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Expression of Modulators of Extracellular Matrix Structure After Anterior Cruciate Ligament Injury

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Abstract

The ability of the anterior cruciate ligament (ACL) to heal after injury declines within the first two weeks after ACL rupture. To begin to explore the mechanism behind this finding, we quantified the expression of genes for collagen I and III, decorin, tenascin-C, and α smooth muscle actin, as well as matrix metalloproteinase (MMP)-1 and -13 gene expression within multiple tissues of the knee joint after ACL injury in a large animal model over a two week post-injury period. Gene expression of collagen I and III, decorin and MMP-1 were highest in the synovium, while the highest MMP-13 gene expression levels were found in the ACL. The gene expression for collagen and decorin increased over the two weeks to levels approaching that in the ligament and synovium; however, no significant increase in either of the MMPs were found in the provisional scaffold. This suggests that while the ACL and synovium upregulate both anabolic and catabolic factors, the provisional scaffold is primarily anabolic in function. The relative lack of provisional scaffold formation within the joint environment may thus be one of the key reasons for ACL degradation after injury.

Keywords

Anterior cruciate ligament; extracellular matrix; matrix metalloproteinases; failed healing response; qPCR

INTRODUCTION

Previous work has shown that a delay between anterior cruciate ligament (ACL) injury and enhanced primary repair in a large animal model has a significantly negative effect on the functional performance of the repaired tissue (1). As most patients would likely have a delay between injury and ACL repair, there is a need to understand the source of this negative effect.

Previous studies have shown that wound healing following injury entails a complex series of events resulting in cell activation and extracellular matrix (ECM) production and remodeling (2). One potential reason for the impaired healing ability of the ACL over time may be detrimental changes in gene expression for genes regulating collagen balance, such as a decrease in collagen gene expression or an increase in gene expression of matrix metalloproteinases (MMPs). These changes could be within the ACL or within cells in other

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tissues within the joint that are able to communicate with the ACL via synovial fluid, such as those residing in the synovium or the provisional scaffold which forms in the ACL wound. Changes leading to a negative collagen balance could lead to inferior repair of the injured ligament.

Key anabolic elements of ligament healing include extracellular matrix production, as well as extracellular matrix organization and myofibroblast activation. Extracellular matrix production is a key element of successful ligament healing. Wound healing typically starts with type III collagen (Col III) production predominating and shifts to production of type I collagen (Col I) as the wound matures (3–5). Decorin is also a key element, and helps to control collagen fibril diameter and organization (6, 7). Tenascin-C is most commonly transcribed in dense connective tissue, including ligament and tendon (8), and is associated with fibroblast migration (9) and wound healing (10). Fibroblasts which become activated following injury are believed to migrate into the damaged tissue where they differentiate into myofibroblasts. These activated myofibroblasts contribute to tissue repair and can be identified through alpha smooth muscle actin (α SMA) expression (4).

On the catabolic side, MMPs are involved in several normal physiological processes, including embryonic development, reproduction, and tissue remodeling. MMP-1 is thought to be principally responsible for breaking down interstitial collagens, including types I, II, and III, and degrading these fibrillar collagens in their triple-helical domain. This leaves the collagen molecules in an unstable thermal state, allowing them to unwind and form gelatin, whose structure is capable of degradation by other MMPs (11). A variety of cell types, including fibroblasts, macrophages, and endothelial cells, are capable of MMP synthesis, however MMP-1 levels are usually low in normal adult tissue and is mainly expressed during physiological and pathological tissue remodeling (12). MMP-1 has therefore been shown to preferentially cleave type III collagen, an important constituent of the wound healing process, while MMP-13 can cleave the fibrillar collagens in addition to other substrates, including gelatin, aggrecan, and fibronectin (13, 14).

Tissue damage often results in the formation of a blood clot within the site of injury. This clot serves as a provisional scaffold, bridging the wound site and providing a route of cell migration into the injured area (2). Over time, fibroblasts remodel this provisional matrix into an ECM that more closely resembles the native tissue. However, for the ACL, while a small amount of provisional scaffold may form over the ends of the torn ligament, it does not bridge the entire wound (15). We have previously hypothesized that it is this lack of mechanical joining of the two torn ends of the ligament which prevent successful healing of the ACL. In this study, we included gene expression analysis of the small amounts of provisional scaffold that were found on the torn surface of the ACL in an effort to find out what effect, anabolic or catabolic, this non-bridging provisional scaffold might have on the adjacent ACL and the joint milieu after an ACL injury.

In this study, we hypothesized that 1) the gene expression of collagen I and III within the ligament decreases and that 2) the gene expression of MMPs increases in one of the three studied joint tissues, the ligament, synovium, or provisional scaffold formed at the site of injury, in the initial post-injury period. We also hypothesized that increases in MMP gene expression in the ligament, synovium, or provisional scaffold would result in increased MMP activity in the synovial fluid and would be associated with degradative changes in the injured ACL tissue.

MATERIALS AND METHODS

Thirty adolescent Yucatan minipigs (CoyoteCCI, Douglas, MA), aged 12–15 months, were obtained for use in this study and were handled according to approved IACUC protocols at Animal Resources at Children's Hospital (ARCH, Boston, MA). Minipigs were acclimated to the ARCH environment for a minimum of 3 days then assigned to one of five groups, with each group assigned six minipigs. Minipigs in four of the groups were subjected to ACL transection, then tissue harvested at 1, 5, 9, or 14 days post-injury, while a fifth group of six non-operated Yucatan minipigs were reserved as intact ACL controls.

Surgical Procedures

Twenty-four minipigs underwent an ACL transection procedure on one knee as previously described (16, 17). ACL transection was performed on alternating knees, resulting in 3 subjects with injured right and 3 with injured left ACLs in each group. Briefly, the ACL was exposed by performing a medial arthrotomy and partial resection of the fat pad. A scalpel blade was used to cut the ACL at the junction of the proximal and middle thirds. Functional loss of the ACL was verified using the Lachman maneuver, a clinical indication of ligament integrity, and then the knee was closed in layers (1). Following surgery, minipigs were allowed normal nutrition and *ad lib* activity throughout the experimental period.

Tissue Collection

The ACL, including any provisional scaffold matrix found on the end of the injured ligament, and medial synovium from each minipig were harvested after the specified time points from the injured knee of each subject (day 1, 5, 9, or 14, n=6 for each time point). The provisional scaffold within the ACL wound site was separated from the ligament tissue. Each tissue specimen was then divided, with one portion submerged in a cryovial containing RNA Later (Ambion, Austin, TX, USA), and flash frozen in liquid nitrogen then stored at –80 °C until analysis. Another portion of the tissue was embedded within OCT medium (Sakura Finetek, CA, USA), frozen, and stored at –80 °C for histological analysis. Synovium and intact ACL tissue were also harvested from six control subjects. Systemic blood of control minipigs was clotted to serve as a provisional scaffold control for the intact ACL group.

Synovial fluid collection

Twelve animals belonging to the groups which were sacrificed at day 9 (n=6) and 14 (n=6) were also subjected to serial synovial fluid draws at alternating time points. IACUC protocols demanded a break between anesthesia events, therefore one group was sampled pre-transection, then 3 h, 1 day, 3 days, 7 days and 9 days post-injury. The second group was sampled pre-transection, then 1 h, 5 days, 12 days, and 14 days post-injury. Synovial fluid was centrifuged at 3000×g for 10 min to remove any cells. The supernatant was removed and stored in 120 µL aliquots in cryovials at –80 °C until analysis.

qPCR and data analysis

The ligament, synovium, and provisional scaffold were examined for mRNA expression of several genes using real-time reverse transcriptase polymerase chain reaction (qPCR) run in duplicate. Total RNA was extracted from the frozen tissue using the Pure Link RNA Mini Kit (Ambion, Austin, TX, USA), treated with DNase I (Pure Link DNase, Invitrogen, Life Technologies, NY, USA) according to the manufacturer's protocol and quantified. Total RNA was reverse transcribed to generate cDNA using the RETRO script kit (Ambion, Austin, TX, USA). For use in qPCR, previously reported primers were validated by sequencing the PCR product and performing a BLAST search with these results. Primers are

summarized in Table 1. Sybr Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA) (10 μ L), nuclease-free water, forward and reverse primer (2 μ L each), and 0.5 μ L of the 1 ng cDNA were mixed and quantified in a reaction volume of 10 μ L. Non-template controls (NTC) were included to indicate contaminants or non-specific amplification. An Applied Biosystems 7900 HT (Applied Biosystems, Foster City, CA, USA) was used for amplification and detection. Level of gene expression was normalized to the housekeeping gene, GAPDH. Relative gene expression was calculated using the $2^{-\Delta C_t}$ method (18). The MMP-1 and MMP-13 gene expression data is adapted from a subset of previously described data (19).

MMP activity assay

MMP activity in the synovial fluid was determined using the MMP Activity Assay Kit (ab112147, Abcam, Cambridge, MA) according to the manufacturer's protocols. Changes in MMP activity are reported in relative fluorescence units (RFU).

Histology

Ligament specimens which had been embedded within OCT medium were cut in 6 μ m sections and stained with hematoxylin and eosin (Mass Histology, MA, USA). The collagen structure of the ACL was analyzed by imaging ligament sections using a Zeiss Axio Imager M1 microscope fitted with an Axio Cam HRC digital camera and analyzed using Axio Vision Imaging software. Collagen organization was assessed using photomicrographs taken under polarized light. A minimum of three images per sample were obtained, with a greater number of images taken for larger tissue slices to ensure representation across all tissue zones. Crimp length was quantified using calibrated Image J software (National Institutes of Health, MD, USA). A minimum of ten measurements per image were recorded. A second 6 μ m section of OCT embedded ligament specimens was stained for the C1, 2C antibody (IBEX Technologies Inc., Quebec, Canada), indicative of collagen type I and II degradation, and cell nuclei counterstained with hematoxylin (Mass Histology, MA, USA).

Western blotting

Synovium obtained from day 0, 5, and 14 time points (n=3 per time point) was homogenized in RIPA buffer (Sigma-Aldrich, St. Louis, MO) to extract the protein. Protein extracts were separated on a Nu PAGE 3–8% Tris-Acetate Gel (Novex, Life Technologies, Grand Island, NY) and transferred onto a PVDF membrane (Invitrogen, Life Technologies, Grand Island, NY) followed by incubation in a 1:5,000 dilution in the primary antibodies (anti-collagen I antibody (ab34710) and anti-GAPDH antibody (ab9484), abcam, Cambridge, MA) then 1:10,000 dilution in the secondary antibodies (Goat anti-rabbit IgG conjugated with alkaline phosphatase and goat anti-mouse conjugated with alkaline phosphatase, Thermo Scientific Inc, Rockford, IL). Antibody binding was visualized by AP Chemiluminescent Substrate (Novex, Life Technologies, Grand Island, NY) and developed on HyBlot CL Autoradiography Film (Denville Scientific Inc., Metuchen, NJ). GAPDH served as the loading control.

Statistical methods

Gene expression was summarized as mean levels of relative gene expression (log transformed), with error bars representing standard error of the mean. Differences in relative gene expression levels between the three tissue groups over the entire two week study period were determined using a two-way ANOVA followed by post-hoc Bonferroni-Dunn analysis, with statistical significance determined at $P < 0.0167$. Differences in individual relative gene expression between each time point within each tissue were determined using an ANOVA split by tissue type, with statistical significance determined at $P < 0.005$. Comparisons of

MMP activity were also determined via ANOVA followed by post-hoc Bonferroni-Dunn analysis, with statistical significance from baseline determined at $P < 0.0011$. Changes in crimp length were determined through ANOVA and significance determined via post-hoc Bonferroni-Dunn analysis, with statistical significance determined at $P < 0.005$. All statistical analyses were performed using StatView (version 5.0.1, SAS Institute Inc., Cary, NC).

RESULTS

Col I gene expression

Expression of Col I was significantly higher in the synovium when compared to the ligament and provisional scaffold ($P < 0.0001$ for both comparisons), with at least 4× higher levels in the synovium than in the ligament or provisional scaffold at days 5 and day 9 after injury ($P < 0.0009$ for both comparisons) (Figure 1A). The gene expression for Col I in the synovium also increased over time, with a 4 fold increase by day 5 ($P = 0.0018$), and a 6.5-fold increase by day 9 ($P < 0.0001$). No significant changes in Col I gene expression were noted in the ligament throughout the two week period; however, a greater than 8,000-fold increase in expression from day 0 was found in the provisional scaffold at days 9 and 14 ($P < 0.0049$).

Col III gene expression—Col III gene expression levels were significantly higher in the ligament and synovium compared to the provisional scaffold ($P < 0.0001$) (Figure 1B). An initial decrease in Col III gene expression was noted between days 0 and 1 in the synovium ($P = 0.742$), followed by a 10-fold increase in gene expression between days 1 and 5 after injury ($P = 0.0037$). However, no significant changes were observed from Day 0 at any time point. The ligament had a 4.5-fold decrease in Col III gene expression from day 0 to day 1 ($P = 0.0042$), while the provisional scaffold had a 28-fold increase in expression from day 0 to day 14 ($P = 0.0017$).

Decorin gene expression

The synovium exhibited significantly higher decorin gene expression levels than both the ligament and provisional scaffold over the two week period ($P < 0.0096$), while the ligament expression was higher than the provisional scaffold ($P < 0.0001$). In the synovium, an 11-fold decrease in expression was observed between day 0 and day 1 ($P = 0.0002$), moving to a 3-fold deficit by day 5 ($P = 0.0020$). Decorin gene expression levels in the ligament at days 1, 5, and 9 were approximately 3-fold less than levels at day 0 ($P < 0.0003$). The provisional scaffold had a 600-fold increase in decorin gene expression from day 1 to day 14 ($P = 0.0004$).

Tenascin-C gene expression

There were no significant differences in the level often as cin-C gene expression among the three different tissue types ($P > 0.0403$) (Figure 2B). While no significant changes from baseline expression were observed in the synovium over time, a 44-fold decrease in expression was seen between day 1 and day 9 and a 16-fold decrease in expression seen between days 1 and 14 ($P < 0.0009$). A 30,000-fold increase in tenascin-C gene expression was seen in the ligament between day 0 and day 14 ($P = 0.0046$). No significant changes from baseline in tenascin-C gene expression were seen in the provisional scaffold at any time point.

αSMA gene expression

No significant differences in αSMA gene expression was noted between the three tissue types ($P > 0.0828$) (Figure 2C). αSMA gene expression levels increased 16-fold in the

provisional scaffold at days 14 compared to baseline expression levels ($P=0.0046$). Though a 5-fold increase in α SMA gene expression was seen in the ligament between day 0 and day 14, this change was not significant ($P=0.1489$). α SMA gene expression levels in the synovium did not vary significantly at any time point.

MMP-1 gene expression

MMP-1 gene expression levels were significantly greater in the synovium than both the ligament and provisional scaffold ($P<0.0002$), with the greatest overall increase observed at day 14 ($P<0.0001$) (Figure 3A). MMP-1 gene expression levels in the synovium increased 8-fold between day 0 and day 14 ($P=0.0001$). MMP-1 gene expression levels in the ligament also increased 5-fold from baseline levels at day 14 ($P=0.0038$). Though mean MMP-1 gene expression levels in the provisional scaffold were increased, levels were not significantly elevated compared to baseline at any single time point.

MMP-13 gene expression

MMP-13 gene expression levels were significantly elevated in the ligament compared to both the synovium and provisional scaffold ($P<0.0039$) (Figure 3B). A 2100-fold increase in MMP-13 gene expression was seen in the ligament between day 0 and day 14 ($P<0.0001$). Greater than 50-fold increases in MMP-13 gene expression were seen in both the synovium and provisional scaffold by day 5 ($P<0.1056$), however, significant changes from baseline were not observed in either tissue at any of the time points.

MMP activity in synovial fluid

General MMP activity (Figure 4) in the synovial fluid significantly increased immediately following injury, with a 2.7-fold increase observed at 3h post-injury ($P=0.0003$). MMP activity levels remained slightly (1.3-fold) elevated above normal levels at day 14, though this increase was not significant ($P=0.4492$).

Crimp Length

Average porcine ACL crimp length was found to be $31.8 \pm 2.0 \mu\text{m}$ in the uninjured ligament (Figure 5A). A significant increase in crimp length from the day 0 uninjured controls occurred by day 9 ($P=0.0017$) and remained significantly increased, with an average length of $83.7 \pm 16.7 \mu\text{m}$, at day 14 ($P=0.0004$). A general loss of ligament integrity was also noted at day 14, as evidenced by separation of the collagen fibers (Figure 5D).

Collagen Degradation in the ACL

C1, 2C antibody staining of collagen degradation fragments in the ligament revealed an increase in staining near the site of ligament injury which gradually increased in intensity following ACL injury (Figure 6). Very little C1, 2C staining was evident in the intact specimens, however, staining increased through day 9. Diffuse staining throughout the tissue remained evident at day 14, with the highest intensity observed along the outer edges of the ligament.

Western blotting

The results of the semi-quantitative western blot analysis indicate an increase in the alpha 1 chain of type I collagen is present at day 14 (Figure 7).

DISCUSSION

Results from this study supported the hypothesis that ACL injury initiates a wound healing cascade consisting of both matrix generating and matrix degrading proteins. One interesting

observation was that while the highest levels of Col I, Col III, decorin and alpha-SMA were seen in the synovium and ligament, these levels remained relatively unchanged while the levels in the provisional scaffold increased during the two week period to levels approaching those seen in the synovium and ligament. In contrast, the expression of MMP-1 and MMP-13 increased in the ligament itself, but didn't significantly increase in the provisional scaffold. This suggests that on balance, the most anabolic structure is the provisional scaffold, a structure known to be present in smaller quantities for ACL injuries than for injuries which heal successfully like the MCL (15).

Healing of the injured ligament requires collagen production followed by remodeling of the collagen fibrils. Due to the limited healing capacity of the injured ACL, we hypothesized a lack of collagen gene expression may play an important role in the observed failed healing response of that ligament. Interestingly, no decrease in Col I gene expression was found within the ACL itself after injury, but the level of Col I gene expression steadily increased within the provisional scaffold, to a level similar to that in the ligament by day 14. This is consistent with a prior study which reported a significant increase in Col I expression at 2 weeks in the entire excised ligament (ligament plus provisional scaffold) subjected to isolated ACL transection (20). An increase of the alpha1 chain of type I collagen at the protein level at day 14 was also observed in the synovium, suggesting a slight lag time exists between gene expression and protein accumulation in the tissue (21). Similarly, while we did not see a significant decrease in Col III expression after injury in the ligament, we did find a steadily increasing expression of Col III within the provisional scaffold. This is consistent with prior reports of increasing type III collagen within the provisional scaffold of a healing MCL in the first two weeks after injury (4, 5). The combination of these findings suggest that although gene expression for collagen does not change within the ligament itself, the adjacent provisional scaffold has a significant increase in collagen production within the first two weeks after injury.

Decorin is also known to control the size of collagen fibrin formation, and to facilitate cell migration, proliferation, and protein synthesis (6, 7). Protein levels of decorin has previously been found to be decreased at day 5 of healing of the MCL (4), a finding which supports our observations in this study of suppressed early decorin gene expression in the synovium and ligament after ACL injury. Interestingly, while decorin expression was suppressed in the ligament itself, it rapidly increased over 1000-fold in the provisional scaffold to levels approaching that in the normal ligament. This finding was similar to that seen for collagen production, and the combined findings here and in the literature suggests that cellular processes including upregulation of collagen gene expression and decorin expression, while not present in the ACL proper, do occur in the provisional scaffold after MCL or ACL injury.

In contrast, tenascin-C was found to be upregulated in the ACL itself, but remained low in the provisional scaffold during the two-week period. This is consistent with previous studies which have reported tenascin becoming strongly expressed in the healing wound (10), particularly in association with native collagen fibers (10). As tenascin is thought to play a major role in fibroblast migration, the results reported here suggest the injured ligament itself may play a large role in the coordination of fibroblast migration to the wound site.

The expression of α SMA gene expression was not found in the ACL or synovium, but was significantly upregulated in the provisional scaffold by 100 $\times$, to levels approaching that of the synovium and ligament. This is consistent with reports of increased α SMA gene expression one week following ACL transection in mature New Zealand White rabbits (3). α SMA gene expression was not found here to be significantly upregulated in the synovium at any time point, thus indicating fibroblast recruitment may be isolated to the site of

ligament injury. However, significant increases in α SMA gene expression in the provisional scaffold suggest it may play a crucial role in fibroblast recruitment and differentiation at the site of injury.

On the catabolic side, significant upregulation of MMP-1 and MMP-13 were found in the ACL tissue itself, but not in the provisional scaffold. The increase in MMP expression in the ligament is consistent with prior reports of increased expression of MMP-1 in massive rotator cuff tears compared to isolated supraspinatus tendon tears (22). It is also consistent with prior reports of significantly increased MMP-13 gene expression levels at 3, 7, and 14 days in the ACL tissue following ACL injury in New Zealand White rabbits (3, 20). These results confirm our hypothesis that the gene expression of MMPs increases in the ACL itself after ACL injury.

The observed increase in MMP activity in the synovial fluid early on after injury is also consistent with prior studies. In an *in vitro* injury model using human ACL fibroblasts exposed to injurious stretch, global MMP activity was also elevated compared to non-injured controls at 3 days post-injury (23). MMP activity of synovial fluid is of particular interest as the ACL is consistently exposed to synovial fluid. In addition, the increased MMP activity may have a detrimental effect on the provisional scaffold found to be such an important source of anabolic changes. MMP-13, for example, is known to degrade fibrinogen (24), an important constituent of the provisional scaffold. Conversion of fibrinogen into a fibrin clot was shown to be greatly reduced following fibrinogen/MMP-13 incubation, and results indicated the fibrinogen α -chains were almost completely cleaved after 1 minute, and evidence of β - and γ -chain degradation was apparent at 3h (24). An upregulation in both MMP-13 gene expression and general MMP activity following ACL transection may lead to an accelerated breakdown of the provisional scaffold.

Crimp length of the ACL collagen fibers was also found to increase during the first two weeks after ACL injury. The crimp length of the intact porcine ACL ($31.8 \pm 2.0 \mu\text{m}$) was found to be similar to that of the previously reported intact human ACL (25 ± 2 to $33 \pm 22 \mu\text{m}$)(25). However, by the two week time point fraying of the ligament was evident, with increased gaps observed between the collagen fibers. In addition, collagen fiber degradation, as indicated by positive C1, C2 antibody staining, increased over time after injury. Such morphological changes in the ligament may be due, in part, to degradation of the collagen structure through increased MMP activity and loss of tensile strength following rupture.

A few study limitations should be noted. First, an animal model was required to provide tissue and repeated synovial fluid draws which allowed us to assess changes over time. The porcine model was chosen in part due to its size, which is similar to that of a human knee. The porcine model also exhibits a similar dependence on the ACL and overall similarities to human gait biomechanics. However, even the best animal model cannot exactly replicate the human condition. A second potential limitation of this study is the method of ACL injury. A transection model was chosen to insure ligament injury was isolated to the ACL and did not involve the other ligaments of the joint or the surrounding cartilage. This is clearly different from a human ACL injury, which is typically associated with other injuries including bone bruising and meniscal injury.

Failure of the ACL to heal following injury has been attributed to a variety of factors, including resorption of the ACL due to an increase in matrix metalloproteinases and in myofibroblast activity. In this study, we found that although there exists a coordinated effort by various tissues to promote the formation of new collagen matrix, there is a simultaneous production of matrix metalloproteinases which may have a negative effect on both the ACL and the provisional scaffold structure. The provisional scaffold was found to be a major site

of anabolic processes, suggesting a paucity of provisional scaffold after ACL injury may not be detrimental only from a mechanical standpoint, but from a biologic standpoint as well. Future studies aim to capitalize on the healing capacity of the joint by reducing the initial degradative response at early time points, thus shifting the balance towards a reparative process.

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Figure 1.

Relative gene expression of collagen I (**A**) and collagen III (**B**) in the synovium, ligament, and provisional scaffold following ACL injury. Note the log 10 scale on the y-axis. Bars represent standard error and asterisks indicate significance determined at $p < 0.005$ from baseline levels (Day 0).

Figure 2.

Relative gene expression of decorin (A), tenascin-C (B), and α SMA (C) in the synovium, ligament, and provisional scaffold following ACL injury. Note the log 10 scale on the y-axis. Bars represent standard error, asterisks indicate significance determined at $P < 0.005$ from baseline levels (Day 0), and + indicates significance difference from day 1 at $P < 0.005$.

Figure 3.

Relative gene expression in MMP-1 (A) and MMP-13 (B) gene expression in the synovium, ligament, and provisional scaffold following ACL injury. Note the log₁₀ scale on the y-axis. Bars represent standard error and asterisks indicate significance determined at $P < 0.005$ from baseline levels (Day 0).

Figure 4. Mean general MMP activity in the synovial fluid over the first two weeks following ACL injury. Bars represent standard error of the mean and significance from baseline was determined at $P < 0.0011$.

Figure 5.

(A) Change in mean crimp length $\pm$ standard deviation observed in the ligament before (day 0) and after injury (days 1, 5, 9, and 14). Significant changes from baseline (Day 0) were determined at $P < 0.005$. Representative light microscopy images of control ACL tissue (B) and ACL tissue obtained 14 days after injury (C and D) showing increase in collagen crimp length and fiber separation. Scale bar represents 50 μm .

Figure 6. Representative light microscopy images of C1, 2C antibody staining of the (A) intact ligament and the ligament (B) 1, (C) 9, and (D) 14 days after injury. Scale bar represents 20 μm.

Figure 7.

Presence of the alpha1 and alpha2 chains of type I collagen in the synovium following ACL injury. Qualitatively, the staining was more intense at day 14 than at day 0 or day 5, suggesting an increase in the presence of both the alpha1 and alpha2 chains of type I collagen over time after injury. GAPDH was used as a loading control.

Table 1

Sequences of porcine-specific qPCR primers.

GAPDH (26)	GGG CAT GAA CCA TGA GAA GT	GTC TTC TGG GTG GCA GTG AT
Col 1 (26)	CAGAAC GGC CTC AGG TAC CA	CAG ATC ACG TCA TCG CAC AAC
Col 3 (26)	CCT GGA CTT CCT GGT ATA GC	TCC TCCTTC ACC TTT CTC AC
MMP-1	CCA GAG AAG ATG TGG ACC GTG CC	CCT GGG CCT GGC TGA AAA GCA
MMP-13	CAC GCC TGA TTT GAC TCA TT	CAT CAA AAT GGG CAT CTC CT
Decorin	CTC TCT GGC CAA CAC TCC TC	GCG GGC AGA AGT CAT TAG AG
Tenascin-C (27)	(F) CGG ATC CGT TTG GAG ACC GCA GAG AAG AA	(R) CGC AAG CTT TGT CCC CAT ATC TGC CCA TCA
αSMA (27)	(F) CGG GAT CCA AAC AGG AAT ACG ACG AAG	(R) CGC AAG CTT CAG GAA TGA TTT GGA AAG GA