Novel Biological Approaches to Enhance Primary Repair of the Anterior Cruciate Ligament

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Intra-articular tissues, including the anterior cruciate ligament, meniscus and articular cartilage, often fail to heal following primary repair. In this report, we outline recent progress in enhancing the biological repair of the anterior cruciate ligament (ACL). This translational research project combines advances in cell biology, molecular biology, orthopaedic surgery and materials science into novel regenerative strategies, and illustrates the advantage of clinicians and scientists working closely together to address pressing clinical problems.

CLINICAL SIGNIFICANCE OF ACL INJURY

With increased participation in sports, ACL ruptures are rapidly increasing. Presently over 100,000 patients rupture their ACL each year. The ACL fails to heal after rupture, and loss of ACL function leads to knee instability, loss of proprioceptive function,[1] and osteoarthritis in over 60% of patients.[2] Since primary repair of the ligament has been found to fail in greater than 50% of patients,[3-5] the recommended treatment for the ACL deficient knee with instability is ligament reconstruction with biologic grafts like autologous patellar tendon or hamstring tendon. However, this operation does not restore the complex architecture and biomechanics of the ACL, and more than 50% of patients will have radiographic changes consistent with early osteoarthritis at only 7 years after surgery.[6,7] Thus, ACL rupture is a clinically important problem, and there remains a need for improved treatments. Our research focuses on a novel treatment method to enhance healing of the ACL after rupture.

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HOW DO LIGAMENTS HEAL AND WHY DOES THE ACL FAIL TO HEAL AFTER RUPTURE?

There have been two major arguments as to why the ACL fails to heal: failure of the intrinsic cells to respond effectively to injury, and failure of formation of a provisional scaffold that is the basis of functional scar formation. Recent work, including our reports published in The Journal of Bone and Joint Surgery and The Journal of Orthopedic Research, has revealed that the cells of the ACL are able to proliferate after ligament rupture,[8] are able to migrate to an adjacent provisional scaffold in vitro,[9,10] and continue to make extracellular matrix proteins as long as one year after rupture.[11,12]

An additional finding in our original studies was that the provisional scaffold, one of the key components of wound healing in other connective tissues, was absent from the gap between the ruptured ACL ends.[8] In connective tissues that heal, such as the medial collateral ligament (MCL), a blood clot forms as the original provisional scaffold. This scaffold is gradually invaded by surrounding cells that proliferate and produce extracellular matrix proteins[13] and form a vascular[14] functional scar which becomes increasingly similar to the normal ligament.[15] However, in the ACL, no stable blood clot forms in the gap between the ruptured ligament ends.[8] This deficiency may be due to the presence of fibrinolytic enzymes in synovial fluid.[16] We hypothesize that, without this clot or alternative provisional scaffold, there is no basis for formation of a functional scar, and thus, no foundation for the healing process (**Figure 1**).

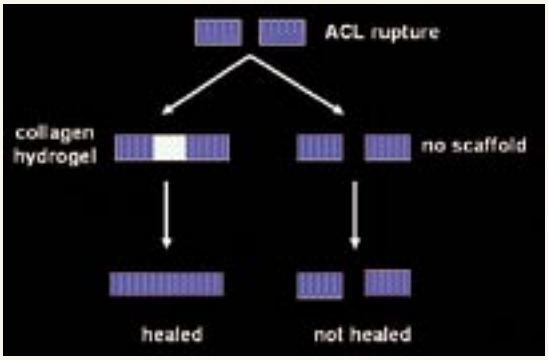

Figure 1: A schematic of the proposed model for ACL healing. Where no clot is formed between the ligament ends, as in the torn ACL, there is no provisional scaffold present to facilitate functional scar formation and maturation (right). Our research suggests the use of a collagen hydrogel which is placed into the gap and allows invasion by surrounding cells and remodeling until a functional scar is formed in the gap (left). Alternatively the hydrogel can be supplemented with cells and/or viral vectors encoding beneficial growth factors.

Thus, even when the ligament ends are re-approximated with a suture repair, healing does not occur, and the suture repair eventually fails.

DESIGN OF AN INTRA-ARTICULAR SUBSTITUTE FOR THE BLOOD CLOT

Our work for the last three years has included the development of a substitute provisional scaffold that can stimulate the production of functional scar in the ACL. Our initial study published last year in the Journal of Orthopaedic Research demonstrated the effectiveness of a collagen bridge in stimulating ACL cell migration into the gap between ligament fascicles in vitro[8]. Based on these findings we presently focus on the insertion of a collagen hydrogel between the severed ends of a ruptured ACL and hypothesize that this will engender a primary healing response (Figure 1).

CYTOKINES AND THEIR ROLES IN THE HEALING OF LIGAMENTS

To further stimulate cellular ingrowth, proliferation and protein production within the hydrogel, we began incorporating growth factors and extracellular matrix proteins into the scaffold. Our rationale for this was that the provisional scaffold in other tissues is blood clot, which contains additional growth factors, such as TGF-ß$_1$ and PDGF, which stimulate functional scar formation. Furthermore, based on our knowledge about the beneficial effects of different growth factors, including TGF-ß$_1$, IGF-1 and PDGF, on monolayer cultures of ligament fibroblast in terms of cell proliferation and matrix synthesis,[17-19] we reasoned that the optimal basis for stimulating ACL healing might involve similar growth factors. We have investigated the effect of several growth factors on the ability of human ACL cells to migrate, proliferate and produce collagen in the gap between the ends of the ACL. This study revealed that ACL cell proliferation and collagen production could be stimulated by the addition of TGF-ß$_1$, and also suggested that certain growth factors can alter the biologic functions of human ACL cells in a collagen scaffold implanted as a bridge at the site of an ACL rupture.[20]

Our data suggest that healing is accelerated and improved by the application of appropriate growth factors and other gene products. However, delivery remains a major impediment to the eventual clinical application of these and related products, because of the rapid efflux and metabolism of these recombinant proteins. We hypothesize that transfer of the relevant genes, rather than the gene products, is the most expeditious method of harnessing such factors for clinical use.[21] Thus we added an additional line of research focusing on the development of clinically appropriate methods for gene transfer to the healing ACL.[22]

GENE TRANSFER APPROACHES TO ENHANCE THE HEALING OF LIGAMENTS

In studying gene transfer to ACL cells, we initially wanted to confirm the susceptibility of ACL cells to gene transfer and test their response to the transgenes they express. In an initial experiment, ACL fibroblasts were transduced with a recombinant adenovirus carrying a cDNA encoding green fluorescent protein (Ad.GFP), and the number of green fluorescent cells in the monolayer was counted. The number of green cells increased in a dose dependent fashion (Figure 2). Similar cultures were then transduced with a recombinant adenovirus carrying TGF-ß$_1$ cDNA (Ad.TGF-ß$_1$). This resulted in increased TGF-ß$_1$ production in a dose-dependent manner as measured by ELISA. Cell number and DNA content of the monolayers also increased.[22]

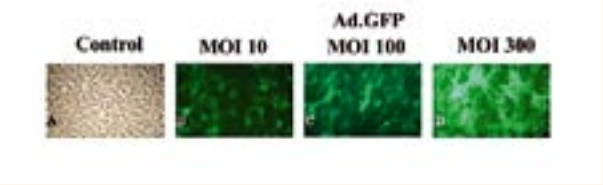

Figure 2: Adenoviral-mediated gene delivery to bovine ACL cells in monolayer culture. Phase contrast microscopy (A), and fluorescence microscopy of GFP transduced cultures at 10 (B), 100 (C) and 300 (D) multiplicities of infection (MOI; number of infectious viral particles per cell) after 3 days. The increasing number of green cells is indicating a dose dependent expression of the GFP transgene. Original magnification: X10. (Reproduced with permission from Pascher A, Steinert AF, Palmer G *et al.* Mol Therapy (in press)[22]).

Once we had demonstrated the ability of adenoviral vectors to transduce the ACL cells in monolayers, we investigated how transduced ACL cells would behave when incorporated into collagen hydrogels. ACL cells were transduced with recombinant adenovirus carrying luciferase cDNA (Ad.Luc) and seeded into collagen hydrogels. The cells showed elevated levels of transgene expression throughout the 3 weeks of culture, with the highest level of expression at day 3 and a subsequent decline over time. However, even after three weeks, luciferase expression remained 5-6 fold above background levels. Cultures transduced with Ad.GFP showed a similar pattern of transgene expression. When ACL cells in hydrogels were transduced with Ad.TGF-ß$_1$, expression of TGF-ß$_1$ transgene was initially high, and then declined gradually, with moderately elevated levels after a month, compared to the Ad.GFP controls. On histologic examination of the ACL cell seeded hydrogels, those constructs with Ad.TGF-ß$_1$ transduced ACL cells (Figure 3B) were more cellular, in particular on the surface of the construct, when compared to controls (Figure 3A). This indicates that transgene expression resulted in stimulated proliferation or survival of the ACL cells in the hydrogel.[22]

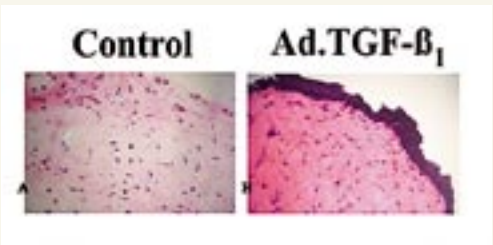

Figure 3: Adenoviral-mediated TGF-ß$_1$ gene transfer to ACL cells cultured in collagen hydrogels. Histologic evaluation of ACL-cell-seeded control (A) and Ad.TGF-ß$_1$ (B) transduced collagen hydrogels after 4 weeks in culture. H&E staining revealed that the hydrogels seeded with ACL cells modified to express TGF-ß$_1$ (B) showed a higher cellularity than the controls (A) after 4 weeks in culture. (Reproduced with permission from Pascher A, Steinert AF, Palmer G *et al.* Mol Therapy (in press)[22]).

Transduction of ACL Cells Migrating into a Collagen Hydrogel

Our next experiment was designed to determine whether ACL cells migrating into a hydrogel containing an adenoviral vector would be transduced. To address this question we placed ACL tissue pieces into culture with a collagen hydrogel containing Ad.GFP (See Figure 4A). During the first week, ACL cells

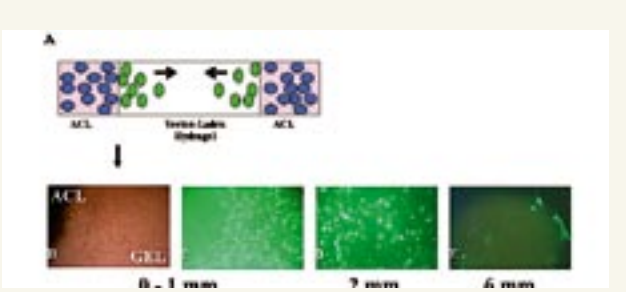

Figure 4: Fluorescence microscopic view of the interface between ACL explant cultures and vector-laden collagen hydrogel. The schematic illustrates how ACL cells (blue) migrated into collagen hydrogels loaded with adenoviral vectors encoding GFP, and become transduced (green) by these vectors as they migrated into the hydrogel (A). The lower panels show a fluorescence microscopic view of GFP$^+$ cells (B-E). The number of GFP$^+$ cells increased over the 21 days in culture and suggests that ligament cells migrate into the gel, and aquire and express the GFP transgene. Migration of Ad.GFP infected ACL cells in the hydrogel occurred up to 6mm in 21 days (B-E). Original magnifications: X10. (Figures B-E were reproduced with permission from Pascher A, Steinert AF, Palmer G et al. Mol Ther (in press)[22]).

had successfully migrated from the explants into the gel and began expressing GFP. The number of GFP+ cells increased progressively until the experiment was terminated (See Figure 4B-E). These data suggest that cells migrating into the hydrogel engage the recombinant adenovirus within the gel, and become transduced by it.

Based on the migration results above, we next tested the cell response to an adenovirus carrying the gene for TGF-ß1 (Ad.TGF-ß1) placed into the hydrogel. Histologic and immunohistochemical analyses revealed that the Ad.TGF-ß1 transduced constructs were much more cellular and revealed enhanced collagen production compared to the Ad.Luc controls.[22]

Conclusions

In summary, the clinical problem of the ruptured ACL remains significant. In the light of the long-term problems of knee laxity and osteoarthritis associated with loss of ACL function and reconstruction failure, new treatment methods that preserve as much of the complex structure and function of the ligament as possible hold some considerable promise. The study of the cell biology of the ACL, the response to rupture in the ligament, and the migration potential of cells of the ACL provides a starting point for investigating guided tissue regeneration as a potential treatment method for ACL rupture. Future directions of research include optimization of the regeneration template, both in terms of substrate and additives such as growth factor encoding genes, determination of the effect of synovial fluid on substrate integrity, and in vivo testing of this method. Translational research of this type should lead to better treatment methods for patients with ACL rupture.

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