■ REVIEW ARTICLE

The biology of integration of the anterior cruciate ligament

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The anterior cruciate ligament (ACL) is a short, stout, intra-articular, extrasynovial structure which acts to control rotational movements and anterior translation of the femur upon the fixed tibia.¹ Rupture is a traumatic event, often as a consequence of twisting upon the weight-bearing limb. Dissolution of any local clot by the action of synovial proteases and lack of contact between the two ends prohibits successful healing. Surgical replacement using a graft, often an autogenous central-third patellar tendon or a four-strand hamstring construct, is often required to reduce the feeling of instability and the frequency of injury to the menisci and load-bearing chondral surfaces.²⁻⁴ ACL reconstruction is not a universally successful procedure, with reported rates of laxity failure at one year of up to 17%.⁵ There are traumatic, iatrogenic and atraumatic reasons for recurrent laxity. Failure of integration of the graft may be responsible in a significant number of such cases. This review will discuss the biology of the anterior cruciate ligament, the cellular biology of reconstruction and graft integration and current strategies for the enhancement of early graft function.

lateral femoral condyle. It is approximately 35 mm long, 11 mm in diameter, and derives its blood supply from the middle geniculate artery. Through mechanoreceptors, the ACL plays a role in proprioception at the knee.

Microscopic anatomy. The ACL is composed of multiple fascicular collagen bundles interlaced and grouped into fibres, large numbers of which together form subfascicular units, 100 to 250 μ m in diameter. Subfascicular units have a surrounding endotenon. Groups of approximately 20 such units coalesce to form fascicles surrounded by an epitenon. These fascicles may lie in the longitudinal axis of the ACL or spiral around the longitudinal axis of the central ligament. The ACL has an enveloping paratenon with vascular and neural elements. Microscopic examination of the sites of its bony attachment shows that interdigitation of the collagen fibres with bone has a transitional zone of non-mineralised, and then mineralised, fibrocartilage. This allows a graduated change in stiffness and dissipates stress at any one point along the ligament. This zone is thought to act as a barrier to endosteal-derived vascular ingrowth.⁷

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Anatomy of the native ACL

The limb bud is visible at the fourth week of embryonic life. By six weeks, there is an identifiable bend anteriorly in the midpoint of the limb with the knee being seen as a concentration of mesenchyme.⁶ The medial and lateral compartments are initially separated by an intercondylar septum. Within this blastema, the ACL forms as a condensation at about seven weeks. Through a process of involution and maturation, the ACL is formed and lies in an extrasynovial environment from its initiation. It elongates and moves posteriorly at its proximal portion as the distal femoral condyles develop. It originates from a broad anterior intercondylar area of the tibia, passes from anterior to posterior, and inserts into the most posterior part of the medial aspect of the

Mechanism of injury

Disruption of the ACL is usually as a result of a non-contact injury. This may be a flexion-valgus-external rotation movement, flexion-varus-internal rotation loading, forced external rotation or hyperextension. Other rarer mechanisms have been described. Acute trauma may result in a tense haemarthrosis and temporary impairment of weight-bearing.⁸ The absence of an acute effusion may be associated with significant capsular disruption.

Rationale for surgery

The aim of reconstruction is to restore stability to the knee, maintain the range of movement and thereby minimise injury to both the chondral surfaces and the menisci. Basic surgical principles assert that the femoral tunnel should be placed at 11 o'clock in the right knee and

one o'clock in the left knee, at the back of the intercondylar notch.⁹⁻¹¹ An anterior femoral tunnel may cause loss of flexion and lead to early graft failure. The tibial tunnel should lie in the posterior third of the ACL tibial footprint on direct inspection and at an angle of approximately 45°. Anterior positioning of the tibial tunnel may block extension and again lead to early graft rupture.

Ideal graft substitute. In current surgical practice, the majority of procedures are performed using either a hamstring or patellar tendon autograft.¹²⁻¹⁵ The ideal ACL graft should possess a microscopic structure and biomechanical characteristics similar to those of the native ACL. The normal site of insertion is a highly specialised zone which transmits stress from hard to soft tissue. The native ACL attaches to the surface of bone through a direct insertion, which contains four distinct histological zones: 1) ligament, 2) uncalcified fibrocartilage, 3) calcified fibrocartilage and 4) bone.¹⁶ There is a tidemark between the zones of calcified and uncalcified cartilage, which represents the mineralisation front. There are no sites in humans where a tendon goes into a bone tunnel, and there is, therefore, no native situation analogous to a tunnel-hamstring graft.¹⁷⁻¹⁹ Healing of the tendinous portion of the graft is important even when bone-tendon-bone grafts are used, because with current endoscopic techniques, and the increasing desire for aperture fixation, the tendinous portion of the graft usually extends into the upper part of the tibial tunnel. On the basis of normal ACL structure and the known function of the site of insertion, the ideal ACL graft should allow for early rehabilitation while protecting the anchorage points and avoiding graft slippage.^{20,21} Fuller discussion of the rationale for and timing of surgical intervention lies outside the scope of this review.

Causes of failure

Reconstruction of the ACL usually involves the use of a tendon/ligament autograft which is transplanted into bone tunnels at its femoral and tibial insertions. The site of attachment is often seen as the weak link in the early healing period, necessitating a delay in return to function. As the most important site for secure healing may be at the tunnel opening into the joint (aperture fixation), it is obvious that even bone-tendon-bone grafts require both tendon-to-bone and bone-to-bone healing.²² It is believed that there are biomechanical and biological causes for failure of graft function.

Biomechanical causes include poor tunnel placement, a poor press fit, excess movement at the graft bone interface and further trauma. Such failure may be seen on the tibial side, in association with radiological evidence of widening of the tunnel, suggestive of a marked osteoclastic response.²³

However, failure has also been reported, albeit in an important minority, where there is no discernible cause from either the patient's history or an analysis of the radiographs for tunnel placement, and an adequate fixation has

been used. This failure may be because of either failure^{9,24-26} of initiation of the anchorage process or an arrest of incorporation before adequate bonding of the graft bone construct has occurred, with the resulting persistent movement at the bone tunnel aperture and a return of subjective instability.^{8,23-25}

Biology of graft healing

Biology of integration. Based upon histological study, the ACL graft is seen to undergo four phases of integration. In the early, acute inflammatory process, ischaemic necrosis occurs. With cell recruitment and chronic inflammation there is revascularisation, cell proliferation and finally collagen remodelling.

Acute inflammation. Upon release of the tourniquet, the knee is immediately filled with blood from the drilled, exposed bone. This generates an acute inflammatory response with mesenchymal cell recruitment, proliferation and matrix synthesis. There is aggregation and degranulation of circulating platelets within the forming fibrin clot, resulting in controlled release of cytokines.²⁷ In particular, transforming growth factor beta (TGF- β) and platelet-derived growth factor (PDGF), act in a coordinated manner to regulate this early response. TGF- β 1 modulates tissue healing, matrix deposition and remodelling through the chemotaxis of neutrophils and monocytes to the wound site.²⁸ The expression of TGF- β 1 by leucocytes and fibroblasts, in turn, induces them to generate additional cytokines, including tumour necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β) and PDGF, as well as chemokines, as components of a pro-inflammatory cytokine cascade. Neutrophil recruitment typically peaks at about 24 to 48 hours after operation, followed by an increasing representation of monocytes which are essential for clot maturation, tissue adherence to bone and the early formation of granulation tissue.²⁹

Chronic phase. The monocyte drives the angiogenic phase, thereby providing nutrients and oxygen, a process which is enhanced by hypoxia and high local concentrations of nitric oxide (NO), vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF-2). Fibroblasts will degrade the provisional matrix through matrix metalloproteinases (MMPs) and respond to cytokine/growth factors by proliferating and synthesising new extracellular matrix (ECM) to replace the injured tissue with a connective tissue scar. Matrix synthesis may last for several weeks. TGF- β 1 acts synergistically to this fibrotic process by recruiting fibroblasts and stimulating their synthesis of collagens I, III, and V, proteoglycans, fibronectin and other ECM components. *In vivo* studies have confirmed that exogenous TGF- β 1 increases granulation tissue, collagen formation, and wound tensile strength when applied locally or given systemically in animal models. The remodelling phase, when collagen is synthesised, degraded and re-organised as it is stabilised by molecular crosslinking into a scar, is also cytokine-mediated. Degradation of fibrillary col-

lagen and other matrix proteins is driven by serine proteases and MMPs under the control of the cytokine network. MMPs not only degrade matrix components, but also function as regulatory molecules by driving enzyme cascades and processing cytokines, matrix and adhesion molecules to generate biologically active fragments.³⁰ Tissue inhibitors of metalloproteinases (TIMPs) counterbalance MMPs and disruption to this orderly balance can lead to excess or insufficient matrix degradation and ensuing tissue pathology. The co-ordinated regulation of enzymes and their inhibitors ensures tight control of local proteolytic activity. In physiological circumstances, these molecular brakes limit tissue degradation and facilitate the accumulation of matrix and repair.³¹⁻³⁴

Current state of knowledge of *in vivo* work

Animal studies. Various animal models have been used to examine the biological response of graft placement and interaction with host bone.³⁵⁻³⁷ These experiments have involved dividing the ACL, reconstructing it with autologous graft and killing the animals at various stages of healing. Histological analysis of sections taken from the graft-bone interface allows an examination of the integration process at a defined point after surgery. Hunt et al³⁸ validated an ovine model of ACL reconstruction using a digital flexor tendon and anatomical graft fixation. Martinek et al³⁹ studied the role of bone morphogenetic protein-2 (BMP-2) gene transfer in a rabbit model of ligament reconstruction by transecting semitendinosus autografts with adenovirus-BMP-2 before implantation and Weiler, Schefler and Sudkamp⁴⁰ examined the biomechanical properties of interference screw fixation in a calf model of ACL fixation.

Bone-patellar tendon-bone autograft. The use of a patellar tendon graft offers the advantage of direct bone to bone contact. The sequence of healing of a bone-patellar tendon-bone graft within a bone tunnel has been studied histologically in a canine model. Full incorporation was reported by 12 weeks after surgery. The original tendon attachment in the graft maintained the features of direct attachment throughout the healing process with Sharpey-like fibres found at the interface between the patellar tendon and bone tunnel.⁴¹ The structure of the graft-bone interface resembled that of the normal ACL. Strong, early anchorage of the bone to bone, may replicate the natural transition zone and thereby allow for early, aggressive rehabilitation. Similar work in a rabbit model suggests that there may be a substantial delay before any histological evidence of graft-bone bonding is seen. It is only by 38 weeks after implantation of a bone-patellar tendon model that the four layers of graft-bone integration were apparent.⁴²

Hamstring autograft. The use of hamstring autograft is becoming increasingly popular. The reasons cited include the absence of potential damage to the extensor mechanism, less anterior knee pain and a lower incidence of fixed flexion.⁴³ In the majority of studies a form of indirect heal-

ing between host bone and soft tissue is identified.⁴⁴ In a study of canine digital extensor tendons sutured into bone, collagen fibres were seen between the tendon and the wall of the bone tunnel within four weeks. Sharpey's fibres were identified by 12 weeks and this tissue was felt to have reached maturity by 26 weeks from implantation. Before 12 weeks, pull-out testing found that the construct failed by separation of the graft from the bone. However, after 12 weeks interstitial failure was more likely to occur, suggesting that anchorage was completed by this point.⁴¹

Human studies. Little work exists to date on human ACL reconstruction. Pinczewski et al⁴⁵ obtained tissue from the graft-bone interface at the time of surgery in two patients who required revision for traumatic mid-substance rupture within 15 weeks of the primary reconstruction. This study, based upon observed collagen fibre continuity, concluded that osteointegration occurred between tendon tissue and host bone between six and 15 weeks after surgery. It was argued that the tight contact between bone and graft achieved by an interference screw was crucial for graft integration. It is proposed that anatomical fixation with interference screws may promote direct healing and help tendon-bone incorporation.⁴⁰ Ishibashi et al⁴⁶ examined the histological changes at the tibial tunnel in ten patients undergoing revision surgery after an initial patellar tendon implantation. They arbitrarily divided the patients into early (less than one year) and late revision cases.⁴⁶ They argued that bone-tendon integration could continue for several months after implantation with clear differences in interface architecture. Song et al⁴⁷ proposed that failure of adherence of the soft-tissue graft to bone caused early failure and correlated clinical findings with the absence of graft adherence on histological examination. Haus and Refior⁴⁸ examined the topographical relationships of the implanted ACL with the host bone in 16 human ACL grafts retrieved at autopsy. They described a subsynovial layer which was found to be composed of a tight fibrous layer peripherally, adjacent to loose connective tissue that contained a variety of blood vessels, collagenous strands and nerves.

One of the biological goals of reconstructive surgery is to achieve stable adherence of the graft to the host tunnel over as large a surface area as possible.⁹ Aperture fixation is considered crucial to the long-term functional stability of any graft. This translates on a cellular level to early cross-linking of collagen, from the soft-tissue construct to the host cancellous bone. At a molecular level, the balance between resorption and degradation of a local haemarthrosis, removal of cellular debris led by the synovial fluid, promotion of collagen hypertrophy, and growth factor expression from both host and graft tissue, will ultimately determine the success of graft integration. Healing appears to begin with proliferation of fibrovascular tissue in the interface between tendon and bone. There is ingrowth of monocytes, macrophages, and marrow-derived pluripotential stem cells. Healing progresses through the formation of a new matrix at the tendon-bone interface. Proliferation of new

bone trabeculae along the edge of the tunnel is seen as early as three weeks. This pattern is not uniform, as some areas exhibit a cartilaginous interface between tendon and bone. This zone of fibrocartilage may persist and represents a form of direct healing by tissue which may undergo endochondral bone formation. The distribution of graft-bone anchorage is also variable. This may be because of a bend in the graft as it exits the femoral aperture, the presence of an interference screw, the timing of the biopsy (representing incomplete maturation), micromotion (suspensory fixation) or as a result of intrusion or pulsing of synovial fluid.

The pattern of change within the body of autograft tendon when it is transplanted into a human recipient has been classically described as 'ligamentisation'.^{49,50} This reflects the morphological changes within the tendon, which shows an increase in fibroblast ingrowth and MRI changes which may represent enhanced shear resistance and increased vascularity. Histologically, there is predominantly fibroblastic ingrowth for the first two months, followed by graft remodelling with neovascularity and areas of necrosis over the next ten months. Finally, there is steady maturation of the graft over the next two years. The transplanted graft has been found to undergo a process of complete metaplasia to a ligamentous structure within three years of implantation. It is important to realise that this biological sequence of events may not necessarily be mirrored at the graft-bone tunnel interface and that studies based on samples taken from this particular junction may incorrectly reflect the true process of integration.

Potential new modulatory agents

Matrix metalloproteinases. The MMPs are members of a family of at least 15 zinc-dependent endopeptidases which function in both an extracellular environment and through transmembrane and intracytoplasmic domains. The TIMPs are the natural inhibitors of MMP activity and act to regulate these highly physiological mediators.⁵¹ It is now recognised that MMPs usually degrade multiple substrates, with considerable substrate overlap between individual MMPs. Collagenase (MMP-1) can degrade fibrillary collagen and elastin. MMP-12 is highly active against type IV collagen. In general, MMPs are capable of breaking down any extracellular matrix component and contribute to normal tissue remodelling.⁵² Inflammatory cytokines such as IL-1, TNF and TGF- β are required to initiate the transcription and processing of MMPs from the inactive zymogens to the active enzymes. Once active, these MMPs will induce further cellular production of pro-inflammatory cytokines. TIMPs and IL-4 reduce this destructive, catalytic response. We have studied the tissue expression and transcription rates for mRNA for a variety of MMPs in autograft tissue taken from the femoral tunnel at the time of revision ACL surgery. Preliminary work has found high levels of MMP-9, -13 and -1. This would lend weight to the theory that there is an ongoing degradative process at the graft-bone interface and may help to explain the failure of bonding of soft

tissue to bone and atraumatic failure in a proportion of patients.

Exogen. Exogen (Smith & Nephew, Andover, Massachusetts) is a pulsed, low-intensity ultrasound delivering a controlled, focused micromechanical force. It is applied topically for up to 30 minutes each day to adjacent tissue. It has been shown to stimulate production of the angiogenic factors IL-8, FGF and VEGF in osteoblasts, fibroblasts and monocytes,⁵³ expression of the immediate early response gene c-fos and COX-2 and elevation of m-RNA for bone matrix protein.⁵⁴ Parvizi et al⁵⁵ found that exogenous ultrasound, when applied to cultured rat chondrocytes, caused increase aggrecan gene expression and proteoglycan synthesis. Clinical trials have confirmed its efficacy for recalcitrant nonunions of long bone.⁵⁶ More recently, clinical work has focused upon its role in abating tendonitis of the tendo Achillis. We are currently undertaking a prospective, blinded study to determine its efficacy for the treatment of recalcitrant patellar tendinopathy. Through its pre-angiogenic potential it has been studied as an adjunct to encouraging graft ingrowth on the femoral side in an animal model of ACL reconstruction on the basis of confirmed acceleration of mineralisation.⁵⁷ Using an ovine model with indirect femoral fixation with an endobutton, histology of the bone-tunnel interface found increased neo-angiogenesis and vascularity. This was associated with increased cell viability and enhanced bone ingrowth through Sharpey-like fibres.

Nitric oxide. This highly reactive free radical agent is synthesised from L-arginine by nitric oxide synthase. It acts in both the intercellular and extracellular environment⁵⁸ and is believed to be a regulatory molecule in a variety of soft tissues including articular cartilage, ligament, tendon, skeletal muscle and bone. It is induced during tendon healing *in vitro*.⁵⁹ It appears that there is a dose-dependent effect upon its contribution to fibroblast production of collagen. There is also a site-specific effect with the anterior cruciate ligament-derived fibroblasts capable of producing more nitric oxide than from cells derived from the medial collateral ligament.⁶⁰ Manipulation of nitric oxide production has been thought to help accelerate repetitive overuse tendon injury and tendinosis. The role of nitric oxide in the incorporation of an ACL graft remains under investigation through studies using transfection of cDNA for nitric oxide synthase.

Growth factors

These are small polypeptides produced by a variety of cell types which act by binding to specific cell surface receptors, thereby activating intracellular signal transduction pathways. They are known to induce cell migration, proliferation, differentiation and matrix synthesis, thereby modulating local tissue healing.⁶¹

Targeted cell-specific locally-applied growth factors offer the potential to stimulate successful graft ingrowth. Indeed, Rodeo et al⁶² have successfully used recombinant bone

morphogenetic protein-2 (rhBMP-2) in an animal model to augment bone and fibroblast activity adjacent to the site of the graft-tunnel nexus.

In vitro effects. FGF, TGF- β 1 and PDGF- α may act in isolation or synergistically to stimulate fibroblast proliferation, collagen synthesis and cell outgrowth.⁶³ The effect of such growth factors has been found to be dose-dependent and related to the age of the tissue model, with younger tissue exhibiting a greater response.^{64,65}

In vivo effects. The influence of these same growth factors *in vivo* has been found to be highly variable.⁶⁶ Such variability may be due to the method of delivery and the period over which delivery of the agent occurs.^{67,68} In general, it can be said that PDGF tends to enhance fibroblast proliferation and TGF- β 1 promotes collagen synthesis.⁶⁹ To date, there does not appear to be one particular agent which either offers a reproducible effect or confers a greater single degree of efficacy over other similar pro-inflammatory agents.

Delivery systems. Growth factors have short half-lives as they are subject to the action of tissue-derived local proteinases and may be cleared by the local circulation.⁷⁰ This has limited their efficacy and may not allow for critical induction of surface receptor production. Therefore, work has been undertaken to produce safe and reproducible delivery systems which allow for minimal contamination or dilution of the growth factor. Such scaffolds include collagen, cellulose sponges and fibrin sealant.⁷¹ Refinement of these methods remains experimental.

Gene transfer

Limitations of exogenous growth factors include accuracy of delivery at appropriate concentrations, maintenance of therapeutic concentrations, the local lavage effect of joint fluid and the short half-lives of such agents.⁷²

Augmentation of the process of graft integration may be improved through growth factor modification of fibroblast behaviour. TGF- β , epidermal growth factor (EGF) and PDGF may all stimulate ligament healing through collagen production. Gene therapy involves injection of transduced cells, either myoblasts or fibroblasts, with reporter genes which will result in high local production of growth factors for up to six weeks. There are both direct and indirect methods of gene transfer. Direct transfer involves the use of naked DNA from mammalian tissue and a one-step delivery of genes into host cells *in vivo*. Indirect transfer involves transfection of desired genes into cells followed by implantation of the cellular tissue into the host. Gene transfer offers the opportunity for a longer half-life of delivery of the target molecule. Gene expression for up to six weeks after direct transfer has been demonstrated.⁶⁶ However, there may be poor control of both the timing and the delivery of the concentrations produced. Such experimental work does not directly influence local control mechanisms and presumes adequate concentrations of cell surface receptors for these key growth factors to work. Indirect

transfer may also induce an immunogenic response with associated local and systemic damage.⁷³ Strategies for minimising local response and controlling the length of gene expression remain under intense study.⁷⁴

In summary, with greater understanding of the biology of ACL autograft reconstruction, there should be fewer atraumatic failures and a reduced revision workload. The results after revision surgery for recurrent pathological laxity are far inferior to those after primary intervention.^{14,75-77} Controlled modulation of the early graft-host interaction in ACL reconstruction offers the exciting possibility of a reproducible, accelerated clinical response to surgical intervention and may reduce the incidence of clinical failures. Many mechanical and biological agents are now under investigation. We have provided a simplified overview of the current knowledge of the biology of the graft-host bone interaction for hamstring and patellar tendon autografts. We have not addressed the complex subject of allograft or synthetic agents, as the majority of primary ACL reconstructions in the United Kingdom use either patellar tendon or hamstring tendon autografts.

The specific use of highly potent agents to enhance graft ingrowth requires careful clinical *in vivo* study through randomised controlled trials. It is clear that any one of these agents may, if incorrectly administered, result in deleterious local and even systemic effects.

Studies of the basic mechanisms of tendon-to-bone healing may lead to new methods of treatment which improve healing. These could apply to any situation in which a tendon graft is placed into bone tunnels, such as reconstruction of the anterior or posterior cruciate ligaments, ankle ligaments reconstruction, or ulnar collateral ligament stabilisation at the elbow. Knowledge of gene expression at healing tendon insertion sites may suggest ways to manipulate the chemical and molecular signals in order to improve healing. The use of chemical messengers presumes an expertise in delivery systems which does not currently exist *in vivo*. It is possible that the use of topical adjuvant therapy may herald the widespread interest and use of such novel treatment as this is controllable and does not appear to have any deleterious side effects. This area of interest is in its infancy.

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