Effect of Pro- and Anti-Angiogenic Factors on Vascular Response in the Rat Achilles Tendon after Injury

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Introduction

Tendons are hypovascular tissues that become hypervascular after injury. While vascular ingrowth is necessary for tendon healing, hypervascularization following tendon injury is not always believed to be beneficial[1], as degenerated tendons are also highly vascularized[2]. Modulating the vascular response during healing could ultimately improve tendon healing. However, a method for vascular modulation, as well as the optimal vascular response during tendon healing, is unknown. Therefore, the objective of this study was to evaluate the effects of delivery of both pro- and anti-angiogenic factors on the rat Achilles tendon vascular response after injury using in vivo ultrasound imaging and ex vivo histological measures. We hypothesized that vessel properties such as vessel density, vessel size, and blood flow velocity will be increased due to the pro-angiogenic factor and decreased due to the anti-angiogenic factor.

Materials & Methods

Study Design

Under IACUC approval, 56 Sprague Dawley rats were used. All animals underwent a bilateral Achilles incisional injury, followed by injections of VEGF, anti-VEGF, or saline on 3 consecutive days. Ultrasound imaging was performed on days 7, 10, and 14 after injury and photoacoustic imaging was done for the anti-VEGF groups on days 7 and 14. Animals were sacrificed at either day 7 or 14 for histological evaluation.

Surgical Protocol

A 1.5mm scalpel blade created a mid-substance incisional injury in the center of the Achilles tendon width and the tendon was left unrepaired.

Angiogenic Factor Injections

To evaluate pro-angiogenic factor delivery, 5ug VEGF in 20ul saline (or 20ul saline only as control) was injected bilaterally intratendinously on either days 0-2 (early) or 4-6 (late) after surgery. To evaluate anti-angiogenic factor delivery, 50, 250, or 500ug anti-VEGF antibody (B20.4-1-1, Genentech) in 30ul saline was injected bilaterally intratendinously on days 4-6 after surgery (or 30ul saline as control).

Color Doppler Imaging

Imaging (n = 4-8) was performed using a Vevo 2100 ultrasound system (VisualSonics) with a 40 MHz transducer. Animals were anesthetized and positioned with the transducer parallel to the long axis of the tendon. The mean color level (MCL—average blood flow velocity), the fractional area (FA—% area of Doppler signal), and the color weighted fractional area (CWFA—weighted average of blood flow velocity/unit area) were quantified over the tendon area. Data was compared using a 2-way (treatment, time) ANOVA followed by post hoc t-tests.

Photoacoustic Imaging

Photoacoustic imaging (n=6-8) was performed with the Vevo LAZR Photoacoustics Imaging System (VisualSonics) using the same transducer and positioning. Images were taken at two wavelengths (750 and 850 nm) based on the absorption spectrum of oxygenated (HbO_2) and deoxygenated hemoglobin (Hb), respectively. Blood oxygenation (sO_2 Avg), total hemoglobin (Hb Total), and relative tissue oxygenation (sO_2 Tot) were determined. Again, data was compared using a 2-way ANOVA followed by t-tests.

Histological Analysis

After sacrifice, Achilles tendons were dissected and processed. Sections were stained with hematoxylin-eosin (H&E) and graded by 3 blinded, independent graders for cell shape (1 = spindle to 3 = round) and cellularity (1 = less cells to 3 = more cells). Additionally, sections underwent immunohistological staining for CD34, a vascular marker, and graded by 3 blinded, independent graders for vessel density (1 = less dense to 4 = more dense) and vessel size (1 = small diameter to 4 = large diameter). Data was compared using Mann-Whitney t-tests (n=4-8).

Results

VEGF Delivery

There was a significant increase in FA at days 7 and 14 in the late group and a trend towards a decrease in FA at day 14 in the early group when

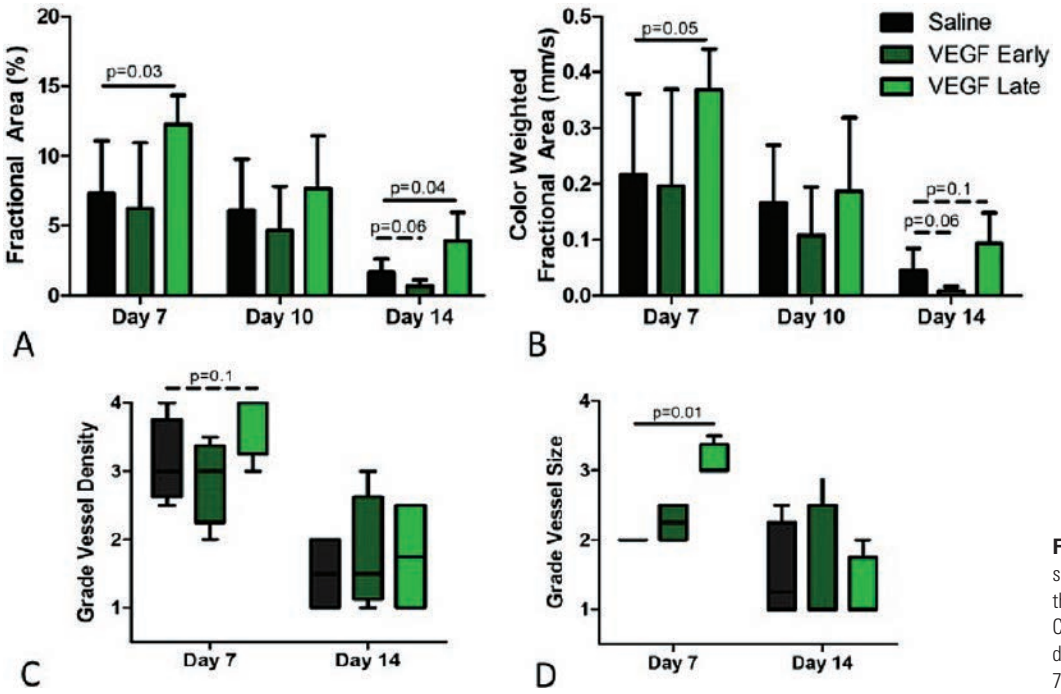

Figure 1. Color Doppler analysis of VEGF delivery showed an increase in **(A)** FA and **(B)** CWFA in the late group at days 7 and 14 compared to saline. CD34 staining showed an increase in **(C)** vessel density and **(D)** vessel size in the late group at day 7 compared to saline.

compared to saline (Figure 1A). There were no changes in MCL (not shown). There was a significant increase in CWFA in the late group at day 7, a trending increase in the late group, and a trending decrease in the early group at day 14 compared to saline (Figure 1B). Histology shows a increasing trend in vessel density (Figure 1C), a significant increase in vessel size (Figure 1D), and significantly more rounded cell shape (not shown) in the late group compared to saline. There were no changes in cellularity (not shown).

Anti-VEGF Delivery

There was a significant decrease in FA on days 7 and 10 in the mid B20 group, and a significant increase in the low B20 group compared to saline (Figure 2A). There were no differences in MCL (not shown). There was a trending decrease in CWFA in the mid B20 group at day 7, a significant decrease in the mid B20 group at day 10, and a significant increase in the low B20 group at day 14 compared to saline (Figure 2B). There were trending and significant decreases in Hb total

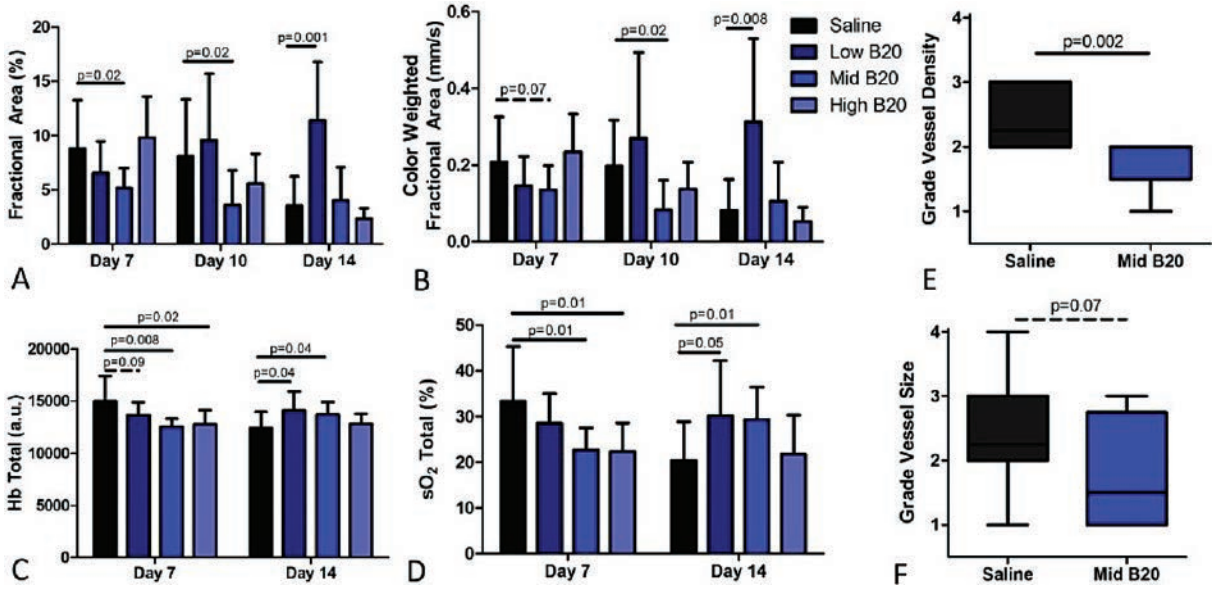

Figure 2. Color Doppler analysis of B20 delivery showed a decrease in **(A)** FA and **(B)** CWFA in the mid B20 group compared to saline on days 7 and 10. Photoacoustic analysis shows a decrease in **(C)** Hb Total and **(D)** sO2 Total in the mid and high B20 groups at day 7 compared to saline. CD34 staining on day 7 demonstrated a decrease in **(E)** vessel density and a trend towards a decrease in **(F)** vessel size in the mid B20 group.

in the B20 groups at day 7, and a significant increase at day 14 in the low and mid B20 groups (Figure 2C). There were no changes in sO_2 avg (not shown). There was a significant decrease in sO_2 total in the mid and high B20 groups on day 7, and a significant increase in the low and mid B20 groups on day 14 (Figure 2D). Finally, there was a significant decrease in vessel density (Figure 2E), and a trending decrease in vessel size (Figure 2F) in the mid B20 group on day 7, with no change in cell shape or cellularity (not shown).

Discussion

This study demonstrated that tendon vascular response after injury could be increased through the delivery of VEGF and decreased through delivery of anti-VEGF. Importantly, both dosage and timing are important factors in regulating the vascular response. The delivery of VEGF was only effective when delivered 4-6 days after injury, during the time when VEGF expression is naturally at a peak[3]. Additionally, the increase in vascularity seen with the delayed VEGF delivery coincided with a more rounded cell shape, suggesting a more active cellular state. When delivered early, vascular response was not increased, and trended toward a decrease at day 14, suggesting that this delivery may have shifted the VEGF expression time period earlier than normal. For delivery of anti-VEGF, the largest reduction in the vascular response was with the mid dosage. The lower dosage caused a compensation effect, with increased vascular measures at later time points. Histological measures of vascular size and density supported the changes seen with ultrasound.

Conclusions

This study establishes a model system for vascular modulation in a rat tendon injury model that can be used to evaluate the role of vascularity in tendon injury or degeneration, and potentially determine therapeutics for improved tendon healing.

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