Chapter 18
Ligament Tissue Engineering

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Abstract Ligaments are commonly injured in the knee joint, and have a poor capacity for healing due to their relative avascularity. Ligament reconstruction is well established for injuries such as anterior cruciate ligament rupture, however the use of autografts and allografts for ligament reconstruction are associated with complications, and outcomes are variable. Ligament tissue engineering using stem cells, growth factors and scaffolds is a novel technique that has the potential to provide an unlimited source of tissue. In this chapter we discuss the role of tissue engineering in dealing with ligament injuries and provide an overview of in vitro and in vivo studies.

18.1 Introduction

The knee joint with its long lever arms is subject to significant forces, and sporting and other daily activities can put it at a higher risk of injury. Ligament injuries account for a significant proportion of musculoskeletal injuries and result in disability and morbidity in patients worldwide [1]. Ligament injuries may ultimately lead to pain, articular cartilage injury, meniscal injury and early osteoarthritis [87]. It has been estimated that the incidence of injuries involving knee ligaments could be as high as 1,193 per 100,000 person-years, with surgery performed in 3.9 % of ligament injury cases [33]. Anterior cruciate ligament (ACL) rupture is one of the most common injuries of the knee [70]. More than 200,000 ACL reconstructions are performed yearly in the United States and the number being performed is increasing in frequency [75]. The estimated cost of an ACL surgical repair and subsequent rehabilitation is between $17,000–$25,000 per injury [22]. Over 200,000 ACL reconstructions per year are carried out in the US [75]. The total

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expenditure on ACL reconstructions in a year has been estimated as exceeding $5 billion [83].

In this chapter we will discuss limitations of current treatment strategies, ligament structure and ligament healing before taking an in depth view of tissue engineering for ligament repair and reconstruction.

18.2 Limitations of Current Treatment Options

Current treatment strategies for ligament injuries depend on the degree of injury and the patient's activity level and symptoms [57]. Grade I ligament injuries are mild sprains that are not associated with ligament laxity. Grade II injuries demonstrate moderately increased joint laxity. Grade III injuries are severe and associated with complete ligament disruption and significant laxity [88]. Non-operative management consists of pain relief and rehabilitation. Ligaments are poorly vascularized and have a limited capacity for healing. When healing does occur the composition of the healed tissue is different to normal tissue and the biomechanical properties of the healed tissue are usually inferior [72]. Operative management with hamstring or patellar tendon autografts, allografts and synthetic grafts is often undertaken [36] but the reconstructive surgery also may be associated with disadvantages. Autografts are associated with donor site morbidity, weakness, reduced range of movement and anterior knee pain in the case of patellar tendon donor tissue. Laboute et al. [47] found a re-rupture rate of 12.7 % for ACL reconstructions performed with hamstring tendon autografts. Allografts carry the risk of immunological reactions, disease transmission and infection. Synthetic grafts including carbon fibre, polypropylene, Dacron and polyester are associated with a high failure rate due to wear debris, foreign body tissue reactions and synovitis [23, 72].

18.3 Ligament Structure

Ligaments passively stabilize joints by connecting one bone to another and allow smooth motion. They are subject to multidirectional forces depending on activities. They also have a role in joint proprioception. The four main ligaments around the knee are the cruciates and the collaterals. Microscopically the ligament is composed of specialized fibroblasts that account for approximately 20 % of the tissue, and produce the extracellular matrix (ECM) that accounts for approximately 80 % of the tissue. The ECM consists of approximately 70 % water and 30 % organic tissue. The collagen accounts for 75 % of the dry weight with the remaining 25 % consisting of proteoglycans, elastin and other proteins and glycoproteins such as actin, laminin and integrin. Although there are 16 types of collagen, type I collagen accounts for 85 % of the collagen in ligaments. Type I collagen has an enormous

tensile strength enabling fibrils to be stretched without being broken. Less than 10 % of the collagen in ligaments is type III. This is more often found in healing tissues before most of it is converted to type I collagen. Very small amounts of collagen types IV, V, XI and XIV are also present. The basic structural unit of collagen is a triple-stranded helical molecule packed together side by side. The collagen bundles are aligned along the long axis into bundles of parallel fibres. The fibres have a periodic change in direction along the length known as the crimp pattern. It is likely that with increased loading, some areas of the ligament 'uncrimp' which allows the ligament to elongate without sustaining damage. The ligaments are covered by an outer layer known as the epiligament. This merges into the periosteum of the bone around the attachment site of the ligament. The epiligament is more vascular and cellular with a greater number of sensory and proprioceptive nerves [28, 29, 53, 72].

18.4 Ligament Healing

As mentioned earlier, regeneration and healing of ligaments after injury is often poor due their relatively avascular nature and low metabolic rate. Healing of ligaments can be divided into four stages. Firstly, there is a haemorrhagic stage in which the ligament ends retract and a blood clot forms and fills the gap. Cytokines are released within the clot and a heavily cellular infiltrate of polymorphonuclear leucocytes and lympthocytes appear within several hours. The macrophages appear by twenty-four to forty-eight hours in the inflammatory stage. By seventy-two hours the wound also contains platelets and multipotential mesenchymal cells. Macrophages phagocytose necrotic tissues as well as secreting growth factors such as basic fibroblast growth factor (FGFβ), transforming growth factor alpha and beta (TGFα and TGFβ) and platelet derived growth factor (PDGF) that stimulate fibroblast proliferation and synthesize types I, III and V collagen and non-collagenous proteins, as well as inducing neovascularization and formation of granulation tissue. During the proliferative stage, fibroblasts produce dense, cellular, collagenous connective tissue binding the torn ligament ends. This 'scar tissue' is initially disorganized and contains more type III and type V collagen, and smaller diameters collagen fibrils. This is followed by remodeling and maturation of the tissue. There is a gradual decrease in the cellularity of the tissue. The matrix becomes denser and longitudinally orientated. The matrix continues to mature for at least a year [28, 72, 88, 90].

The repair tissue never achieves the morphological or mechanical characteristics of normal ligament. There remains an increased vascularity and cellularity, abnormal innervation, decreased collagen fibril diameter, altered relative collagen type proportions with inadequate cross-linking, and altered proteoglycan profiles. The ligaments recover only up to twenty percent of their viscolelastic properties.

The repair tissue also has inferior creep properties (i.e. deformation properties under constant or cyclic loading) that could result in joint laxity. The resultant tissue has half the normal failure load and absorbs less energy before failing [28, 88].

18.5 Tissue Engineering

Tissue engineering involves the use of appropriate cells, growth factors and scaffolds, either in isolation or combination to repair and regenerate tissue, and has a role in musculoskeletal tissue repair and regeneration [41, 43]. Tissue engineering has a potentially useful role in ligament surgery as these structures are often injured and demonstrate limited healing potential [90]. Tissue engineering could be used to repair and regenerate tissue. In vivo injection of appropriate cells into the injured ligament in conjunction with the use of biomimetic scaffolds and bioreactors is a strategy that could potentially accelerate the process of tissue repair [90]. We have previously described a role for stem cells in the tissue engineering of ligaments [18]. Below, we will discuss cell sources, growth factors and scaffolds before considering the role of bioreactors.

18.6 Cell Sources for Ligament Tissue Engineering

Although reparative cells could be recruited from host tissue through the specific attachment of tissue engineered scaffolds, seeding cells could further improve the functionality of tissue engineered constructs [31]. The seeded cells lay down ECM and recruit reparative and/or progenitor cells through chemotaxis through growth factors and cytokines accelerating ligament repair. Additionally, they incorporate and release endogenous growth factors to elicit an immune response [31]. It is important to select the appropriate cell type for the specific application in order for the tissue engineered product to have the best outcome. However, little is known about the optimal cell source for ligament tissue engineering. The cell type selected must show enhanced proliferation and production of an appropriate ECM and must be able to survive in the relevant knee environment, intraarticular in the case of the cruciates [83]. Primary fibroblasts can be derived from ligaments such as the ACL, or from the skin. ACL fibroblasts can be harvested in diagnostic arthroscopic procedures following ACL rupture. The medial collateral ligament (MCL) is extraarticular, and it could be easily harvested partially without impairing its function in the long term [31]. Mesenchymal stem cells (MSCs) have the ability to proliferate and differentiate into a variety of mesenchymal cell phenotypes including osteoblasts, chondroblasts, myoblasts and fibroblasts [76, 90]. Culture conditions can be designed to direct MSC differentiation into the desired mesenchymal phenotype [83].

18.6.1 Fibroblasts

Fibroblasts are a choice of cells that can be harvested from different sources. Bellincampi et al. [10] investigated skin fibroblasts as a potential source for ligament tissue engineering as skin fibroblasts are known to have a greater healing potential and may be easily retrieved in a clinical setting. ACL and skin fibroblasts were harvested, cultured, labeled, seeded on collagen fibre scaffolds in vitro and implanted into the autogenous knee joint in a rabbit model. The cells remained viable for at least four to six weeks after implantation. They concluded that both skin and ACL fibroblasts survived in an intraarticular environment, but the potential of ACL fibroblasts to improve neoligament formation may be limited by a poor intrinsic healing capacity. Cooper et al. [20] investigated the cellular response of primary rabbit connective tissue fibroblasts from four sources (Achilles tendon, patellar tendon, MCL and ACL) to a novel three-dimensional poly-L-lactic acid (PLLA) braided scaffold for ACL tissue engineering. The fibroblasts from all four sources had similar morphological appearances on tissue culture polystyrene. However, the cellular growth differed for cell sources. They concluded that ACL fibroblasts were the most suited for ACL tissue engineering. Tremblay et al. [81] implanted a bioengineered ACL graft seeded with autologous living dermal fibroblasts into goat knee joints for six months. Histological and ultrastructural analysis demonstrated a highly organized ligamentous structure with vascularization, innervation and organized Sharpey's fibres and collagen at the osseous insertion sites of the grafts. Morbidity associated with harvesting of the skin is a potential limitation of using skin fibroblasts as a source for ligament tissue engineering. Additionally, the performance of skin fibroblasts for ligament tissue engineering may be affected as the physiological environment of skin fibroblasts is different to that of ligaments [31].

18.6.2 Mesechymal Stem Cells

Although the use of primary fibroblasts for ligament tissue engineering is a logical approach, the use of MSCs may be more efficient [67]. MSCs are naturally occurring cells that have the ability to both self-replicate as well as differentiate into another cell types [43]. Their capacity to repair is due to the secretion of factors that alter the tissue microenvironment. MSCs may be isolated from a variety of adult tissues including the bone marrow, adipose tissue, cord blood and synovial fluid [24, 45, 54]. MSCs are easily obtainable form bone marrow by a minimally invasive approach and can be expanded in tissue culture and encouraged to differentiate into the desired lineage [77]. Although Cheng et al. [17] reported benefits of stem cells derived from the ACL compared to bone marrow derived MSCs, the ligament has fewer MSCs. Most studies look at MSCs of an earlier passage before they lose their ability to proliferate and differentiate [74]. MSCs are positive for a

set of cell surface markers including CD105, CD73, and are negative for the haematopoietic markers CD34, CD45, and CD14 [44]. There is evidence that pericytes may represent MSC in different tissues, and indeed tissues that are vascular have a higher proportion of MSCs [42]. The differentiation into desired lineages is achieved by the use of bioactive signaling molecules, specific growth factors and appropriate environmental conditions [43, 80]. An alternative approach is the use of embryonic stem cells which are derived from the inner cell mass of the blastocyst and are capable of unlimited undifferentiated proliferation and have been shown to differentiate into all types of somatic cells. However, the use of embryonic stem cells is associated with several disadvantages including technical difficulties, immunogenicity, tumour formation in vivo, uncertainty regarding the long-term outcome and ethical considerations [19]. Adult MSCs possess immunomodulatory properties, making them potential candidates for cellular therapy in an allogeneic setting. Transplantation of MSCs into an allogeneic host may not require immunosuppressive therapy. Adult MSCs express intermediate levels of class I major histocompatibility complex proteins but do not express human leucocyte antigen (class II) antigens on the cell surface [14, 48]. MSCs have been shown to have an indirect inhibitory effect on T cells which is mediated by regulatory antigen-presenting cells with T cell suppressive properties [11].

Oe et al. [66] studied ligament regeneration in rats following intra-articular injection of either fresh bone marrow cells (BMCs) or cultured MSCs 1 week after partial ACL transection. At 4 weeks donor cells were detected within the transected ACLs in both the groups and the ACLs exhibited almost normal histology. They concluded that direct intra-articular bone marrow transplantation is an effective treatment for partial ruptures of the ACL. Lim et al. [52] performed ACL reconstructions in adult rabbits using hamstring tendon autografts which were coated with MSCs in a fibrin glue carrier. At 8 weeks good osteointegration was observed and they performed significantly better on biomechanical testing than the controls.

18.6.3 Studies Comparing Fibroblasts and Mesenchymal Stm Cells

There are few studies comparing fibroblasts with MSCs. MSCs may differentiate into ligament fibroblasts after two weeks [90]. It has been shown in a rabbit model, that MSCs have a significantly higher proliferation rate and collagen production than ACL and MCL fibroblasts, and that MSCs could survive for at least six weeks in the knee joint [31]. Van Eijk et al. [82] seeded bone marrow stromal cells, skin fibroblasts and ACL fibroblasts at different seeding densities onto braided poly(L-lactide/glycolide) scaffolds. The cells were cultured for up to 12 days. All cell types readily attached to the scaffold. On day 12, the MSC-seeded scaffolds showed the highest DNA content and collagen production. Scaffolds seeded with ACL fibroblasts showed the lowest DNA content and collagen production. The

ideal cell type selected for ligament tissue engineering must be readily available, have excellent proliferative and differentiation ability, be capable of producing an organized ECM, and have a good affinity for the scaffold.

18.7 Growth Factors Through Gene Transfer Technology

Growth factors are polypeptides that support various terminal phenotypes and regulate stem cell differentiation and proliferation. Growth factors such as transforming growth factor-β (TGF-β), bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), epidermal growth factor (EGF), insulin like growth factor (IGF-I), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and growth and differentiation factor (GDF) can, in isolation or various combinations, expedite the MSC proliferation and fibroblast differentiation [40, 90].

Gene transfer technology may be used to deliver genetic material and information to cells to alter their synthesis or function [90]. Genes can be introduced into cells using retroviral and adenoviral vectors as carriers, liposomes or with a gene gun. The genes can be placed in the cell ex vivo or in vivo. The target cells can be made to produce or increase expression of growth factors or suppress the synthesis of endogenous proteins or growth factor within the local tissue [88]. When Wei et al. [85] surgically implanted bone marrow derived MSCs transfected with adenovirus vector encoding TGF-β1, VEGF or TGF-β1/VEGF into experimental ACL grafts in rabbits, this significantly promoted angiogenesis compared to non-transfected control cells, and improved the mechanical properties. Hildebrand et al. [35] used a retroviral ex vivo and an adenoviral in vivo technique to introduce and express the LacZ marker gene in the MCL and ACL of rabbits. LacZ gene expression was detected and shown to last between 10 days and 3 weeks in the MCL and ACL with the use of the retrovirus and between 3 and 6 weeks in the MCL and at least 6 weeks in the ACL with the adenoviruses. Menetrey et al. [63] showed the feasibility of gene transfer to a normal ACL using direct, fibroblast mediated and myoblast mediated approaches. Either adenoviral particles were directly injected into the ACL of rabbits, or myoblasts or ACL fibroblasts transduced with recombinant adenoviral particles carrying the LacZ reporter gene were injected. Direct and myoblast mediated gene transfers demonstrated persistence of gene expression up to 6 weeks, but fibroblast mediated gene transfer showed gene expression for only 1 week. A number of other studies have indicated that using gene therapy to improve ligament healing is a promising approach [56, 71, 78, 91].

We conducted a review of growth factors in ACL reconstruction [12]. When TGF- β is added directly to the canine tibial bone tunnel it increases the ultimate load complex and richly generates perpendicular collagen fibres connecting the tendon graft and bone as early as 3 weeks post operatively [89]. As predicted, TGF-β increases proliferation and migration of ACL fibroblasts and stimulates matrix protein deposition thus enhancing wound repair [84] and significantly increasing maximum load and stiffness compared to control groups in rabbits [46]. In humans,

TGF- β increases cell number, increases collagen production and increases expression of alpha-smooth muscle actin in ACL defects [62]. The direct application of a virus vector mediated gene transfer of bFGF (both in vitro and in experimentally injured human ACLs) significantly enhances levels of type I and type II collagen production [58]. This could be the result of enhanced neovascularization and the formation of granulation tissue in injured ACLs in response to bFGF. Weiler et al. [86] found that the local addition of PDGF coated sutures in hamstring tendon ACL reconstruction lead to significantly higher load to failure, crimp length, vascular density and collagen fibril at varying time periods (3–12 weeks) post operatively. PDGF does not however appear to affect inflammatory parameters, MRI appearance of the graft or clinical evaluation scores when injected into the graft and tibial tunnel [64]. Letson and Dahners [50] compared PDGF alone or in combination groups (PDGF + IGF-1, PDGF + bFGF) and found that all three groups improved strength, stiffness and the breaking force of ligaments, suggesting that it is PDGF that is the most important growth factor in ligament healing. Indeed other studies confirm that PDGF has a role in accelerating ligament and tendon healing [6]. VEGF is highly expressed in the early post-operative phase (2–3 weeks) of patellar tendon ACL reconstruction implying VEGF is predominantly involved in the graft remodeling process at this stage [92]. However in trials with rabbits, it seems to work by promoting angiogenesis in the grafts rather than directly affecting the mechanical properties such as anterior-posterior translation, tensile strength, cross-sectional area or strain at failure [38].

18.8 Scaffolds

Biomaterial scaffolds provide a structural and logistic template for new tissue formation and remodeling [83]. Scaffolds are designed to support cell attachment, survival, migration and differentiation as well as control transport of nutrients, growth factors, metabolites and regulatory molecules to and from the cells [18]. A scaffold should be made of a biocompatible, biodegradable material and should be able to bridge any complex three-dimensional anatomical defect. The scaffold should ideally possess adequate strength post-implantation to be effective as a load-bearing construct and degrade at a rate matching the rate of new tissue deposition. The scaffold should have sufficient pore sizes to allow cell infiltration, and sufficient void volume to allow extracellular matrix formation to promote gradual load transfer from the scaffold to the neotissue [79]. Porous scaffolds enhance tissue regeneration by delivering biofactors, however pores that are too large could compromise the mechanical properties of the scaffold [90]. Polymers used in ligament tissue engineering [32] may be naturally derived e.g. gelatin, small intestine submucosal extracellular matrix and silk, or synthetic e.g. polyesters such as polyglycolic acid [59].

18.8.1 Natural Scaffolds

Collagen used in laboratories is usually derived from the bovine submucosa and intestine from rats tails in small quantities. The derived collagen requires processing to remove foreign antigens, improve its mechanical strength and sometimes to slow down the degradation rate by crosslinking. The crosslinking can be performed using chemical agents e.g. glutaraldehyde, formaldehyde, polyepoxy compounds, acylazide, carboiimides and hexmethylenediisocyanate risking potential toxic residues, or physically using drying, heat or exposure to ultraviolet or gamma radiation [32]. The resorption rate and mechanical properties of scaffolds can be altered through cross-linking. Fibroblasts have been shown to attach, proliferate and secrete new collagen when seeded on collagen fibre scaffolds [25]. In vivo, it has been demonstrated that fibroblast seeded collagen scaffolds may remain viable after implantation into the knee joint for prolonged periods [10]. Examples of commercially available biological collagen-based scaffolds include Restore (derived from porcine small intestine), Graftjacket (from human cadaver dermis), Permacol (from porcine dermis) and Bio-Blanket (from bovine dermis) [15]. The scaffolds demonstrate an early decrease in mechanical strength followed by tissue remodeling resulting in a strength gain similar to autografts by 20 weeks [29].

Silk has the advantage of possessing good biocompatibility, slow biodegradability and excellent tensile strength and toughness [91]. Silk fibroin is a protein excreted by silkworms and isolated from sericin [91], and has similar mechanical properties to functional ACL when organized into appropriate wire-rope geometry. Silk scaffolds also support cell attachment and spreading by providing an appropriate three-dimensional culture environment. Silk fibres lose the majority of their tensile strength within one year in vivo and fail to be recognized in two years [32]. Silk-fibre matrices have been shown to support adult stem cell differentiation towards ligament lineages [2]. A composite scaffold fabricated from silk and collagen tested in a rabbit MCL defect model was shown to improve structural and functional ligament repair by regulating ligament matrix gene expression and collagen fibril assembly [16]. Fan et al [26] examined ACL regeneration with MSCs on silk scaffolds in vivo. The lapine model demonstrated that the regenerated ligaments exhibited essential ligament ECM components including collagen I and collagen III in significant amounts, and direct ligament–bone insertion was reconstructed exhibiting the four zones typical of native ACL–bone insertions; bone, mineralized fibrocartilage, fibrocartilage and ligament. The tensile strength of the regenerated ligaments was assessed to be biomechanically adequate.

18.8.2 Synthetic Scaffolds

Synthetic polymers that have been investigated for ligament repair include poly glycolic acid (PGA), polylactic acid (PLA), their copolymers and poly caprolactone

(PCL) [55]. PLA is a commonly used synthetic scaffold that easily degrades within the human body by forming lactic acid. PCL and PGA degrade in a similar way to PLA but exhibit different rates of degradation. Synthetic polymers are not limited by donor source, have no risk of disease transmission and are designed to degrade over time. Their mechanical properties may be controlled by altering the degree of polmer crystallinity, changing the polymer molecular weight or changing the ratio of each polymer in the copolymer [29].

18.8.3 Preclinical and Clinical Studies Using Scaffolds

We performed a systematic review [13] to examine and summarize the preclinical in vivo studies and limited clinical studies on the use of scaffolds in the treatment of ligamentous injuries. We identified eight studies looking at collagen platelet composite (CPC), two studies on collagen in isolation, two on silk and one study each for Poly-L-Lactic acid (PLLA) and small intestinal mucosa. The studies involving CPC were on porcine or canine models of ACL injuries, and all had variable time frames for examination from one to fifteen weeks. The concentration of PRP varied from two to five times the physiological level. All found that CPC had a significant effect on healing. Mastrangelo et al. [61] found in their porcine model that higher PRP concentrations produced greater cellular densities at thirteen weeks. They also found that skeletally immature animals had a greater intrinsic capacity to heal compared to adolescents and adults. Palmer et al. [69] determined that increased temperature of CPC decreased strength and yield load in the porcine model and Magarian et al. [60] determined that decreased yield load occurred when the repair was delayed either two or six weeks, with no difference between the delay groups. Joshi et al. [37] performed ACL repairs in twenty-seven immature pigs, with 14 having a repair augmented with CPC. The CPC augmented group had better functional, load, and stiffness measurements in addition to improved structural properties at 3 months. Nishmoto et al. [65] treated rabbit medial collateral ligament defects using PLLA scaffolds. Fibrocartilage alignment and morphology increased in a time dependent manner, but PLLA fibres were not absorbed after the sixteen-week assessment, raising potential concerns regarding synovitis. Badylak et al. [8] reconstructed goat ACL injuries with either a porcine small intestinal sub mucosa scaffold or a more conventional patella tendon graft and a found no difference in functional testing. The small intestine submucosa group did however show transient weakening early with variable strength over time in comparison to the patella tendon group which increased in strength. We identified three studies on synthetic scaffolds. Cooper et al. [21] presented data on a comparison between seeded and unseeded biomimetic ligament generated by using 3D braiding technology to reconstruct rabbit ACL defects and determined that seeding ligaments with ACL cells resulted in better histological and mechanical evaluation. Liljensten et al. [51] assessed poly urethane urea (PUUR) in thirty-five rabbits and two pigs and found that at six, twelve, and twenty-four months there was no synovial

reaction or joint damage in any knees and all had an integrated ligament. Argona et al. [7] demonstrated that carbon fibre polylactic acid polymer ligaments allowed for more stable medial collateral ligament constructs with time related collagen ingrowth in the beagle model at a maximum of twenty-six weeks. There were no biological studies available. Four studies assessed the effects of absorbable copolymer carbon fibre ligaments (ABC) and three found that while tissue ingrowth into the ligaments was found there were unacceptable short and long-term failure rates even after a change in technique in the early 1990's. Petrou et al. [73] prospectively followed up seventy-one patients for a minimum of five years and found that while there was evidence of recurrent synovitis and stiffness there was a one hundred percent survival rate. This discrepancy may have been due to relatively short follow up as it has been previously noted that after a technique and equipment change in the early 1990's the early failure rates of the ABC ligament were replaced by mid to late term failures.

18.9 Bioreactor Systems

The differentiation of MSCs into fibroblasts may be accelerated by the use of a bioreactor that provides a controlled biomimetic optimum environment for cell functions. Bioreactors are a key component of tissue engineering [3, 68]. They use various combinations of chemical, mechanical, electrical or magnetic stimulation to guide differentiation, proliferation and tissue development. In the case of ligament tissue engineering, a bioreactor may be used to accelerate the process of differentiation of MSCs into the fibroblastic lineage [90]. The body may be used as a bioreactor when a cell-scaffold composite is implanted directly into the injured site. Another approach is to culture the cell-scaffold composite in a bioreactor ex vivo for a period of time before transplantation [34, 91].

In order for a bioreactor to function successfully, there are several basic design principles that need to be fulfilled. Firstly, a bioreactor should maintain precise control of the physiological environment of the tissue culture, including control of variables such as temperature, oxygen concentrations, pH, nutrients, media flow rate, metabolite concentrations and specific tissue markers within close limits. Bioreactors should also be able to support the culture of two or more cell types simultaneously particularly when engineering complex tissues. It is also essential that the bioreactor is designed to operate under strict aseptic conditions in order to prevent any contamination of the tissues by influx of microorganisms [68].

Chemical stimulation techniques are employed by using chemicals such as growth factors described in the section above. Mechanical stimulation techniques involve subjecting a scaffold to mechanical stresses resembling the in vivo environment. Intracellular signaling cascades are activated by triggering the cell surface stretch receptors leading to synthesis of the necessary extracellular matrix proteins [90]. The effects of mechanical stimulation are dependent on the magnitude, duration and frequency of mechanical stress [49]. Additionally, mechanical

stimulation has been shown to affect extracellular matrix synthesis and remodeling. Enzyme activity and growth factor expression, collagen type I, collagen type III, elastin and tenascin-C expression in MSCs have been shown to be increased with the application of mechanical loads [91]. Electromagnetic stimulation has been shown to have positive results. For example, Fung et al. [30] showed that low energy laser therapy can enhance the mechanical strength of healing MCL in rats and increase collagen fibril size. Co-culture may also be used to induce differentiation of MSCs because of its ability to promote cell communications [90]. Direct co-culture of MSCs with fibroblasts induces MSCs to differentiate into fibroblast-like cells [9]. Cell-to-cell interactions in the microenvironment play a key role in regulating the differentiation of MSCs in the healing process. Additionally, specific regulatory signals released from fibroblasts have been shown to support the selective differentiation of MSCs towards ligament fibroblasts in a two-dimensional transwell insert co-culture system [49]. Fan et al. [27] demonstrated that specific regulatory signals released from fibroblasts in a three-dimensional co-culture also enhanced the differentiation of MSCs for ligament tissue engineering.

Although various commercial bioreactor systems are available, some may not be applicable to ligament tissue engineering as the design lacks the specificity to meet the requirements for engineering of ligament tissue [83]. Altman et al. [4, 5] designed a bioreactor to permit the controlled application of ligament-like multi-dimensional mechanical strains to undifferentiated cells embedded in a collagen gel. They used mechanical stimulation in vitro to induce the differentiation of mesenchymal progenitor cells from bone marrow into a ligament cell lineage in preference to bone or cartilage cell lineages. Kahn et al. [39] designed a bioreactor for tissue engineering of ligament tissue that imposed mechanical conditions close to the physiological movement of the ACL. The bioreactor consisted of a mechanical part allowing movement to be applied on scaffolds, two culture chambers, a perfusion flow system to renew nutrients in the culture medium, a heating enclosure as well as an electronic component to manage movement and to regulate heating.

18.10 Conclusions

Ligament injuries are common in the knee, and can be challenging to treat with the current nonoperative and operative treatment options available. Considerable progress has been made in generating tissue engineered ligaments. The following requirements were noted by Vunjak-Novakovic et al. [83] as being key to the success of tissue engineered ligaments:

- Autologous source of MSCs that is easily accessed with no associated morbidity to eliminate concerns such as infection, immune rejection or disease;
- Biomaterial scaffold with mechanical properties matching the native ligament, biodegradation to match tissue formation, and porosity to allow for cell infiltration;

- Biochemical and biophysical regulation of MSC differentiation;
- Quantitative methods of measuring success.

Studies on the generation of tissue engineered ligaments have generally been in vitro preliminary studies or trials in animal models. At this stage we appear to be moving closer to achieving the above aims, but human trials need to be conducted in addition to a cost benefit analysis to determine the appropriateness of treatment. Engineering ligaments that have the appropriate mechanical properties is the significant challenge. However, advances in cell biology, understanding of the roles of growth factors, scaffold engineering and mechanical conditioning using bioreactors may be able to provide a viable long-term alternative to current autografts and allografts in the future. The use of tissue engineered ligaments would potentially have significant health care implications. In view of the more active aging population, the number of patients who will benefit from the use of tissue engineered ligaments is likely to increase with time.

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