Tissue engineering of knee ligaments

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Knee ligament injuries are the most common injury sustained in major sports with anterior cruciate ligament (ACL) and medial collateral ligament (MCL) tears accounting for more than 27% of the knee injuries combined. The gold standard for treatment of ligament injuries remains controversial. Non-operative methods can result in decreased strength and prolonged recovery while surgery does not ensure graft incorporation and return to pre-injury function. Tissue engineering technology incorporates biological, chemical, engineering and materials science principles, and can facilitate ligament healing. The purpose of this article is to review current tissue engineering approaches for knee ligament injuries. Intra-articular ligaments, such as the ACL and posterior cruciate ligament (PCL) experience a very different environment than extra-articular ligaments such as the MCL and LCL. Therefore, the approach to healing in these two areas are different. Tissue engineering related to ligament healing consists of three components: cells, scaffolds, and the healing environment. So far, neither an ideal cell source or optimal scaffold has been established. Biological environment modification is in its clinical infancy, and is currently being investigated to address the appropriate stimulation through the application of growth factors. The mechanical environment, in turn, can be reasonably optimized by performing ligament reconstruction surgery in an anatomic fashion. Through advances in tissue engineering, surgeons may one day be able to offer solutions to help patients return to their desired activities faster and stronger.

Key words: **Tissue engineering - Knee - Ligament, injuries.**

Ligaments can be defined as dense bands of collagenous tissue that connect bones across a joint. The ligaments function mainly as static stabilizers of joints throughout their range of motion when a tensile load is applied. They also provide proprioceptive sensory feedback to motor control.[1-3] Ligaments generally exhibit tensile strengths of 60-80 MPa and failure strains of less than 10% of their lengths. Ligaments behave as non-linear viscoelastic structures that exhibit small amounts of stress-relaxation as well as creep when exposed to fixed stresses.[4] These structures tend to be resistant to fatigue damage, perhaps because of a combination of these characteristics, native healing and remodeling processes.

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The knee contains the anterior cruciate ligament (ACL) and posterior cruciate ligament (PCL), which are intra-articular, while the medial collateral ligament (MCL) and lateral collateral ligament (LCL) are extra-articular. They are oriented such that they stabilize the knee against anterior, posterior, valgus, and varus forces, respectively, as well as provide resistance against rotational forces.[5, 6] Knee ligament injuries are the most common injuries sustained in major sports.[7] The ACL is most frequently injured, accounting for 20.3% of all athletic knee injuries, followed by the MCL (7.9%), LCL (1.1%), and PCL (0.65%).[8] Injuries to these ligaments can frequently lead to instability and subsequent changes in the kinematics and biomechanics about the joint. Potential long-term consequences include clinical instability, meniscal and chondral damage if left untreated, and osteoarthritis.[9, 10]

The preferred method of treatment is different amongst the four ligaments, particularly between the intra- and extra-articular ligaments. Each ligament varies in its intrinsic healing potential and function. In general, ACL and grade III PCL injuries benefit from surgical treatment to restore the integrity of the ligament and minimize the sequelae from chronic instability. On the other hand, isolated MCL and LCL injuries are usually treated non-operatively, with operative management reserved for cases when they are disrupted in combination with other ligamentous injuries or exhibit gross instability.

Spontaneous healing cannot be expected with complete rupture of the intra-articular ligaments. The gap between the ruptured ends and the disruption of the synovial sheath cannot hold blood at the site of healing,[11] depriving the damaged ligament of the cellular source and various growth factors derived from blood that are critical for the normal healing process. Additionally, the relative lack of vascularity,[12] intrinsic cell deficiencies,[13] and cell loss after rupture [14] are additional factors associated with a poor healing response of the intra-articular ligaments. Clinically, conservative treatment of the ACL does not re-establish the integrity of the ligament [15] and often places the patient at increased risk for meniscal tears.[16] Some active individuals are able to cope with a deficient ACL due to contributions from other ligaments as well as the bony morphology; however this complex relationship is poorly understood. Therefore, in active patients an injured ACL is usually reconstructed. Similarly, PCL injury is often indicated for reconstruction surgery in the presence of other ligamentous injuries.[17] Conservative treatment has been widely accepted for the treatment of isolated PCL injuries.[18] However, long-term studies of PCL deficient patients demonstrate detrimental effects on the articular cartilage on the knee.[19]

Isolated injuries to the extra-articular ligaments are more likely to be treated non-operatively.[20] The MCL has the best intrinsic healing potential amongst the four major knee ligaments,[21] but the native mechanical properties cannot be fully restored with conservative treatment.[22, 23] An isolated LCL injury is rare, and is primarily treated non-operatively.[24] However, current research has not concluded whether or not the healed ligament is restored biologically and biomechanically to its pre-injury state by non-operative treatment. The LCL is often associated with other ligamentous injuries requiring operative treatment; in these scenarios it can either be repaired in the setting of an acute bony avulsion, or reconstructed for midsubstance or chronic tears.

Tissue engineering can be defined as a multidisciplinary field that incorporates the application of biological, chemical, engineering and materials science principles, and facilitates the improvement of ligament healing in a variety of ways.[25] Tissue engineering combines the design, production or culture and application of materials to augment tissues of the body. These technologies encompass many forms, including the production of scaffolds designed to direct growth of new tissue to membranes intended to modulate the pattern and magnitude of the native healing response. This emerging field of biotechnology has the potential to drastically improve treatment modalities across many fields, including orthopedic surgery. To date,

few engineered solutions have reached clinical acceptance, but the increased research efforts in this field may result in significant treatment improvements in the future.

Targeted therapies utilizing tissue engineering principles could revolutionize the treatment of knee ligament injuries. An engineered substitute for the torn ligament would obviate the need for either tendon autograft or allograft reconstruction. Autograft harvest carries an intrinsic morbidity to the donor sites,[26], whereas allograft tissue has the potential for viral infection. Moreover, techniques to sterilize allografts have been proven to compromise the structural integrity of the graft.[27] In addition, a recent study of ACL reconstructions demonstrated that allografts when used for reconstruction in younger patients had higher failure rates compared to autografts.[28] Problems with this modality remain, including the extended recovery time after reconstruction surgery, which usually takes up to 6 months to 1 year,[29] undesirable biological reaction, delayed incorporation [30] and finally tunnel enlargement.[31] Future tissue engineering techniques may provide a solution in the form of a controlled and/or enhanced healing response. As for conservative treatment of the injured MCL/LCL, biological enhancement can help expedite the healing process and achieve the native ligament mechanical properties.

Tissue engineering related to ligament healing can be divided into three components: cells, scaffolds, and the healing environment. The purpose of this review is to introduce current tissue engineering approaches for knee ligament injuries with respect to each component of ligament healing. Knowledge of both the potential and limitations of current tissue engineering can contribute to the clinicians' consideration of the tissue engineering approach and help direct further research for enhancing treatment.

The ligament healing response

Ligaments are composed of two major components: cells and extracellular matrix. Approximately two-thirds is made up of water, while the remainder consists of solid components, mainly collagen (types I, III, VI, V, XI and XIV), which accounts for up to 75% of the dry weight of the ligament structure. The remainder is composed of proteoglycans (<1%), elastin and glycoproteins[1]. The majority (85%) of the collagen is type I.

The internal structure of ligaments has been described as a set of fascicles formed by a longitudinally oriented group of collagen fibers. When evaluated histologically, each of these fascicles contains a hypocellular and hypovascular appearance, suggesting that ligament cells could be functionally isolated and relatively inert. However, it has been demonstrated that the ligament cells are connected in a complex three-dimensional network allowing them to coordinate their behavior similarly to other tissues.[3] Of note, ligament fibroblasts are metabolically active showing constant, yet slow, cell renewal and matrix turnover;[32] this behavior will have a direct impact on the speed and pattern of the ligament healing process.

Ligament morphology is not the only factor that impacts the ability of ligaments to heal. The native environment is also a variable that influences the healing potential of a ligament. Extra-articular ligaments, such as the MCL, respond to injury in three overlapping phases: I) hemorrhage and inflammation; II) cellular proliferation and matrix deposition; and III) long term remodeling and maturation.[23, 25, 33]

Phase I takes place within the initial 72 hours of the injury, as a hematoma forms and fills the gap between the torn ends of the ligament. This hematoma functions as a bridge and chemotactic scaffold where inflammatory cells such as leukocytes, monocytes and macrophages secrete cytokines, including growth factors, to initiate the healing process.[25] Then, a considerable hypertrophic response takes place between the disrupted ends increasing both, the vascularity and blood flow to the ligament.[1]

Phase II is characterized by the arrival of fibroblasts that gradually occupy the injured area and synthesize predominantly type III collagen, which leads to vascular scar tis-

sue formation, and, a small amount of type I collagen. The fibroblasts & inflammatory cells combine to form granulation tissue; this scar tissue is initially quite disorganized, with more defects when compared to normal ligament matrix.[1]

Phase III consists of synthesis of a higher quantity of type I collagen, which is oriented along the functional axis of the injured ligament. Through the remodeling process, these collagen tissues will be oriented along lines of stress, similar to the normal ligament.[23, 33].

During phases I and II, the infiltrated cells produce various cytokines. The expression of these endogenous growth factors such as platelet-derived growth factors (PDGF) and transforming growth factor beta (TGFβ) achieve a higher level within the first 7 days after injury and return to their baseline level after 2 or 3 weeks.[34] The sequence of expression of growth factors in the initial phases of the healing response is essential to fill the gap between the torn ends of the injured ligament as well as to restore the ligament function.

On the other hand, the ACL and PCL have a less successful healing response, partly secondary to their intra-articular environment. Because the native ACL is surrounded by synovial fluid, when it is injured, the ACL dissipates its bleeding into the joint space after the disruption of the thin synovial sheath. Dissipation of blood into synovial fluid does not allow for the formation of a localized hematoma, which prevents the subsequent chemotactic cytokine expression, thereby limiting the secretion of growth factors. In contrast, an intact synovial sheath facilitates the initial healing response and scar formation. Hefti et al. demonstrated in animal models that an injured ACL with an intact sheath can result in partial ligament healing by preserving its resulting hematoma.[11] Other studies have shown that ACL cells can multiply and produce matrix for up to 1 year after rupture and can migrate to a scaffold *in vitro*.[35] These findings demonstrate that cells of the native ACL are more limited by their environment than by their own intrinsic healing capacity. However it is also known that the fibroblasts in the ACL have a low proliferation rate, lower mobility, decreased metabolic activity and lower matrix production, when compared with the other ligaments.[12, 36] Additionally, the stumps of the torn ACL significantly shrink because of high residual strain experienced by its remaining fibers, rendering it even more difficult for the initial hematoma to overcome this gap.[37]

Cells

Tissue engineering has the potential to improve the characteristics of damaged ligaments, and is dependent on the presence of specific cells. Without the proper cells in place, the potential for enhanced healing is less likely. Therefore the selection of the cellular source to be employed in the new ligament construct is vital for success. The ideal cell for tissue engineering must have robust proliferative potential and the capacity to produce sufficient quantities of extracellular matrix (ECM). Transplanted cells must posses these qualities to effectively replace and remodel the injured tissues. In addition, cells must be compatible and have the capacity for adhesion to the scaffold and/or the surrounding tissues in order to locally target its effects. Depending on the cells in question, biological or mechanical stimulation can also facilitate the healing process induced by tissue engineering products. Additionally, the availability of the cell source must also be considered when constructing tissue engineering technologies.

Fibroblasts have been utilized in combination with bio-scaffold ACL substitutes[38,39] but their effectiveness in ligament healing is controversial. Fibroblasts extracted from ACL tissue can migrate into the ECM,[40] and can change shape and deliver collagen fibers in response to mechanical stress.[41] Bellicampi *et al.*[38] transplanted the fibroblast from the ACL and skin with a collagen scaffold into the rabbit knee and demonstrated the enhanced viability of the transplanted ligament by the fibroblasts. They also found

Figure 1.—Muscle derived stem cells. The marker PAX7 is stained red within the stem cell nuclei using an immunocytochemistry technique. Each non-stem cell nucleus is stained blue with DAPI.

superior proliferation of the skin fibroblast compared to the ACL fibroblast. Although the concept of using skin fibroblasts may seem counterintuitive because the skin does not function like the ACL, these cells are quite similar to ACL fibroblasts because they share the capability of producing both type I and type III collagen.[42] Furthermore, they are significantly easier to harvest when compared to ACL fibroblasts. Tremblay *et al.*[43] recently used the skin fibroblast with a bovine type I collagen scaffold to create ACL grafts *in vitro* that were surgically implanted into a goat model. The authors observed rich cell proliferation, type I collagen synthesis, graft vascularization, innervation and incorporation. This study demonstrated that bioengineered grafts are capable of participating in the *in vivo* remodeling process; however future studies have yet to demonstrate (through mechanical testing) that the quality of the tissue is equivalent to autografts and/or allografts.

An alternative to fibroblasts are the marrow stromal cells or mesenchymal stem cells (MSC), which possess the potential for differentiation into nonhematopoietic cells, such as fibroblasts, osteoblasts, chondroblasts, adipocytes and myoblasts.[44] The most commonly used origin of the MSC is the bone marrow, which can be collected using a needle biopsy;[45] this process yields "bone marrow stem cells" (BMSC). There are a variety of other stem cell sources, reported in the literature, including adipose cells, periosteum, synovial cells, umbilical cord, hamstrings, muscle, and the ACL.[46] All MSCs (Figure 1) are able to differentiate into various cells with healthy proliferative capacity, however there are subtle differences between the cells from the different donor sites.[47] MSCs have primarily drawn attention as a promising cell source because of their superior proliferative capacity [48] and ability to differentiate into different cell types[44], particularly when compared to fibroblasts.[49]

The proper method for directing stem cells in the ligament healing process is not fully understood. Altman *et al.*[50] demonstrated collagen fiber formation from bone-marrow cells resulting from mechanical stimulation. Other studies have reported that growth factors induce the differentiation of the stem cell into fibroblasts[51]. In an ACL reconstruction model, BMSCs were shown to improve ligament healing after ACL reconstruction, but the mechanism responsible for this enhanced healing is not clearly elucidated.[52] This topic is explored further in the section entitled "Environment".

Human ACL-derived stem cells also have been shown to enhance tendon-bone healing via enhancement of angiogenesis and osteogenesis in a rat model.[53] Cheng *et al.*[54] compared the proliferation and the ECM production between the BMSC and the ACL derived MSC, and showed the superiority of the ACL derived MSC in regards to both the proliferation potential and ECM productivity. This suggests that specific functions of stem cells may vary as a function of the stem cell origins and applied stimulations, although all MSCs seem to exert a positive effect on ligament healing due to their abundant versatility.

Many different cell types have been used in tissue engineering constructs. Based on their superior proliferation and pluripotent differentiation capability compared to fibroblasts, MSCs have great potential as a cell source in tissue engineering applications for ligament reconstruction. However,

there are subtle variations among the different sources of MSCs regarding their capability to heal, as well as their migratory and adhesion potential. Therefore, the appropriate choice of the cell source must be considered when planning stem cell constructs.

Scaffolds

When healing begins in damaged human connective tissues, cells at the border of damaged tissue and free space begin to secrete extracellular matrix and produce collagen as outlined in the ligament healing section. Although the human body's own healing capabilities are adequate, cells in the mature human do not seem to build organized structures effectively in free space. Fibroblasts are not able to reconstruct a ligament into an empty joint space; there simply is no mechanism to guide the growth of unbound, new tissue into a specific shape. However, the body is very adept at utilizing existing structures as a blueprint for remodeling. The gap between borders of an injured structure can limit cell migration and material production. Scaffolds are structures that provide cells with a geometric space for construction and can bridge gaps between separated tissue borders. Scaffolds prepared *in vitro* for use as ligament replacements or for augmentation in surgery have been eagerly anticipated. Unfortunately, no current implant exists that translates theories from the research world to standard clinical practice. Scaffolds can be constructed in an infinite array of shapes and sizes and are used in many other joints and organ systems in the body. In the knee, research has focused on either augmenting the healing process on the surface of extra-articular ligaments through the use of a small, implanted patch or in efforts to design an intra-articular graft to be used in ligament reconstruction procedures.

Certain parameters are important to consider in the engineering of tissue scaffolds. These include the bulk material used, the three-dimensional architecture of the scaffold, the mechanical properties and surface chemistry of the material, the microenvironment maintained within the scaffold, and whether the scaffold can support cellular seeding.[55, 56] Each parameter impacts the ability of the body to use the scaffold as a guide to build and remodel the neoligament. Scaffolds ideally function as a complete replacement ligament in the short term and promote neoligament growth. The structure of the scaffold allows the new and weaker structures to experience appropriate stresses to promote remodeling, and eventually will degrade over time as the neoligament completes its formation.

Bulk material

The bulk material of the scaffold has implications for how the scaffold will function. Historically, human tissue was the first scaffold to be used in surgery; every allograft used to reconstruct ligaments acts as a scaffold that becomes remodeled by fibroblasts along lines of mechanical stress. Animal tissue, in the form of porcine small intestinal submucosa (SIS), has been used in many applications through the body to try to augment the native healing process. Musahl *et al.*[57] investigated the use of SIS to enhance the healing of the MCL in rabbits. A surgical defect was created in the MCL of 20 rabbits that was subsequently either repaired with a strip of SIS sutured onto the two ends of the MCL or left untreated. The contralateral legs underwent sham surgery as a control. The stiffness and ultimate load of the SIS treated group were significantly higher than the non-operative group but less than the intact controls. SIS is, however, a controversial material and is not universally accepted due to concern for its potential to cause an immunologic reaction.

Another bulk material option comes in the form of biological polymers, such as collagen, hyaluronan and fibrin. Collagen provides strength in native ligaments, and so it is a logical choice for scaffolding. Its polymeric structure allows for chemical processing and crosslinking *in vitro* and it is easily seeded with fibroblasts. Murray *et al.* investigated the use of collagen hydro-

gels soaked in platelet-rich plasma (PRP). The collagen-platelet composite scaffolds have been shown to increase healing histologically in canine ACL defect models.[58] Additionally, this group has pioneered an ACL repair model in mini-pigs using primary suture repair at the transected ends of the ACL and wrapping the opposed ligament ends in a collagen-platelet scaffold.[59] This repair-scaffold construct resulted in significantly improved yield strength and displacement compared to suture repair alone as well as greater cell density on histology. It remains to be seen how the technique of suture repair performs against traditional tunnel based reconstruction techniques. Hyaluronan is a normal component of the extracellular matrix in ligaments but also can be crosslinked in polymeric chains. Funakoshi *et al*.[60] demonstrated that a chitosan-hyaluronan hybrid scaffold was capable of hosting rabbit ACL fibroblasts and promoting collagen production *in vitro*. Fibrin is perhaps the most natural scaffold material as it is the core structural material in blood clots. Meniscal repairs can be augmented with fibrin clots.[61] Currently, some institutions have begun to investigate the use of fibrin clots to augment grafts in ACL reconstruction,[62] however, no results have been published to date.

Finally, chemically produced synthetic polymers can be shaped into ligamentous structures. The first synthetic scaffolds were constructed of non-absorbable, static materials similar to polyester or gore-tex™. These grafts have fallen out of favor because of poor results and the increased understanding of the breakdown of non-biologic fibers into wear particles due to cyclic mechanical stress [63] and also because of their association with granulomatous synovitis.[64] Additionally, synthetic, non-resorbable fibers are not able to repair themselves as native ligaments can, and they experience creep without the potential for remodeling.[65] The second line of synthetic polymers were designed to be resorbable by the body, and are based on polymers such as polyglycolic acid (PGA), poly-L-lactide (PLLA), and poly-lactic-co-glycolic acid (PLGA). Silk fibers have many of the ideal ligament mechanical properties.[66] Bioengineered fibers can be made from polymers that resemble the structure of natural silk and can be designed to be resorbable, but also share the strength and flexibility that silk is known for, and are an emerging possibility for ACL reconstruction.

Three dimensional architecture

Scaffolds are complex structures with pores and channels of many sizes that provide open internal volume or "void space" within the structure. The largest pores must be of the appropriate size to facilitate cellular migration, tissue ingrowth and vascularization of the new tissue.[56] Smaller nanopores may be useful to aid in the movement of nutrients and water to facilitate cellular processes out from the interior of the structure. Additionally, the porosity of the material can affect the surface adhesion mechanics of proteins at the exterior of the scaffold, which impacts the hydrophilicity of the material. The three dimensional architecture dictates these processes as the size of the void spaces limits the rate of fluid movement and, therefore, material flow in and out of cells. There is evidence that calcified tissue ingrowth can occur at a lower limit pore size of 100 nm, while a minimum pore size of 150 nm has been suggested in the literature for bone and 200–250 nm for soft tissue.[67]

Properly structured scaffolds allow for rapid cellular infiltration,[68] promote the formation of ligamentous tissue,[55] provide appropriate internal surface area and structure for cell attachment, proliferation and differentiation, as well as promote tissue organization and remodeling.[69, 70] As a result of these goals, many scaffolds are designed using linear, fibrous constructs because of the dual benefit of significant intra-fiber void space as well as appropriate mechanical properties. However, they are not ideal, as the braided configuration of these fibrous materials appear to contribute to problems with nutrient transmission, cellular seeding, infiltration and matrix production.[70] Recent-

ly, James *et al.*[71] shared their technique for developing scaffolds using electrospun nanofibers, which they hope will solve some of these concerns and provide a synthetic ligament to be used in cruciate reconstruction surgery.

In ligament reconstruction, the process of graft healing is much different in the bone tunnels than it is intra-articularly. Because of these differential interactions, the neo-ACL has needs that vary along its length. Bourke *et al.*[72] attempted to address this issue by using poly-methyl-methacrylate plugs to anchor a poly (DTE carbonate) based scaffold for testing in a rabbit model of ACL reconstruction. This scaffold exhibited comparable tissue ingrowth *in vivo*, but mechanical testing revealed weakness in the construct at the plug-scaffold interface and failure below the magnitude of native force levels. Cooper *et al.*,[67] in turn, used controlled braiding techniques in order to achieve different braiding angles for the intraarticular segment and bone tunnels to achieve appropriate porosity in both regions.

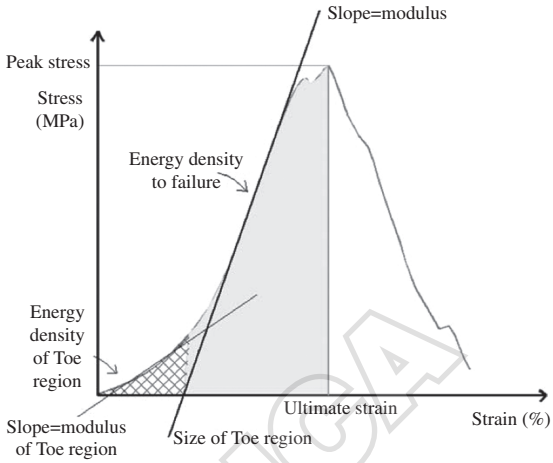

Figure 2.—Schematic of a stress-strain curve generated for a collagen scaffold[73], demonstrating the three regions of behavior. The toe region experiences changing modulus as force is initially applied to the ACL. The linear region represents the behavior of the construct through much of the loading profile. Finally, the stress-strain curve drops beyond peak stress, representing failure of the construct.

Mechanical properties

To effectively behave as a replacement ligament, scaffolds must resemble the mechanical properties of the native ligament. The important parameters to match from native ligaments include stress-strain relationships, stiffness, yield strength, performance under fatigue, creep behavior, and viscoelasticity. The stress-strain curve of native ACL ligaments has three separate zones of behavior (Figure 2):[69] a "toe region" of changing slope; a linear region, in which collagen fibers are engaged and resist deformation proportionally to the force applied; and an abrupt loss of load, in which the collagen fibers fail and the fibers stretch completely.[69] The toe region experiences great changes in slope before the linear region; this is thought to be either from bundled fibers transitioning from slack to taut [69] or from nonlinear stress-strain mechanics of collagen due to non-simultaneous recruitment of individual collagen fibers.[73] Woo *et al.*[74] demonstrated that the ultimate load and stiffness of the native femur-ACL-tibia complex decreased as the age of the subject increased. The ultimate load in the specimens examined aged 22-35 was 2 160 (±157) N, while the stiffness was 242 (±28) N/mm. These values are in contrast to the ultimate load and stiffness at middle age of 1 503 (±83) N and 220 (±24) N/mm, respectively, as well as at older age 658 (±129) N and 180 (±25) N/mm, respectively. Noyes *et al.*[75] reported an elastic modulus of 111 (±26) MPa of the ligament alone in younger patients. The precise viscoelastic behavior of ACL fibers is more difficult to characterize. Kwan *et al.*[76] described that the ACL demonstrated a reduction of more than 50% of the peak value, but did so in a non-linear fashion. Comparison of the performance of many scaffolds relative to these basic parameters are difficult, as many authors do not normalize the material properties to specimen cross-sectional area.[73] Most of the physiological force experienced by the ACL is in the toe and linear regions of the curve [73] and perhaps matching the shape and magnitude in this region is most important.

A secondary goal of scaffold implantation is to trigger neoligament growth and

remodeling. As this process occurs, the mechanical demands on the scaffold change with time.[65, 70] At time zero, when the scaffold is implanted, the mechanical properties of the scaffold are a function of the bulk material used in construction and the three dimensional microstructure. As new tissue grows into the scaffold, the material properties change and reflect contributions from both the bulk material of the scaffold as well as the emerging neoligament. The scaffold material may degrade over time if it has bioresorptive properties, further transferring stress to the neoligament. Additionally, fatigue damage to the scaffold is accelerated as it loses mechanical integrity.[55] Managing this transition of stress application is extremely complex. As outlined in the Environment section, fibroblasts must experience an appropriate stress profile in order to direct remodeling and the proper orientation of the new collagen. If the scaffold degrades too slowly, or not at all, as in the earliest synthetic materials, the scaffold can actually stress shield the new fibers from the mechanical signal necessary for remodeling.[65]

Scaffold environment

The intrascaffold space is the site of tissue ingrowth and important cellular processes. Additionally, any cellular seeding with fibroblasts or stem cells places these cells in the interior of the scaffold. As a result, the interior environment must be able to support cellular life. The osmolarity and pH of these spaces are important to provide an environment compatible with fibroblastic function. This is especially an issue in scaffolds constructed of the biodegradable polymeric acids such as PLA, PGA and PLGA. Agrawal *et al*.[77] demonstrated that this pH change occurred when the polymer chains were cut into chains small enough to exit the polymer matrix, at which time the pH dropped from 7.0 to 3.0 in a span of 10 days. The pH changes were significantly reduced by the addition of basic compounds to the scaffold structure. This study was carried out *in vitro* and the authors hypothesized that future *in vivo* studies would demonstrate a reduced effect due to the buffering potential of human body fluids. Cellular seeding with fibroblasts or stem cells remains the most promising avenue to enhance and trigger the process of tissue ingrowth. However, much of the research on this process has been completed *in vitro*, and we have a poor understanding of the performance of cellular adhesion in a knee that actually experiences cyclic physiological stresses.[78]

Environment

Ligament healing is a tremendously complex process. The cellular interactions are affected and guided by both mechanical and biological stimuli. In order to best manage this process, the environment in which healing takes place must be understood and controlled. This involves knowledge of the mechanical loading and biological signaling of the environment. Without proper mechanical loading of the healing ligament, collagen fibers may not be introduced in ideal orientations, depriving the ligament of its maximum potential strength. Without proper biological signaling, the inflammatory response can be incorrectly targeted in its magnitude or location and can place the new construct at risk. Tissue engineering solution involving ligaments must take these factors into account.

Biologic environment

The biologic environment consists of complex interactions at the molecular and cellular level. At the core of the healing pathway are growth factors, which are molecules that trigger cellular growth responses through cellular signaling pathways. Researchers remain optimistic that isolation and application of growth factors could enhance both the intensity and the rate of the native healing response. The ultimate goal of this work is to produce normal tissue with native properties. Several growth factors directly affect the ligament

healing process. These include fibroblast growth factor (FGF), transforming growth factor (TGF-β), platelet derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), growth and differentiation factor (GDF) and nerve growth factor (NGF). These factors have been shown to improve vascularization and new tissue formation.[79-81]

Kondo *et al*.[80] have studied the effects of TGF-β1 and PDGF-ββ in an *in vivo* overstretched ACL injury model in rabbits. This study showed that the application of 4 ng of TGF-β1 around the ACL significantly increased the stiffness of the injured ACL substance, whereas 20 ng of PDGF-ββ did not affect the structural properties of the injured ACL. In a similar study performed by Hildebrand *et al*.,[79] but using a "Mop End" MCL injury model in rabbits, the same amount of TGF-β1 (4ng) and PDGF-ββ (20ng) produced very different results. The injured MCL showed a significantly enhanced healing response with PDGF-ββ. On the other hand, TGF-β1 seemed to enhance healing, but was not statistically significant. These different results in similar models reflect the variations amongst each of the ligaments. Therefore, quantities of growth factors and application strategies should be individualized to each ligament in order to reach the optimal result.

Vavken *et al*.[82] studied the association between vascular endothelial growth factor (VEGF) receptor expression associated with functional healing of the ACL. In their porcine *in vivo* ACL transected model, Vavken *et al*. suggested that higher expression of the VEGF receptor was associated with a more compliant scar, which may lead to knee laxity and poor functional results. They also stated that there is a correlation with the expression of these receptors with age, similar to other growth factors. Thus, the rate limiting step of cellular stimulation could be receptor expression and not the concentration of growth factors.[82]

Platelet Rich Plasma (PRP) has been widely used to treat many soft tissue musculoskeletal problems, including knee ligament injuries.[83] PRP contains both alpha and dense granules, which are rich sources of growth factors. The utility and proper administration of PRP is still unclear. The different proprietary application methods, such as injectable activated liquid concentrate and implantable fibrin scaffolds, as well as the heterogeneity of the solutions make drawing conclusions about the usefulness of this therapy challenging. In a recent study, Castillo *et al*.[84] characterized the composition of single-donor PRP produced by three different separation systems (MTF Cascade, Arteriocyte Magellan, Biomet GPS III) available in the market. Their analysis showed that there was no significant difference in mean PRP platelet, red blood cell, active TGF-β1 or fibrinogen concentrations, but a significant difference regarding the concentration of white blood cells (WBC), PDGF-αβ, PDGF-ββ and VEGF was found. Advocates for and against the presence of WBC in PRP preparation are used to justify the quality of each system. For the high concentration of WBC it is advocated that those preparations can prevent infection in the site of the injection. On the other hand, high WBC concentration could lead to an increasing inflammatory response resulting in pain[85] and a more difficult tissue recovery.[86]

Despite the increased enthusiasm and use of the PRP preparations, there is no scientific endorsement with level I randomized control trials (RCT) supporting its advantages in the treatment of knee ligament injuries.[84] Nin *et al*.[87] showed no statistically significant difference concerning inflammatory parameters, resonance imaging appearance of the graft and clinical evaluation scores at 2 years follow up between the group with application of PRP gel to the tendon segment of a bone patellar tendon bone (BPTB) allograft versus the control group in a RCT. In a prospective study, Silva *et al*.[88] demonstrated that ACL reconstruction with hamstring allograft was not affected by the presence of PRP in terms of signal intensity of the fibrous interzone in the femoral tunnels when analyzed by MRI after three months postoperatively. These findings argue against the theorized

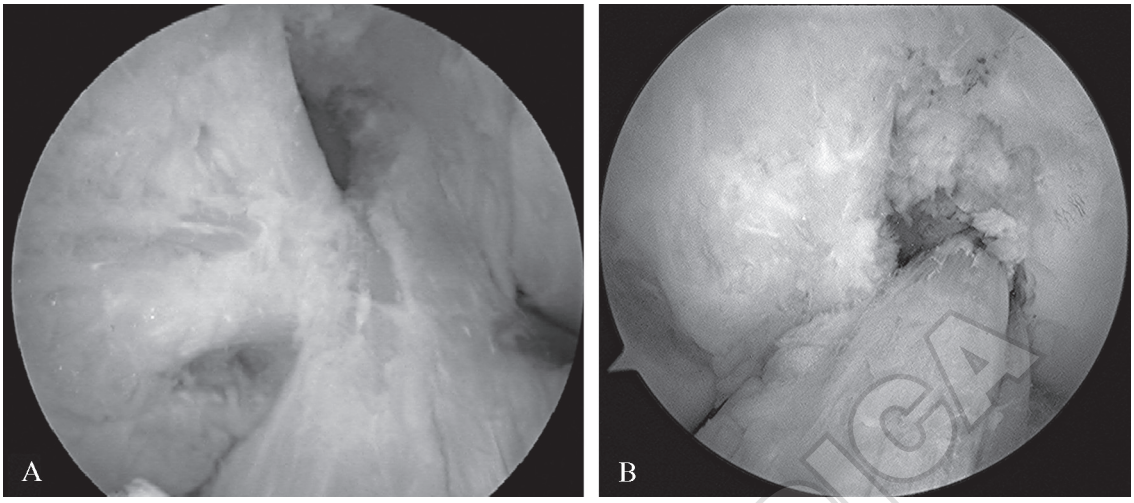

Figure 3.-A) Native cadaver ACL and PCL; B) postanatomic ACL reconstruction, demonstrating restoration of the native ACL anatomy.

beneficial effect of PRP in clinical applications for ACL reconstruction.

On the other hand, some basic science studies have demonstrated that PRP is effective in aiding MCL healing in animal models.[79] However, this was not an RCT and so there are some limitations to the conclusions. Overall there continues to be a lack of results from RCT's to justify the clinical usage of PRP in extra-articular ligament injuries.[87]

Mechanical environment

The mechanical environment impacts and directs proper cell and tissue differentiation as well as fiber orientation during the ligament healing response. Ligaments experience significant loads as part of their normal function to withstand the forces applied to the knee; these forces provide information to cells that help to guide ligament homeostasis and healing. Native ligaments are complex and dynamic [89, 90] and are no longer viewed as static structures. These mechanical factors must be taken into account, especially if ligaments are surgically repaired or reconstructed; the native anatomy must be respected with the goal of anatomic reconstruction or repair.

Ligament fibroblasts are affected by dynamic stress.[25] This has been demonstrated quite effectively in the MCL. Woo *et al.* demonstrated the benefits of exercising and dynamic loading on the biomechanical properties of the MCL in various animal models [91] and the deleterious consequences of knee immobilization after MCL injury in a rabbit model.[21] They observed that immobilization led to stiffness due to synovial adhesions and proliferation of fibrofatty connective tissue. Woo *et al.*[21] also demonstrated in a rabbit model that the tensile load required to cause MCL failure in rabbit knees immobilized for 12 weeks after injury to be up to 31% of the failure load demonstrated by the control group, which was immobilized for 9 weeks and then mobilized for another 9 weeks. Each of these studies demonstrated that the clinical outcome after MCL injury is improved by the addition of a dynamic, native loading environment during the healing period.

The ACL is different than the MCL; as an intra-articular ligament, the healing response is not as robust or successful in restoring its preinjury function. So far, the gold standard treatment for this ligament is surgical reconstruction. In the 1980s, the surgical technique was much different than the procedure performed today. At this time, knee arthroscopy was in its infancy and the field

evolved rapidly. Initially, surgeons aimed to place the ACL in the "isometric" position, or the position that prevented length change of the ACL during flexion and extension.[92] This principle was advocated based on the concept that the native ACL was thought to exhibit an isometric length change pattern.[93] However, recent anatomic studies have revealed that the native ACL in fact exhibits a different length change pattern depending on the position of the knee,[94] and that isometric placement of the graft does not replicate native loading patterns.

Anatomic reconstruction of the ACL graft is a concept built around the goal of restoring each patient's native anatomy. The principles for anatomical ACL reconstruction are to functionally reestablish the ACL to its native dimensions, collagen orientation and insertion sites (Figure 3).[95] There is significant controversy in the field of sports medicine regarding the definition of "anatomic". The authors choose to observe the definition as recorded by Forsythe *et al.*[96] Anatomic ACL reconstruction is an alternative that allows the new ACL graft to experience native joint mechanics in multiple degrees of freedom rather than just AP translation.[97]

Anatomic reconstruction has been hypothesized to improve long-term knee health as a result of the restoration of native kinematics, but long-term studies do not exist yet to corroborate this hypothesis. However, Ekdahl *et al.*[98] demonstrated enhanced bone-tunnel healing of the ACL graft in anatomically placed reconstructions vs. non-anatomic placement in a goat model. In this study, the anatomic groups demonstrated less tunnel enlargement, fewer osteoclasts, more vascularity, less A-P translation, and greater in-situ force when compared to goat knees that had been reconstructed using traditional transtibial drilling techniques, which place the femoral tunnel higher in the intercondylar notch. Zavras *et al.*,[94, 99] in turn, showed that the stresses experienced by the ligament are dependent on the placement of the tunnels. They found that even small changes in tunnel placement could significantly affect tensioning and laxity patterns. Finally, several robotic studies measuring in situ force in the ACL demonstrated that an anatomic reconstruction better restores the in situ force of the graft to native levels.[100] These studies support the idea that anatomical reconstruction better reproduces the physiological stress in the graft, which leads to a favorable environment for healing.

Bedi and colleagues demonstrated that the quantity and timing of mechanical loading in the post-operative knee impacts the course of the healing process.[101] In this study, 156 rats underwent ACL reconstruction using allograft, and were grouped according to rehab protocol in which the rats were either treated with immobilization or supervised cyclic loading beginning on post-op day 0, 4 or 10. Delayed application of the cyclic loading protocol resulted in improved mechanical and biological parameters of the knee compared to immobilization or immediate loading. This study is interesting because illustrates that both deprivation of the mechanical environment and over-induction of mechanical forces impairs construct strength.

Knee ligaments and reconstruction grafts have the best chance for success in the proper environment that maximizes the body's healing potential. This encompasses both the biological environment, which can be enhanced by the supplementation of growth factors, and the mechanical environment, which is affected by the technique of the surgical reconstruction. In both cases, the final construct is adversely affected by improper maintenance of both biological and mechanical factors.

Conclusions

The multidisciplinary approach of tissue engineering could generate enhanced treatment of knee ligament injuries. Despite recent progress in the field, further advances are needed before this technology is fully developed. Optimization of the ligament healing process requires appropriate management of many factors. The proper scaffold needs to be determined; the ap-

propriate stem cells need to be selected for seeding onto the scaffold to encourage proliferation and ECM production. Additionally, in order to control the healing process after the injury or surgery, the environment around the ligament should be optimized. The natural pattern of up- and down-regulation of growth factors needs to be extensively investigated to produce appropriate biological stimulation through the application of various growth factors. The mechanical environment can be reasonably controlled by performing the surgery as anatomic as possible, ensuring that the neoligament is loaded appropriately to orient new fiber production. The future of tissue engineering has great potential for the treatment of knee ligament injuries.

Riassunto

Engineering tissutale per i legamenti del ginocchio

Le lesioni dei legamenti del ginocchio sono le lesioni più frequenti in ambito sportivi, rendendo conto la rottura del legamento crociato anteriore (LCA) e del legamento collaterale mediale (LCM) di più del 27% delle lesioni combinate del ginocchio. Il gold standard nel trattamento delle lesioni del legamento è tuttora controverso. I metodi non-operatori possono esitare in una riduzione della forza ed in un recupero prolungato mentre l'intervento chirurgico non garantisce l'incorporamento della protesi e il recupero completo della funzionalità. La tecnologia dell'engineering tissutale comprende principi di biologia, di chimica, di ingegneria e di scienza dei materiali e può favorire la guarigione delle lesioni. Lo scopo di questo articolo è di rivedere gli attuali approcci di engineering tissutale per le lesioni dei legamenti del ginocchio. I legamenti intra-articolari, come il LCA e il legamento crociato posteriore (LCP) si trovano in un ambiente completamente differente rispetto ai legamenti extra-articolari come il LCM e il LCL. Pertanto, l'approccio per la guarigione in queste due aree corporee sono differenti. L'engineering tissutale relativo al trattamento delle lesioni dei legamenti consiste in tre componenti: cellule, "ponti", e l'ambiente. Fino ad ora, non è ancora stato trovato una sorgente cellulare ideale o un "ponte" ottimale. Le modificazioni dell'ambiente biologico si trovano nelle loro fasi iniziali, e si sta attualmente studiando la stimolazione appropriata mediante l'applicazione di fattori di crescita. L'aspetto meccanico, a sua volta, può essere ottimizzato effettuando la chirurgia ricostruttiva dei legamenti in una maniera anatomica. Grazie ai progressi compiuti in ambito di engineering tissutale, i chirurghi potranno un giorno offrire soluzioni utili ad aiutare I pazienti a tornare alle loro attività abituali più rapidamente e con maggior forza.

Parole chiave: Ingegneria tissutale - Ginocchio - Legamento, lesione.

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