded in 96-well plates (well diameter approximately 6.4 mm). For each confined group, 4 matrices were seeded wet and 4 seeded filter dried. After 2 hours in an incubator, the samples were terminated for DNA assay.

4.2.1.3.3 Seeding of matrices for *in vitro* or *in vivo* investigation

In experiments where matrices were seeded on an agarose bed, agarose-lined tissue culture plates wells (12, 24, and 96-well plates, Falcon #’s 3043, 3047, and 3072, Fisher #’s 08-772-29, 08-772-1 and 08-772-2C) were prepared at least several hours prior to the experiment. Two percent agarose (Bio-Rad #062-0100, Cambridge, MA) was autoclaved and aliquotted to cover the bottom of the wells being used (the amount depending on the size of the wells). Matrices were first sterilized for 10 minutes in 70% ethanol. Ethanol was rinsed by $5 \times 10$ minutes in PBS, and $2 \times 10$ minutes in culture medium. Cells were then collected by trypsinization of cell culture flasks and stored temporarily in complete culture medium. The pre-wet matrices were placed in the center of the tissue culture plate wells in which they would be seeded. In some cases they were first partially dried by placing them briefly on sterile filter papers. Cells were resuspended at the desired concentration (typically at 25 million cells/ml) and placed on the matrices by drops from a micropipettor. When many matrices were seeded, the order of seeding matrices was chosen to ensure that matrices in each experimental group received aliquots spaced relatively evenly throughout the seeding process. Matrices in some experiments were seeded on both sides. In these instances, after one side of all matrices were seeded, the matrices were flipped over in their wells and the second side then seeded. The seeding of the second side was performed in reverse order of the first side. Seeded matrices were placed in the tissue culture incubator for 1-3 hours before they were transferred to tissue culture 24-well plates into wells with 1.5 to 2 ml complete tissue culture medium.

**Seeding conditions for *in vitro* testing of type I and type II matrices:** Second passage canine chondrocytes were obtained and resuspended at a concentration of $2 \times 10^7$ cells / ml. The sponges were pre-wet, placed directly into their wells, and seeded with $50 \mu l$ of the cell suspension containing one million chondrocytes on the more visibly porous side to provide optimal penetration of cells into the sponge. Pilot studies in our laboratory showed that a sponge at the size of 4 mm diameter can hold 50-80 $\mu l$ of fluid, and that
there was a trend to a higher seeding efficacy in the type II sponge. Culture medium (1.5 ml) was added 3 hours after seeding and changed every day.

Seeding conditions for *in vitro* degree of cross-linking pilot study. Cores were prepared by pre-wetting, filter drying, and placement in agarose-lined wells. Cells were resuspended at 25 million/ml and the cores were seeded with approximately 1 million cells in 40 μl on the porous side of the matrix.

Seeding conditions for *in vitro* degree of cross-linking long-term study. Cores were prepared by pre-wetting, filter drying, and placement in agarose-lined wells. Cells were resuspended at 40 million/ml and the cores were seeded in two steps. In the first step, 1.6 million cells in 40 μl were placed on every matrix. Approximately 10 minutes after seeding the first surface, half the matrices were turned over, and the opposite side was seeded with 1.6 million cells in 40 μl aliquots. 40 μl complete medium was added the matrices that received cells on only 1 surface. Seeded matrices were transferred to wells with medium after 2 hours.

Seeding conditions for *in vitro* testing of matrix pore size: The seeding conditions for seeding of type I matrices of different pore size (experiment #1), were identical to those of *in vitro* testing of type I and type II matrices as described above.

For the second experiment, seeding in type II matrices of varying pore size, the following conditions were used: canine chondrocytes were resuspended at a concentration of 25 million/ml. The matrices were pre-wet, dried on filter paper, and placed into agarose-lined 24-well plates. Both surfaces were seeded with 40 μl of cell suspension. The matrices were added to culture medium 2 hours after seeding.

Seeding conditions of initial experiments with type I and type II matrices for *in vivo* implantation: The matrices were prepared only by pre-wetting and placement in 24-well plates. Cells were suspended at a concentration of 20 million/ml. Seeding was performed by placing a 50 μl drop containing 1 million cells on the more porous surface of the matrix. After 1 hour, 0.5 ml medium was added to the well.

Seeding conditions of engineered type II matrices for *in vivo* implantation: Five-mm diameter cores of matrix were filter dried and placed in agarose-lined 96-well plates. Cells were suspended at 25 million/ml. A total of 1.5 million cells were seeded by 30 μl aliquots of 750,000 cells to each surface of the core. One hour after seeding, the matrices were placed in 24-well plates with 1 ml medium.
4.2.1.4 Frozen chondrocyte procedures

Freezing: Cell for freezing were first resuspended at a concentration of roughly 2 million/ml complete medium. 5% sterile filtered DMSO (0.45µ acrodisc filter) was added, and up to 3 ml aliquots placed in tubes for freezing (Nalgene 5 ml cryovial, #5000-0050). Vials were frozen in a tilted position in -20°C freezer, then transferred to a -70°C freezer after about 3 hours.

Thawing: Frozen tubes were removed from the -70°C freezer and disinfected. The tubes were placed in 37°C bath until the outside just turned liquid (less than 1 minute), then the tubes opened and add drops of pre-warmed complete medium slowly added to help thawing. When the tube was all liquid, it was re-capped and shaken to mix and suspend the cells. The resulting suspension was pipetted into a 50 ml tube and rinsed and spun three times before use.

4.2.1.5 Measurement of matrix contraction

Matrix contraction was assessed by measurement over time of the diameter of matrices cored as discs. A millimeter scale ruler was placed above the matrix core and read to the nearest half millimeter. Two readings were taken per matrix, along orthogonal diameters. For matrices which displayed shrinkage into an oblong shape, the measurements were taken along the short and long axes.

4.2.2 Biochemical analysis

Biochemical analyses of cell-seeded matrices in vitro included: 1) radiolabeled sulfate uptake (to measure rate of GAG synthesis at a given time point), 2) total GAG content (to measure the net GAG accumulated in the sample over a given time period), and 3) total DNA content (to estimate the number of cells in the constructs at a given time). For each assay, it was necessary to first digest the matrix to break down the large collagen molecules to allow binding of assay dyes their substrates, and to prevent interference from large molecules in the readings. Before digestion, all samples were freeze-thawed three times in the -70°C freezer to assist in lysing of cell membranes and releasing DNA from the nucleus. Samples were then frozen until the end of the experiment for DNA and GAG analysis.

4.2.2.1 Digests for biochemical analysis

Two different digests of cell-seeded matrices (or controls) were performed in this study. In the majority of experiments a papain digest was used. Papain was prepared from a commercially available 2x suspension in 0.05 M sodium acetate, pH 4.5 with 0.01% thymol, 24 mg protein/ml; 32 U/mg protein. (Cat. #P-3125, Sigma, St. Louis, MO). The complete protocol for DNA digestion is presented in Appendix D. In summary, 50 µl papain, 1 ml digestion buffer (17.54 ml 0.5M NaH₂PO₄, 2.46 ml 0.5M Na₂HPO₄, 80 ml dH₂O, 87.82 mg L(+)}
Cysteine HCl, and 186.12 mg Disodium Ethylenediaminetetraacetate (EDTA), pH 6.2), and the sample are combined and set overnight in a 65°C water bath.

For experiments investigating the effects of seeding parameters and the degree of cross-linking in type II matrices, a protease digest was used. Protease (Pronase E, Cat. #P 5147, Sigma Chemical Corp., St. Louis, MO) was prepared in TBS buffered at a concentration of 2.5 mg/ml. 1 ml of this solution was added to each sample and allowed to digest in a 55°C water bath overnight.

4.2.2.2 DNA quantification and cell counting

The detailed protocol for DNA assay is found in Appendix D. In summary, a TNE 10x buffer solution (100 mM TRIS, 10 mM EDTA, 1.0 M NaCl, pH 7.4) and concentrated Hoechst dye stock solution (1 mg/ml) were prepared. On the day of the assay, a fresh working solution working solution of dye was prepared with 90 ml dH2O, 10 ml TNE 10x buffer, and 10 to 100 µl concentrated Hoechst dye stock solution (dependent on the cell concentrations expected in the samples). Samples were centrifuged at 3000 rpm for 10 minutes, then a 100-200 µl aliquot of from the supernatant added to a glass cuvette and brought to 2.0 ml total with working dye solution. The sample was read immediately in a fluorometer (model TKO 100, λex=365 nm, λem=460 nm, Hoefer Scientific Instruments, San Francisco, CA). The fluorometer was scaled with standards of calf thymus DNA.

4.2.2.3 GAG determination

The same digests used for DNA assay were used for GAG quantification. Samples were vortexed to completely mix the samples, and a 100 µl aliquot was placed in a polystyrene disposable cuvette. 3 ml dimethylmethylene blue dye solution (32 mg 1,9-dimethylmethylene blue (DMB), 10 ml 100% ethanol, 2 L distilled H2O (d H2O), 4 ml 90% formic acid, pH 3.5, see Appendix D) was added to the cuvette. Samples were read for absorbance at 535 nm in a spectrophotometer (LKB Biochrom Ultrospec 4050, Pharmacia, Piscataway, NJ). Standards were obtained from shark chondroitin sulfate in dH2O.

4.2.2.4 Radiolabeled sulfate incorporation

The rate of synthesis of proteoglycan was assayed with the use of radiolabeled sulfate. 35-SO4 was obtained from New England Nuclear, Bedford, MA. Samples in 24-well plates (both seeded and unseeded matrices) were prepared by removing medium. 1.5 ml of complete medium containing 35-SO4 (10 µCi/ml) were added and the samples incubated (37°C, 5% CO2) for 4 hours. Unincorporated sulfate was removed by rinsing with a solution of culture medium (DMEM/F12) supplemented by 0.6 mM cold sulfate (from Na2SO4) for a total of 1.0 mM cold sulfate. A total of 12 rinses were performed over a period of 200 minutes, with the samples incubated an average of 10 minutes
between rinses. Confirmation of rinsing was documented by scintillation
counting of 100 μl aliquots of the recovered rinse solution. Rinsing was
stopped when the pooled rinse medium from each group read less than 50
cpm. Samples were freeze-thawed three times, digested in papain (section
4.2.2.1) and assayed for 35-SO\textsubscript{4} within 2 days. 100 μl of digest was combined
with 5 ml scintillation fluid (Scintiverse II, cat. # SX12-4, Fisher Scientific, Fair
Lawn, NJ) and counted for 2-5 minutes in a liquid scintillation counter
(Packard Tri-Carb 4640, United Technologies Packard, Downer’s Grove, IL).
Counting times were adjusted so the standard deviation of all counts more
than five times the background values was less than 5%.

Verification of sulfate incorporation into macromolecular forms: In order to
verify that scintillation counting was recording macromolecular GAG chains
(as opposed to unincorporated sulfate), several samples were analyzed by
chromatography to demonstrate the approximate molecular weight of the
radioactive species. Sephadex PD-10 columns (Pharmacia Biotech, Piscataway,
NJ) were used. The column is run by gravity, with all solutions pipetted
manually. The buffer consisted of 150 mM TRIS (Tris-(Hydroxymethyl)-
Aminomethane, C\textsubscript{4}H\textsubscript{11}NO\textsubscript{3}) pH’d to 6.6 with HCl (referred to as column buffer).
Columns were equilibrated with 25 ml buffer, then allowed to run almost dry
before adding 0.5 ml of sample. The sample was chased with 12 ml buffer
while fractions were collected manually in 1.5 ml microcentrifuge tubes,
typically 0.5 ml to 1 ml per fraction.

Aliquots of each fraction (100-300 μl) were mixed with 5 ml scintillation fluid
and counted for 4-5 minutes in a liquid scintillation counter. The larger
aliquots were used for groups with lower expected counts. Specifically, the
following columns were run:

Control column #1 (Unincorporated sulfate with high molecular weight dye): Sample consists of 400 μl saturated solution of Blue Dextran 2000, molecular
weight 2,000,000 (Pharmacia, Uppsala, Sweden) plus 100 μl of 35-SO\textsubscript{4}, 10
μCi/ml in DMEM/F12 culture medium.

Control column #2 (Unincorporated sulfate): Sample consists of 100 μl of
35-SO\textsubscript{4}, 10 μCi/ml in DMEM/F12 culture medium + 400 μl column buffer.

Sample verification columns: One sample from each time period (1 day, 8
days, 15 days, and 29 days) was used to demonstrate the molecular weight
of the labeled fractions. The medium pore size, seeded group was chosen in
each case. The sample consisted of 100 μl aliquots from each of the 4 seeded
samples, plus 100 μl column buffer.
4.2.3 Cell morphology

Cell morphology was evaluated in certain seeded matrices. Cells were assigned to one of three categories:

1) elongated: cells with greater than a 2:1 ratio of length to width
2) round or ovoid: cells with a round or elliptical shape with length to width ratio less than 2:1, and
3) not assigned: cells which had a length to width ratio very close to 2:1 and therefore could not be confidently assigned to groups 1 or 2, or in rare cases, cells with odd shapes in which neither categories 1 nor 2 seemed appropriate.

Methods of selecting and counting cells: Histological cross-sections of cell-seeded matrices, generally taken from near the central diameter of the matrix cores, were stained with hematoxylin and eosin. Areas for analysis were selected by “randomly” placing a microscope eyepiece grid insert on the section to be evaluated. The grid was moved until an area with a suitable number of cells for analysis was found. Only cells within the interior pore structure of the matrix were counted; cells in surface layers were excluded. Therefore the grid was always placed at least 80 μm from the surface of the matrix. The grid was used to outline the area for counting. Each area was selected at an objective magnification of 20X (the grid corresponding to an analysis area of 300 x 300 μm), however, morphology was generally graded using the 40X objective. Generally, the grid was placed on five different areas of a given histological section. In addition, several (3 or 4) sections from each sample were evaluated to improve the sampling and statistical analysis.

4.3 The animal model

4.3.1 Surgical technique and postoperative care

Dogs involved in the study were adult mongrel or hound dogs, each weighing approximately 25-30 kilograms. Prior to operation, the knee joints were examined roentgenographically to exclude animals with degenerative joint disease. All operations were performed under general anesthesia and sterile conditions. The basis for most experiments was a 4-mm diameter defect created in the trochlear groove of the stifle (knee) joint. A 4-mm diameter dermal punch was used to outline the defect. Using loupe visualization, an attempt was made to remove all non-calcified cartilage from the defect by scraping the calcified cartilage surface with a customized curette, without fissuring the calcified cartilage. The objective was to remove as much articular cartilage as possible so as not to leave residual articular cartilage which might interfere with healing or affect the validity of the histomorphometric measurement. Defects were placed approximately 1.25 and 2.25 centimeters proximal to the intercondylar notch, each slightly lateral or medial to the
midline (Figure 4.3). Before closing the capsule, bleeding vessels were clamped and cauterized. The knee joint was closed by zero point suturing.

Figure 4-3. Schematic depicting the location of the proximal (P) and distal (D) defects in the canine trochlea. The defects are 4 mm in diameter.
The procedure for manipulating autologous cultured chondrocytes is shown in Figure 4.4. In animals receiving cultured autologous chondrocytes, articular cartilage was removed from the lateral and medial margins just outside the femoral groove of the left femur, and was used for chondrocyte culture. The dogs were allowed free cage activity after the harvest procedure. The cartilage from the defects and margins was either sent to Genzyme Tissue Repair (Cambridge, Massachusetts), where the cells were isolated and expanded in culture, or the isolation and expansion was performed in our laboratory according to our cell culture protocols (section 4.2.1). Approximately two to three weeks later, the cultured autologous chondrocytes were provided for reimplantation in a variety of implants and treatment conditions (section 4.3.2).

Defects for treatment, whether or not they included cultured cells, were prepared in the contralateral right femur as described above. One of a variety of treatments were applied (see below), the patella replaced resting roughly between the defects, the incision was closed, and the knee was immobilized by external fixation (IMEX Veterinary, Longview, Texas) for ten days to prevent dislodging of the graft or reparative tissue.

4.3.2 Treatments applied to animal model

4.3.2.1 Untreated defects and defects with autologous transplanted cells

4.3.2.1.1 Use of periosteum

Periosteum was harvested from the proximal tibia, and the defect was covered with a four-millimeter diameter circular periosteal flap, with the cambium layer facing the base of the defect, and sutured to the articular cartilage surrounding the defect using 8-0 sutures. Cultured autologous chondrocytes were injected under the periosteum in culture medium (2x10^6 cells/defect).

4.3.2.1.2 Fibrin glue

The defect was sealed with autologous fibrin. This procedure utilized autologous fibrinogen isolated from the animal's blood by cryoprecipitation several weeks before surgery. At surgery, the frozen cryoprecipitate was thawed and 1 cubic centimeter drawn into a syringe. One cubic centimeter bovine thrombin, (Parke-Davis, Morris Plains, New Jersey) at a concentration of 5 units per milliliter with 0.5 per cent calcium chloride and 10 milligrams per milliliter aminocaproic acid, was provided in a separate syringe. After drying the defect area, the contents of both syringes were co-injected into a sterile container, mixed, and spread to cover the entire surface of the periosteal cover and immediately adjacent cartilage.
Figure 4-4. Procedure for manipulation of cultured autologous chondrocytes. Surgeries on the left and right knees performed approximately 3 weeks apart.
4.3.2.1.3 Beta-galactosidase labeling

Two dogs in both the 1.5-month and 3-month groups were selected at random for beta-galactosidase labeling. In these four animals, the harvested chondrocytes were transfected with the gene for beta-galactosidase (performed by Genzyme Tissue Repair) \textit{in vitro} before re-implantation.

4.3.2.2 Use of fascia cover

In animals receiving a fascial cover to the matrix, a separate incision was made in the lateral aspect of the thigh before opening the knee. A piece of the tensor fascia latae, approximately 1 cm square, was removed. Covers were cored out on a tongue depressor using the 4-mm dermal punch. The covers were hydrated with saline until the defects were prepared.

4.3.2.3 Use of collagen sponge cover

Collagen type II sponge covers were made from one of the same Chondrocell matrix batches (#915905) that were used for \textit{in vitro} and \textit{in vivo} seeding experiments. The matrices were compressed manually, cross-linked under UV light for 12 hours, and recompressed before use. The sponge covers were placed dry on the defect for suturing.

4.3.2.4 Use of collagen film cover

Type II collagen films were produced by allowing melted Chondrocell slurry to air dry in stainless steel pans. Molds were formed by sanding the bottom from 50-mm or 85-mm plastic petri dishes and taping them to the pans. The thickness of the film can be controlled by the amount of slurry placed in the mold. For this work, 42 ml of slurry was used in the 85-mm petri dish. Air drying took from one to several days, depending on the amount of slurry. Flat bottomed plastic weigh boats also were suitable molds. Films were sterilized and cross-linked by UV radiation. The films were placed approximately 30 cm from the UV source (Philips Sterilamp #G10T5 1/2 L, $\lambda = 253.5$ nm), and remained for 8 hours total, 4 hours on each side. 5 mm cores were made with a dermal punch. The covers were pre-wet in saline for several minutes before being sutured to the defect.

4.3.2.5 Type II matrix

Two different type II matrices were used in these experiments. These matrices, described in greater detail in section 4.1.1, were Geistlich matrix, batch 915905, and a medium-pore size matrix produced in our laboratory. When matrices were employed for implantation in the model, the cover was first partially sutured into place, then the matrix placed into the defect, suturing completed, and the joint closed and immobilized.
4.3.2.6 Microfracture technique

Microfracture was performed with the straight pointed end of a microsurgical pick. Once the defect was completely prepared, the pick was forced into the subchondral bone with a twisting motion to a depth of approximately 1.5-2.0 mm. The width of the pick at this depth was approximately 0.8 mm. Bleeding from the subchondral bone varied, both from one pick hole to another within a defect or knee, and from one animal to another. The orthopedic surgeon who developed this procedure has emphasized the importance of a light scraping of the calcified cartilage to facilitate integration of the reparative tissue to the underlying structure.

4.3.2.7 Seeding of matrices

Matrix seeding was performed from 4-12 hours before surgical implantation of the matrices, as described in section 4.2.1.3. Seeded constructs were placed in the tissue culture incubator until being moved to the operating room 30 minutes to 3 hours before implantation.

4.3.2.8 Matrix implantation

Seeded matrices were brought to the operating room in a sterile container. After the joint was opened and the defects prepared, the cover was first partially attached by 2 or 3 sutures. The seeded implant was then placed under the cover and suturing was completed. Care was taken in replacing the patella into the patellar groove so as not to disrupt the implant in closing the joint.

4.3.3 Animal sacrifice and tissue retrieval

Animals were sacrificed by injecting 10 cubic centimeters Pentobarbital Sodium CII euthanasia solution (0.6 milligrams per milliliter). At necropsy, the defects were examined grossly and photographed. The distal femurs were excised and placed in 10 per cent neutral buffered formalin. Several hours later the trochlea was carefully dissected with a fine tooth coping saw and placed into formalin for four days. The trochlea was rinsed of formalin, further dissected to individual samples of approximately five millimeter thickness by sharp blows from a hammer to a razor blade, and placed into 15 per cent disodium ethylenediamene tetracetate decalcifying solution, pH 7.4. The sample was placed on a shaker at 4 degrees Celsius with three changes of the decalcifying solution each week for approximately four weeks. Samples were rinsed thoroughly, dehydrated, and embedded in paraffin at 60 degrees Celsius. Seven-micrometer thick sections were prepared and stored at 4 degrees Celsius.
4.3.4 Methods of evaluation

4.3.4.1 Histology

Samples of tissue or cell-seeded matrices were embed either plastic or in paraffin using standard techniques (Appendix E). Samples containing bone were first decalcified in 15% EDTA decalcifying solution, pH 7.4 (Appendix E). Decalcification took approximately 4-6 weeks, depending on the amount of bone remaining on the sample. Histological sections were cut at 7 μm for paraffin sections, and 5 μm for GMA. Slides were coverslipped and sealed with Cytoseal 60 cell-mounting medium (Stephens Scientific #8310-16, Riverdale, NJ).

Samples were stained with one or more of the following stains, listed with their primary uses (complete protocols are found in Appendix F):
- hematoxylin and eosin (general tissue and cell morphology)
- safranin O/fast green (stains sulfated proteoglycans deep red)
- Masson’s Trichrome (reveals collagen fiber structure)
- aniline blue (used to stain matrix structure for pore analysis)

4.3.4.2 Immunohistochemistry

Selected paraffin embed specimens of both in vivo and in vitro experiments were stained with antibodies to type I collagen, type II collagen, link protein, fibronectin, and a-smooth muscle actin. All staining protocols used the ABC colorimetric staining method. Protocols for each of these procedures are found in Appendix G).

4.3.4.3 Histomorphometric evaluation

4.3.4.3.1 Quantitative evaluations

Overview of defects with healing tissue: Quantitative analysis of the entire defect area of the selected sections was performed with the aid of an eyepiece reticle inscribed with a 10 line by 10 line square grid. An overview of the process is given in Appendix H. In the evaluation process, the geometry of the defect was first defined, then certain healing characteristics were determined as an areal or linear percent of the appropriate parameters. These percentages refer only to the representative histological cross-section through the middle portion of the defect. They do not imply values equivalent to the actual volume percents of tissues in the defects. Due to edge effects (regenerating tissues tended to form at the periphery of the defect), only sections representing 60% or more of the defect diameter were analyzed. Sections taken too close to the edge of the defect could preferentially show regeneration, which could give misleading data.
Grading was performed at a magnification of 100 times (a 10 X objective and 10 X eyepiece), with each grid opening measuring 60 micrometers on each edge. The defect area was calculated as a rectangle of base, \( b \), equal to the distance along the base of the defect at the tidemark, and height, \( h \), equal to the average height of the articular cartilage 600 micrometers lateral to the two edges of the defect (see Appendix H). The height was recorded away from the margins in this standard manner to reflect the original height of the defect; the height of the cartilage at the edge of the healing defect was normally slightly depressed relative to the original height of the cartilage, a finding associated with rounding of the surface at the defect edge and flow of material into the defect during healing. (The average dimensions of a defect in histological cross-section were approximately 0.64 millimeters by 3.4 millimeters.) The area of each specific type of tissue within the defect was measured by counting the number of grid openings that fell on that specific tissue type. The actual defect shape was that of a trapezoid, as the heights of the cartilage on either side of the defect were not necessarily identical. Dividing the area of the specific tissue type by the total defect area yielded an areal percent.

Additional quantitative measures included the percent of the total length of the base of the defect (0 to 100 per cent) that was bonded to the healing tissue, and the percent of the total length of the edges of the defect (0 to 100 per cent) at which repair tissue integrated with the adjacent cartilage. The final parameter recorded was the percentage of the length of the calcified cartilage with a normal appearance (0 to 100 per cent).

4.3.4.3.1.1 Definition of tissue types

Foreword on matrix flow: Matrix flow, e.g. apparent bulging of adjacent articular cartilage into the space created by the defect, is thought to represent a mechanical flowing of adjacent tissue into the defect (as opposed to proliferation of this adjacent tissue). This flow did not extend laterally into the defect beyond a distance approximately equal to the thickness of the cartilage layer. The flowing matrix occupied space in the defect, but did not represent newly synthesized repair tissue. Matrix flow material could usually be readily identified in areas at the periphery of the defect. Areas of flow appeared as hyaline tissue continuous with the adjacent cartilage. This flowing material often displayed a variety of degenerative changes such as cloning and loss of matrix staining. The extent of these changes was reflected in the grading of adjacent cartilage (see below, semiquantitative grading). The collagen fibers under polarized light and the columnar architecture appeared continuous with the adjacent cartilage, but bent over, leaning into the defect. The collagen fibers bent over as much as 90°, with some parts touching the base of the defect, but they did not attach to the calcified cartilage. Matrix flow was recorded separately in grading of tissue types within a defect.
Five tissue types (fibrous tissue, fibrocartilage, hyaline cartilage, articular cartilage, and bone) were distinguished on the basis of the appearance of cells and matrix when examined by normal microscopy and polarized light microscopy. Representative micrographs of selected tissues are shown in Figure 4-5. Fibrocartilage was further subdivided into three distinct subsets. Safranin O staining was used to identify the presence of sulfated glycosaminoglycans. A summary of the major characteristics of each soft tissue type is presented below:

a) **Fibrous tissue** consisted of spindle-shaped cells with bipolar, tapered ends and elongated nuclei. The cells were intimately associated with surrounding collagenous matrix and did not reside in lacunae.

The matrix contained type I collagen fibers, oriented parallel to each other, which formed bundles roughly parallel to the surface. The collagen fibers were clearly visible at low power under polarized light, and the matrix did not stain positively for glycosaminoglycans.

b) **Fibrocartilage** had characteristics intermediate between fibrous tissue and hyaline cartilage. Some matrix domains stained for glycosaminoglycans. The visibility of fibers in the matrix distinguished this tissue from hyaline cartilage. Cells were rounded, and often resided in lacunae, thus distinguishing fibrocartilage from fibrous tissue. There were different three versions of fibrocartilage. Often, these tissues were closely juxtaposed, with indistinct borders between them due to gradual changing of cell or matrix characteristics:

i. Type 'a' resembles fibrous tissue, except that cells are ovoid or rounded instead of spindle shaped. The cells lack lacunae. The matrix is usually highly oriented and stains for type I collagen, but does not stain with safranin O or type II collagen.

ii. Type 'b' fits the traditional definition of fibrocartilage, such as that seen in meniscus. The matrix is fibrous, but often disoriented. The cells are chondrocyte-like, including lacunae. The matrix sometimes stained for type I and/or type II collagen.

iii. Type 'c' is similar to hyaline tissue, except that the matrix retains some fibrous characteristics. While not completely fibrous like types 'a' and 'b', the matrix has some visible fibers and often has a patchy or cloudy appearance, with variable safranin O staining. There is normally much more staining for type II vs. type I collagen. The cells normally resemble chondrocytes, complete with lacunae, although some may appear slightly immature.

c) **Hyaline cartilage** was identified primarily by the characteristic appearance of its type II collagen matrix. No individual collagen fibers or bundles were
Figure 4-5. Light micrographs of tissue types found in articular cartilage and healing defects. E) Normal articular cartilage stained with safranin O/fast green. D) Hyaline cartilage stained with safranin O/fast green. Note the complete absence of red safranin O staining for glycosaminoglycans. C) A mixture of hyaline cartilage (bottom of figure) and fibrocartilage (top of figure). This fibrocartilage is nearly hyaline in appearance. B) Fibrocartilage stained with Masson’s Trichrome. Note the fibrous nature of the matrix and chondrocytic appearance of the cells. A) Fibrous tissue, hematoxylin and eosin stain. The collagen fibers and elongated cells are aligned parallel to the tissue and joint surface.
visible in the matrix. The hyaline matrix also had a distinctive appearance under polarized light, with diffuse transmission through the matrix except where the large collagen bundles were seen inserting into the calcified cartilage at the tidemark.

Cells displayed a spherical morphology (except near the surface where they were usually elongated as in normal articular cartilage). Normally, the cells in hyaline cartilage have well-developed lacunae, and pericellular staining by safranin O is more intense than interterritorial matrix staining. However, tissue lacking normal safranin O stain or cellular appearance was seen and graded as hyaline. Portions of hyaline cartilage which met the histological criteria of articular cartilage, including complete matrix staining and columnar arrangement of cells, were recorded as a subset of hyaline cartilage.

d) **Articular cartilage** was defined as a specialized subset of hyaline tissue which displays the distinctive organized articular structure including: columnar arrangement of cells at the base; an arcuate collagen fiber structure originating with thick fibers rising perpendicularly from the calcified cartilage, then arching to becoming thin fibers running parallel to the surface; a limited region lacking safranin O staining at the surface; the flattening of chondrocytes in a horizontal plane near the surface; and other characteristic variations in chondrocyte density and staining intensity from superficial to deep regions. These histological features, as well as cell density, matrix staining, and appearance under polarized light were required to be near normal, as made by comparison with adjacent healthy cartilage. Due to the variations with depth, reparative tissue was judged according to its position relative to the base of the defect. While attachment to the calcified cartilage was required for tissue to be deemed "articular cartilage", continuity with other adjacent tissues was not. In addition, the overlying tissue did not need to complete the articular structure as long as the deep portions were intact.

e) **Bone** was defined as any tissue with a histological appearance more closely resembling bone than cartilage.

4.3.4.3.1.2 Descriptions of quantitative categories examined

Total defect filling: given as an areal percent of the original defect site filled with any type of tissue, but NOT matrix flow. Filling was measured by counting grid boxes filled by reparative tissues (Appendix H). Material which may have been left over from surgery was included. Only material in or above the original defect site (not below) was counted. Tissue overflowing the surface could result in greater than 100% filling.
Individual tissue types (percentage of defect area that was articular cartilage, hyaline cartilage, fibrocartilage, fibrous tissue, or bone): given as an areal percentage of total defect area. Because the very surface of the cartilage often had a more fibrous appearance, when thin layers of fibrous material (3 cells or less) were found at the surface of the defect and appeared continuous with another tissue type just below the surface, this tissue was not graded as fibrous tissue. It was instead included with the underlying tissue type.

Bonding of repair tissue to the calcified cartilage: For reparative tissue to be bonded to the base of the defect, the calcified cartilage layer was required to be intact. Bonding criteria were based on collagen fiber continuity from reparative tissue to calcified cartilage, not simply by apposition of the two surfaces. Very thin bonded hyaline regions—about 20 μm or 1/3 box—were presumed left from creation of the defect and were excluded. All larger areas of bonded tissue are counted, even those that may have never been removed during surgery at the defect margin.

Attachment of repair tissue to adjacent cartilage: given as a percentage of the vertical length (perpendicular to the base—NOT along any contours of flow or damage) of both edges of the defect. The height of the attached tissue from the two edges were totaled, then converted to a percent by dividing by the total defect height (the two edge heights added together). This grade was somewhat subjective, judging the useful integration of repair tissue to the adjacent cartilage. This judgment is based primarily on the appearance of at least some collagen fibers integrated at the interface. Polarized light was used as much as possible. Tissue was excluded from the “attached” category if the appearance of features or borders (other than a change in matrix staining) parallel to the defect edge suggested a lack of integration.

Percentage of length of calcified cartilage with normal appearance: given as a linear percentage of the entire length of the original defect. Abnormalities in the calcified cartilage included pits, large cracks, fissures, resorption, thinning, or invasion by vasculature. Microfractures (small cracks not more than 20 μm wide and 30 μm deep) were distinguished from larger cracks and were judged as normal. Microfractures occurred in almost all samples at surgery.

4.3.4.3.1.3 Method of performing measurements

To ensure reproducibility in grading, markings were made on photographs of each defect to define the defect margins (edges and, if not clear due to significant bone remodeling, the base). Photographs were taken with a 4 X objective lens, with 2 exposures needed to capture the entire defect.

Identification of defect margins: The edges of the defect were not always obvious, especially in the case where tissue presumably left at the defect edge at surgery was not completely separated from the adjacent cartilage. In
general, the fissures made by the dermal punch were distinguishable at least by higher power microscopy (20-40 X) or with the use of polarized light. The lateral most fissure consistent with the operation to create the defect was chosen as the edge. Fissures and damage to the defect periphery sometimes made it difficult to distinguish matrix flow from small amounts of remnant material in the corners of the defect.

The base of the defect was defined by the tidemark with two exceptions. First, when there was significant bone remodeling, the calcified cartilage and tidemark were often resorbed. Second, in rare cases after resorption, bone reformed within the defect area clearly above the level of the adjacent tidemark. In these cases the path of the base was estimated with straight or slightly curved paths (reflecting the anatomy of the specific defect being analyzed) between points of the actual tidemark (points clearly connected to the adjacent cartilage at the correct level).

**Recording measurements:** All measurements were made without photo eyepiece magnifiers engaged. The units of measurement were either square grid areas (for areal measurements) or grid edge lengths (for linear measurements). Heights were always measured perpendicular to the base. Base measurements were made along the actual or estimated path of the tidemark.

**Grading of mixed tissue types:** Certain regions of tissues seen in healing were not clearly assignable to a single tissue type. These regions were either intermediate between two tissue types, or an intermingled combination of two tissues. In cases such as these the grader made a judgment on the relative contributions of the contributing tissue types, and assigned relative percentages of the area of these tissues accordingly. Example 1: 40 grid areas with a fibrous matrix contains about 80% elongated cells and 20% rounded cells. The grader marked 32 units fibrous tissue and 8 units fibrocartilage, type 'a'. Example 2: 40 grid area of tissue with rounded cells in lacunae display a matrix which was neither clearly blurry nor clearly hyaline. The grader split the tissue types and marked 20 units of fibrocartilage, type 'c' and 20 units of hyaline cartilage.

4.3.4.3.1.4 **Assessment of interobserver error**

A separate experiment was conducted to evaluate interobserver error in grading of tissue types. Three observers took part in this study: 1) The author, HB, who developed the criteria; 2) a graduate student, CL, previously unskilled in histology/pathology who was trained in depth on how to use the scale by HB, and 3) a pathologist, QW, given minimal training on the scale by the author. Ten histological sections were selected to provide a broad range of defect appearance with respect to tissue types, filling, geometry, integrity of surrounding tissues, and perceived ease of grading. Each defect was graded for geometry and tissue types blindly by each of the three authors. Interobserver
agreement was assessed by computing the mean, standard deviation, and covariance among the three observers for each category on each sample.

4.3.4.3.2 Semiquantitative grading of pathological changes to surrounding structures

Semiquantitative grading was based on a qualitative assessment: scores ranged from 0 to 3, with 3 representing tissue similar to normal. A score of 0 represented severe damage or degenerative change; 1, moderate degenerative change; and 2, slight degenerative change. Categories examined included the state of the subchondral bone and the effects on the adjacent cartilage: glycosaminoglycan staining, cloning, and the integrity of the surface and deeper tissues.

4.3.5 Characterization of the initial lesion

Six fresh defects were processed for histology immediately after their creation and examined to demonstrate the extent of the original lesion. These defects, identical to those used for the treatment groups, were created in three dogs immediately after the animals were euthanized. Each original, untreated defect was serially sectioned through approximately sixty percent of the defect width. At least eight sections, spaced approximately 250 micrometers apart, were examined.

The amount of articular cartilage remaining in the defect was determined in three transverse sections made through the middle of the lesion. The values were recorded as the percent of the total area of the defect and included: 1) cartilage remaining in the corners of the defect, and 2) cartilage remaining elsewhere on the base of the defect. Additional parameters, related to the inability to remove all cartilage to the tidemark, were expressed as the percent of the length of the defect base and included: 3) the percentage of the base of the defect covered by residual articular cartilage, and 4) the percent of the base with damaged calcified cartilage. Cartilage was described as damaged if there was evidence of thinning, or if there were large fractures, more than 20 micrometers wide or 30 micrometers deep from the tidemark. Fractures smaller than the stated size were termed microfractures and were not included as damage (see Figure 5-23).

4.4 Statistics

The predominant methods for evaluating quantitative data were ANOVA and the students t-test. ANOVA was used for all experiments with more than two experimental groups. One-way ANOVA and balanced 2-way ANOVA were performed on Microsoft Excel software, while unbalanced 2-way ANOVA was
performed with BMDP software at the Orthopedic Biomechanics Laboratory, Beth Israel Deaconess Medical Center, Boston, MA.

T-tests were used for comparison of data with 2 groups. All t-tests were performed on Microsoft Excel with the assumption of equal standard deviations. In certain cases, such as animal with empty defects in one leg and treated defects in the contralateral leg, a paired t-test was employed.

Statistical analysis of semiquantitative data was performed using the Mann-Whitney ranked order test, computed by hand.

The number of samples chosen for the study of the effect of microfracture and engineered type II matrices was determined by a power calculation based on results from previous studies in the model (Appendix I). This calculation was performed by hand.
5. Results

5.1 Chemical composition and pore characteristics of the collagen-GAG analogs of extracellular matrix

In many of the matrices, the pore structure of the two surfaces differed in appearance, and sometimes also from the bulk. It was common for at least one surface to contain material aligned primarily with the surface. This structure created a sheet across the surface (sometimes with smaller pores than the bulk), and was normally found on the surface which was exposed to air in freezing. It was also normal for the other side of these matrices, exposed to the stainless steel pan during freezing, to have more open (and sometimes larger) pores. An example of the pan and air surfaces of a matrix are shown in Figure 5-1.

5.1.1 Type I matrices

In these matrices, the surface contacting the pan during freezing was judged to have a more open pore structure, and is therefore described as the more porous surface (Figure 5-1).

The average pore diameter of the two different type I matrices made from bovine hide and previously analyzed were:

- 27 μm ("small-pore")
- 83 μm ("large-pore")

![Figure 5-1. Photomicrograph showing the air (top- less porous) and pan (bottom- more porous) surfaces of a type I collagen-GAG matrix. The matrix is approximately 2 mm thick. Aniline blue stain.](image-url)
The calculated and measured GAG content of type I matrices are shown in Table 5-1.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>GAG content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>measured (mean ± SD)</td>
</tr>
<tr>
<td>Type I hide</td>
<td>7.7% ± 1.3%</td>
</tr>
<tr>
<td>Type I tendon</td>
<td>8.7% ± 0.1%</td>
</tr>
</tbody>
</table>

Table 5-1. Glycosaminoglycan content of type I collagen matrices as a percentage of total dry weight (mean ± SD). n=3 samples from each group were analyzed. For type I materials, calculated values are from dry weights of purified components used to make the slurry.

5.1.2 Type II matrices

Type II matrices differed from type I matrices in that they contained remnant block-like features of varying size that contained features consistent in appearance with a hyaline matrix and empty cell lacunae. These blocks ranged from slightly thicker than pore walls (approximately 10 μm) to as large as 120 μm by 1000 μm or 300 μm by 400 μm. The pore structures of these matrices varied considerably with controlled changes in the processing conditions.

Analysis of matrices received from Geistlich Biomaterials. Two different primary batches of Geistlich matrices were evaluated. The first batch was used in the \textit{in vitro} experiment comparing matrices of varying chemical composition and in early canine implant studies (batch #915905). The second batch was used in \textit{in vitro} experiments of the effect of cross-linking and cell-seeding density. Five sub-batches of these matrices, numbered 914X06 (X=1 to 5), were intended to differ only in degree of cross-linking. The pore characteristics of two different sub-batches (not cross-linked, #914106, and cross-linked 2 days, #914306) were evaluated. These data are presented in Table 5-2.

<table>
<thead>
<tr>
<th>batch</th>
<th>average pore diameter (μm)</th>
<th>standard deviation</th>
<th>percent porosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>not cross-linked</td>
<td>(914106)</td>
<td>297</td>
<td>66</td>
</tr>
<tr>
<td>2-day cross-linked</td>
<td>(914306)</td>
<td>298</td>
<td>138</td>
</tr>
<tr>
<td>early implants, test of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chemical composition</td>
<td>(915905)</td>
<td>86</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5-2. Pore characteristics of Geistlich matrices.
The GAG content of these matrices is found in Table 5-3 under the heading "Geistlich", measured from batch 914306. Measurements of matrices from batch 915905 also gave values of approximately 2%.

<table>
<thead>
<tr>
<th>n=3</th>
<th>Matrix</th>
<th>GAG content (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type II, “Geistlich”</td>
<td>2.0% ± 0.1%</td>
</tr>
<tr>
<td></td>
<td>Type II, small pores</td>
<td>3.1% ± 0.1%</td>
</tr>
<tr>
<td></td>
<td>Type II, medium pores</td>
<td>3.0% ± 0.1%</td>
</tr>
<tr>
<td></td>
<td>Type II, large pores</td>
<td>2.9% ± 0.03%</td>
</tr>
</tbody>
</table>

Table 5-3. Glycosaminoglycan content of type II collagen matrices as a percentage of total dry weight (mean ± SD). n=3 samples from each group were analyzed.

Analysis of matrices synthesized with different pore diameters. The dry thickness of the small-pore matrix (approximately 3.3 mm) was slightly greater than that of the medium- and large-pore matrices (approximately 3.0 mm). Small- and medium-pore matrices had a fairly homogenous appearance (Figure 5-2a), although a region of the small-pore matrices very close to the edge of the specimen had even smaller pores. Since this was a minor portion of the specimen, these pores were not analyzed. The large-pore matrices, however, were divided roughly into thirds, exhibiting a "sandwich" type structure. The outer surfaces (the 'air' surface and 'pan' surface) had very large pores, while the center third had much smaller pores. The transition between these areas was generally abrupt (Figure 5-2b). The pore diameters of these two different regions within the larger-pore matrices were graded separately. The pore diameters for all three types of matrices are given in Table 5-4.

<table>
<thead>
<tr>
<th>structure</th>
<th>average pore diameter (µm)</th>
<th>percent porosity</th>
</tr>
</thead>
<tbody>
<tr>
<td>small-pore</td>
<td>52 ± 9</td>
<td>91</td>
</tr>
<tr>
<td>medium-pore</td>
<td>88 ± 14</td>
<td>90</td>
</tr>
<tr>
<td>large-pore (outer pores)</td>
<td>257 ± 41</td>
<td>86</td>
</tr>
<tr>
<td>large-pore (center pores)</td>
<td>44 ± 2</td>
<td>87</td>
</tr>
<tr>
<td>canine implants</td>
<td>86 ± 8</td>
<td>93</td>
</tr>
</tbody>
</table>

Table 5-4. Pore characteristics of type II matrices of varying pore size. Pore diameter given as mean ± S.D.

The pore size of the type II implants for the canine study of the effect of microfracture closely matched that of the medium pore matrices produced under identical conditions in different batches (Table 5-4).
Figure 5-2. Scanning electron micrographs of a) small-pore and b) large-pore type II matrices. The small-pore matrix (a) has a homogenous structure. The large-pore matrix (b) has very large pores near the surface (bottom-right of micrograph), and very small pores in the middle region (upper-left of micrograph). The transition between the two different regions is abrupt and clearly seen (arrow).
5.2 In vitro experiments

5.2.1 Parameters related to seeding

The specific results of the experiments designed to optimize seeding of matrices are presented below:

Effect of medium addition. The number of cells remaining in each construct before and after medium addition is shown in Figure 5-3. Across all groups, the number of cells remaining in the matrix at one day were comparable. Up to three hours, about 70% of the cells delivered to the matrix were retained after addition of medium. The percentage retained was increased by letting the cells equilibrate to the matrix for 6 hours. The change of medium at one day did not wash out additional cells.

Effect of matrix type and experimenter. The quantitative results of this experiment are shown in Figure 5-4. Under identical seeding conditions, the type II matrices absorbed significantly more cells than type I matrices of approximately the same volume. This difference was consistent for the two experimenters.

Effect of cell number and surface(s) seeded. Doubling the number of cells seeded to one side (1 million vs. 2 million) significantly increased the number of cells retained by the matrix (p=0.002, 2-tailed t-test), but did not double this value (1.12 million vs. 0.73 million) (Figure 5-5). Seeding 1 million cells on each surface also increased the number of cells contained in the matrix (p=0.00002, 2-tailed t-test), and did approximately double this value to 1.44 million. Finally, the two-sided seeding with 2 million cells delivered more cells to the matrix than the one-sided seeding with 2 million (p=0.001, 2-tailed t-test).

Effect of coating of culture wells: Seeding in agarose-coated culture wells was technically easier than with other conditions, primarily because the agarose coating raised the matrix to a position where it was easier to manipulate. In addition, flipping matrices in the agarose was easier, and the agarose formed a slightly dimpled surface which collected some of the excess medium from seeding. The flipped matrix easily settled into this depression, and spread out due to the slight pool of medium. The number of cells retained in each matrix as a function of well coating and seeding condition is shown in Figure 5-6. In both conditions, there was a uniform increase in number of cells in going from uncoated to polyHEMA-coated to agarose-coated wells. These trends were found significant by ANOVA in both the 5-mm matrices (p=0.016) and in the 9-mm matrices (p=0.003). The increase from uncoated to agarose-coated wells was approximately 75% (174,000 ± 13,000 to 305,000 ± 58,000, mean ± SEM) in the 5-mm matrices and 36% (418,000 ± 54,000 to 568,000 ± 47,000, mean ± SEM) in the 9-mm matrices.
Figure 5-3. Cells retained in matrices when medium was added at different times based on DNA analysis; n=4 samples per group. Data given as mean ± SEM. The number above each column indicates the percent of cells lost from medium addition and culture to 1 day.

Figure 5-4. Effect of experimenter and matrix type on cells retained in chondrocyte seeding; n=4 when not indicated. Data given as mean ±SEM.
Figure 5-5. Effect of cell number and surface seeded on cells retained in type I collagen matrices 3 hours after seeding; n=4. Data given as mean ± SEM.

Figure 5-6. Effect of well coating and matrix size on cells retained in chondrocyte seeding of type I matrices; n=4 except as noted. Data given as mean ± SEM.
Effect of filter drying: The results of the two experiments examining the effect of filter drying are presented in Figures 5-7 and 5-8. When matrices were unconfined (Figure 5-7), filter drying increased the number of cells seeded into all three sizes of matrices, but with a p-value of only 0.252 by 2-way ANOVA. When small matrices were partially confined in small wells (Figure 5-8), there was no effect from filter drying (same result with all coatings). One unexpected finding in these experiments was the higher number of cells found in 7 mm vs. 9 mm matrices. This may in part be attributed to the larger standard deviations in this experiment, as it was one of the first performed and the experimenters technique was not yet well-practiced.

Effect of confinement of matrices: The number of cells retained in 5-mm cores under unconfined and partially confined conditions are shown in Figure 5-9. More cells were found in the confined condition for both wet and filter-dried matrices. This effect was found significant by 2-way ANOVA (p=0.014).

Summary of seeding results: The following parameters were found to improve the number of cells delivered to a matrix: removing excess medium on filter paper ('drying' the matrices), seeding on agarose lined wells, constraining the matrix, and dividing cells aliquots to seed on both surfaces. Approximately 70% of delivered cells were retained after normal patterns of medium addition.

5.2.2 Interaction of chondrocytes with matrices of different chemical composition

Comparison of pore structure: The pore structures of the type I and type II matrices were found to be comparable (Table 5-5). The primary difference in appearance was the finding of block-like structures throughout the type II material (see section 5.1.2).

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Pore Diameter</th>
<th>Percent Porosity</th>
<th>Pore Wall thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>83 μm</td>
<td>87 %</td>
<td>11 μm</td>
</tr>
<tr>
<td>Type II</td>
<td>86 μm</td>
<td>85 %</td>
<td>13 μm</td>
</tr>
</tbody>
</table>

Table 5-5. Comparison of key pore characteristics of the predominantly type I and type II collagen matrices used for chondroctye culture.

Chondrocyte culture in agarose. Passaged chondrocytes always adopted a flattened morphology on plastic substrates, and, when cultured in agarose, reverted to a rounded morphology. This experiment was repeated several times with canine chondrocytes after varying number of passages and different cell density with similar results.
Figure 5-7. Effect of filter drying and matrix size on cells retained in seeding of type I matrices; n=4. Data given as mean ± SEM.

Figure 5-8. Effect of filter drying and matrix coating on cells retained in seeding of partially constrained type I matrices; n=4. Data given as mean ± SEM.
Gross observations, contraction measurements: After 7 days, the surface of cell-seeded type I specimens had fibrous appearance and the specimens had decreased in diameter from 4 mm to an average size of 2.8 mm ± 0.08 with curling at the edges of the implants. The type II sponges maintained a flat surface and displayed no shrinkage after 7 days (4.0 mm ± 0.03). The difference in diameter was found statistically significant (p<0.05, 2-tailed t-test).

Cell morphology: At all time periods, a significantly greater percentage of cells in the type II sponge displayed the spherical morphology consistent with chondrocytic phenotype, when compared with cells in the type I material (Table 5-6).

<table>
<thead>
<tr>
<th></th>
<th>3 hours</th>
<th>7 days</th>
<th>14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type I</td>
<td>Type II</td>
<td>Type I</td>
</tr>
<tr>
<td>rounded</td>
<td>22 ± 1</td>
<td>68 ± 4</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>elongated</td>
<td>69 ± 1</td>
<td>25 ± 2</td>
<td>63 ± 3</td>
</tr>
<tr>
<td>not assign.</td>
<td>9 ± 1</td>
<td>7 ± 2</td>
<td>10 ± 1</td>
</tr>
</tbody>
</table>

Table 5-6. Percentage of chondrocytes with different cell morphologies in matrices of different chemical composition, predominantly either type I or type II collagen. Data given as percent of total cell number, mean ± SEM.
By three hours after the seeding the cells in the type I matrix spread along the walls of the sponge and 69 ± 1 % of the chondrocytes took on a fibroblast-like morphology. At the same time period, 68 ± 4 % of the cells in the type II matrix displayed spherical cell shape. In the type II sponge, the cells were initially more uniformly dispersed in the porous structure. After one week some pericellular matrix had formed around spherical cells in the type II matrix (Figure 5-10a), whereas in the type I material the cells mostly maintained fibroblastic appearance (Figure 5-10b). By two weeks, clusters of round shaped cells with small amounts of surrounding matrix had formed (in the type II matrix. In contrast, at two weeks in the type I matrix, cells attached in a flattened morphology and formed a dense fibroblast-like layer of elongated cells on the surface.

Inside the type I construct, an increasing percentage of spherical cells were observed with time. However, after 14 days, 60 ± 1 % of the cells in the type II sponge continued to display spherical shape, compared with 30 ± 4 % in the type I. Statistical analysis by ANOVA showed a significant difference in cell morphology between the two matrices (p<0.01). There was no significant difference between the two types of material in the amount of cells, that were not assigned to a category of cell morphology. The percentage of undetermined cells ranged from 5-10 %.

**Histochemistry and Immunohistochemistry:** There was no safranin O-staining matrix in the intercellular area of chondrocyte-like cells in either type of sponge after three hours. Safranin O stained lightly the pericellular matrix around spherical cells in the one- and two-week implants in the type II sponge. But there was no significant increase of the stained areas of the two-week samples compared to the one-week specimen. Positive staining was only seen in cell aggregates and was distinctive in the intercellular area of the spherical cells.

Immunohistochemistry revealed positive type II collagen antibody staining of pericellular matrix of spherical cells in the type II sponge, but this was often difficult to distinguish from positive staining of the degrading pore walls. There was no staining for type II collagen in the type I sponge.

**Biochemical Assay:** One sample was lost in the type I group at the 14-day time point. There was a higher amount of DNA in the unseeded type II collagen sponge than in the type I sponge: 0.83 μg ± 0.02 vs. 0.61 μg ± 0.02 (mean ± SEM). The net values of DNA content, after subtracting the values for unseeded sponges, are presented in Figure 5-11. At three hours, more cells were retained in the type II matrix (p< 0.10, 2-tailed t-test). Both groups showed increases in DNA content from 3 days to 7 weeks (type I: p<0.03; type II: p<0.04, 2-tailed t-test). However, from 7 to 14 days, cells continued to proliferate in type II matrix (p<0.13, 2-tailed t-test) while there was no change in type I matrices.
Figure 5-10. Light micrographs of chondrocytes in predominantly type I (a) and type II (b) collagen matrices 7 days after seeding (hematoxylin and eosin stain). (a) Cells in the type I matrix are primarily elongated along the matrix walls. (b) Cells maintain a more rounded morphology in the type II matrix. Small amounts of pericellular matrix can be detected around cell clusters (arrow).
Figure 5-11. DNA content of chondrocyte-seeded matrices of varying chemical composition. Unseeded values have been subtracted; n=3. Data given as mean ± SEM.

Figure 5-12. Net glycosaminoglycan accumulation in chondrocyte-seeded matrices of varying chemical composition with time. Three-hour values have been subtracted; n=3. Data given as mean ± SEM.
The 14-day result showed more cells in the type II matrices compared to type I matrices (p<0.13, 2-tailed t-test).

The mean GAG contents of the unseeded type I and II sponges were 5.05 ± 0.36 and 6.35 ± 0.07 μg/sponge, respectively. The increase in GAG content in both types of matrices relative to the value of the seeded matrix at 3 hours is shown in Figure 5-12. All values were significantly increased compared to the 3-hour value (maximum p value =0.007, 2-tailed t-test). 14-day values were greater than 7 day values in both groups (type I: p<0.06; type II: p<0.09, 2-tailed t-test). The increase in GAG from 3 hours was greater in type II matrices versus type I matrices at 7 days (p<0.03, 2-tailed t-test) and at 14 days (p<0.17, 2-tailed t-test).

5.2.3 Effect of cross-linking treatment and cell seeding density

Gross observations of degradation: The ability to handle matrices was used as a practical measure of resistance to degradation. Not cross-linked, unseeded matrices were already noticeably soft and somewhat difficult to pick up with forceps at one day in culture. At one week, not cross-linked, unseeded matrices completely fell apart into small pieces when disturbed. Seeded matrices could not be picked up whole by forceps, however, although they were extremely soft, they could be moved with a spatula. The not cross-linked matrices were not suitable for longer culture experiments.

Matrices DHT cross-linked for 1 to 4 days, whether seeded or unseeded, all were easily picked up by forceps for up to 2 weeks. Seeded matrices at these times seemed slightly more stiff than unseeded matrices. There was no gross difference in size of the seeded and unseeded matrices at these times. Only matrices cross-linked by UV, 1 day DHT, and 4-day DHT were cultured for 4 and 6 weeks. All of these matrices could be picked up by forceps at 4 weeks, however unseeded matrices were noticeably softer. Also, the UV-cross-linked matrices began to delaminate in a plane parallel to the surfaces of the cores (although they maintained stiffness at least that of the 4-day DHT matrices). Some pieces came completely apart by 6 weeks, although the seeded matrices could still be picked up with forceps. By 6 weeks, only the 4-day DHT, seeded samples could be handled by forceps. One-day DHT seeded samples were noticeably softer and had to be handled with a spatula. Small pieces were breaking off the matrix at this time. Unseeded matrices cross-linked by DHT were softer still, and had to be handled very carefully with the spatula to avoid breaking them up.

Biochemical data: A biochemical analysis of unseeded matrices is included in Appendix J. This analysis measured the degree of loss of matrix components over the 6 weeks in culture, and provided another type of estimate of the resistance to degradation.
The DNA content of seeded matrices (seeded at two different densities) is shown in Figure 5-13. The values for unseeded matrices were subtracted. The DNA content of all groups decreased with time. For either seeding density, the patterns seen in the 1-day and 4-day DHT groups were essentially the same, with slightly higher values in the 4-day group vs. the 1-day group. However, for a given degree of cross-linking, the trends for the two different seeding densities differed. The DNA content in the high seeding density group was initially higher, but fell much more rapidly than in the low seeding density group. By 6 weeks, the DNA content of all groups was very similar, equal to approximately 300,000 to 400,000 cells.

Figure 5-13. Net DNA content of chondrocyte-seeded type II collagen matrices of varying degree of cross-linking in culture up to 6 weeks. Three different cross-linking treatments are indicated in the legend. Unseeded values have been subtracted. Data given as mean ± SEM. Initial numbers of cells delivered were 3.2 million (high seeding density, solid lines) and 1.6 million (low seeding density, dashed lines).
The GAG content of these matrices is shown in Figure 5-14. Again, for each seeding density, values for the 4-day DHT groups were higher than those for the corresponding 1-day DHT groups. Given a seeding density, the patterns of the corresponding changes with time were identical in the 1-day and 4-day groups. In contrast, for each degree of cross-linking, patterns based on seeding density differed (although not statistically significantly): matrices seeded at low density continued to increase the accumulated GAG content from 4 to 6 weeks, while those seeded at high density exhibited an overall loss of GAG from 4 to 6 weeks.

Figure 5-14. Net accumulated glycosaminoglycan content of chondrocyte-seeded type II collagen matrices of varying degree of cross-linking in culture up to 6 weeks. Three different cross-linking treatments are indicated in the legend. Unseeded values have been subtracted. Data given as mean ± SEM. Initial numbers of cells delivered were 3.2 million (high seeding density, solid lines) and 1.6 million (low seeding density, dashed lines).
5.2.4 Effect of matrix pore diameter

5.2.4.1 Pore diameter in type I matrices

Comparison of pore structure: The predominant difference in pore structures of the small- and large-pore type matrices was pore diameter (Table 5-7).

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Pore Diameter</th>
<th>Percent Porosity</th>
<th>Pore Wall thickness</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I, small pore</td>
<td>20 µm</td>
<td>83 %</td>
<td>7 µm</td>
</tr>
<tr>
<td>Type I, large pore</td>
<td>83 µm</td>
<td>87 %</td>
<td>11 µm</td>
</tr>
</tbody>
</table>

Table 5-7. Comparison of key pore characteristics of small- and large-pore collagen matrices used for chondroctye culture.

Matrix contraction: The large- and small-pore type I matrices underwent a comparable amount of shrinkage: 30% for the large-pore diameter matrix (from 4.1 ± 0.1 mm at three hours to 2.8 ± 0.1 mm after 7 days) and 35% for the small-pore material (from 4.9 ± 0.1 to 3.2 ± 0.2 mm).

Morphological data: The percent of cells graded as rounded, elongated, or not assigned is shown in Table 5-8. At 3 hours, there were more rounded cells (and fewer elongated cells) in the small-pore matrices than the large-pore matrices (p<0.0001, 2-tailed t-test). By 7 days, there was no difference in rounded cells.

<table>
<thead>
<tr>
<th></th>
<th>3 hours</th>
<th>7 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>pores:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rounded</td>
<td>43 ± 1</td>
<td>27 ± 1</td>
</tr>
<tr>
<td>elongated</td>
<td>48 ± 1</td>
<td>68 ± 1</td>
</tr>
<tr>
<td>not assigned</td>
<td>8 ± 1</td>
<td>5 ± 1</td>
</tr>
</tbody>
</table>

Table 5-8. Percentage of chondroctyes with different cell morphologies in type I collagen matrices of different pore size. Data given in percent of total cells, mean ± SEM.

Biochemical results: Unseeded small-pore matrices had a higher DNA content (1.03 ± 0.02 µg vs. 0.61 ± 0.02 µg) and GAG content (11.9 ± 0.1 µg vs. 5.0 ± 0.4 µg) than unseeded large-pore matrices. The data for DNA content with unseeded values subtracted are shown in Figure 5-15. DNA content increased from 3 hours to one week in both matrices, with slightly higher increases in the small pore matrices. Two-way ANOVA gave p-values of p=0.001 with respect to time and p=0.23 with respect to pore size.

The change in GAG content from 3 hours to 7 days for these matrices is shown in Figure 5-16. Small pore matrices exhibited a greater increase in GAG content (p<0.11, 2-tailed t-test).
Figure 5-15. DNA content of chondrocyte-seeded type I collagen matrices of varying pore diameter. Unseeded values have been subtracted; n=3. Data given as mean ± SEM.

Figure 5-16. Net glycosaminoglycan accumulation in chondrocyte-seeded type I collagen matrices of varying pore diameter. The change is from 3 hours to 7 days; n=3. Data given as mean ± SEM.
5.2.4.2 Pore size in type II matrices

During seeding, additional aliquots of cell suspension were saved for DNA analysis at six times throughout the seeding process (from beginning to end) to verify the variability in the number of cells delivered to each matrix. The coefficient of variation in DNA content of these aliquots was 3.7% (readings of $573 \pm 21$ units, mean ± SD).

Matrix contraction. All seeded matrices showed visible contraction by 1 week in culture, and continued to shrink as long as 6 weeks. Shrinkage was accompanied by the curling of the edges of the matrix. There was no discernible difference in the amount of shrinkage among the varying pore sizes (Figure 5-17). Each group shrank to roughly 55% of the original diameter. Unseeded matrices showed no shrinkage.

Figure 5-17. Shrinkage of chondrocyte-seeded type II collagen matrices of varying pore size. Data given represents mean average diameter ± SEM; n= 2 to 8.
Histology. Histology showed varying degrees of cell penetration and distribution related to the time in culture and pore size of the matrix seeded (Table 5-9). At 2 hours very few cells penetrated into small-pore matrices, slightly more penetrating medium-pore matrices, and many more cells in large-pore matrices (Figure 5-18). All samples showed many cells attached to the surface of the matrix. There was not a complete layer of cells on the surface of any sample at this time.

<table>
<thead>
<tr>
<th>time</th>
<th>pore size</th>
<th>penetration evaluated by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>depth</td>
</tr>
<tr>
<td></td>
<td>small</td>
<td>0</td>
</tr>
<tr>
<td>2 hour</td>
<td>medium</td>
<td>1</td>
</tr>
<tr>
<td>(n=1)</td>
<td>large</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>small</td>
<td>0</td>
</tr>
<tr>
<td>1 day</td>
<td>medium</td>
<td>0</td>
</tr>
<tr>
<td>(n=3)</td>
<td>large</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>small</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>medium</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>large</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 5-9. Semiquantitative assessment of chondrocyte penetration into type II collagen matrices of varying pore size. Data given as mean value. 0 = minimal penetration, 3 = maximum penetration.

At one day, there was still little penetration of cells in small- or medium-pore matrices. In large-pore samples, cells were found throughout the thickness in the matrix, except in the central areas with the very small pores. Cell numbers appeared slightly reduced in medium- and large-pore samples. The cells on the surface began to align and form layers, generally only 1 cell thick at this time. The cellular morphology varied but was generally ovoid to elongated. The layers were slightly more complete on small- and medium-pore surfaces, in part due to the highly irregular surface of the large pore matrices.

At one week, there was increased cell penetration of the matrix in small- and medium-pore matrices, although still much less than in large-pore matrices. Large pore matrices maintained a fairly even distribution of cells, however cells still did not penetrate well into the smallest pore regions in the center. Cells in the interior of the matrix were often found clustering, with visible extracellular matrix surrounding them. This matrix did not stain measurably for safranin O or type II collagen. At this time, there was a complete or nearly complete layer of cells surrounding all surfaces in small- and medium-pore matrices. This layer was thickest in small-pore matrices, often more than 5 cells thick on one surface, while typically 2-4 cells thick in medium pore matrices. In several samples, the cells were concentrated on one surface with contraction causing a curling toward that surface. In large-pore matrices, the
Figure 5-18. Light micrographs of chondrocyte-seeded large- and medium- pore matrices cultured for 2 hours and 2 weeks. At 2 hours (A and B), virtually all cells are rounded in morphology. In the medium-pore sample (A), cells are seen predominantly on the surface, or at most at a depth of several pores. Cell penetration is much deeper in the large-pore sample (B). At 2 weeks (C and D), a layer of cells has formed at the surface of both matrices (open arrows). Cells have now penetrated slightly deeper in the medium-pore matrix, and most of the cells in the interior are now elongated (C, arrowheads). Cells at the surface of the large-pore matrix appear to be contracting the adjacent structures and curling the surface (D). (Small-pore matrices, not shown, appear similar to medium-pore matrices.)
cell layer surrounding the matrix was slightly less complete, but greater than 1 cell thick on at least most of one surface.

By two weeks, there was a complete layer of cells more than 1 cell thick surrounding all matrices (Figure 5-18). The layers were nearly the same thickness around the periphery of each sample, generally slightly thicker for small-pore matrices (about 5 cells) and medium-pore matrices (about 4 cells) than large-pore matrices (about 2-3 cells). In all matrices, there were thicker layers covering over pores or holes in the surface, as well as areas where the cells appeared to be drawing or pinching the adjacent regions of the surface together (Figure 5-18).

By four weeks, for all pore sizes, the cell layer was slightly more thickened on one surface (6-10 cells for small-pore, 5-7 cells for medium-pore, and 4-5 cells for large-pore), while the opposite surface maintained a cell layer roughly 2-4 cells thick.

**Cell morphology:** At 2 hours, over 90% of the cells in each size matrix were spherical in morphology. By 1 day, quantitative analysis showed that fewer than 50% of the cells remained spherical (Table 5-10). At one day, there were no differences among the pore sizes by ANOVA. The results at one week were similar to those at 1 day, except that the large pore size group had fewer spherical cells, and more elongated and not assigned cells. 2-way ANOVA analysis for time and pore size gave the following results:
round cells: p (time)<0.10, p (pore size)=0.12;
elongated cells: p (time)<0.34, p (pore size)=0.31.

<table>
<thead>
<tr>
<th>time:</th>
<th>1 day</th>
<th>1 week</th>
</tr>
</thead>
<tbody>
<tr>
<td>pore size:</td>
<td>small</td>
<td>medium</td>
</tr>
<tr>
<td>rounded</td>
<td>39 ± 1</td>
<td>36 ± 6</td>
</tr>
<tr>
<td>elongated</td>
<td>49 ± 2</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>not assigned</td>
<td>11 ± 1</td>
<td>9 ± 1</td>
</tr>
</tbody>
</table>

Table 5-10. Morphology of chondrocytes cultured in type II collagen matrices of varying pore size. Data given as a percent of total cells, mean ± SEM. n=3 for all groups. Note that observations at 2 hours, not reflected in this table (with n=1) showed greater than 90% round cells for each pore size.

A biochemical analysis of unseeded matrices is included in Appendix J. These results describe the rate at which matrix components are eluted to the medium during culture.

**DNA content of cell-matrix constructs:** The DNA content of cell-seeded matrices are shown by time and treatment group in Figure 5-19. In this figure, the value of unseeded matrices have been subtracted, and the data is converted from counts to cell equivalents based on our standard curve. There was a
Figure 5-19. DNA content of chondrocyte-seeded type II collagen matrices of varying pore size. Unseeded values have been subtracted. DNA values have been converted to cell equivalents.

Figure 5-20. Net accumulated GAG in chondrocyte-seeded type II collagen matrices of varying pore size. Unseeded values have been subtracted.
slight proliferation of cells over the first week (found significant by 2-way ANOVA of time and pore size, \( p(\text{time})=0.03 \)), however, this was followed by loss of cells during the remaining time in culture. The loss of cells in the small matrix took place earlier than in the medium and large pore matrices (from 1-2 weeks vs. 2-4 weeks), however the long term number of cells retained was very similar and not found significant by ANOVA \( (p=0.294) \).

**GAG content of construct:** The net amount of GAG accumulated in the matrix over each time in culture is presented for each treatment group (Figure 5-20). This total GAG represents the value of seeded matrices minus those of unseeded controls, and is a function of the number of cells in the matrix, their rate of GAG synthesis, and the amount of GAG lost to the medium. There is a consistent increase in GAG content over time, however, the rate of accumulation decreases after the first week in culture. The results are very similar for all pore sizes.

**Demonstration of high molecular weight fractions:** The running of PD10 columns was able to establish that the counts for radioactive sulfate measured in the digested matrix reflected high molecular weight GAG chains or proteoglycans manufactured and retained by the cell-matrix constructs. Figure 5-21 depicts the elution of column fractions from two sets of samples:

1) Control samples of high and low molecular weight fractions, and
2) Representative experimental samples from each of the time periods.

The appearance of radiolabeled unincorporated sulfate fractions to the right indicates the presence of low molecular weight fractions which are eluted more slowly by the column. In contrast, high molecular weight fractions, as estimated by the visual observation of dextran blue 2000 (molecular weight 2,000,000), move more quickly through the column. Uniformly, in the experimental samples, any radioactive species were eluted in the high molecular weight fraction, and therefore must have represented sulfate incorporated into long chains.

**Normalized sulfate incorporation:** The amount of sulfate incorporated into the cell-matrix construct during the four hour labeling period (Figure 5-22) was expressed as counts per minute of incorporated sulfate divided by the number of cells as determined by the DNA assay. The normalized incorporation increased in all groups at each time. In addition, the small pore matrices always demonstrated the highest synthetic rate, albeit by a small amount. The effect of pore size was not found significant in a 2-way ANOVA of pore size and time \( (p=0.11) \).
Figure 5-21. Demonstration of incorporation of 35-S radiolabeled sulfate into high molecular weight molecules. All experimental samples coincide with the high molecular weight region (as visualized by blue dextran), and do not overlap the low molecular weight region in which counts from unincorporated 35-S-labeled sulfate were measured.

Figure 5-22. Normalized uptake of 35-S-labeled sulfate by chondrocytes in type II matrices of varying pore diameters. The counts per minute (cpm) for the sample has been normalized by the mean DNA measurement for the group (Figure 5-21).
5.3 The animal model

5.3.1 Description of the lesion

The description of the original lesion is taken from the six fresh defects examined immediately after their creation. The average amount of residual cartilage left in these defects was 1.7 ± 2.1% (mean ± standard deviation, range: 0.2 to 6.0%). Of this, 0.7 ± 0.6% (range: 0.2 to 1.8 per cent) was in the corners, and 1.0 ± 1.6% (range: 0 to 4.2%) was elsewhere along the base of the defect. The percentage of the length of the base that was covered by residual cartilage was 16 ± 26%. The high coefficient of variation was due to an outlying value of 68% (Figure 5-23a), one of 17%, and four of under 4% (Figures 5-23b, c, d).

Histological analysis of serial sections of the original, fresh defects revealed three types of injury to the calcified cartilage: fracture at the defect edge, thinning, and microfracture. In four of the six defects, at least one large fracture (50 to 100 micrometers wide) at the extreme edge was noted. This damage was consistent with overpenetration by the dermal punch in circumscribing the defect. Two of these fractures extended through the calcified cartilage, but only superficially into the subchondral bone (Figure 5-23c). A second type of damage was thinning of the zone of calcified cartilage, presumably from scraping the base of the defect with the curette. Calcified cartilage thinning was inconsistent, varying from sample to sample (Figure 5-23a-d), and even within a given sample. The percentage of thinned or fractured calcified cartilage was 22 ± 14% (range: 2 to 39%). The final type of damage was microfracturing of the calcified cartilage surface (Figure 5-23c). Each of five defects which contained little or no residual articular cartilage showed microfractures ranging from one or two per slide to ten or more per slide. In no case did the microfractures penetrate into the underlying bone.

In summary, one defect showed no thinning of the calcified cartilage, but had much more residual cartilage than the other defects (Figure 5-23a). Two defects showed thinning of the calcified cartilage; a portion of the calcified layer was completely removed on these samples (up to 700 micrometers along the base), with consequent damage to the underlying bone (Figure 5-23b). In the remaining three samples the defect depth generally extended to the tidemark, with little residual cartilage and thinning (if present) limited to the superficial portions of the calcified cartilage (Figure 5-23d). Some of these samples had a corner fracture and all had microfractures.

It is important to note that the technique used for this study was that for the 18 month study of the effect of CAC and periosteum, as well as the two studies including type I matrices. The observation of corner fractures prompted a change in technique which involved less penetration by the dermal punch. Thus, in the first study with type II matrices, as well as in the examination of
Figure 5-23. Light micrographs of original, acute, untreated full-thickness chondral defects in the canine trochlear groove. Adjacent cartilage, stained red with safranin O, is visible to one side of each defect (arrows). The subchondral bone (SCB) stains blue with fast green. A, B, and D (next page) were taken at the same magnification. A) This sample was the outlier, with the most residual cartilage left at the base of the defect (RC). B) One of two samples with injury through the calcified cartilage caused by the curette. The marrow space has been violated near the defect edge (open arrow). (continued on next page)
Figure 5-23, continued (light micrographs of original, acute, untreated full-thickness chondral defects in the canine tricohlear groove). C) This defect shows the maximum of residual cartilage in a corner, as well as a corner fracture (open arrow) through the calcified cartilage. In addition, the calcified layer to the right of the residual cartilage is thinned and exhibits several microfractures (small arrows). D) Virtually all articular cartilage has been removed to the level of the tidemark.
engineered type II matrices and microfracture, the defects likely experienced no
damage from corner fractures.

5.3.2 Interobserver error

Coefficients of variation (COVs) for recorded measurements of defect geometry
(base and heights) were all under 5%, indicating that the geometry of the
defect could be accurately determined by a single observer.

The coefficient of variation between the observers for identification of tissue
types varied somewhat from tissue to tissue, and was related to the absolute
value of the quantity recorded. In general, the higher the value measured, the
lower the coefficient of variation.

Key results of the analysis of interobserver error are shown in Figures 24 a-c.
These figures plot the coefficient of variation of total repair tissue, hyaline plus
articular cartilage, and fibrocartilage versus the average value of the
measurement. Fibrous tissue, bone, and matrix flow are not plotted due to
their minor contributions to the total repair in most samples (and
consequently large COVs). The downward slope of the data with increasing
abscissa indicates the inverse relation between absolute measurement and
coefficient of variation. Also plotted on these graphs is the interdog error,
shown on a similar scale; each data point represents an experimental group
with a given treatment and time point. Thus, the importance of the
interobserver error can be compared to the interdog error for a given mean
value of measurement. In total repair tissue, all values for interobserver error,
as measured by the interobserver coefficient of variation, are below those for
interdog error. For hyaline cartilage, the result is similar, with all but one
interobserver value less than all of the interdog values. In the case of
fibrocartilage, there is slightly more overlap, however, the interobserver values
are in general lower than the interdog values.

5.4 In vivo experiments

5.4.1 Overview of gross results for all in vivo experiments

All animals ambulated normally after removal of external fixation and
survived the planned period to sacrifice. In the vast majority of animals,
when the joints were opened, synovial tissue appeared grossly normal,
although there was evidence of slight hypertrophy or, less commonly, atrophy
in certain joints. Similarly, the joint fluid was grossly within a normal range,
with some slightly more wet or more dry. In two cases, the gross appearance of
the joint suggested significant pathology. These observations included
significant effusion with turbid synovial fluid, pronounced synovial
hypertrophy, or lateral patellar displacement with denuded cartilage
Figure 5-24. Comparison of interobserver to interdog error for grading of healing full-thickness chondral defects. Errors given as coefficients of variation. Each interobserver error represents 3 observers on one sample. Each interdog error represents a group of dogs with a given sacrifice time and treatment. Categories: (a) total filling; (b) hyaline cartilage; (c) fibrocartilage.
consistent with patellar subluxation. Defects from these two knees were excluded from subsequent analysis.

All defects were grossly distinguishable from surrounding cartilage, although there was wide variability in the amount of filling and general appearance (Figure 5-25). Repair tissue usually had a whiter, more opaque, and less glistening appearance than normal cartilage. In some cases the appearance of the reparative tissue more closely resembled the surrounded cartilage, however, the margins of the defect were always visible. Variability was generally found within treatment groups as well as between groups. Histologic examination confirmed the variable filling of the defects observed grossly, however visual estimates did not always correlate well with the histological appearance. Certain defects were relatively unfilled, while others were filled with repair tissue to or above the level of the surrounding cartilage.

5.4.2 Healing of untreated chondral and microfracture defects

5.4.2.1 Histological results

Representative histology of untreated defects from 1.5 months to 12 months (Figure 5-26a-d) demonstrate a changing profile of tissues in the defect. These specimens are chosen from samples which are at least half-filled. Some specimens not pictured were mostly empty. At 1.5 months, the tissue filling the defect was predominantly fibrous in nature, either fibrous tissue or fibrocartilage (Figure 5-26a). In this micrograph, resorption of much of the calcified cartilage and a portion of the underlying bone has occurred. This
Figure 5-26. Light micrographs of healing untreated full-thickness chondral defects in the dog trochlear groove. Defect margins are shown by arrowheads. A) 1.5 months, hematoxylin and eosin stain. The reparative tissue consists of fibrous tissue (elongated cells) and fibrocartilage (type ‘a’, rounded cells). A large portion of the calcified layer has been remodeled by a severe resorptive process allowing direct communication between the bone and defect (150 X). B) 3 months, hematoxylin and eosin stain. The reparative tissue seen in this defect is almost all fibrocartilage, type ‘a’ near the center, and type ‘c’ near the periphery (50 X). (continued, C and D on next page)
Figure 5-26, continued (light micrographs of healing untreated full-thickness chondral defects in the dog trochlear groove). C) 6 months, hematoxylin and eosin stain. Hyaline cartilage has formed on the base of the defect near the center and at the edge of the defect next to the adjacent cartilage. The remaining reparative tissue is fibrocartilage (60 X). D) 12 months, trichrome stain. Hyaline cartilage is still visible on the base in the center of the defect and at the extreme edge. Fibrocartilage, primarily type 'b', is variably attached to the hyaline cartilage. This tissue is fibrillating and coming detached from the calcified cartilage (75 X).
reaction normally consisted primarily of dense round or ovoid or rounded cells, and was not inflammatory in nature. The communication between the defect and the bone varied considerably at this time- this would be graded as a severe reaction. Many other reactions were much less subtle, with damage to the calcified cartilage limited to sections 300 µm or less in with. In some sections, even with serial sectioning across the defect, no communication was found. The finding of breaks in the subchondral plate was associated with the formation of blood vessels in the defect area in some samples: 1 of 6 six-week samples and 2 of 11 9-week samples.

By 3 months, little fibrous tissue was found (Figure 5-26b). Furthermore, the calcified cartilage layer was substantially more intact than at 1.5 months. In this sample, fibrocartilage near the edge of the defect has started to take on a more hyaline appearance. In some samples, significant levels of hyaline cartilage did form by this time. No vessels were found in the defect at 3 months or later times, although two samples at 3 months demonstrated vessels just below the level of the defect where the calcified layer was resorbed. Figure 5-26c shows a mixture of hyaline cartilage and fibrocartilage in the defect at 6 months. As was typical, the hyaline cartilage in this defect formed preferentially at the base of the defect, especially at the periphery. Through 6 months, most fibrocartilage was of types 'a' or 'c', those similar to fibrous tissue or hyaline cartilage. Meniscus-like type 'b' fibrocartilage was not as common. At 12 months (Figure 5-26d), the reparative tissue again consisted primarily of fibrocartilage and hyaline cartilage. However, the amount of type 'b' fibrocartilage was greatly increased, shown in the figure by trichrome stain fibrillating and coming detached. At 12 and 18 months, much of the hyaline cartilage exhibited reduced staining by safranin O for glycosaminoglycans (not shown).

Microfracture defects were only observed at 15 weeks (Figure 5-27). Compared to untreated defects, these defects were generally more filled with reparative tissue and had greater reactions in the subchondral bone. The reparative tissue was predominantly fibrocartilaginous. While approximately half of the untreated chondral defects at 3 months were found to have a perforated subchondral plate, with at least some associated remodeling, perforation was universal in microfracture defects and degree of the remodeling was often more notable in microfracture specimens (but not in Figure 5-27). Further description of microfracture defects is given in section 5.4.4.
5.4.2.2 Quantitative results and statistical analyses

Reporting of proximal and distal defects: Quantitative data were analyzed by two methods:
1) all defects treated independently (results reported with n = number of defects examined).
2) the proximal and distal defects were averaged to give one value for each knee (results reported with n = number of knees examined).

In cases where all samples are successfully recovered and graded, the means are necessarily identical in using these two methods. Means will vary between these two methods only if one of the two defects in a knee does not yield data. However, the two methods will normally give different results for measures of variance and statistical significance.
Based on an analysis summarized in Appendix K, there was little correlation among proximal and distal defects for the most important grading categories. Thus, the method of reporting proximal and distal defects independently has been used for quantitative results and statistics below. In one special case (that of hyaline and articular cartilage in study of untreated defects and defects treated with autologous cultured chondrocytes alone, see Appendix K), additional statistics are reported for the method of averaging the defects by knee. In addition, a complete listing of the alternate data format (averaging the two defects in each knee), is given in Appendix L.

The contributions of different tissue types to the healing of chondral defects (from 6 weeks to 18 months) and from chondral defects also undergoing microfracture (15 weeks only) are shown in Table 5-11. The values for attachment of reparative tissue to adjacent structures and the amount of intact calcified cartilage are found in Table 5-12.

The quantitative data reflect the trends reported in histological assessment. There was significant variation with time in all tissue types filling the defect and total filling (Table 5-13). The trends matched those seen histologically (Table 5-11). Fibrous tissue was at a maximum at the shortest time (1.5 months), fibrocartilage at the longest time (18 months), and hyaline plus articular cartilage in the intermediate times (3 to 12 months). The evolution of the profiles of tissue types can be shown by plotting the normalized contribution of the major tissue types as a percent of the total amount of reparative tissue (Figure 5-28). In addition, the amount of intact calcified cartilage and bonding of reparative tissue to this calcified layer were at a minimum at 1.5 months. The ANOVA values reflect increases by three months,

<table>
<thead>
<tr>
<th>time</th>
<th>defect type (n)</th>
<th>fibrous tissue</th>
<th>fibrocartilage</th>
<th>hyaline cartilage</th>
<th>articular cartilage</th>
<th>bone</th>
<th>total filling</th>
<th>matrix flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 wk</td>
<td>C (6)</td>
<td>24.5 ± 4.0</td>
<td>20.5 ± 4.8</td>
<td>3.1 ± 0.8</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>48.0 ± 5.6</td>
<td>0.6 ± 0.4</td>
</tr>
<tr>
<td>9 wk</td>
<td>C (9)</td>
<td>11.7 ± 2.0</td>
<td>18.0 ± 6.9</td>
<td>5.3 ± 2.0</td>
<td>0.7 ± 0.6</td>
<td>0 ± 0</td>
<td>35.7 ± 5.8</td>
<td>5.1 ± 1.4</td>
</tr>
<tr>
<td>6&amp;9 wk</td>
<td>C (15)</td>
<td>16.8 ± 2.6</td>
<td>19.0 ± 4.4</td>
<td>4.4 ± 1.3</td>
<td>0.4 ± 0.3</td>
<td>0 ± 0</td>
<td>40.6 ± 4.3</td>
<td>3.3 ± 1</td>
</tr>
<tr>
<td>3 mo</td>
<td>C (9)</td>
<td>1.2 ± 0.9</td>
<td>13.5 ± 5.5</td>
<td>15.9 ± 3.7</td>
<td>3.3 ± 1.4</td>
<td>0 ± 0</td>
<td>33.9 ± 6.9</td>
<td>1.5 ± 0.5</td>
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<tr>
<td>15 wk</td>
<td>MF (8)</td>
<td>10.9 ± 3.5</td>
<td>52.4 ± 8.2</td>
<td>0.7 ± 0.3</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>64 ± 17.9</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>6 mo</td>
<td>C (5)</td>
<td>3 ± 1.7</td>
<td>14.8 ± 6.7</td>
<td>11.4 ± 3.1</td>
<td>2.1 ± 1.2</td>
<td>0 ± 0</td>
<td>31.2 ± 10.1</td>
<td>7.6 ± 3.0</td>
</tr>
<tr>
<td>12 mo</td>
<td>C (8)</td>
<td>1.3 ± 1</td>
<td>17.2 ± 5.9</td>
<td>19 ± 2.7</td>
<td>3.7 ± 1.3</td>
<td>0.2 ± 0.2</td>
<td>41.4 ± 4.7</td>
<td>2.5 ± 0.9</td>
</tr>
<tr>
<td>18 mo</td>
<td>C (6)</td>
<td>8.4 ± 2.9</td>
<td>51.8 ± 10.5</td>
<td>9.6 ± 2.1</td>
<td>0 ± 0</td>
<td>5.8 ± 5.8</td>
<td>75.7 ± 14.2</td>
<td>1.6 ± 0.6</td>
</tr>
</tbody>
</table>

Table 5-11. Components of healing full-thickness chondral defects in an adult canine model. C = untreated chondral defect. MF = chondral defect with microfracture. Values given as an areal percent of total defect area seen in a histological cross-section from the center diameter of the defect, mean ± SEM (data averaged by defect).
Figure 5-28. Composition of reparative tissue in untreated defects. Mean contributions of each category are normalized by the total percent of defect filling at the time point.
### Table 5-12. Integration of reparative tissue and status of the underlying calcified cartilage in healing full-thickness chondral defects in an adult canine model. One group, as indicated, was treated with microfracture. Values given as a linear percent of the length of the defect base or height, as seen in a histological cross-section from the center diameter of the defect, mean ± SEM (data averaged by defect).

<table>
<thead>
<tr>
<th>time</th>
<th>defect type</th>
<th>Integration with adjacent AC</th>
<th>Bonding to calcified cartilage</th>
<th>Intact calcified cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 week</td>
<td>chondral</td>
<td>0 ± 0</td>
<td>6.3 ± 2.1</td>
<td>56.8 ± 16.2</td>
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<tr>
<td>9 week</td>
<td>chondral</td>
<td>15.4 ± 4.5</td>
<td>27.5 ± 10.9</td>
<td>60.4 ± 13.4</td>
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<tr>
<td>6 + 9</td>
<td>chondral</td>
<td>9.2 ± 3.3</td>
<td>19 ± 7</td>
<td>58.9 ± 10</td>
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<td>3 months</td>
<td>chondral</td>
<td>11.7 ± 3.4</td>
<td>50.8 ± 10.7</td>
<td>95.4 ± 2.2</td>
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<td>15 weeks</td>
<td>microfracture</td>
<td>25.3 ± 8.7</td>
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<td>35.6 ± 11.1</td>
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<tr>
<td>6 months</td>
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<td>44.4 ± 13.3</td>
<td>87.2 ± 10</td>
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<tr>
<td>12 months</td>
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<td>88.6 ± 8.6</td>
<td>92.3 ± 7.1</td>
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<tr>
<td>18 months</td>
<td>chondral</td>
<td>24.6 ± 6.2</td>
<td>40.7 ± 14.2</td>
<td>73.4 ± 12</td>
</tr>
</tbody>
</table>

Table 5-13. ANOVA values for quantitative data in untreated defect series from 1.5 to 18 months. Data is reported as individual defects. **ANOVA value of hyaline plus articular cartilage when grouped by knee: p<0.09**

<table>
<thead>
<tr>
<th>p value</th>
<th>category</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0012</td>
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<tr>
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<td>fibrocartilage</td>
</tr>
<tr>
<td>0.0083</td>
<td><strong>hyaline plus articular cartilage</strong></td>
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<tr>
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<td>total filling</td>
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<tr>
<td>0.59</td>
<td>integration with adjacent</td>
</tr>
<tr>
<td>0.0055</td>
<td>bonding to calcified cartilage</td>
</tr>
<tr>
<td>0.056</td>
<td>intact calcified layer</td>
</tr>
</tbody>
</table>

however there were no clear trends from 3 to 18 months. No significant variation in integration with adjacent cartilage was detected with time.

**Statistical comparison of untreated defects with microfracture defects.**

Untreated defects from the 3 month time point (actual sacrifice time: 16 weeks) were compared with 15 week microfracture defects by t-test (Table 5-14). The microfracture defects had more total reparative tissue, more fibrous tissue, more fibrocartilage, and less hyaline cartilage. Untreated defects had a substantially more intact calcified cartilage layer, and also, more bonding to
<table>
<thead>
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<th>p value</th>
<th>category</th>
</tr>
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<td>fibrous tissue</td>
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<td>fibrocartilage</td>
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<td>integration with adjacent</td>
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<tr>
<td>0.0006</td>
<td>bonding to calcified cartilage</td>
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<tr>
<td>5.1E-05</td>
<td>intact calcified layer</td>
</tr>
</tbody>
</table>

Table 5-14. T-test comparison of untreated defects and microfracture defects at approximately 15 months in a canine model. Data is reported as individual defects. ** t-test value of hyaline plus articular cartilage when grouped by knee: p<0.05.

the base of the defect. Integration with the adjacent cartilage was higher in the microfracture defect, but low in both cases.

5.4.2.3 Pathologic changes in surrounding structures

Changes in the surrounding structures were revealed through semiquantitative grading (Table 5-15). Application of microfracture to the defect caused substantial loss of bone support under the defect. While such reactions were seen in untreated defects, they were much less common. Changes in other adjacent structures were similar in all groups, with some loss of safranin O staining and cloning common. There were often slight disruptions in the smoothness of the surface of adjacent tissue, but the deep tissue adjacent to the defect was generally intact in structure.

5.4.3 Effect of cultured autologous chondrocytes and a periosteal flap in chondral defects

Results presented in this section will compare the experimental group of defects treated with cultured autologous chondrocytes under a periosteal flap with two control groups: defects treated with periosteum alone, and untreated chondral defects (same data as presented in section 5.4.2). Several defects were excluded from analysis due to pathology such as subluxated patella or pannus formation in the joint. In addition, several additional samples were not processed in a satisfactory manner and are not reported.
5.4.3.1 Histological results

Histologic examination confirmed the variable filling of the defects observed grossly in all treatment groups and times. Histological micrographs of sections that are substantially filled and contain representative reparative tissues are shown for untreated defects (section 5.4.2, Figure 5-26a-d) and for cultured-autologous chondrocyte-treated (CAC) defects (Figure 5-29 a-d).

The histological description of healing was broken into early, intermediate, and later times. The appearance of all groups was relatively similar to the untreated defects at early and later times, with some differences at intermediate times as described below:

Early times (1.5 months): By this time reparative tissue formed in defects in all groups. The average filling was about half of the defect, although the amount varied significantly from sample to sample. Most of the tissue filling the defect was fibrous tissue or fibrocartilage (Figure 5-29a). A limited number of cells, most often found at the sides of the defect, displayed various stages of formation of lacunae and type II collagen production. But there was little

Table 5-15. Pathologic changes in structures surrounding full-thickness chondral articular cartilage defects in an adult canine model. Data given as the number of observed grades by defect. 3 = little or no change, 2 = slight change, 1 = moderate change, 0 = severe change. For all categories except bone, separate grades were given to the two different lateral edges. Mean values approximate the average of all observations. *6 and 9-week data combined in 1.5 month EC group.
Figure 5-29. Light micrographs of healing CAC-treated full-thickness chondral defect in the dog trochlear groove up to 18 months. Defect margins are shown by arrowheads. A) 1.5 months, hematoxylin and eosin stain (60 X). The reparative tissue consists of primarily fibrous tissue (elongated cells), with some fibrocartilage (type 'a', rounded cells) at the base of the defect (arrow). The tissue does not bond to the calcified layer. B) 3 months, safranin O/fast green stain (50 X). The reparative tissue is primarily hyaline cartilage bonded to the defect base, with some fibrocartilage superficially (arrows). The right margin of the defect is pictured and virtually indistinguishable. (continued, C and D on next page)
Figure 5-29, continued (light micrographs of healing CAC-treated full-thickness chondral defect in the dog trochlear groove up to 18 months). C) 6 months, safranin O/fast green stain (50 X). This was the best-healing defect of the experiment. The defect is nearly filled with hyaline cartilage staining intensely for glycosaminoglycans. However, integration with the adjacent cartilage is not complete, and the repair surface is somewhat rough. D) 12 months, safranin O/fast green stain (60 X). Hyaline cartilage is still visible on the base of the defect, but stains weakly for glycosaminoglycans and is continuous with type 'c' fibrocartilage (thin arrow). The superficial region of the defect is filled with fibrocartilage type 'b' that is fibrillating and coming detached from the other reparative tissue (open arrow).
hyaline cartilage and very little articular cartilage. Most hyaline cartilage that was found was consistent with small "islands" of material left over from surgery. This assessment was based on previous studies which examined identical defects at the time of creation and similar defects at 2 and 4 weeks [169]. The remnant tissue typically showed poor integration with surrounding fibrous tissue. While the findings were generally comparable among groups, the CAC treated lesions appeared to have slightly less fibrous tissue and slightly more hyaline cartilage than the periosteum or empty control groups. In the several histological sections examined from the center of the defect, about half of these samples from all groups showed evidence of resorption of the underlying bone with fracturing of the subchondral plate. Reparative tissue was clearly seen flowing between the bone and the defect site. Serial sectioning of the entire defect may have revealed an even greater prevalence of bone damage. Infrequently (less than 20% of samples in each treatment group), neovascularization was found in the defect area.

Intermediate times (3 and 6 months): These times were characterized by a chondrogenic change in tissue composition. Compared with 1.5-month samples, less fibrous tissue and more hyaline and articular were found in these defects (Figures 5-29b). Fibrocartilage tended to have a more hyaline appearance and more staining with safranin O. Both hyaline and articular cartilage were concentrated in the corners and at the base of the defect. Much of the hyaline tissue stained at or near normal for safranin O, although some regions had reduced matrix staining. The regions with reduced staining tended to be at the fringes of larger pieces, or thin regions in the defect center, especially the "islands" that may have been remnant material. Some of the islands also displayed surface fibrillations. In certain samples, the regions that appeared to be degenerating tissue left over from defect creation were clearly distinguished from adjacent regions which appeared to be regenerating cartilage.

Fibrocartilage was found superficial to the hyaline tissue, and the small amounts of fibrous tissue was almost always on the surface. Bone formed in small quantities at the base of one defect; however, no neovascularization was found in the defect area at this time.

During this remodeling phase, the CAC treated group contained more hyaline and articular cartilage, and less fibrous tissue than empty controls (no periosteal controls were included at this time). The defect which displayed the most regeneration of hyaline cartilage (Figure 5-29c) was from the CAC group (this was not the typical appearance of these defects). Both CAC and EC samples appeared to have a more intact calcified cartilage layer than 1.5-month samples, and breaches of the subchondral bone were found less frequently. The appearance of adjacent cartilage was similar to the earlier times, except that there seemed to be increased cloning in the vicinity of some defects.
Later times (12 and 18 months): Degenerative changes in the reparative tissue and/or in the adjacent cartilage characterized most specimens after one year (Figure 5-29d). For all treatments at 12 months, the tissue profile appeared similar to the EC group at 3 and 6 months. At 18 months there was an increase in fibrocartilage (mostly type ‘b’) and decreased hyaline and articular cartilage. It appeared that fibrocartilage was able to displace previously formed hyaline cartilage. In addition, other transitional tissue which did not progress to hyaline instead appeared likely to form fibrocartilage. There was little fibrous tissue consistent with scar in the long-term defects, mostly limited to the superficial layers.

Overall, there was no discernible difference among CAC, P, and EC samples with respect to reparative tissues. While degradative changes were observed in a few specimens as early as 3 months, by one year they were more widespread. Most of the hyaline cartilage did not stain normally with safranin O, was acellular, contained abnormal cloning, or appeared fibrillated (especially at the surface). Also, bone appeared more frequently in reparative tissue at the base of the defect. This occurred in 5 of 44 samples, with no preference among the treatment groups. Only 1 of 62 defects from 1.5 to 6 months exhibited bone in the defect area.

Observations of damage in adjacent cartilage: Samples which were covered with periosteum (P and CAC groups) displayed disruption of the adjacent cartilage where sutures cut through (Figure 5-30). In certain cross-sections, the suturing was found to create clefts and completely separated the superficial layers from deep layers of articular cartilage (Figure 5-30). Regions surrounding suture marks often displayed increased cloning, decreased safranin O staining, and occasionally, complete disruption of normal architecture.

Furthermore, many specimens exhibited degenerative changes in the adjacent cartilage which may have been attributable to the operation or harvest procedure, but not necessarily to suturing. These changes were most pronounced after one year. At 12 and 18 months, in the cultured autologous chondrocyte and periosteum alone groups this included cloning and loss of safranin O staining beyond the suture marks (several millimeters from the defect edge). In addition, almost every defect in the empty control group (subjected to cartilage harvesting, but not suturing) showed at least slight cloning in the cartilage far from the defect. Three of the eight untreated knees, including a knee excluded from grading, showed severe cloning, loss of safranin O stain, and disruption of the normal collagen architecture across at least several millimeters of the articular surface. Furthermore, in two of these knees, pannus formed over the cartilage surface, in some locations invading the superficial zone. In the excluded knee, the pannus completely covered the defects.
Figure 5-30. Light micrograph of a defect showing damage to adjacent cartilage from suturing of the periosteum cover. The defect is empty, except in the corner where there is a small amount of hyaline cartilage (open arrow). The adjacent cartilage still shows the path of the suture (solid arrow), with obvious mechanical disruption and some loss of safranin O staining. The damage to subchondral bone is an artifact of processing.

Figure 5-31. Light micrograph of a 6-week specimen containing cultured autologous chondrocytes labeled with the gene for β-galactosidase. The cells toward the base of the defect stain only with safranin O (red), while those at the surface stain only with X-gal (blue). This section was taken from the center portion of the defect. The tidemark is shown by the arrowhead.
Beta-galactosidase results: Of the four defects at the six-week time point, two contained cells stained blue for beta-galactosidase. One contained numerous cells in all sections (Figure 5-31), while the second had very few cells, with some sections containing no visible labeled cells. At three months, only one of four defects was found to contain labeled cells. The stained cells were encountered very infrequently (again absent in certain sections), and staining was very faded.

5.4.3.2 Quantitative results and statistical analysis

Tissue types filling the defect: Defect filling by all tissue types in all groups at all times are shown in Table 5-16.

<table>
<thead>
<tr>
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<th>n</th>
<th>Fibrous Tissue</th>
<th>Fibrocartilage</th>
<th>Hyaline Cartilage</th>
<th>Articular Cartilage</th>
<th>Bone</th>
<th>Total Repair</th>
<th>matrix flow</th>
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<td>15</td>
<td>16.8 ± 2.6</td>
<td>19 ± 4.4</td>
<td>4.4 ± 1.3</td>
<td>0.4 ± 0.3</td>
<td>0 ± 0</td>
<td>40.6 ± 4.3</td>
<td>3.3 ± 1</td>
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<td>6</td>
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<td>5.4 ± 2.9</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>56.5 ± 13.2</td>
<td>0.9 ± 0.4</td>
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<td>1.6 ± 0.7</td>
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</tr>
<tr>
<td>P</td>
<td>6</td>
<td>2.2 ± 1.4</td>
<td>27.8 ± 10</td>
<td>14.7 ± 7.6</td>
<td>1.7 ± 1.7</td>
<td>1.6 ± 1.3</td>
<td>48 ± 7.8</td>
<td>2.7 ± 1.2</td>
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<tr>
<td>CAC</td>
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<td>1.5 ± 1</td>
<td>19.1 ± 6.5</td>
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<td>0.5 ± 0.3</td>
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<td>35.9 ± 7.7</td>
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<tr>
<td>P</td>
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<td>9.6 ± 2.1</td>
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<td>5.8 ± 5.8</td>
<td>75.7 ± 14.2</td>
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<tr>
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<td>2.7 ± 2.7</td>
<td>45.9 ± 7.3</td>
<td>1.7 ± 0.7</td>
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</table>

Table 5-16. Components of healing full-thickness chondral defects in an adult canine model. Defects are untreated (EC), treated with periosteum alone (P), or treated with periosteum and cultured autologous chondrocytes (CAC); n = number of defects. Values given as an areal percent of total defect area seen in a histological cross-section from the center diameter of the defect, mean ± SEM (data averaged by defect). *1.5 month EC group includes both 6-week and 9-week defects.

Statistics of tissue types: early and intermediate results: (less than 1 year): At 1.5 months ANOVA for the three treatments showed no difference with respect to total filling (p=0.33) or with filling by fibrous tissue (p=0.54), fibrocartilage (p=0.18), or combined hyaline and articular cartilage (p=0.17). Furthermore, there were no significant differences in bonding to adjacent tissue (p=0.33) or
calcified cartilage (p=0.18), or the amount of calcified cartilage remaining intact (p=0.42).

At 3 and 6 months, no periosteal controls were included. Comparisons between groups were made by 2-tailed paired t-test. At 3 months, CAC-treated defects were found to have more total filling (p=0.04) and combined hyaline and articular cartilage (p=0.0003, p=0.005 when proximal and distal defects were averaged) than empty controls, but no difference in fibrous tissue (p=0.72) or fibrocartilage (p=0.96). The trends appeared the same at 6 months, but with lower "n", higher p-values were found in the categories where differences were found at 3 months: total filling, p<0.15 and combined hyaline and articular cartilage, p=0.12, p=0.26 when proximal and distal defects were averaged. There was still no difference in fibrous tissue, p=0.43, and fibrocartilage, p=0.38.

Statistics of tissue types: long term results: The results of the 12 and 18 month time points are of special interest, as they include all treatment groups. It was desired to know if the treatment had any effect at these times. Two-way analysis of variance with respect to treatment and time showed that there were no significant findings with respect to treatment group. Analysis of variance did, however, indicate several differences between twelve- and eighteen-month groups, the trends (from twelve to eighteen months) of which included:

1) increasing fibrous tissue (p=0.016)
2) increasing fibrocartilage (p<0.003)
3) increasing total repair tissue (p=0.016)
4) decreasing hyaline and articular cartilage (p=0.032, p=0.087 for averaging proximal and distal defects)

For completeness, the twelve- and eighteen-month results were separated and each examined for differences among treatment groups in the critical healing categories (viz., total filling and hyaline cartilage) by t tests. T test was chosen over analysis of variance to take advantage of the paired comparison of the empty control and cultured autologous chondrocyte groups, representing contralateral legs of the same animal. T tests with the periosteum alone group were unpaired. When t tests were performed with proximal and distal defects considered independent, twelve-month EC defects had more combined hyaline and articular cartilage than twelve-month CAC defects (p=0.05). With defects averaged by knee, the corresponding value was p=0.13.

Overall, the evolution of the profiles of tissue types was very similar in all groups to that already presented for the untreated group (Figure 5-32). This figure shows that the initial contribution from hyaline and articular cartilage in CAC-treated defects is slightly higher than that for the other groups. Also, the changes in tissue types which follow (intermediate times: increasing hyaline tissue, decreasing fibrous tissue and fibrocartilage; and longer times:}
Figure 5-32. Composition of reparative tissue in three types of defects. Mean contributions of each category are normalized by the total percent of defect filling for the category at that time point. CAC=cultured autologous chondrocyte, P=periosteum alone, EC=untreated, empty control.
decreasing hyaline tissue and increasing fibrocartilage) are generally even more pronounced in the CAC-treated group.

**Attachment, bonding, and calcified cartilage**: Attachment to adjacent tissues and preservation of the calcified cartilage are shown in Table 5-17.

<table>
<thead>
<tr>
<th>time</th>
<th>treatment</th>
<th>Integration with adjacent AC</th>
<th>Bonding to calcified cartilage</th>
<th>Intact calcified cartilage</th>
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<td>9.2 ± 3.3</td>
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<td>P</td>
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<td>59.1 ± 18.9</td>
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<tr>
<td></td>
<td>CAC</td>
<td>16.4 ± 3.9</td>
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<td>77.5 ± 8.6</td>
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<tr>
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<tr>
<td></td>
<td>CAC</td>
<td>20.8 ± 5.3</td>
<td>75.5 ± 12.2</td>
<td>96.4 ± 2.6</td>
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<tr>
<td>6 months</td>
<td>EC</td>
<td>15 ± 6.4</td>
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Table 5-17. Integration of reparative tissue and status of the underlying calcified cartilage in healing full-thickness chondral defects in an adult canine model. Defects are untreated (EC), treated with periosteum alone (P), or treated with periosteum and cultured autologous chondrocytes (CAC). Values given as a linear percent of the length of the defect base or height, as seen in a histological cross-section from the center diameter of the defect, mean ± SEM (data averaged by defect). *1.5 month EC group includes both 6-week and 9-week defects.

**Statistics of attachment, bonding, and calcified cartilage**: At 1.5, 12, and 18 months, ANOVA found no differences among all three treatment groups with respect to bonding to adjacent cartilage or the base, or in the amount of intact calcified cartilage. When periosteum was omitted analysis was performed by 2-tailed paired t-test. In cell-treated samples vs. empty controls there was more bonding to adjacent cartilage (p=0.067 and p=0.015) and the calcified cartilage (p=0.004 and p=0.078) at 3 and 6 months respectively, but there were no differences in the amount of calcified cartilage remaining intact (p=0.81 and p=0.86).
Like the untreated group, the periosteum and CAC groups also displayed minimal values for intact calcified cartilage and bonding to calcified cartilage at 1.5 months.

5.4.3.3 Pathologic changes in surrounding structures

As previously described, reactions at the base of the defects sometimes resulted in disruption of the calcified cartilage layer, penetration into the subchondral bone, and resorption of the subchondral bone resulting in decreased bone support under the defect (Table 5-18). In all groups, the bone support was graded lowest at 1.5 months, corresponding to the greatest amount of damage to the calcified cartilage in Table 5-17. At all times, the empty control group had approximately as much or more safranin O staining than CAC- treated defects. This was consistent with damage that was seen to have occurred from suturing. However there did not appear to be consistent differences in cloning reactions to the treatment or with time. Excluding damage from sutures, the superficial and deep tissue integrity was similar in all times and groups, with these structure substantially intact in most specimens.
<table>
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</tbody>
</table>

Table 5-18. Pathologic changes in structures surrounding full-thickness chondral articular cartilage defects in an adult canine model. Defects are untreated (EC), treated with periosteum alone (P), or treated with periosteum and cultured autologous chondrocytes (CAC). Data given as the number of observed grades by defect. 3= little or no change, 2= slight change, 1= moderate change, 0= severe change. For all categories except bone, separate grades were given to the two different lateral edges. Mean values approximate the average of all observations. *6 and 9-week data combined in 1.5 month EC group.
5.4.4 Effects of matrix treatments

This work included two series of experiments involving matrix implantation in the canine model, all evaluated quantitatively at 15 weeks. In the first series, implants included fascia alone, fascia-covered type I matrix (seeded and unseeded), and seeded type II collagen matrix. In the second series, implants/treatments included seeded type II matrix, microfracture treatment, and microfracture with unseeded type II collagen. The gross appearance of defects at sacrifice was variable, resembling that of the original series of dogs for testing autologous chondrocyte transplantation.

**Overall histological impression:** Histologically, there were several clear differences between these series and the original series. First, communication with the subchondral bone occurred more frequently and to a more significant extent in all of these groups compared to the original series at this time. This was especially true for the second series. The reactions in the underlying bone often resorbed nearly all of the calcified layer. Also, unlike the original series in which vascularized reparative tissue was limited to a few samples at 1.5 months and none at all at 3 months, vascularized tissue was seen in the defect area in several of the specimens in these series.

**Original defect area:** While the level of filling was increased in most groups compared to untreated defects (excluding fascia and type I matrix alone), the vast majority of the tissue forming was fibrous in nature, either fibrous tissue or fibrocartilage. Safranin O and type II collagen staining were found in the original defect area in many of these specimens. This staining was very preferentially in the corners of the defect (at the periphery and base). These locations corresponded to regions which were graded as fibrocartilage types 'b' or 'c', or with the slight amounts of hyaline cartilage. In some specimens where the calcified cartilage was resorbed, staining in remodeling tissue in the underlying bone (see below) extended upward into the defect, in some cases almost to the surface. When there was safranin O staining, there was also type II staining. However, some specimens stained with type II in areas that did not stain with safranin O.

Vascularization was always found in defects in which there was increased communication with the bone, and with vessels extending into the underlying bone region. This was the case primarily in the second series, which had the most communication and in which 7 of 24 defects contained vessels in the original defect area. In the first series, only 1 of 22 defects had neovascularization in the defect area, with two exhibited new vessels just below the defect area. Tissue with vessels did not stain for safranin O or type II collagen.
Underlying bone: Bone remodeling took place in a variety of manners. In some sections, there was evidence of remodeling despite an intact overlying plate. In others, there was a small crack (30-200 μm) in the calcified cartilage with a teardrop-shaped area of resorption immediately beneath. These areas varied in size, from almost nothing to over 1 mm in depth and width. In other samples, the resorptive process included nearly the entire width of the defect. The depth varied from barely below the calcified layer to over 1.5 mm.

Because of the reaction in the bone, observations were also made of the tissue filling this region. The appearance of tissues in the subchondral region varied significantly (Figure 5-33). In many samples in which a remodeling reaction in the underlying bone was visible despite an intact overlying plate, or in which communication was limited to one small break in the plate, the tissue filling the marrow spaces was primarily very dense rounded or ovoid cells lacking lacunae (Figure 5-33a). Some elongated cells were also seen. These areas did not stain for safranin O or type II collagen. In some sections regions of highly vascularized tissue were found in the subchondral region (Figure 5-33b). Vascularized areas did not stain for safranin O or type II collagen, even in defects which also included other types of tissue that did (see below).

Other samples contained regions with a variety of appearances from fibro-cartilaginous (Figure 5-33c) to cartilaginous, distinguished by subtle differences in matrix appearance. The characteristic feature of these regions was chondrocyte-like cells in lacunae. Pericellular matrix was often visible, and there was often staining for both safranin O and type II collagen. Again, type II collagen staining was always at least as prevalent as safranin O staining. Type II and safranin O staining were most intense around the most cartilaginous appearing areas (less fibrous matrix with chondrocytic, rounded cells with lacunae). There was a tendency for the most intense pericellular and intercellular matrix staining in the deepest portions of the defect. In virtually all specimens with staining for safranin O above and below the original location of the calcified layer (which was mostly resorbed), the extent and intensity of the staining was greater below this level.
Figure 5-33. Light micrographs showing a range of appearances of tissue found in remodeling subchondral bone in the canine trochlea (type II collagen/hematoxylin stain). (a) Fibrous, vascularized tissue, with dense ovoid and elongated cells. Numerous vessels are found (arrows) and there is no visible brown staining for type II collagen. (b) Fibrous tissue with dense cells of more ovoid and rounded appearance. Some bone is visible (open arrow). (continued on next page)
Quantitative and semiquantitative results: The quantitative and semiquantitative data for the two series are summarized in Tables 5-19 though 5-24. General discussion of these results is contained within this section. Results specific to comparison between specific groups follow. The amount of matrix flow was similar in all treatment groups (approximately 2%). No bone was found in the defect site in any of these groups.
In both series, all implanted matrices increased the amount of defect filling compared to fascia or untreated controls. However, the untreated controls had the highest level of hyaline and articular cartilage. Fibrous tissue contributed about the same amount to defect filling in all matrix implants (15-19%), however, the most prominent tissue type was fibrocartilage.
Table 5-21. Integration of reparative tissue and status of the underlying calcified cartilage in healing full-thickness chondral defects in an adult canine model. Defects were treated with collagen matrix implants or controls as indicated. Values given as a linear percent of the length of the defect base or height, as seen in a histological cross-section from the center diameter of the defect, mean ± SEM (data averaged by defect).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bonding to calcified cartilage</th>
<th>Attachment to adjacent cartilage</th>
<th>Percent of intact calcified cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeded Type II (initial)</td>
<td>6 ± 2</td>
<td>52 ± 7</td>
<td>43 ± 14</td>
</tr>
<tr>
<td>Seeded Type I + fascia</td>
<td>22 ± 13</td>
<td>33 ± 4</td>
<td>58 ± 14</td>
</tr>
<tr>
<td>Unseeded Type I + fascia</td>
<td>14 ± 5</td>
<td>32 ± 8</td>
<td>67 ± 11</td>
</tr>
<tr>
<td>Fascia</td>
<td>5 ± 4</td>
<td>16 ± 13</td>
<td>71 ± 13</td>
</tr>
<tr>
<td>Untreated controls</td>
<td>55 ± 12</td>
<td>23 ± 4</td>
<td>87 ± 11</td>
</tr>
</tbody>
</table>

Bonding to the calcified cartilage was much less in treated defects than controls. This appeared to be directly related to the criterion that a prerequisite for bonding was an intact calcified cartilage layer. The calcified layer was substantially damaged in all implant groups, ranging from 18 to 67% intact. The calcified cartilage in untreated controls were approximately 90% intact, and the fascia group 71%. The lack of bonding to the calcified layer also matched the lack of formation of hyaline tissue and was reflected in semiquantitative grading (Tables 5-23 and 5-24, below). These results were similar to those in the study of untreated defects and the effect of CAC in this respect. The attachment to adjacent cartilage was increased in most matrix treated groups compared to controls, with attachment levels ranging from 25 to 50% of the interface with adjacent cartilage.

Table 5-22. Integration of reparative tissue and status of the underlying calcified cartilage in healing full-thickness chondral defects in an adult canine model. Defects were treated with collagen matrix implants and/or microfracture, or left untreated. Values given as a linear percent of the length of the defect base or height, as seen in a histological cross-section from the center diameter of the defect, mean ± SEM (data averaged by defect).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bonding to calcified cartilage</th>
<th>Attachment to adjacent cartilage</th>
<th>Percent of intact calcified cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeded engineered type II</td>
<td>1 ± 1</td>
<td>34 ± 8</td>
<td>39 ± 13</td>
</tr>
<tr>
<td>Unseeded type II + microfracture</td>
<td>3 ± 1</td>
<td>24 ± 6</td>
<td>18 ± 5</td>
</tr>
<tr>
<td>controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microfracture</td>
<td>2 ± 1</td>
<td>25 ± 9</td>
<td>36 ± 11</td>
</tr>
<tr>
<td>Untreated</td>
<td>51 ± 11</td>
<td>12 ± 3</td>
<td>95 ± 2</td>
</tr>
</tbody>
</table>
The grades assigned to the semiquantitative categories in the two series dogs is shown in Tables 5-23 and 5-24. For the adjacent cartilage section, the two grades given to the two adjacent edges are both recorded. For each group, the mean grade is given. Note that this mean does not have full quantitative significance due to the ordinal nature of the scale. Also, the semiquantitative grading from this series may not be directly compared to results from the first series because the grading was performed by a different observer.

Table 5-23. Pathologic changes in structures surrounding full-thickness chondral articular cartilage defects in an adult canine model. Defects were treated with collagen matrix implants or controls as indicated. Data given as the number of observed grades by defect. 3= little or no change, 2= slight change, 1= moderate change, 0= severe change. For all categories except bone, separate grades were given to the two different lateral edges. Mean values approximate the average of all observations. *6 and 9-week data combined in 1.5 month EC group.

<table>
<thead>
<tr>
<th>category</th>
<th>bone</th>
<th>adjacent articular cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>saf O-stain</td>
</tr>
<tr>
<td>Seeded II</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>mean</td>
<td>0.75</td>
<td>1.43</td>
</tr>
<tr>
<td>Seeded I</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>mean</td>
<td>1.13</td>
<td>1.78</td>
</tr>
<tr>
<td>Unseeded I</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>mean</td>
<td>1.00</td>
<td>1.35</td>
</tr>
<tr>
<td>Fascia</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>mean</td>
<td>1.83</td>
<td>1.19</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>mean</td>
<td>2.11</td>
<td>1.90</td>
</tr>
</tbody>
</table>

Table 5-23. Pathologic changes in structures surrounding full-thickness chondral articular cartilage defects in an adult canine model. Defects were treated with collagen matrix implants or controls as indicated. Data given as the number of observed grades by defect. 3= little or no change, 2= slight change, 1= moderate change, 0= severe change. For all categories except bone, separate grades were given to the two different lateral edges. Mean values approximate the average of all observations. *6 and 9-week data combined in 1.5 month EC group.

Note on semiquantitative tables: the semiquantitative results for pathology of specimens used for studying the effect of matrices on healing in Tables 5-23 and 5-24 were recorded by different observers. The interobserver reliability of the semiquantitative assessment was not tested and is not known. Thus, within each table, ordinal comparison of data is valid. However, data in one table CANNOT be directly compared to that in other tables due to the changes made between experiments.
Table 5-24. Pathologic changes in structures surrounding full-thickness chondral articular cartilage defects in an adult canine model. Defects were treated with collagen matrix implants and/or microfracture, or left untreated. Data given as the number of observed grades by defect. 3= little or no change, 2= slight change, 1= moderate change, 0= severe change. For all categories except bone, separate grades were given to the two different lateral edges. Mean values approximate the average of all observations. *6- and 9-week data combined in 1.5 month EC group.

<table>
<thead>
<tr>
<th>category:</th>
<th>bone</th>
<th>saf O stain</th>
<th>cloning</th>
<th>surface integrity</th>
<th>deep tissue integrity</th>
</tr>
</thead>
<tbody>
<tr>
<td>grade:</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>0 1 2 3</td>
</tr>
<tr>
<td>Engineered type II + CAC</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>2 1 4 9</td>
</tr>
<tr>
<td>mean</td>
<td>0.75</td>
<td>2.25</td>
<td>1.88</td>
<td>2.56</td>
<td>2.12</td>
</tr>
<tr>
<td>Engineered type II+ microfracture</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4 2 4 6</td>
</tr>
<tr>
<td>mean</td>
<td>0.12</td>
<td>1.75</td>
<td>1.50</td>
<td>2.56</td>
<td>2.06</td>
</tr>
<tr>
<td>microfracture</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>2 1 5 8</td>
</tr>
<tr>
<td>mean</td>
<td>0.50</td>
<td>2.19</td>
<td>2.06</td>
<td>2.69</td>
<td>2.88</td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>6</td>
<td>0 0 15 3</td>
</tr>
<tr>
<td>mean</td>
<td>2.56</td>
<td>2.17</td>
<td>1.67</td>
<td>2.53</td>
<td>2.83</td>
</tr>
</tbody>
</table>

**Series I:** The results of the adjacent cartilage reflect the damage done in suturing in the four implant groups. Untreated defects retain more safranin O stain, as well as integrity of the superficial and deep adjacent cartilage relative to the implant groups. However, cloning is more similar in all groups.

The grading of subchondral bone indicates that severe damage occurred only in the defects receiving matrix implants. The untreated and fascia groups had less bone damage, with untreated the only group with an average score indicating only slight damage.

**Series II:** The most striking effect was that seen in the subchondral bone. Approximately three quarters of the samples in each of the three treatment groups had severe changes in the bone, while no untreated defects registered this score.

In the adjacent cartilage, the two groups which received suturing registered more changes in deep tissue integrity due to the presence of the suture tracks. However, close to the defect site, the changes in safranin O, cloning, and the surface of the tissue were similar in all groups.
5.4.4.1 Effects of unseeded type I matrix in chondral defects

All 4 week and 15-week defects containing fascia only or fascia-covered matrix were observed histologically. Only 15-week defects were evaluated quantitatively for tissue types (Table 5-20).

Histological observations showed wide variation in defect filling from sample to sample. Fifteen-week samples appeared to be slightly more filled with repair tissue than 4 week samples. At 15 weeks, matrix implanted samples showed a trend toward more reparative tissue than fascia only controls (p<0.10). In each group, several defects had almost no filling, while others were filled over 50% by repair tissue, but more fascia only defects were essentially unfilled. The repair tissue was similar in all groups, with the vast majority fibrous in nature. In each treatment group, in selected samples, small areas of fibrocartilage, type 'c' or hyaline cartilage were seen, usually in the corners of the defect next to the adjacent cartilage. These areas stained faintly with safranin O, indicating the presence of GAGs. However, even at 15 weeks, hyaline and articular cartilages were essentially absent compared to controls.

At 4 weeks, tissue that was consistent with remodeling fascia was found in several defects in both groups. It was not proven that this tissue was originally fascia, however, in numerous studies in groups without fascia, no tissue similar in appearance was seen. At 15 weeks, tissue that resembled fascia was only seen in 1 of 6 defects in each group (with and without matrix). No matrix was found even at 4 weeks.

As expected the fibrous tissue did not bond to the calcified cartilage (judged by polarized light). Attachment to adjacent cartilage was good in defects which were filled, but the overall attachment was low and there was no statistical difference between groups. Still, some samples showed obvious clefts between the adjacent cartilage and repair tissue.

Approximately three out of every four defects at each time showed at least one break in the subchondral plate. The reactions in the subchondral bone varied, with the most severe reactions found in the matrix implanted group. There was no statistical difference in the amount of remaining intact calcified cartilage. The presence of calcified cartilage, repair tissue, sutures, and fascia in resorbed areas below the level of calcified cartilage in certain samples seem to indicate a sudden collapse of a large area into the bone. In some cases there was a clear distinction between fibrous tissue derived from the subchondral area and fibrous tissue presumably from the joint space. In one case, the subchondral material seemed to be displacing the overlying tissue.
5.4.4.2 Effect of unseeded type II matrix in microfracture defects

Micrographs of healing defects treated by microfracture alone and with microfracture and engineered type II matrix are shown in Figures 5-27 and 5-34. The calcified cartilage and subchondral plate were violated in all such specimens, however the reaction in the bone of the matrix-implanted group was much greater. Figure 5-34 shows a severe reaction in the matrix-implanted group. The deeper portions of the defect contain more cartilaginous tissue than the surface, staining at least lightly for safranin O throughout. Hyaline cartilage is forming at the periphery of the defect above the small section of calcified cartilage that has not resorbed.

More matrix implanted specimens were filled to the top of the defect, however the trend toward more total filling was not found statistically significant (p=0.20). Although the reparative tissue was predominantly fibrocartilage in both groups, there was more safranin O and type II collagen staining in the matrix implanted group. Two samples in each group had vessels forming in the defect area.

The subchondral bone reaction was visually more severe in the matrix implanted microfracture defects compared to the untreated defects. While the corresponding amount of intact calcified cartilage was greater in the microfracture only group, the difference was not found to be highly statistically significant (p=0.17). While only one microfracture defect had at least an intermediate amount of safranin O staining in remodeling bone below the defect, most of the matrix implanted specimens had large subchondral regions staining for safranin O. Furthermore, while three of four microfracture defects with significant bone remodeling contained newly vascularized tissue in this region, only two of eight matrix implanted specimens had similar vessel formation.

5.4.4.3 Effect of seeded type I matrix in chondral defects

Defects implanted with seeded type I matrices were more filled than defects implanted with unseeded type I matrices. Statistically this difference in total fill was not significant by t-test (p=0.37). Reparative tissue was fibrous and fibrocartilaginous, and no vessels were seen in the defect area in either group (although some new vessels were very close to the defect). The appearance of the adjacent cartilage was similar, with both groups demonstrating damage from suturing.

The reaction in the subchondral bone appeared slightly more severe with the seeded implant group, although the amount of intact calcified cartilage could not be distinguished statistically. In addition, there was more staining for safranin O and type II collagen in the cell-seeded group. In each group, in one
Figure 5-34. Light micrograph of a healing full-thickness chondral defect treated with microfracture and unseeded type II collagen matrix in the dog trochlear groove at 15 weeks (safranin O/fast green stain, 40 X). There is severe subchondral bone remodeling in this defect, with cartilaginous tissue staining for safranin O in deeper regions (arrow). The fibrocartilaginous tissue in the defect stains for safranin O primarily in the corner. Near the adjacent cartilage at the defect edge, near-hyaline tissue is forming.

Figure 5-35. Light micrograph of a healing full-thickness chondral defect treated with CAC-seeded type II collagen matrix in the dog trochlear groove at 15 weeks (safranin O/fast green stain, 40 X). There is severe subchondral bone remodeling in this defect, with cartilaginous tissue staining for safranin O in deeper regions (closed arrow). The adjacent cartilage is severely damaged by suturing, with mechanical disruption and near complete loss of safranin O staining (open arrow).
of eight specimens newly formed vessels were found in the remodeling bone. No differences in attachment or bonding could be distinguished.

5.4.4.4 Effect of implant chemistry in chondral defects

The appearances of defects implanted with seeded type I or type II collagen matrices were very similar, with approximately the same total fill and tissue profiles (predominantly fibrocartilage, Table 5-19). The type II group was found to have better attachment to adjacent cartilage \( (p<0.03, \text{ two-tailed t-test}) \). One type II-implanted defect contained vessels at the very base of the original defect.

The reaction in the underlying bone varied significantly across each group, with slightly more reaction in the type II group. In the defects in which the bone remodeling was most severe, there tended to be more subchondral staining for safranin O and type II collagen in each group. Only one defect in each group displayed newly formed vessels in the subchondral bone.

5.4.4.5 Comparison of cell source with type II matrices

For comparison with the microfracture plus type II matrix group (Figure 5-34), a representative sample from the engineered, CAC-seeded type II matrix group is depicted in Figure 5-35. The figure shows that in the CAC-treated group, there is also an extensive reaction in the underlying bone in many samples. In this case there is intense staining for safranin O, mostly at the level of the underlying bone, but extending somewhat into the defect area. However, this finding was more consistent across the microfracture with matrix group. In Figure 5-35, also note the damage in the adjacent cartilage from suturing including mechanical disruption, tissue forming in the suture track, and near complete loss of safranin O from the matrix.

In comparing the treatments involving implanted type II and different cell sources (subchondral cells from microfracture vs. cultured autologous chondrocytes in chondral defects), the microfracture defects (Table 5-20) contained more reparative tissue \( (p=0.27) \). In each case, the predominant tissue types filling the defect were fibrous in nature, with similar contributions from all tissue types. The treatment with microfracture caused more damage to the calcified cartilage layer, as evidenced by less remaining intact calcified cartilage \( (p=0.15) \).

5.4.5 Comparison of all 15 week groups

Selected data from all canine groups at 15 weeks are presented for comparison in Figures 5-36 and 5-37. Figure 5-36 plots total defect filling in decreasing order and the corresponding percent of intact calcified cartilage. These data have an inverse correlation with \( R^2 = 0.61 \) (linear regression analysis, \( R = - \)).
Figure 5-36. Graph depicting the inverse relationship between total defect filling and remaining intact calcified cartilage for 15 week canine implant groups or controls. Linear regression analysis of correlation of total filling with intact calcified cartilage: $R^2=0.61$.

Note that the two points which appear to not fit the trend (CAC alone and untreated defects) are two of three groups in which a matrix was not implanted.

In Figure 5-37, the normalized contribution of tissue types for these same groups are plotted. This graph shows clearly the inability of implant treatments to match the contribution of hyaline cartilage found in CAC and empty control defects. These same treatment groups exhibited the lowest contributions to reparative tissue by fibrocartilage and fibrous tissue. These same groups had a substantially more intact calcified cartilage layer (Figure 5-36) and the least reaction in the subchondral bone, however the level of filling was relatively low (Figure 5-36).
Figure 5-37. Composition of reparative tissue in all groups at 15 weeks. Mean contributions from each tissue type are normalized by the total percent defect filling for the group. μFx=microfracture, CAC=cultured autologous chondrocytes. type I and type II refer to collagen matrices.
6. Discussion

The novel findings of this work were as follows:
- This is the first work to develop a quantifiable adult canine model of a full-thickness chondral articular cartilage defect, a defect that is being created clinically in treating some cartilage lesions.
- The model was used to study the time course of healing, up to 18 months, of defects treated with cultured autologous chondrocytes injected under periosteum. Untreated controls were included at all times and periosteum controls at selected times. The finding of no long-term effect differed from previous studies. This was also the first work in any model to document the nature of the initial appearance of a full-thickness chondral lesion.
- These are the first reports of implantation of a resorbable matrix (seeded or unseeded) and of microfracture treatment applied to a full-thickness chondral defect in an animal model.
- The use of an insoluble porous matrix made of predominantly type II collagen has not been reported in previous work in tissue engineering of articular cartilage. This construct generated several notable findings:
  1) A difference in chondrocyte behavior in vitro in the type II material versus one made of predominantly type I collagen.
  2) The implantation of these matrices in the animal model increased the amount of reparative tissue, albeit with less hyaline tissue at 15 weeks.
  3) The implants of these matrices were associated with a biological reactivity that caused resorption of the calcified cartilage and underlying bone.

It is important to note that this work has not yet resulted in the regeneration of articular cartilage. While limited areas of healing tissue appeared similar to articular cartilage, the complete articular structure, from base to surface, was not found. Furthermore, there was no demonstration that tissue which histologically resembled articular cartilage was, in fact, biochemically and biomechanically identical to normal tissue. On the positive side, engineered matrices did result in increased amounts of reparative tissue relative to untreated controls and sites treated with cultured autologous chondrocytes alone. Longer term studies will be required to determine if this reparative tissue matures to articular cartilage.

6.1 Type II collagen-GAG analogs of extracellular matrix

Although only the few constructs described in this thesis were extensively studied, a wide range of pore sizes were produced in flat sheets of matrix by varying the temperature of the freeze drier or freezing tray/mold. This is consistent with previous work using the same equipment with type I collagen-GAG matrices. It also appeared that several other variables could affect the resultant pore structure, including slurry temperature, size of the mold,
contact between the tray and shelf, and position within the freeze drier. Thus, if a consistent pore structure is desired, care must be taken to ensure that all processing conditions are exactly reproduced.

The lowest temperature obtainable in our freeze drier was approximately -45°C. In preparing matrices in sheets at a range of temperatures down to this level, the lower limit on pore size for a sample large enough to be used in these experiments (5 mm diameter) appeared to be approximately 50 µm. Work with type I collagen materials suggest that much pores as small as 5 µm can be made in sheets under different freeze-drying conditions including much lower temperatures (-200°C) [41]. It is also known that pores as small as 5 µm in type I matrices can be produced by lowering tubes (1.5 mm diameter) into liquid-nitrogen cooled oil baths [30], and that the pore structure can be modified by the rate of immersion and graft diameter [101, 102]. These methods were not used with type II materials in this work, but could be used if smaller pores were desired. However, because the minimum pore size used in this work (27 µm) approaches the size of the a chondrocyte (approximately 15-20 µm), it is not anticipated that smaller pores would allow cell penetration into the material.

Type II matrices made in our laboratory grossly had physical properties similar to those of type I matrices made under similar conditions. However, the properties of the matrices in our lab appeared different from type II matrices produced commercially from a similar starting material. The reasons for this difference are unknown, as the processing conditions for the commercial product were not revealed. Pore diameter analysis indicated that the pore structure was more closely controlled in our laboratory than in the commercial product.

6.2 In vitro experiments

6.2.1 Recommendations for seeding procedures

Several experimenters in our laboratory agreed that it was technically easier to seed the type II matrices. Relative to the type I matrices, the type II matrices, were, stiffer, collapsed less when wet, and were able to maintain an expanded form when dried on filter paper. In contrast, it was difficult to dry the type I matrices to the same degree due to the collapse of the matrix. The drier type II matrices also appeared to absorb the cell suspension more readily.

Stiffer matrices, as evidenced by type II seeding vs. type I, were easier to seed. Based on the results of the seeding experiments, the following were concluded:
- Seeding on both sides increased the number of cells in the matrix and improved the distribution.
- Seeding on an agarose bed and drying the matrices on filter paper are both recommended to increase the number of cells delivered to the matrix.
- If it is possible to constrain the matrix laterally, this will also increase the percentage of cells delivered to the matrix.

- The time at which the seeded constructs are added to medium is not critical for the first few hours. It is recommended to add the matrices to medium quickly (within one or two hours) to provide the cells with nutrition. The finding of less washout of cells from the 6 hour sample suggested that after several hours more cells may attach to the matrix and reduce the washout effect. However, washout is only reduced from approximately 30% to 12%, and leaving the matrices without a source of nutrition for this long may cause cell death.

The result that the use of agarose improved seeding agreed with previous work in which Louie seeded similar matrices with tenocytes [102]. Additional methods which may improve seeding, such as agitation [35, 51], injection [191] and vacuum seeding [134, 186] were not studied in this work. Modification of the matrix to be seeded with extracellular matrix components or synthetic factors which may bind the cells has also been used in other work to attempt to improve seeding with other cell types [84, 98] and chondrocytes [170].

6.2.2 Matrix chemistry effects

There was a significant effect of the type of matrix used in the study on cell morphology in matrices comprised primarily of type I and II collagen. This study was repeated up to three weeks with similar results [130]. The majority of cells in predominantly type II matrices were rounded, while those in predominantly type I matrices were elongated.

This was the first study reporting the effect of insoluble matrices made of types I and II collagen on chondrocytes in vitro. Because the constructs consisted of at least 90% collagen, it is likely that collagen type was a primary regulator of chondrocytes. As early as the 1970’s, it was demonstrated that type II collagen could affect chondrocyte phenotype, at least in developing tissues [96]. More recently that a type II collagen coating helped promote adult chondrocyte attachment to synthetic polymers [170]. Another recent study directly comparing the effects of types I and II collagen in soluble form indicated that different collagen types had different effects on chondrocyte DNA and proteoglycan synthesis in a specific culture system [149].

In the study by Qi et. al. [149], at least part of the collagen type effect could be explained by matrix modulation of growth factor activity. Thus, although in the present study the effect of growth factor modulation was not studied, it is one possible mechanism for the effects seen. Another possible mechanism for differential effects of collagen type is that of integrin binding. Tuckwell has shown that integrins may react with different types of collagen [183]. The
differences in peptide sequences in the different type collagens can determine which collagens act as ligands to specific integrins [181].

Furthermore, the effect of matrix chemistry may also depend on collagen conformation. Two studies with type II collagen have shown such a difference. Tuckwell found that different integrins mediated chondrosarcoma cell attachment to type II collagen with intact native structure versus denatured collagen [181]. This study was supported by of changes in chondrocyte biosynthetic activity when cultured with solubilized intact versus denatured type II collagen [149]. The change in the levels of collagen structure encountered in the production of type I matrices has been extensively reviewed by Yannas [199]. While the type I material has been shown to retain some elements of native collagen banding (quaternary structure), the structure of the type II material received from Geistlich Biomaterials has been shown to be less organized [130]. This finding may explain some of the difference in behavior between the materials in the present study.

However, it cannot be conclusively stated that only the collagen type was responsible for the observed behavior, as there may have been other subtle differences in matrix chemical or mechanical properties which could contribute to these differences. Thus, a clear limitation of this work is the lack of a complete biochemical analysis of the materials used to produce the matrices. Other extracellular matrix components which have been implicated in regulating chondrocyte behavior include proteoglycans [96] hyaluronic acid [197] and link protein [148]. These reports indicated that several additional factors, including concentration and molecular weight may be critical determinants of cell behavior. In addition, the other extracellular matrix components found in small quantities may have differential effects on chondrocyte behavior. For example one study found that one purified, component cartilage oligomeric matrix protein, promoted chondrocyte attachment, while a second, thrombospordin 1, did not [43].

One known difference between the matrices in the present work was that of glycosaminoglycan content. The type I material had 3 to 4 times as much GAG as the type II material. Because there were differences in producing the slurries, the GAG may also have been incorporated in different forms. It has been shown in production of the type I material that the GAG is covalently linked to the collagen by dehydrothermal treatment [199]. In the type II material the state of the GAG was not studied. Many studies have demonstrated effects of GAG-like substances on chondrocytes or, more generally, the synovial environment [10, 11, 103, 114]. It is not known if the GAG in the form or forms found in the present work may effect chondrocyte behavior.

The findings of morphology in one study of type II materials (the comparison with type I materials) did not match those in a second study of type II
materials (comparison of varying pore size in type II materials). In the first study, over 60% of cells in type II matrices were rounded even after as long as 2 weeks in culture. This result was duplicated by another experimenter in our laboratory using the same materials [130]. In the second experiment, while well over 90% of the cells were initially rounded, fewer than 40% remained rounded at times from 1 day to 1 week. It should be first noted that caution should be used in comparing in vitro experiments using different batches of cells. Although the passage number was the same, there could have been subtle differences in cell origin or culture conditions which altered the phenotype of the cells. In addition, the type II matrices used in the study were made by different processes, and may therefore have had subtle structural differences. Because the first study was duplicated in one case with the same material, but not in the second study with a different material, this is a strong possibility. One possible mechanism may have been a change in collagen conformation (banded vs. non-banded) from differences in processing.

Other differences in this series of experiments may not be completely due to matrix chemistry (see discussion of matrix contraction, 6.2.5).

6.2.3 Effects of matrix pore structure

In this work, it appeared that the most important effect of the pore structure of the matrix was in determining cell penetration into the matrix. Grossly, surfaces with more open structures allowed more cells to penetrate into the matrix. In surfaces with few pores, cells still managed to attach to the outer layer. For relatively open pores, the pore diameter was grossly related to the degree of penetration, with cells penetrating further into matrices with larger pores. A pore diameter of at least 100 μm was needed to obtain fairly uniform seeding throughout a 2-3 mm thick sample. This finding is in agreement with the quantitative finding of Louie with tenocytes in type I collagen-GAG matrices [102].

It is assumed that the principal mode of penetration in our system was gravitational settling, where the cells are passive. This finding agrees with work that examined distribution of passive particles injected into matrices of varying pore size [191]. In the present work only after one week in culture was there evidence of active cell migration into the construct.

The measured effects of the pore diameter of a matrix on cell behaviors in vitro was limited. In type I matrix, there was an effect on cell morphology at three hours, but not at one week. In type II matrix, there was no significant difference in quantitative analyses at 1 day and 1 week, or in qualitative analysis at three hours. It is more difficult to make conclusions about the pore diameter effects on the proliferative and biosynthetic behaviors of these cells because the assays included both cells within the pores and those in layers on
the surface of the matrix. With many matrices exhibiting thick surface layers of cells, the contribution of the cells within the pores was not known.

Previous analysis of the effect of pore size in tissue regeneration has concentrated on in vivo experimentation [30, 93, 202]. Because in the current work no differences were seen in vitro in the pore diameter range 50-250 μm, it is not believed that the pore diameter will be an important variable in vivo.

6.2.4 Effects of degree of cross-linking

There was a clear difference in the ability of matrices to resist degradation in culture based on the degree of cross-linking to which they were exposed. This is most meaningful to the surgeon or tissue engineer who is interested in providing a construct that can be easily handled and transplanted from culture to a biological recipient.

However, this experiment was not able to detect quantitative differences in cell behavior based on degradation rate. While cell-breakdown products may affect cell-behavior [149], it was not evident in this work. It is possible that such products were not able to accumulate to significant enough levels in the medium under the conditions of the current experiment.

In vivo, only one degree of cross-linking was used. It has been hypothesized that the degradation rate of an implant, as controlled by the degree of cross-linking, may be an important determinant in the biological activity [199]. Further work would be needed to determine whether the time course of healing in the cartilage defects studied here could be altered by varying the degree of cross-linking of the implants.

6.2.5 Matrix contraction

The observation of matrix contraction indicates that, when seeded into matrices of either type I or II collagen, chondrocytes are capable of adopting a contractile, or “myofibroblastic” phenotype [48, 163]. In one other study reporting contraction of a collagen gel by chondrocytes, the chondrocytes were described as “dedifferentiated” [143], also suggesting that in order for chondrocytes to contract a matrix, they may have to undergo a change of phenotype.

Little is known about chondrocytes contracting matrices. Most work in contraction has focused on the behavior of fibroblasts in contracting collagen gels or lattices. This work has suggested that collagen type may be one mediator of cell contraction. Initially, one set of experiments using collagen gels of differing collagen type showed differences in matrix contraction by fibroblasts [44]. It should be noted that these experiments were not controlled for the mechanical properties of the matrix (see below). Other studies which
implicate integrins in contraction would support the hypothesis that a
difference in contraction is due to different integrin recognition by different
collagen types. These studies concluded that gel or lattice contraction was
mediated only by a subset of the chondrocyte integrins [66, 177], and that
integrin expression by the cells was in turn regulated by exposure to the matrix
[91, 162].

But based on the present work, collagen type alone cannot be solely responsible
for contractile phenomena. In this work, two different type II matrices
behaved differently with respect to the ability of the chondrocytes to contract
the matrix. One explanation might be that these two observations were not
made in a single controlled experiment, and thus the number and nature of
the chondrocytes used may have varied somewhat. In addition, the matrices
were processed differently and may have had different conformations of
collagen. However, it is most likely that the differences in matrix shrinkage
were due to matrix stiffness. The type II Chondrocell matrices provided by
Geistlich were by far the stiffest matrices and showed the least shrinkage. In
contrast, type II (and type I) matrices produced in our laboratory were similar
to each other (both significantly softer than the Geistlich matrices), and
underwent comparable and significant shrinkage. This effect is currently
under investigation in our laboratory.

6.3 Animal model

This is the first use of a “full-thickness chondral” defect in the canine. It is
also the first work in any animal documenting the appearance of a lesion
made to the level of the calcified cartilage including scraping of the tidemark
to remove all non-calcified cartilage. Previous work in the rabbit did not
report such a study of the initial lesion [23, 64]. Because this type of defect is
prepared clinically to treat certain cartilage defects, there is a need to
determine more specifically the effects of creating this lesion.

6.3.1 Limitations of the model

The chondral defect applied in this work, which in some cases became
osteochondral over time, should be distinguished from more shallow defects in
which the bone is always preserved. Complete removal of cartilage may
subject the subchondral plate to mechanical forces high enough to cause
fractures during the healing period. In addition, it might be suspected that
the very thin calcified layer and limited subchondral plate in the canine are
more susceptible to damage at surgery. In our study, damage to the calcified
layer and reactions in the bone were not predictable, and significantly affected
the repair mechanisms of individual defects. These uncertainties in the
animal model raise questions about whether or not, and to what degree the
same events occur in clinical treatments.
It is not known how well the results in the canine predict those in the human. It should first be noted that in current practice, in many, if not most, of the clinical preparations of these defects, the subchondral bone is visibly violated during the preparation of the defect [80, 120, 129]. This may be a reflection of the use of the procedure on patients with chronic lesions who have already experienced damage to the bone. Because this procedure is invasive, in a clinical context it is being used under limited conditions that do not match the idealized isolated chondral lesion in many cases.

Also, in this study the primary outcome variable was histology, whereas in clinical work it is pain relief. In addition, the activity level and conditions of loading can differ from the dog to the human. While it is hoped that the canine model provides an accelerated reflection of healing in the human, there is no assurance that this is the case. As with any animal model, the results from this work should be interpreted with caution and efforts should be made to verify them in humans.

6.3.2 Source of reparative tissue

The source of the cells involved in the observed healing is unknown. Possible sources include the transplanted cells or periosteum, the adjacent cartilage, the underlying bone, or the synovial environment, including bleeding into the joint during surgery. The beta-galactosidase labeling demonstrated that transplanted cells may be retained in this type of lesion. However, with the autologous cell transplantation procedure, the participating surgeons felt that it is likely that the number of these cells implanted and/or retained in the lesion in the canine varied significantly from defect to defect due to variability in surgical technique. Study of seeded matrices documented a low degree of variability in the number of cells retained in such matrices after seeding (a coefficient of variation of 4% in one such study, section 5.2.4.2), and suggested that this is likely a more reproducible method of delivering cells to a chondral defect.

In many defects, a contribution of healing tissue from the subchondral bone was clearly established by the histological observation of breaks in the subchondral plate. However, some defects, including several at 1.5 months when communication with the underlying bone was most common, appeared to fill without a break in the subchondral plate. This observation was based on numerous (at least 8) sections from across the sample. It is possible that fractures in the subchondral plate occurred in these defects, but were not found due to insufficiently frequent sectioning, or because they had already healed.
While the depth of the lesion was well-documented in this work (section 5.3.1), it is not known what contribution this made to the reparative response in the calcified layer and bone. The resorption of large areas of calcified cartilage and bone was important because it was correlated with increasing reparative tissue, decreasing hyaline cartilage in the defect, and formation of vessels in the defect area. Possible mechanisms for reaction in the calcified cartilage and bone include:

1. Damage from the surgery (documented)
2. Activity of cells which enter the joint from damage in 1.
3. Mechanical forces in the joint
4. Other soluble factors in the joint
5. Reaction to the implanted matrix or tissue
6. Factors released by cells transplanted to the defect

In absence of any treatment, there is still damage to calcified cartilage and associated response in the bone. Factors 1 through 4 may all contribute to this process. We have most control over factors 1 and 2, direct consequences of the damage done in surgery. With current mechanical methods of creating the defect, at least slight damage to the calcified layer (i.e., microcracks) is inevitable. In addition based on our analysis of acute defects, further damage to the calcified layer occurred in up to one third of specimens. We do not know to what degree this damage contributes to reaction in the bone, but it is possible that even the slightest disturbance of the calcified layer, such as the microfractures, may predispose the bone to resorptive processes.

Thus, the chondral defect applied in this work, which in some cases became osteochondral over time, should be distinguished from more shallow defects in which the bone is always preserved. In addition, it might be suspected that the very thin calcified layer and limited subchondral plate in the canine are more susceptible to damage at surgery. In our study, such processes were not predictable, and significantly affected the repair mechanisms of individual defects. These uncertainties in the animal model raise questions about whether or not the same events occur in clinical treatments.

There is also indication that communication can occur in similar animal models. An initial investigation of full-thickness chondral defects in the rabbit femoral condyles suggested a similar degree of communication with the subchondral bone as in our study [1]. Grande reported that in one of five control defects in the rabbit patella evaluated at 6 weeks, there was a subchondral fracture [64]. Thus, subchondral fractures and communication may be a normal consequence of defects made by scraping to the level of the calcified cartilage. The prevalence of fractures and the intensity of reactions in the bone is unknown, but is likely to vary with many factors including species, defect location, and post-operative motion and loading.
The biological reaction in the bone varied considerably across the treatments applied in this study. These experiments with matrix implants showed increased reaction of the bone compared to autografts and control treatments. Thus, there appeared to be a certain “biological activity” associated with the implants. Furthermore, the degree of this activity appeared to vary. Because of the limited histological results in the only other study which implanted a matrix in this type of defect [23], it is not possible to make comparisons with other implantable materials. This may have important implications for treatment. If the calcified layer can reform and hyaline cartilage form in the defect, this reactivity may be desirable. However, there was also evidence in this work that greater communication allowed vessels to form in the defect, and it was clear that hyaline cartilage formation was at least delayed, if not prevented. Because implants were evaluated only at 15 weeks, it was not known what the long-term effect would be. If this effect were found to inhibit healing due to the increased reaction in the bone, a less-reactive matrix might be chosen to better preserve the calcified layer.

6.3.3 Sources of variability

Variability in similar biological models with penetration of the bone is common [24, 27]. This study was no exception, with some defects in a given treatment group showing excellent healing, and others showing almost no healing. Two major sources of this variability could be: 1) the state of the calcified cartilage (whether broken and or healed, discussed above), and 2) dislodgment of the graft or reparative tissue early in healing. The prevalence of dislodgment of grafts is unknown. Study of several animals implanted with matrix grafts for only 30 minutes established that the graft was not dislodged in closing the joint or from minimal joint motion (greater than that assumed to occur with the external fixation device). However, in defects which received grafts, but later displayed minimal filling, the graft and/or reparative tissue must have detached at some time during healing. This may have resulted from early loss of the graft, or from incomplete bonding of reparative tissue and loss with wearing away over time. In several defects viewed up to 15 weeks, histological observations were consistent with large pieces of reparative tissue, in some cases nearly filling the defect, coming dislodged. While the immediate effect may have resulted from tissue processing, this tissue was clearly not well-bonded to the defect and may have been subject to similar results from ongoing wearing in the joint.

Because the finding of an empty or nearly empty defect may have been attributable to failure of the inherent reparative mechanism (as opposed to the effectiveness of attaching the graft) all defects were included in reporting of group totals for filling. While the variation is reflected in data through the standard error of the mean, a more direct presentation of the number of defects with minimal filling is included in Appendix M. To summarize, in most groups the large majority of defects had greater than 20% filling. The
one exception was the fascia group, in which four of six defects had less than 5% filling. The only other group in which more than one defect was found with less than 10% filling was the 6 month untreated defects.

It should also be noted that because untreated defects showed some filling, the finding of a filled defect does not prove that a graft was retained. With the exception of the eight defects for beta-galactosidase labeling, no grafts in this work were labeled to establish whether or not they became incorporated into the reparative tissue.

Collectively, these results suggest that, with the exception of the fascia control group, graft detachment probably did not frequently occur.

6.4 In vivo experiments

6.4.1 Phases of healing

Histological evaluation of chondral defects in canine articular cartilage over time revealed phases of healing characterized by proliferation and remodeling, comparable to those seen in the healing of other tissues [5, 36]. Through 6 months, significant portions of articular cartilage regenerated. In certain defects, fibrous tissue was replaced by hyaline tissue and damage to the subchondral bone was healed. However, by one year most samples appeared to have entered a phase of degradation characterized by decreased safranin O staining or fibrillated surfaces of reparative tissue, and by degenerative changes in the adjacent cartilage. Due to the complexity of healing, several of the three phases could occur simultaneously in close apposition to each other. The overall process mirrors that found in osteochondral defects in articular cartilage [168].

Several observations suggested a time dependent conversion or remodeling (degradation followed by formation) of one tissue type to another: the evolution of the tissue type profiles, and the histology which often showed indistinct, mixed borders between otherwise distinct tissue types. It is likely that reparative tissue can follow the regenerative sequence: fibrous tissue - fibrocartilage - hyaline cartilage - articular cartilage. This would be one of few examples in which an organized fibrous tissue is not a terminal scar, but instead a precursor to another tissue type. Occurrence of the reverse transitions were not certain, except for articular to hyaline. However, it is strongly suspected that the transformation of hyaline cartilage to fibrocartilage does occur, and that this is in fact the end point of healing. In some cases hyaline cartilage appeared to degrade by fibrillation and gradual wearing away (as in osteoarthritis), and there was also histological evidence at 18 months that transitional tissue (specifically fibrocartilage) could replace hyaline cartilage. If fibrocartilage is the terminal stage in repair, it would be the cartilage analog to scarring in other tissues.
Previous authors have also reported degradation following healing in various models of cartilage defects [24, 124, 139, 168]. In our model, the initiating event for degradation may have been a) opening of the joint b) the surgical treatment to the defect (including suturing), or c) failure of immature reparative tissue subjected to high biomechanical forces. Likely, all of these factors were involved. Once initiated, the degradation appeared to encompass the entire joint. Clearly, steps should be taken to prevent such occurrence. Development of a less invasive, arthroscopic procedure is preferable to an open surgery. In addition, an alternative method of graft fixation should be sought. The idea that failure can be traced to breakdown of reparative tissue suggests that long-term success in treating articular cartilage may require near perfect regeneration in the remodeling phase and carefully controlled post-operative care.

6.4.2 Microfracture

Although widely used clinically, this is the first report of microfracture in an animal model. Several defects showed very good filling with limited bone damage, while others had near complete resorption of the of the underlying bone, similar to that seen often with collagen matrix implants. While the filling was encouraging, no hyaline cartilage was found in the defects. Longer term studies are necessary to establish the long term fate of healing in the underlying bone and possible maturation of the reparative tissue.

6.4.3 Effect of matrices

This is the first study of the effect of a resorbable collagen matrix in either full-thickness chondral defects or microfracture defects in any model. The only other reported study of implants in full-thickness chondral defects involved carbon fiber implants in the patella of a rabbit [23]. Thus, it is difficult to compare the findings of this study to other published work. Many studies have implanted matrices in osteochondral defects in several animal models (Tables 2-1 and 2-2). These experiments may provide some basis for comparison.

Implanted matrices had mixed effects on healing in this model. Both seeded and unseeded matrices did increase the amount of reparative tissue relative to control groups without matrices in both normal chondral defects, and those treated with microfracture. The statistical significance of these findings was only $0.10 < p < 0.20$ due to large variability in the data. For the comparison of microfracture with and without matrix, statistical significance is extremely difficult to prove as the hypothetical limit of approximately 100% fill is approached. The finding of increased filling when matrices are used is consistent with the findings for a variety of matrices in osteochondral defects [52, 53, 67, 93, 145, 172]. When used as a carrier for autologous cell transplantation, the matrix delivers cells capable of participating in repair to
the defect. However matrix effects are also found in unseeded matrices. In this case, the matrix may perform one of several functions. First, the matrix may serve as an attachment site and support for cells migrating into the defect from the underlying bone, synovium, or adjacent cartilage. Second, the matrix may regulate the effect of growth factors on the healing defect by binding or inactivating them [81, 149]. Finally, the effect of the matrix may be indirect, causing increased filling of the defect via increased resorption of the underlying bone and the consequent flowing of undifferentiated mesenchymal tissue into the defect. This mechanism is supported by the data from all of the present experiments with matrices, in which the total filling of the defect universally increased with the amount of damaged calcified cartilage.

On the other hand, the nature of this tissue at 15 weeks was less mature than that found in untreated defects or autologous cell transplantation. It is possible that this was also due to the greatly increased damage to the calcified cartilage which prevented hyaline cartilage from forming. In the many defects examined in this work, hyaline cartilage clearly formed preferentially on an intact calcified cartilage layer. This suggests that the reformation of the calcified layer and tidemark will be critical to regenerating articular cartilage. It would be interesting to follow up these experiments at longer times to determine whether a normal tidemark is reconstituted. It may also be hypothesized that the biological activity of the implant will be critical in determining how the matrix affects these processes in the bone. Similar findings in the bone were not reported in work in similar defects (12 defects at 12 weeks) with carbon fiber matrices in the rabbit patella [23]. It is possible that the animal species, defect location, and type of implant may all help explain this difference.

The timing of the effects of the matrix is unknown. Both type I and type II remnants were virtually completely degraded by 15 weeks (only a trace of a large block-like type II matrix was found at 15 weeks). Presumably, once the matrix is resorbed, it ceases to cause direct effects. However, the degree to which the presence of the matrix has changed the cell populations, chemical milieu, and resorption processes and mechanical integrity of the calcified layer and underlying bone is unknown.

It is important to note that the composition of reparative tissues found filling the defect from seeded type II matrices was similar in two separate trials with separate sources of matrices and a slightly different seeding procedure.

Collagen matrices may have contributed to healing through two mechanisms. First they provided a support to more consistently deliver cells to the defect compared with autologous cell transplantation. Second, as discussed above, they were associated with a biological activity in the subchondral bone that created communication that can explain the high degree of filling found.
6.4.4 Effect of cover materials

The effect of cover materials was not studied in a controlled manner in all experiments. The cover controls included fascia at 15 weeks and periosteum at 1.5, 12, and 18 months. No controls for type II sponges or films were included because of a need to limit the scope of the experiments.

All covers were resorbed or at least significantly remodeled within the first few months. Type II collagen covers (compressed sponges or films) were resorbed or remodeled to the point where the original tissue or material could not be recognized at 15 weeks. Fascia was seen remodeling at 4 weeks, and may have been evident at 15 weeks, although this was not proved. Periosteum could not be distinguished clearly even at 6 weeks.

It is not certain to what extent the covers may have contributed to healing. When used alone as a control, periosteum results mirrored those of periosteum with transplanted CAC. However, the periosteum control was omitted at the times when the CAC plus periosteum treatment had a measurable effect. Periosteum has been used in previous work in full-thickness chondral defects with methods similar to those in the present work. In the one study that used periosteum controls [23], there was no indication that periosteum improved healing. However, in a series of experiments in which periosteum was used in a different manner, including osteochondral defects, reversed orientation of the periosteum in the defect, and continuous passive postoperative motion, the treatment was able to significantly improve healing [89, 127, 139, 140, 159].

Fascia may have hindered repair. First, many fascia-implanted control defects were nearly empty. In addition, despite a substantially intact calcified cartilage layer, there was almost no bonding to the defect base. Thus it is appeared that fascia 1) did not stimulate repair, and 2) the fascia itself was eventually detached from the defect due to its inability to integrate with the surrounding structures.

6.4.5 Effect of autologous cultured chondrocytes

Based on comparison with periosteal controls at 1.5, 12, and 18 months, there was no clear evidence in this work that cultured autologous chondrocytes improved regeneration. However, at the intermediate times (3 and 6 months) when the treatment was most effective, no periosteal controls were employed. There did appear to be a benefit to the cell and periosteum treatment compared with empty controls at these times. However, in the longer term studies (12 and 18 months), the reparative tissue in all groups appeared similar. One significant difference at these times was additional damage to the adjacent cartilage in defects which were sutured (P and CAC groups).
It is important to note that the earlier findings of filling of defects with hyaline cartilage does not correlate with the long-term success (beyond 1 year). It is likely that long-term success depends on many important factors, including a higher degree of defect filling, formation of mature (hyaline) reparative tissue, and complete integration with adjacent structure (both calcified cartilage and adjacent cartilage). The findings of this study indicated that hyaline cartilage forming in the defect readily bonded to calcified cartilage, but not always to the adjacent cartilage. It may be necessary to involve growth factor or enzymatic treatments in efforts to stimulate better integration of the reparative tissue [78].

This experiment did not reach the same conclusions as some previous work with respect to the effect of transplanted cells. Experiments in a rabbit model showed a positive effect of CAC in the amounts and types of reparative tissue forming up to one year [23]. In addition, the rabbit did not exhibit generalized degradation in the treated defects at the 12-month time point. Finally, significantly more spontaneous regeneration is reported in the canine in this work than in previous work in the rabbit. While the rabbit response could also be described by the phases of proliferation and remodeling, the time course and intensity of each phase differed from the canine. The different results from work in the rabbit and the dog indicate that the effect of the cells may be sensitive to several variables including: a) species, b) subject age, c) defect location (patella vs. trochlea), d) surgical skill in the treatment, and e) post-operative animal activity.

The current canine results in the trochlear defect do not mirror clinical experience in chondrocyte transplantation in other sites. Thus, while positive clinical results of cell treatment in human subjects [22] warrants continued investigation, it is not possible to conclusively attribute the promising effects to the transplanted chondrocytes based on this study. The present results do, however, suggest that the long-term performance in humans must be assessed to determine the fate of the reparative tissue. Histological and biomechanical analyses revealing normal or near normal articular cartilage structures and properties would be of great value in confirming the efficacy of this novel and promising procedure.
7. Conclusions

The following are conclusions that relate to the hypotheses of this thesis and can be drawn from the present work:

- The chemical composition of porous collagen-GAG matrices, made principally of types I and II collagen, influences canine chondrocyte morphology, proliferation, and accumulation of glycosaminoglycan in culture; a greater percentage of cells in the type II collagen matrix display a rounded morphology.

- The pore diameter of type I collagen-GAG matrix, when varied from 25 μm to 85 μm, affects chondrocyte morphology 3 hours after seeding, but not after one week. The pore diameter does not affect chondrocyte proliferation or glycosaminoglycan accumulation.

- The pore diameter of type II collagen-GAG matrix, when varied in the range of 50-250 μm, has only slight effects on chondrocyte morphology and proliferation, and no effect on glycosaminoglycan synthesis or accumulation for up to four weeks in culture.

- A canine model, using quantitative measures of defect filling by distinct tissue types, can discriminate repair processes in the healing of full-thickness chondral articular cartilage defects in the trochlea.

- Treatment of full-thickness chondral articular cartilage defects in the canine trochlea with cultured autologous chondrocytes under a periosteal flap, while increasing the amount of reparative tissue and hyaline cartilage at three and six months relative to untreated controls, does not influence the long-term healing of full-thickness chondral defects in this model (at 12 and 18 months).

- Relative to untreated controls, application of microfracture in treating full-thickness chondral articular cartilage defects in the canine trochlea increases the total amount of reparative tissue, but decreases the amount of hyaline cartilage filling the defect at 15 weeks.

- In untreated, periosteum-treated, and CAC-treated defects, reparative tissue follows similar trends in remodeling, beginning with predominantly fibrous tissue (up to 1.5 months), progressing through a phase of maximum hyaline cartilage filling (3 and 6 months), and tending toward fibrocartilage by 18 months.
- Relative to untreated controls, implantation of chondrocyte-seeded type I or type II collagen-glycosaminoglycan matrices in full-thickness chondral articular cartilage defects in the canine trochlea increases the total amount of reparative tissue, but decreases the amount of hyaline cartilage filling the defect at 15 weeks.

- The implantation of a porous type II collagen-GAG matrix in a microfracture-treated full-thickness chondral articular cartilage defect in the canine trochlea increases the total amount of reparative tissue, but not the amount of hyaline cartilage filling the defect.

- In this defect model, there is an inverse correlation, by linear regression, between total defect filling and the amount of calcified cartilage remaining intact. This suggests that the primary means of filling of these defects is from cells derived from the subchondral bone.
8. Future work

The results from matrix implantation in the animal model at 15 weeks suggest the need for follow-up studies at longer time periods. While total filling of defects at this time was favorable, little hyaline cartilage existed at a time when maximum hyaline cartilage as seen in untreated defects and defects treated by cultured autologous chondrocyte transplantation. In addition, more severe reactions in the underlying bone were noted. It would be of great value to determine the long-term result in these defects and to determine whether the mechanism of perforation of the plate, with bone reaction and filling of the defect, is consistent with long-term success in healing. Specifically, this future work should answer the following questions: Will the subchondral plate and tidemark reform? Will the reparative tissue remain in the defect? Will hyaline cartilage form in the defect? Will the tissue be integrated with adjacent structures? If the long-term result from perforation is poor, efforts should be made to develop treatments which cause less reaction in the underlying bone.

This thesis did not study the effects of growth factors on healing. Future work should investigate the contribution of growth factors in the healing process and how the use of matrices may affect these processes. In addition, growth factors should be investigated as an important component of tissue engineering strategies, i.e., to augment treatments with cells and/or matrices.

Research should also continue to focus on which cell sources (i.e., transplanted chondrocytes or periosteal cells, or cells supplied from the joint or underlying bone) can be most useful in healing. In addition, there is a need to firmly establish the conditions of the treatment (e.g., postoperative conditions such as motion and loading) which may make the procedure more effective. Because only one defect location was used in this study, it is not possible to generalize to all defects. It would also be useful to determine the response of defects in different locations, and whether they respond similarly to the same treatments.

One means of improving the reparative process with cultured autologous chondrocytes might focus more closely on the cells used for repair. In this work, the articular cartilage was harvested without regard to the depth relative the surface, and thus the origins of the cells that survived culture and transplantation were not known. However, it is known that there can be significant variations in chondrocyte activity with distance from the surface [8, 9]. Selective isolation of more active cells might improve repair.

Another important element not studied in this work is that of mechanical properties of reparative tissue. It would especially be of interest to determine if the reparative tissue described as hyaline or articular cartilage in this defect has mechanical properties similar to normal cartilage. In addition, the
mechanical properties of the reparative tissue in this work could be compared to previous studies reporting the properties of tissue engineered cartilage.

This work indicated that significant damage to the adjacent cartilage occurred in suturing the grafts or covers to the joint surface. This suggests the need to identify an alternate means of fixing grafts to chondral defects. Several adhesives have been used in work in cartilage in the past. Fibrin glue has been used as a sealant in our canine model, and has been used to fix perichondrial grafts in defects [74, 75, 121], although graft delamination sometimes occurs. One study has suggested mussel adhesive protein a stronger alternative to fibrin glue [147]. Most recently, the use of transglutaminase has been found superior to fibrin for cartilage-cartilage bonding, and may be useful in fixing collagen grafts [86]. It is likely that it is easier to fix a collagen matrix to the calcified layer than creating a cartilage-cartilage bond, as glycosaminoglycan chains which can detract from bonding are absent in the former case [86].

The present work touched on the gross and histological effects of the various treatments on adjacent areas of the treated joint. In some cases, pathology was noted. Thus, this work should be extended to determine if there are any adverse effects on the biochemical or mechanical properties of other important joint structures, and especially the opposing or unininvolved cartilage.

Future work may also investigate the development of a primate model. The primary advantage will be in allowing arthroscopic investigations to be performed. Because of the many adverse effects of open joint surgery, there is a general trend in orthopedic surgery is to develop arthroscopic techniques wherever possible. This is currently the case in autologous chondrocyte transplantation, and it is desired to have a model that will be able to mimic the clinical situation. In addition, arthroscopy can be used as a means of multiple follow-ups on a single lesion, especially with the ongoing development of new non-destructive arthroscopic testing methods. The primate model would also have thicker cartilage and the cell biology may more closely resemble that of human cartilage.

It is also important to determine if the defect response in the animal can be used to predict the success of treatment of specific types of defects in the human. For example, future work may be used to answer the following questions: Is the clinical observation that CAC-treatment is more effective in the femoral-tibial articulation than the patellar-trochlear articulation mirrored in the canine? Can other conditions of treatment, such as patient age, previous surgery, and relative joint stability be studied in the animal and applied to the human?
Further *in vitro* work may focus on two observations made in this study. First, it would be of great interest to determine which elements of matrix chemistry are responsible for the differences in chondrocyte behavior in matrices of varying chemical composition. In addition, the mechanism of chondrocyte-mediated contraction of sponges should be investigated.
Appendices

Appendix A  Methods of production of collagen-GAG matrices

**Integra type I bovine tendon collagen** (for matrices HB95-1 and all others)

1. Defrost wet tendon collagen (Integra Life Sciences, 2/2/95, 26.3% solids)
2. Cool blenders for 30 min. to 4°C
3. Fill one blender with 600 ml of 0.05 M acetic acid
4. Weigh 13.69 g wet tendon collagen
5. Place collagen in blender
6. Blend for 90 minutes on high setting
7. Add 120 ml of chondroitin 6-sulfate (Sigma catalog # C-4384, St. Louis, MO) solution over 15 minutes using peristaltic pump. (0.32 g C-6-S in 120 ml 0.05M acetic acid)
8. Blend additional 90 min. on high
9. Pour out slurry and refrigerate in plastic storage bottle
10. De-gas in vacuum flask immediately before moving. Slurry is ready to pour into freeze drying tray.

**VirTis freeze drier protocol:**

1. Set shelf temperature.
2. Turn on freeze and heat switches.
3. Turn on condenser switch. Condenser should reach -50°C before pulling any vacuum.
4. Wait for shelf to cool to desired temperature.
5. Prepare tray with molds or dividers. Pre-cool freeze-drying tray if desired.
6. Add slurry to tray.
7. Wait until all slurry in tray is frozen.
8. Once frozen, turn on vacuum (make sure door is sealed). (Make sure condenser is colder than -50°C.)
9. Wait for vacuum to reach less than 200 mtorr. Set temperature to 0°C and leave overnight. Larger samples may need longer times to completely sublimate.
10. Set temp to 20°C and turn off freeze switch.
11. When sample reaches 20°C, turn off heat, vacuum, and condenser. Release vacuum and recover sample from chamber. Turn power off.
Appendix B  Description of pore characterization

Embedding and sectioning for pore analysis

1. Embed in JB-4 plastic (Appendix E).
2. Section to 5 μm on microtome and stain with aniline blue (Appendix F).

Pore Characterization Protocol

Capture Image:

1) Macintosh computer set up:
   • Under the Apple menu, choose Control Panels, then Monitors.
   • choose 32-bit addressing and 24-bit color
   • turn off screen savers

2) Video camera set up
   • Attach video camera to microscope
   • Arrange sliding eyepiece selector knobs (at level of eyepieces) for TV:
     - Left side slider in "out" position
     - Right side slider in "middle" position
   • Turn Gain and Offset knobs all the way down (counter clockwise)
   • Turn on Hitachi video camera

3) Using the computer to capture the image:
   • Several programs can be used: Scion Image, Ultimage, or Digit
   • Using Scion Image 1.60c to grab video:
     - Adjust light level on microscope so green light on Hitachi is on
     - Choose Start capturing in the Special menu.
     - Turn up gain and offset to get approximate picture on screen.
     - Fine tune image and focus, then click on the image to freeze video.
     - Save image by selecting Save as in the File menu.
     - Use TIFF format. Each image file is about 325K.

Image Clean-Up:

1) Open Scion Image 1.60c and load image.

2) Ambiguous parts in the matrix need to be manually drawn in or deleted. It is helpful to view the specimen under the microscope for reference points.

3) Additions can be made by clicking on the Pen Icon in the Tools box. Double click on this same icon to change the pen width. Click on the Eraser in the Tools box to make deletions. For more detailed modifications to the image, it is suggested that you magnify the region of interest. To do this, select
the *Magnifying Glass* in the *Tools* box. Then click on the appropriate area of the image. To return to the original scale, select *Unzoom* in the *Edit* menu.

4) Click on *Density Slice* in the *Options* menu. This will highlight the non-obvious matrix in red, in addition to some extraneous background. Areas that are red should be manually filled in with the pen, unless of course it is not matrix. It is not necessary to remove background noise at this point.

5) Click on *Threshold* in the *Options* menu. Click on *Smooth* or *Sharpen* in the *Process* menu. If outcomes are not desirable, click on *Undo* in the *Edit* menu. Do not overuse these techniques!

6) Click on *Binary* under the *Process* menu. Click on *Make Binary*. Click on *Erode* which is underneath *Binary* in the *Process* menu. This is helpful in systematically removing background pixels. Repeat if necessary.

7) When all modifications are complete, and you are satisfied with the image, click on *Apply LUT* in the *Process* menu. This will provide an image of only black and white (no gray) which will facilitate analysis. Save image with a different filename in TIFF format. Be sure to indicate in the filename that this is the final modified file. Quit Scion Image.

**Pore Analysis:**

1) Load Digit program 11-July -93 version. It will be necessary to install the SoftwareFPU program in the control panel to run this program on a PowerMacintosh. Failure to do so will result in a math coprocessor error.

2) Open final modified image file to be analyzed. Digit cannot be used to alter the image.

3) Setting the Materials

   Click on *Materials* in *Options* menu. The materials can either be loaded from a saved file (if previously set), or edited on the spot. The following are instructions for the correct settings for pore analysis:

   Set the number of materials to 2.
   The material to edit should be 1 (first material, or matrix). Click on *Edit Material*.
   Adjust to these settings:
   
   **Region number, solid = 1**
   **Data source = pixel**
   **Region number, void = 0**
   **Threshold = gray level; low=0; high=100**
   **Label = _matrix.**

   Click on *Next Material*. This is material 2 (pores). Adjust to these settings:
   
   **Region number, solid = 1**
   **Data source = pixel**
   **Region number, void = 0**
   **Threshold = gray level; low=101; high=255**
Label = _pores

Click on Okay. Save this file (by clicking Save to File) if you wish to use again.

4) Setting the Scale

Set Scale from the Options menu. Note that the “known distance” has no units. You must know your units use them consistently (generally microns). The microscope is calibrated as follows:

<table>
<thead>
<tr>
<th>Objective magnification</th>
<th>Known Distance (microns)</th>
<th>Pixels</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x</td>
<td>4.15</td>
<td>1</td>
</tr>
<tr>
<td>10x</td>
<td>1.66</td>
<td>1</td>
</tr>
<tr>
<td>20x</td>
<td>0.83</td>
<td>1</td>
</tr>
<tr>
<td>40x</td>
<td>0.415</td>
<td>1</td>
</tr>
<tr>
<td>100x</td>
<td>0.166</td>
<td>1</td>
</tr>
</tbody>
</table>

5) Choosing the Region

Set the Region from the Options menu. A circular region of interest must be used. Set by clicking Circle and dragging the mouse. If region drawn is not acceptable, click on Clear, and repeat. Regions may be Saved. Click on Close when done.

6) Click on Threshold in the View menu. Change high setting to 100.

7) Stereological Analysis

Click on Stereology in the Analyze menu. For analysis of matrix only, set material number to 1. For analysis of pores only, set material number to 2. For analysis of both materials, enter 0. The time of analysis will depend on the size of the region and speed of the computer, and can be more than 30 minutes for a PowerMacintosh 7200/90 and a full screen region. Check box for append results to existing file if you have already started a file (see below). Click on Okay and either create a new stereology data file, or select the stereology data file to append to. As soon as the file is opened or created, the program will begin calculation (Apple watch icon appears on screen) and you will not be able to work on the computer. Appearance of a blue ellipse within the yellow region circle indicates completion of calculation for material number 1. The ellipse indicates pore orientation (data for the ellipse is contained in the data file). Clicking anywhere on the screen will either a) start analysis of the next material if there is more than one, or b) reset to the original file and region if this is the last or only material. This data file can later be viewed with Excel, but do not make any modifications to the file. This will corrupt the file so that Digit will be unable to use it in attempts to append later data.
Notes on data output:
MIL=minimum intercept length and is taken as the pore size (Maximum and Minimum estimates given)
Area Solid = Area of material reported
Area Total = Total area within region
Extent Anis = eccentricity of the blue ellipse showing pore orientation. 0 means isotropic, 1 means highly oriented in one direction

**Estimating Pore Size with Eyepiece Grid**

When using the grid eyepiece (10 x 10) in the microscope to estimate pore size, use the following conversion:

Note: Microscope must NOT have photo cross-hairs engaged!!

If using 4x objective, each square has length of 150 microns.
If using 10x objective, each square has length of 60 microns.
Appendix C  Cell isolation and seeding protocols

Isolation of chondrocytes

- Prepare 50 ml-tubes to store cartilage specimens: 40 ml of D-PBS + 0.4 ml Pen./Strept. (100 U or µg/ml) + 0.2 ml fungizone. Weigh tube. Keep at 4°C if you expect a delay between harvest and processing the tissue. Digestion should not start later than 4-6 hours after the harvest.
- Autoclave: cutting board, 3 petri dishes, 2 forceps, scalpel, spatula, ruler, special blue tips (see below). (optimal forceps- 1 fine point curved, 1 micro point straight)
- Prepare collagenase at 0.10 to 0.15% in DMEM/F12. The activity of collagenase will vary. Generally try a bit higher concentration with lower activity. At this time, we are using Sigma Collagenase, activity 370 U/mg. This will take some playing depending on tissue and other, sometimes unpredictable acts of the supernatural.

  100 ml DMEM/F12
  0.1 to 0.15 g collagenase
  2 ml pen/step (20,000 U or µg)
  1 ml fungizone

Sterile filter complete solution through 45 µm corning filter system (cellulose nitrate).
Store in T75 flask or sterile bottle or 50 ml tube.
- Retrieve tissue in sterile operation. Weigh tubes with tissue to get tissue weight for future reference. Set up sterile area in hood with drape and autoclaved stuff.
- Fill first petri dish with the specimen (dump in with PBS).
- Fill second petri dish with PBS (10 ml) for washing the specimen or placing waste in.
- Fill third petri dish with about 20-30 ml of the collagenase-media solution.
- The cartilage chips are taken from the first dish, washed (dipped) in the second dish (PBS), and then placed on the cutting board and manually diced into pieces of the size 1x1 mm (much easier to cut on board than in dish). If pieces are very thin (as in thin cartilage shavings), they might not need to be so small. Scoop or use forceps to move the small pieces into the third petri-dish with collagenase.
- Transfer pieces plus collagenase to spinner flask. The following procedure has been found to work well: Use 1000 µL micropipettor. Cut off end of sterile blue to make large (about 5-6 mm) opening (best to prepare ahead of time and autoclave). Use pipettor to suck up pieces and excess collagenase, and squirt into spinner flask. Once pieces are gone, remove the rest of the collagenase from the petri dish (by serological pipet). Add more collagenase to spinner flask, washing down the arm of the flask. Must be enough medium in spinner flask so that it will stir.
• Put the spinning flask in the 37°C incubator with the stir plate. Stir very slowly, just enough to move the fluid. Digestion times can vary widely with tissue type, size, and collagenase activity and concentration. Monitor as needed- at least every few hours. At the end, just about everything should be digested; only small remnants of adjacent tissue may remain. But don't digest too long, because of the decreasing viability of the cells.

• Digested cartilage is filtered through nylon cell strainers from Falcon (70μm) directly into 50 ml tubes. 2 methods may be used to get the solution from the spinner flask to the cell strainers:
  1) Pour directly out of spinner flask arm. Flame the opening from time to time.
  2) If not removing all of the contents of the spinner flask, remove what you need with a 10 ml serological pipet. Pipet directly through the cell strainers into the 50 ml tubes.

• Spin filtered solution at 1500 rpm for 10 minutes in centrifuge at room temperature.

• Decant supernatant (via vacuum suction- be careful not to suck up cells) and resuspend the pellet in 25 ml (total of all tubes from previous step) of complete medium (or serum-supplemented PBS), then spin again at 1500 rpm for 10 minutes. Do a total of two to three rinses.

• Before final spin, count cells in hemacytometer and determine viability (Trypan blue staining)

• Resuspend final clean pellet in full culture media (5-10 ml usually works well) (recount if desired).

• Put cells into a T25 or T75 flask (6.5 or 17 ml of media cell solution) and incubate at 37°C in a humidified 5-10% CO2 environment. T25: put about 5e5-1e6 cells. T75: put about 2-5e6 cells. Number of cells per flask depends on cell type and expansion desired. For cells that don't like to attach or may not be healthy, put more, as many may be lost. For chondrocytes, I would put lots.

• Allow cells to attach before adding medium. This will depend a lot on cell type and viability. For cells that may have been damaged in digestion or don't like to attach (like chondrocytes), be patient. It is possible that only a few out of many millions will attach. Wait to change medium and have faith.

Cell-seeding protocols

1. Sterilizing/prewetting matrices: (all steps/instruments sterile)

   A. Wet/sterilize with ethanol
      - pour 70% ethanol into 85 mm petri dish (enough to cover matrices)
      - add matrices by forceps
      - squeeze out air bubbles with curved forceps (be careful not to tear delicate matrices)
- soak 10 min.
- squeeze out bubbles again before changing solution

B. Rinse out ethanol
- Remove all ethanol with pasteur pipette (if using vacuum suction, take care not to suck up matrices). Tilt dish to allow last bits to collect along edge.
- Add PBS to cover matrices
- Squeeze out air bubbles
- soak 5-10 min.
- squeeze out air bubbles
- REPEAT 4 more times with PBS

C. Pre-wet with medium
- Remove all PBS with pasteur pipette
- Add medium to cover matrices (can be complete or incomplete medium)
- Squeeze out air bubbles- let medium penetrate matrix
- soak 5-10 min.
- squeeze out air bubbles
- REPEAT 1 more time with medium
- Cover and place matrices in medium in incubator until ready to seed

2. Collect cells.
- Follow protocol for cell passage/subculture to collect cells
- Count and resuspend in medium at 25 million/ml for seeding
- Store cell suspension in incubator until matrices are prepared

3. Seeding
- place sterile barrier in hood
- on barrier, spread out sterile filter papers for partially drying matrices
- with sterile forceps, remove matrices from pre-wetting petris
- drop matrices on filter papers and move or flip until "sufficiently" dry
- place matrices into agarose covered wells: center them, away from walls
- remove suspension from incubator, resuspend with 1 ml pipette
- use micropipettor with extension for tips for aliquotting
- place aliquot of cell suspension (usually 32-40μl) on each matrix
- shake or resuspend cell suspension often during seeding
- allow several minutes for drop to settle into matrix
- flip matrix, placing again in center of well
- seed second side with drops from micropipettor
- place in incubator for 1 hour to allow cells to attach
- while seeded matrices are in incubator, prepare final plates with medium and also place in incubator. 1.5 to 2 ml/24 well.
- with forceps, carefully move matrices from agarose to final wells
Appendix D  Protocols for biochemical assays

GAG assay

1. Prepare DMB stock solution:
   32 mg 1,9-dimethylmethylene blue (DMB) dye
   10 ml 100% ethanol
   Mix gently with pipet (no vigorous shaking)

2. Prepare formic acid solution:
   2 L distilled H\textsubscript{2}O (d H\textsubscript{2}O)
   4 ml 90% formic acid
   pH to 3.5 with 10M NaOH

3. Prepare final DMB dye solution:
   Combine DMB stock solution with formic acid solution
   Store in dark bottle
   Shelf life approximately 2 months

4. Assay:
   Collagen samples must be digested by papain or protease
   Vortex sample or mix completely
   Add 100 \mu l aliquot of sample to cuvette
   Add 3 ml final DMB dye solution to cuvette. Mix.
   Read immediately in spectrophotometer at 535 nm.
   Read standards of shark chondroitin sulfate in dH\textsubscript{2}O

DNA assay

1. Prepare TNE 10x buffer solution: (100 mM TRIS, 10 mM EDTA, 1.0 M NaCl)
   800 ml dH\textsubscript{2}O
   3.7 g Disodium Ethylenediamine Tetraacetate (EDTA)
   12.1 g TRIS
   58.4 g NaCl
   pH to 7.4 with concentrated HCl
   dH\textsubscript{2}O to 1000 ml  (approximately 165 ml)
   store 4°C

2. Prepare concentrated Hoechst dye stock solution:  (1 mg/ml)
   10 mg Hoechst dye #33258
   10 ml dH\textsubscript{2}O
   Store 4°C, shelf life: 6 months
   Protect from light: fluorescent!
3. On day of assay, prepare working solution of dye:
   90 ml dH₂O filtered through 0.45 μm
   10 ml TNE 10x buffer filtered through 0.45 μm
   At latest possible time before working, add 100 μl concentrated Hoechst
dye stock solution (unfiltered- do not allow dye to bind to filter)
This concentration is good for reading DNA in final concentrations 10-
400 ng. Increase dye concentration for higher DNA concentration

4. Scale fluorimeter:
   Warm up fluorimeter for 15 minutes
   Prepare standards of calf thymus DNA
   Zero with 2 ml working dye solution
   Scale with calf thymus DNA, typically 250 ng

5. Assay:
   Collagen samples must be digested by papain or protease and
   freeze/thawed 3 times to lyse cells to release DNA
   Centrifuge samples at 3000 rpm for 10 minutes (leaves DNA in solution
   but pelletizes protein and collagen fibers)
   Add 100 μl aliquot of sample (from supernatant only) to cuvette
   Add 1.9 ml final working dye solution to cuvette. Mix.
   Read immediately in fluorometer

Papain digestion

1. Prepare stock solutions:
   Monobasic stock: 6.9 g 0.5M NaH₂PO₄ in 100 ml dH₂O
   Dibasic stock: 7.1 g 0.5M Na₂HPO₄ in 100 ml dH₂O

2. Prepare papain buffer:
   2.46 ml dibasic stock solution
   17.54 ml monobasic stock solution
   80 ml dH₂O

3. On day of digestion, complete papain buffer with:
   87.82 mg L(+) - Cysteine HCl
   186.12 mg Disodium Ethylenediaminetetraacetate (EDTA)
   pH to 6.2 with 10M NaOH

4. Digestion:
   Place specimen in 1.5 ml microcentrifuge tube
   Add 1 ml complete papain buffer
   Add 50 μl papain. Vortex or mix thoroughly.
   Float tubes in covered 65°C bath overnight
Appendix E  Sample embedding and sectioning protocols

Decalcification:

Samples containing bone were first decalcified in 15% EDTA decalcifying solution, pH 7.4. This solution was mixed as follows (makes ~1800 ml):

- 1570 ml PBS (0.01 M phosphate buffer)
- 44 g NaOH (Fisher Scientific, Cat. #S-318, Fair Lawn, NJ)
- 270 g EDTA (Disodium Ethylenediamine Tetraacetate, Fisher Scientific, Cat. #S311-3, Fair Lawn, NJ)
- ~27 ml concentrated HCl

Instructions:
1) Make PBS, 10 mM. Either with dissolvable tablets (Sigma #P-4417, St. Louis, MO. 1 tablet/200 ml dH2O), or with packets (Sigma #P-3813, 1 packet/L dH2O).
2) Add NaOH. Dissolve completely. It is difficult to get EDTA into solution without NaOH.
3) Add EDTA
4) pH adjustment to 7.4

Plastic embedding:

Embedding in plastic was performed using Polysciences JB-4 embedding kit (Polysciences, Inc., Warrington, PA). This process included the following items:
- JB-4 embedding solution A (Cat. #0226A, Acrylic monomer n-Butoxyethanol)
- Catalyst (Cat. #02618, benzoyl peroxide, 70% wet).
- JB-4 embedding solution B (Cat. #0226B, N,N-Dimethylaniline Poly[Ethylene glycol]).
- Catalyzed solution A: dissolve 9 g catalyst in 1000 ml solution A.

1. Preparation. Samples should be decalcified and rinsed well of fixative with water.

2. Dehydration.
   A. Tissue specimens were dehydrated by machine (Tissue-Tek VIP 1000, model 4617, Miles Scientific, Mishwaka, IN). In this experiment, the following dehydration program (program #9) was used, with solutions changed automatically as follows:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% EtOH</td>
<td>1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>70% EtOH</td>
<td>1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>80% EtOH</td>
<td>1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>1 hour</td>
<td>room temp</td>
</tr>
</tbody>
</table>

177
95% EtOH 1 hour room temp
100% EtOH 1 hour room temp
100% EtOH 1 hour room temp
100% EtOH 3 hour room temp

B) Fragile collagen matrix samples were dehydrated by hand. Water and alcohol solutions were pipetted in and out of 24-well plates as follows:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled H2O</td>
<td>30 min</td>
<td>room temp</td>
</tr>
<tr>
<td>distilled H2O</td>
<td>30 min</td>
<td>room temp</td>
</tr>
<tr>
<td>distilled H2O</td>
<td>30 min</td>
<td>room temp</td>
</tr>
<tr>
<td>50% EtOH</td>
<td>30 min</td>
<td>room temp</td>
</tr>
<tr>
<td>70% EtOH</td>
<td>30 min</td>
<td>room temp</td>
</tr>
<tr>
<td>80% EtOH</td>
<td>30 min</td>
<td>room temp</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>30 min</td>
<td>room temp</td>
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<tr>
<td>100% EtOH</td>
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<td>room temp</td>
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<tr>
<td>100% EtOH</td>
<td>30 min</td>
<td>room temp</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>30 min</td>
<td>room temp</td>
</tr>
</tbody>
</table>

(Dry, unused matrices were placed directly in 100% alcohol). Leave overnight at 4°C.

3. Equilibration. Samples were stored in tissue cassettes and equilibrated overnight at 4°C in a solution of 50% ethanol/50% catalyzed JB-4 solution A.

4. Vacuum infiltration. Under house vacuum, 4°C, infiltrate in 100% catalyzed JB-4 solution A, 1-4 days.

5. Embedding. Combine JB-4 catalyzed solution A : JB-4 solution B at a ratio of 20:1. Mix well and pipet into plastic molds. Place samples face down in plastic molds. Ensure that sample orientation is maintained. Solution begins to harden in approximately 30 minutes. Before hardening completed (careful- polymerization may progress rapidly once started), place labeled metal embedding blocks in molds. Note: label should be written in standard pencil marking only!

Paraffin embedding:

1. Samples should be decalcified and rinsed well of fixative, and placed in plastic tissue cassettes (Tissue Tek unicassettes, #4170 or #4173, Miles, Inc., Elkhart, IN).
2. Dehydration and infiltration.

A) Tissue specimens were dehydrated and infiltrated by machine (Tissue-Tek VIP 1000, model 4617, Miles Scientific, Mishwaka, IN). In this experiment, dehydration program #4 was used, with solutions changed automatically as follows:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% EtOH</td>
<td>1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>70% EtOH</td>
<td>1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>80% EtOH</td>
<td>1 hour</td>
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<tr>
<td>clearing solution</td>
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</tr>
<tr>
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</tr>
<tr>
<td>paraffin</td>
<td>1 hour</td>
<td>59°C</td>
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<td>59°C</td>
</tr>
<tr>
<td>paraffin</td>
<td>30 min</td>
<td>59°C</td>
</tr>
</tbody>
</table>

Clearing solution: Americlear histology clearing solvent, Baxter Healthcare Corp. #C4200-1, Deerfield, IL
Paraffin: Paraplast Cat. #8889-501006, melting temp 56°C, Oxford Labware, St. Louis, MO

B) Fragile collagen matrix samples were dehydrated by hand. Water and alcohol solutions were pipetted in and out of 24-well plates as follows:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>distilled H₂O</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>distilled H₂O</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>distilled H₂O</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>50% EtOH</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>70% EtOH</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>80% EtOH</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>95% EtOH</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
<tr>
<td>100% EtOH</td>
<td>30 min- 1 hour</td>
<td>room temp</td>
</tr>
</tbody>
</table>

The matrices were then placed in tissue cassettes in clearing solution (Americlear) for 1 hour, then placed in molten paraffin to pre-infiltrate for a minimum of 2 hours (as long as overnight) with 1-2 changes of solution.
3. Embedding. Specimens were embed with the aid of Tissue-Tek II paraffin embedding center, model 4603 (Lab-Tek Products, Westmont, IL). The machine provided a molten source of paraffin and cooling plate. Paraffin was placed in stainless steel molds of varying size, the specimen placed in the mold, and the mold placed on a cooling plate. The tissue cassette was placed on the mold and additional paraffin was added to affix the plastic cassette to the mold.

Sectioning and storage:

Histological sections were cut on a microtome (Reichert-Jung model 2050 Supercut, Leica Instruments, Nussloch, Germany). Thickness were 7 μm for paraffin sections and 5 μm for plastic. The sections were placed on glass slides (Fisherbrand Superfrost Plus, Cat. #12-550-15, Fisher Scientific, Pittsburgh, PA) for paraffin, and S/P Micro Slide, Baxter Scientific Cat. #M6156, McGraw Park, IL for plastic). Slides with plastic sections were dried on a hot plate at low temperature, then stored at room temperature. Paraffin slides were placed in an oven at 50°C to melt off excess paraffin overnight, then stored at 4°C.
Apppendix F  Histological staining protocols

Note: Slides were coverslipped and sealed with Cytoseal 60 cell-mounting medium (Stephens Scientific #8310-16, Riverdale, NJ).

Masson Trichrome Staining
Formalin-fixed, paraffin-embedded tissue

0. Deparaffinize and hydrate
   Xylene: 2 x 5 min.
   100% ethanol: 10-20 dips
   100% ethanol: 10-20 dips
   95% ethanol: 10-20 dips
   80% ethanol: 10-20 dips
   70% ethanol: 10-20 dips
   dH₂O: 10-20 dips

1. Mordant in Bouin's solution 1 hour at 56°C or overnight at RT
2. Cool and wash in running water until yellow color disappears
3. Rinse in distilled water
4. Weigert's iron hematoxylin solution for 10 min. Wash in running water 10 min
5. Rinse in distilled water
6. Phosphomolybdic-phosphotungstic acid solution for 10 to 15 min. Discard solution.
7. Aniline blue solution for 5 min. Save solution
8. Rinse in distilled water
9. Glacial acetic solution for 3 to 5 min. Discard solution
10. Dehydrate in 70, 80, 95, and 100% EtOH
11. Clear in xylene
12. Coverslip with Permount

Safranin O - Fast Green staining protocol
Formalin-fixed, paraffin-embedded tissue

0. Deparaffinize and hydrate
   Xylene: 2 x 5 min.
   100% ethanol: 10-20 dips
   100% ethanol: 10-20 dips
   95% ethanol: 10-20 dips
   80% ethanol: 10-20 dips
   70% ethanol: 10-20 dips
   dH₂O: 10-20 dips
1. Safranin O, 10 min (30 min for GMA sections)
2. Rinse with distilled water
3. Counterstain with working solution Fast Green 10-15 sec
4. Rinse with distilled water
5. Dehydrate (70%-80%-95%-100% (x2)-xylene (x2))
6. Air dry sections
7. Coverslip with Permount

Solutions:

**Safranin O**
0.2 gm Safranin O
100 ml distilled water
1 ml glacial acetic acid

**Stuck Fast Green- stock solution:**
0.2 gm Fast Green
100 ml distilled water
1 ml glacial acetic acid

**Fast Green working solution:**
dilute stock solution 1:5 in distilled water

**Hematoxylin and Eosin staining**
Formalin-fixed, paraffin-embedded tissue

1. **DEPARAFFINIZE AND REHYDRATE**
   - Xylene: 2 x 5 min.
   - 100% ethanol: 10-20 dips
   - 100% ethanol: 10-20 dips
   - 95% ethanol: 10-20 dips
   - 80% ethanol: 10-20 dips
   - 70% ethanol: 10-20 dips
   - dH2O: 10-20 dips
2. Harris hematoxylin, 10 min.
3. Rinse in tap water, approx. 1 min. running or swishing until almost clear
4. Acid alcohol. Dip quickly 5-10 times. 20-30 sec total.
5. Rinse in tap water. Until foaming stops, maybe 30 sec.
6. Ammonia water. Quick dips (5 or so) until blue.
7. Rinse in tap water. About 1 min.
8. Eosin, 45 sec.
9. Rinse in tap water, 2 min.
10. **DEHYDRATE**
    - 70% ethanol: 10-20 dips
    - 80% ethanol: 10-20 dips
    - 95% ethanol: 10-20 dips
    - 100% ethanol: 10-20 dips
100% ethanol: 10-20 dips
Xylene: 2 x 5 min.

11. Air dry, coverslip with Permount.

Solutions:

HEMATOXYLIN: Filter 200 ml of stock solution into staining dish. Sigma Harris Hematoxylin Solution, Catalog HHS-128, Modified: Hematoxylin, 7.5 g/L; sodium iodate, aluminum and ammonium sulfate, stabilizers and preservative.

ACID ALCOHOL: 200 ml of 70% ethanol (in dH$_2$O) + 0.5 ml HCl

AMMONIA WATER: 200 ml dH$_2$O + 5-10 drops ammonium hydroxide pH should be roughly around 10.0 - use pH paper.

EOSIN: 100 ml stock solution + 100 ml dH$_2$O + 1.0 ml glacial acetic acid Sigma Eosin Y Solution Aqueous, catalog HT110-2-128.

---

**Hematoxylin and Eosin staining**
Formalin-fixed, plastic-embedded tissue

1. Dry slides
2. Harris hematoxylin, 60 min.
3. Rinse in tap water, approx. 1 min. running or swishing until almost clear
4. Acid alcohol. Dip quickly 5-10 times. 20-30 sec total.
5. Rinse in tap water. Until foaming stops, maybe 30 sec.
6. Ammonia water. Quick dips (5 or so) until blue.
7. Rinse in tap water. About 1 min.
8. Eosin, 5 min.
9. Rinse in tap water, 5 min.
10. Air dry, coverslip with Permount.

---

**Aniline Blue Staining**

Solution: 2.5 gm aniline blue
100 ml dH$_2$O
2 ml glacial acetic acid

For **GMA**: 2-4 min. Rinse in alcohol
For **paraffin**: deparaffinize and hydrate. Stain 10 sec. Rehydrate and clear in xylene.
Appendix G  Immunohistochemical staining protocols

Immunohistochemistry Supplies

**Anti-Type II collagen antibody**
cIIcI (mouse anti-chick type II collagen monoclonal antibody)
Developmental Studies Hybridoma Bank
Iowa City, IA

**Anti-type I antibody**
Rabbit anti-human type I collagen
catalog #: IgG : D20111

Dr. Daniel Hartman
Institut Pasteur de Leon
Avenue Tony Garnier
69365 Leon CEDEX 7, France
011-33-72-73-43-25
FAX 011-33-78-61-05-03

**Protease XIV** (protease type XIV: Bacterial (pfs), also Pronase E)
Sigma cat # P 5147, St. Louis, MO

**DAB** (3,3’-diaminobenzidene) **KNOWN CARCINOGEN! HANDLE WITH CARE!**
Sigma cat # D-5637, St. Louis, MO 63178

**DTT** (Dithiothreitol, (Cleland's reagent, DL-DTT), 99%)
Sigma cat # D 0632, St. Louis, MO

**Iodoacetic Acid**
Sigma cat # I 4386, St. Louis, MO

**Horse serum**
Sigma cat # H 0146, St. Louis, MO

**Donkey serum**
Sigma cat # D 9663, St. Louis, MO

**Donkey anti-rabbit secondary antibody** (biotinylated donkey anti-rabbit Ig)
Amersham catalog #: RPN 1004, Arlington Heights, IL

**Horse anti-mouse secondary antibody** (biotinylated horse anti-mouse Ig)
Vector Laboratories catalog #: BA-2000, Burlingame, CA

**ABC staining kit** (Standard Vectastain ABC kit)
Vector Laboratories Catalog #: PK 4000, Burlingame, CA

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Control Tissue:

Chicken: Mayflower Poultry Co, Cambridge, MA
Bovine: Arena Brothers, Hopkinton, MA

TRIS (Tris[hydroxymethyl]aminomethane): 250 g white container from Sigma, catalog # T-1378

Anti-Type I Collagen

Immunohistochemical Staining (ABC Method)
Rabbit anti-human type I collagen from Daniel Hartman:

Handle all washes as gently as possible to preserve tissue adherence. Minimize dipping.

Hydration (for paraffin sections)
1. Xylene, 2 X 2 min. Wipe off excess paraffin.
2. Alcohol: 100% -> 95% -> 80% -> 70%, 2 min each
3. Wash in TBS pH 7.4, 2 X 2 min. Wipe slides.

Blocking Serum (Donkey serum)
1. Dilute 1:20 in TBS pH 7.4
2. 30 min at RT
3. Do NOT wash slides (simply pipette off excess serum)

Primary Antibody (rabbit anti-human type I collagen IgG)
1. Dilution 1:50 in TBS pH 7.4
2. Incubate at RT x 60 min
3. Wash in TBS pH 7.4 2 X 2 min. Wipe slides.

Secondary Antibody (Donkey anti-rabbit IgG)
1. Dilution: 1:200 in TBS pH 7.4
2. 45 min at RT (*)
3. Wash in TBS pH 7.4 2 X 2 min. Wipe slides.

Endogenous peroxidase quench
1. 3% H₂O₂ in distilled water
2. 10 min at RT
3. Wash in TBS pH 7.4 2 X 2 min. Wipe slides.

ABC (Vectastain Labs)
1. Use pre-prepared ABC reagent (as outlined below (*)
2. 30 min at RT
3. Wash in TBS pH 7.4 2 X 2 min. Wipe slides.

**DAB**

1. Prepare fresh DAB as outlined below (#)
2. 8 min at RT (or until dark)
3. Wash in TBS pH 7.4 2 X 2 min

**Counterstain with Hematoxylin (paraffin protocol)**

1. Hematoxylin for 10 min. Rinse thoroughly in water.
2. Dip in acid alcohol (5 sec). Rinse in water.
3. Dip in ammonia water (5 sec). Rinse in water.

**Dehydrate and Mount (paraffin)**

1. Alcohol: 70% -> 80% -> 95% -> 100%, 2 min each.
2. Xylene, 2 X 2 min. Let dry overnight.
3. Mount and coverslip.

(*) Make up ABC reagent at this point:

Add 1 drop reagent A to 5 ml of cold TRIS/HCl buffer (pH 7.6) and mix.
Add 1 drop reagent B and mix thoroughly. Allow to stand for 30 min at room temperature before use.

(#) DAB reagent:

Add 100 µl of 30% hydrogen peroxide to 2.9 ml distilled water. Add 50 µl of this to a prepared solution of 2.5 mg DAB in 5 ml TRIS/HCl buffer (pH 7.6). Check the efficacy of this by adding one drop of this to one drop of ABC reagent on a blank slide or coverslip.

**Anti-type II Immunohistochemical Staining (ABC Method):**

Handle all washes as gently as possible to preserve tissue adherence. Minimize dipping.

**Hydration (for paraffin sections)**

1. Xylene, 2 X 2 min. Wipe off excess paraffin.
2. Alcohol: 100% -> 95% -> 80% -> 70%, 2 min each
3. Wash in TBS pH 7.4, 2 X 2 min. Wipe slides.

**Digestion:**

1. Enzymatically digest specimens in protease type XIV (Sigma) 1.0 mg/ml in TBS pH 7.4 for 60 min.
2. Wash in TBS pH 7.4 2 X 2 min. Wipe slides.
Blocking Serum (Horse serum)
1. Dilute 1:20 in TBS pH 7.4
2. 30 min at RT
3. Do NOT wash slides (simply pipette off excess serum)

Primary Antibody (Mouse anti-chick type II collagen IgG)
1. Dilution 1:20 in TBS pH 7.4
2. Incubate at RT x 60 min or overnight at 4°C in hydration chamber.
3. Wash in TBS pH 7.4 2 X 2 min. Wipe slides.

Secondary Antibody (Horse anti mouse)
1. Dilution: 1:200 in TBS pH 7.4
2. 45 min at RT (*)
3. Wash in TBS pH 7.4 2 X 2 min. Wipe slides.

Endogenous peroxidase quench
1. 3% H₂O₂ in distilled water
2. 10 min at RT
3. Wash in TBS pH 7.4 2 X 2 min. Wipe slides.

ABC (Vectastain Labs)
1. Use pre-prepared ABC reagent (as outlined below (*)
2. 30 min at RT
3. Wash in TBS pH 7.4 2 X 2 min. Wipe slides.

DAB
1. Prepare fresh DAB as outlined below (#)
2. 8 min at RT (or until dark)
3. Wash in TBS pH 7.4 2 X 2 min.

Counterstain with Hematoxylin (paraffin protocol)
1. Hematoxylin for 10 min. Rinse thoroughly in water.
2. Dip in acid alcohol (5 sec). Rinse in water.
3. Dip in ammonia water (5 sec). Rinse in water.

Dehydrate and Mount (paraffin)
1. Alcohol: 70% -> 80% -> 95% -> 100%, 2 min each.
2. Xylene, 2 X 2 min. Let dry overnight.
3. Mount and coverslip.

(*) Make up ABC reagent at this point:
Add 1 drop reagent A to 5 ml of cold TRIS/HCl buffer (pH 7.6) and mix.
Add 1 drop reagent B and mix thoroughly. Allow to stand for 30 min at room temperature before use.
(1) DAB reagent:
Add 0.1 ml 30% hydrogen peroxide to 2.9 ml distilled water. Add 50μl of this to a prepared solution of 2.5 mg DAB in 5 ml TRIS/HCl buffer (pH 7.6). Check the efficacy of this by adding one drop of this to one drop of ABC reagent on a blank slide or coverslip.
Appendix H Methods of quantitative grading of articular cartilage defects

Schematic guide to QUANTITATIVE grading of chondral articular cartilage defects (acute and healing defects)

**All grading must be performed with a 10x objective without the photo knobs engaged**

Schematic of normal articular cartilage

![Schematic of normal articular cartilage](image)

Normal appearance of a fresh chondral defect

![Normal appearance of a fresh chondral defect](image)

Schematic of a fresh chondral defect showing how to define the defect base, defect height, and the area of negative matrix flow

![Schematic of a fresh chondral defect](image)
Typical appearance of a healed defect filled with reparative tissue

Schematic of a healed chondral defect showing how to define the defect base, defect height, and the areas of reparative tissue and matrix flow

STEP 1: Grid placed outside defect to measure height
- measure height 10 small boxes from corner of defect
- here the height is about 8.5 boxes (then do other side-- 9 boxes)
STEP 2: begin sweeping grid across to measure defect base and repair tissues (it may be easiest to do the base completely first, then go back to do the repair tissues).

Here the grid covers:
- about 55 boxes of tissue (15 matrix flow and 40 repair tissue)
- exactly 10 boxes along the base of the defect

NOTE! The repair tissue can extend slightly outside the defect area, as it does here.

---

STEP 2, CTD: Sweep to next spot (b), then continue to end (f).

(b) contains about 42 boxes of repair tissue
(f) contains 5 boxes of repair tissue, 5 boxes of flow
The base is 51 boxes
Measuring bonding of repair tissue to the calcified cartilage and apposition of repair tissue to adjacent cartilage is next. This schematic shows how you might look at a defect to determine bonding/apposition. First, bonding to the CC is checked by polarized light. You can make a mental picture like the schematic below. Bonding to the adjacent cartilage is also measured. Rips and cracks (like the one shown below do not count.

**STEP 3:** Measuring bonding to the base
- first, identify the bonded areas by polarized light (above)
- then sweep the grid across the bonded area to get a total:
- here the total = 37  (a=9, b=10, c=10, d=8)
STEP 4: Judging tissue attachment is done by recording the amount height of the repair tissue which is bonded to the adjacent cartilage.

On the left side, repair tissue goes to a height of 5.8 boxes. All is attached. On the right side, the tissue goes all the way to the top (9 boxes vertically), but 4.5 boxes are subtracted for the crack.

Total height attached: $5.8 + 4.5 = 10.3$

This schematic can be used for the final quantitative step, grading the amount of intact calcified cartilage.

In this example there has been some damage, as indicated:

corner fracture  
resorption

STEP 5: Measuring the intact calcified cartilage and subchondral plate
This is done by sweeping the grid across the intact sections as shown:

The total intact is 27 boxes ($a=10$, $b=5.5$, $c=10$, $d=1.5$)
Appendix I  Power calculation

The number of animals to be used in each group was determined by a power calculation. The procedure for a power calculation is an iterative one. Some values must be based on hypothesized results or previous work. In our case, we based the calculation on the results of our preliminary study of healing of untreated and CAC-treated defects in our animal model.

Specifically, our calculation was based on our previous results for the most important outcome variable, the combined amount of hyaline and articular cartilage found in the defect site at the equivalent time period. These values were $34.9 \pm 16.1\%$ for the CAC treated group, and $19.2 \pm 13.0\%$ for the empty control (EC) group. We wished to detect differences approximately 50% greater than that between these groups in order to identify a clinically meaningful improvement. Thus, we required the minimum detectable difference between groups to be 1.5 times that found previously, i.e. $1.5 \times (34.9-19.2) = 26.4\%$.

Assuming the same standard deviation found in the CAC treated group (16.1%) for both groups, a power of 0.80 ($\beta=0.20$) and a level of significance, $\alpha=0.05$, n = 6 defects was required. Eight defects were chosen to provide a margin of safety in the event of the loss of an animal or other pathology which would prevent analysis of certain specimens.

The steps of the power calculation were as follows. This treatment assumes that the experimenter is searching for the value of $n_2$, the number of samples in a treatment group, to allow detection of significant difference between the treatment group and a control group. The case considered does not assume that the sample numbers or variance in the control and treatment groups are the same. It is assumed that if $n_2$ is chosen for the treatment or experimental group, than $n_1$ for the control group is the same, or is otherwise known.

1) Determine the desired or expected difference between sample means which is the minimum you wish to detect between groups. This value will be based on the best available information, such as that in the second paragraph above.

2) Guess the value of $n_2$ needed for the experimental group.

3) If groups have different variances, calculate the pooled variance. The pooled variance computed from the variances of each group is the best estimate the variance of the entire population (eq. 1).
equation 1: 
\[ s_p^2 = \frac{v_1 s_1^2 + v_2 s_2^2}{v_1 + v_2} \]

\( s^2 = \text{variance} \)

\( s_p^2 = \text{pooled variance} \)

\( v = \text{degrees of freedom} \)

subscripts: '1' or '2' refer to the two samples compared, here the control (1) and experimental (2) groups. Note: \( v = n-1 \)

4) Calculate the equivalent sample size, \( n \). In order to compare the means of the control and experimental groups, a single sample size, representative of both groups, must be calculated (eq. 2).

\[ \text{equation 2: } n = \frac{2n_1 n_2}{n_1 + n_2} \]

\( n = \text{sample size} \)

5) Given these values, equation 3 will calculate the minimum detectable difference, \( \delta \), between the means of the two samples:

\[ \text{equation 3: } \delta \geq \sqrt{\frac{2s_p^2}{n}} (t_{a(2), v} + t_{\beta(1), v}) \]

\( \delta = \text{minimum detectable difference in means} \)

\( t_{a(2), v} = \text{critical value of 't' for given parameters} \)

\( a(2) = \text{two-tailed probability of significance } \alpha \)

\( \beta(1) = \text{one-tailed probability of significance } \beta \)

The power, 1-\( \beta \), and the level of significance, \( \alpha \), are chosen by convention. If the desired or expected difference in mean, as in step 1), is larger than \( \delta \), then the calculation is repeated with smaller \( n_2 \). If the desired difference is smaller than \( \delta \), the calculation is repeated with larger \( n_2 \). The iteration continues until the critical \( n \) is determined.
Appendix J  Elution of components of unseeded matrices in culture

In two sets of experiments with longer term cultures (at least 4 weeks), measurements on unseeded matrices indicated the approximate rate at which the matrix was degraded and components were eluted to the matrix. Both experiments used type II collagen matrices which are known to contain both GAG and DNA. Thus, the standard assays for DNA and GAG (section 4.2.2) were used to monitor their loss over time in culture. These two series included 1) matrices of varying degree of cross-linking, and 2) matrices of varying pore diameter. It should be noted that the values for DNA content include an unknown background component due to interference by other matrix molecules. Thus the absolute DNA values were not determined, and GAG/DNA plots are not presented because they may not accurately indicate the true ratio of GAG to DNA.

The results of the experiment where type II matrices were subjected to varying degree of cross-linking are shown in Figures ApJ-1 and ApJ-2. These matrices were evaluated at 1 day, 4 weeks, and 6 weeks. The results of the experiment in which type II matrices of varying pore diameter were cultured up to 4 weeks are shown in Figures ApJ-3 and ApJ-4.

Matrices of varying degree of cross-linking: The results of the experiment where type II matrices were subjected to varying degree of cross-linking are shown in Figures ApJ-1 and ApJ-2. There was no statistical significance between 4-week and 6-week values in either DNA or GAG content. Thus, most of these components were lost over the first four weeks. Approximately half of the GAG content in each DHT’d matrix was lost. Slightly more DNA and GAG were lost from the 1-day DHT group vs. the 4-day DHT group. This effect of cross-linking treatment was not highly significant by 2-way ANOVA (p=0.15 for DNA, p=0.38 for GAG). When UV cross-linked matrices were also evaluated at 6 weeks, the three groups had the same amount of DNA, but differing amounts of GAG (p=0.01, single factor ANOVA). The UV-cross-linked matrices had the least GAG at 6 weeks.

Analysis of matrices of varying pore diameter: Biochemical assay of the DNA and GAG contents of unseeded type II matrices of varying pore size show the loss of the matrix to the medium over time (Figures ApJ-3 and ApJ-4). 2-way ANOVA showed that the DNA content of unseeded matrices was significantly dependent on both time (p<10-4) and pore size (p<10-7). The pore size effect was evident as slightly less DNA in small pore matrices. The time effect was a very slight decrease in DNA over time. This loss was most noticeable for the small pore matrices. The loss of GAG from the matrices over time was much more obvious. Both time and pore size effects were highly significant by 2-way ANOVA (p values < 10-6). This loss had a similar profile in all three pore sizes, but was slightly greater in the small pore matrices. These results showed that the GAG was eluted from the matrix more rapidly than the DNA.
Figure ApJ-1. DNA content of unseeded type II matrices of varying degree of cross-linking with time in culture; n=3. Data given as mean ± SEM.

Figure ApJ-2. GAG content of unseeded type II matrices of varying degree of cross-linking with time in culture; n=3. Data given as mean ± SEM.
Figure ApJ-3. DNA content of unseeded type II matrices of varying pore size with time in culture; n=4. Data given as mean ± SEM.

Figure ApJ-4. GAG content of unseeded type II matrices of varying pore size with time in culture; n=4. Data given as mean ± SEM.
Appendix K. Evaluation of relationship of proximal and distal defects

In this work, it was important to establish any relationship between the proximal and distal defects. Two analyses were performed to this end:

1) A correlation analysis, to determine any positive or negative correlation between the defects. Three separate analyses were performed, based on the three series of animal experiments: series 1, untreated defects, periosteum, and autologous cultured chondrocyte treatment (CAC); series 2, preliminary implants of seeded and/or unseeded type I or II collagen; and series 3, engineered type II collagen implants and microfracture. Within each series all proximal and distal defects were grouped for all quantitative categories. A correlation with linear regression was performed, and the coefficient of determination ($R^2$) obtained for all combinations of groups. The coefficients of determination for the most important categories in healing in the three series are reported in Table Appendix K-1:

<table>
<thead>
<tr>
<th>series (n)</th>
<th>total filling</th>
<th>fibrous tissue</th>
<th>fibrocartilage</th>
<th>hyaline + articular</th>
<th>intact calcified layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (50)</td>
<td>0.03 (-)</td>
<td>0.04 (+)</td>
<td>0.06 (-)</td>
<td>0.42 (+)</td>
<td>0.10 (+)</td>
</tr>
<tr>
<td>2 (14)</td>
<td>0.15 (+)</td>
<td>0.01 (-)</td>
<td>0.06 (+)</td>
<td>0.03 (-)</td>
<td>0.02 (+)</td>
</tr>
<tr>
<td>3 (12)</td>
<td>0.08 (-)</td>
<td>0.03 (+)</td>
<td>0.14 (-)</td>
<td>0.00</td>
<td>0.18 (+)</td>
</tr>
</tbody>
</table>

Table Appendix K-1. Coefficients of determination from correlation analysis of proximal vs. distal defects for selected categories from three series of experiments in a canine model. The sign of the correlation is also given. Defect locations were identical in all series. $n =$ number of knees in which comparison of proximal and distal defects was possible.

The coefficient of determination represents the portion of the value one variable (distal data) which can be predicted by knowing values of the second variable (proximal data). Thus, the results of this correlation indicate that knowing the results of one defect in a knee gives very little information about the other. This suggests that the two defect locations could be treated independently. For this reason, the quantitative data for the animal series are reported with each defect treated independently. In one case, that of hyaline + articular cartilage in the first series, this assumption is less substantially justified. In this case, a fair portion of the data in one defect (42%) can be predicted by the other. For this reason, in analyzing this data, a second approach was added for completeness: the data from the proximal and distal defects in each knee were averaged. This method results in the loss of some of the information contained in the defects, but yields a more conservative value for statistical significance. For completeness, the entire set of data, when averaged by knee, is presented in Appendix L. Secondary statements of statistical significance for the hyaline + articular category in the first series are included in the main text for the data treated in this manner.
2) The second evaluation was designed to determine if there were any consistent differences between proximal and distal defects. This analysis examined all quantitative categories including defect geometry, tissue types filling the defect, attachment to adjacent structures, and the status of the calcified cartilage. The difference in values for proximal and distal defects was calculated and the resulting statistic compared to a value of zero by t-statistic (one sample hypothesis). The same three series of animals as in the correlation analysis were used in this manner. The results for selected categories are presented in Table Appendix K-2.

<table>
<thead>
<tr>
<th>series</th>
<th>total filling</th>
<th>fibrous tissue</th>
<th>fibro-cartilage</th>
<th>hyaline + articular</th>
<th>intact calcified layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;&gt;0.5</td>
<td>&lt;0.2</td>
<td>&gt;0.2</td>
<td>&gt;0.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>(50)</td>
<td>+1%</td>
<td>-3%</td>
<td>+1%</td>
<td>-1%</td>
<td>+9%</td>
</tr>
<tr>
<td>2</td>
<td>&lt;0.1</td>
<td>&lt;0.5</td>
<td>&lt;0.2</td>
<td>&gt;0.2</td>
<td>&lt;0.5</td>
</tr>
<tr>
<td>(14)</td>
<td>-20%</td>
<td>-5%</td>
<td>-12%</td>
<td>-3%</td>
<td>+8%</td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.2</td>
<td>&lt;0.005</td>
<td>&gt;0.5</td>
<td>&gt;0.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>(12)</td>
<td>-27%</td>
<td>-25%</td>
<td>-3%</td>
<td>+1%</td>
<td>+23%</td>
</tr>
</tbody>
</table>

Table Appendix K-2. Significance level of difference in value of proximal vs. distal defects for selected categories from three series of experiments in a canine model. The absolute difference, proximal-distal (as a percentage of the defect area or linear measure) is also given. Defect locations were identical in all series. n = number of knees in which comparison of proximal and distal defects was possible.

There were few differences in the first series of experiments, with only the suggestion that the calcified layer was slightly more intact in the proximal defects. The third series did show some differences in proximal and distal defects: proximal defects had a substantially more intact subchondral plate and less total filling. The extra tissue in distal defects was almost entirely fibrous tissue. In the second series, the results were roughly similar although the statistical significance and magnitude of the differences were lower. In addition, in the second series, the additional tissue in the distal defects was more fibrocartilage.

In most categories not presented, including matrix flow, integration with adjacent cartilage, and bonding to the calcified cartilage, differences between proximal and distal defects were not highly significant, and not consistent among the three series. However, there was a consistent finding that proximal defects were slightly larger than distal defects. The differences, as a percent of total area were 6%, 1%, and 9% in the three series, with significance between proximal and distal in each series ranging from p<0.1 to >>0.5.
Appendix L  Quantitative data for in vivo chondral defect experiments with data averaged by knee

<table>
<thead>
<tr>
<th>time</th>
<th>defect type (n)</th>
<th>fibrous tissue</th>
<th>fibro-cartilage</th>
<th>hyaline cartilage</th>
<th>articular cartilage</th>
<th>bone</th>
<th>total filling</th>
<th>matrix flow</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 wk C</td>
<td>3</td>
<td>24.5 ± 2.9</td>
<td>20.5 ± 2.1</td>
<td>3.1 ± 1.1</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>48.0 ± 3.0</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>9 wk C</td>
<td>6</td>
<td>11.5 ± 2.2</td>
<td>20.6 ± 9.2</td>
<td>5.2 ± 1.6</td>
<td>0.6 ± 0.4</td>
<td>0 ± 0</td>
<td>37.9 ± 7.9</td>
<td>4.7 ± 1.5</td>
</tr>
<tr>
<td>6 &amp; 9 wk C</td>
<td>9</td>
<td>15.8 ± 2.7</td>
<td>20.6 ± 6.0</td>
<td>4.5 ± 1.1</td>
<td>0.4 ± 0.3</td>
<td>0 ± 0</td>
<td>41.3 ± 5.4</td>
<td>3.3 ± 1.2</td>
</tr>
<tr>
<td>3 mo C</td>
<td>5</td>
<td>1.1 ± 0.7</td>
<td>12.3 ± 4.2</td>
<td>14.4 ± 4.6</td>
<td>3.0 ± 1.8</td>
<td>0 ± 0</td>
<td>30.8 ± 8.8</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>15 wk MF</td>
<td>4</td>
<td>10.9 ± 3.3</td>
<td>52.4 ± 16.6</td>
<td>0.7 ± 0.3</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>64 ± 13.7</td>
<td>1.8 ± 0.7</td>
</tr>
<tr>
<td>6 mo C</td>
<td>3</td>
<td>2.5 ± 1.3</td>
<td>12.9 ± 5.4</td>
<td>10.0 ± 4.7</td>
<td>1.8 ± 1.3</td>
<td>0 ± 0</td>
<td>37.1 ± 10.3</td>
<td>8.8 ± 4.4</td>
</tr>
<tr>
<td>12 mo C</td>
<td>4</td>
<td>1.3 ± 0.9</td>
<td>17.2 ± 8.0</td>
<td>19 ± 3.7</td>
<td>3.7 ± 1.7</td>
<td>0.2 ± 0.2</td>
<td>41.4 ± 4.5</td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>18 mo C</td>
<td>3</td>
<td>8.4 ± 3.0</td>
<td>51.8 ± 8.4</td>
<td>9.6 ± 2.0</td>
<td>0 ± 0</td>
<td>5.8 ± 5.8</td>
<td>75.7 ± 17.5</td>
<td>1.6 ± 0.5</td>
</tr>
</tbody>
</table>

Table 5-11a. Components of healing full-thickness chondral defects in an adult canine model. C = untouched chondral defect. MF = chondral defect with microfracture. Values given as an areal percent of total defect area seen in a histological cross-section from the center diameter of the defect, mean ± SEM (data averaged by knee).

<table>
<thead>
<tr>
<th>time</th>
<th>defect type</th>
<th>Integration with adjacent AC</th>
<th>Bonding to calcified cartilage</th>
<th>Intact calcified cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 week chondral</td>
<td>0 ± 0</td>
<td>6.3 ± 2.9</td>
<td>56.8 ± 18.6</td>
<td></td>
</tr>
<tr>
<td>9 week chondral</td>
<td>17.2 ± 6.2</td>
<td>24.9 ± 8.5</td>
<td>62.5 ± 12.4</td>
<td></td>
</tr>
<tr>
<td>6 + 9 chondral</td>
<td>11.4 ± 4.9</td>
<td>18.7 ± 6.3</td>
<td>60.6 ± 9.7</td>
<td></td>
</tr>
<tr>
<td>3 months chondral</td>
<td>12.8 ± 3.1</td>
<td>46.1 ± 15.6</td>
<td>94.9 ± 2.9</td>
<td></td>
</tr>
<tr>
<td>15 weeks microfracture</td>
<td>25.3 ± 4.1</td>
<td>1.8 ± 1</td>
<td>35.6 ± 15.4</td>
<td></td>
</tr>
<tr>
<td>6 months chondral</td>
<td>13.2 ± 4.5</td>
<td>39.5 ± 16.5</td>
<td>89.3 ± 8.7</td>
<td></td>
</tr>
<tr>
<td>12 months chondral</td>
<td>15.6 ± 8.2</td>
<td>88.6 ± 9.7</td>
<td>92.3 ± 7.4</td>
<td></td>
</tr>
<tr>
<td>18 months chondral</td>
<td>24.6 ± 9.1</td>
<td>40.7 ± 16.3</td>
<td>73.4 ± 12.9</td>
<td></td>
</tr>
</tbody>
</table>

Table 5-12a. Integration of reparative tissue and status of the underlying calcified cartilage in healing full-thickness chondral defects in an adult canine model. One group, as indicated, was treated with microfracture. Values given as a linear percent of the length of the defect base or height, as seen in a histological cross-section from the center diameter of the defect, mean ± SEM (data averaged by knee).
<table>
<thead>
<tr>
<th>time treatment (mo.)</th>
<th>n</th>
<th>Fibrous Tissue</th>
<th>Transition Tissue</th>
<th>Hyaline Cartilage</th>
<th>Articular Cartilage</th>
<th>Bone</th>
<th>Total Repair</th>
<th>matrix flow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*EC</td>
<td>9</td>
<td>15.8 ± 2.7</td>
<td>20.6 ± 6.0</td>
<td>4.5 ± 1.1</td>
<td>0.4 ± 0.3</td>
<td>0 ± 0</td>
<td>41.3 ± 5.4</td>
<td>3.3 ± 1.2</td>
</tr>
<tr>
<td>CAC</td>
<td>6</td>
<td>10.6 ± 4.8</td>
<td>15.2 ± 6.7</td>
<td>10.4 ± 4.7</td>
<td>1.9 ± 1.8</td>
<td>0.1 ± 0.1</td>
<td>38.2 ± 9.2</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>5</td>
<td>1.1 ± 0.7</td>
<td>12.3 ± 4.2</td>
<td>14.4 ± 4.6</td>
<td>3.0 ± 1.8</td>
<td>0 ± 0</td>
<td>30.8 ± 8.8</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAC</td>
<td>5</td>
<td>0.7 ± 0.5</td>
<td>12.5 ± 3.7</td>
<td>30.2 ± 6.1</td>
<td>4.7 ± 3.6</td>
<td>0 ± 0</td>
<td>48.1 ± 4.9</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>3</td>
<td>2.5 ± 1.3</td>
<td>12.9 ± 5.4</td>
<td>10.0 ± 4.7</td>
<td>1.8 ± 1.3</td>
<td>0 ± 0</td>
<td>37.1 ± 10.3</td>
<td>8.8 ± 4.4</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAC</td>
<td>3</td>
<td>6.1 ± 4.9</td>
<td>10.9 ± 5.9</td>
<td>18.7 ± 6.1</td>
<td>18.6 ± 16.7</td>
<td>0 ± 0</td>
<td>54.3 ± 15.3</td>
<td>1.3 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>4</td>
<td>1.3 ± 0.9</td>
<td>17.2 ± 8.0</td>
<td>19 ± 3.7</td>
<td>3.7 ± 1.7</td>
<td>0.2 ± 0.2</td>
<td>41.4 ± 4.5</td>
<td>2.5 ± 1.1</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAC</td>
<td>3</td>
<td>2.2 ± 1.4</td>
<td>27.8 ± 13.6</td>
<td>14.7 ± 11.5</td>
<td>1.7 ± 1.7</td>
<td>1.6 ± 1.1</td>
<td>48 ± 10.5</td>
<td>2.7 ± 1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>4</td>
<td>1.5 ± 0.9</td>
<td>19.1 ± 8.5</td>
<td>14.9 ± 3.0</td>
<td>0.5 ± 0.3</td>
<td>0 ± 0</td>
<td>35.9 ± 10.3</td>
<td>2.8 ± 1.0</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAC</td>
<td>3</td>
<td>8.4 ± 3.0</td>
<td>51.8 ± 8.4</td>
<td>9.6 ± 2.0</td>
<td>0 ± 0</td>
<td>5.8 ± 5.8</td>
<td>75.7 ± 17.5</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC</td>
<td>3</td>
<td>1.6 ± 0.8</td>
<td>41.4 ± 7.7</td>
<td>9.5 ± 2.2</td>
<td>0.4 ± 0.4</td>
<td>0 ± 0</td>
<td>53 ± 5.8</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAC</td>
<td>4</td>
<td>2.5 ± 1</td>
<td>27.5 ± 7.5</td>
<td>12.9 ± 4.5</td>
<td>0.3 ± 0.3</td>
<td>2.7 ± 2.7</td>
<td>45.9 ± 8.0</td>
<td>1.7 ± 0.6</td>
</tr>
</tbody>
</table>

Table 5-14a. Components of healing full-thickness chondral defects in an adult canine model. Defects are untreated (EC), treated with periosteum alone (P), or treated with periosteum and cultured autologous chondrocytes (CAC). Values given as an areal percent of total defect area seen in a histological cross-section from the center diameter of the defect, mean ± SEM (data averaged by knee). *1.5 month EC group includes both 6-week and 9-week defects.
### Table 5-15a. Integration of reparative tissue and status of the underlying calcified cartilage in healing full-thickness chondral defects in an adult canine model. Defects are untreated (EC), treated with periosteum alone (P), or treated with periosteum and cultured autologous chondrocytes (CAC). Values given as a linear percent of the length of the defect base or height, as seen in a histological cross-section from the center diameter of the defect, mean ± SEM (data averaged by knee).

<table>
<thead>
<tr>
<th>time</th>
<th>treatment</th>
<th>Integration with adjacent AC</th>
<th>Bonding to calcified cartilage</th>
<th>Intact calcified cartilage</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 months</td>
<td>*EC</td>
<td>11.4 ± 4.9</td>
<td>18.7 ± 6.3</td>
<td>60.6 ± 9.7</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>8.3 ± 5.4</td>
<td>27.3 ± 16.9</td>
<td>59.1 ± 28.8</td>
</tr>
<tr>
<td></td>
<td>CAC</td>
<td>15.0 ± 3.8</td>
<td>41.9 ± 13.5</td>
<td>79.3 ± 10.4</td>
</tr>
<tr>
<td>3 months</td>
<td>EC</td>
<td>12.8 ± 3.1</td>
<td>46.1 ± 15.6</td>
<td>94.9 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>not studied</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAC</td>
<td>20.8 ± 4.4</td>
<td>75.5 ± 14.8</td>
<td>96.4 ± 2.7</td>
</tr>
<tr>
<td>6 months</td>
<td>EC</td>
<td>13.2 ± 4.5</td>
<td>39.5 ± 16.5</td>
<td>89.3 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>not studied</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CAC</td>
<td>35.1 ± 5.5</td>
<td>87.4 ± 7.5</td>
<td>91.2 ± 7.6</td>
</tr>
<tr>
<td>12 months</td>
<td>EC</td>
<td>15.6 ± 8.2</td>
<td>88.6 ± 9.7</td>
<td>92.3 ± 7.4</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>32.4 ± 4.1</td>
<td>43.6 ± 28.7</td>
<td>65.1 ± 17.9</td>
</tr>
<tr>
<td></td>
<td>CAC</td>
<td>21.2 ± 4.5</td>
<td>60.1 ± 12.1</td>
<td>80.1 ± 11.1</td>
</tr>
<tr>
<td>18 months</td>
<td>EC</td>
<td>24.6 ± 9.1</td>
<td>40.7 ± 16.3</td>
<td>73.4 ± 12.9</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>16.1 ± 7.4</td>
<td>46.7 ± 14.5</td>
<td>98.3 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>CAC</td>
<td>21.6 ± 5.2</td>
<td>59.3 ± 20.4</td>
<td>79.9 ± 15.4</td>
</tr>
</tbody>
</table>
Appendix M Description of defects with minimal filling

This table describes the number of defects which had less than 20% filling. The data are given as the number of defects in each group with less than the given percentage of total repair tissue, i.e. less than 5, 10, 15 or 20% of the cross-sectional area. Also recorded are the remaining low value for the group (outside of those reported for <20%), and the absolute minimum value for the group. In the one experiment at multiple time points, the group totals for all times are also given.

<table>
<thead>
<tr>
<th>time</th>
<th>group</th>
<th>&lt;5%</th>
<th>&lt;10%</th>
<th>&lt;15%</th>
<th>&lt;20%</th>
<th>low (%)</th>
<th>min (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 wk</td>
<td>CAC+typell</td>
<td>1/8</td>
<td>1/8</td>
<td>1/8</td>
<td>1/8</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>15 wk</td>
<td>μFx+typell</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>48</td>
<td>48</td>
</tr>
<tr>
<td>15 wk</td>
<td>μFx only</td>
<td>1/8</td>
<td>1/8</td>
<td>1/8</td>
<td>2/8</td>
<td>44</td>
<td>5</td>
</tr>
<tr>
<td>15 wk</td>
<td>CAC</td>
<td>0/10</td>
<td>0/10</td>
<td>1/10</td>
<td>1/10</td>
<td>32</td>
<td>15</td>
</tr>
<tr>
<td>15 wk</td>
<td>harvest</td>
<td>1/9</td>
<td>1/9</td>
<td>1/9</td>
<td>2/9</td>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>15 wk</td>
<td>fascia</td>
<td>4/6</td>
<td>4/6</td>
<td>4/6</td>
<td>4/6</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>15 wk</td>
<td>typel only</td>
<td>0/6</td>
<td>0/6</td>
<td>1/6</td>
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<tr>
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<td>harvest</td>
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<td>0/9</td>
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<tr>
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<tr>
<td>periosteum</td>
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<td>1/18</td>
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<tr>
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<td>0/43</td>
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</table>

Figure ApM-1. Number of defects in animal series with minimal filling with reparative tissue. CAC=cultured autologous chondrocytes. Type I and II are collagen matrices. μFx=microfracture. Harvest groups are empty control defects. Remaining low is next lowest value above 20%.
References


