Chapter 19
Regenerative Engineering of the Anterior Cruciate Ligament

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Abstract Anterior cruciate ligament (ACL) injuries, both acute and chronic, are common in sport injuries. The presence of the synovial fluid in the knee joint inhibits the spontaneous healing of the ACL, thus requiring surgical intervention. Although current methods to reconstruct the ACL can stabilize the knee joint, the progression of osteoarthritis is not halted. This chapter describes the current clinical methods to reconstruct an injured ACL and new methods to enhance the healing process. Three therapeutic strategies will be discussed in this chapter on the repair of ACL: (1) single bundle versus double bundle surgical techniques, (2) biodegradable matrices for ACL repair, and (3) biological adjuvants to enhance ACL repair. These strategies are promising clinically translatable methods to allow patients to return to normal activity levels and to alleviate pain and discomfort caused by osteoarthritis.

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19.1 Introduction

The human skeletal system is a joint assembly, and the linchpins of it are ligaments. Comprised of nine hundred ligaments, six hundred in the arms and legs, two hundred and thirty in the torso, and seventy above the shoulder, ligaments allow for the integration of two hundred and six bones to form the internal framework of the body. By forming linkage points, ligaments limit the degrees of freedom of the skeletal system and stabilize joints, preventing damage of soft tissue through inhibition of unnecessary movement. Disruption of this internal framework, due to ligament lesions, may lead to osteoarthritis. Thus, therapeutic strategies to heal damaged ligaments are necessary to allow patients to return to normal activity levels and to alleviate pain and discomfort caused by osteoarthritis.

A common ligament that is injured is the anterior cruciate ligament (ACL). An ACL injury is a momentous event in the career of athletes and overall health of non-athletes. Typically, an ACL injury is associated with sports injuries where the ACL is overloaded in tension or the knee twists causing a high torsional load, which predominately results in a ruptures of the intra-articular region. As the major intra-articular ligament of the knee, the ACL stabilizes the knee by controlling the anterior to posterior translation of the femur and tibia. The loss of ACL function causes joint instability, which leads to damage of meniscus and cartilage due to mechanical distortion.

Annually, approximately 400,000 ACL injuries occur, necessitating surgical intervention [1]. Unlike other ligaments in the body, a torn ACL does not have the capacity to heal due to the presence of the synovial fluid in the knee joint. In the case of a medial cruciate ligament tear, a blood clot forms and serves as a scaffold to allow the healing of the lesion without the need of surgery. However, in the knee joint the synovium environment inhibits the formation of a blood clot, leaving patients with an unstable knee. Therefore, ACL reconstruction is performed in order to regain the proper kinematic function of the knee with the overall goal to recapitulate the native ACL biomechanical properties. Although surgical reconstruction of the ACL is routinely performed and does allow for the stabilization of the knee, recovery of an ACL injury is a long process (approximately 8 months), and patients are at high risk for osteoarthritis. This is due to anterior subluxation of the tibia, leading to compression of the posterior lateral tibial plateau against the anterior lateral femoral condyle [2]. For these reasons, new methods to enhance the healing process of an ACL and to prevent osteoarthritis are of high interest. Three strategies will be discussed in detail: (1) single bundle versus double bundle surgical techniques, (2) biodegradable matrices for ACL repair, and (3) biological adjuvants to enhance ACL repair.

19.1.1 Structure of the Anterior Cruciate Ligament

On average, the human ACL is approximately 27–32 mm in length and has a cross-sectional area of 44.4–57.5 mm^2 [2, 3]. Macroscopically, the gross structure of the ACL appears as a band-like structure, which connects the femur and the tibia. From the femur, the ACL travels anteriorly, medially, and distally to its attachment at the tibia, and is characterized by a 180° twist between its bony attachment ends and its flexible collagenous intra-articular region (Fig. 19.1) [4]. The structure of the ACL is irregular in that the cross-sectional area is not a simple geometric shape and experiences deformation when the knee undergoes flexion [5]. Furthermore, the ACL is defined by two bundles, the anteromedial (AM) and posterolateral (PL) bundle, which act as the functional components of the ligament [6, 7]. The AM and PL bundle are characterized by the location of their insertion into the tibial tunnel. The AM bundle originates in the most proximal part of the femoral origin and inserts at the anteromedial tibial insertion site, whereas, the PL bundle originates distal to the femoral origin of the AM bundle and inserts into the posterolateral part of the tibial insertion site. These two bundles have contrasting orientations, which are dependent on the extension or flexion of the knee. In the case of knee extension, the PL bundle is seen to be in tension while the AM bundle is moderately relaxed (Fig. 19.1a). During flexion of the knee, a 110° bend, the AM

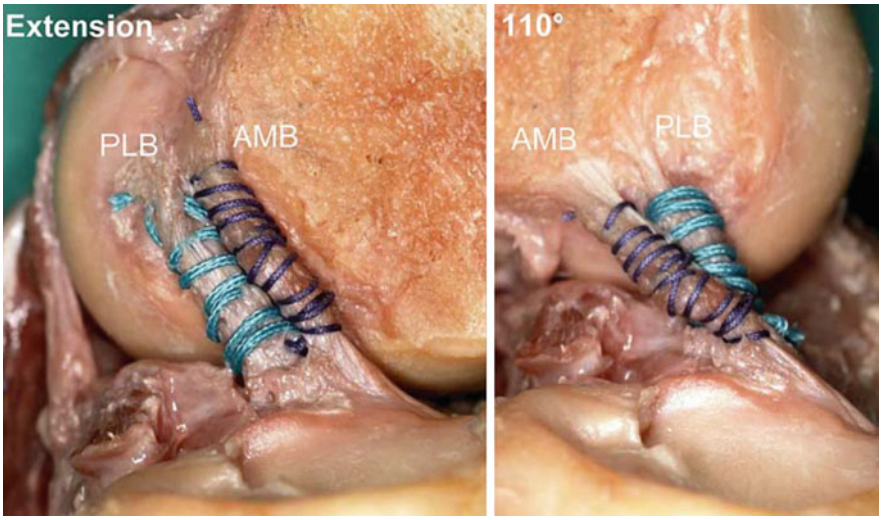

Fig. 19.1 *Diagram of the anteromedial and posterolateral bundle of the ACL.* In extension, the PL bundle is seen to be in tension while the AM bundle is moderately lax. The opposite effect is seen when the knee is in flexion at 110°. (Reprinted from [4] with permission form Elsevier)

bundle is in tension and the PL bundle becomes relaxed (Fig. 19.1b). During passive knee flexion the two bundles experience different patterns of length change and are not isometric in either flexion or extension. Furthermore, the two bundles are distinguished by their individual structures. In comparison to the AM bundle, the PL bundle is comprised by a larger number of fascicles [8]. The structure and anatomical placement of these two bundles help to stabilize the knee joint in differing physiological movements and loads.

19.1.2 Constituent Components of Ligaments

Ligaments are dense, complex, tissues composed of collagens (type I, III, and V), elastin, proteoglycans, water, and cells [4, 9]. Ligaments display a hierarchical structure with collagen molecules, fibrils, fibril bundles, and fascicles that are arranged parallel to the longitudinal axis of the ligament [10]. Microscopically, the ACL has been categorized into three sections: proximal, middle, and distal. Each of these sections are comprised of differing cellular and extracellular matrix components, and are instrumental in the healing of the surgically reconstructed ACL, as well as, the biomechanics of the ACL [4, 5, 7, 8]. The proximal section is characterized by greater cellularity in comparison to the other sections, and is thus less solid. The main components of the proximal part are fibroblasts, type II collagen, and glycoproteins. The middle part contains spindle shaped fibroblast, has a high density of collagen fibers, and a special zone of cartilage and fibrocartilage, which is located at the ligament to bone interface [4]. Furthermore, the importance of the middle zone is that the fusiform and spindle-shaped fibroblasts are longitudinally oriented. This longitudinal organization of the cells contribute to the organization of the deposited collagen fibrils, which is important for the non-linear stress-strain response of the ACL. Finally, the distal part of the ligament is the most solid of the three and is rich in chondroblasts and has a lower density of collagen bundles. In the anterior portion of the ACL, a layer of dense fibrous tissue engulfs the ligament and corresponds to the zone where the ligament is compressed by the anterior rim of the femoral intercondylar fossa. The sections of the ACL correspond to the complex anatomical structure of the tissue, which give rise to the variety of properties necessary for the ACL to comply with the kinematics of the knee.

The bone to ligament interface of the ACL is essential for the motion of the knee. This interface has a unique transitional zone that is defined as the chondral apophyseal enthesis, that guides to transition the ligamentous component of the ACL to rigid bone [8]. This transitional zone allows the ACL and bone tissue to function properly together. During ACL reconstruction the proper healing of this bone to ligament interface is essential for the knee to withstand physiological loads and joint motion. The chondral apophyseal enthesis consists of four layers: the ligament proper, non-mineralized fibroblasts, mineralized fibroblasts, and the subchondral bone plate. The first layer is composed of collagen fibrils that is followed by a second layer of non-mineralized fibroblast cells that are aligned within the

collagen bundles. The third layer is composed of mineralized cartilage and facilitates the transition to the subchondral bone plate. The transition zone from ligament to bone serves to distribute the stresses at the insertion site, and therefore decreases the rise of stress concentrations [8, 11, 12]. As such, the transition zone allows for a graduated change in stiffness at the attachment site, and is critical for the stress distribution of the ligament under loads.

The constituent components of the ACL microstructure are similar to that of other soft connective tissues [5]. Of the ACL constituent components, collagen is the major ECM protein that comprises its structure. Collagen fascicles are bundled together to form the band like structure of the ACL. These fascicles range from 250 μm to several millimeters and are connected by paratenon connective tissue. The lateral growth of collagen fascicles are regulated by two extra cellular matrix proteins, decorin and fibromodulin. Within the fascicles are subfascicular groups on the order of 100–250 μm and are enclosed by epitenon tissue. In all, fascicles are composed of approximately 3–20 subfascicular units. The subfascicular groups are undulated, and therefore provide the organization fundamental to the biomechanical response of the ACL. Furthermore, the subfascicular groups are a family of fibers that are composed of collagen fibrils. These collagen fibrils are approximately 25–250 nm in diameter and are the primary component of the ACL structure.

Collagen fibrils have been categorized into two types: fibrils with varying diameter, and uniform diameter fibrils [4, 5]. The inhomogeneous fibrils have varying diameters that peak at 35, 50, and 75 nm and account for 50.3 % of the entire ACL. Biomechanically, the inhomogeneous fibrils have been stated to specialize in resisting high tensile stresses. On the other hand, homogenous fibrils have uniform diameters with a peak diameter of 45 nm and account for 47.3 % of the ACL. The three-dimensional organization of the ligament is provided by these homogenous fibrils, which also serve a critical role in modulating the biomechanical response of the ACL.

The remainder of the ACL is composed of cells and matrix components. The matrix components of the ACL are formed by collagen, glycosaminoglicans (GAGs), glyco-conjugates, and elastic components [4, 5]. There are four types of collagen found in the ACL, type I, III, IV, and VI, of which type I and III collagen are the primary components that affect the biomechanical response of the ACL. At the molecular level, collagen protein are composed of two collagen alpha 1 chains and one collagen alpha 2 chain. These three chains interact together to create a triple helix structure. Collagen type I fibrils are oriented along the longitudinal axis of the ACL, provide the tensile strength of the ligament, and are the aforementioned homogenous fibrils of the ligament. On the other hand, type III collagen is the connective tissue that connects the type I collagen bundles, serves as the main ground matrix of the ACL, are fundamental in fibril assembly, and are inhomogeneous throughout the ligament. Morphologically, type III collagen is either a single or multi-strand, with a diameter of 2 and 9 μm, respectively [1]. Maximal concentrations of type III collagen are seen near the bony attachment end of the ACL, and biomechanically type III collagen is important for the pliability of the ACL [1].

In addition to collagen, GAGs play an essential role in the biomechanics of the ACL. GAGs are important for the viscoelastic properties of the ACL, and since GAGs are highly negatively charged and contain a large proportion of hydroxyl groups, they attract water through hydrogen binding. Thus, GAGs recruit water into the ACL, which comprises 60–80 % of the total wet-weight of the ACL. The GAGs retain water in the ACL, which is released when tensile loads are placed on the ligament. In comparison with tendon, ligaments have a higher proportion of GAGs, approximately two to fourfold greater. Importantly, these GAGs act as a shock-absorber in the ligament. These constituent components work together to allow for proper knee kinematics, yet when the ACL is ruptured and reconstruction of the ACL is needed other factors need to be considered to understand the biomechanics of the healing process. Further insight into the genetic make-up of the ACL is needed to further delineate the components that regulate the post reconstruction biomechanics of the ACL.

Gene analysis of the ACL has been conducted to elucidate the differences between the ACL and tendon. To date, the discrepancy between ligament and tendon is not well understood. Therefore, in engineering approaches to study ligament many of the cellular properties of the tissue are neglected and simplified to describe the ligament as tendon. Ligament and tendon share a common progenitor marker, scleraxis, a transcription factor that promotes the production of collagen extra cellular matrix [13]. To describe the differences between ligament and tendon, Pearse II et al. conducted a microarray analysis of porcine ACL, posterior cruciate ligament (PCL), medial cruciate ligament (MCL), patellar tendon (PT), and Achilles tendon (AT). In the ACL and PCL, it was found that the genes tenascin-C and aggrecan core protein were upregulated in comparison to the PT and AT [14]. Tenascin-C functions as an adhesion-modulatory extracellular matrix protein and plays a fundamental role in regulating fibroblast extra cellular matrix deposition and the ability of fibroblasts to contract their matrix. Relative to ACL reconstruction, tenascin-c is known to be greatly upregulated in extra cellular matrix remodeling during wound repair and neovascularization, and therefore plays an impactful role in the restoration of ACL biomechanics post reconstruction [15].

Aggrecan core protein is a critical component of cartilage structures and has been noted to affect the stiffness of cartilage. In the context of the ACL, aggrecan is found in the bone insertion site. At the bone-ligament interface, aggrecan is most prominent in the mineralized fibrocartilage region. Given that the transitional zone plays a key role in distributing stresses, aggrecan would be important in the biomechanical response. Interestingly, Majima et al. conducted tensile and compressive tests on the MCL and found that the mRNA levels of aggrecan were elevated due to cyclic hydrostatic compression and cyclic tension [16]. Through the comparison of gene expression novel insights on the constituent components of the ACL led to a greater understanding of the cellular components and their role in the biomechanics of the ligament.

19.1.3 ACL Reconstruction: Graft Placement and Their Role in the Kinematics of the Knee

The surgical reconstruction techniques and their outcome are affected by the placement of graft fixation into the femur and tibia. Research has been conducted to determine the correct placement of the ACL. The anatomy of the femoral insertion site is characterized by its length and width, which has been found to be approximately 18 mm in length, 10 mm in width, and 4 mm from the articular cartilage (Fig. 19.2a). In addition, the insertion site is characterized in the sagittal plane, where it is rotated relative to the axis of the femur and reflects the insertion sites congruity to the posterior border of the femoral condyle. Generally, the rotation is 25°–35° relative to the femur (Fig. 19.2a). The AM and PL bands are characterized for their insertion site based on a 90° flexion of the knee. In this reference, the proximal and distal margins of the ACL are approximated at 11 and 10 o'clock, respectively (Fig. 19.2b). In the tibial insertion site, the ACL inserts into the intercondylar eminence of the tibia [17]. Studies have reported measurements for the precise insertion site of the ACL into the tibia with an oval geometric shape. The approximate length and width of the oval site is 18 and 10 mm, respectively. The midline of the oval attachment site can be described from the posterior tibial plateau, and its distance from the plateau is approximately 6 mm (Fig. 19.3) [17]. In ACL reconstruction, it is desirable to mimic the characteristics of the native insertion site to preserve the kinematics of the knee.

A debate on whether a single bundle technique adequately maintains the kinematic integrity of the knee began in the early 2000s. As previously stated, the native ACL exhibits two bands, the AM and PL. Therefore, the use of a double bundle

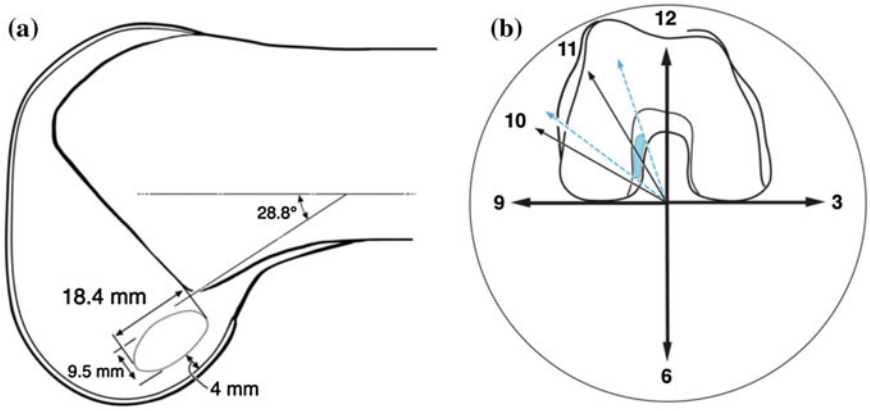

Fig. 19.2 **a** Sagital view of femoral insertion site. **b** Anterior view of the knee at 90° flexion. (Adapted from [17] with permission from SAGE Publications Inc.)

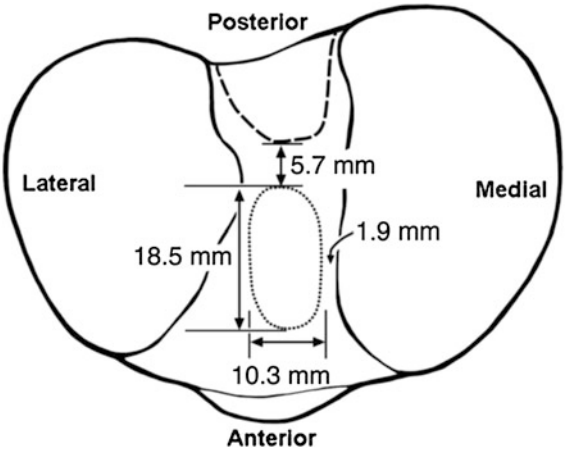

Fig. 19.3 Cross-sectional view of the tibial insertion site for ACL reconstruction. (Reprinted from [17] with permission from SAGE Publications Inc.)

graft for ACL reconstruction has been investigated and used clinically, so as to recapitulate the gross anatomy of the ACL. In the double bundle technique the femoral and tibial insertion sites are reamed. The femoral insertion site allows for the reaming of two tunnels with ease, however due to the geometry of the tibial insertion (Fig. 19.3), considerable variability occurs due to the obliquity of the line separating the two bundles [17, 18]. Although variability will occur in the tibial insertion site, studies have shown the efficacy of a double bundle reconstruction technique. Cadaveric studies on the kinematics of single and double reconstructions demonstrated that the double bundle technique provides better stabilization of the knee in the anterior translation direction when exposed to valgus-internal rotation [19]. Furthermore, Koga et al. recently conducted a clinical trial to compare the results of single bundle versus double bundle ACL reconstruction. Seventy-eight patients were included in the study, in which the ruptured ACL were replaced with an autologous semitendinosus tendon. Of the seventy-eight patients, fifty-three were evaluated for 3 years. The results demonstrated that the double bundle technique lead to greater results in the Lachman, pivot-shift test, and KT-1000 arthrometer measurements [20]. The Lachman test is a physical examination, performed by a clinician, which gains insight in the anterior translation of the tibia in comparison to the tibia. The pivot-shift test is also a physical examination in which a clinician tests the instability of the knee. Finally, the KT-1000 measurement provides an objective means to measure the anterior tibial motion relative to the femur, and this test validates the clinician's assessment. The research in the field of graft placement and reconstruction technique, single versus double bundle grafts, provide insight in choosing a surgical technique to replace a ruptured ACL for a more desirable clinical outcome in regards to the knees function.

19.2 Regenerative Engineering Approaches to Enhance Ligament Regeneration

19.2.1 Criteria for Ligament Scaffolds

Ligament scaffolds should seek to mimic the architecture and behavior of native ligament structures, and as such these scaffolds must be biocompatible, biodegradable, and mechanically competent. With regard to ligament regeneration, biocompatibility implies that fibroblasts can adhere, proliferate, and secrete natural extracellular matrix (ECM) components. The production of native ECM components (such as collagen type I, elastin, and proteoglycans) is especially important for biodegradable scaffolds, which act as a temporary structure to provide mechanical strength and appropriate cellular interactions as fibroblasts create natural ligamentous tissue. The development of a suitable scaffold for ligament regeneration also requires careful observation of the mechanical properties of the natural ligament tissue in its native environment. The mechanical properties of the ACL will be highlighted within because of the extensive research in this area. ACL tissue must be able to provide differential load support [21], resist plastic deformation, and have high tensile strength. It has been discovered that high tensile strength is not sufficient to recapitulate the mechanical behavior of the natural ACL because the ligaments of the knee encounter a variety of different forces and torsions during normal knee movement [8]. Notably, the ACL has been subdivided into an anteromedial (AM) band and a posterolateral (PL) band [22]. These distinct bands are named according to their tibial insertion points, and are generally accepted in the literature in spite of their anatomical simplification.

Natural ACL tissue has a unique microstructural feature, typically referred to as the crimp pattern, which endows the ligament with a non-linear stress-strain behavior [23]. Figure 19.4 displays the three regions of this mechanical behavior. The toe region represents the application of force to collagen fibrils within the

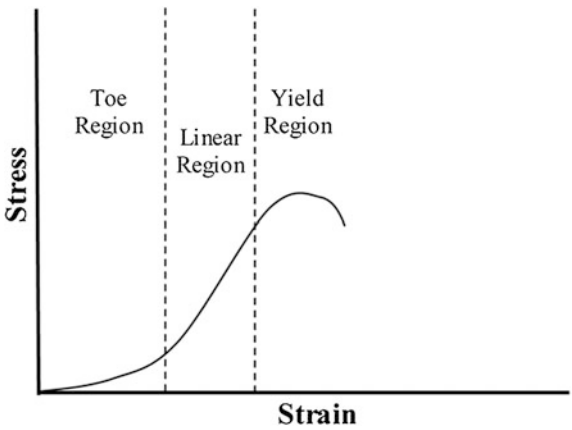

Fig. 19.4 Stress-strain behavior of natural ACL tissue. (Reprinted from [9] with permission from Elsevier)

ligament, causing a straightening of the crimp pattern. The linear region starts once the crimp is fully elongated, and is representative of collagen molecular strain [9]. Finally, the yield region signifies the failure of the ligament as the collagen fibers defibrillate. The understanding of natural ligament tissue properties has enabled researchers to fabricate natural and synthetic ligament replacement materials.

Autografts and allografts were explored due to the inherent mechanical strength and biocompatibility that was offered from natural human ligamentous tissue. Each of these systems has its own drawbacks. Autografts are taken from the body of the patient, and as such require at least two surgeries. This leads to the issue of donor site morbidity, the possibility that the first surgery may impair the viability of the surrounding donor site tissue. Allograft tissue is not harvested from the patient, but there is a limited supply of healthy human ligament tissue. In addition, the ligament from another human may cause an immunogenic response in the patient receiving the allograft, ultimately leading to graft rejection. The shortcomings of autografts and allografts have led researchers to study natural and synthetic scaffolding materials that mimic the properties of the native ligament.

19.2.2 Natural Scaffolds

Ligament scaffolds comprised of naturally occurring materials are attractive due to the biocompatible microenvironment provided by such materials. As the predominant component of native ligament tissue, collagen type I has been extensively researched in the literature [24, 25]. Dunn et al. showed that fibroblasts harvested from rabbit ACL tissue were able to orient along the long axis of collagen fibers and synthesize collagen to a greater degree than fibroblasts adhering to tissue culture plastic [24]. Subsequent in vivo studies confirmed the viability of the fibroblast-seeded collagen constructs, but complete resorption by week 8 limited to impact of the collagen construct [26]. In addition, collagen scaffolds cannot match the mechanical properties of native ligaments, leading groups to search for ways to enhance said properties.

Common techniques reiterated throughout this chapter are the braiding, twisting, and weaving of fibers to enhance the mechanical properties of the respective materials. Walters et al. braided and crosslinked Sprague-Dawley-harvested collagen type I fibers to match the mechanical behavior of native ligaments [25]. The collagen fibers were crosslinked using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry, a standard amide-bond forming reagent. Walters et al. reports a Young's modulus of 148 ± 17 MPa and an ultimate tensile strength (UTS) of 19.3 ± 3.1 MPa for the braided and crosslinked scaffolds. Though these values are close to results obtained from native human ACL by Noyes and Grood (Young's modulus: 111 ± 26 MPa, UTS: 37.8 ± 9.3 MPa) [27], there are always concerns about the immunogenicity of harvested materials and the use of harmful crosslinking agents in implanted scaffolds.

Alternative natural materials that exhibit sufficient mechanical integrity without the need for crosslinking have also appeared extensively in literature. Silk fiber is a well-known clinical suturing material with a high UTS and favorable biocompatibility [28]. Altman et al. developed a ligament scaffold using twisted silk fibers arranged into a 6-cord matrix. This silk scaffold is reported to have an UTS of 2337 ± 72 N, a stiffness of 354 ± 26 N/mm, a yield point of 1262 ± 36 N, and an elongation percentage of 38.6 ± 2.4 %. These mechanical properties are similar to those reported for a human ACL. Woo et al. reported that native human ACL tissue has an UTS of 2160 ± 157 N, a stiffness of 242 ± 28 N/mm, a yield point of approximately 1200 N, and an elongation percentage of approximately 33 % [29]. The silk scaffold may not be able to induce infiltration of human bone marrow stromal cells (BMSCs), but the comparable mechanical properties suggest that silk could be a viable material for ligament scaffolds. Chen et al. modified the same silk scaffold with arginylglycylaspartic acid (RGD) peptides to promote cell attachment and collagen production, suggesting that future modification technologies could enhance certain properties of materials that are initially unfavorable [30]. Collagenous matrix production via fibroblast seeded on silk scaffolds should be designed to match the rate of degradation of silk, which is reported to degrade within 1–2 years [28]. The efficacy of proteinaceous fibers for ligament regeneration has led to the development of various naturally derived non-protein fibers.

Chitin is a natural polysaccharide obtained from crustacean shells. Chitosan is formed when chitin is sufficiently deacetylated (>50 % deacetylation), and as such a copolymer containing N-acetylglucosamine and N-glucosamine remains. Polysaccharide fibers have not received the same attention as protein fibers in ligament regeneration applications because proteinaceous polymers are more mechanically competent. However, Irie et al. have produced chitosan-hyaluronan (chitosan-HA) hybrid polymer fibers that match the failure load of rabbit MCLs. The chitosan-HA fibers were produced using a wet-spinning technique [31]. The resulting fibers were then braided into a scaffold using a 30° angle between the braided fibers and the longitudinal line. The chitosan-HA scaffolds had the most favorable mechanical properties when seeded with Achilles tendon fibroblasts (isolated from the same rabbit to be used for subsequent surgeries). Surgical insertion of the cell-seeded scaffolds was followed by surgical removal at 3, 6, and 12 weeks. After 12 weeks, the cell-seeded constructs had a failure load of 125.2 ± 28.4 N and stiffness of 31.5 ± 8.7 N/mm (compared to a failure load of 106.1 ± 27.5 N and stiffness of 92.8 ± 26.5 N/mm for natural rabbit MCL tissue). Chitosan-HA ligament scaffolds cannot match the stiffness of natural ligament tissues, which will eventually lead to unfavorable deformation in vivo. These cell-seeded scaffolds were able to enhance type I collagen production (when compared to similar non-cell-seeded scaffolds) and were not shown to elicit any inflammatory response 12 weeks after surgery. In addition, this group claims that chitosan-HA scaffolds support cell proliferation and extracellular matrix production due to significant swelling of the scaffold cross sections after surgery.

Tamura et al. experimented with chitosan-coated alginate filaments using the aforementioned wet-spinning technique [32]. Increasing chitosan content led to

increased tensile strength, but alginate braided constructs were not compared to other leading materials for ligament engineering. As such, further work needs to be done to identify whether alginate has any promising properties to contribute to the regeneration of ligament tissues.

19.2.3 Synthetic Scaffolds

Synthetic scaffolds are an attractive opportunity for ligament tissue engineering approaches because synthetic materials can be tailored to suit the properties of the desired scaffold. Many early ligament replacements utilized non-degradable materials, such as poly(tetrafluoroethylene) (GoreTex), polypropylene (Kennedy Ligament Augmentation Device), and polyethylene terephthalate (Leeds-Keio ligament) [33–35]. Although initially mechanically competent, permanent plastic deformation of these devices is prevalent following surgical implantation. Additional failure mechanisms related to these devices include creep, fatigue, fragmentation, and stress shielding [36].

Next generation synthetic materials for ligament engineering include poly (L-lactic acid) (PLLA), poly(glycolic acid) (PGA), poly(lactic-co-glycolic acid) (PLGA), and polycaprolactone (PCL). These materials improve upon past scaffold designs by better mimicking the architecture and mechanical properties of natural ligaments. Ligament scaffolds composed of these materials also tend to incorporate chemical modifications, such as fibronectin or RGD sequences, to improve cell proliferation and ECM production.

PLLA is a synthetic polymer composed of lactic acid monomers. This polymer is reported to take approximately 2 years to degrade within the body, which is an ideal length of time to permit tissue ingrowth of implanted constructs composed of this material while also maintaining mechanical integrity. Laurencin et al. have developed a braided scaffold with controllable porosity and mechanical properties similar to that of a natural ACL [3]. The braided scaffold has two bony attachment ends to resist bone tunnel-associated wear, and an intra-articular region that is sandwiched by the bony attachment ends. The intra-articular region has pore diameters on the range of 200–250 μm to allow for soft tissue ingrowth. PLLA ligament scaffolds are reported to have favorable cell growth and collagen type I production with the addition of fibronectin [3, 37].

Scaffolds utilizing materials derived from glycolic acid have previously been reported in the literature [38]. Lin et al. have developed a scaffold composed of Dexon II, a material made up of PGA homopolymer coated with polycaprolate. The scaffold was capable of supporting early cell growth of human ligament fibroblasts, especially when these cells were seeded with growth factors. Recent literature has been increasingly critical of growth factor-assisted cell proliferation because of the inherent instability of proteins and immunogenic reactions of such growth factors [39]. The United States Food and Drug Association has strict standards for the approval of growth factors for clinical use, and therefore treatments involving

growth factors are not yet clinically viable. Additional concerns associated with PGA-based ligament scaffolds include rapid degradation (complete degradation within 1 month) and loss of mechanical strength.

PLGA has been extensively studied in the literature because of the tunable properties associated with this material. The degradation and mechanical properties can be modified by changing the ratio of lactic acid to glycolic acid monomers when synthesizing the polymer. As such, the degradation of glycolic acid based polymers can be delayed by incorporating more lactic acid into the polymer product. Slow degradation of ligament scaffolds is preferable, but certain scaffolds may benefit from regions of faster degradation. Lu et al. report that PLGA scaffolds have decreased strength and lower rates of ACL fibroblast proliferation compared to PLLA scaffolds, which appears to be a direct result of the incorporation of glycolic acid monomers [37].

The importance of ligament scaffold degradation cannot be overstated. Despite having the slowest degradation of all of the previously discussed synthetic materials (approximately 4 years), PCL has received increased attention in tissue engineering applications because of the superior rheological and viscoelastic properties of the material [40]. Leong et al. report using a PCL scaffold with 31.3 % of the stiffness and 28.2 % of the peak load of native ACL tissue [41]. The stiffness and peak load values were reported to improve when basic fibroblast growth factor (bFGF) was incorporated into the PCL grafts. PCL is commonly characterized as a bioinert material, which implies that the material lacks the ability to induce cell proliferation. Many groups have surface modified PCL to improve the hydrophilicity of the polymer and therefore increase the proliferation of cells seeded onto this scaffolding material [42–44]. Surface modification approaches have been shown to enhance cell proliferation with varying degrees of success, but the implication of such modifications has yet to be translated into a PCL-based ligament scaffold with favorable properties that match native ligaments.

Any tissue engineered ligament scaffold must meet an extensive list of criteria to optimally rejuvenate the injured ligament site. Overall, synthetic materials have a promising future for ligament scaffold applications because of the favorable mechanical properties possessed by these materials. More extensive in vivo studies will be required to verify the efficacy of synthetic scaffolding materials and confirm the clinical potential of the aforementioned tissue engineering constructs.

19.3 Biological Adjuvants for Enhanced Ligament Regeneration

ACL reconstruction contains two main biological processes, ligamentization of the intra-articular region and graft-to-bone healing in the femoral and tibial tunnels. Efforts have been made to enhance the healing of the gold standard ACL reconstruction (bone-patellar tendon-bone graft), through the use of stem cell populations

and platelet rich plasma. Efficacy of biological adjuvants to enhance ligamentization and graft-to-bone healing has been heavily researched. Two methods that can be utilized in the operating room is the use of platelet rich plasma and bone marrow aspirate from the iliac crest, without the need to go through further FDA approval. Additionally, there has been further interest in the use of adipose-derived stem cells to aid in the healing of musculoskeletal injuries. Herein, the use of these therapeutic strategies to aid in ACL healing are discussed.

19.3.1 Platelet Rich Plasma to Enhance ACL Repair

Platelet Rich Plasma (PRP) is an autologous plasma suspension enriched with platelets. Normally it is prepared from peripheral blood through a two- phase centrifugation process called plasmapheresis, in which liquid and solid components of anti-coagulated blood are separated, leading to a concentrate with 3–5 times as many platelets as normal blood [45].

PRP contains platelets and a high concentration of the fundamental growth factors proved to be actively secreted by platelets to initiate wound healing, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epithelial growth factor, basic fibroblast growth factor, and Transforming Growth Factor-β1 and β2 (TGF-β) [46]. Additionally PRP also contains three proteins found in blood, fibrin, fibronectin, and vitronectin, which are known to act as cell adhesion molecules for osteoconduction and as a matrix for bone, connective tissue, and epithelial migration [45].

The success of ACL reconstruction depends heavily on biological processes that could improve the outcomes and ensure optimal clinical results [47]. Most of the factors released by PRP are involved in the repair of tendon and ligament injuries, and high concentrations of these growth factors are considered to accelerate tendon/ligament healing [47–49]. During the past decades, the application of PRP has been used as a strategy to enhance the healing of injured tendons and ligaments.

The first study regarding PRP in ACL surgery was published by Ventura et al. in 2005 [50]. In the study, 20 patients were randomly assigned to receive ACL hamstring reconstruction with or without PRP. Three milliliters of PRP was placed in both tunnels directly with autologous thrombin, though the concentration of PRP used was unclear. Clinical outcomes showed that the transformation from autologous quadrupled hamstring tendon graft to new ACL was faster in the PRP treated group than in controls. This suggests that growth factors contained in PRP could accelerate the integration of the new ACL in the femoral and tibial tunnels.

Although many studies have been carried out with PRP in ACL repair, the effectiveness of PRP is still up for debate. Recent works from Murray and associates have analyzed the efficacy of PRP treatment in combination with ACL suture repair [51, 52]. The use of PRP in combination with an extracellular matrix protein scaffold containing collagen, referred to as the bio-enhanced ACL repair, has been evaluated in a porcine model and compared with ACL reconstruction using an

allograft tendon. Both models reported no significant difference in mechanical properties at 3 months and 1 year after the surgery. However, the reason for the lack of statistical significance is due to the inconsistent methodologies of the two groups. Though suture repair of the ACL is not improved with the use of PRP alone, the ACL can be effectively repaired with the use of whole blood containing a physiological concentration of platelets in an extracellular matrix-based scaffold. The results of this bio-enhanced repair technique are similar to ACL reconstruction in terms of the mechanical properties of the healing tissue and graft, but the bio-enhanced repairs resulted in less post-traumatic osteoarthritis in large animals. The data from ACL reconstruction study using bio-enhanced ACL in goat and porcine models with whole blood and 5X platelets, respectively, provide encouragement regarding the efficacy of the platelet-enhanced ACL reconstruction approach in immature animals.

Generally speaking, the various systems used to obtain PRP lead to disparities in platelet collection efficiency and repeatability, final leukocyte count, platelet activation and ease of use [53]. Any one of these disparities could lead to controversial results. There is need for standardization of PRP preparation methods, but more evidence is needed to support the routine use of PRP for treating ACL injuries.

19.3.2 Adipose-Derived Stem Cells

Mesenchymal stem cells (MSCs) are adult stem cells from various sources, being multipotent and having the capacity of self-renewal. MSCs can differentiate into mesoderm-associated cell types such as chondrocytes, adipocytes or osteoblasts [53]. Due to ease of harvest and abundance, adipose-derived mesenchymal stem cells (ADSCs) are an attractive, readily available adult stem cell source that has become increasingly popular for use in orthopedic applications [54].

Eagan et al. looked at the in vitro utility of ADSCs for ligament engineering. They treated ADSCs for 4 weeks with TGF-β1 or IGF1 not showing any significant and consistent upregulation in the expression of collagen types I and III, tenascin C, and scleraxis. While treatment with EGF or bFGF resulted in increased tenascin C expression, increased expression of collagens I and III were never observed. Therefore, simple in vitro treatment of human ADSC populations with growth factors may not stimulate ligament differentiation [55]. Little et al. prepared novel ligament derived matrix by mixing phosphate-buffered saline or 0.1 % peracetic acid with a collagen gel. Over 28 days, the matrices were found to promote ADSC differentiation into a ligament fibroblast phenotype [56]. Proffen et al. co-cultured stem cells from both the retropatellar fat pad and peripheral blood, and the results showed stimulated ACL fibroblast proliferation and collagen production in vitro [57]. Further investigation was carried out by adding MSCs obtained from the adipose tissue or peripheral blood to see the in vivo biomechanical properties of bioenhanced ACL repair. After 15 weeks of healing, there were no significant improvements in the biomechanical or histological properties with the addition of

ADSCs. The only significant change with the addition of peripheral blood MSCs was an increase in knee anteroposterior laxity when measured at 30° of flexion, suggesting that the addition of adipose-derived or peripheral blood MSCs to whole blood—before saturation of an extracellular matrix carrier with the blood—did not improve the functional results of bioenhanced ACL repair after 15 weeks of healing in a porcine model [58]. These MSC studies suggest the potential of ADSCs in tendon and ligament repair, but more evidence is needed to fully substantiate these claims.

19.3.3 Alternative Methods for ACL Reconstruction

Murray and associates explored a new paradigm in primary ACL repair. Previous studies for primary repair of the ACL after traumatic rupture have reported unacceptable rates of failure after primary surgical repair, and the poor rate of primary healing is believed to be due to the intra-articular environment and synovial fluid that surrounds the ACL [59]. Through a canine, central ACL wound model, Murray and associates demonstrated the differences in intra-articular (i.e., ACL) versus extra-articular (i.e., MCL) healing. Ligaments which exist outside of joints (extra-articular) heal with an orderly progression of events. The first basic process is bleeding and then formation of a fibrin–platelet clot within the wound site, which fills in the gap between the torn ends of the tissue and forms a provisional scaffold for the surrounding cells to move into and remodel into a functional scar. However, in the intra-articular environment, after an injury, there is an upregulated production of urokinase plasminogen activator by synoviocytes, which converts the inactive plasminogen molecule present in synovial fluid into its active form, plasmin. Plasmin quickly degrades fibrin. Therefore, if a tissue is exposed to synovial fluid after injury, the ends may bleed, but the fibrin is unable to form a stable clot as it is degraded too quickly. The early loss of this provisional scaffold has been thought to be a major reason why tissues within joints, such as the ACL or meniscus, fail to heal after the injury [59–61]. The lack of a scaffold in the intra-articular ligament wounds was also associated with decreased inflammatory cytokines needed for the healing response, including fibrinogen, PDGF, TGF-b, and FGF. However, replacement of the central intra-articular ligament void with a collagen-platelet-rich plasma scaffold resulted in increased filling of the wound with repair tissue that had similar profiles of protein expression to matched, extra-articular ligament wounds. Biomechanical studies of suture ACL repair augmented with a collagen-platelet–rich scaffold in a porcine model have shown significant improvement in load to failure and linear stiffness at 4 weeks compared with control repairs, which lack the collagen-platelet-rich scaffold [58].

19.4 Future Trends

The gold standard to treat ACL ruptures is the use of bone-patellar tendon-bone grafts, and it is understood that the current treatment results in donor site morbidity due to the harvest of the graft. In addition, the current gold standard does not inhibit the progression of osteoarthritis. Given these knowns, researchers have aimed to develop engineered ACL scaffolds that are mechanically competent, biodegradable, and inhibit the progression of osteoarthritis. Current generation engineered scaffolds have shown the capacity to allow for the regeneration of a natural ACL in the intraarticular region after 1.5 years. The next steps are to evaluate the ability of these engineered matrices to inhibit the progression of osteoarthritis and to enhance the ligamentization such that patients can return to their prior levels of activity.

Enhancing the properties of ACL scaffolds can be realized by utilizing biological adjuvants. Current clinical use of PRP and bone marrow aspirate allows for a quicker route to translating new surgical treatment strategies. Further research in the addition of PRP on mechanically competent scaffolds is needed. Additionally, research in prolonging the effects of PRP treatment through the use of carriers may also aid to enhance and accelerate ACL healing.

The use of MSCs from various sources for ACL reconstruction is in its infancy. Bone marrow aspirate and adipose tissue are two sources that are abundant and have stem cells that may aid in the enhancement of ACL healing post reconstruction. Cytokines from these stem cells can help to inhibit inflammation, and these molecules can also provide signals to promote ligamentization and graft-to-bone healing. That being said, the synovial environment presents challenges in delivering these stem cells. Future studies on carriers for stem cells which maintain their stemness and prolong the release of pro-healing cytokines is needed to increase the efficacy of this therapeutic strategy.

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