THE LOCALIZATION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN A REPETITIVELY LOADED TENDON IN VIVO: AN IMMUNOHISTOLOGICAL STUDY

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INTRODUCTION:

Vascular Endothelial Growth Factor (VEGF) is one of the most important angiogenic components of tissue healing. I*n vitro* and *in vivo* studies have shown that VEGF is responsible for stimulating the proliferation of microvascular endothelial cells, inducing angiogenesis and rendering the microvasculature hyperpermeable.[1,2] In the tendon, expression of VEGF can be up-regulated by both mechanical and biochemical stimuli that includes hypoxia and the presence of other growth factors.[3,4] Recent studies have shown that in the injured tendon, the highest levels of VEGF occur after inflammation[5] when it acts as a potent stimulator of angiogenesis. The ingrowth of new blood vessels towards the repair site from within the healing tendon appears necessary for healing to occur.[5]

Currently, little is known about the role that VEGF and other growth factors play in tendon loading and injury. Elucidating the cellular and molecular pathways that occur during this period may lead to a better understanding of mechanisms behind tendon healing and remodeling. Our laboratory has developed a rabbit model of epicondylitis in which the flexor digitorum profundus (FDP) muscle is repeatedly stimulated against a load. The purpose of this study was to evaluate the regional variation of VEGF staining cells in the FDP tendon at the epicondyle in response to repetitive loading.

METHODS:

Eight female, New Zealand White Rabbits weighing 2.45 kg (± 0.35) were used. Under general anesthesia, the FDP muscle on one limb was electrically stimulated to contract repetitively for 2 hours per day, 3 days a week, for 10 weeks (60h of cumulative loading). The contralateral limb, although restrained in the same manner as the loaded limb, did not receive a stimulus and as a result served as the control. This study was approved by the University's Committee on Animal Research.

After inducing anesthesia with isoflurane, the rabbit was placed in a supine position with the forearms loosely secured to supports. A muscle stimulation needle (33G) was inserted subcutaneously in the middle forearm region over the central region of the FDP and the needle tip was pushed back through the skin. A needle was similarly inserted in the contralateral limb, but was not stimulated. A fingertip glove was slipped over digit 3 and connected to a load cell to measure flexion force of the finger about the metacarpophalangeal joint. The muscle was stimulated (Grass) with a train of pulses at 1Hz, with a train duration of 200ms and a pulse rate of 100 pulses/s. The stimulation voltage was adjusted [6-12V] to maintain a peak fingertip force of 0.42N (15% of P_o). After two hours of repetitive loading, the stimulation electrode and fingertip glove were removed.

At 10wks, the animals were euthanized and the epicondyles were dissected with the FDP tendon and muscle attached. Samples were fixed, decalcified and sectioned. Serial sections were analyzed by immunohistochemistry for the presence of VEGF using a mouse monoclonal antibody directed against VEGF (2μg/ml) and then incubated with a biotinylated horse anti-mouse 2° antibody. Sections were stained with the Vectastain ABC system, and developed with DAB. Six areas of interest were digitally photographed at 20x: 3 areas along the enthesis (classified as inner, center and outer) and 3 corresponding areas 1500μm distal to the tidemark (TM). The inner area is that part nearest the bone. Positive staining cells were manually counted in each region ($300x300\mu m^2$) and normalized by the area observed (Figure 1). Tissue preparation and cell counting was performed blinded.

RESULTS:

Examinations of the wrist, forearm and elbow revealed no limping, reduction in gross claw flexion strength, or skin breaks.. Mean rabbit weight at the end of the study was 3.15 kg (± 0.30). Samples from 3 animals were eliminated due to low section yield.

The data was analyzed using a paired t-test. With the exception of the outer region of the enthesis, all areas had significantly higher densities of VEGF staining cells when compared to the control limb ($p < 0.043$). The greatest differences were observed at the outer and inner region of the tendon 1500μm from the enthesis (Table 1). No significant differences were found between control and loaded limbs ($p < 0.107$) at the outer region of the enthesis.

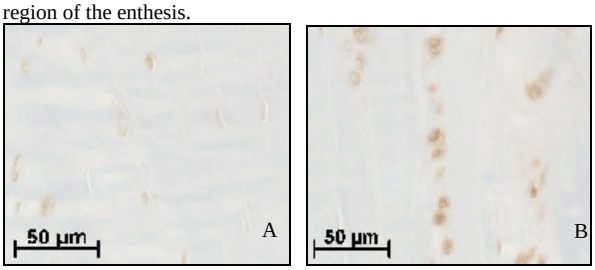

Figure1A. Control specimen with few positive staining cells. **B**. Loaded specimen showing dark, cellular staining within tendon.

	Differences in the Means (cells per mm^2)					
	Enthesis			1500μm from Enthesis		
Rabbit	Inner*	Center*	Outer	Inner*	Center*	Outer*
1	n/a	114	-95	749	446	987
2	515	386	586	105	204	1405
3	329	86	33	380	331	585
4	94	222	405	673	189	220
5	572	373	377	253	230	198
mean	378	236	261	432	280	679

Table 1. Differences in the mean number of cells per mm^2 between loaded and control limbs within each matched pair. *Indicates a significant difference ($p < 0.043$).

DISCUSSION:

This in vivo model demonstrates that the density of cells staining with VEGF is increased in tendons experiencing cyclical loading in comparison to control limbs. Although this trend was observed at all 6 regions of interest at the epicondyle, significant findings occurred at only 5 regions. A limitation of the study was the small sample size.

The FDP tendon experiences both tension and compression as it wraps around the epicondyle and relative differences in the two types of loads may explain regional variation in protein production. In this study, the greatest difference in cell density was observed at the outer region distal to the enthesis, a region where the dominant load is in tension.

These results suggest that the angiogenesis observed in tendon biopsies of patients with epicondylitis may by mediated by VEGF. Further studies may investigate regional differences and determine the relative roles of tension, compression, or other factors (e.g., microtrauma, strain) in increasing VEGF.

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