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Tendon properties in interleukin-4 and interleukin-6 knockout mice

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Abstract

Cytokines are known to play an important role in normal tendon development, function, and maintenance through interactions with fibroblasts and extracellular matrix proteins. However, the role of interleukins on normal tendon activity remains poorly understood. Previous studies that have researched the role of specific cytokines by exogenously applying them have often reported conflicting results. Therefore, a knockout mouse model was used to investigate the role of interleukins 4 and 6 on normal tendon organizational and biomechanical properties. It was hypothesized that interleukin-6 knockout (IL6 -/-) mice will display more organized collagen orientation and greater cross-sectional area and mechanical properties when compared to that of control mice. In addition, interleukin-4 knockout (IL4 -/-) mice will display the most disorganized collagen orientation and lowest cross-sectional area and mechanical properties. As hypothesized, IL6 -/- mice show a trend towards lower angular deviation (more organized) ($p<0.1$) when compared to IL4 -/- mice. In addition, the IL6 -/- mice show a trend towards a higher percent relaxation ($p<0.1$) and a significantly higher modulus ($p<0.01$) when compared to CTL and IL4 -/- mice. Unexpectedly, the IL6 -/- mice exhibited no significant differences in collagen fiber distribution and maximum stress from the other groups and actually had a smaller cross-sectional area than CTL mice ($p<0.1$). This study supports transgenic mice as an animal model for investigating how cytokines affect normal tendon properties. In addition, this study demonstrates that interleukins may play an important role in tendon development, function, and maintenance.

Keywords: Tendon; Cytokines; Interleukins; Transgenic mice; Knockout mice

1. Introduction

Normal tendon development, function, and maintenance are dependent upon a balance of activities between tendon cells and the surrounding extracellular matrix proteins. Cytokines have been shown to interact with both fibroblasts and specific ECM proteins and play an important role in these tendon activities (Arai et al., 2002; Robbins et al., 1997). For example, when implanted ectopically in vivo, growth and differentiation factors (GDFs) 5, 6, and 7 induced neotendon formation in rats (Wolfman et al., 1997). Vascular endothelial growth factor (VEGF) expression was shown to be crucial in inducing angiogenesis in fetal tendon development, and basic fibroblast growth factor (bFGF), another potent angiogenic stimulator, was detected in uninjured canine flexor tendons (Petersen et al., 2002; Duffy et al., 1995). Although the effect of various cytokines in tendons has been studied, the role of pro- and anti-inflammatory cytokines has not been investigated. Interleukin-6 (IL6), a pro-inflammatory cytokine, and interleukin-4 (IL4), an anti-inflammatory cytokine, may also play important roles in normal tendon development and maintenance.

IL6 is a multifactorial cytokine that regulates various aspects of the immune response and hematopoiesis. In addition, IL6 has been implicated in differentiation of B-cells, T-cells, and macrophages, stimulation of keratinocyte growth, and inhibition of fibroblast proliferation (Hirano, 1992; Van Snick, 1990). Elevated levels of IL6 have also been detected in peritendinous tissue as a result of prolonged exercise (Langberg et al., 2002). IL4 has been shown to stimulate the synthesis of extracellular matrix proteins, specifically collagen types I and III and fibronectin, by human dermal fibroblasts in vitro (Postlethwaite et al., 1992; Gillery et al., 1992). IL4 is also overexpressed in a wide variety of fibrotic conditions, such as scleroderma, radiation-induced

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pulmonary fibrosis, liver fibrosis, and potentially in keloids (Salmon-Ehr et al., 2000). Because of their observed effect on fibroblast proliferation and ECM protein synthesis, the role of IL6 and IL4 in normal tendon function and development requires further investigation.

In order to study the role of cytokines on tendon function, previous studies have exogenously applied cytokines to tendons. Although exogenous application of cytokines is straightforward, these studies have often reported conflicting results due to the many factors that must be considered, such as the dosage, timing, order, and combination of cytokines that should be added. To circumvent these problems in this study, knockout mice were utilized to investigate the role of specific cytokines. The creation of mice with knockouts of pro- and anti-inflammatory cytokines allows for a more precise and targeted approach (Kopf et al., 1994; Kuhn et al., 1991). Therefore, the overall goal of this study was to use transgenic mice with knockouts of interleukin-6 (IL6 -/-) and interleukin-4 (IL4 -/-) to study their role in tendon function and development. Tendon properties from control mice and both types of knockout mice were characterized. Because of the reported roles of interleukin-6 and interleukin-4 in inflammation and cellular activity, we hypothesize that IL6 -/- mice will display more organized collagen orientation and greater cross-sectional area and mechanical properties when compared to that of control mice. In addition, IL4 -/- mice will display the most disorganized collagen orientation and lowest cross-sectional area and mechanical properties.

2. Materials and methods

Studies were approved by the University of Pennsylvania IACUC. Twelve mice from each of the three groups of mice, C57BL/6 control (CTL), C57BL/6J – $IL6^{tm1Kopf}$ (IL6 -/-), C57BL/6J-$IL4^{tm1Cgn}$ (IL4 -/-) were included in this study (The Jackson Laboratory, Bar Harbor, ME). For all three groups, mice were sacrificed at 10 weeks of age by CO_2 inhalation, weighed, and their tendons were designated for either organizational or biomechanical assessment. Specimens were stored at $-20^{\circ}C$ and thawed at room temperature before dissection. The patellar tendon was chosen for this study not only because of its geometry and superficial location, but it is also proximally and distally inserted into the patella and tibia respectively, which facilitated mechanical testing of the small mouse tendon.

For organizational analysis, four mice from each group were randomly selected, and the right patellar tendons were dissected free from the patella and tibia and processed with standard histological techniques. Sections of 7 μm were cut parallel to the tendon fibers, stained with hematoxylin and eosin, and analyzed using a quantitative polarized light microscopy method as previously described (Thomopoulos et al., 2003; Gimbel et al., 2003). Briefly, using a green bandpass filter (BP 546 nm), grayscale images of the tendon were taken at 5° increments with crossed analyzer and polarizer and simultaneously rotated through 90°. Subsequently, the filter was removed and a rotatable λ compensator was rotated through 90° along with crossed analyzer and polarizer. Custom-designed software was then utilized to determine collagen fiber orientations and to generate histograms of collagen fiber distributions, and the angular deviation of the collagen orientations, a measure of fiber distribution spread, was calculated. Collagen fiber distributions were statistically compared using a Chi-squared method to test goodness of fit with a circular statistics package (Oriana version 1.06). Angular deviations within each of the three groups were averaged, and a one-way ANOVA followed by Fisher's post-hoc test was used to evaluate differences between groups with statistical significance set at $\alpha = 0.05$. A trend was defined at $\alpha < 0.1$.

For geometric and biomechanical analysis, 12 mice from each group were designated, and the left patellar tendons were dissected and cleaned, leaving only the patella, patellar tendon, and tibia as one unit. The central area of interest in the patellar tendon was prepared as a standardized dumbbell-shaped specimen using well-established techniques (Soslowsky et al., 2000). Two Verhoeff stain lines were placed on either end of the dumbbell shape on the tendon (Fig. 1). Tendon width and thickness were then quantified, and cross-sectional area was calculated as the product of the two. Tendon width was measured using an optically based image processing system, and tendon thickness was quantified by lowering an indenter probe attached to a high resolution linear variable differential transformer used previously in our lab (Soslowsky et al., 1994). The tibia was then embedded in polymethylmethacrylate in a custom-designed fixture and secured in place with a metal pin. The patellar was held in place with a

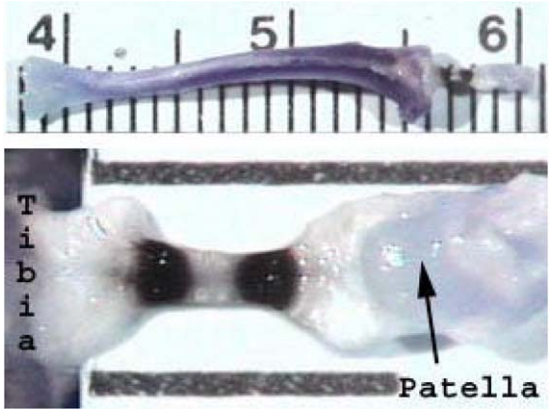

Fig. 1. Testing unit comprised of patella, patellar tendon, and tibia (top). Magnified picture of dumbbell-shape stamped patellar tendon with Verhoeff stain lines (bottom). Ruler in both pictures is in millimeters.