Modulation of Vascular Response after Injury in the Rat Achilles Tendon Alters Healing Capacity

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Introduction

Although vascular ingrowth is necessary for tendon healing, hypervascularization following tendon injury is not always considered beneficial[1] as, for example, degenerated tendons are also highly vascularized. We demonstrated that delivery of VEGF and anti-VEGF antibody locally to tendons can increase and decrease the vascular response after injury, respectively[2]. However, the effect of altering the vascular response after healing in the tendon is unknown. Therefore, the objective of this study was to define the alterations to tendon healing following injection of angiogenic factors. We hypothesize that reducing the vascular response will result in reduced scar tissue formation and reduced failure properties while increasing the vascular response will result in the opposite. Further, we hypothesize that in vivo gait and joint functional measures will not be significantly impacted by vascularity changes.

Methods

Study Design: 90 Fischer 344 rats (4 months old, IACUC approved) underwent a bilateral Achilles incisional injury, followed by local injections of vascular endothelial growth factor (VEGF) (Peprotech), anti-VEGF antibody (B20.4-1-1, Genentech), or saline (SAL). In vivo functional measures and ultrasound imaging were performed, and animals were sacrificed at 7, 14, and 28 days after injury. *Injury:* A 1.5mm incisional injury in the Achilles tendon mid-substance was created without repair. *Injections:* On days 4-6 after surgery, each animal received either 5μg VEGF or 250μg anti-VEGF antibody (B20) in 20μl saline, or 20μl saline only, injected bilaterally intratendinously. *Imaging:* Imaging was performed on days 7, 14, 21, and 28 (n=12/group) after injury using a Vevo LAZR ultrasound system (MS550D and MS250 transducers, VisualSonics). Animals were anesthetized and positioned with the transducer parallel to the tendon long axis. For contrast-enhanced ultrasound imaging, a 200 sec ultrasound clip was initiated at the start of a bolus injection of 100μl Definity (Lantheus Medical Imaging) microbubble contrast agent. The echo-power vs. time data was fit to a perfusion model[3]. Color Doppler images were taken to quantify percent area of signal and blood flow velocity measures[4]. Photoacoustic images were taken at wavelengths of 750 and 850 nm based on the absorption spectrum of oxygenated and deoxygenated hemoglobin, respectively[5]. *In Vivo Measures:* Gait, passive ankle joint range of motion (ROM), and stiffness measures were evaluated on days 7, 10, and 14 (n=12/group). *Histology:* Tendon sections from 7, 14, and 28 days after injury (n=6/group) were stained with hematoxylin-eosin (H&E) and graded for cell shape (1=spindle to 3=round) and cellularity (1=less cells to 3=more cells), and stained for CD34 and graded for vessel density (1=less to 4=more dense) and vessel size (1=small to 4=large diameter). *Mechanics:* Tendons from 14 and 28 days after injury (n=12/group) were prepared for tensile testing with preconditioning, stress-relaxation, frequency sweeps at 0.1, 1, 5, and 10 Hz, and a ramp to failure at 0.1% strain/sec. *Statistics:* Ultrasound, mechanical, and functional measures were analyzed using Student's t-tests, and histology analyzed using Mann-Whitney t- tests, all with Bonferroni corrections. Significance was set at $p<0.05$ and trends at $p<0.1$, and all comparisons were made to saline control.

Results

Ultrasound: The B20 group demonstrated a decrease in contrast peak enhancement, wash-in rate, and wash-in perfusion index at day 14. When evaluating only the injury area, this group also had a decreased wash-in area under the curve at day 14 and decreased rise time at day 28 (Fig1A,B). The VEGF group showed no changes when evaluating the whole tendon, but an increase in rise time (Fig1A) at days 7 and 14 and a decrease in wash-in rate at day 7 in the injury region. There was a decrease in the Doppler mean color level at day 14 (Fig1C), corresponding to blood flow velocity, but an increase at day 21 in this group. Similarly, there was a decrease in Doppler fractional area (Fig1D) and color weighted fractional area in the B20 group in both the whole tendon and at the injury site at 7 and 14 days, but an increase in these properties at day 21 in the whole tendon. Finally, mean color level and fractional area increased in the VEGF group in the whole tendon at day 21 (Fig1C,D). Photoacoustics imaging (data not shown) revealed an increase in blood oxygenation in the B20 group at day

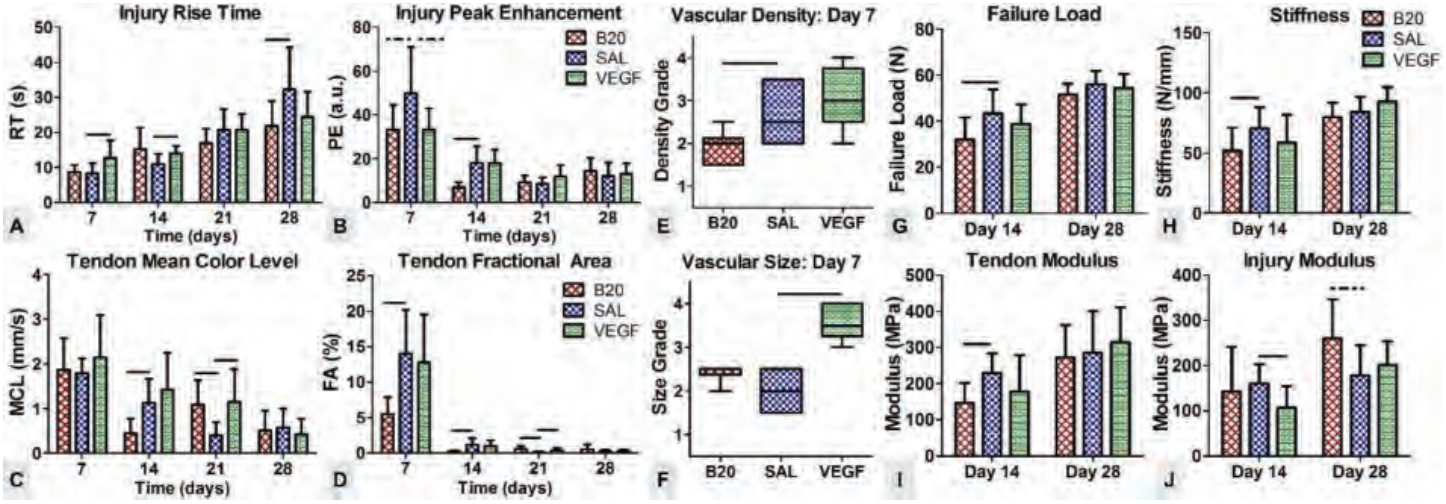

Figure 1: Contrast-enhanced ultrasound showing **(A)** rise time increased with VEGF and **(B)** peak enhancement decreased with B20. Doppler ultrasound **(C)** mean color level and **(D)** fractional area. Histological measures of CD34 showing **(E)** decreased vascular density with B20 and increased vascular size with VEGF. Mechanical measures of **(G)** failure load, **(H)** stiffness, **(I)** tendon modulus, and **(J)** injury modulus showing overall changes at early time points.

21. There was an increase in tendon tissue oxygenation level at day 28 in the B20 group, but no other differences. *In Vivo Measures:* There were no changes in the passive joint stiffness, range of motion, or gait analyses between groups (data not shown). *Histology:* CD34 staining for vascular endothelial cells demonstrated a decrease in vascular density in the B20 group at days 7 (Fig1E) and 14. Additionally, there was an increase in vascular size in the VEGF group at day 7 (Fig1F). There were no significant changes in H&E histology. *Mechanics:* The B20 group demonstrated a decrease in failure load (Fig1G), max stress, stiffness (Fig1H), and tendon modulus (Fig1I) at day 14. The VEGF group had decreased modulus at the site of injury (Fig1J) and % relaxation, but no changes in tendon quasi-static properties. There were no differences in cross-sectional area, dynamic modulus, or Tan(δ) in either group. At day 28 after injury, there were no differences for either group in any parameter compared to saline.

Discussion

Alterations in vascular response after injury impacted healing outcome in the rat Achilles tendon. Decreases in all ultrasound measures of vascularity with the delivery of the anti-VEGF antibody were supported by a decrease in histological measures of vessel density at early time points. The decrease in vascularity caused a reduction in mechanical properties 14 days after injury. However, all negative mechanical changes returned to control levels by day 28. Additionally, tissue oxygenation, as well as the Doppler fractional area and blood flow velocity, were increased in this group at late time points, which could explain the improvements in mechanics. Surprisingly, we found only mild changes in our ultrasound and mechanics data with the delivery of VEGF. We found an increase in vessel size, but no change in vessel density from our histology. While we previously demonstrated that VEGF increased the vascular response after injury, these smaller changes could be due to the use of younger animals, potentially with an already robust vascular response to injury. It is also possible that our ultrasound measures are more sensitive to changes in vessel density than vessel size. Ongoing work includes implementing these vascular modifications on aged animals, which could yield larger changes in the case of VEGF delivery.

Significance

A decrease in vascular response after injury reduced mechanical outcome in early healing, which recovered over time. Future studies will evaluate the effect of vascular modulation after injury with aging to potentially determine therapeutics for improved tendon healing in this population.

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