ALTERED COLLAGEN MRNA LEVELS AFTER SUBFAILURE LIGAMENT INJURY: REAL-TIME QUANTITATIVE PCR ANALYSIS

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INTRODUCTION

Ligament sprains occur more often than complete tissue rupture and can be substantially debilitating, frequently causing persistent joint instability, prolonged pain, and progressive joint degeneration. Sprains are typically classified by severity based upon clinical examination or imaging and are defined as follows [1]: Grade I sprains are mild stretches with no discontinuity of the ligament and no clinically detectable increase in joint laxity; Grade II sprains are moderate stretches in which some collagen fibers are torn and detectable joint laxity is present, yet enough fibers remain intact so that the damaged ligament has not failed; Grade III sprains are severe and consist of a complete or nearly complete ligament disruption and result in significant joint laxity. Although numerous studies have examined Grade III ligament injuries, further study is required to describe the mechanics and biology of the collagenous extracellular matrix after a subfailure damage (sprain) injury.

A prospective study of patients with partial tears of the anterior cruciate ligament (ACL) revealed clinically unstable knees in over 50% of the patients after a mean follow-up of 17 months [2]. This instability is likely caused by increased tissue deformation during physiologic loading. Panjabi and co-workers [3] reported increased deformations within the physiologic strain-stiffening toe-region of the load-deformation curve after a subfailure injury was induced in rabbit ACLs. This behavior has also been reported in rat medial collateral ligaments (MCLs) after a damage threshold of approximately 50-60% of the failure strain has been crossed [4]. In collateral ligaments, the initial increase in tissue laxity is likely caused by collagen fiber damage and/or rupture [5, 6] to fibers which are first recruited during tissue loading [7]. In addition, the initial injury to the tissue results in substantial cellular damage [4] followed by apoptosis [8]. Hence, it is reasonable expect a substantial healing response to remove cell and tissue debris and repair the damaged extracellular matrix. Yet this repair is imperfect. Although tissue strength increases with healing time after a subfailure injury, tissue laxity persists [6]. An in vivo study of rat ligament healing showed a significant increase in tissue ultimate stress (to 80% of control) two weeks after a subfailure injury,

yet there was no significant improvement in the tissue laxity over two weeks. Further study of the causal mechanisms of tissue damage (and the resulting laxity) as well as the in vivo healing response after subfailure injury are required.

METHODS

This study was approved by the Institutional Animal Use and Care Committee. Twelve male Sprague-Dawley rats were used as an animal model. Under general anesthesia, one randomly selected MCL was approached via skin incision without damage to other joint structures. The MCL received a subfailure (Grade II) injury in the same manner as previously described in lateral collateral ligament [6]. The contralateral MCL received a sham surgery. The incision was closed in a routine manner. Animals were euthanized at 1, 3, or 7 days (n=4 rats/group).

Primer sets for collagen I, collagen III, and glyceraldehyde-3phosphate dehydrogenase (GAPDH) have been developed and tested for specificity in our laboratory. After tissue harvest, MCLs were homogenized and total RNA was isolated from the homogenate using a protocol which combines the $TRIzol^{TM}$ method with the column fractionalization steps of the RNeasy® Total RNA kit (Qiagen Inc., Valencia, CA). Tissues were pooled in groups of two in order to insure quantifiable RNA. Yield and purity of RNA were quantified by spectrophotometric measurement. RNA was reverse transcribed using Oligo-dT₁₅ primers and the Superscript II reverse transcriptase in a total volume of 20 µL at 37°C. Real-time quantitative PCR was performed using a BIO-RAD iCycler iQ Real-time PCR system (BIO-RAD, Hercules, CA) in a 20µL reaction mix containing 1X Platinum Quantitative PCR Supermix (Life Technologies), 10 nM Fluorescein, 200 nM forward primer, 200 nM reverse primer, 0.25X Sybr Green, 5µL template, and 3.95µL DEPC-treated H₂O. O-PCR standards were prepared from purified PCR products of the target sequences. The copy numbers of the respective cDNAs in the samples were determined relative to the standard curves and the target gene normalized to the copy number of GAPDH. Statistical analysis was performed using the Student's t-test to analyze differences in gene expression within a time group.

RESULTS

Induction of a subfailure (Grade II) ligament injury resulted in substantial changes in collagen gene expression (Figs 1 and 2). Collagen I expression showed a trend of initial suppression after 1 day, followed by an increasing trend at 3 days. By day 7 collagen I gene expression was significantly increased over the sham side MCL (p<0.05). Type III collagen gene expression also revealed a suppressed trend at 1 day which by 3 days was virtually identical to sham values. After 7 days type III collagen showed a trend of increasing, yet this was not statistically significant. Collagen I expression in injured tissue was significantly larger than type III expression (p<0.05) at 7 days.



Figure 1. Type I collagen gene expression following a subfailure (Grade II) MCL injury (mean ±s.d).



Figure 2. Type III Collagen gene expression following a subfailure (Grade II) MCL Injury (mean ±s.d).

DISCUSSION

Numerous studies have examined wound healing after a complete rupture or transection. However, few studies have examined the biologic response after a subfailure injury in which a localized concentration of scar tissue does not form. In contrast to Grade III ligament injuries in which scar tissue forms at the rupture site through a largely extrinsic inflammatory response, Grade II injury healing appears to be largely intrinsic [9]. To date this intrinsic healing data has not been verified nor rigorous study of the individual cell types undertaken. However, if correct it has important implications for understanding wound healing and fibroblast behavior. Since the inflammatory response (as a source of growth factors and cytokines) plays are large role in wound healing by inducing cell migration and proliferation and controlling extracellular matrix scar formation [10, 11], its absence would leave the responsibility of directing tissue healing largely to the fibroblast. This potential difference in extrinsically versus intrinsically dominated healing response may account for the relatively low levels of type III collagen expression. In a more typical wound model type III expression is substantial and precedes type I expression [10, 11]. Herein type I collagen appears to be upregulated at an earlier stage with a more substantial response. Further work is required to validate and understand the behavior of a potentially intrinsic healing response which differs in behavior from the more extensively studied extrinsic healing response

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REFERENCE

- Scuderi, G.R. and W.N. Scott, *Classification of knee ligament injuries, vol 1, 3rd ed.*, in *Surgery of the Knee*, J.N. Insall and W.N. Scott, Editors., Churchill Livingstone: Phil. Pa. p. 590, 2001.
- Fruensgaard, S. and H.V. Johannsen, Incomplete ruptures of the anterior cruciate ligament. *Journal of Bone and Joint Surgery*. *British Volume*, 71(3): p. 526-30, 1989.
- Panjabi, M.M., E. Yoldas, T.R. Oxland, and J.J. Crisco, 3rd, Subfailure injury of the rabbit anterior cruciate ligament. *Journal* of Orthopaedic Research, 14(2): p. 216-22, 1996.
- Provenzano, P.P., D. Heisey, K. Hayashi, R. Lakes, and R. Vanderby, Jr., Subfailure damage in ligament: a structural and cellular evaluation. *J Appl Physiol*, **92**(1): p. 362-71., 2002.
- Yahia, L., Brunet, J., Labelle, S., Rivard, C.H., A scanning electron microscopic study of rabbit ligaments under strain. *Matrix*, 10: p. 58-64, 1990.
- Provenzano, P.P., K. Hayashi, D.N. Kunz, M.D. Markel, and R. Vanderby, Jr., Healing of subfailure ligament injury: comparison between immature and mature ligaments in a rat model. *J Orthop Res*, 20(5): p. 975-83., 2002.
- 7. Viidik, A., Simultaneous mechanical and light microscopic studies of collagen fibers. *Zeitschrift fur Anatomie und Entwicklungsgeschichte*, **136**(2): p. 204-12, 1972.
- 8. Provenzano, P.P., P. Muir, Z. Hao, and R. Vanderby Jr. *Apoptosis in ligament after a subfailure stretch*. in *Proc. of the World Congress on Biomech*. 2002. Calgary, Ca.
- Laws, G. and M. Walton, Fibroblastic healing of grade II ligament injuries. Histological and mechanical studies in the sheep. *Journal of Bone and Joint Surgery. British Volume*, **70**(3): p. 390-6, 1988.
- Eckes, B., M. Aumailley, and T. Krieg, *Collagens and the reestablishment of dermal integrity*, in *The molecular and cellular biology of wound repair*, 2nd ed., R.A.F. Clark, Editor., Plenum Press: New York. p. 493, 1996.
- 11. Riches, D.W.H., Macrophage involvement in wound repair, remodeling, and fibrosis, in The molecular and cellular biology of wound repair, 2nd ed., R.A.F. Clark, Editor., Plenum Press: New York, p. 95, 1996.