Chapter 22
Stem Cells in Ligament Tissue Engineering

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Abstract Injured ligaments 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 may be associated with a number of complications, and outcomes are variable. Ligament tissue engineering using stem cells is a novel technique that has the potential to provide an unlimited source of tissue. The process of tissue engineering involves the use of stem cells, growth factors, mechanical loading, a bioreactor, a biomimetic scaffold and gene therapy. In vitro and in vivo studies on ligament tissue engineering have shown some promising results; however, clinical research in this field is needed.

Keywords Ligament injury • Ligament reconstruction • Anterior cruciate ligament rupture • Ligament tissue engineering • Cell therapy

Abbreviations

ACL	Anterior cruciate ligament
BMPs	Bone morphogenic proteins
EGF	Epidermal growth factor

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FGFβ	Basic fibroblast growth factor
GDF	Growth and differentiation growth factor
IGF	Insulin-like growth factor
MCL	Medial collateral ligaments
MSCs	Mesenchymal stem cells
PCL	Polycaprolactone
PDGF	Platelet-derived growth factor
PGA	Polyglycolic acid
PLA	Polylactic acid
PLLA	Poly-L-lactic acid
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
VEGF	Vascular endothelial growth factor

22.1 Introduction

Ligament injuries account for a significant proportion of musculoskeletal injuries and result in disability and morbidity to patients worldwide [1]. Ligament injuries are commonly associated with sporting or overuse injuries [2]. For example, a tear or rupture of the anterior cruciate ligament (ACL) is one of the commonest sports injuries (particularly in football) [3]. Seventy percent of ACL tears occur as a result of repeatedly performed noncontact mechanisms such as sudden deceleration, landing and pivoting manoeuvres [3]. More than 200,000 ACL reconstructions are performed yearly in the United States, and the number being performed is increasing in frequency [4–6]. The cost of treating injuries to the cruciate ligaments is relatively high and has previously been estimated to be almost US $3,000 per patient [7]. The total expenditure on ACL reconstructions in a year has been estimated as exceeding $5 billion [8, 9].

Current treatment regimens for ligament injuries depend on the degree of injury and the patient's activity level, symptoms and effect on quality of life. There are three stages of ligament injury. Grade I injuries are mild sprains that are not associated with ligament laxity. Grade II injuries show moderately increased joint laxity. Grade III injuries are severe and associated with complete ligament disruption and significant laxity [10]. Treatment may consist of nonoperative management with pain relief and rehabilitation. However, operative management with autografts, allografts and synthetic grafts is often undertaken [11]. 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 [2]. Despite appropriate treatment, the ligament may not necessarily achieve its pre-injury characteristics or function and outcomes are variable. Additionally, the reconstructive surgery itself may be associated with disadvantages. Autografts may be associated with donor site morbidity. Allografts carry the risk of immunological reactions and infection. Synthetic grafts may be complicated by foreign body reactions [2].

Tissue engineering has a potentially very useful role in the specialty of orthopaedic surgery in general, as musculoskeletal tissues are often injured or lost in trauma and disease and may demonstrate limited healing potential [12]. Tissue engineering could be used to repair and regenerate tissue such as bone, cartilage, tendon as well as ligament. 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 [12].

This chapter discusses the characteristics of ligamentous tissue and approaches that are being developed to repair and regenerate ligament such as stem cell therapy, use of growth factors, gene therapy and mechanical stimulation.

22.2 Ligament Function Structure and Healing

Ligaments span a joint and connect one bone to another. Ligaments passively stabilize joints and help in guiding joints through their normal range of motion when a tensile load is applied. Ligaments also play a role in joint proprioception. When ligaments are strained they invoke neurological feedback signals that activate muscular contraction, and this appears to play a role in proprioception. Ligaments consist of dense bands of collagenous tissue. The surface of a ligament is often covered by an outer layer known as the epiligament. The epiligament merges into the periosteum of the bone around the attachment site of the ligament. Beneath the epiligament the ligament is organized into bundles of parallel fibres. The epiligament is more vascular and more cellular with more sensory and proprioceptive nerves than the underlying ligament [13].

Microscopically the ligament is composed of cells and an extracellular matrix. The cells are fibroblasts and account for approximately 20 % of the tissue. The extracellular matrix accounts for approximately 80 % of the tissue. The fibroblasts are responsible for synthesis of the matrix which consists of approximately 70 % water and 30 % collagen, ground substance and elastin. 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 VI, V, XI and XIV are present. The collagen accounts for 75 % of the dry weight. The remaining 25 % consists of proteoglycans, elastin and other proteins and glycoproteins such as actin, laminin and integrin [2, 13].

The collagen bundles are aligned along the long axis of the ligament and have a periodic change in direction along the length known as the crimp pattern. Crimp is thought to play a biomechanical role. It is likely that with increased loading, some areas of the ligament ‘uncrimp’ which allows the ligament to elongate without sustaining damage [13, 14].

As mentioned earlier, regeneration and healing of ligaments after injury is often poor due to their relatively avascular nature. 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 lymphocytes appear within several hours [2, 10, 12, 13].

The second stage is the inflammatory stage in which macrophages appear by 24–48 h. By 72 h 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). Platelets release PDGF, TGFβ and epidermal growth factor (EGF). These growth factors are chemotactic for fibroblasts and other cells, stimulate fibroblast proliferation and synthesize types I, III and V collagen and non-collagenous proteins. The growth factors also induce neovascularization and formation of granulation tissue [10, 13].

During the proliferative stage (stage 3), fibroblasts produce dense, cellular, collagenous connective tissue binding the torn ligament ends. This 'scar tissue' is initially disorganized. Capillary buds begin to form. After a few weeks, the collagen becomes quite well aligned with the long axis of the ligament. However, this tissue contains more type III collagen in relation to type I and more type V collagen. The collagen fibrils also have smaller diameters [10, 13].

The fourth stage consists of remodelling and maturation of the tissue. There is a gradual decrease in the cellularity of the tissue. Defects in the scar become filled in and the matrix becomes more dense and longitudinally orientated. The matrix begins to become more like normal ligament and continues to mature for at least a year. However, this tissue never achieves the morphological or mechanical characteristics of normal pre-injury ligament. There is a persistently decreased collagen fibril diameter and failure of collagen cross-links to mature as well as altered proteoglycan profiles (increased biglycan and decreased decorin protein and mRNA levels). There are also differences in the collagen types, altered cell connections, increased vascularity, abnormal innervation and increased cellularity and vascularity [10, 13].

During the remodelling stage, the viscolelastic properties recover to up to 20 % of normal. The tissue also has inferior creep properties (i.e. deformation properties under constant or cyclic loading). A rabbit model looking at healing of the medial collateral ligament demonstrated that ligament scars creep tissue as much as normal medial collateral ligaments (MCL) during cyclic and static loads that are only a fraction of the loads. Extensive creep could result in joint laxity. The resultant tissue has half the normal failure load and absorbs less energy before failing [10, 13].

22.3 Cell Sources for Ligament Tissue Engineering

Reparative cells could be recruited from host tissue through the specific attachment of tissue-engineered scaffolds. However, seeding cells could further improve the functionality of tissue-engineered constructs [15]. Cellular interaction between local tissue host cells and donor cells while extracellular matrix is being excreted

may result in accelerated ligament healing. The seeded cells are involved in attracting reparative and or progenitor cells through chemotaxis signals. They also lay down extracellular matrix which results in initiation of further recruitment of reparative and/or progenitor cells. Additionally, they incorporate and release endogenous growth factors to elicit an immune response [15].

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 extracellular matrix and must be able to survive in an intraarticular environment in the patient's knee [16]. Mesenchymal stem cells (MSCs) have the ability to proliferate and differentiate into a variety of mesenchymal cell phenotypes including osteoblasts, chondroblasts, myoblasts and fibroblasts [12]. Culture conditions can be designed to direct MSC differentiation into the desired mesenchymal phenotype [9]. The potential use of mesenchymal stem cells to regenerate ligament tissue will be discussed in Sect. 22.4.

Primary fibroblasts derived from ligaments such as the ACL or MCL are another option. ACL fibroblasts can be harvested in diagnostic arthroscopic procedures after ACL rupture. As the MCL is extraarticular, it could be easily harvested partially without impairing its function in the long term [12, 15].

A study by Cooper et al. investigated the cellular response of primary rabbit connective tissue fibroblasts from four sources (Achilles tendon, patellar tendon, medial collateral ligament and anterior cruciate ligament) 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 in culture on tissue culture polystyrene. However, the cellular growth is different according to the cell source. They concluded that ACL fibroblasts were the most suited for ACL tissue engineering [17].

Bellincampi et al. 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, labelled, 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 4–6 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 [18]. Tremblay et al. implanted a bioengineered ACL graft seeded with autologous living dermal fibroblasts into goat knee joints for 6 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 [19]. 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 [12, 15].

22.4 Mesenchymal Stem Cell Therapy

Although the use of primary fibroblasts for ligament tissue engineering is a logical approach, the use of stem cells may be more efficient. 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 6 weeks in the knee joint [15]. Eijk et al. 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 scaffolds seeded MSCs showed the highest DNA content and collagen production. Scaffolds seeded with ACL fibroblasts showed the lowest DNA content and collagen production [16].

MSCs may differentiate into ligament fibroblasts after 2 weeks [12]. MSCs may be isolated from a variety of adult tissues including the bone marrow (obtained from aspiration of the iliac crest). Other potential sources of MSCs include adipose tissue (see Fig. 22.1), cord blood and possibly synovial fluid in ligament regeneration [21]. An alternative approach is the use of embryonic stem cells which are derived from

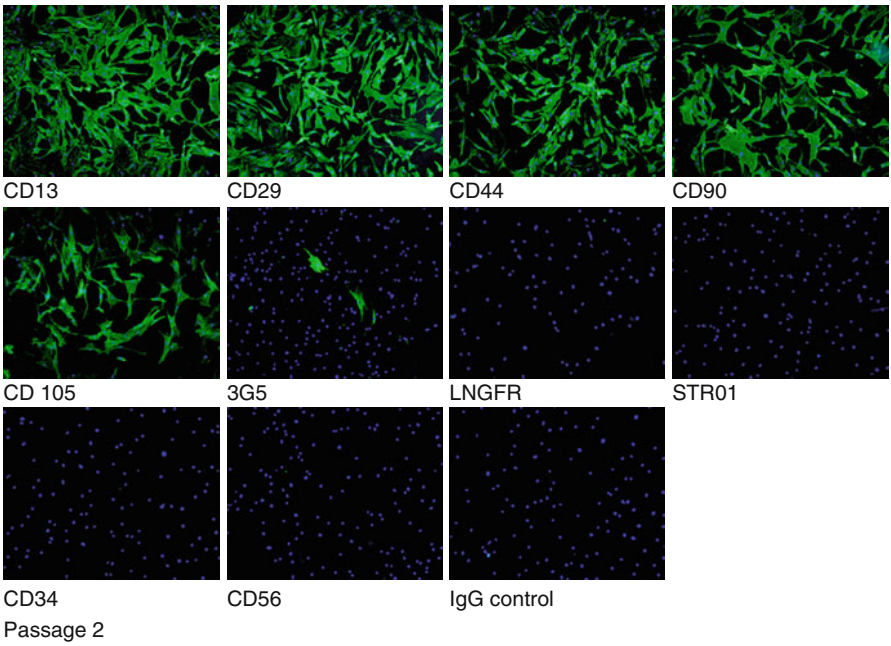
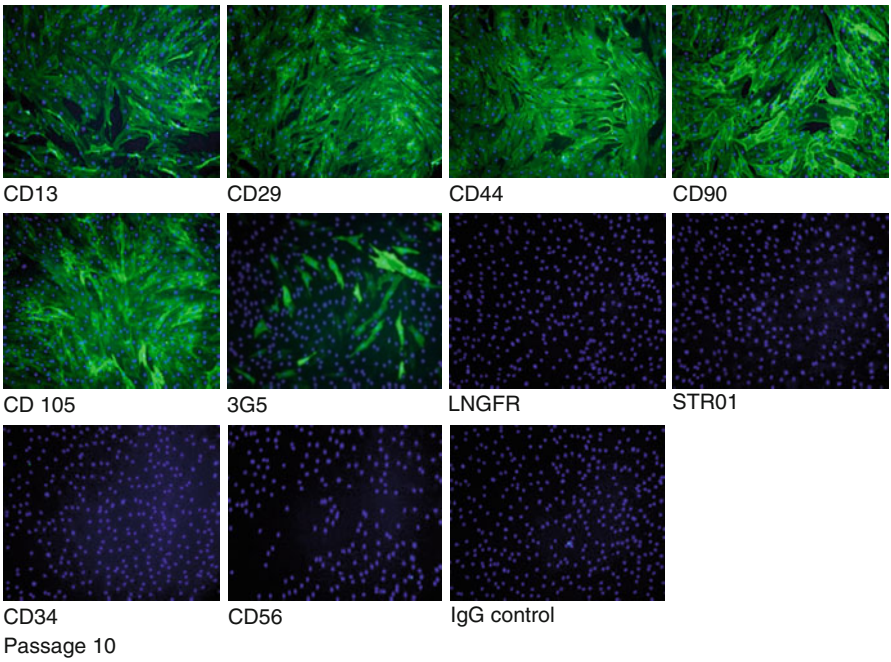


Fig. 22.1 Cell surface epitope characterization of passage 2 (**a**), passage 10 (**b**) and passage 18 (**c**) fat pad-derived MSCs using a panel of antibodies. Cell surface staining using FITC-conjugated secondary antibody (*green*) and DAPI (*blue*) shows that the cells stained strongly for CD13, CD29, CD44, CD90 and CD105 and poorly for LNGFR, STRO1, CD34 and CD56. Occasional cells stained positively for 3G5. No staining was observed for the IgG control [20]



b

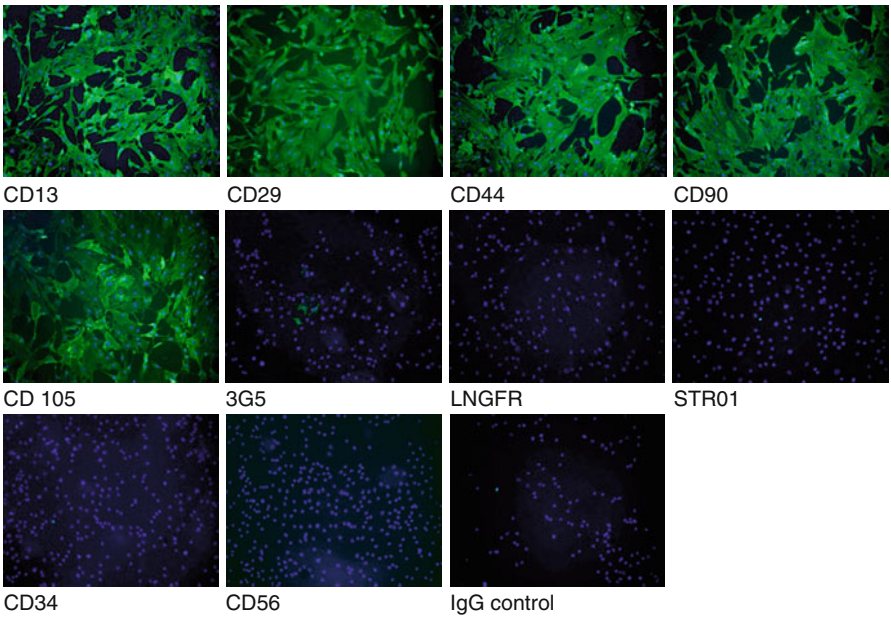


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Fig. 22.1 (continued)

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 [12, 22].

Adult mesenchymal stem cells have the advantage of possessing immunomodulatory properties. Although these immunomodulatory properties have not been fully explained, they make MSCs 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 [12, 23–25]. 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 [24].

22.5 Bioreactor Systems

The differentiation of MSCs into fibroblasts may be accelerated by the use of a bioreactor which provides a controlled biomimetic optimum environment for cell functions. Bioreactors are a key component of tissue engineering [26]. 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 [12]. 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 [27, 28].

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 [29].

Chemical stimulation techniques are employed by using chemicals such as growth factors. Growth factors are polypeptides that support various terminal phenotypes and regulate stem cell differentiation and proliferation. Examples of growth factors include TGFβ, bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), EGF, vascular endothelial growth factor (VEGF), PDGF, growth and differentiation growth factor (GDF) and insulin-like growth factor (IGF) [12, 27, 29].

Mechanical stimulation techniques involve subjecting a scaffold to mechanical stresses resembling the in vivo environment. It is used to induce differentiation of

MSCs into the fibroblast lineage. Intracellular signalling cascades are activated by triggering the cell surface stretch receptors leading to synthesis of the necessary extracellular matrix proteins [12]. The effects of mechanical stimulation are dependent on the magnitude, duration and frequency of mechanical stress [30]. Additionally, mechanical stimulation has been shown to affect extracellular matrix synthesis and remodelling. 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 [28].

Coculture may also be used to induce differentiation of MSCs because of its ability to promote cell communications [12]. Direct coculture of MSCs with fibroblasts induces MSCs to differentiate into fibroblast-like cells [31]. 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 coculture system [30]. Fan et al. demonstrated that specific regulatory signals released from fibroblasts in a three-dimensional coculture can also enhance the differentiation of MSCs for ligament tissue engineering [32].

Electromagnetic stimulation has been shown to have positive results. For example, Fung et al. showed that low-energy laser therapy can enhance the mechanical strength of healing MCL in rats and increase collagen fibril size [33].

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 [9]. Altman et al. designed a bioreactor to permit the controlled application of ligament-like multidimensional 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 [26, 34]. Kahn et al. 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 [35].

22.6 Scaffolds

Biomaterial scaffolds provide a structural and logistic template in which new tissue formation and remodelling can occur [9]. Scaffolds are designed to support cell attachment, survival, migration and differentiation as well as to control transport of nutrients, metabolites and regulatory molecules to and from the cells [22]. A scaffold should be made of a biocompatible, biodegradable material and should be able to bridge any complex three-dimensional anatomical defect. This may be achieved using surgical experience or through sophisticated computer mapping systems [12].

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 also have sufficient void volume for cell infiltration and extracellular matrix to promote gradual load transfer from the scaffold to the neotissue [36]. Porous scaffolds enhance tissue regeneration by delivering biofactors. However, pores that are too large would compromise the mechanical properties of the scaffold [12]. Currently all materials used in ligament tissue engineering are polymers [37]. Polymers may be naturally derived, e.g. gelatin, small intestine submucosal extracellular matrix or silk. Synthetic polymers include polyesters such as polyglycolic acid.

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, to improve its mechanical strength and sometimes to slow down the degradation rate by cross-linking. The predominant chemical cross-linking agents used in research are glutaraldehyde, formaldehyde, polyepoxy compounds, acyl azide, carbodiimides and hexamethylene diisocyanate. Potential toxic residues are a disadvantage. Physical methods include drying, heating or exposure to ultraviolet or gamma radiation [37]. Fibroblasts have been shown to attach, proliferate and secrete new collagen when seeded on collagen fibre scaffolds [38]. In vivo, it has been demonstrated that fibroblast-seeded collagen scaffolds may remain viable after implantation into the knee joint for prolonged periods [18]. 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) [39]. Advantages of collagen include the ability to alter resorption rate and mechanical properties of scaffolds through cross-linking and low antigenicity. The scaffolds experience an early decrease in mechanical strength followed by tissue remodelling between by 20 weeks resulting in a strength gain similar to autografts [14].

Silk has the advantage of possessing good biocompatibility, slow biodegradability and excellent tensile strength and toughness [9, 28]. Silk fibroin is a protein excreted by silkworms and isolated from sericin [28]. Silk fibroin has similar mechanical properties to functional ACL when organized into an 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 1 year in vivo and fail to be recognized in 2 years [37]. Silk-fibre matrices have been shown to support adult stem cell differentiation towards ligament lineages [40]. 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 [41].

Synthetic polymers that have been investigated for ligament repair include polyglycolic acid (PGA), polylactic acid (PLA), their copolymers and polycaprolactone (PCL). PLA is a commonly used synthetic scaffold which 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. An advantage of using a synthetic polymer is that there is no limit to the supply of grafts and no risk of disease. These polymers

are designed to degrade over time. Their mechanical properties may be controlled by altering the degree of polymer crystallinity, changing the polymer molecular weight or changing the ratio of each polymer in the copolymer [12, 14].

22.7 Gene Transfer Technology

Gene transfer technology may be used to sustain sufficient quantities of growth factor within the local tissue [12]. Gene transfer is a method to deliver genetic material and information to cells to alter their synthesis or function. 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 outside 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 [10]. Wei et al. surgically implanted bone marrow-derived MSCs transfected with adenovirus vector encoding TGF-β1, VEGF or TGF-β1/VEGF into experimental ACL grafts in rabbits. They found that this significantly promoted angiogenesis compared to non-transfected control cells. The best mechanical properties were achieved at 24 weeks [42]. Hildebrand et al. 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 [43]. Menetrey et al. showed the feasibility of gene transfer to a normal ACL using direct, fibroblast-mediated and myoblast-mediated approaches. Adenoviral particles were directly injected into the ACL of rabbits. Rabbit myoblasts and ACL fibroblasts were transduced with recombinant adenoviral particles carrying the LacZ reporter gene, and these were also injected into the ACL of rabbits. The persistence of gene expression lasting up to 6 weeks was observed for the direct and myoblast-mediated gene transfers. Fibroblast-mediated gene transfer showed low efficiency with gene expression persisting for 1 week in the ligament and 2 weeks in the synovial tissue surrounding the ligament. Only a few cells located in the synovium were positive for the marker gene at 3 weeks post injection [44]. A number of other studies have indicated that using gene therapy to improve ligament healing is a promising approach [28, 45–47].

22.8 Conclusion

Ligament injuries may be challenging to treat. Results of ligament reconstruction with grafts are variable. Considerable progress has been made in generating tissue-engineered ligaments. Important areas for future development include improving the biomechanical properties of tissue-engineered ligaments, improving the characteristics of scaffold materials and increasing the strength of ligament-bone junctions

of implanted engineered ligament. Studies on the generation of tissue-engineered ligaments have generally been in vitro preliminary studies or trials in animal models. In the future, large clinical trials, in particular randomized controlled trials, assessing tissue-engineered ligaments should be performed. The use of tissue-engineered ligaments would potentially have significant health-care implications. In view of the ageing 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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