THE UNIVERSITY OF CALGARY

The Cellular Changes in Medial Collateral Ligament Autotransplantation

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF

MEDICAL SCIENCE

CALGARY, ALBERTA

SEPTEMBER, 1990

ISBN 0-315-66857-1

THE UNIVERSITY OF CALGARY

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Due to the incidence of ligament injuries, clinical experience has led to a growing belief that connective tissue grafts have some potential for replacing ligament functions. From previous studies, some surgical factors have been isolated which appear to affect the overall outcome of connective tissue transplants. Two of these factors are (i) the importance of maintaining the original cells in a graft and (ii) the importance of graft tension at the time of implantation. The purpose of this investigation was to define the importance of these two factors by attempting to define how cell indices may be altered in the midsubstance of a medial collateral ligament autograft under conditions which may be controlled by surgery. More specifically, I wanted to define how the variables of graft tension and graft freeze-thawing would affect the numbers and the "normality" of cells in the midsubstance of ligament autografts at equal points in the healing process. The purpose of gaining a better understanding of the role ligament cells play in graft tissues stems from knowledge that cells are responsible for the repair and maintenance of the matrix structure. The specific aim of this investigation was to compare totally cellular (fresh) autografts with what was initially believed to be totally acellular (multiply frozen-thawed) ligament-bone autografts, under three tensions at time of implantation; anatomic (normal pretension), loose (no pretension) and tight (excessive pretension). Thus, this study involved quantifying with computerized histomorphometry, the cellular changes in MCL autografts under initial "cellular and mechanical control". The hypotheses of this investigation were that for optimal transplant healing, the graft should have a full complement of original cells (fresh) and should have anatomical tension at the time of implantation.

In order to study the MCL transplant model, a baseline was first established for the cell biology of normal rabbit MCL that resulted in a complete description and quantification of MCL fibroblast numbers, distributions, shapes and viability. Pilot studies showed that multiply frozen-thawed autografts were not totally "acellular" but were metabolically damaged at the time of implantation. After 12 weeks of healing, both the fresh and frozen-thawed autografts displayed good healing with no detectable differences. Before determining the effects of tensioning on MCL autografts, the effects of tensioning on normal ligament cells was determined. The tensioning results were the first to ever demonstrate *in situ* changes in cell shape with ligament loading. Despite any of the three standardized tensions placed on the MCL autografts, after 12 weeks of healing all transplanted MCLs had similar cellular characteristics and were morphologically indistinguishable from normal MCL.

To summarize the results on MCL autotransplantation, the autograft model appeared successful at an early healing period. All the MCL autografts appeared to heal and be populated with an abundance of cells. These cells had an appearance consistent with normal ligament cells by our morphological definition. In conclusion, despite the treatment of MCLs at implantation, all autografts postoperatively at 12 weeks were morphologically indistinguishable from normal ligament. This implies that at least in terms of cellular appearances, that the surgical variables of tension and cryopreservation have little effect. The mechanism by which all grafts become repopulated with normal looking ligament fibroblasts remains to be determined.

ACKNOWLEDGEMENTS

I would like to thank Dr. Cy Frank for his supervision, support and continued enthusiasm for medical research which stimulated me throughout the course of my graduate studies. I acknowledge my committee members Drs. Hart, Schachar and Shrive whose helpful suggestions and recommendations aided the progress of my project, never allowing me to lose sight of the "big picture". I also thank Dr. Russell for serving as my external examiner.

Without the excellent technical knowledge and support of Mrs. Donna McDonald and Dr. John Matyas this work would not have been possible. I thank Dr. Graham King whose surgical expertise and collaboration contributed to the success of this project, as well as Mr. Rohit Joshi's computer knowledge which assisted me on several occasions. I would also like to acknowledge everyone associated with the Joint Injuries and Disease Research Group who made research an enjoyable experience.

I am grateful to my parents who have continuously supported me in all my educational pursuits and who supported my decision of a career in science. Lastly, I thank Mr. Peter Scott for his understanding and editorial skills that were significant in encouraging me to pursue my goals.

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CHAPTER 1
INTRODUCTION

THE HUMAN KNEE

The human knee is a synovial joint composed of the femoral and tibial long bones, whose articulating surfaces are covered by hyaline cartilage and separated by two fibrocartilagenous pads known as menisci. These articulating surfaces are held in apposition by several stabilizing structures which cross the knee joint. Tendons are dense connective tissues which connect muscles to bones, often across joints, where they help provide stability. The second major tissue which more passively stabilizes diarthrodial joints is known as ligament. The word "ligament" is derived from the Latin word *ligare* meaning "to bind together" (Gamble et al., 1984). Skeletal ligaments have been described anatomically as short bands of tough but flexible fibrous connective tissue that bind bones together (Amiel et al., 1984); in the case of the knee joint, binding the tibia to the femur. While the major role of ligaments is clearly one of mechanical support there is additional evidence that ligaments may serve some function in neuromuscular feedback (Brand, 1986).

There are four major ligaments that stabilize the knee. Two of these four ligaments cross roughly in the centre of the joint and are known as the anterior cruciate ligament (ACL) and the posterior cruciate ligament (PCL). The other two distinct ligaments are situated on either side of the knee joint and are called the collateral ligaments. The lateral collateral ligament (LCL) is located on the outer side of the knee, while the medial collateral ligament (MCL) is located on the inner side of the knee (Figure 1).

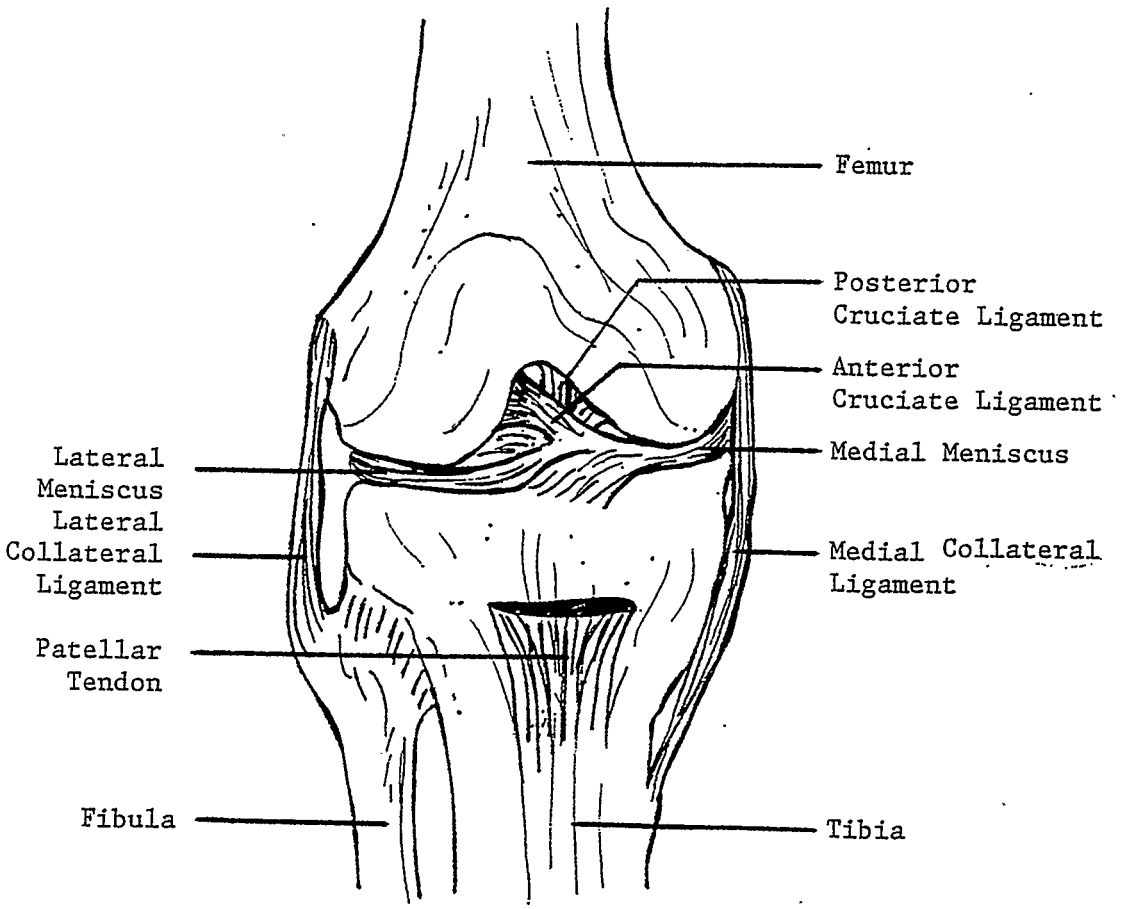

Figure 1 Anterior view of a right human knee joint. Note that the patellar tendon is cut.

The MCL is a broad, flat band attaching proximally to the medial epicondyle of the femur and distally to the medial condyle and medial surface of the tibial shaft (Williams and Warwick, 1980).

LIGAMENT INJURIES

A high incidence of ligament injuries has created a need for a better understanding of ligament structure and function. The knee ligaments in particular are very susceptible to injuries ranging from slight sprains to total avulsions (Quigley, 1954; DeHaven et al., 1980). Ligament sprains account for 25% to 40% of all knee injuries (Andriacchi et al., 1988). Of these, MCL sprains are probably the most frequent (Palmer, 1938), sometimes alone, but more often combined with damage to other knee structures (O'Donoghue, 1950; Gillquist, 1977; Noyes et al., 1980). Severe knee ligament injuries have been found to cause permanent disability and degenerative arthritis when left untreated (O'Donoghue, 1955; Marshall and Olson, 1971; Jacobsen, 1977; Sullivan et al., 1984; Garrick, 1985). This poor prognosis has inspired surgeons to attempt a variety of surgical solutions to knee ligament deficiencies both acutely and chronically.

Surgical Treatment

Three surgical options which have evolved for stabilizing a ligament-deficient knee are to repair, repair and augment, or to replace damaged ligaments. Many types of ligament replacements have been attempted using either synthetic materials (Gart and Rostrup, 1959; Rostrup, 1964; Feagin et al., 1972; Cho, 1975) or biological materials. Biologic replacements are used far more commonly and usually involve surgically removing and replacing a dense connective tissue structure taken either from the host (autogeneic) or from another individual of the

same species (allogeneic) and using it to replace the damaged ligament in question.

Of these biologic options, the most commonly used in a chronic knee ligament deficiency is the replacement of damaged ligaments by a tendon or fascial autograft (tissue taken from somewhere else in the body and used to replace the ligament in question). Hey Groves (1920) was the first to describe the use of fascia lata to replace ligaments in man. Twenty years later it was suggested that part of the quadriceps tendon could be used to replace the ACL (Campbell, 1939), followed by the possibility of using part of the infrapatellar tendon (Jones, 1963). More recently, many other tendons have been used for these biologic reconstructions (O'Donoghue, 1963; Cho, 1975), but the most popular of all ligament autografts has become the replacement of the ACL by the patellar tendon (PT) (Eriksson, 1976; Puddu and Ippolito, 1983; Jonsson et al., 1989). While some autografts are clearly successful for many years, these same types of replacements have been shown to degenerate sometimes within four to five months postoperatively (Alm et al., 1976). Such failures have been attributed to such things as disruption of graft blood supply (Noyes et al., 1980), improper tension on a graft after implantation (Butler et al., 1989) and graft cell injury and death caused by improper graft handling (Kleiner et al., 1986).

ANIMAL MODELS OF LIGAMENT INJURY

Animal models have been used extensively to derive information on the variables that potentially cause failure in ligament healing and ligament replacement. For example, totally transected cruciate ligaments in dogs heal poorly if at all, when left untreated. Collateral ligaments in rabbits, sheep and dogs appear to possess the ability to form scar bridges at the injury site (Jack, 1950; O'Donoghue et al., 1971; Frank et al., 1985; Laws and Walton, 1988), but these

bridges are usually inferior to the original structure and have remained inferior even with surgical repairs (O'Donoghue et al., 1971; Kappakas et al., 1978; Cabaud et al., 1980). Since disrupted ligaments which have either been left untreated or directly repaired seem to be substantially weaker than normal ligaments, it is necessary to have alternate strategies to replace ligament functions.

Biologic Ligament Replacements

There is a long history of experimental study with biologic replacements of connective tissue. The ultimate goal has been to find the tissue which best reproduces the original tissue's structure and function and yet is still practical and feasible for surgery. As previously noted, the two biological options for ligament replacements involve the use of tissues from other individuals (allografting) or from patients themselves (autografting).

Allografts

While some studies have found encouraging results with tendon and ligament allografts (Nikolaou et al., 1986) with no evidence of immune reactions (Shino et al., 1984), others have shown that many of these grafts are mechanically inferior to normal ligaments (Shino et al., 1984, Jackson et al., 1987; Sabiston et al., 1990b), sometimes even showing secondary immune reactions and damage to articular surfaces (Thorson et al., 1987; Jackson et al., 1987). Several investigators have attempted to diminish the chance of allograft rejection by trying to damage the histocompatibility antigens on the cell surface by freezing or freeze-drying the graft before implantation (Graham et al., 1955; Barad et al., 1982; Shino et al., 1984; Thorson et al., 1987; Jackson et al., 1988). This would at least theoretically remove one of the reasons for graft failure, by removing immunogenic cells. On the other hand, however, it may also be removing or altering intrinsic graft cells

which may be helpful in post-transplantation graft repair or maintenance. In fact, it is not clear if allograft cells survive transplantation or if they are important for a grafted tissue to successfully replace host ligaments.

Due to the inadequate attention given to the cell morphological results of these experimental allografts, it remains undetermined whether allografts can ever regain normal ligament cell characteristics (presumed to be essential for optimal graft repair and function). Since transplanting allogeneic tissues potentially involves many immunologic variables, investigation of the cellular characteristics of biological replacements for ligaments has shifted to a more controlled autograft model.

Autografts

An autograft involves the replacement of one tissue with a tissue taken from a different location within the same individual. Similar to the allograft studies noted above, there has been an emphasis on investigating the biomechanical behaviour of autograft ligament replacements and very little study of their cell populations. For example, the biomechanical behaviour of patellar tendon autografts has consistently been shown to be inferior to the anterior cruciate ligament that it has been intended to replace (O'Donoghue et al, 1971; Cabaud et al, 1979; Clancy et al, 1981, 1982; McFarland et al, 1986; Nikolaou et al, 1986; Thorson et al, 1987; Ballock et al., 1989; Butler et al., 1989) for cellular reasons which remain obscure.

The best mechanical recovery of a ligament autograft involves a recent study of a rabbit MCL (Sabiston et al., 1990a) which was simply returned immediately to its original environment and very close to its normal position. The biomechanical quality of the healing response was found to be excellent, possibly since the graft was "fresh" (containing its original population of cells), possibly

because it was placed in an extraarticular environment where extrinsic cells could easily repopulate and repair the graft, or possibly since the graft was tensioned optimally under its normal *in vivo* load. Unfortunately these factors were not addressed specifically in that work.

Despite the fact that no one has ever addressed autograft cell morphology in detail, many investigators have concluded that autografts were "biologically successful" since grafts appeared to have become (or remained) cellular. While most grafts appear to be absent of cells in the first few days after transplantation, many have been said to take on "normal ligament cellular appearances" as early as 30 weeks post-transplantation (Mason and Shearon, 1932; Flynn et al., 1960; Andreeff et al., 1967; Chiroff, 1975; Alm et al., 1976; Clancy et al., 1981; Arnoczky et al., 1982; Puddu and Ippolito, 1983; Amiel et al., 1986a,b; Kleiner et al., 1986; McFarland et al., 1986; Nikolaou et al., 1986; Ballock et al., 1989; Kleiner et al., 1989). When these observations are studied more critically, however, it is clear that "normal ligament cell appearances" have never been quantified and that autograft cell repopulation has at best, been studied subjectively. From these studies, it is obvious that researchers have understood the importance of having a viable cell population in healing connective tissue grafts, since the cells are responsible for the repair and maintenance of the tissue matrix. Yet, the qualitative observations of grafts have failed to determine what the source of the cells is in graft tissues (cells intrinsic to the graft or invading, extrinsic cells) and whether the graft cells are functioning in a manner which is beneficial for healing. A clearer understanding must be obtained on how variables involved in transplantation, such as graft handling and graft loading environments, could affect the original graft cell population, thus implicating the role of cells in determining the outcome of grafts.

Regardless of the type of connective tissue being transplanted, therefore, the first question of importance is: Do any intrinsic cells survive the transplantation of a graft? Secondly, do extrinsic cells repopulate grafts, particularly if intrinsic cells have been damaged or removed by freezing? Evidence from the literature is inconclusive regarding these questions. Some groups have proposed that transplanted tissue "remains alive" (Mason and Shearon, 1932), while other investigators have held the contrary belief that grafted tissue does not maintain original, viable cells after transplantation and that they must be populated with other cells from the recipient (Andreeff et al., 1967; Kleiner et al., 1986). The methods by which these replacement cells are described as extrinsic, however, are not totally convincing. Kleiner et al. (1986) for example, did not analyze thoroughly or quantitatively the cellularity in the frozen-thawed tendon graft prior to implantation to establish the initial cellular state of the graft. As well, their studies did not define the cellularity of normal tendon in order to serve as a control for the analysis of the frozen-thawed tendons. Further, while they showed that freeze-thawing of tendon grafts renders the grafts acellular *in vitro* after 21 days in culture, other investigators have been able to culture cells from tendon immediately following even multiple freeze-thawing (Jackson et al., 1989; Bush-Joseph et al., 1990). Such discrepancies imply that it is not known if frozen-thawed grafts are initially acellular or, in fact, have surviving cells which may contribute to graft healing. This is the first deficiency addressed in this thesis.

A second factor which could alter cell contents of ligament autografts could be due to differences in the external load placed on the connective tissue grafts. This speculation, that graft tissues respond to tension, is supported by extensive evidence suggesting that tissues respond to loading conditions, such as during exercise and immobilization. Recent transplantation studies (Gomez and Woo, 1989; Bush-Joseph et al., 1990) have suggested that graft positioning could cause

abnormal external loads on the graft during joint motion. If graft tissues respond to this tension, it could be speculated that tension, in turn, could have an affect on the graft cells. Thus, the possibility that graft cell characteristics may be affected by ligament loading is the second deficiency addressed in this thesis.

RATIONALE OF PRESENT STUDY

The preceding review has shown that connective tissue grafts have some potential for replacing ligament functions. I speculated that the best material to use in a ligament reconstruction would be a ligament-bone complex. Such a complex would initially reproduce the original ligament's morphological, biochemical and mechanical properties. By definition, this would require allografting of a ligament complex since there are no "spare ligaments" in the body. Since allografting would involve the potentially devastating effects of immune rejection superimposed on the many other variables of a surgical transplant, I have chosen to instead study the simpler situation of a ligament autograft. In this situation, a bone-ligament-bone complex is simply removed and replaced in its host, modeling the surgical factors which could influence the graft.

From previous studies, I selected two surgical factors which may affect the healing of the autograft. These factors are (i) the importance of maintaining the original cells in a graft at the time of transplantation and (ii) the importance of controlling graft tension at the time of implantation. The first factor was chosen since it is not clear if a tissue harvested for transplantation should be transplanted fresh or may be stored for future use by freezing. Specifically, it is not known if a fresh, cellular graft presumably containing normal ligament cells, will lead to better healing than a graft in which cells are killed or are absent (ie. killed by freezing). The second factor, graft tension at implantation, has been speculated to be important due to extensive evidence that loading of dense connective tissues

can have dramatic effects on tissue mechanical behaviour and on cell metabolism. Since tension is clearly one controllable variable at the time of surgical implantation due to graft position and potential graft preloading, I chose this as the second variable in my study.

This thesis therefore separates the two surgical factors and attempts to define their effects on the cell morphology in ligament autografts. The transplant model selected in this ligament replacement study was the rabbit MCL autograft. The main rationale for selecting MCL autografts was that they can be nearly a "perfect fit" (bone graft) in a "privileged" extraarticular environment, thus potentially representing an "ideal transplant". Additionally, due to its relatively superficial anatomical position, the MCL is probably the simplest ligament structure in which to study transplant variables. The reason that I chose to study cell morphology and cell numbers is simply the fact that ligament cells play an important role in graft healing by continually synthesizing and degrading the ligament matrix structure. Clearly, ligament cells are the mechanism by which homeostasis or change is induced in connective tissues; thus the "normality" of cells is presumably critical to a tissue's maintenance and function.

Specific Aim and Hypotheses

Specifically, the aim of my studies was to quantitatively compare the midsubstance cellular characteristics of totally cellular (fresh) ligaments, with what I initially assumed to be totally acellular (multiply frozen-thawed) adult rabbit ligament-bone autografts, under three standardized tensions at the time of implantation; anatomic (normal tension), loose (no pretension) and tight (excessive pretension). My hypotheses were that for grafts to retain or recover normal ligament cell appearances and numbers, they should contain a full complement of original cells (ie. be fresh) and should have anatomical tension at

the time of implantation. I speculated that all other groups would have abnormal cell numbers and/or cell shapes at the 12 week interval of healing.

In order to study the cell morphology of MCL autografts, it was necessary to first have a quantitative definition of normal MCL cell morphology. No quantitative definition exists for normal ligament fibroblast shape, number or distribution. This baseline fibroblast definition would allow for better quantitative descriptions of the cellular changes in ligament grafts over healing time and may be useful in determining origins of cells at the same time. It may be equally important to correlate certain cell characteristics with specific cell functions. If cell shape is found to be a good indicator of cell identification and function, this information could later be used to define various processes within the entire tissue. Hence, tissues that are healthy and normal could be distinguished from injured or diseased tissues on the basis of the cell shape.

In this thesis, I developed a database of cell morphology of normal and transplanted MCLs with respect to their cell shape, cell distribution, cell number and cell viability. The rationale for selecting each method will be discussed separately.

Cell Shape, Distribution and Number

Cell shapes are used frequently by investigators and pathologists to determine cell types and the integrity of tissues. This concept was applied to my MCL autograft study, where the "normality" of cell shape was compared. Ligament cell shapes were measured by a computerized histomorphometry system that visualized and defined the shape of cells by projection of thin-sectioned and stained MCL. Following the quantification of cell shapes, the same program counted the number of cells present and the distribution of the cells in the ligament collagen matrix.

Cell Number

It is possible that variables involved in the transplantation of connective tissues cause changes in graft function by altering graft cell populations. Changes in graft cell numbers could help to explain the mechanism(s) responsible for specific transplant results, thus cell numbers were quantified in my MCL autografts. In addition to the histomorphometric method for determining cell number, a biochemical assay for DNA was used to determine the cellularity of MCL. DNA concentration was used as an index of ligament cell numbers per unit mass of ligament matrix to determine the relative tissue cellularity.

Cell Viability

The intracellular synthesis of collagen was used to define the viability of ligament cells in normal MCL in order to test the effect of freeze-thawing on MCL viability. For the subsequent MCL autografts, I decided to base all comparisons of the cell populations strictly on morphological information, because viability measures using hydroxyproline synthesis were too variable and qualitative.

The cell parameters of shape, distribution, number and viability were used in the analysis of the cell morphology of rabbit MCL autografts implanted under different graft treatments (fresh and frozen-thawed) and graft tensions (anatomic, loose and tight). In order to test the hypothesis previously described, a sequence of studies was required and the rationale for each study will be discussed separately.

I. A New Baseline of Normal Rabbit MCL Cell Morphology

In order to address the deficiencies identified in the preceding sections, studies were performed first on normal MCLs. A complete analysis of the cell

morphology of transplanted ligaments would be futile without first defining and quantifying the ligament cells in normal MCLs. Therefore, the purpose of studying the normal cellularity and viability of the adult rabbit MCL was to develop a proper baseline for the model.

The study of normal MCL was focused on defining and quantifying the ligament cell populations both biochemically and histologically at "time zero". Time zero is defined as the point in time when one year old NZW rabbits would normally undergo autografting procedures in the autograft series (ie. equivalent to "zero healing time"). In order to develop a sufficient definition of normal MCL "fibroblasts", ligament cells in normal MCLs from control animals were characterized by nuclear shape, number and distribution. Further, I attempted to define the viability of the cells within normal MCLs using one metabolic assay.

II. The Effect of Freeze-Thawing on Autograft Healing

Tendon and fascial allografts are frequently frozen or freeze-dried before implantation to help diminish an immune attack on donor cells. Grafts may be injured by this preservation, yet it has been shown that some graft cells can survive harsh treatment, such as freezing, for several weeks (Frank et al., 1988a). Our laboratory (Sabiston et al., 1990a,b) showed that the freeze-thawing and cryopreservation protocol used allowed some cells to survive. Some cells, however, were also obviously killed. Either the loss of normal cells, or an alteration in cell function caused the grafts to then deteriorate. This would also presuppose that migration of "non-ligament" cells from the host into grafts are incapable of maintaining the ligament complex.

The results of Sabiston's work (Sabiston et al., 1990a,b) formed the basis of my investigation on frozen-thawed MCL autografts where the effects of the initial presence or absence of cells on autograft healing was tested. It was recognized

that to quantify and define the sources and effects of any subsequent cellular changes in normal MCL autograft fibroblasts, ideally cells would be marked in some way to recognize "new" from "old" cells, or alternatively get rid of all cells in grafts at the time of their implantation ("time zero"). Due to the lack of any definitive cell marker, the latter approach was chosen, to attempt to remove cells from grafts by multiple freeze-thawing. It was believed that freeze-thawing would create an acellular, non-viable graft at time zero such that any subsequent cells seen in grafts could clearly be identified as being extrinsic.

The assumption was that frozen-thawed MCL would be a totally acellular tissue (ie. cells would be ruptured by freezing and their debris removed from the matrix during the thaw in saline).

III. The Effect of Tension on Autograft Healing

As noted above, previous studies have speculated that one reason that allografts may have inferior recovery as compared to autografts (Sabiston et al., 1990a,b) is "mechanical". The aim of my autograft tension study was to therefore determine if any association existed between the graft cell parameters outlined earlier and graft tension. In other words, could ligament cell numbers, distributions and shape factors in MCL autografts be influenced by the initial graft tension at implantation?

In order to test whether the tension on MCL autografts at implantation affects the cell content and the outcome of transplant healing, it was interesting to speculate that cell nuclear shape could be associated with the graft tension. In order to assess the effect of tension on cell indices in MCL transplants, it was first necessary to know the effect of tensioning on normal MCL cells at time zero. To define the effect of tension at time zero, various tensions, ranging from "no tension" to "excessive tension", were applied to the normal ligament complex. A

control study therefore was focused on measuring the range of normal fibroblast shape factors in the MCL and the effect of tension on normal ligament cells at time zero and was a necessary control for analyzing cell changes in 12 week autografts. The hypotheses were that there would be quantifiable cellular changes in normal MCL under different tensions and that for optimal MCL autograft outcomes, the grafts would be implanted at an anatomical tension.

CHAPTER 2
METHODS AND MATERIALS

ANIMAL MODEL

The model selected was the medial collateral ligament (MCL) of the stifle (knee) joint in skeletally mature female NZW rabbits. All rabbits were obtained from one supplier (Reimens Fur Ranches, St. Agatha, Ont., CAN.), were 12 months old and whose state of health was strictly controlled (Matyas et al., 1990). All animals were housed in stainless steel cages (450 mm wide x 370 mm high x 610 mm deep), fed standard rabbit chow and had consistent care and supervision.

THE NORMAL RABBIT MEDIAL COLLATERAL LIGAMENT

As background, a brief review of the model, MCL biochemistry and morphology will be given, with some standard definitions of cells that will be used throughout the remainder of this thesis.

The rabbit MCL is composed of cells and matrix (water, collagen, proteoglycans, elastin and other glycoproteins). Water, is by far, the most abundant component comprising roughly two thirds of the total ligament structure. The dry ligament is 80% collagen, less than 1% proteoglycan and the remaining 19% consisting of several non-collagenous proteins, deoxyribonucleic acid (DNA), elastin and minerals (Amiel et al., 1984; Frank et al., 1985). The unique arrangements and interactions of these components gives the ligaments their complex functional properties.

The MCL is designed primarily to resist tensile stress through interactions of the collagen fibers with the non-collagenous components. Different portions of the tissue take up loads at different stress levels, thus the complex exhibits a nonlinear mechanical behaviour. (Akeson et al., 1985; Frank et al., 1985). The

nonlinear mechanical behaviour of the MCL is well suited to its functional role, allowing some joint displacement to occur while providing increased resistance to larger tensile loads.

MCL Morphology

MCLs are often described as "cord-like" due to their dense, white, hypovascular, homogeneous appearance. They are easily distinguished from surrounding tissues as distinct collagenous structures stretched between two bony attachments. On gross inspection, ligaments are surrounded by a loose, vascular "epiligamentous" tissue. (Arnoczky, 1983). The fibrous appearance of the MCL is produced from the parallel collagen fibres which extend between the two ligament insertions into bone. It is the undulating collagen fibres which give the MCL a longitudinal wavy appearance ("crimp") under polarized light (Frank et al., 1988c). A closer look at ligament substance reveals that MCLs actually possess a modest blood supply. Associated with these sparse intraligamentous vessels are nerve endings which enter the ligament from the superficial layers (Bray and Fisher, 1988).

Ligament Cells

Standard histological staining of ligaments with hematoxylin and eosin allows the pink-staining ligament matrix to be distinguished from the blue-staining nuclei of ligament cells. Resolution of the cell cytoplasm at this level is poor since the cytoplasm stains pink making the cytoplasm indistinguishable from the collagen matrix. For this reason, researchers have consistently described all ligament cells in the matrix solely by the shape of their nucleus. Endothelial cells are identified when nuclei are seen adjacent to blood vessel lumina or when nuclei are connected end to end forming a steady stream of cells in a ligament.

Blue-staining ligament cells are classified as fibroblasts when their nuclei are located between collagen bundles away from any vessel lumina and have a "cigar-shape" or ovoid appearance. Fibroblasts are almost always oriented along the long axis of the ligament and are scattered at infrequent intervals, either individually or in groups. (Amiel et al., 1984; Gamble et al., 1984; Akeson et al., 1985; Frank et al., 1985, 1988c). The main body of fibroblasts, as seen in electron micrographs, have thin, lamellar cytoplasmic extensions that spread through the collagen fibrils over great distances and are interconnected by junctions between cell processes (Bray et al., 1990b).

"Fibroblasts"

It is believed that a population of ligament cells is responsible for the maintenance of the collagen matrix of the MCL. These cells have been classified as one cell type and are known as "fibroblasts". Historically, the generic name "fibroblast" has been assigned to any cell within the MCL that has an "ovoid" shape, but the exact definition of ovoid has been very subjective. For clarity in this thesis, the term "fibroblasts" will be used for cells which have a defined ovoid nuclear shape (which will be defined histomorphometrically) within the ligament matrix. The term "ligament cells" will be used to describe the entire population of cells found in and around the MCL including these fibroblasts and endothelial cells.

METHODS OF ANALYSIS

MCL Dissection and Sampling

In order to analyze and compare within and between the ligaments, a strict protocol was developed for dissection, tissue preparation and sampling of the MCL. Euthanasia was performed on the animals at the assigned time interval by a

lethal injection (Euthanyl, MTC Pharmaceuticals, Cambridge, Ont., CAN.) into the posterior ear vein. Under sterile conditions, the stifle joints were disarticulated from the hips and splinted at 70 degrees of flexion. Anteromedial incisions through skin and fascial layers exposed the MCL. Particular attention was given to thorough removal of the very cellular ligament superficial layer ("epiligament") and disruption of the fibrous tissue connection between the MCL and the medial meniscus. All musculature and loose tissue were cleared from the stifle joint to expose both insertion sites of the MCL (Figure 2).

A small rotary saw was then used to remove the ligament insertions reproducibly as rectangles of bone from both the tibia and femur, without disrupting the ligament. Sterile saline was used liberally during dissection to avoid tissue drying.

The midsubstance MCL cell characteristics under investigation were divided into three categories; cell number, cell distribution and shape, and cell viability. Appropriate methods were selected to define each cell category and will be described separately.

1. Cell Numbers

In order to attempt to determine the cellularity in MCL, two different quantitative methods were used. One method involved a biochemical assay for determining DNA concentrations and the other a histomorphometric method relying on image analysis to determine the number of cell nuclei present. Each method will be discussed separately below.

<u>DNA Concentrations</u>

Ligament DNA concentrations were measured in accordance with a modified procedure of Bonting and Jones (1957). The advantage of this method is

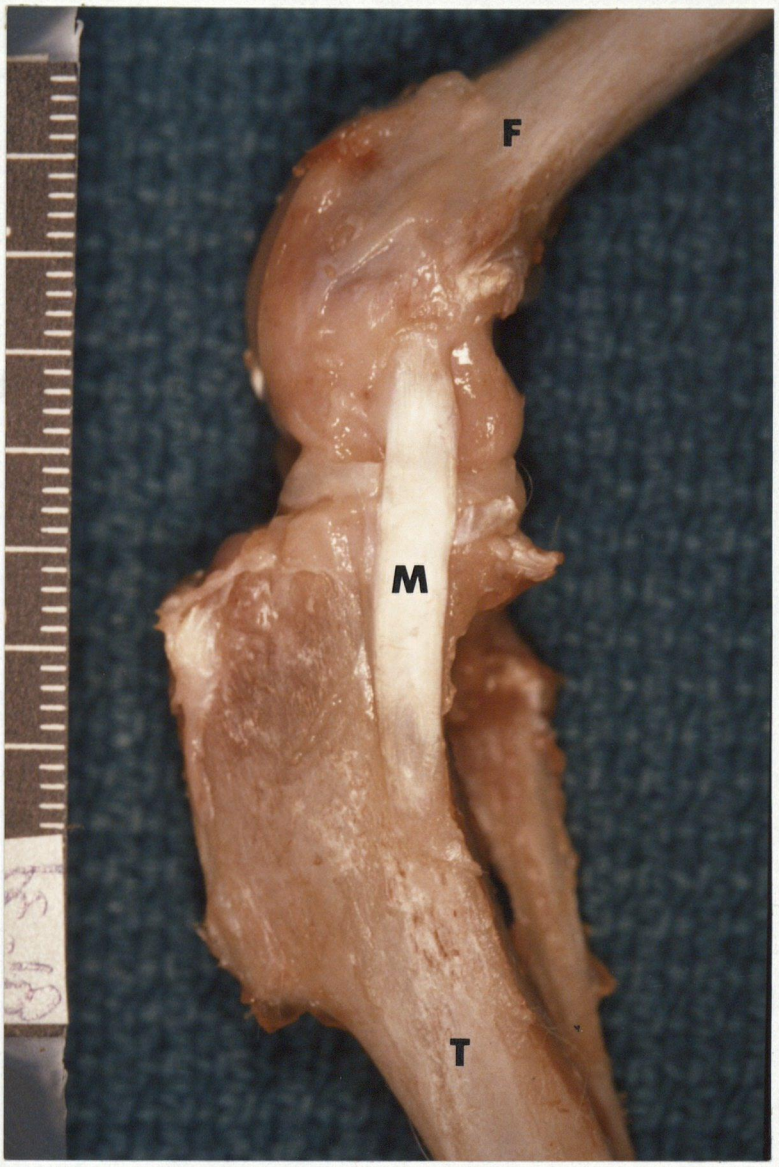

Figure 2 Medial view of a stifle joint from a one year old NZW rabbit. (F) Femur, (T) Tibia, (M) MCL

that it allows determination of DNA in very small quantities of tissue, leaving enough material for other studies. The ultramicro method consisted of an indole reaction with DNA to produce a colored product measured at 490 nm. The MCLs were weighed wet, lyophilized for 48 hours and weighed in duplicate when dry. After each sample was homogenized in 1 Normal NaOH, one ml of each filtered supernatant was placed in a conical centrifuge tube and had 1 ml of indole-HCL reagent (indole: Fisher #I-17) added. After 10 minutes of incubation at 100°C, the tubes were cooled and had 5 ml of chloroform added, vortexed, spun, and extracted. The chloroform acted to remove any insoluble, interfering substances. After a second chloroform extraction, the water phase was read spectrophotometrically (Beckman, DU8). DNA standards (Sigma type I - DNA sodium salt) and blanks (1 Normal NaOH) were processed with the MCL samples. Results were expressed in micrograms of DNA per milligram of dry tissue for each ligament.

Number of Nuclei

To calculate the number of nuclei in midsubstance MCL, the tissue cellularity was quantified by computerized histomorphometry. Visualization and automated counting of cell nuclei was possible with projection of stained thin sections of MCL under the light microscope.

Histological Process

After euthanasia and during ligament dissection, the MCLs were drip fixed *in situ* with 10% neutral buffered formalin. Following a fixation period of several minutes, the attachment between the MCL and the medial meniscus was severed. Each MCL was cut transversely with a scalpel blade, 3 mm proximal to the medial meniscus attachment site and 3 mm proximal to the ligament tibial insertion, producing approximately a 7 mm long sample. The dissected sample was termed

"midsubstance" and was further processed for eventual histomorphometric analysis (Figure 3).

The midsubstance samples were fixed overnight in 10% neutral buffered formalin at 4°C. This was followed by dehydration in a graded series of ethanol (70%, 95% and 100%), infiltration of catalyzed JB4 and embedded in JB4 resin with the medial MCL surface orientated so that it was first to be cut. Parasagittal sections were cut on a microtome at a 3 micron thickness, sequentially from the medial surface to the deeper MCL substance. The plastic sections were stretched in a distilled water-bath and adhered to glass slides. Representative sections were stained with acidified Gill's hematoxylin and acid-precipitated eosin, cover-slipped and allowed to dry.

Presently, there is no reported method for specifically staining the cytoplasm in fibroblasts within the collagen matrix, thus making the cell nucleus the only source of cellular information with light microscopy. Sections were stained with standard hematoxylin and eosin to produce the best contrast between ligament cells and matrix. Hematoxylin has a high affinity for cell nucleus, producing excellent resolution of the nuclear shape under the light microscope, allowing its quantitative image analysis.

Image Analysis

Before detailing the image analysis process for the midsubstance MCL, a preliminary study will be described. This preliminary study was performed with image processing of normal MCL midsubstance to determine how many frames should be analyzed in order to achieve a representative sample of the nucleus parameter studied. The parameter arbitrarily selected was a measure of nuclear roundness, which will be discussed in detail later in this chapter. An accurate and reproducible nuclear roundness measurement was obtained for normal fibroblasts after approximately 16 frames (each frame was 125 x 125 microns) were analyzed

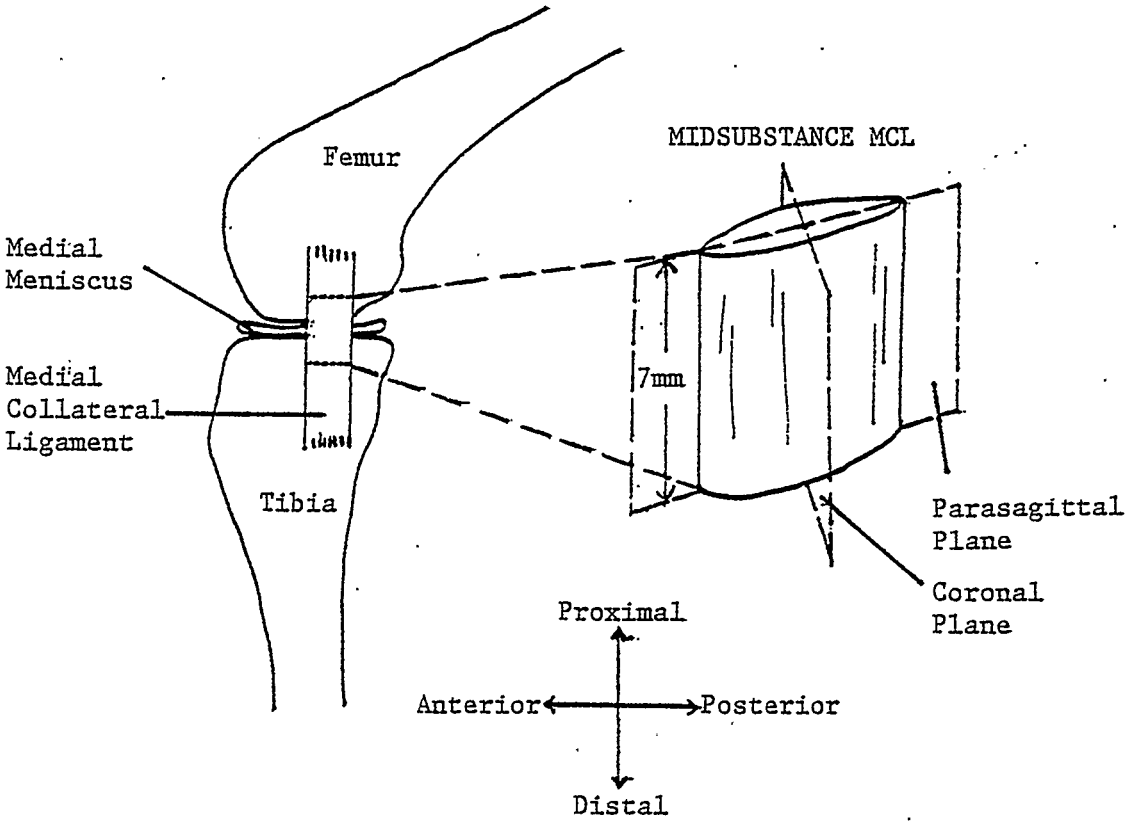

Figure 3 Drawing of the medial aspect of the rabbit knee joint and dissection of the MCL midsubstance sample. Note that 3 micron sections were cut in the parasagittal plane for all histomorphometry and autoradiography and in the coronal plane for the Tension Controls. Note the drawing is not to scale.

per ligament, or the equivalent of 50 nuclei. To detect a difference in nuclear roundness of 0.2 (on a scale 0.0 to 1.0), sample size estimations determined that 4 frames must be analyzed, whereas, to detect a difference of 0.1, 16 frames must be analyzed.

From the above preliminary study, it was decided that for an accurate measure of normal nuclei, 16 frames per ligament must be processed. For the histomorphometry studies on midsubstance MCL, it was determined that one section from each MCL should be selected for analysis that was 150 microns into the ligament substance. Therefore, all nuclear sampling was reproducibly chosen from the interior midsubstance of the MCL, eliminating the possibility of sampling a surface "epiligament" cell population. One stained section for each ligament was coded and image processed by one investigator in a blinded and randomized fashion for nuclear image processing.

The computerized histomorphometry system consisted of a video camera mounted on a microscope (Leitz Orthoplan), a frame-grabber image processor (Oculus 300, Coreco Inc.) and image analysis software (Image Measure IP2500IP and IM5100IP, Microscience Inc.). Predetermined image analysis software settings were selected to specify the desired light intensity threshold and edge detection criteria. Following the calibration of the microscope and camera lucida using a stage micrometer, the video image from the microscope was calibrated before being captured by the image processor. After calibration, a low power camera lucida drawing, using the 2.5X objective, was made of each section. The low power drawing allowed selection of a starting point for analysis. This ensured that only pure midsubstance was sampled and no ligament edges were included. The objective was then switched to 40X since it gave better nuclear detail. All cell nuclei were measured by the image program with the 40X objective, yielding a final magnification of 1040X.

The first 125 by 125 micron image frame was captured and converted into a camera image consisting of 250 levels of grey. The image measure program scanned the television monitor detecting objects at certain specified intensities. Since computerized image processing did not have the ability to distinguish automatically and delete analysis of any non-fibroblast nuclei, there was a need for manual operation. Whenever cell nuclei were lining what was clearly a blood vessel lumina, they were identified as endothelial cells and were excluded from study. In addition, artefact-laden tissue areas (ie. tears, folds, gaps), most likely the result of the histological process, were not scanned. Thus, the software allowed for human intervention whenever an object other than a nucleus (ie. artefact) was detected or when nuclei were omitted because they were slightly under threshold value. These desirable (fibroblasts) or undesirable (non-fibroblasts) objects could then be included or deleted from the frame analysis.

After scanning the frame, the number of cell nuclei detected in the specified area of the midsubstance matrix was recorded. A drawing of each analyzed frame was made, making it possible to coordinate the movement of the microscope so that continuous frames were scanned and preventing nuclei from being counted twice. A total of 16 frames were scanned, moving from the distal end of the midsubstance to the proximal end, resulting in analysis of a total area of 250,000 microns^2.

2. Cell Distribution and Shape

Midsubstance MCL cell shapes and cell distributions were defined by quantifying the full range of ligament cell nuclear shapes and their distribution in the midsubstance collagen matrix. The cell nuclei shapes and distributions were measured by the computerized histomorphometry system previously described for cell number analysis.

The following measures were taken by the image measure program for midsubstance MCL cell populations:

Minimum Distance - is a measure of the cell nuclei distribution within the midsubstance matrix. The image measure program took numerous measures of one nuclear centroid and its distance to all other nuclei centroids in the frame being scanned. The image measure program then selected for each cell nucleus the closest distance to another cell nucleus. The minimum distance measure is the average of all the smallest distances between nuclei. The minimum distance measure thus gives a relative value for the cellular density in the midsubstance MCL area sampled.

Roundness - is a measure for the shape of cell nuclei. The nuclear shape was described using a roundness coefficient defined by the equation: 4 pi * nuclear area / nuclear perimeter^2. This shape parameter, based on the ratio between area and perimeter, describes perfectly round shapes as 1.0 and any shape becoming more ovoid as less than 1.0.

Maximum Diameter - is a measure of the long axis length of the nucleus.

Minimum Diameter - is a measurement taken halfway along and perpendicular to the maximum diameter of the nucleus. Both the maximum and minimum diameters give information on the relative size of the cell nucleus.

3. Cell Viability

Two methods were used to determine a measure of viability of ligament cells in the study on freeze-thawing effects on MCL cellularity. First, a metabolic assay for collagen synthesis was used to determine the amount of new collagen synthesized per unit mass of matrix and second, autoradiography was used to localize metabolically active ligament cells in ligament midsubstance.

Collagen Synthesis

An intact and viable fibroblast is necessary for the synthesis and degradation of the ligament collagen matrix. The amino acid, hydroxyproline, is a metabolic product of the complex process of hydroxylation of peptide bound proline (Stetton, 1949). Uptake and conversion of tritiated proline to hydroxyproline in the MCL is evidence of metabolically active cells. Hydroxyproline was used to assess one metabolic index of ligament cell viability with an *in vitro* assay for collagen synthesis, previously described by Frank et al. (1988a). Bone-MCL-bone complexes were removed immediately following euthanasia and subjected to an *in vitro* cold-proline incubation procedure for 7 hours at 37°C in a shaker water-bath. Ligament complexes were immersed in 2.5 ml Basal Eagle culture medium with Earles salts and L-glutamine (Gibco #320-1015) containing 125 U penicillin, 125 ug streptomycin (Gibco #600-5140), 125 ug ascorbate (Sigma #D-1819) and 21 ug proline (Sigma). The purpose of an initial cold-proline incubation was to form a baseline *of in vitro* fibroblast synthetic activity in the MCL. Cold-proline incubation was followed by a 5 minute wash with 30 ml distilled H_2O at 4°C. MCL complexes were then immersed in vials containing the media described earlier, but cold-proline was replaced with 50 uCi ^{3}H-proline (New England Nuclear # NET-323). Basal Medium Eagle was chosen because it contained neither proline nor hydroxyproline (both interfering substances in the assay), while still supplying all other essential amino acids to the MCLs. After a 20 hour incubation at 37°C, media were discarded and the MCL complexes underwent a 4 hour cold wash that was efficient enough to remove all unincorporated isotope without extracting collagen (Frank et al., 1988a).

Following the wash, the ligaments were dissected from their bony insertions. Ligaments were weighed wet, lyophilized for 48 hours and weighed twice when dry. The MCLs were hydrolyzed in sealed ampules with 2.5 ml 6

Normal HCL for 16 hours at 100°C. A one ml aliquot of each MCL hydrolysate was run on an ion exchange chromatography column to separate the proline and hydroxyproline peaks. All collected fractions from the column were counted on a liquid scintillation counter for 1 minute. A separate 100 ul aliquot of the MCL hydrolysates was used to determine the total hydroxyproline content of the ligaments (original, cold and new, radioactive hydroxyproline) by the automated method of Blumenkrantz and Asboe-Hansen (1974). The metabolic activity for each MCL was expressed as disintegrations per minute (DPM) ^{3}H-proline/ug hydroxyproline.

Autoradiography

Autoradiographs were processed to localize the metabolically active cells in the ligament midsubstance. MCLs were removed and incubated in ^{3}H-proline as previously described. After the washing procedure, each MCL was cut transversely with a scalpel blade, 3 mm proximal to the medial meniscus attachment site and 3 mm proximal to the ligament tibial attachment. The dissected sample of MCL midsubstance was then processed for autoradiography.

The midsubstance samples were fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol (70%, 95% and 100%), infiltrated in catalyzed JB4 and embedded in JB4 resin (Polysciences, Inc., Analychem Corp., Markham, Ont., CAN.). Sample processing all took place in a 4°C cold room. The radiolabelled ligaments were cut parasagittally (parallel to the long axis of the MCL) at a 3 micron thickness, stretched in a water-bath and dried on slides at approximately 50°C. Slides were dipped in a photographic emulsion containing silver bromide crystals (Kodak Emulsion) at 37°C in the dark. Dry emulsion-coated slides were stored in sealed boxes at 4°C for 2 weeks. It was during this time interval that the emulsion was activated by radiation originating from the

decay of radioactive material in the ligament sections. The activated crystals became the sites for deposition of silver, thus locating the radioactive label in the sections. After 2 weeks, the emulsion was developed (Kodak D-19) and fixed (Kodak Fixer) and stained with hematoxylin and eosin. Negative and positive control slides were processed at the same time. Estimates for the number of metabolically active cells present in the MCL were performed by observation of sections under the light microscope.

Table 1: Number of MCLs used for each method of analysis

Group	DNA Assay	Histomor.	Collagen Assay	Autorad.
A. Normal MCLs	6	6	1	2
B. Frozen-Thawed Control MCLs	3	2	3	2
C. Tension Control MCLs	--	8 rabbits (16 MCLs)^{T}	--	--
D. Autograft MCLs	--	18 rabbits (36 MCLs)*	--	--

^{T}, * = right MCLs are experimentals, left MCLs are contralateral controls

THE FOUR MODELS OF STUDY

Four studies were carried out which were designed to define normal rabbit MCL cellularity (shape and number), to determine how multiple freeze-thawing affects these cell indices along with cell viability in otherwise normal MCLs, to

determine how tension affects cell indices in normal MCLs and finally, what cell shapes and cell numbers are found in the midsubstance of MCLs which had been autografted 12 weeks previously under different conditions of tension and different conditions of viability (ie. fresh versus multiply frozen-thawed). My goal in these studies was to attempt to define how cell indices may be altered in the midsubstance of an MCL autograft under conditions which may be controlled at the time of surgery. More specifically, I wanted to define how the variables of graft tension and graft freeze-thawing would affect the numbers and the "normality" of cells in the midsubstance of MCL autografts at equal points in the healing process. Further, I speculated that multiple freeze-thawing may eliminate all viable cells from an autograft at the time of its implantation, such that any cells seen in its substance subsequently could be identified as extrinsic. Alternatively, if all cells were not removed from these grafts initially, I suspected that intrinsic cells may have their shape altered in such a way that they could be identified and again separated from extrinsic cells during the healing process. Collectively, I therefore wanted to identify the source of cells and their "normality" (relative to normal MCL midsubstance cells) in specific MCL autografts during their healing.

A. NORMAL MCLs

To obtain a better definition of cells in normal rabbit MCL, the ligament cell population was defined and quantified both biochemically and histologically at time zero. The cell characteristics of 15 normal midsubstance MCLs were assessed by the three categories; cell number, cell distribution and shape, and cell viability.

B. REMOVAL OF VIABLE MCL CELLS BY FREEZE-THAWING

The development of frozen-thawed controls was necessary in order to assess the effects of multiple freeze-thaw on normal ligament cells at "time zero" to

determine if cells could be effectively removed by freeze-thawing, or if their cell shapes, numbers or distributions would be changed in some way that would allow them to be characterized as being intrinsic in the subsequent model of autografting. Thus, information was obtained on the cellular characteristics of both the fresh and frozen-thawed MCL autografts at the time of implantation.

Ten MCL-bone complexes to be frozen-thawed were handled consistently. Immediately after dissection, the MCL-bone complex was placed in a small container of liquid nitrogen (-196°C), followed by a thaw in warm saline (37°C). The duration of each procedure was 1 minute and the entire freeze-thaw sequence was repeated five times. This harsh freezing protocol was used since previous study has shown some ligament cells to be able to survive a single episode of freezing at -70°C for several weeks (Frank et al., 1988a).

Multiple freeze-thaw was measured by its effect on the cell number, cell distribution and shape and cell viability as compared to normal (fresh) MCLs.

C. THE EFFECT OF TENSION ON MCL CELLS

In order to assess the effect of tension on the normal ligament cells at time zero, cell characteristics were measured while under ligament loads ranging from "no load" to "excessive load". As with the freeze-thaw experiment noted above, I wanted to identify how cell numbers and shapes may be changed by changing tensions on a fresh MCL - potentially allowing cells to be identified subsequently in an autograft and potentially identifying a mechanism by which cell behaviours in a graft may be altered. Quantification of the cell characteristics were made through computerized histomorphometry for cell number, cell distribution and shape.

Eight normal adult rabbits were used for this tension control study, with all the right hindlimbs classified as experimentals and all lefts as contralateral

controls. After euthanasia, each hindlimb was disarticulated at the hip joint and all musculature and joint capsule was removed, ensuring that the collateral ligaments, the cruciate ligaments, and both menisci remained intact. The tibia and femur were removed, leaving approximately 4 to 5 cm of long bone on either side of the stifle joint.

All eight contralateral controls, were securely splinted at 70 degrees of flexion and fixed overnight in 10% neutral buffered formalin. Of the eight experimental joints, four subgroups were identified. In group 1, two stifle joints were fixed overnight in 10% neutral buffered formalin in an Instron (materials testing machine) at -2 mm extension (no tension/Loose). Group 2, 3, and 4 each consisted of two stifle joints Instron-fixed at 0 mm extension (0% strain/Anatomic), 0.7 mm extension (approximately 3% strain), and 1.4 mm extension (approximately 6% strain/Tight), respectively.

All eight experimental stifle joints were secured into a materials testing machine (Model 1122, Instron Corp., Canton, MA, U.S.A.) with a knee flexion angle at approximately 70 degrees. A starting position known as "ligament zero" was determined for each joint (Lam, 1988). At this point, each joint was set at its designated experimental tension (ranging from no tension to excessive tension) and the environmental tank surrounding the clamping system was filled with 10% neutral buffered formalin. After fixation overnight (approximately 16 hours), the tank was drained and with the stifle joint still clamped, the MCL was dissected out. The left contralateral controls were fixed overnight and the midsubstance samples were procured similarly as the rights.

D. MCL AUTOGRAFTS

The autograft study was designed to determine the importance of both maintaining the original cells in a graft and controlling graft tension at the time of

implantation. Since these two factors are potentially controllable and may grossly alter graft outcomes, my autograft study was designed to determine the importance of these two factors on the cell number, cell distribution and cell shape in MCL autografts (as compared with normal MCLs and control MCLs) by computerized histomorphometry. The decision to use only histomorphometry was made because DNA measures could not be used to quantify cellularity since it was discovered in pilot experiments that normal quantities of DNA were still present in the frozen-thawed MCL. My original speculation that freezing would disrupt cells and that washing during thawing would remove cell contents (including DNA) from the matrix was therefore not realized. Therefore, no biochemical determination of DNA concentration was performed on the MCL autografts.

Transplantation Model

A total of 18 right MCL complexes were divided equally into two series of autograft transplants. One group was designated the "Fresh Autos" since the grafts were implanted fresh immediately following harvest. These grafts were thus considered "as viable as possible", containing original bone and ligament cells which were as undamaged as any surgical procedure would allow. The second series was called the "Frozen-Thawed Autos", because grafts were multiply frozen-thawed (by a procedure which will be described) prior to implantation. Both the Fresh and Frozen-Thawed Autos were then divided equally into three groups of autografts implanted under different tensions. The three standardized tensions were; Anatomic, Loose and Tight. After a post-transplantation interval of 12 weeks, all animals were euthanized for study. The 12 week interval was selected based on previous finding that 12 weeks is the earliest graft healing interval that displays good bone healing (Sabiston et al., 1990a,b). The left MCL complex of all animals remained unoperated and served as contralateral controls.

Surgical Procedure

All animals undergoing surgery were sedated with Atravet (Ayerst Laboratories) and received general anesthesia (halothane 1% and oxygen 1 litre/minute). The right hindlimb was shaved and prepared with iodine. The lower limbs of the animal were stabilized at a fixed position of 70 degrees of flexion. An anteromedial incision through skin and fascia exposed the MCL. All musculature and loose tissue was cleared from the immediate area of the stifle joint. A 1.2 mm drill was used to drill two holes through the cortices of the femur and tibia; one hole approximately 4 mm proximal to the femoral insertion and the other, 4 mm distal to the tibial insertion of the MCL. Two additional holes were made on the tibial shaft approximately 4 mm and 30 mm distal to the first tibial drill hole, respectively. These two holes allowed a calibrated outrigger to be temporarily fixed to the medial tibial cortex to enable graft tension to be controlled precisely ($\pm$ 0.01 N) (Figure 4).

A small rotary saw (Dremel, Div. of Emerson Electric Co.) was then used to cut a three-quarter rectangle of bone around the tibial insertion, not disrupting the most proximal edge of bone nearest the joint surfaces. A scalpel cut was made distal to the ligament tibial insertion which was to serve as a marker for the original position of the bone graft. With the calibrated outrigger secured into the medial tibial cortex, retractors gently displaced the MCL and subligamentous cuts were performed using a microrouter tip to release the MCL tibial insertion complex. At this time, a reading was taken of the amount of force on the ligament *in situ* at 70 degrees flexion. Sharp dissection was used to separate the MCL from the underlying medial meniscus and a second force measurement was taken. The second quantified force was defined as the normal *in situ* force on the MCL. Both *in situ* force measurements were taken when the scalpel markers were lined up on the bone graft and the graft bed. The outrigger was then removed and the femoral

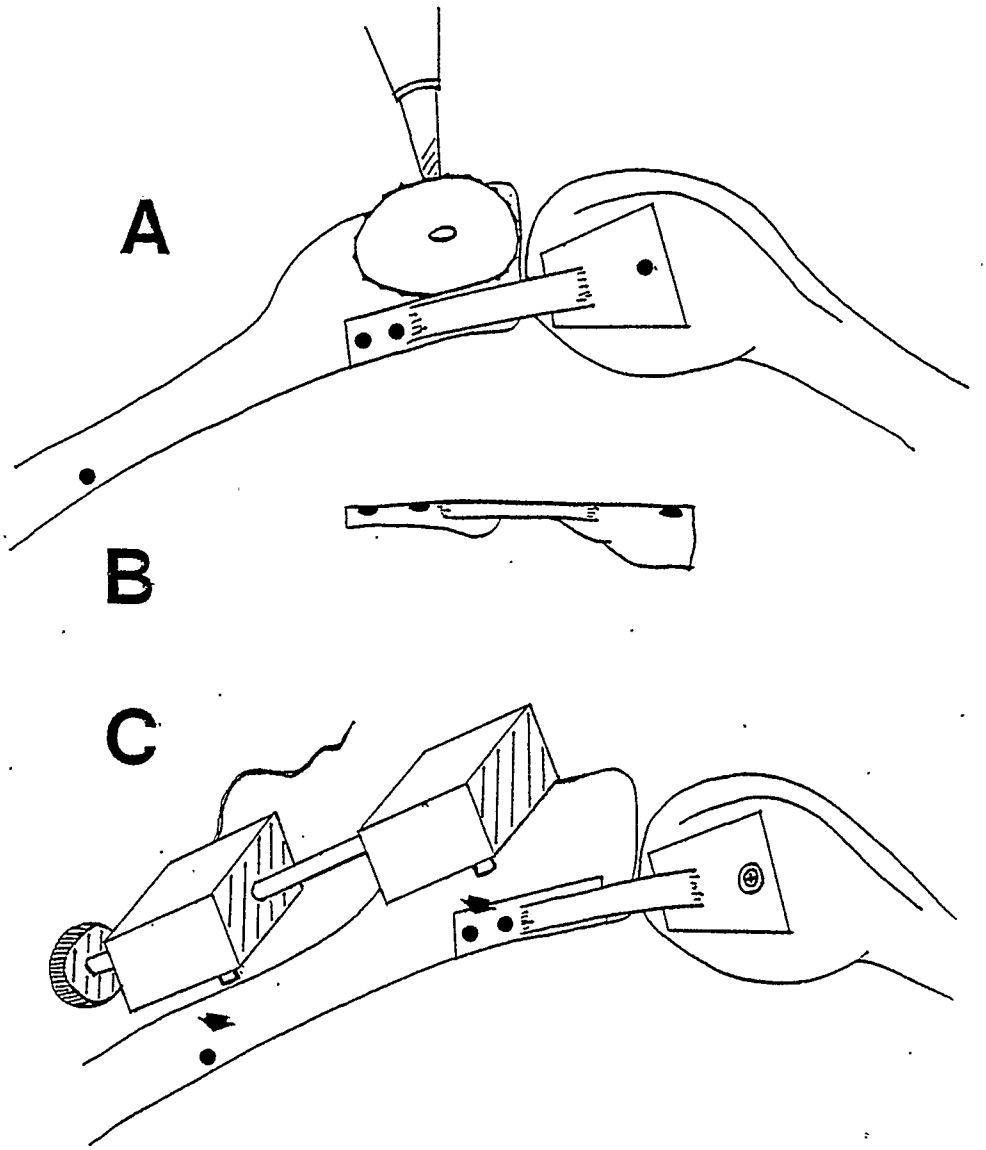

Figure 4 Schematic drawing of autograft medial collateral ligament transplant procedure: (A) removal of bone-MCL-bone graft with a circular saw and drilled screw holes; (B) free MCL graft; (C) reinserted graft to original graft bed with calibrated outrigger attached to control MCL graft tension.

insertion was cut out, while not disturbing the ligament substance. With the use of a drill, all graft corners were rounded to minimize the risk of fractures.

Grafts assigned to the Fresh Autos group were rinsed in sterile phosphate buffered saline (PBS) and immediately re-implanted to their original graft beds. Grafts assigned to the Frozen-Thawed Autos were placed in liquid nitrogen (-196°C), followed by a thaw in warm sterile PBS (37°C). The duration of each procedure was 1 minute and the entire freeze-thaw sequence was repeated five times. Immediately following the multiple freeze-thaw, the MCL grafts were re-implanted into their original beds.

All grafts had their femoral bony insertions fixed with a 2.0 mm cortical screw and a small washer, as close as possible to their original location. The outrigger was reattached to both the graft tibial insertion and the distal tibial shaft. The graft complex was then loaded to the *in situ* force measured when harvested. At this point, all grafts assigned to the Anatomic tension group had their tibial boney insertions fixed with a 2.0 mm cortical screw (Figure 5). Grafts to be fixed at a Loose tension (no pretension) had their tibial bone block displaced 2 mm proximally in the graft bed, closer to the joint surface. Grafts to be fixed "too Tight" had an additional 12 N force added to the *in situ* force and were allowed to stress-relax for 20 minutes. After a constant force level was obtained from stress-relaxation in these Tight grafts, they were fixed in place. Anatomic grafts were simply put back in under the same loading conditions as defined at their removal. Sterile PBS was used liberally during surgery to prevent drying of tissues.

The outrigger was removed to allow wound closure. Fascial layers were closed using interrupted 6-0 nylon suture and 4-0 running suture (Ethilon) was used to close skin wounds. The animals were returned to their cages and allowed to move their joint freely with unrestricted cage activity. Frequent post-surgery examination of stifle joints allowed maintenance of operated leg and wounds for a

Figure 5 X-ray of medial view of an MCL autograft one day postoperative. Note the two outrigger drill holes on the tibial shaft.
(F) Femur, (T) Tibia

good recovery. Both pre-surgical and pre-euthanasia body weights were also taken as a measure of animal health.

Contralateral Left Control MCLs

The 18 unoperated left legs could not be described as normal controls, due to the possibility of secondary effects (such as a change in cell behaviour) resulting from the operated right leg. The left MCL complexes were therefore defined as contralateral controls.

STATISTICAL METHODS

The six normal MCLs analyzed by histomorphometry were compared for each cell parameter measured; number of nuclei, minimum distance and roundness. The right and left ligament samples of each animal were related and termed "paired". The appropriate test to compare means and variances of two related samples was the paired T-test. The null hypothesis was that the mean difference between paired samples was equal to zero. The intra-animal variability was also determined from the normal controls.

The normal and frozen-thawed MCLs were compared using the Student's T-test, which compares two group means for one factor, for DNA concentration, number of nuclei, minimum distance and roundness.

For the study of tension effects, a one-way analysis of variance (ANOVA) was performed to compare the means for each cell parameter measured for the four tension groups, and then multiple comparisons (Tukey's test) identified which group means were different, if any.

For the study of autografts, I first wanted to determine if all contralateral left control MCLs were similar enough to be pooled or if they must be considered

as separate groups. Stated another way, I wanted to determine if there an effect on the left leg due to surgery on the right leg. One-way ANOVA compared all the independent means of the left MCLs for a single factor with the null hypothesis that the means were equal. If there was no evidence of a difference between the contralateral left controls and the normal controls, the means in each control group were averaged together. Since the two control groups were independent and had equal variances, the two-sample T-test with equal variances was used to compare the means and variances of the two samples.

To determine if all the experimental right MCLs (pooled fresh and frozen-thawed autografts) were the same and if they were similar to their contralateral left control MCLs, the data were stratified by three categorical variables; graft treatment, graft tension, and leg. It must be made clear that all variables in the autograft model were used in the design of the statistical analysis. Instead of using a three-way ANOVA, a two-way ANOVA was used, where both the right and left MCLs had two-way ANOVAs performed separately and then tested together for the effect of leg. A two-way ANOVA compares the effects of each variable on the means after controlling for the effects of the other variable. The right and left MCLs were classified for the two-way ANOVAs by two variables; the effect of graft treatment and the effect of graft tension. The right and left MCLs were then brought together and tested for the effect of leg. The justification for separating the right and left MCLs for testing the effects of surgery was to ensure a correct error term was used in testing the right MCL, since the left MCLs never actually underwent surgery.

All comparisons (contrasts) arranged in the two-way ANOVAs were pre-planned. The initial contrast examined the effect of tension by testing the means between grafts implanted at an Anatomic tension versus a Loose tension. If there was no evidence of a difference (similarly, no evidence against averaging), then the

averages of the two tension groups were pooled and tested against the Tight tension group. The basis for these pre-planned comparisons stemmed from the preliminary image analysis study on Tension Time Zero Controls. This study found only the 1.4 mm extension, the tightest tension, to have any effect on changing the normal cell nuclear shape. That conclusion allowed the *a priori* hypothesis in the autograft study to state: of the three different tension groups, the Anatomic and Loose tensions would be most similar.

Statistical significance for all tests was determined at an alpha level of 0.05. All analyses were performed using the Minitab computer package (Minitab, version 5.1).

CHAPTER 3
RESULTS AND DISCUSSION

In this chapter, results will be presented for each of the studies presented previously and each will be followed by a brief discussion, focused on the specific interpretation and significance of those results to the overall goal. An overall discussion of all four studies will follow as a separate chapter.

A. NORMAL MCLs

The results presented in this section are from the analyses of Normal MCLs. All results presented in the text, table and graphically will be in the form of means with standard errors. Statistically significant data will be represented by symbols accompanied by an explanation.

Gross Observations

All normal MCLs had a similar gross appearance after dissection from the normal adult rabbit stifle joint. The band-like structures were dense, white, glistening and had an overall homogeneous appearance. On closer inspection, the ligaments tapered from the midsubstance to form the long, flat tibial insertion into bone. The proximal end of the MCL appeared slightly thicker, ending by an abrupt femoral insertion.

Cell Number

DNA Concentrations

The cellularity of normal MCL was determined by measuring DNA concentrations in six normal MCLs. It was determined that normal MCL had an average 2.27 $\pm$ 0.14 ug DNA / mg dry weight (Table 2).

Number of Nuclei

The histomorphometry results for the six normal control MCLs showed that the ligament midsubstance had an average of 59 $\pm$ 8 nuclei per standardized area analyzed (250 000 microns^2). The cell populations of the six normal MCLs ranged from 43 to 88 nuclei. No statistical differences were found within the three pairs of normal control MCLs ($p > 0.05$) (Table 2).

Cell Distribution and Shape

Minimum Distance

The average minimum distance found between cell nuclei located in the parasagittal plane of midsubstance normal control MCLs was 33.0 $\pm$ 2.3 microns (Table 2). There was a small range of minimum distances found between the nuclei, from 28 to 43 microns. There was no statistical difference found between the normal control MCL pairs for the minimum distances between their nuclei ($p > 0.05$).

Roundness

As noted in the methods section, the nuclear roundness scale ranged from 0.0 to 1.0, with a circle having a value of 1.0. When normal control nuclei were analyzed they had an average roundness of 0.45 $\pm$ 0.03 signifying a fairly long, ovoid shape (Table 2). One of the three pairs of control MCLs showed evidence

of a right-left statistical difference in the shape measurement of their nuclei ($p = 0.004$). The other two control pairs had an extremely strong correlation with no evidence of a statistical difference between their right and left MCL nuclear shapes ($p > 0.10$).

Table 2: Normal Medial Collateral Ligament

(n = no. of ligaments analyzed)

Group	n	Mean + SE
Cell Number:		
DNA Concentration	6	2.27 ± 0.14 ug DNA/mg dry wt
Number of Nuclei	6	59 ± 8 nuclei
Cell Distribution and Shape:		
Minimum Distance	6	33.0 ± 2.3 microns
Roundness	6	0.45 ± 0.03*
Cell Viability:		
Collagen Synthesis	1	12.2 DPM/ug hydroxyproline
Autoradiography	2	75% cells M.A. 25% cells not M.A.

* = significant difference found in one right-left MCL pair, ($p = 0.004$).

M.A. = metabolically active

Cell Viability

Collagen Synthesis

The normal MCL was shown to have cellular activity *in vitro*, since radiolabelled hydroxyproline was isolated at 12.2 DPM / ug hydroxyproline (Table 2). Combined with evidence published elsewhere in this model (Frank et al., 1988a) that blockage of protein synthesis with cyclohexamide prevents all synthesis of hydroxyproline, these results prove a functioning MCL cellular metabolic process and confirmed that the normal MCL matrix was penetrated by ^{3}H-proline, allowing cellular conversion to ^{3}H-hydroxyproline.

Autoradiography

Autoradiography gave further evidence of cell viability through the presence of associated label with the cells. All the normal ligament midsubstance samples had a small and consistent, nonspecific background deposition of label, similar to that observed in the negative controls, but showed a dense localization of label over approximately 75 per cent of the ligament cells. These "hot" regions were evidence of metabolically active ligament cells. A few patchy areas showed cells with no associated label (Figure 6).

DISCUSSION

This study is the first report containing quantitative descriptions of normal, adult MCL fibroblast numbers, shapes and distributions within their collagen matrix. Normal adult MCL (away from blood vessels and deep within the midsubstance) appears to contain a single population of only slightly variable fibroblasts and provides a quantitative baseline for all other studies on MCL cellularity.

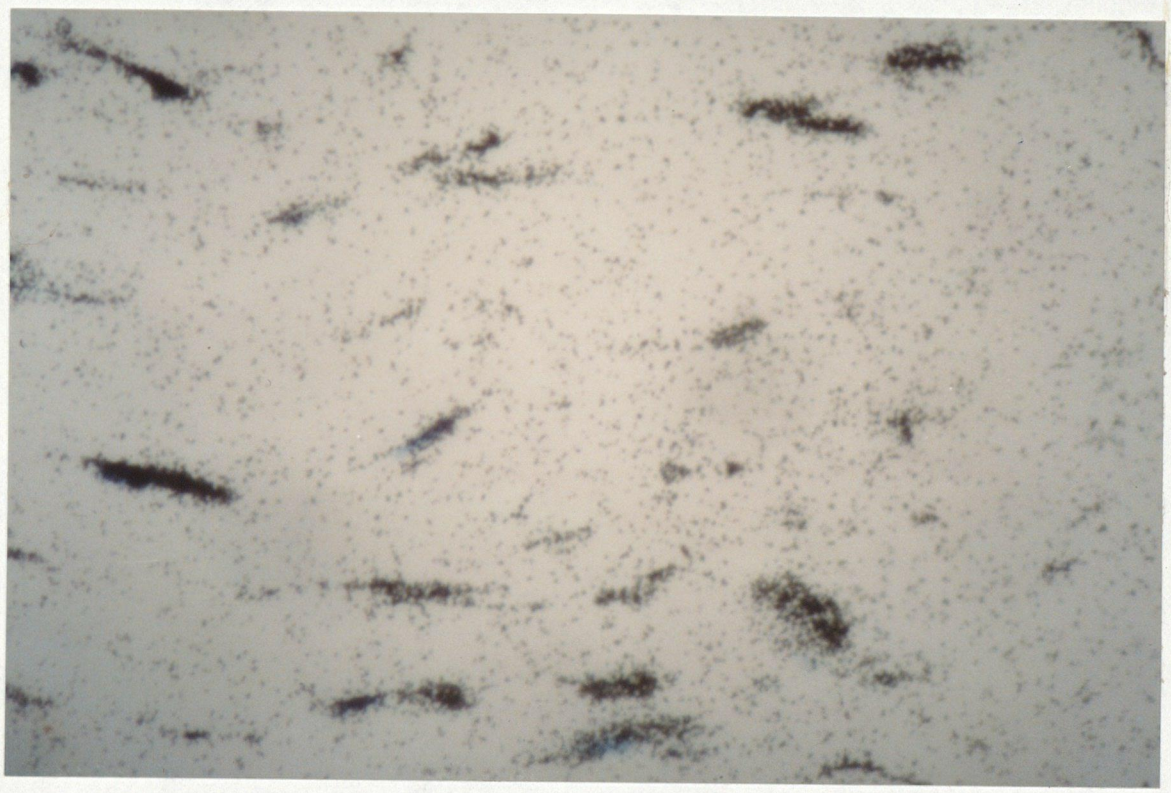

Figure 6 High power view of autoradiography of normal midsubstance MCL. Note the localization of silver granules with the cell nuclei which is evidence of metabolically active cells.

The tissue cellularity of midsubstance MCLs showed a close correlation between MCLs from the same animal, as well as between animals. The nuclear distribution in ligament substance was also consistent across animals, with nuclei generally having a long, ovoid shape. The inspection of the nuclear shapes within each individual MCL suggested there was only one cell type present in normal MCL. All normal MCL nuclei had a narrow range of nuclear roundness, yet one of the six MCLs had a slightly lower roundness average with a small variance, thus being different from its contralateral MCL. The detection of this MCL with a slightly flattened overall cell shape could be attributed to the knee joint being placed at a larger joint angle during tissue fixation, thus increasing ligament tension, possibly causing subtle changes in nuclear shape. It did not become obvious that ligament tension was an important determinant of cell shape until later in the project (see section C on tension).

The cellularity of normal MCL was also determined quantitatively by a biochemical assay for DNA, with DNA concentration being slightly lower, but similar to previous published work (Frank et al., 1983a,b; 1988a). The small difference in DNA concentration is probably caused by an age-related change, as the previous studies used immature and adolescent animal models. Combined with the histomorphometric results for cell numbers, this suggests that normal rabbit MCL midsubstance contains a fairly consistent number of cells per volume of matrix and that this number can be considered a normal MCL characteristic.

There are several possible explanations for the larger variance resulting from the histomorphometric method versus the biochemical determination for the number of nuclei in normal MCL. The histomorphometric method involved a two-dimensional measure of the midsubstance which analyzed a small proportion of the overall ligament mass, compared to the DNA assay which sampled the entire MCL. There is also the possibility there were technical problems, such as

inconsistencies in staining the nuclei and that midsubstance MCL sampling was not performed in the identical location in each MCL for histomorphometry. These factors likely contribute to the error and account for the large variance involved with the histological method for determining cell numbers.

Metabolic results in this study suggest that adult MCL cells synthesize less collagen than previously described for adolescent rabbit MCL (Frank et al., 1988b), possibly due to an aging effect, but more likely due to the cold-proline incubation used before pulsing MCLs with radio-labelled hydroxyproline. The purpose of this cold proline was to allow all ligament cells to achieve the same metabolic state *in vitro* before testing, but it also probably had the effect of saturating the cells and competitively inhibiting cell synthesis of the radioactive precursor.

Autoradiography also provided evidence that approximately 75% of fibroblasts in the midsubstance were metabolically active, however, all samples also contained "non-hot" cells displaying no evidence of metabolic activity. Previous studies have reported similar observations (Frank et al., 1988b), possibly since some cells are simply damaged by the histological procedure, or possibly since some permanent or transient subpopulations of midsubstance cells are actually quiescent and in a "non-functional" state at any point in time.

These findings from the normal adult MCLs were similar to previous descriptions made by other investigators (Arnoczky et al., 1983; Frank et al., 1988b) in that well-defined, blue-staining nuclei were seen embedded in pink-staining collagen fibers. At higher magnification, it was not possible to detect the cell membrane or any cell organelles, so only nuclear-derived information was used as an indicator of ligament cell numbers, cell distribution and cell shape. Nonetheless, these results form a reproducible baseline of several normal

"fibroblast characteristics" which in turn allow quantitative comparisons with MCLs treated in other ways.

B. REMOVAL OF VIABLE MCL CELLS BY FREEZE-THAWING

This study was conducted in order to define changes in midsubstance cell characteristics between the time of implantation of MCL Autografts and their sacrifice 12 weeks later. Fresh (normal) MCLs were compared with frozen-thawed MCLs at "time zero" (the time of their implantation and at sacrifice). Frozen-Thawed Control MCL results will be presented first in this section followed by the results for MCL Autografts implanted fresh and frozen-thawed. Autograft contralateral control results will also be presented separately.

I. Time Zero Frozen-Thawed Control MCLs

Gross Observations

After an MCL had been multiply frozen-thawed at time zero, there were some obvious changes to its appearance compared with a normal (fresh) MCL. The frozen-thawed MCL had lost all translucency, appearing a dull, stark white color. The overall thickness of the MCL had also apparently increased due to swelling, yet both insertions into bone felt firm on palpation.

Using standard light microscopy, the normal ligament displayed a structure similar to the frozen-thawed ligament. Both tissues demonstrated tightly packed, longitudinally oriented, roughly parallel collagen fibres. Also observed was the previously described sinusoidal waves of the fibrillar matrix. Both tissue types had darkly stained cell nuclei distributed at infrequent intervals between the fibres, with the nuclear long axes also running roughly parallel to the long axis of the ligament. These nuclei were found end to end, clustered in small rows or scattered

individually throughout the fibres. All ligaments displayed occasional and partial cross-sectional and longitudinal sections of blood vessels.

There were small but noticeable histological differences between the normal and frozen-thawed ligaments. The nuclei from the normal MCL midsubstance tended to be more rounded cigar shapes while frozen-thawed cell nuclei differed by appearing more flattened.

Cell Number

DNA Concentrations

Multiple frozen-thawed ligament had an average of 2.41 $\pm$ 0.54 ug DNA / mg dry weight, which was in the same range as normal ligament of 2.27 $\pm$ 0.14 ug DNA / mg dry weight (Figure 7). No significant difference ($p=0.73$) was found in cellularity between frozen-thawed and normal MCL tissue.

Number of Nuclei

Image analysis of frozen-thawed MCL showed that the ligament midsubstance had an average of 56 $\pm$ 12 nuclei per total area scanned. The frozen-thawed MCL was unchanged from normal MCL which had 59 $\pm$ 8 nuclei in the same total area (Figure 8).

Cell Distribution and Shape

Minimum Distance

The average minimum distance between cell nuclei located in the midsubstance of frozen-thawed MCLs was 39.6 $\pm$ 1.6 microns. This distance was only slightly increased from the average 33.0 $\pm$ 2.3 microns found between nuclei located in normal MCLs (Figure 9) and differences were again not statistically significant ($p > 0.05$).

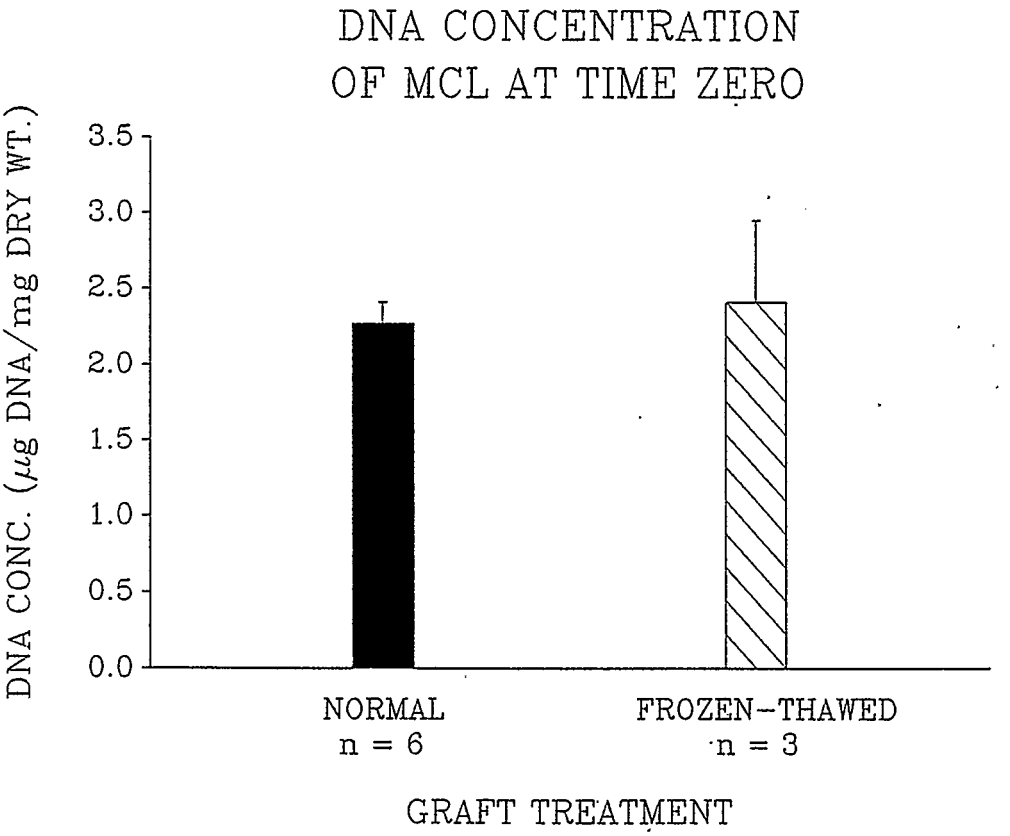

Figure 7 Cell Number: Plot of DNA concentration of normal and multiple frozen-thawed MCL at time zero. No evidence of statistical significance ($p = 0.73$).

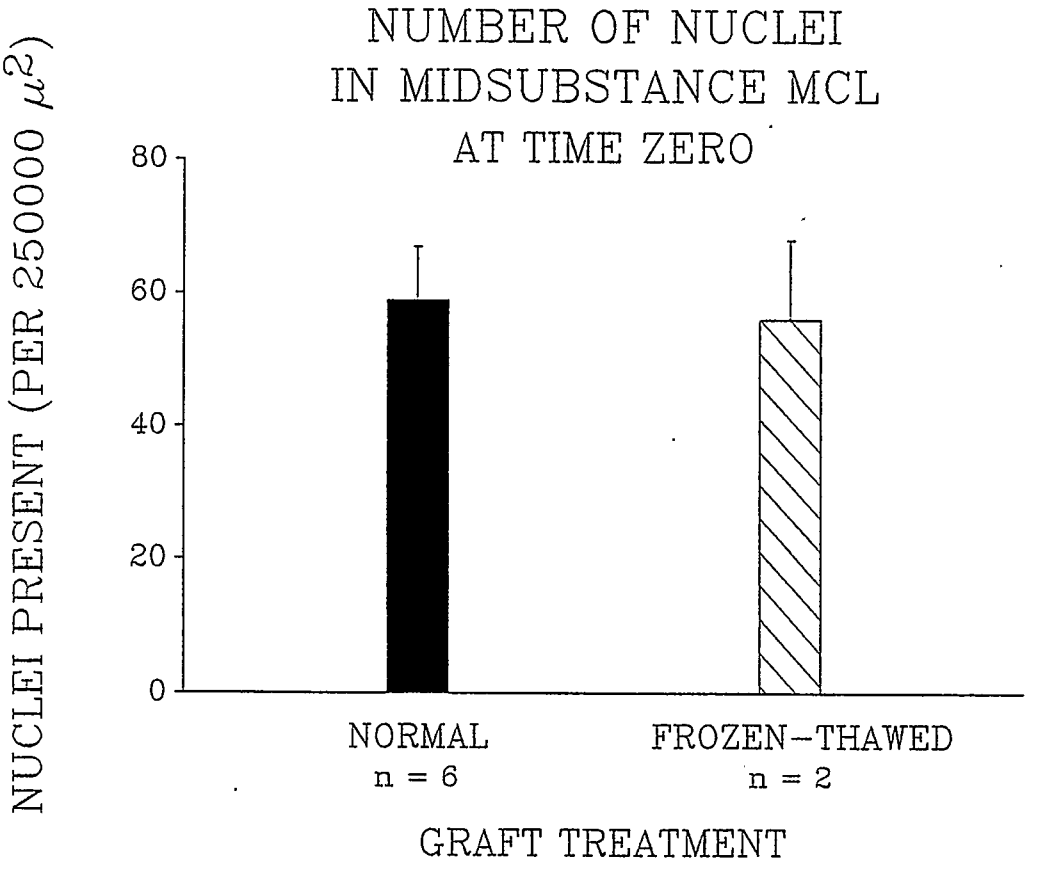

Figure 8 Cell Number: Plot of number of nuclei in midsubstance normal and multiple frozen-thawed MCL at time zero. Note there is no significant difference in number of nuclei between the two groups ($p > 0.05$).

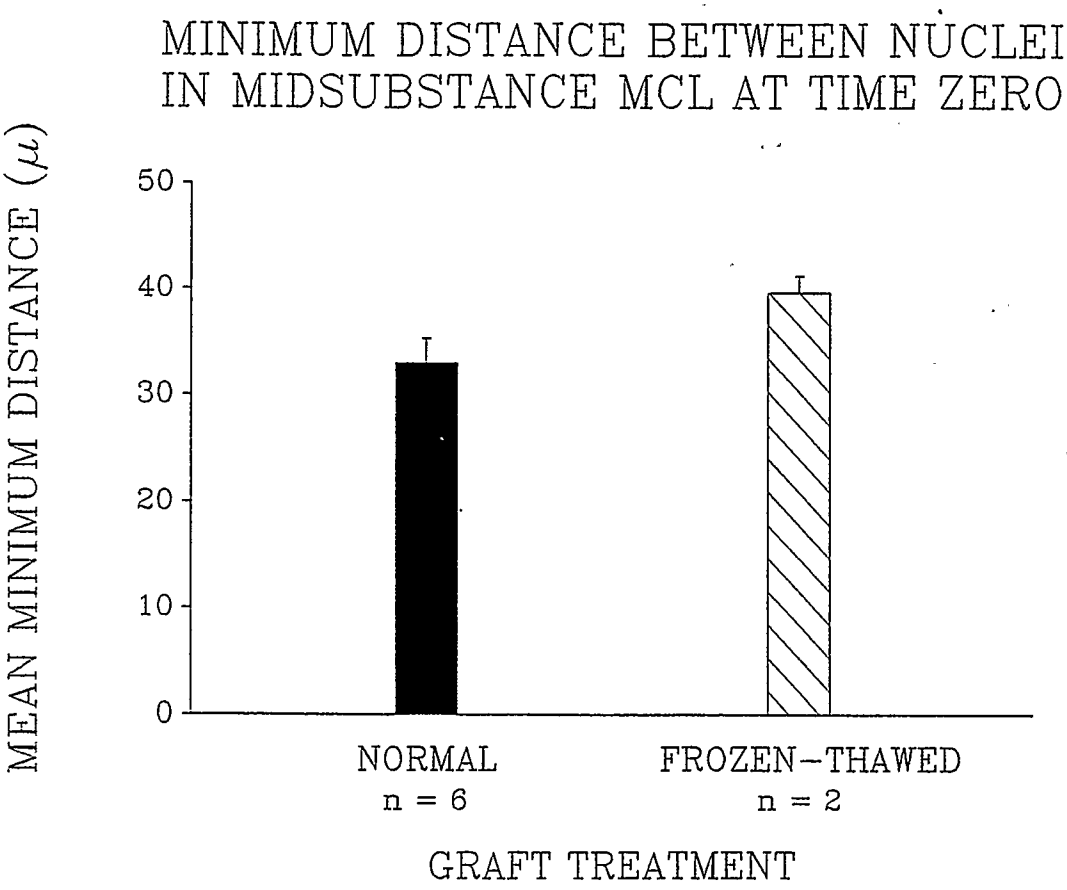

Figure 9 Cell Distribution: Plot of the minimum distance between nuclei in midsubstance normal and multiple frozen-thawed MCL at time zero. No evidence for statistical significance ($P > 0.05$), but note how nuclei in frozen-thawed MCL tend to be further apart than in normal MCL.

Roundness

Cell shape analysis determined that nuclei from frozen-thawed MCL had a roundness of 0.37 $\pm$ 0.02. These frozen-thawed nuclei were flattened compared to the characteristic shape of normal MCL nuclei of 0.45 $\pm$ 0.03 (Figure 10), but these differences again were also not statistically significant ($p > 0.05$).

Cell Viability

Collagen Synthesis

Frozen-thawed ligament cells displayed no evidence of collagen production since there was no synthesis of ^{3}H-hydroxyproline in any of the three ligaments tested (see Table 1). In contrast, the normal MCL was shown to have cellular activity in this *in vitro* test, because of the isolation of radiolabelled hydroxyproline of 12.2 DPM / ug hydroxyproline.

Autoradiography

Frozen-thawed ligament midsubstance from both of the ligaments sampled had a small and consistent nonspecific radioactive background which was similar to that observed in the normal ligaments and the negative controls. There was no activity seen over any cells in multiply frozen-thawed ligament suggesting the absence of cell viability. This was distinct from the normal MCL, which as noted previously, showed some presence of metabolically active ligament cells (Figure 11).

DISCUSSION

To allow for a proper analysis of the cellular changes of frozen-thawed MCL autografts at 12 weeks, it was first necessary to define the cellular

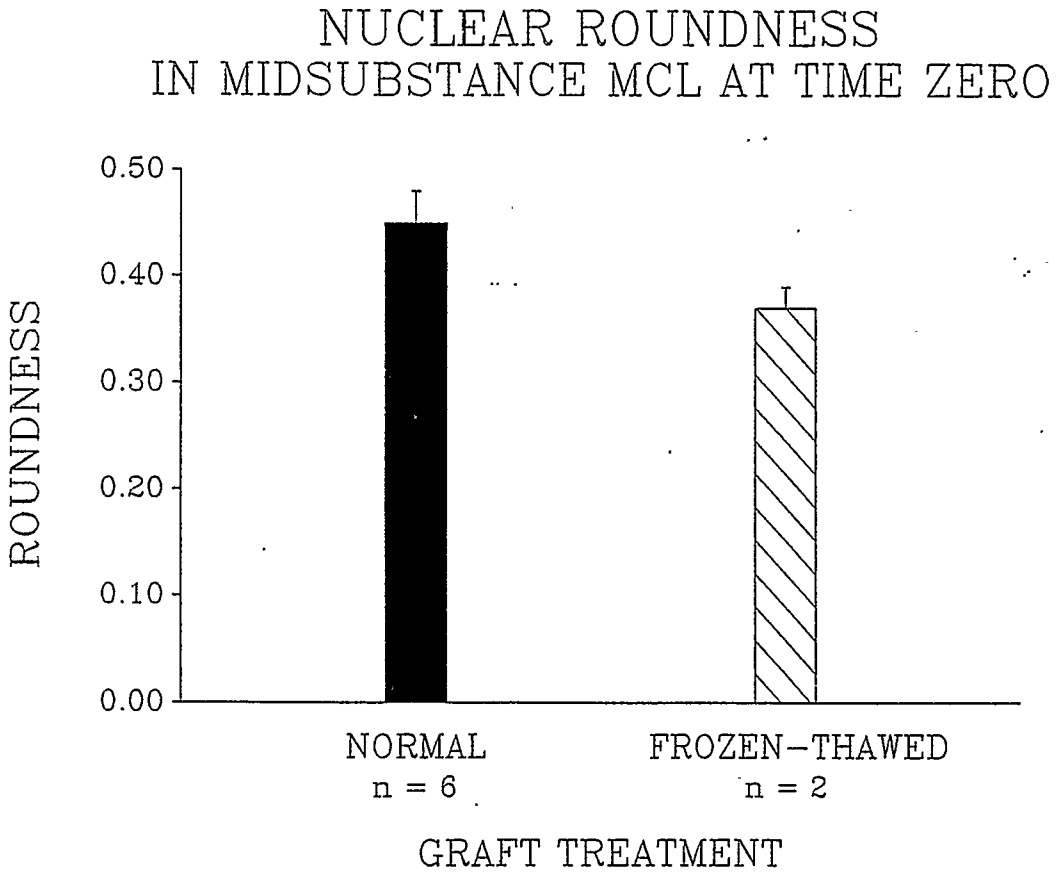

Figure 10 Cell Shape: Plot of the roundness of nuclei in midsubstance normal and multiple frozen-thawed MCL at time zero. No evidence for statistical significance ($p > 0.05$), but note the subtle change in frozen-thawed MCL nuclear shape to a less round appearance.

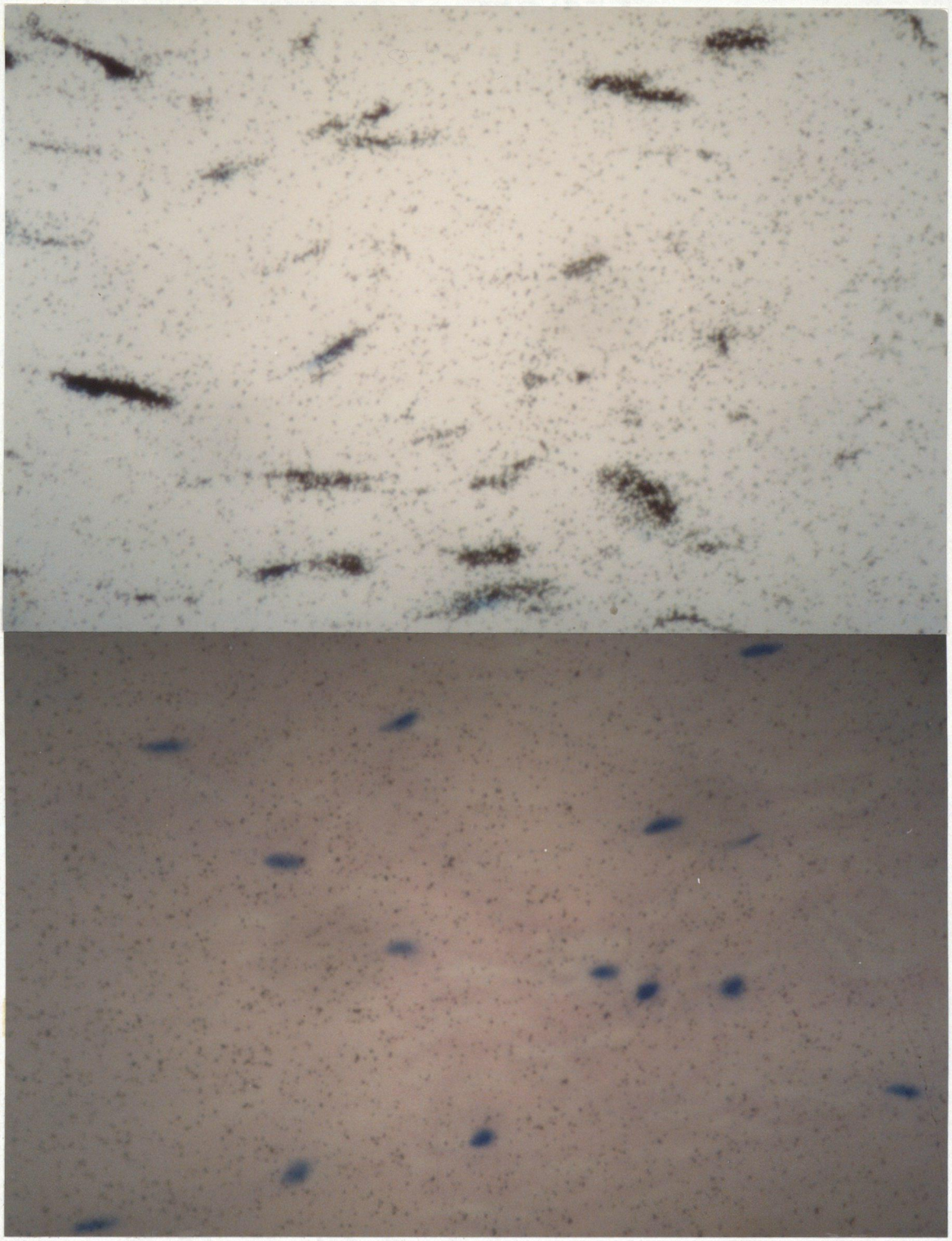

Figure 11 Autoradiography of normal (top) and frozen-thawed (bottom) MCL at time zero. Note that frozen-thawed MCL has no localization of granules associated with the blue-stained nuclei.

characteristics of the grafts at time of implantation. In other words, there was a need to develop frozen-thawed MCL controls and compare them to normal MCLs.

Some recently published research suggests that the procedure of multiple freeze-thaw renders connective tissue acellular (Amiel et al., 1986a,b; Kleiner et al., 1986). Contrary to this report, my results showed that multiple freeze-thawing has had almost no effect on the nuclei in the ligament cell population. Cell numbers, cell distributions and cell shapes have all remained within normal cell limits, making it virtually impossible to determine exactly which tissues have been frozen based on morphology alone. Metabolic studies revealed a difference (frozen-thawed tissues showed almost no cellular activity), but this difference was unfortunately not very useful. Since even fresh MCLs contain a subpopulation of inactive cells (by the parameter of collagen synthesis), it would be impossible to use the parameter of activity alone as an indicator of "normality". Since cells in frozen-thawed grafts were essentially no different from some normal MCLs, therefore, it became obvious that it would be impossible to use either nuclear morphology or cellular metabolism to identify the source of cells in grafts. In other words, my goal of removing all cells by freeze-thawing such that all cells in a graft could be proven to be extrinsic in origin was therefore not realized.

Essentially, I found that frozen-thawed MCL cannot be classified as "acellular" as determined by either the histological methods used or biochemically. Equal quantities of DNA were found in both frozen-thawed MCL and normal MCL. It was not surprising that the DNA content had not changed after freeze-thawing since the saline thaw was not adequate to remove large cell remnants from the ligament matrix. What did come as a surprise was the near normal appearance of the frozen-thawed cells, suggesting that either the cell membranes were ruptured, leaving preserved nuclei, or that nuclei were somehow totally resistant to freezing. Interestingly, the only measure that proved to be different from normal

MCL was that frozen-thawed MCL showed no evidence of metabolic activity as measured by its lack of *in vitro* collagen synthesis. Thus, this investigation's hypothesis that multiple freeze-thawing would render MCL void of identifiable cells was proven wrong. Overall, it is suggested that the harsh multiple freeze-thaw procedure did not render MCLs acellular, as described by the histological methods or by measuring the DNA content of the ligament, but it did render the intrinsic cell population as "non-functional", at least as determined by the metabolic index of collagen synthesis. Thus, it can be suggested that the technique of freeze-thawing used appears to have had a damaging effect on rabbit MCL fibroblasts (by at least temporarily altering the cells' metabolism), but not one of rendering the tissue absolutely acellular.

It should also be mentioned that small variances were found for all measures so that it seems unlikely that the lack of statistical differences found between the fresh and the frozen-thawed MCLs were due to a type II error.

II. Fresh vs. Frozen-Thawed Autografts

In this section, midsubstance characteristics of fresh and frozen-thawed autografts at 12 weeks of healing will be presented to allow their direct comparison.

Gross Observations

All post-transplantation rabbits appeared healthy and had an average mass gain of 0.2 Kg over the 12 weeks following surgery. The animals appeared to be bearing full weight on the operated leg and displayed a normal gait pattern. Following euthanasia, all autograft hindlimbs could be almost fully flexed and were stable with manual valgus testing.

During dissection, it was apparent that the fascial layers had scarred down onto the autograft. A deeper inflammatory membrane enveloped the entire ligament-bone complex. With sharp dissection, it was possible to remove this fibrous membrane to expose the autograft. All ligaments had reattached to the underlying medial meniscus with fibrous tissue. Compared to the contralateral control MCLs, all autografts had increased in both length and width by a few millimeters. All screws and washers had remained in position and the graft bone at each insertion appeared to be united with the femoral and tibial bone forming the graft bed. There was no obvious evidence of damage to the articular surfaces or menisci, with the exception of an occasional bone growth present on the tibial condyle under the MCL (Figure 12).

Microscopically, the autografts were more cellular than the contralateral left controls. The Frozen-Thawed Autos appeared the most cellular, with the highest cell density occurring at the ligament surface layers. This dense cell population tended to decrease more towards the midsubstance matrix. All autografts had a noticeable difference in their nuclear shape, which was more round compared to normal spindle-shaped fibroblasts. Additionally, the autograft matrix close to the ligament surface layer had collagen fibers in periodic whorling or circular patterns.

Cell Number

Number of Nuclei

There was no statistical difference ($p = 0.083$) found between the Fresh Autos and the Frozen-Thawed Autos in terms of number of cell nuclei present in the midsubstance after 12 weeks of healing (Figure 13). Yet, with the p-value just over the alpha level of 0.05, there was a trend for the Frozen-Thawed Autos to be more cellular (94 ± 15) than the Fresh Autos (69 ± 13). Tests for sample size

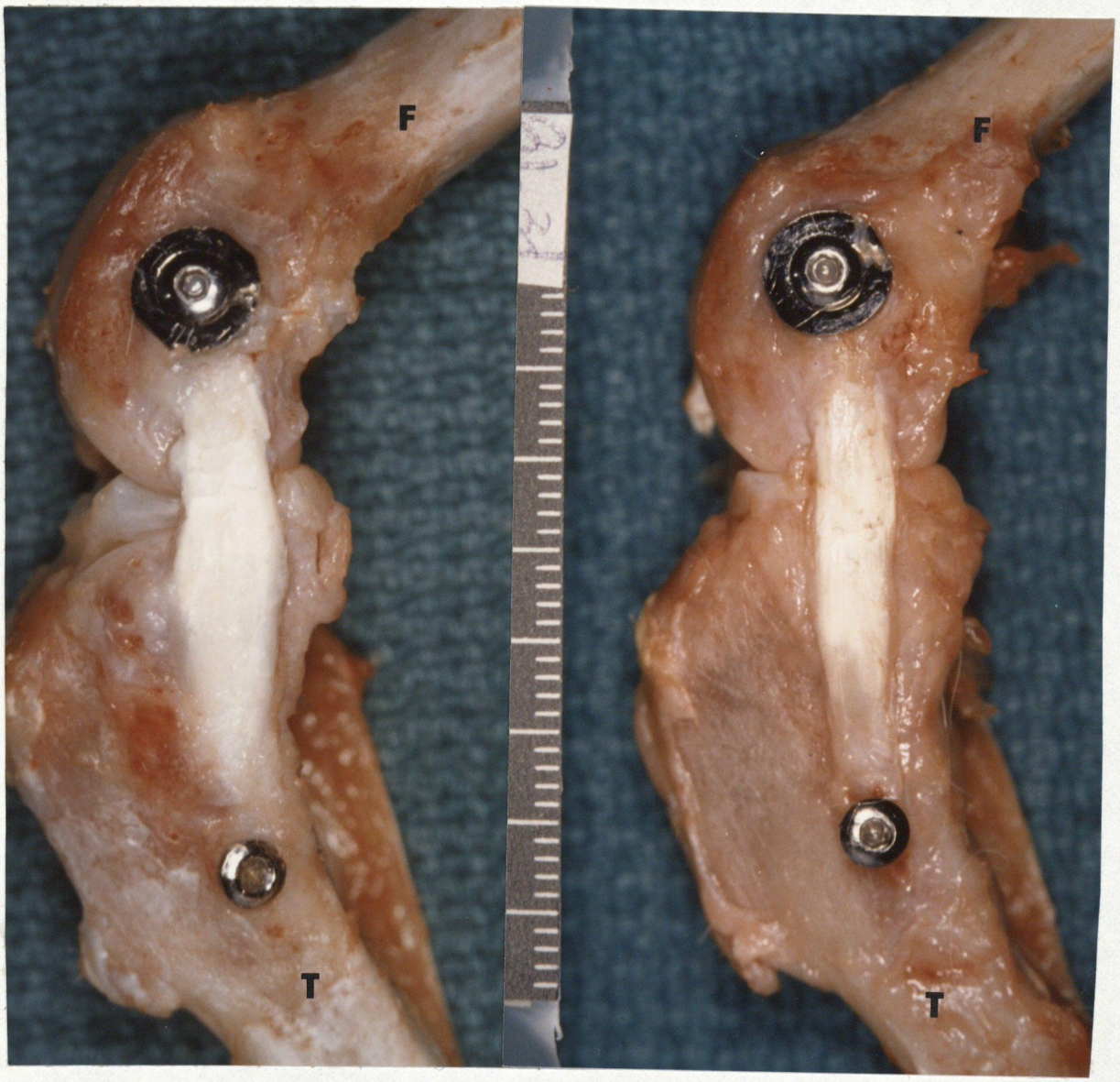

Figure 12 Medial view of frozen-thawed (left) and fresh (right) MCL autografts after 12 weeks of healing. Note the more swollen appearance of the frozen-thawed MCL.

estimation indicate that a total of 32 MCLs would have to analyzed in order to show statistical significance at alpha of 0.05.

Cell numbers in fresh MCL autografts also did not increase significantly from the time of implantation (time zero) to the 12 week healing interval. In contrast, the grafts that were frozen-thawed had a larger increase in cell number over the 12 week healing interval from the time of implantation.

Cell Distribution and Shape

Minimum Distance

The minimum distance between nuclei in the Fresh Autos was not found to be significantly different from the Frozen-Thawed Autos at 12 weeks ($p = 0.319$). The average minimum distance for the Fresh Autos was 34.8 ± 3.1 microns, compared to 31.4 ± 3.6 microns for Frozen-Thawed Autos (Figure 14).

While the 12 week Fresh Autos remained virtually unchanged from time of implantation (time zero), the nuclei in the 12 week Frozen-Thawed Autos were again found in a slightly higher density than when the autografts had been implanted (time zero).

Roundness

The results of the two-way ANOVA, comparing the nuclear roundness of autografts implanted fresh versus frozen-thawed and at various tensions, proved to show significant ($p < 0.001$) interaction (see section C, Figure 25). Therefore, there was an effect of freeze-thaw, but it depended on the effect of tension. Thus, it was not possible to determine the main effects of either freeze-thaw or tension independently and this interaction will be discussed in the next chapter.

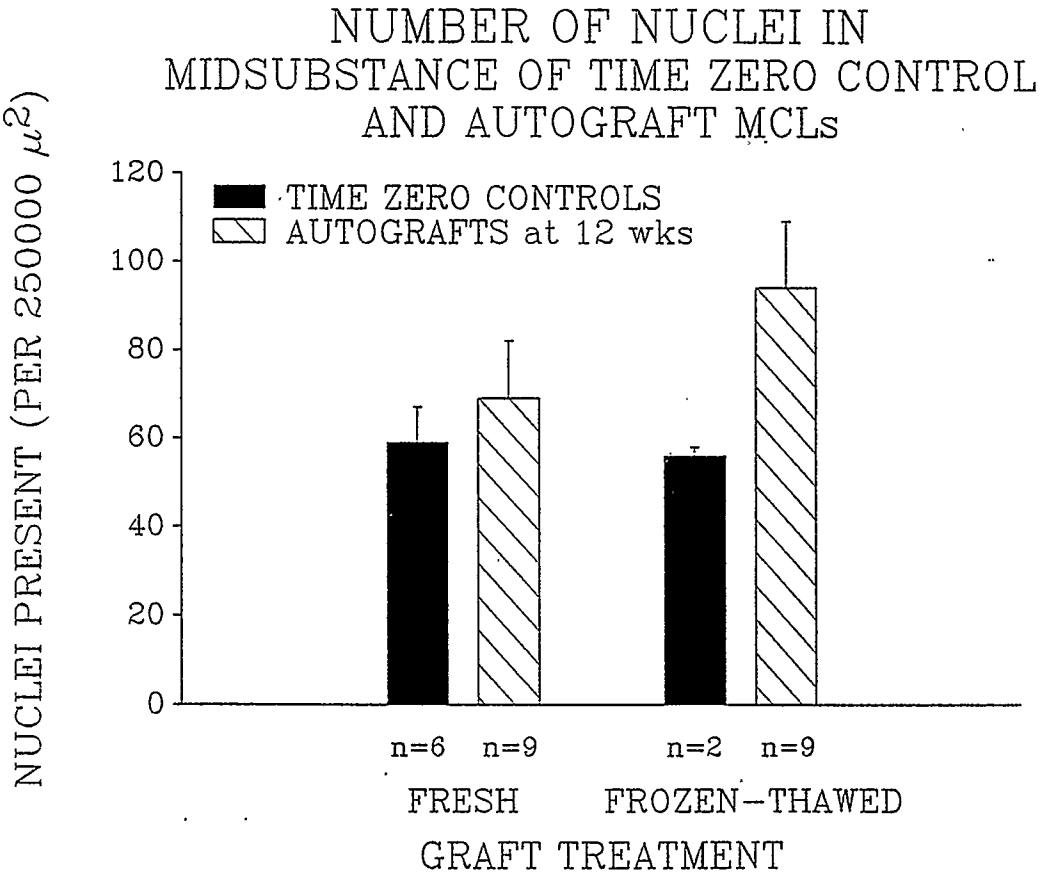

Figure 13 Cell Number: Plot of the number of nuclei in 12 week autograft midsubstance MCL treated Fresh or Frozen-Thawed as compared with their time zero controls. There is no evidence of a difference in cell number between the Fresh and Frozen-Thawed MCLs at 12 weeks ($p = 0.083$). Note that both groups have increased cell numbers from time zero.

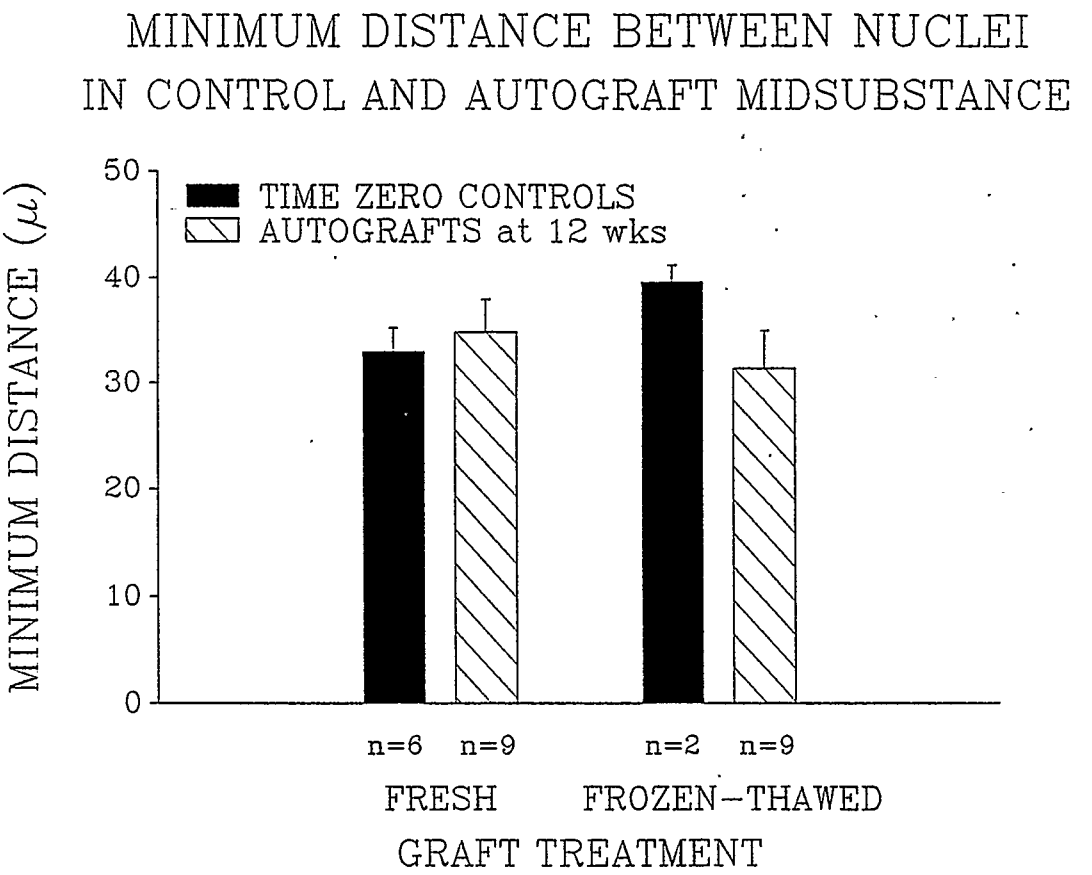

Figure 14 Cell Distribution: Plot of the minimum distance between nuclei in autograft midsubstance MCL at 12 weeks treated Fresh or Frozen-Thawed as compared with their time zero controls. No significant difference was found between the Fresh and Frozen-Thawed autografts ($p = 0.319$). Note the trend for a decrease in distance in the Frozen-Thawed experimentals from their time zero state.

DISCUSSION

From gross observations at 12 weeks, the MCL autograft model appeared successful in that all animals were healthy and displayed signs of healing in the operated stifle joint. A closer look at the MCL grafts revealed that no graft was acellular at 12 weeks. In fact, all grafts appeared hypercellular compared to normal MCL, an observation similar to that made previously by Sabiston et al. (1990b).

The surface ligament layer, in the frozen-thawed grafts in particular, had an extremely dense population of cells. This density of cells decreased and became more organized within the internal matrix of the ligament (the area which we analyzed). This qualitative observation of a gradient of cells from surface to deep in the midsubstance, however, may be important in determining the source of replacement cells in grafted ligament and will be discussed further in the section on overall discussion.

Findings from histomorphometry of MCL autograft midsubstance did not detect any significant differences in the cell numbers, shapes or distributions between fresh and frozen-thawed autografts. Both graft types showed an increase in cellularity from that measured in controls at time of implantation, with the frozen-thawed having a trend to be the most cellular. Two possible explanations exist for this observed increase in cell to matrix ratio in both autograft groups. First, the number of cells in the ligament could be increasing either by proliferation of ligament cells or due to ingrowth of extrinsic cells. Alternatively, there could be a decrease in the quantity of collagen matrix, thereby allowing the cells to move closer together. A combination of these explanations is likely, since diffuse proliferation of cells (both intrinsic plus extrinsic) and some removal of autograft matrix are expected early post-surgery results based on what has been observed in similar models of ligament healing (Sabiston et al., 1990c). If, in fact,

our freezing protocol inhibited intrinsic cells from proliferating (as it obviously had inhibited their metabolism), then the vast majority of new cells in the freeze-thaw grafts could be said to be of extrinsic origin. Exactly why frozen-thawed grafts would be more cellular than fresh grafts is not clear, but at least two possibilities exist. This could be due to addition of extrinsic cells to the preexisting intrinsic population which was seen in time zero frozen-thawed controls and which have remained *in situ* over the duration studied. Alternatively, the freeze-thaw may have incited an abnormally high proliferation of either extrinsic or intrinsic cells (or both) due to some type of biochemical signal or excessive "injury response".

Perhaps the most important observation made was that the analysis of cell distribution and shape, identified the cell populations in both fresh and frozen-thawed autograft midsubstance as being similar to the morphological definition of normal MCL fibroblasts. The observation is partly due to the survival of at least some original graft cells following transplantation, which is likely in the fresh autografts. Survival of original graft cells could also be possible in the frozen-thawed grafts since the freeze-thaw procedure did not really eliminate the ligament cells before implantation. Some of the cells, however, are almost certainly extrinsic in origin and it is of major interest that they too may have taken on the shape characteristics of normal MCL fibroblasts. This is highly speculative, since we have no direct evidence for the presence of a new cell population (ie. inflammatory cells) other than the apparent gradient of cells seen surface to deep in the grafts. Additionally, since metabolic studies were not performed on autografts, we do not have proof that intrinsic cells (especially in frozen-thawed grafts) were just temporarily "stunned" and eventually "woke up". From the histomorphometry it was therefore unfortunately impossible to determine old from new cells as originally hoped, because it was discovered that freeze-thawing does

not change cells enough to distinguish them. These points will be discussed further in chapter 4.

III. Contralateral Control MCLs

The results for each cell parameter measured for the contralateral left control MCLs will be presented in three ways. First, the results within the contralateral left control group will be analyzed. Second, if no differences could be found for a particular cell parameter, the contralateral left control data will be pooled and compared to the pooled right and left normal control MCLs. Comparing the contralateral controls to the normal controls will reveal any secondary effects of the right leg surgery on the unoperated left leg. Third, the contralateral left control MCLs served as internal controls for the autograft right MCLs. Thus, a comparison between the contralateral controls and the autografts will be made. This third comparison involves the pooling of all the fresh and frozen-thawed 12 week autografts and comparing the autografts to their pooled contralateral left control MCLs.

Gross Observations

The 12 week contralateral left control MCLs appeared unchanged from the normal MCL characteristics described in previous studies and were morphologically indistinguishable from the normal control MCLs reported in the first results section.

Cell Number

Number of Nuclei

The analysis of the number of nuclei showed no statistical differences ($p > 0.25$) between the contralateral left control MCLs. These control MCLs had cell populations ranging from 30 to 75 nuclei, with an average of 47 $\pm$ 3 nuclei present in the midsubstance.

Since there was no evidence against averaging in either control group, a cell number average was taken for both the contralateral lefts and the normal control (both the right and left) MCLs. No statistical difference ($p = 0.19$) was found between the two control groups for number of nuclei present, but a trend was noted for the contralateral left control MCLs to have fewer nuclei present than the normal controls.

The pooled autograft right MCLs (fresh and frozen-thawed) were found to be highly significantly different ($p = 0.002$) from the contralateral left control MCLs, in terms of number of nuclei present in the ligament midsubstance (Figure 15). The autografts were significantly more cellular than the contralaterals, with 82 $\pm$ 10 and 47 $\pm$ 3 nuclei, respectively.

Minimum Distance

From the analysis of the minimum distance between nuclei there was no evidence for statistical differences ($p = 0.112$) between the contralateral left control MCLs. These left controls had a range of minimum distances from 31 to 47 microns, with the average minimum distance of 38.5 $\pm$ 1.1 microns.

Since there was no evidence for differences within the contralateral left and normal control groups, each group had their minimum distances pooled in order to test the normal (both the right and left MCLs) and contralateral left control groups against each other. The control groups showed no evidence of a difference

($p = 0.067$), yet there was a trend for the contralateral left control MCL nuclei to be slightly further apart than the nuclei in the normal control MCLs..

The pooled autograft right MCLs (fresh and frozen-thawed) had a smaller minimum distance between nuclei than the unoperated left MCLs. The minimum distances were found to be significantly different ($p = 0.035$). The minimum distance between nuclei in the autograft right MCLs was 33.1 $\pm$ 2.4 microns, versus 38.5 $\pm$ 1.1 microns for the contralateral left control MCLs (Figure 16).

Roundness

There was evidence of a significant difference ($p < 0.001$) in the shape of the nuclei found in the contralateral control left MCLs. The variability in nuclear roundness was also observed, as previously mentioned, in the normal control MCLs. The statistical significance is believed to be real since each roundness average for every MCL had a small variance therefore increasing the likelihood of a difference being detected. Yet, the overall range of nuclear roundness was quite narrow, most likely describing only one cell population. It is possible that the measure of nuclear shape is sensitive enough to detect subtle differences in a dissection variable, such as joint angle, that produces various tensions on the ligament thus affecting the roundness measure of nuclei.

Statistical differences were found within both the contralateral left and normal (time zero) control groups. Any group that has statistical differences within it cannot be averaged. Therefore, neither control group could have been averaged thus no comparisons between them was possible. It should be noted that the normal control nuclear shapes tended to be more round than the long, thin nuclear shapes found in the contralateral MCLs.

There was a highly significant difference ($p < 0.001$) between the roundness of the pooled autograft right MCL (fresh and frozen-thawed) nuclei and the

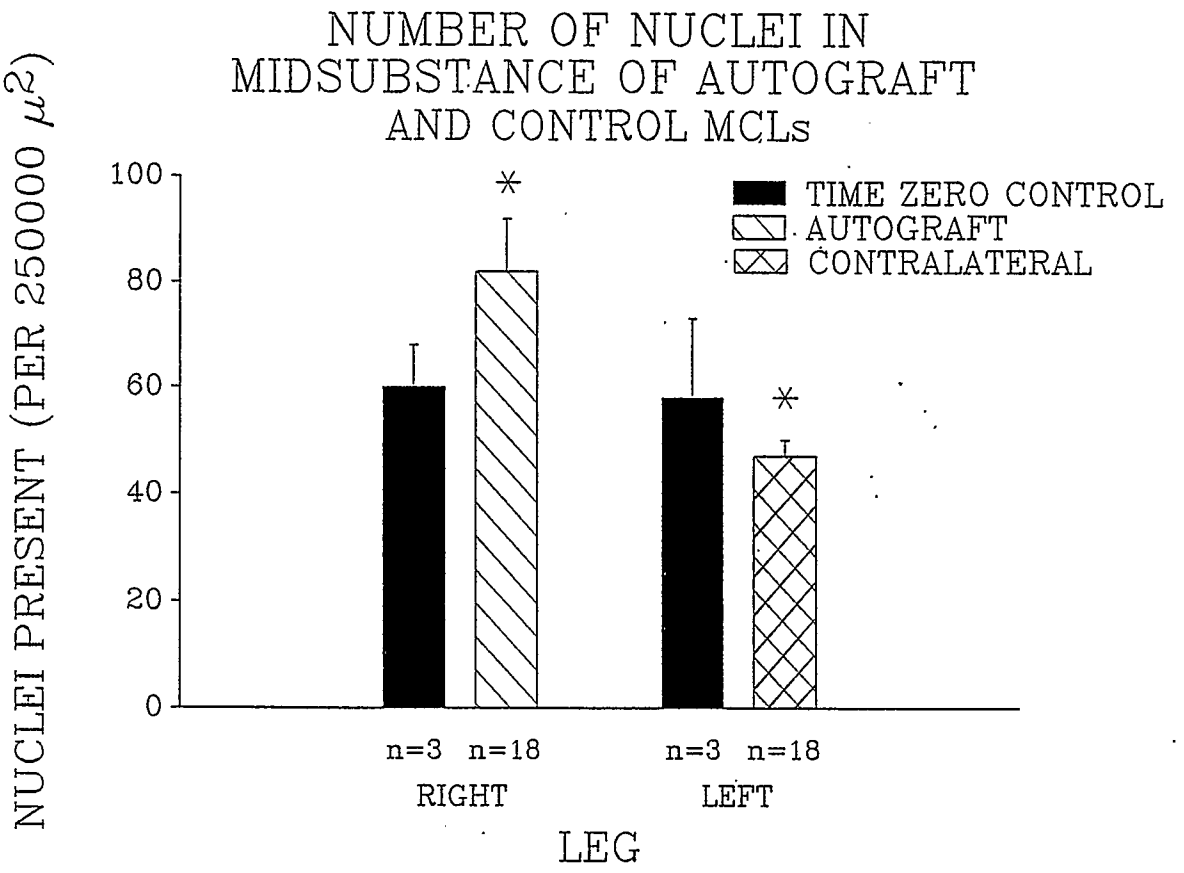

Figure 15 Cell Number: Plot of the number of nuclei in all 12 week autograft (pooled fresh and frozen-thawed) right and contralateral left control MCLs as compared to their time zero controls. The autograft right MCLs were significantly more cellular than the contralateral left control MCLs (* $p = 0.002$). There is no evidence of a difference between the cellularity in contralateral left and normal control MCLs ($p = 0.19$).

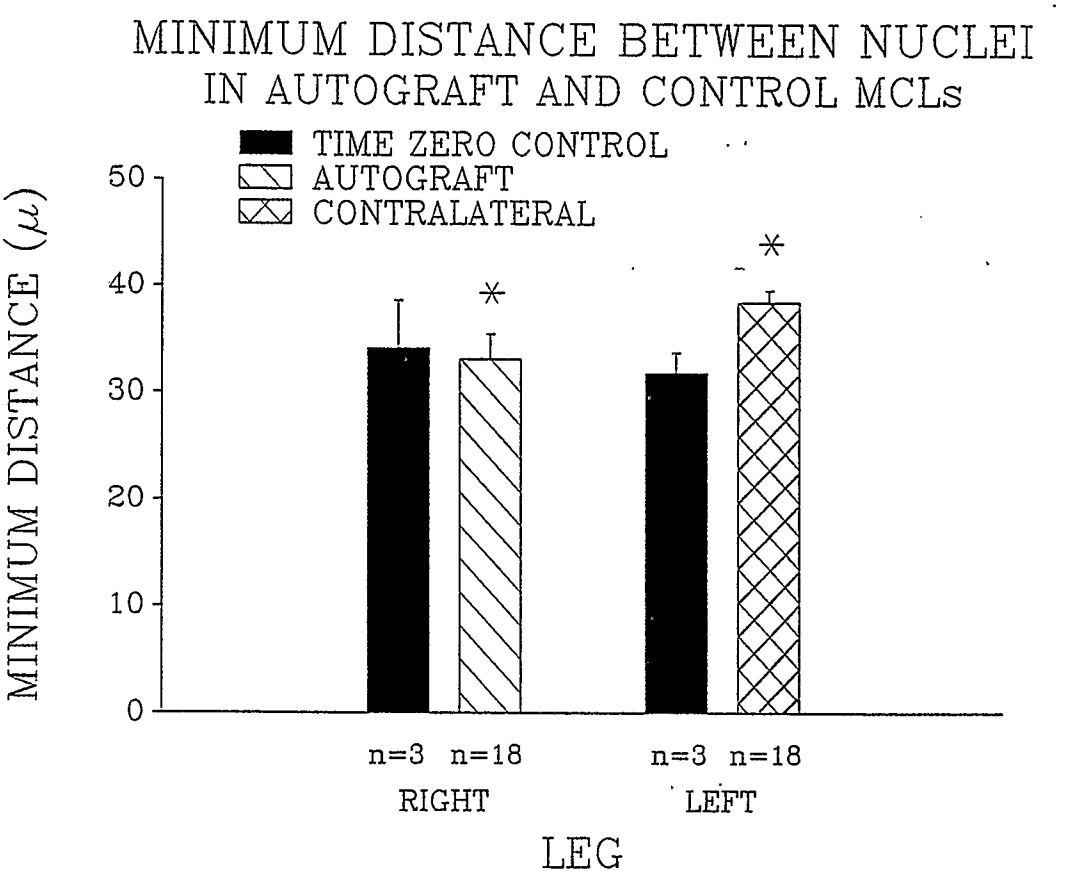

Figure 16 Cell Distribution: Plot of the minimum distance between nuclei in all 12 week autograft (pooled fresh and frozen-thawed) right and contralateral left control MCL as compared to their time zero controls. The minimum distance between nuclei in autograft right MCLs was significantly smaller than in the contralateral left controls (* $p = 0.035$). There was not a significant difference in cell distribution between the two control groups ($p = 0.067$).

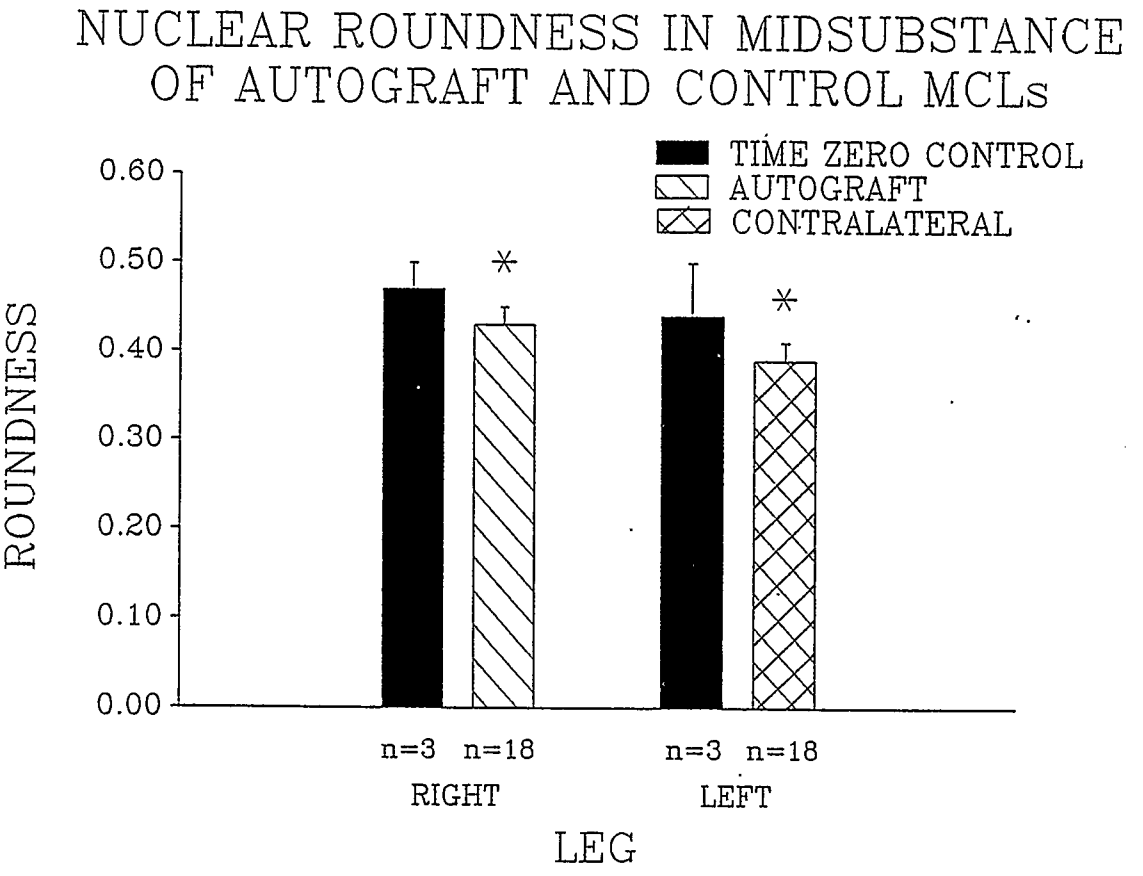

Figure 17 Cell Shape: Plot of the nuclear roundness in all 12 week autograft (pooled fresh and frozen-thawed) right and contralateral left control MCL as compared to their time zero controls. The autograft right nuclei are significantly more round than the contralateral left nuclei (* $p < 0.001$). There is evidence of differences within both the contralateral left and normal control groups ($p < 0.05$).

unoperated contralateral left control MCL nuclei (Figure 17). The autograft nuclei had a more round shape than the contralateral controls.

DISCUSSION

The analysis of all contralateral left MCLs, which served as internal controls for the autografts, showed no statistical differences from normal MCL cell characteristics. Yet, some trends existed in cell number, cell distribution and cell shape that suggested that contralateral controls may actually contain some subtle differences from normal MCL. If true, this trend would suggest that the cells in contralateral control MCLs may somehow be affected by the surgical procedure performed on the autograft right MCLs. These speculations correspond with some evidence which is accumulating suggesting that there are subtle effects on contralateral ligament cell behaviours in a number of animal models of injury (Bray, 1990) and immobilization (Walsh, 1988; Weir, 1990).

Most importantly, however, results show that similar to differences between autografts and external normal control MCLs, definite differences also exist between the autograft right MCLs and their contralateral left MCLs. Autografts definitely contain a higher number of normal appearing fibroblasts in the midsubstance. Since the contralateral left and normal control MCLs were found to be similar, the differences observed between the autografts and the contralateral lefts, are the same as those discussed in the previous section.

C. THE EFFECT OF TENSION ON MCL CELLS

The cell shapes and cell numbers of normal MCLs under different tensions and the same cell indices of MCL Autografts which have been healing under a

similar range of tensions are presented in this section. The comparison of these data allowed me to examine how cells in MCL autografts may be affected by tension, both at the time of implantation (to be discussed first) and after 12 weeks of healing.

I. Time Zero Tension Control MCLs

Cell Distribution and Shape

Minimum Distance

There was no significant effect of tension on the distribution of nuclei in midsubstance ligament matrix ($p = 0.110$). Generally, cell nuclei had a minimum internuclear distance of 27 to 37 microns (Figure 18).

Roundness

All ligament nuclei analyzed from MCLs fixed at -2 mm (Loose) and 0 mm (Anatomic) extension, and the contralateral controls had a similar roundness score of approximately 0.40. This score represents a fairly ovoid appearance. When MCLs were fixed under increasing extensions of 0.7 and 1.4 mm (Tight), the nuclear roundness decreased substantially (Figure 19). A significant difference in nuclear roundness was found between ligaments fixed at -2 mm extension (Loose) versus 1.4 mm extension (Tight), as well as, between those fixed at 1.4 mm extension (Tight) versus the contralateral controls ($p = 0.004$).

Maximum Diameter

The smallest lengths (ie. the diameter along the long axis of the oval) were found in tissues fixed at -2 mm extension (Loose) and in contralateral controls. There was a gradual increase in these nuclear lengths as tension increased on the ligament (Figure 20). Similar to the roundness measure, significant differences in

this nuclear maximum diameter were found between -2 mm extension (Loose) and 1.4 mm extension (Tight), and 1.4 mm extension (Tight) and the contralateral controls ($p = 0.004$).

Minimum Diameter

Generally, the MCL nucleus was unaffected by varying ligament tension for the measurement of minimum diameter, with the exception of the highest tension. The approximate minimum diameter of MCL nuclei was 3.5 microns. The only tension that clearly altered this measurement was 1.4 mm extension (Tight), which resulted in a smaller diameter of 2.5 microns (Figure 21). A significant difference was observed between 1.4 mm extension (Tight) and the contralateral controls ($p = 0.042$).

DISCUSSION

Using computerized histomorphometry, the nuclei from otherwise normal fresh MCLs were analyzed for potential nuclear changes due to tension. My results have shown, for the first time, that a relationship exists between the *in situ* shape of midsubstance fibroblast nuclei and the loading conditions on a ligament. In fact, the nuclear shapes appear to be quite sensitive to ligament loading, since fractions of millimeters of change in ligament length did produce significant changes in cell shape even in the midsubstance of the MCL. This finding suggests that tensile strains, in what we believe are physiological strains (0-6%), most likely affects cells throughout the ligament.

Due to ligament matrix architecture, tension will almost certainly affect cells throughout the ligament and not just in the midsubstance. The finding that midsubstance cells are affected by small tensile displacements suggests that cells

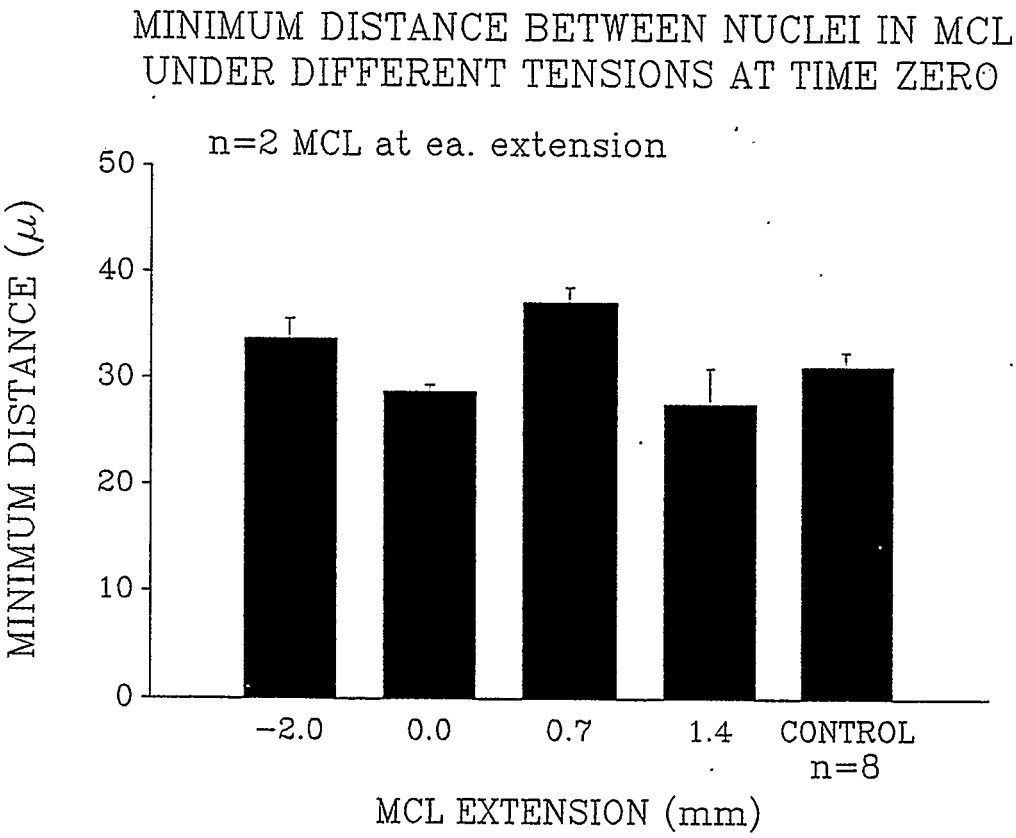

Figure 18 Cell Distribution: Plot of the minimum distances between nuclei in midsubstance MCL under different tensions at time zero. No groups were statistically significant ($p = 0.110$).

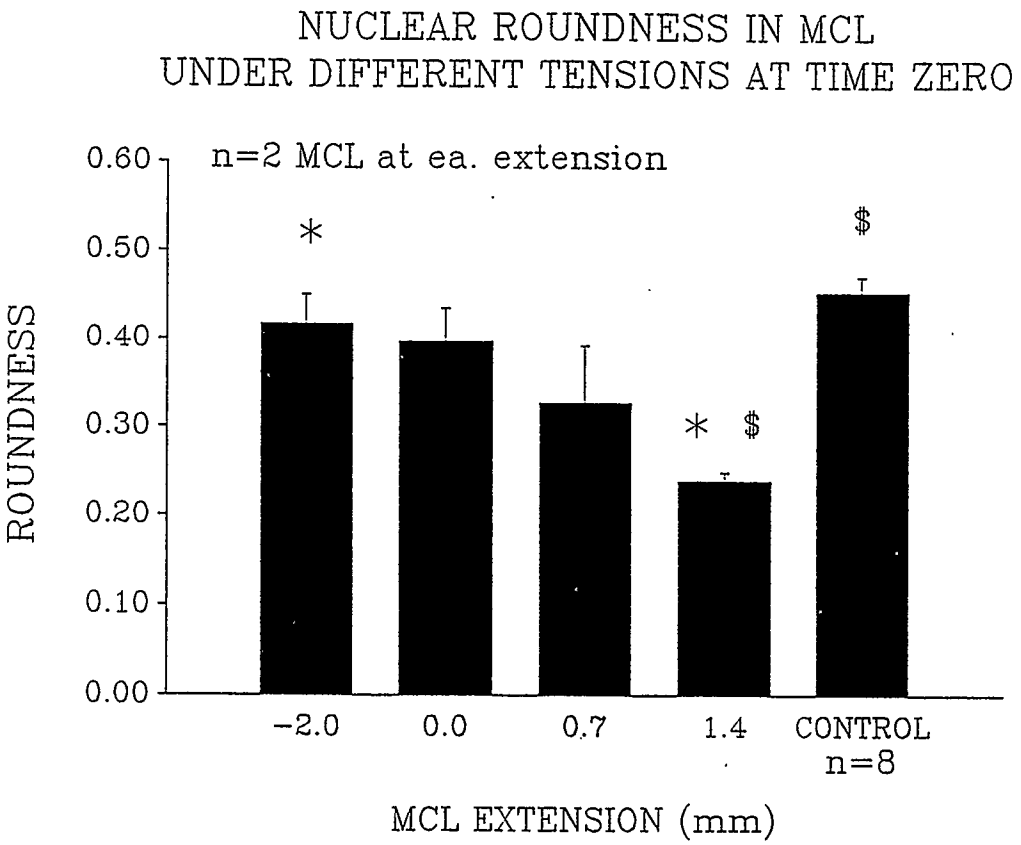

Figure 19 Cell Shape: Plot of nuclear roundness in midsubstance MCL under different tensions at time zero. Nuclear shape is significantly less round in MCLs under 1.4 mm extension compared to -2.0 mm extension (* $p < 0.005$). The 1.4 mm extension is also significantly less round compared to the contralateral controls ($ $p < 0.005$).

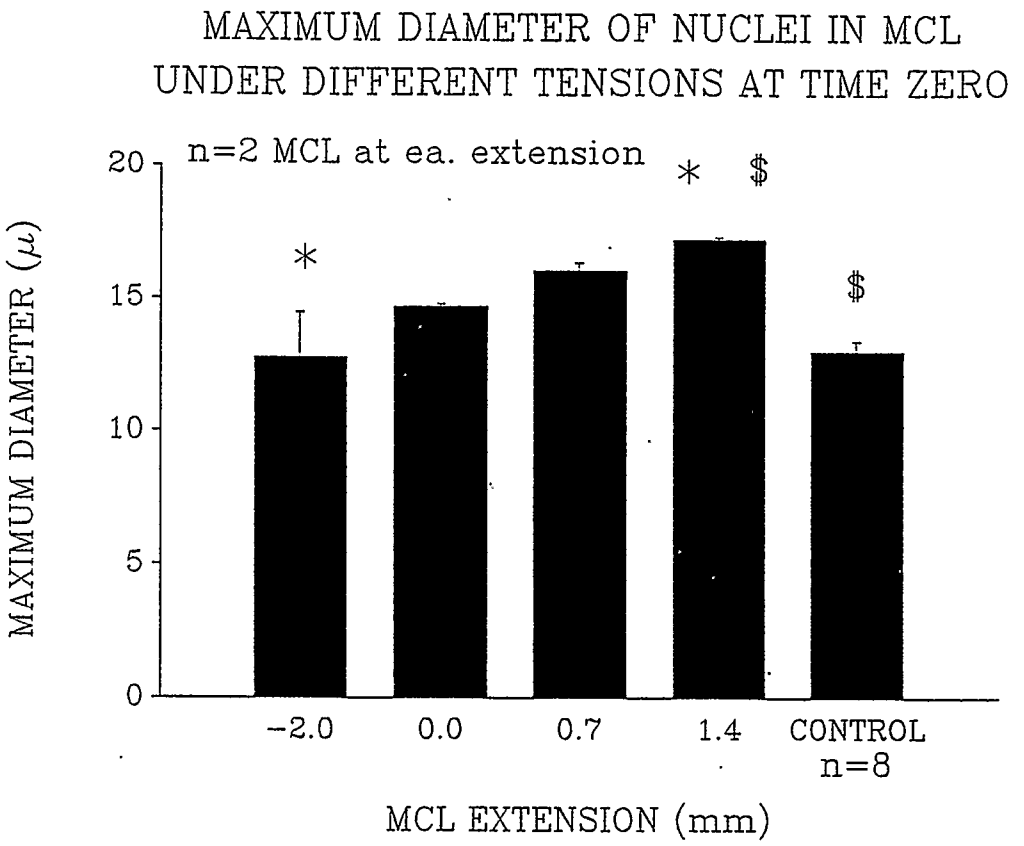

Figure 20 Cell Shape: Plot of the maximum diameter of nuclei in midsubstance MCL under different tensions at time zero. There is a statistically significant difference between MCLs under -2.0 mm extension and 1.4 mm extension (* $p < 0.005$), as well as MCLs under 1.4 mm extension and the contralateral controls ($ $p < 0.005$). The ligament under the tightest tension, 1.4 mm extension, have nuclei with the largest diameters along their long axis.

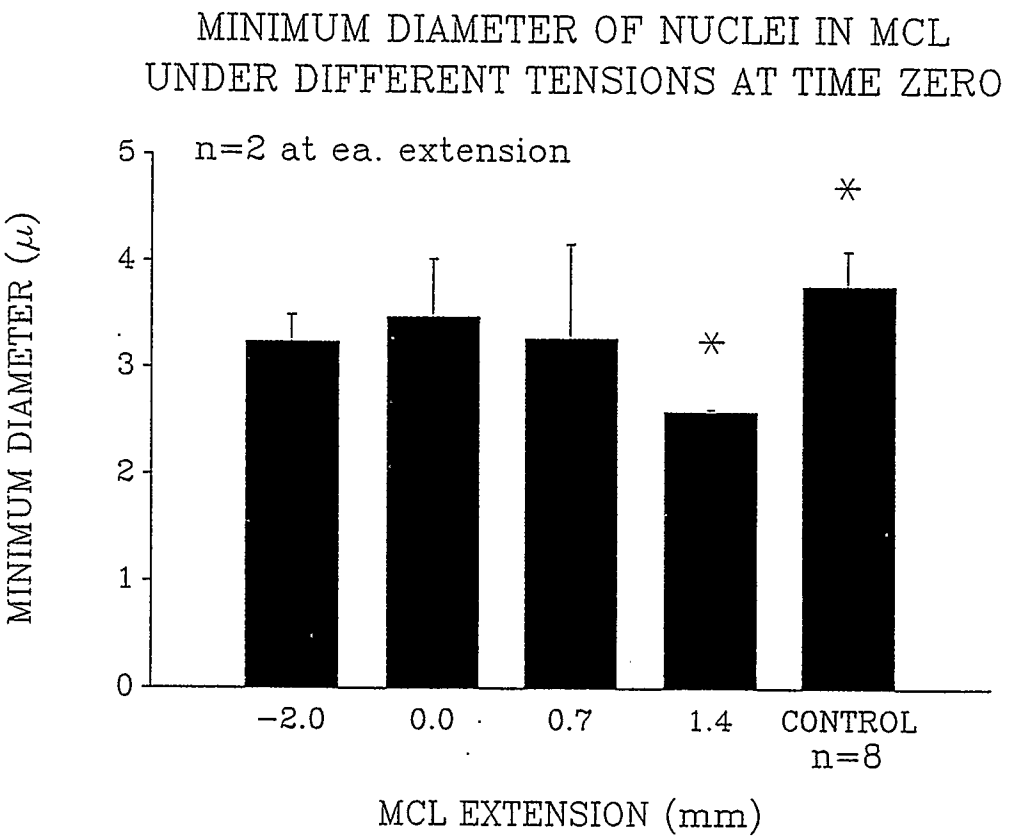

Figure 21 Cell Shape: Plot of the minimum diameter of nuclei in midsubstance MCL under different tensions at time zero. The minimum diameter of nuclei at 1.4 mm extension were significantly smaller than the contralateral controls (* p = 0.042).

throughout the ligament are probably deforming even more, as strains have been shown to be even higher near the insertions for the same displacement.

Specifically, as tension increased on the MCLs, nuclear roundness of fibroblasts substantially decreased, due mainly to increases in nuclear maximum diameters at all tensions and decreases in minimum diameters at the highest tensions. In the minimum diameter situation, nuclear volumes appear to have decreased, but it must be remembered that the shapes measured represent only two-dimensional averages. While it is possible that nuclei are being squeezed into the third dimension (ie. perpendicular to the plane of sections taken), this seems unlikely since there is no reason to suspect that loading affects cells in one particular plane. If sections were taken perpendicular to those quantified in this study, I therefore suspect that an identical pattern of nuclear change would be seen (slight increasing nuclear lengths and decreasing nuclear width with increased longitudinal ligament tension). Thus, this speculation supports the concept that increasing ligament loads caused the volume of nuclear material to change.

It is interesting to speculate on the mechanism by which a dose-response relationship exists between increasing tension and nuclear shape. Assuming that differences are not a function of variable fixation artefacts, the fact that longitudinal cell dimensions more or less varied directly with tensile strain, it may be suggested that cells may be attached to the ligament matrix by their ends. These points of attachment and the nuclear deformation registered may therefore represent some part of the elusive structure-function chain of events by which matrix loading influences cell behaviour.

While the mechanism of nuclear changes remains obscure it should be emphasized that this study shows for the first time that a relationship exists between tension and nuclear shapes in normal MCLs *in situ*. This tension-shape

relationship has obvious significance in that it implies that shape analysis of any nuclei in tissues must be qualified according to tension.

II. Tension on Healing Autografts

Gross Observations

For a complete description of 12 week autografts at sacrifice, refer to the gross observations of the MCL autografts in the results section B.

All autografts implanted under different tensions looked very similar at 12 weeks with no obvious differences in their laxity. All autografts appeared to be larger than their contralateral left controls. Both the Anatomic and Tight autografts had similarly increased areas, whereas the Loose autografts displayed the largest increase in cross-sectional area (Figure 22). All groups had similar appearances of joint surfaces, including those of the Tight autografts.

Cell Number

Number of Nuclei

At 12 weeks of healing there was no evidence of a difference between the Anatomic tension, 72 $\pm$ 13 nuclei, and the Loose tension, 63 $\pm$ 17 nuclei present in ligament midsubstance ($p = 0.44$). Since there was no evidence against averaging, these Anatomic and Loose data were then pooled for statistical reasons and compared to the Tight tension. This comparison showed a significant difference ($p = 0.007$) between Anatomic and Loose versus the Tight tension (Figure 23), with the Tight grafts being hypercellular in their midsubstance at 12 weeks post-transplantation. As well, 12 week Tight autografts displayed a significant increase in cellularity compared with their time zero controls. Loose and Anatomic grafts did not show this degree of increase.

Cell Distribution and Shape

Minimum Distance

The Anatomic and Loose tensions had no significant effect on the distribution of cell nuclei in 12 week autografts ($p = 0.32$). As with nuclear numbers, due to no evidence against averaging, the Anatomic and Loose experimental groups were averaged *a priori* and compared to the Tight tension. This comparison showed a significant difference ($p = 0.008$), with the Tight graft nuclei being found in a higher density, thus decreasing the minimum distance between nuclei (Figure 24). Compared to their time zero controls, the 12 week Anatomic and Loose autograft nuclei were slightly further apart, whereas the 12 week Tight autograft nuclei were even closer together than their controls.

Roundness

The results of the two-way ANOVA, comparing the nuclear roundness of autografts implanted fresh or freeze-thawed and at various tensions proved to have a significant interaction ($p < 0.001$). Thus, the effect of tension was dependent on the effect of freeze-thaw (Figure 25) and it was therefore not possible to determine the main effects of either tension or freeze-thaw. The general range of all the shape measurements for roundness was 0.26 to 0.58.

DISCUSSION

There were some interesting effects of tension on the gross appearances of grafts at 12 weeks of healing. All autografts displayed an increase in cross-sectional area at the midsubstance, with the loose tension appearing to result in the largest increase. Interestingly, the grafts which were implanted loose had

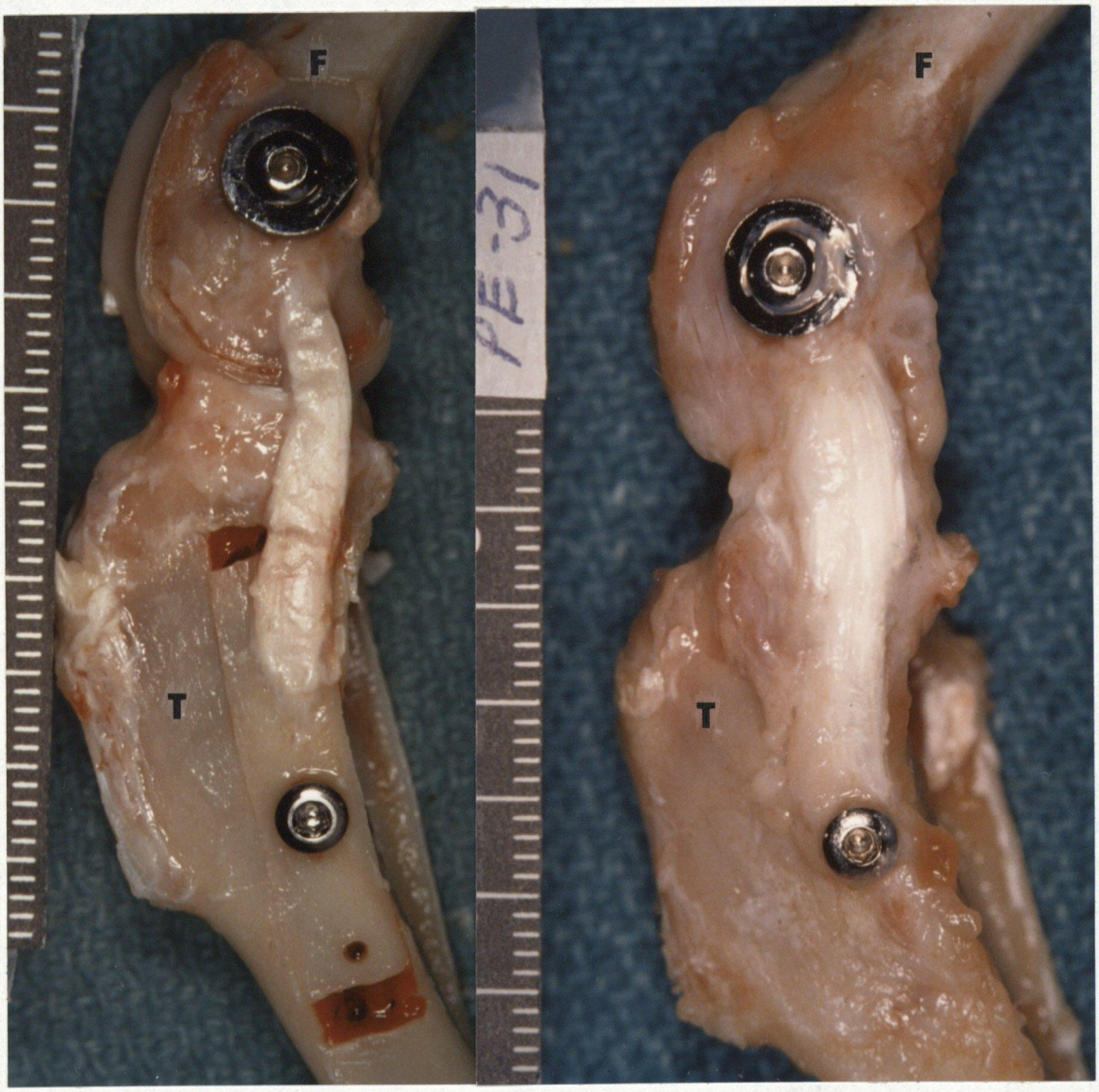

Figure 22 Medial view of "loose" autografts at time zero (left) and after 12 weeks of healing (right). Note the buckled MCL at time zero became taut after 12 weeks.

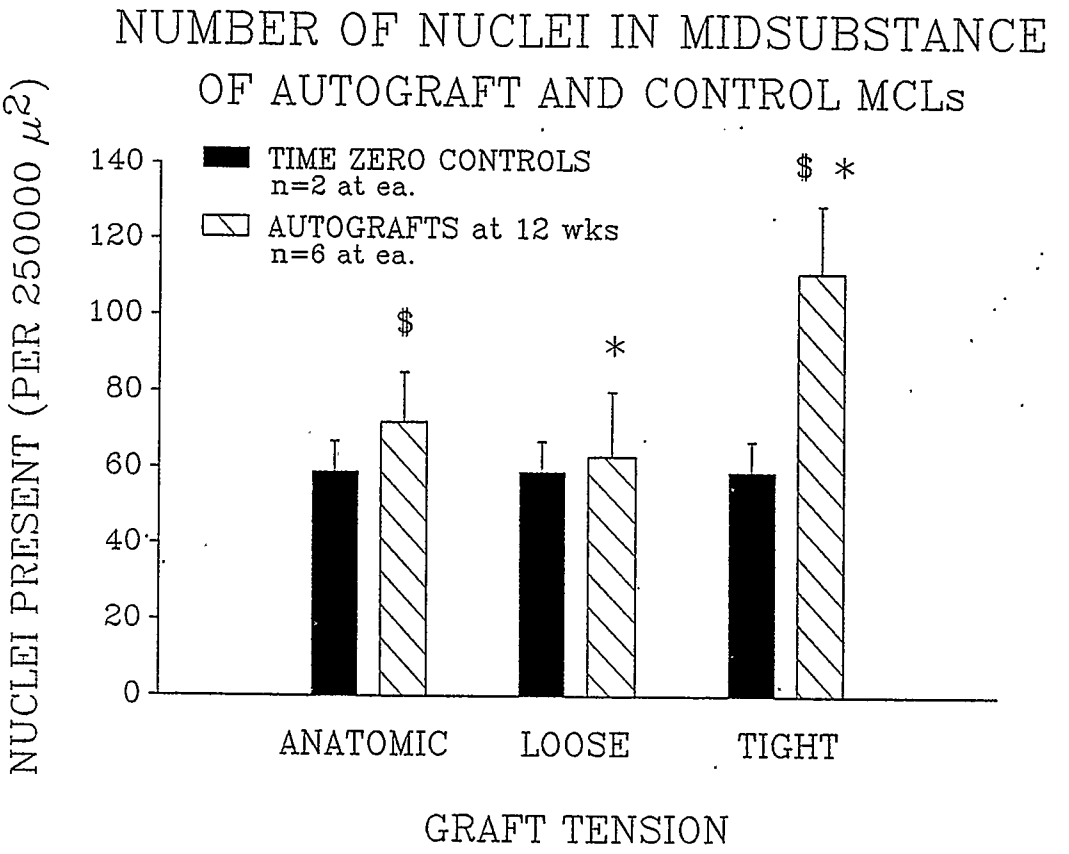

Figure 23 Cell Number: Plot of the number of nuclei in 12 week autograft midsubstance MCL implanted under different tensions as compared to their time zero controls. The Tight tension is significantly more cellular than the Anatomic and Loose tensions ($, * $p = 0.007$). Note that all 12 week autografts under tension are more cellular than their time zero controls.

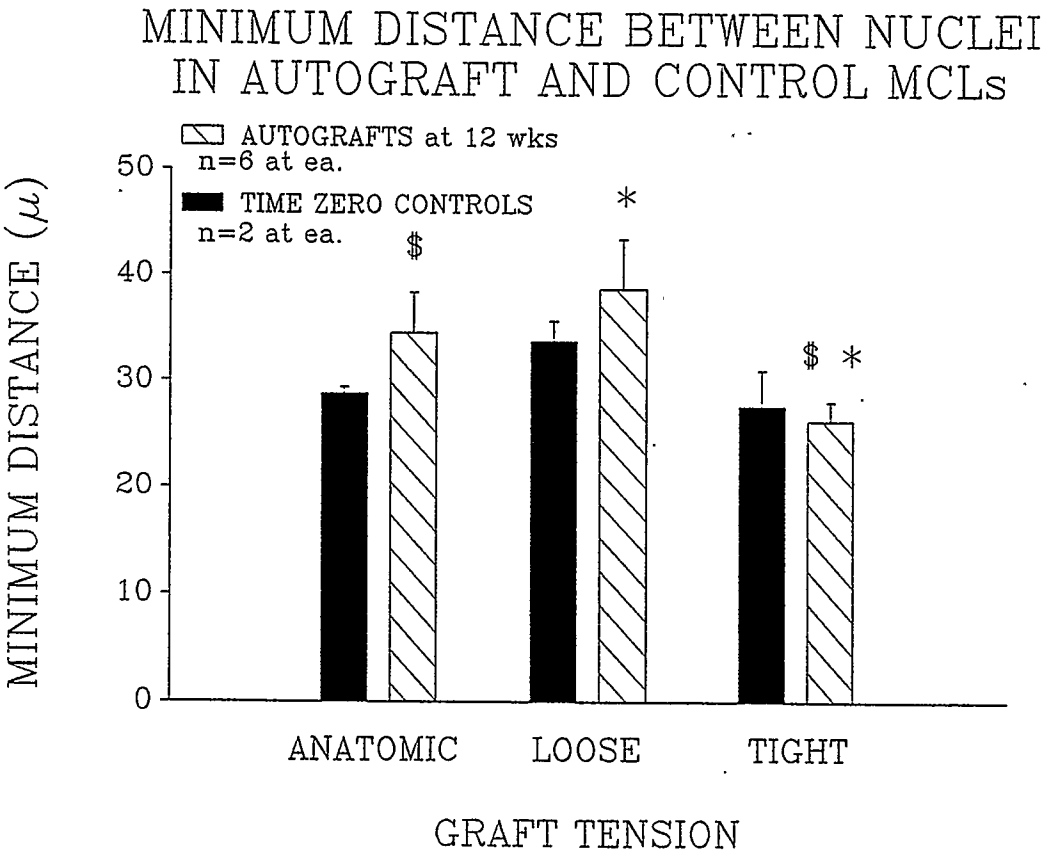

Figure 24 Cell Distribution: Plot of the minimum distance between nuclei in 12 week autograft midsubstance MCL implanted under different tensions as compared to their time zero controls.. The Tight tension has nuclei significantly closer together than the Anatomic and Loose tensions ($, * p = 0.008). As compared to their time zero controls, note that both the Anatomic and Loose autografts have nuclei getting further apart after 12 weeks, while the Tight autografts have nuclei coming closer together.

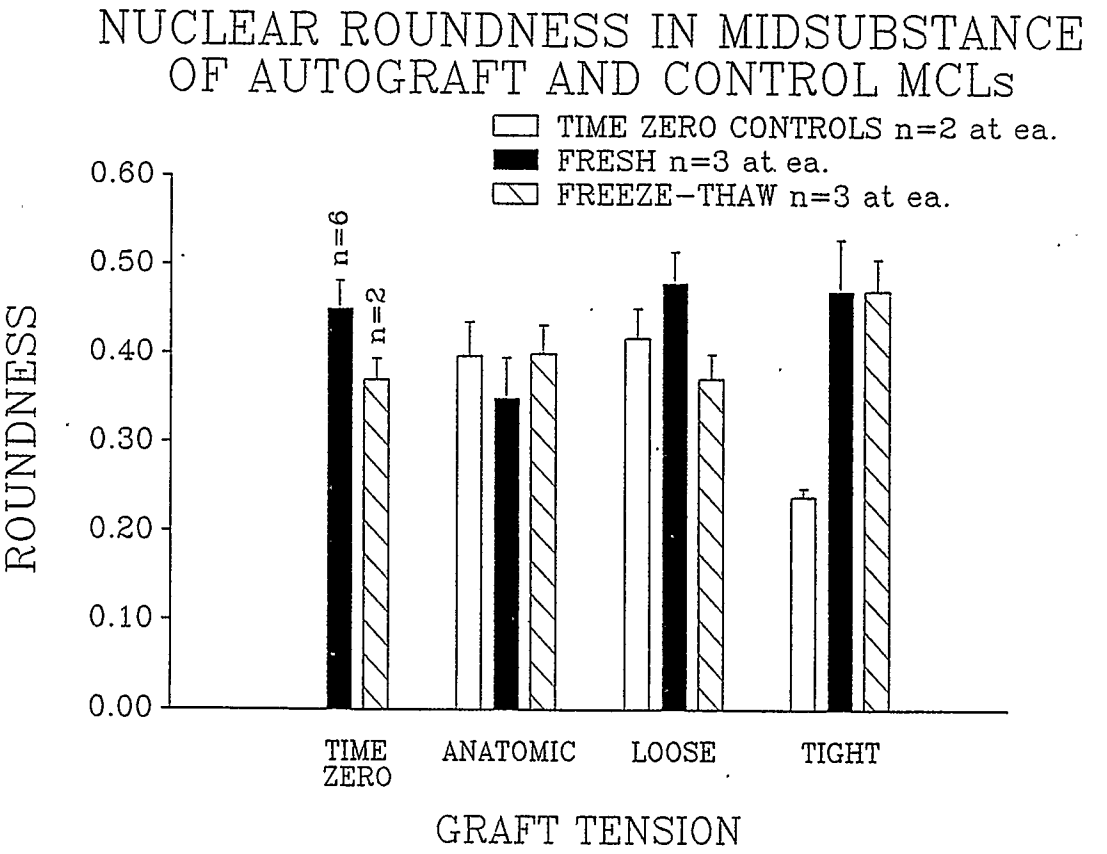

Figure 25 Cell Shape: Plot of nuclear roundness in 12 week autograft midsubstance MCL implanted under different tensions and different cellularities as compared to their time zero controls. There is evidence of an significant interaction for nuclear roundness, $p < 0.001$. Therefore, there is an effect of graft tension on cell shape which depends on the effect of the graft treatment (fresh versus freeze-thaw).

somehow decreased their laxity at 12 weeks (Figure 22). This phenomenon will be discussed further in Chapter 4.

Image analysis of these 12 week autografts showed that while all grafts increased in cellularity, as compared with normal MCLs, some cell parameters measured in ligament midsubstance were differentially affected by the graft tensions. Tight autografts in particular, demonstrated the highest hypercellularity and the minimum internuclear distance measures, confirming that tight autografts had the highest density of cells. Several explanations are possible as to why this was the case. Similar to the frozen-thawed autografts, the increase in number of cells could be the result of cellular proliferation, either intrinsic or extrinsic. Alternatively, tension on the MCL may somehow allow easier penetration of extrinsic cells into the midsubstance. More likely, tension on transplanted tissues may cause degradation of the ligament matrix due to an injury response, thereby forcing cells closer together in the midsubstance.

Despite the differences in cellularity, all autografts appeared to have the similar cell shapes by 12 weeks; that which is indistinguishable from cell shapes in the normal MCL. Autografts at all tensions could therefore be said to be populated with "ligament-like fibroblasts" at this interval of healing, at least according to the nuclear indices which were quantified. It is interesting to note that the cell shape had changed substantially in the "too tight" autografts during healing. This change could have been caused either by intrinsic cells regaining their pretensed shape as the matrix relaxes with time after implantation or by original graft cells being replaced with either intrinsic or extrinsic cells.

In summary, it can be concluded from this study that all MCL autografts displayed good healing at 12 weeks despite differences in implantation tension at time zero. Laxity was removed from loose grafts by some mechanism yet to be determined. All grafts were very hypercellular, but cells in the midsubstance

appeared, by shape and distribution, to be indistinguishable from "normal ligament cells". The largest tension caused the most cellular change, but excessive tension was not "harmful" in the sense that subsequent graft cells still appeared to have normal MCL cell characteristics.

Even the midsubstance of autografts in this model, placed under a range of tensions, therefore, contain an increased number of normal appearing, "ligament-like" cells at 12 weeks of healing. The further implications and qualifications of the above observations will also be discussed in Chapter 4.

CHAPTER 4
DISCUSSION

Several important points for discussion have been raised from the preceding chapters. The overall discussion in this chapter will deal with several significant issues, as well as presenting speculated explanations for the findings described.

To begin with, it should be made clear that I chose to focus on cell morphology in ligament midsubstance as the primary outcome measure studied due to its potential relevance to cell function and ultimately, therefore, to matrix integrity in grafted tissues. This assumption of a connection between cell morphology and function was not addressed specifically in this thesis and remains speculative. Further, it must be pointed out that to establish such a connection, several tissues will probably have to be analyzed for their cell appearances to establish that the range of shapes and distributions that I have attributed to ligament fibroblasts do not overlap with those of other tissues. In the absence of these controls, therefore, I can only refer to the cell parameters described in this thesis as being possibly unique for ligament fibroblasts in the model used. I can, however, compare these indices across the various treatments of the rabbit MCL (freeze-thawing, tension and autografting) to define changes in morphology. The proof of any functional significance of these measures must await other studies (biochemical, biomechanical, etc.).

Before attempting to study the MCL transplant model in detail with respect to cellular populations, it was first realized that no acceptable definition of the normal control MCL fibroblasts existed (Arnoczky, 1983; Frank et al., 1985). To address the deficiency of a proper fibroblast definition, several biochemical and

morphological techniques were used to create a quantitative baseline for defining the cells in the midsubstance of the rabbit MCL. The result was the first complete description and quantification of rabbit MCL fibroblast numbers, distributions, shapes and viability within its normal environment; the ligament collagen matrix. The necessary starting point for the study of changes in cell characteristics during MCL autografting was therefore enhanced by having this quantitative definition of "normal" fresh MCL fibroblasts.

It must be emphasized that in order to quantitatively define normal ligament cells, this study sampled a well controlled area of ligament, the pure midsubstance, thus eliminating the assessment of cells in the epiligament and insertional zones of the MCL. The end result is that instead of determining the entire spectrum of ligament cell shapes, I have defined the shape of a specific population of cells in the MCL midsubstance. From this sampled homogeneous cell population, I was not able to define the outer limits of ligament cell shape.

In the development of a frozen-thawed MCL autograft, I then realized that the literature lacked a consistent quantitative description of the effects of freeze-thawing on the morphology of ligament fibroblasts (Amiel et al., 1986a,b; Kleiner et al., 1986; Jackson et al., 1989; Bush-Joseph et al., 1990). Therefore, freeze-thaw controls at time zero (implantation) for the MCL were developed. Freeze-thaw controls which I initially hoped would be acellular (in order to provide a totally acellular graft at time zero), in fact, had very normal cellular appearances and contents. Evidence from the computerized histomorphometry and quantification of DNA showed that static frozen-thawed MCL cellular measures (ie. cell numbers, shapes and apparent distributions) were almost identical to normal MCL. Yet, when the viability of these frozen-thawed ligaments was measured, cells appeared to be non-functional as determined by a metabolic assay for collagen synthesis and autoradiography of those labelled sections. These results

demonstrated that multiply frozen-thawed MCL autografts were not acellular tissues, but they were at least metabolically altered and likely metabolically inactive. These freeze-thaw results contradict other published results stating that freeze-thawing renders connective tissue "acellular" (Amiel et al., 1986a,b; Kleiner et al., 1986). Those previous studies showed that tendon cultured for 21 days had no evidence of a viable cell population, but failed to include the necessary controls (ie. knowledge of what percentage of a normal fresh ligament cell population can grow in culture conditions).

The analysis of the MCL autografts at 12 weeks proved the autograft model was successful in that all animals appeared healthy and both the fresh and frozen-thawed autografts displayed gross, as well as microscopic signs of healing. These observations of both bony and soft tissue recovery are similar to the previously published work of Sabiston and coworkers (1990b).

Aside from the subjective observations made during dissection of the MCL autografts at 12 weeks, no other assessments were performed on the entire bone-ligament-bone complex. At this point, my investigation was focused solely on the healing process of the MCL midsubstance. It is recognized that the surgical factors under investigation most likely affected the ligament, as well as, both bone grafts. Clearly, this MCL transplant can not be studied wholly without considering the influence that the bone grafts had on the overall performance of the transplant. The reason for selecting the centre of the MCL for analysis was due to the fact that all previous studies of dense connective tissue grafts found the misubstance to be the last area repopulated with cells. Thus, it is crucial to have cells present in the midsubstance since they are responsible for matrix repair when the midsubstance, generally the "weak link" in normal ligament complexes, "stretches under load". Midsubstance MCL was also chosen for study since it is technically the easiest and most homogeneous portion of the MCL to define.

Closer examination of the MCL midsubstance showed no autograft was acellular after 12 weeks of healing and, in fact, all autografts were hypercellular compared to the normal MCLs. This phenomenon of increased cell to matrix ratios in transplanted tissues is well documented in animal models (Liu, 1975; Clancy et al., 1981; Puddu and Ippolito, 1983; Nikolaou et al., 1986; Ballock et al., 1989), as is the gradient of cells decreasing from surface to deep within the graft midsubstance (Sabiston et al., 1990c). Such surface cellularity of autografts makes me suspect that cells are likely inflammatory in origin and may represent a surface repair response of the damaged tissue. This collection of cells would probably be of extrinsic origin and may include white blood cells carried into the transplanted ligament by newly formed blood vessels. Yet, unlike any previous studies, my studies have shown quantitatively that the majority of midsubstance cells in all autografts appear "ligament-like".

Within the ligament midsubstance, there appeared to be two types of mononuclear cells. The first type had the nuclear size and appearance of cells which formed the ligament vasculature, defined as endothelial cells. The second cell type was distributed throughout the midsubstance and were the cells analyzed and defined as "fibroblasts". My findings from image analysis on the cell population showed evidence that the autograft "replacement" cells had similar characteristics of cells found in normal MCL, called fibroblasts. Based on the assumption that other possible mononuclear cells, such as inflammatory and white blood cells which may invade grafts would have different distributions, shapes and sizes of their nuclei, no direct evidence was found for extrinsic cell invasion. A number of investigators (Liu, 1975; Shino et al., 1984; Laws and Walton, 1988) support the observations that there appear to be no migrating inflammatory cells within the ligament autograft.

The similarity of the cell populations between fresh and frozen-thawed autografts may have been due to the survival or at least the preservation of some original graft cells following transplantation. Increases in cell numbers, however, suggest at least some internal cellular proliferation if not the significant addition of extrinsic cells. If this latter situation is the case, then it is clear that extrinsic cells do have the ability of invading ligament matrix and taking on the appearance of normal ligament fibroblasts.

Results of the biomechanical studies that I have reported represent the first time that a relationship has been proven between connective tissue cell morphology and their matrix mechanical environment. More specifically, this study is the first demonstration of an *in situ* change in cell shape with ligament loading. It was found as tension increased on the normal MCL that the shape of the midsubstance nuclei became less round. This finding has important implications for any study attempting to deduce information from cell shape, since the shape of cells could possibly be only the result of the tissue's loading conditions during dissection. In other words, the historical technique of defining cells by their shape may not be legitimate without first knowing the loading environment of the tissue.

In the subsequent study on how initial tension affects the shape of cells in ligament autografts, it was found that at 12 weeks autografts which had been implanted originally under different tensions all had the same cell shape. The only tensioning effect was observed in grafts which were implanted "too tight", in that these grafts had the highest cell densities. Tension therefore appeared to somehow stimulate either cellular proliferation or ingrowth. Freezing similarly stimulated hypercellularity and the combination of tension plus freezing showed an additive effect. This increase in numbers of cells could be the result of cellular

proliferation (intrinsic or extrinsic) or matrix degradation mechanism that forces cells closer together in the midsubstance.

A second unique observation from my studies relates to the fact that autografts implanted under a loose tension, so that the MCL actually buckled, were found to have somehow decreased their laxity by 12 weeks. Simply stated, the loose autografts became tight over time. One possible explanation for this tightening could be that infiltrating or proliferating cells may have produced either "scar" tissue or new ligament tissue to either infiltrate or surround the original collagen fibers. The observation of increased cross-sectional areas in the loose autografts supports this theory. Alternatively, the decreased laxity could be a result of simply increasing water content in autografted ligaments. Biochemical or ultrastructural studies of the matrix will clearly be required to resolve these possibilities.

At this point it is necessary to discuss the accuracy of calculating the mean cell shape for each autograft treatment by obtaining an average shape for each MCL. Since averaging does not always accurately describe a population, the entire distribution of cell shapes for each individual MCL was studied. No MCL autografts were found to have greater than one mode for cell shape. The discovery of only one general cell shape in 12 week MCL autografts was interesting, since a collection of connective tissue studies describes different classes of fibroblast shapes in wound healing. For example, Peach and co-workers (1961) distinguish two types of fibroblasts in 5 day regenerated tendon; type A being elongated and termed "migratory" and type B a rounder cell termed "synthesizing". They speculate these distinguished cell types all merge into one cell shape by six weeks. It can be speculated that the study of 12 week autografts limited the analysis to a stabilized cell population, perhaps missing a more dynamic phase of early recovery involving greater than one cell type.

The results from the frozen-thawed autografts raise some important issues as to the nature of the "replacement" cells in the ligament grafts. The concepts of interest can be identified as normality, source and mechanism of the "replacement" cells. In other words: Normality - what type of cells are they; Source - where did the cells originate; Mechanism - how did the cells arrive in the autograft?

My study did not determine the source of "replacement" cells. In order to identify the origin of the cells as intrinsic or extrinsic, the graft tissue ideally would have been completely acellular at implantation. Therefore, if the graft became repopulated, one could say with absolute certainty, that the cells were of an extrinsic origin. Conversely, if the graft had remained acellular, one could have deduced that "replacement" cells were intrinsic in origin. Unfortunately, neither was the case. Grafts were all populated with fibroblasts at the time of implantation and showed fibroblastic characteristics at sacrifice. I would speculate that there are three possible locations where the cells may have originated. First, the "epiligament" could have been a source of cells that when signalled, caused cells to migrate to the damaged areas of the ligament. This explanation could be supported by the observation that badly damaged frozen-thawed autografts had a hypercellular "epiligament" and an increased cell density from surface to deep. Sabiston et al. (1990) observed this same cellularity pattern in their MCL autografts. A second source of "replacement" cells could be persisting fibroblasts or proliferating intrinsic fibroblasts, as some cells were definitely present in the graft at implantation. I find this second explanation unlikely however since there is little evidence that intrinsic MCL cells can regenerate and it seems unlikely that they could proliferate to the degree required to achieve the high cell density shown in the frozen-thawed autografts. A third source of cells could involve differentiation of either white blood cells or endothelial cells brought in by the grafts revascularizing. Many studies have shown that ligament transplants undergo

early revascularization (Puddu and Ippolito, 1983; Nikolaou et al., 1986; Sabiston et al., 1990a) thus providing considerable opportunity for this route of repopulation by 12 weeks. Further study will be needed however to determine which of these mechanisms is involved in graft cell proliferation.

In reviewing the results on implanting grafts fresh versus the freeze-thaw procedure, no statistical differences could be found between the end products at 12 weeks for these two graft types. However, three important observations suggest that these two graft types had slight differences in cell numbers and cell shapes, thus implying that the frozen-thawed autografts may be distinct from the fresh. First, the cell population at 12 weeks in frozen-thawed grafts appeared to be more fibroblast-like than the original non-functional, slightly flattened cells occupying the frozen-thawed grafts at the time of implantation. Second, normal MCL was surrounded by an outer loose, cellular layer called the "epiligament", which was also found surrounding all the fresh autografts. Unlike the normal "epiligament", the layer surrounding frozen-thawed autografts was thicker and more cellular, thus emphasizing the ligament's surface-to-deep gradient of decreasing cell density. Third, the frozen-thawed autografts appeared to have a more cellular midsubstance than the fresh autografts. Collectively, the results from the quantified similarities and observed differences between the two types of MCL autografts suggests that even MCL grafts treated with multiple freeze-thawing can become ligament-like.

Limitations

As investigators become more interested in the cellular make-up of connective tissues, there will be an increasing need to visualize cells within their matrix. Disappointingly, there have been no cytoplasmic stains identified which can successfully stain these cells in their matrix environment. For this reason, only

information derived from stained nuclei was used to characterize the ligament fibroblast in my studies. I did find that the fibroblast nucleus was measurably altered, but I do feel that more definitive results could probably be obtained if entire ligament cells could be visualized and characterized.

Other limitations in my studies also become apparent when my measures of analysis are scrutinized. Better information on cellular functional states could have been obtained through metabolic studies on cells in all series, rather than just the controls. Instead, my studies focused almost entirely on histomorphometry with only a few parameters being selected to describe nuclear characteristics in a two-dimensional fashion and in a small sampling area. In order to obtain the histological sections for histomorphometry, some errors and artefacts clearly existed in the sampling scheme, dissection and fixation of tissues.

Another limitation of my studies involves the fact that MCL autografts were studied only at 12 weeks of healing. In order to better understand the natural history of the autograft healing process, it would have been helpful to analyze more than one point in time. As well, my studies lacked controls of other cell shapes to validate the measurement scheme over an entire spectrum of shape factors.

A final limitation may be the fact that my studies focused solely on the midsubstance of the MCL. The midsubstance was chosen because it should be the last area of an MCL autograft to become repopulated with cells and should therefore act as a good comparative index of ultimate cellular recovery in the MCL transplant at equal points in time. Unfortunately, it is possible that ligament midsubstance may not be the area of the ligament complex where the effects studied are most dramatic. This factor, of course, can only be investigated by further sampling of the MCL complex.

Future Directions

Further investigation into additional points of healing time is needed, including the long term result of MCL autografting. Studies are also needed which are directed at improving the measures used to determine cellular characteristics in tissues. Perhaps different methods may be found for removing the original cell population of a tissue or develop new techniques for measuring cells. In the more immediate future, metabolic studies should be undertaken to determine the full effects of multiple freeze-thaw on ligament cells and analysis of cell populations should be performed in areas of the MCL complex other than the midsubstance.

Of recent concern affecting all areas of tissue allotransplantation is the possibility of infecting hosts with donor HIV virus infected tissue. This concern was recently expressed at the communication of a very successful study on ACL transplantation in humans (North and Zirschky, 1990). The presentation of this study's good results were overshadowed by the review of another investigator's most recent discovery. Mankin (1990) found it was possible to isolate the HIV virus from previously radiated cartilage and bone tissue. This problem of having to deal with tissue donated from known or suspected carriers of the human immunodeficiency virus (HIV), opens up the whole field of transplantation to scrutiny. Future research will have to address this important problem and develop infection control guidelines for the assessment of HIV virus free graft tissues.

CHAPTER 5
CONCLUSIONS

In order to perform a complete analysis of the cell morphology of transplanted ligaments, it was found necessary first to define and quantify ligament cells in normal MCL. Since the present knowledge on ligament cell morphology was deficient and solely qualitative, my study focused on developing significant measures for the analysis of normal midsubstance MCL cellularity and viability. Ligament cellularity was determined by quantification of DNA and image analysis, ligament viability by a metabolic assay for collagen synthesis and autoradiography and by using image analysis, became the first reporting of fibroblast characteristics as quantified by cell number, distribution and shape. All the qualitative and quantitative study resulted in the formation of a baseline on normal fibroblast characteristics in the rabbit MCL which could then be used in the comparison of abnormal MCL.

The contralateral left MCLs, from animals selected for the autograft study, remained unoperated and served as internal controls. The image analysis results from the contralateral left MCLs were not found to be significantly different and yet not identical to normal control MCL characteristics. The results suggest there are subtle, but not deleterious effects on contralateral ligament cell behaviors resulting from the surgical procedure performed on the right autograft MCLs.

In determining the full effects of multiple freeze-thaw on MCL at time zero, the hypothesis that the MCL would be devoid of any living cells was proven wrong. The study on graft treatment found that frozen-thawed MCL described by morphology and DNA content was unchanged from normal MCL, yet the existing

cell population appeared altered and "non-functional" as measured by cellular metabolism.

The above study showed that frozen-thawed autografts were not totally "acellular" at the time of implantation. Following 12 weeks of healing, both the fresh and the frozen-thawed autografts displayed good healing and were hypercellular with a gradient of cells decreasing surface to deep in the midsubstance. Regardless of treatment, all the cells in the autografts were proven quantitatively to be morphologically consistent with normal ligament cells, except for the cells defined as endothelial cells. No significant differences could be detected in cell numbers, distributions or shapes between fresh and frozen-thawed autografts. There is speculation that both types of autografts had survival of original graft cells following transplantation, plus possibly some internal proliferation or extrinsic cells being added. Additionally, a trend was observed that frozen-thawed autografts tended to have a slightly different cell morphology, possibly caused by a heightened injury response from a more damaged matrix. Though all autografts appeared populated with ligament-like cells, the origin of these "replacement" cells and their mechanism of arriving in the midsubstance clearly requires further investigation.

A second aim of the MCL autograft study was to see if ligament graft cell parameters could be influenced by the initial graft tension at implantation. In order to perform a complete analysis of the effects of tensioning on MCL autografts, the effects of tensioning on normal MCL cells were first determined. The tension study is the first demonstration of *in situ* changes in cell shape with ligament loading. Specifically, as tension increased on normal MCL the nuclei became less round with increased maximum diameters and decreased minimum diameters. Yet, the exact mechanism for how nuclear shape changes remains unknown and requires further study.

Irrespective of the degree of tension placed on the MCL autografts, after 12 weeks of healing, all midsubstances displayed increased cellularity but all cells present appeared to have the shapes of normal ligament cells. It is possible the cells with deformed shapes at the time of implantation, managed to adapt to the applied tension and regained their normal appearance by 12 weeks of healing. On the other hand, perhaps the grafts under tension increased the laxity of their collagen matrix to allow the deformed cells to regain their normal appearance. It is interesting to note that the "too tight" autografts had the highest cell densities, possibly caused by matrix degradation forcing cells closer together. Also, autografts implanted under a "loose" tension were found to have a decreased laxity by 12 weeks, perhaps by the production of "scar" to keep the ligament taut.

In conclusion, the autograft study showed that when implanting ligament grafts fresh versus frozen-thawed, no real differences could be detected between the end products at 12 weeks of healing. Similarly, in determining whether graft implantation tension affects the success or failure of MCL autografts, it was found that despite the tension, by 12 weeks all MCL autografts had similar cellular characteristics. Clearly, the cell morphology of ligament autografts is only one of many important factors involved in determining the overall outcome of transplants. Further study is needed to determine the long term consequences of the factors studied in this investigation and the role they play in affecting graft cell biology in the more complicated situation of MCL allotransplantation.

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