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Chapter 25

THE USE OF BIOLOGICS IN ANTERIOR CRUCIATE LIGAMENT RECONSTRUCTION

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INTRODUCTION

Reconstruction of the anterior cruciate ligament (ACL) is one of the most common orthopaedic surgical procedures performed in the United States [1]. Although the procedure has touted short-term success rates of up to 95% [2], these calculations fail to account for the large proportion of patients who fail to return to their previous levels of sports competition along with the well-documented long-term risk of developing degenerative osteoarthritis as a result of altered knee kinematics. The incidence of graft failure is also thought to be under-reported, especially since this complication may manifest simply as subacute graft elongation, which is often not discovered until the patient develops degenerative osteoarthritis years later.

In an effort to reduce these complications, research has been conducted over the past decade in an effort to refine and perfect the surgical techniques involved in ACL reconstruction such as bone tunnel placement, graft orientation, graft fixation, and graft tensioning, among many other factors. However, the vast majority of new techniques have failed to resolve many of the fundamental issues that often lead to sub-optimal outcomes after ACL reconstruction. Perhaps one of the more troubling factors is that athletes are becoming

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more and more pressured to return to their previous levels of sports competition or intensity before the implanted grafts have fully matured, potentially leading to acute rupture or permanent graft elongation which invariably effects long-term clinical outcomes in a negative manner. In fact, implanted graft tissues are often in their weakest state approximately 6 months after surgery, which unfortunately corresponds to the time at which patients often seek to return to high-level sporting activities.

Although it will always be important to evaluate different surgical techniques for ACL reconstruction, we must also understand that there does not exist a minor technical change in currently accepted surgical techniques that will drastically accelerate graft healing to the point at which patients can safely and consistently return to sports within 6 months after surgery. However, it may be possible to accelerate graft healing using biologic augmentation methods that have not yet been fully explored in the realm of basic science research. The purpose of this chapter is to (1) summarize our current knowledge regarding the use of biologics for knee ligament reconstruction with particular focus on the anterior cruciate ligament (ACL), (2) to review the factors that influence graft healing and maturation, and (3) to identify potential upcoming biologic strategies to improve the clinical outcomes of ACL surgery.

MECHANISMS OF ACL GRAFT FAILURE

In order to fully understand the importance of graft healing after ACL reconstruction, it is appropriate to consider the patterns of graft failure that are commonly observed in clinical practice. Although it is possible for a well-healed graft to rupture in certain circumstances, the vast majority of graft failures that occur following technically well-performed reconstructions (i.e., acute graft rupture or chronic graft elongation) are the result of new injuries to ACL grafts that have not fully matured. For both autografts and allografts (i.e., both soft tissue grafts and bone-tendon-bone [BTB] grafts), failures that occur less than 4 weeks after surgery are most often related to graft pullout from the bone tunnels before adequate bony ingrowth can occur [1, 3-7]. Therefore, surgeons must prioritize fixation strength during the operation as a temporizing measure to prevent early graft pullout during the process of bony integration, a process which requires at least 6 weeks for BTB autografts and often more than 12 weeks for soft tissue autografts (and longer when allografts are used). Considering this information, it stands to reason that intra-articular graft failures (i.e., midsubstance failures) typically occur more than 6 weeks after placement of BTB autografts and more than 12 weeks after placement of soft tissue grafts. This difference is primarily related to the prolonged time required for soft tissue grafts to revascularize and to achieve tendon-bone incorporation [1, 8] when compared to bony union of BTB grafts, which occurs much more rapidly. In addition, Beynnon et al. [9] demonstrated that an implanted graft may lose more than 85% of its initial stiffness and tensile strength up to 6 months after surgery during the process of graft maturation. Unfortunately, this timing also coincides with improved performance at physical therapy, diminished pain, improved knee motion, and a normalized gait pattern which may give some patients (and some clinicians) a false sense of security when they are faced with the decision of whether they should return to their desired sport (Figure 1).

ACL GRAFT INCORPORATION AND MATURATION

Soft Tissue Grafts

Normal tendon/ligament insertions can be either direct or indirect. Indirect insertions are characterized by a sharp transition between the tendon substance and the bony interface with obliquely oriented crossing fibers that insert into bone (i.e., Sharpey's fibers). The superficial medial collateral ligament (MCL) is one example of a naturally occurring indirect insertion site. Direct tendon/ligament insertions are composed of a highly specialized transition zone that, in the case of the ACL, functions as a buffer to protect the ligament substance from excessive stress and strain. This transition zone is composed of 4 distinct layers (ligament, non-mineralized fibrocartilage, mineralized fibrocartilage, and bone). However, after ACL reconstruction, the incorporation of soft tissue grafts within bone tunnels mostly involves the deposition of fibrovascular scar tissue and the appearance of Sharpey-like fibers, thus closely resembling an indirect ligament insertion (as opposed to the 'direct' ligament insertion sites characteristic of the native ACL) [5, 10].

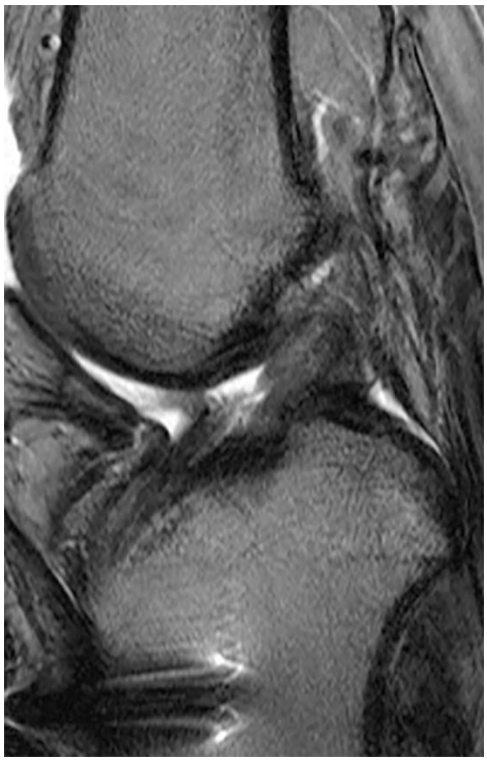

Figure 1. Sagittal MRI obtained from a patient who returned to the clinic with recurrent pain and instability 6 months after ACL reconstruction with hamstring autograft. Complete graft rupture was diagnosed and the patient underwent revision.

During the initial stages of graft maturation (i.e., "ligamentization"), the tendon-bone interface is infiltrated with granulation tissue composed of type III collagen and numerous growth factors that promote chemotaxis of macrophages, fibroblasts, and endothelial cells.

Chondrogenic progenitors begin to migrate into the tendon-bone interface where they begin to replace granulation tissue with type II collagen, thus initiating the process of endochondral ossification (similar to callus formation during fracture healing [11]). Sharpey-like fibers composed of type III collagen then begin to populate the tendon-bone interface to help minimize graft motion until bony ingrowth is completed – a process which often requires 12 weeks or longer to complete. Simultaneous with the above-mentioned process, the tendon graft is gradually decellularized by infiltrating macrophages, thus leaving behind the extracellular matrix (ECM) which functions as a scaffold for infiltrating progenitor cells. This remodeling process explains the substantial decline in tensile strength and stiffness that occurs after graft placement until the decellularized ECM scaffold is repopulated with host cells. In response to the expression of fibroblastic growth factor (FGF) at the tendon-bone interface between 8 and 12 weeks postoperatively, fibroblasts begin to migrate into the tendon substance and deposit type III collagen, originating from the tendon-bone interface and progressing towards the intra-articular portion of the graft [12]. This process continues until the migrating front of repopulating fibroblasts meet in graft mid-substance. The final stage of maturation involves the replacement of type III collagen with type I collagen and the realignment of disorganized collagen fibrils into a more parallel configuration. Most of our understanding of graft healing and maturation is derived from animal studies and limited evidence exists in human subjects; however, magnetic resonance images (MRIs) obtained postoperatively often show significant changes in signal intensity that reflect a decrease in structural integrity 6 months after graft placement (Figure 2).

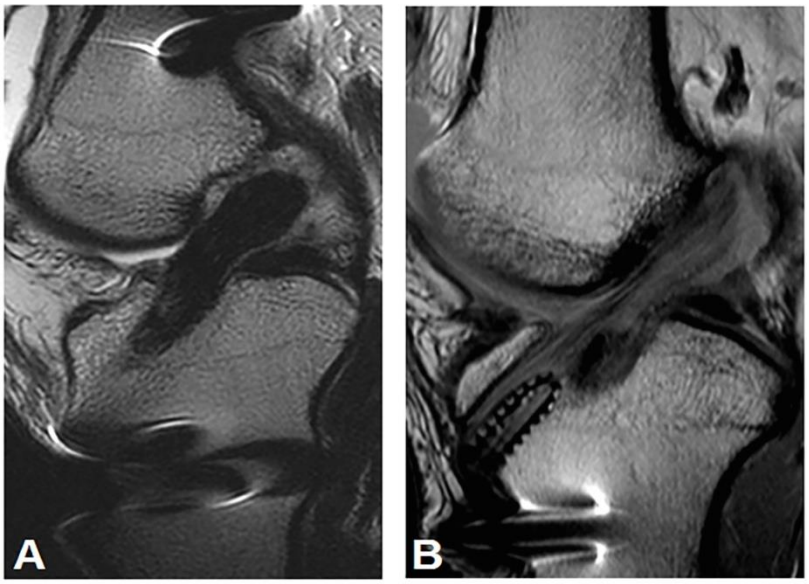

Figure 2. Sagittal MRI slices demonstrating graft appearance at (A) time zero and (B) 7 months after placement of hamstring autograft.

Tendon-bone integration drastically improves pullout strength and is a critical component of long-term graft stability [10, 13]. Graft maturation is required for the long-term maintenance of proper joint kinematics and the prevention of joint degeneration. For soft tissue grafts, the process of bony ingrowth requires up to 12 weeks of relative inactivity and

up to 12 months of physical therapy before full graft maturation is complete (longer if allograft tissue is used). The prolonged rehabilitation that is required after implantation of a soft tissue graft is considered one of the major disadvantages related to their use in ACL reconstruction, especially in young athletes who are anxious to return to competitive sports. However, it should be noted that although bony union of BTB grafts occurs more rapidly, intra-articular graft maturation of BTB grafts undergo the same remodeling process as soft tissue grafts and, therefore, often require a similar overall length of rehabilitation as when soft tissue grafts are used.

There exist a number of strategies that can be implemented at the time of surgery that may improve bony integration of soft tissue grafts within the bone tunnels. Some of these include increasing the length of the bone tunnels [14], matching graft diameter with bone tunnel diameter [15], and ensuring circumferential contact between host bone and the implanted graft tissue without an interference screw (i.e., to maximize surface contact with bone and to prevent leakage of synovial fluid into the bone tunnels) [16]. Rehabilitation protocols can also be catered to the method of graft incorporation – that is, bone-bone healing (as in BTB grafts) or tendon-bone healing (as in hamstring grafts), along with the consideration of whether an autograft or allograft was used. For example, it stands to reason that bone-bone healing would allow for a relatively accelerated rehabilitation protocol (i.e., minimizing the degree of postoperative quadriceps atrophy) when compared to grafts that rely upon tendon-bone healing, simply because of the reduced amount of time required for bony union to occur. However, although the above surgical and rehabilitative modifications can improve tendon-bone integration, there still exists much room for improvement from a biological standpoint.

Bone-Tendon-Bone (BTB) Grafts

In contrast to soft tissue tendon grafts which must heal to the sides of bone tunnels (i.e., producing an indirect-like insertion site), BTB grafts rely primarily upon bony union to achieve fixation which reliably occurs at approximately 6 weeks postoperatively – a time frame that is much shorter than that which is required for soft tissue graft incorporation. The bone block first undergoes osteonecrosis (as a result of infiltrating macrophages) followed by bony ingrowth derived from the cancellous bone that lines each of the tunnels. It has been well established that the use of BTB grafts enhance graft fixation by endochondral ossification (i.e., bony union) which is much more rapid, and perhaps more reliable, than those requiring bony ingrowth into the graft substance (such as soft tissue grafts).

When a BTB graft is used, the site of early failure is almost always located at the bone-bone interface between the bone plug and the bone tunnel until approximately 3 weeks postoperatively, at which point the bone-bone fixation strength begins to increase [1, 17]. As the fixation strength improves at the bone-bone interface, the site of failure shifts to the graft itself, at the transition between the bone plug and the tendon; this is the most common site of failure between 6 weeks and 9 months after implantation of BTB grafts [17]. Graft maturation then occurs in a manner identical to that of soft tissue grafts as a front of fibroblasts begin to migrate towards the intra-articular portions of the graft. It is important to remember that the bone plugs of BTB grafts do not extend the entire length of the tunnel. In other words, it is actually the soft tissue portion of BTB graft that exits from the bone tunnel. As a result, very

high stresses may occur in this area of BTB grafts, particularly before maturation of the soft-tissue portion of the graft has occurred. Therefore, although bone-bone healing occurs more rapidly and provides an impetus for "accelerated" rehabilitation, it must be remembered that, after bony union has occurred approximately 6 weeks after surgery, the remainder of the healing process involves both tendon-bone healing and graft maturation.

Autografts versus Allografts

Although the same general principles of graft healing apply to both autografts and allografts, there are several important fundamental differences that must always be considered. Most notably, it has been well-described that allografts require more time for bony incorporation, revascularization, and host cell repopulation and they also have a decreased failure load for as long as 12 months after surgery when compared to autografts [18-20]. In addition, allografts have also been found to be occupied by inflammatory cells for prolonged periods of time, which effectively prevents graft maturation and remodeling while simultaneously promoting scar tissue formation [18-21]. Other variables that may have an impact on allograft healing such as graft sterilization (i.e., irradiation), freezing, and decellularization have also been well described in the literature [22-29]. When considering the above evidence in light of patient-specific factors, most surgeons recommend autograft reconstruction for younger athletes (i.e., under 35 years of age), most commonly utilizing the central one-third of the patellar tendon (BTB autograft), in order to maximize early fixation strength and to minimize the length of time the athlete must spend away from their sport. Although the majority of existing short- and mid-term clinical outcomes data are inconclusive [30], many surgeons believe that autografts have more favorable long-term biomechanical properties than those of allografts.

Biological Grafts for ACL Reconstruction

There exists a significant push towards the development of strategies that would allow orthopaedic surgeons to avoid the use of autografts or allografts for ACL reconstruction, thus obviating many of their inherent disadvantages. Of particular concern is the procedure required for BTB graft harvest. In this case, the surgeon must excise a sufficiently sized bone block from the patella, which produces stress risers and may predispose to subsequent patellar fracture. Furthermore, removal of the central-third of the patellar tendon may also increase the risk of future patellar tendon rupture, an injury that would be difficult to repair given the lack of reparable tissue. Although the clinical evidence is inconclusive, anecdotal reports suggest that patients who undergo ACL reconstruction with BTB autografts may experience an increased level of postoperative pain during rehabilitation, perhaps due to the cyclic loading and unloading of the midline incision during range of motion exercises. Other logistical problems, such as the lack of available autograft tissue after multiple revision surgeries, can also force surgeons to choose less-optimal grafts. Allografts, on the other hand, require up to 12 weeks of protection to allow for bony integration and often an additional 12 months to fully mature. To avoid many of these issues, much research in the past decade has

focused on the development of scaffold materials that can be used to either replace the torn ligament entirely or to augment primary ACL repair.

In general, the most widely used scaffold materials are composed of decellularized ECM, alginate, chitosan, polyglycolic acid (PGA), poly-L-lactic acid (PLA), polycaprolactone (PCL), hydrogels, and silk, among a variety of others. The use of synthetic scaffolds and ligament substitutes (i.e., carbon fiber, polyester, or polyaramid fibers) for ligament reconstruction have been slow to gain momentum since their premature introduction which initially led to high rates of reactive synovitis from wear particles, early graft rupture, and graft elongation [31-33]. However, because significant advances have been made in other areas of musculoskeletal research since the introduction of synthetic scaffolds, we are now able to integrate new concepts from biomedical engineering with those of molecular biology, genomics, and clinical medicine to generate superior biomimetic scaffold materials that are capable of supporting tissue regeneration. For example, the recent development of silk scaffolds is intriguing because their mechanical, structural, and biological properties can all be easily manipulated [31, 33, 34] Richmond and Weitzel [33] tested their synthetic ligament substitute using a custom-fabricated silk construct for ACL reconstruction in a goat model. Interestingly, histologic sections obtained from the tendon-bone interface 12 months after reconstruction revealed a normal-appearing crimp pattern and the presence of Sharpey-like fibers. Other artificial ligament substitutes that feature mesenchymal stem cells (MSCs), a sustained release of growth factors, and biomechanical properties that are similar to the native ACL are on the rise due to the appearance of favorable and repeatable results in animal studies [35-37]. Although these types of scaffolds have not yet been introduced into clinical practice, they certainly represent some of the most promising advances in biomedical engineering with the potential for widespread clinical impact.

Another interesting recent development involves the use of collagen-platelet composite (CPC) scaffolds which attempt to circumvent the known problems with primary ACL healing due to the presence of synovial fluid which contains several enzymes that are detrimental to ligament healing (i.e., intra-synovial urokinase and plasmin). CPCs are decellularized ECM scaffolds that are permeated with autologous platelet-rich plasma (PRP) obtained at the time of surgery to enhance the capacity for ligament regeneration with the goal of re-introducing the validity of primary mid-substance repair of the ACL [38]. Several studies using animal models have shown that primary ACL repairs with CPCs enhance growth factor expression, histologic appearance, MRI appearance, and biomechanical properties when compared to non-augmented primary ACL repairs (up to 3 months after repair) or untreated ACLs [38-44]. This technique for augmented ACL repair may become clinically relevant when more validated data is published; however, it is easy to imagine that the path towards clinical translation will be prolonged given the major difficulties involving ethical issues and blinding procedures that will arise during the development of prospective comparative clinical trials.

Because ACL reconstruction procedures typically involve implantation of an autograft or allograft (which have many limitations as listed above), artificial ligaments are becoming an attractive alternative to the use of traditional graft materials for ACL reconstruction [45, 46]. However, this technology has primarily focused on the use of non-biodegradable artificial ligaments that have resulted in the development of synovitis and chronic inflammatory reactions that have ultimately led to poor ligamentization and high failure rates [45, 47-50]. New biodegradable acellular synthetic grafts (based on the electrospun, biodegradable synthetic material poly (ester urethane) urea [PEUU] elastomeric scaffold [51, 52]) are

currently being developed and may prove to be a suitable graft material for stem cell delivery at the time of ACL reconstruction, potentially providing dramatic cost savings, accelerating rehabilitation, and improving patient satisfaction [53, 54].

RATIONALE FOR THE USE OF BIOLOGICS IN ACL RECONSTRUCTION

Because tendon-bone incorporation requires an extended amount of time for bony ingrowth, it is inevitable that substantial graft motion will occur during this time which invariably prolongs the duration of inflammation and increases the amount of osteolysis (i.e., tunnel widening) and scar tissue formation at the tendon-bone interface (Figure 3) [3]. Several studies have noted significant correlations between accelerated rehabilitation protocols and slower healing rates, more scar tissue, and tunnel widening, presumably as a result of increased graft motion [3, 21, 55]. Of note, graft motion is not isolated to soft tissue grafts; recent *in vivo* kinematic data suggests that graft motion can also occur after placement of BTB grafts in the early postoperative period following ACL reconstruction [56]. Additional problems include the lack of intrinsic graft vascularity and angiogenesis, the low numbers of progenitor cells near the tendon-bone interface, and the lack of balance between anabolic and catabolic processes that generally results in disorganized fibrovascular scar tissue [57-64].

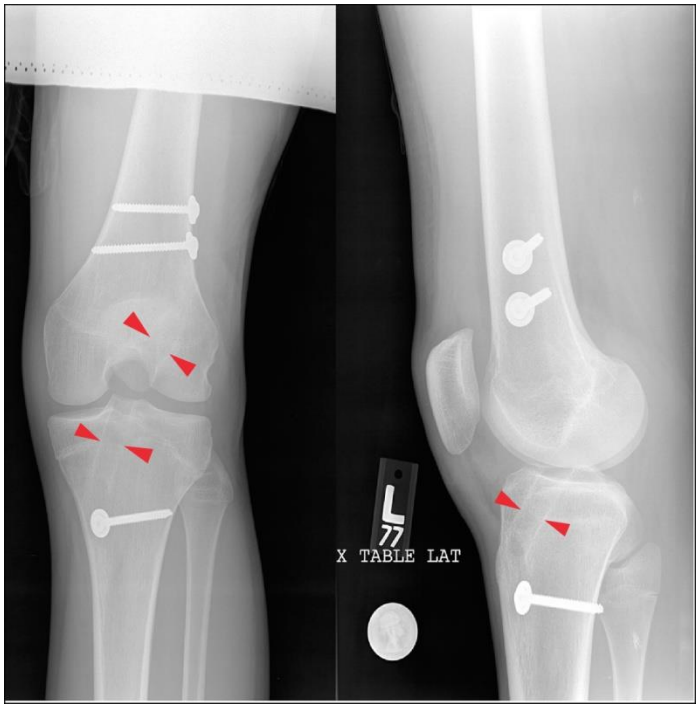

Figure 3. Anteroposterior (AP) and lateral radiographs of the left knee in a patient who returned to clinic with recurrent symptoms two years after primary ACL reconstruction with allograft. Note the significant tunnel widening that is present on both the tibial and femoral sides (red arrows).

Pharmacological strategies to improve tendon-bone healing have been investigated for many years, but the many differences in study designs and the generally conflicting data sets have made it difficult to consolidate the results of these studies into focused clinical applications. For example, some studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) delay graft healing and maturation [65] whereas others have demonstrated that cyclooxygenase-2 (COX-2) inhibitors may actually improve bony ingrowth into soft tissue grafts [13], perhaps as a result of the down-regulation of macrophage activity at the tendon-bone interface which has been reported by other groups [21, 65]. However, still others have found that non-specific COX inhibitors (e.g., NSAIDs) may up-regulate macrophages while simultaneously down-regulating lymphocytes [66], thus heightening confusion on this issue. Other pharmacological strategies include manipulating the bioactivities of receptor activator of nuclear factor kappa-B ligand (RANKL) or osteoprotegerin (OPG), which have shown some evidence of improved bony ingrowth, smaller tunnel diameters, and increased graft stiffness [67, 68]. Overall, although some pharmacological strategies may have some promise for improving tendon-bone integration, there is currently a lack of validated clinical evidence supporting their use for this indication, perhaps due to the shift towards more biologic strategies that may afford additional control of the healing process. With regard to ACL reconstruction, there certainly exists significant amount of interest in the development of new biologic strategies to improve and accelerate the bony integration and maturation of graft tissues.

The intended goals for the use of biologics in ACL reconstruction are primarily geared towards accelerating the return of athletes to high-level activities while also minimizing the risks of graft failure, including early graft pullout, elongation, and late graft rupture. Perhaps driven by the increased visibility of research to the general public, the use of biologics in sports medicine has garnered a great deal of interest; however, unfortunately, none of the presumed reasons for its early introduction are related to overwhelmingly favorable clinical evidence. Therefore, as a result of primarily anecdotal evidence, many elite athletes (including those at the high school, college, and professional levels) express interest in using biologics to accelerate rehabilitation after their injuries or surgical procedures, including after ACL reconstruction. In particular, the ability of young athletes to remain eligible for collegiate scholarships, professional contracts, or similar opportunities is dependent upon their timely return to competition after injury, thus generating additional pressure for the athlete to accelerate their functional recovery. Therefore, combined with the premature enthusiasm for the use of new cutting-edge biologics in the medical field, sports medicine specialists are often pressured to treat their patients with expensive biologics that, although approved by the Food and Drug Administration (FDA), have limited clinical benefit. However, although our current state of research is far behind the current level of social fascination in this area, significant advances have been made in the past decade with regard to biologic treatments that are already FDA-approved and available for use, most notably PRP (a type of growth factor therapy) and bone marrow aspirate concentrate (BMAC; a type of cell therapy).

GROWTH FACTOR THERAPY

Platelet-Rich Plasma (PRP)

Circulating platelets, which contain numerous α granules that house more than 1000 bioactive molecules in physiologic ratios (e.g., growth factors, cytokines, etc.), are the first responders to soft tissue injury owing to the disruption of small capillaries and the leakage of blood into injured soft tissues. Inflammatory cytokines from locally inflamed tissues stimulate platelets to release the contents of their α granules, which then participate in the various cellular and molecular processes involved in the early phases of healing [69, 70].

Researchers have studied PRP and its effects on tissue healing since the 1950s [71], but it wasn't until the 1970s that PRP was investigated as a potential therapeutic modality [72]. With the ongoing development of biologic treatments for a variety of medical conditions over the past few decades, the use of platelet concentrates has garnered significant interest due to the high concentrations of proteins within their α granules that are known to promote tissue regeneration, among other potentially beneficial effects. It has generally been thought that the delivery of supra-physiologic concentrations of platelets to an injured area would accelerate healing while also improving the quality of repair tissues. In support of this contention, it has been shown that in the absence of a platelet-rich concentrate, some of the growth factors released from platelets are functionally impeded by local inflammatory mediators, thus potentially reducing the quality of repair tissues [73, 74]. These findings provided further support for the development of new strategies that utilized some form of autologous platelet concentrate.

However, much of the confusion related to the study of PRP is quite simply that we have a lack of knowledge regarding the identities and functions of the thousands of different growth factors and cytokines that are most likely released in unison after bolus injection. In addition to the widely varying numbers of platelets, our inability to quantify them, and their varying ratios of growth factors (even within the same patient), we also lack standardized methods to prepare marginally consistent PRP samples even after all variables had been controlled [75]. It is clear that any attempt to address all of these issues would be extremely daunting, let alone trying to determine which growth factors are beneficial for tissue healing and which are detrimental.

Numerous prospective clinical studies have been performed in attempts to evaluate the impact of PRP therapy on the clinical outcomes after ACL reconstruction, including several randomized controlled trials [76]. Perhaps expectedly, the overall results of these studies taken together have been equivocal. Nevertheless, some studies have reported favorable results in terms of postoperative remodeling (i.e., ligamentization) [77-79] Sanchez et al. [78] studied the effects of PRP treatment on graft remodeling in 37 patients who eventually required second-look arthroscopy (n=15 without PRP, n=22 with PRP). In the group treated with PRP, each autologous hamstring tendon was injected with PRP several times along its length and then soaked in a PRP solution before implantation. After histologic analysis of biopsy specimens obtained at the time of second-look arthroscopy, the authors reported significantly improved histologic appearances of grafts that were previously treated with PRP, although all of the grafts appeared macroscopically similar. Of note, biopsy specimens in this study were obtained anywhere between 6 and 24 months after graft implantation. One

additional study reported a 48% decreased time for the intra-articular portion of the graft to become homogenous in appearance on MRI after PRP treatment [77].

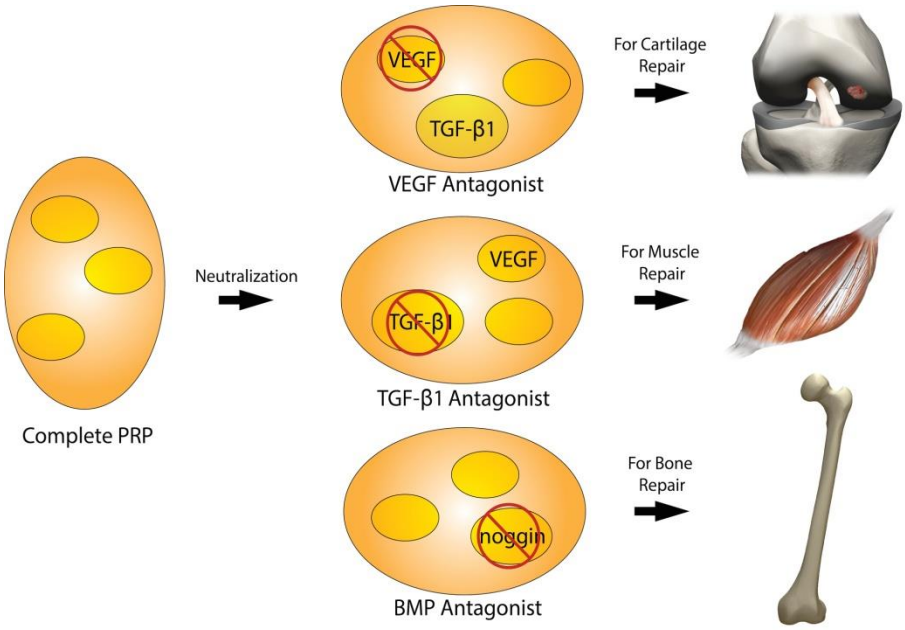

Figure 4. Strategy for the development of personalized PRP preparations according to the type of tissue being treated (also known as second generation PRP). Rather than identifying and characterizing each of the many thousands of growth factors that are present in PRP, it has been proposed that we inhibit the growth factors that we already know are detrimental to tissue regeneration. It is currently thought that neutralizing TGF-β1, VEGF, and noggin would improve the healing of skeletal muscle, articular cartilage, and bone, respectively, after an injury.

Although there is currently a paucity of clinical evidence that favors the use of PRP in clinical practice, recent conceptual developments have allowed us to focus on removing the portions of PRP that are detrimental to certain tissues – a significant step in the direction of personalized medicine. An important concept recently put forth by Terada et al. [80] simplifies the entire matter by suggesting that we simply block the growth factors that are already known to be detrimental for the healing of certain tissue types. For example, we already know from other studies that losartan (an Angiotensin II Receptor Blocker [ARB]) prevents the action of TGF-β1 and inhibits scar tissue formation; therefore, losartan can be added to PRP in cases where the clinician wants to prevent scar formation, such as after muscle injuries. However, inhibition of TGF-β1 can be detrimental in tissues that require TGF-β1 for adequate regeneration, such as articular cartilage. Therefore, the use of neutralizing antibodies to block TGF-β1 in PRP prior to its use may impede regeneration of articular cartilage in focal chondral defects, but the same preparation can be used to enhance muscle regeneration through the avoidance of scar tissue formation [81]. In other words, when used for the treatment of focal chondral defects, it is more desirable to inhibit the pro-angiogenic factors within PRP before injection in order to reduce the ratio of fibrocartilage to hyaline cartilage in the repair tissue [82]. In contrast, it is more desirable to inhibit TGF-β1 within PRP when used for the treatment of muscle injuries to prevent scar tissue formation. It

is also possible to inhibit noggin (i.e., a well-known BMP antagonist) within PRP to accelerate bony ingrowth into soft-tissue grafts within bone tunnels. This concept represents a significant advancement towards the development of more efficacious preparations of PRP that are specifically targeted for the type of tissue being treated (Figure 4).

Bone Morphogenetic Proteins (BMPs)

BMPs (especially BMP-2 and BMP-7) have been studied extensively due to their ability to rapidly promote new bone formation, including that which occurs during soft tissue graft integration within bone tunnels [83-87]. For example, several studies have demonstrated that BMPs increase the thickness of the fibrovascular interface and also increase the depth of bony ingrowth into soft tissue grafts, thus leading to improved graft pullout strength [83-85, 88-90]. The results of these studies have been encouraging and, in some cases, have already been used in the settings of skeletal trauma and non-union to promote healing. However, researchers have yet to consistently demonstrate the regeneration of histologically normal transition zones that are characteristic of direct ligament insertion sites, perhaps due to our current lack of controlled delivery of BMPs into the areas of interest.

Transforming Growth Factor-β (TGF-β)

The Transforming Growth Factor-β (TGF-β) family consists of secreted proteins that regulate the deposition of fibrous tissue during the healing process after connective tissue injury. The three major isoforms of TGF-β (i.e., TGF-β1, TGF-β2, and TGF-β3) work in synchrony with each other and with other cytokines via both autocrine and paracrine signaling pathways to achieve an appropriate balance between catabolic and anabolic processes that ultimately lead to tissue healing. TGF-β1 and TGF-β2 are primarily expressed during the early stages of inflammation following tendon injury to promote fibroblast proliferation and collagen synthesis while also acting as chemotactic stimuli for the infiltration of inflammatory cells [91-94]. With specific regard to tendon-bone healing, TGF-β1 and TGF-β2 are responsible for the deposition of scar tissue at the tendon-bone interface which improves graft pullout strength, but does not closely reproduce the structure or biomechanical function of a direct ligament insertion [95] – rather, the healing interface more closely resembles an indirect ligament insertion with a characteristically sharp transition between tendon and bone along with the appearance of Sharpey-like fibers [5, 7, 10]. TGF-β3, on the other hand, has recently been identified as a significant contributor to the ‘scarless’ healing that occurs during fetal development until shortly after birth [96]. In addition, several animal studies have demonstrated improved strength, collagen type I/III ratios, histologic appearance, and collagen organization when healing insertion sites were exposed to supra-physiologic levels of TGF-β3 [97-99]. In other words, these studies have provided evidence that TGF-β3 promotes the formation of functional transitions zones that are characteristic of direct tendon/ligament insertion sites. Although the potential roles of TGF-β3 in tendon-bone healing have not yet been clearly identified, recent studies have provided encouraging results that may lead to a reduction in surgical complications related to the deposition of disorganized scar tissue and, therefore, an improvement in overall clinical outcomes.

Matrix Metalloproteinases (MMPs)

Although the inhibition of TGF-β1 can be used to effectively prevent scar tissue formation within healing muscle tissue (as mentioned above), this technology has very little effect on the existing scar that is already present in the tissue. To that end, the use of matrix metalloproteinases (MMPs) may become an acceptable strategy to dissolve the existing scar within a previously injured tissue. Indeed, the use of MMPs have been successfully utilized to improve tissue healing by reducing the burden of existing scar tissue within both injured and diseased skeletal muscles [100-102].

In the setting of ACL reconstruction, it is understood that MMPs are up-regulated by Interleukin-1 (IL-1) and TGF-β, both of which exist in high numbers during the inflammatory process [94, 103, 104]. MMPs are generally catabolic and are responsible for the regulation of connective tissue homeostasis and collagen remodeling. However, in addition to the baseline presence of urokinase and plasmin in the synovial fluid (i.e., prevent the formation of intra-articular fibrin clots after injury [43, 105]), the large quantities of activated MMPs that exist in the synovial fluid after ACL rupture also prevent the deposition of ECM components, such as that which occurs during tendon-bone integration and/or bone-bone healing after ACL reconstruction. Therefore, another potential biologic strategy to improve tendon-bone healing is the inhibition of MMP bioactivity, such as with the use of intra-articular doxycycline [106]. With respect to soft tissue grafts, inhibition of MMPs effectively prevents bone resorption (i.e., tunnel widening [18]), improves load-to-failure at both 2 and 5 weeks postoperatively, and improves the organization of interposed Sharpey-like fibers at the tendon-bone interface [25]. Similar results have been shown after rotator cuff repair supplemented with an MMP inhibitor [106, 107]. In the future, this strategy to reduce tunnel widening via MMP inhibition can perhaps be amplified by adding BMPs to increase new bone formation, to reduce the incidence of early graft pullout, and to accelerate the rate of tendon-bone integration.

Insulin-Like Growth Factor-1 (IGF-1)

After ACL rupture, insulin-like growth factor-1 (IGF-1) contributes to the deposition of ECM in the early phases of inflammation by recruiting inflammatory cells and local fibroblasts to the site of injury. In addition, IGF-1 also induces the synthesis and deposition of type I and III collagen along with the secretion of proteoglycans by fibroblasts in a dose-dependent manner [108-110], thus initiating the formation of early granulation tissue. An identical process occurs following the drilling of bone tunnels: IGF-1 recruits inflammatory cells and fibroblasts which then begin to deposit granulation tissue at the tendon-bone interface. In fact, several studies have demonstrated that the absence of IGF-1 significantly hinders the production of stable and functional granulation tissue at the site of acute penetrating injuries [111, 112]. It appears that IGF-1 is a critical component to achieve successful healing of most tissues within the musculoskeletal system, although it is unclear whether the injection of additional IGF-1 into inflamed tissues would accelerate or otherwise improve tissue healing. However, a few preclinical studies have provided encouraging results suggesting that IGF-1 may accelerate healing and improve functional outcomes after tendon repair in both animals and humans without affecting its overall tensile strength [113-115].

Although these studies were not designed specifically for the study of tendon-bone healing, it is possible that their results can be applied to the process of graft incorporation following many types of orthopaedic procedures, including ACL reconstruction.

Fibroblastic Growth Factor (FGF)

Similar to IGF-1, fibroblastic growth factor (FGF) also contributes to the deposition of structural granulation tissue and extracellular matrix (ECM) during the process of tendon-bone integration. However, in contrast to IGF-1, FGF is critical for angiogenesis, the continued deposition of types I and III collagen, and, in the presence of BMPs, the differentiation of local MSCs towards the osteoblastic lineage [8, 116]. Of note, the local concentration of FGF at the tendon-bone interface is dependent upon the stage of healing [8]. More specifically, the presence of scar tissue at the tendon-bone interface is correlated with higher levels of FGF, presumably reflecting active collagen synthesis. As osteoblasts begin to infiltrate the collagenous scar and deposit woven bone at the tendon-bone interface in response to local BMPs, FGF levels begin to decline. Therefore, the coordinated actions of BMPs, IGF-1, and FGF are mutually synergistic – that is, the local secretion of IGF-1 after injury recruits local fibroblasts, which in turn, secrete FGFs to accelerate collagen deposition and strengthen early granulation tissue. The secretion of BMPs by local MSCs then provide a stimulus for their own differentiation into osteoblasts and for the recruitment of more resident fibroblasts and MSCs from the local environment.

Platelet-Derived Growth Factor (PDGF)

In the setting of an acute extra-articular injury, disruption of local blood vessels leads to the exposure of basement membrane collagen upon which platelets normally adhere and contribute to the production of a fibrin matrix, thus preventing further blood loss and initiating the healing process. Platelet-Derived Growth Factor (PDGF) is found within the α granules of platelets and is released when platelets begin to adhere to both the collagenous basement membrane and to one another. After its release into the ECM, PDGF strongly promotes chemotaxis of inflammatory mediators, proliferation of numerous cell types (such as fibroblasts, myoblasts, and glial cells), up-regulation of the local metabolic rate, and potentiation of the extrinsic clotting cascade. With respect to graft healing after ACL reconstruction, PDGF is present within the early granulation tissue that appears at the tendon-bone interface within the first week after injury; however, the production of a fibrin matrix in this area is inhibited by the urokinase and plasmin present within the synovial fluid. When the tendon-bone interface is sealed from the intra-articular environment, the levels of PDGF in the graft substance begin to increase progressively until approximately 6 weeks after graft implantation, at which point the levels of PDGF begin to taper until approximately 12 weeks after surgery, corresponding to the observed time period required for adequate bony integration of soft tissue grafts [117].

The substantial bioactivity of PDGF highlights some of the vital components of healing that are typically absent in tissues that have a tenuous blood supply, such as the ACL. In an intriguing study in rabbits performed by Lee et al. [118] the investigators compared the

growth factor expression profiles between the MCL and the ACL after an injury. They found that PDGF, TGF-β, and basic FGF were present in both ligaments at the site of injury; however, the levels of each growth factor were significantly greater in the injured MCL owing to its substantially increased density of perforating blood vessels. Although the same growth factors were also found in the ACL, they were primarily localized to the torn edges of the ligament remnants in much lower concentrations [118]. The deficiency of inductive growth factors at the site of ACL injury as a result of its poor blood supply and the catabolic intra-articular environment may explain the lack of primary healing seen after mid-substance ACL injuries, even after surgical re-approximation of its torn ends. However, although uncommon, ACL repair can be attempted in carefully selected cases in which the tear occurs at its insertion on the medial femoral condyle where the blood supply to the ligament substance is more robust and when the repair can prevent synovial fluid leakage into the ligament-bone interface.

Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is secreted by local endothelial cells and inflammatory cells in response to inflammatory cytokines (e.g., IL-1 and FGF, among others) and low oxygen tension [119, 120]. VEGF exerts its effects through both autocrine and paracrine signaling pathways that allow for the directional growth of new blood vessels [120]. A relatively recent study by Kohno et al. [8] showed that the levels of VEGF are highest within the bone tunnels along the tendon-bone interface in the early postoperative period after ACL reconstruction. In that study, local osteoblasts continued to express VEGF until 12 weeks after surgery, coinciding with the observed time required for the bony integration of soft tissue grafts [1].

It is important to understand that angiogenesis plays a significant role during the process of tendon-bone integration and graft maturation, although the optimal extent of this vascularity has not been clearly defined. Ju et al. [58] found that the introduction of VEGF into graft tissues improved their vascularity and histologic appearance (i.e., generation of a ‘transition zone’ at the tendon-bone interface). However, the same investigators also found that excessive angiogenesis may actually be detrimental to the healing process by promoting scar tissue formation [58]. Therefore, there likely exists an optimal range of vascular density that may improve tendon-bone healing without becoming deleterious [58, 59].

CELL THERAPY

Bone Marrow Aspirate Concentrate (BMAC)

While PRP is considered a form of growth factor therapy, BMAC is considered a form of cell therapy because it contains MSCs and bone marrow-derived stem cells (BMSCs) in addition to having similar levels of growth factors to those found in PRP [121]. However, BMAC suffers from the same pitfalls as PRP with regard to its use as a therapeutic adjunct largely due to the lack of standardized techniques for its harvest and preparation that can

isolate individual stem cell types or produce BMAC formulations with similar contents. In addition, it is often difficult to harvest a sufficient number of MSCs from the aspiration to allow for immediate re-implantation without the need for extensive *ex vivo* expansion. Similar to PRP, there is insufficient evidence to suggest that BMAC treatment using any of the currently-available techniques provides any clinical benefit, perhaps as a result of the low yield from bone marrow harvest and/or the rapid cell death that may occur at the time of injection [122].

Mesenchymal Stem Cells (MSCs)

MSCs are adult stem cells with specific surface markers that have the ability to differentiate into muscle, bone, cartilage, tendon/ligament, or fat depending on the local environment. MSCs are most often found in fat, skin, and, most important for orthopaedic applications, the periosteum and bone marrow [123]. Numerous studies have been published that evaluate the use and application of MSCs from each of these tissue types for musculoskeletal medicine.

Recent research has explored the possibility of delivering isolated MSCs directly into the healing zones of ACL grafts in order to more closely replicate an organized and functional transition zone, to promote restoration of knee function and stability, and to accelerate graft fixation at tendon-bone interfaces by enhancing bony ingrowth [84, 85, 124-126]. However, it appears that the translation of this basic science data into clinical application will be a challenging endeavor, especially due the desire to develop single-stage procedures that would not require *ex vivo* expansion. In one of the few studies on the use of MSCs for ACL reconstruction, Silva et al. [122] compared the time to tendon-bone healing on the MRIs of 43 patients who underwent ACL reconstructions using hamstring autografts with (n=20) or without (n=23) augmentation using non-cultivated MSCs. Perhaps expectedly, there were no significant differences in any of the MRI parameters between the two groups. Others have attempted to utilize MSCs to regenerate the central one-third of the patellar tendon following BTB harvest [127], but the clinical utility of this strategy also appears limited.

There exist a number of other cell types that have shown some capacity for ligament regeneration *in vitro* following stimulation with growth factors; these primarily include fibroblasts (such as those harvested from the ACL remnants [Figure 5]) and BMSCs that may have potential for future *in vivo* studies [128]. However, problems still exist regarding the low cell numbers obtained during tissue harvest and the limited survivability and migratory capacities of the MSCs after transplantation. It has been recently reported that the walls of blood vessels contain perivascular and endothelial cell markers that are believed to be at the origin of a multitude of stem cell lines [129-131]. Taken together, these results suggest that the blood vessel walls contain stem cells that are likely the origin of various MSC populations, supporting the theory that most adult stem cells originate from well-vascularized tissues, while a paucity of stem cells are associated with poorly-vascularized tissues such as articular cartilage. In fact, it has been demonstrated that the more-vascularized MCL heals much more effectively than the less-vascularized ACL. More importantly, a reduction of angiogenesis within the MCL has been shown to reduce the healing potential of the MCL to a similar degree to that which is observed in the native ACL [132]. Subsequently, blood vessel-derived stem cells have been shown to exist within the ACL (i.e., ACL-derived stem cells)

and, more importantly, these cells can potentially be used to improve the outcomes of ACL reconstruction via intra-articular injection [133, 134]. Improvements in clinical outcomes after ACL reconstruction using stem cell implantation technologies have been primarily attributed to the enhancement of angiogenesis and osteogenesis [133, 135].

One potential adjunctive strategy is gene therapy, which may become valuable for bypassing some of the issues related to MSC survivability and the need for *ex vivo* cell expansion. Rather than attempting to delivery isolated growth factors to an area of injury to promote cell proliferation and differentiation (which runs the risk of rapid washout), gene therapy involves strategically inserting a gene into certain cells (using viral vectors) that is eventually translated into the desired protein or growth factor (such as one of the growth factors described above). Many of the strategies related to genetic manipulation are currently impractical due to the risk of immunogenicity [136], the difficulty in reproducing previous results [137], and the fear of oncogenic transformation, especially when anti-apoptotic genes are up-regulated. Nonetheless, the use of genetically-engineered cells that provide a multitude of growth factors and cytokines that promote tissue repair represent an attractive strategy to accelerate tendon-bone healing and intra-articular graft maturation after ACL reconstruction.

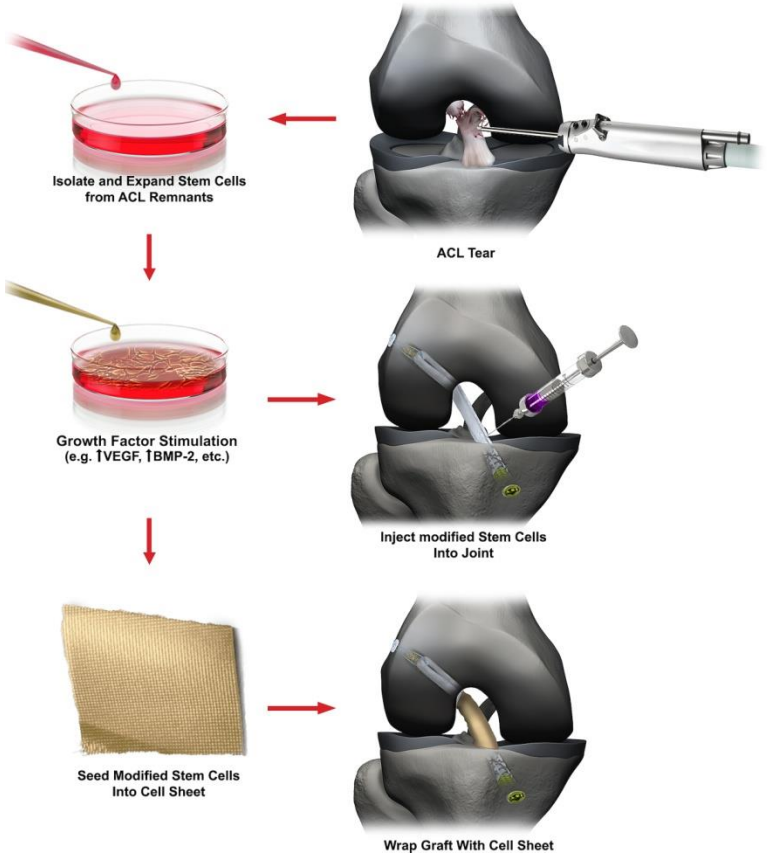

Figure 5. Illustration depicting a potential strategy for improving the healing capacity of the native ACL or graft integration after ACL reconstruction. ACL-derived stem cells can be harvested from the ACL remnants and then injected (with or without genetic modification) into the knee joint to improve ACL healing. Stem cells can also be seeded into a cell sheet scaffold and wrapped around the graft prior to graft placement.

CONCLUSION

Recent advances in the study of biologics for musculoskeletal medicine have allowed us to introduce concepts that have previously seen many years of relative inactivity, including the use of PRP, stem cells, gene therapy, and, potentially, synthetic graft materials. However, one other important change that has occurred involves a shift in our thought processes regarding the manner in which we develop new biologics. More specifically, we are now studying biologics and refining our current techniques in the context of supplementing, rather than replacing, the complex healing mechanisms that already exist in order to enhance tissue recovery following an injury or degenerative process. This new paradigm of strategic thinking will accelerate the development of effective biologic therapies that are highly relevant both in concept and in application.

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