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Combined reflectance and Raman spectroscopy to assess degree of *in vivo* angiogenesis after tissue injury

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ABSTRACT

Background: Angiogenesis, the formation of blood vessels, is a critical aspect of wound healing. Disorders of wound healing are often characterized by lack of angiogenesis, a condition frequently observed in aging and diabetic patients. Current techniques for assessing blood at injury sites are limited to contrast-imaging, including angiography. However, these techniques do not directly observe oxygenation of blood and are not amenable to serial evaluation. A multimodal noninvasive reflectance and Raman spectrometer have been proposed to help clinicians as a point-of-care tool to interrogate local angiogenesis and tissue architecture, respectively. The spectrometer system is a rapid, noninvasive, and label-free technology well-suited for the clinical environment.

Materials and methods: To demonstrate feasibility, the spectrometer system was used to interrogate angiogenesis serially over 9 wk as a result of heterotopic ossification (HO) development in a validated murine model. End-stage HO was confirmed by micro-computed tomography.

Results: Our preliminary results suggest that reflectance spectroscopy can be used to delineate vessel formation and that pathologic wounds may be characterized by unique spectra. In our model, HO formed at sites 1-3, whereas sites 4 and 5 did not have radiographic evidence of HO.

Conclusions: A point-of-care system like that demonstrated here shows potential as a noninvasive tool to assess local angiogenesis and tissue architecture that may allow for timely intervention in a clinical setting.

Introduction

Heterotopic ossification (HO) is a condition of pathologic wound healing in which patients develop ectopic osseous lesions after trauma.[1-3] Patients at risk for HO include those with large surface-area burns and severe musculoskeletal injury. These patients are exposed to massive inflammatory insults, which lead to pathologic cellular differentiation,

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cartilage formation, and ossification. These ectopic lesions are often painful, can lead to new wounds, inhibit joint motion, and may require surgical excision of tissue. After surgical excision, patients are at risk for recurrence due to the resurgence of local inflammation.[4] Furthermore, there exist no early detection or adequate treatment options today for a process closely associated with dynamic changes in local vasculature.

Soft-tissue wound healing is another commonly encountered clinical picture intimately reliant on vascular adaptation. Although early treatment would be beneficial to prevent the chronic effects associated with poor angiogenesis, diagnostic methods are limited.[5,6] Current techniques for real-time vascular imaging are limited to contrast-based techniques including vascular angiography.[6,7] These techniques are often invasive, resource-intensive, and impractical for serial imaging. Noninvasive spectroscopy, including reflectance and Raman spectroscopy, has emerged as a tool to image vessels at the bedside.[8] Reflectance spectroscopy assesses oxygenated and deoxygenated hemoglobin present in local vessels due to variations in the absorption spectra of the respective forms of hemoglobin. Raman spectroscopy assesses the chemical fingerprint of a sample. Clinical applications of reflectance spectroscopy include analyzing malignant tissue[9] and tissue oxygenation.[10,11] Clinical applications of Raman spectroscopy include assessment of bone quality[12] and HO.[13]

We have previously reported the use of Raman spectroscopy to identify changes in the extracellular matrix associated with HO[13-15] and calciphylaxis.[16] However, this technique is limited to the detection of suspicious calcification and does not focus on angiogenesis. Tools to image blood vessels would have utility not only for identifying changes associated with HO but also for evaluating other defects of angiogenesis related to poor wound healing.

Materials and methods

Here, we employ micro-computed tomography (microCT), Raman spectroscopy, and reflectance spectroscopy to assess serial wound healing in a validated mouse model of HO. This study was approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC; Protocol # 00005909). Two experimental groups were evaluated—mice received either tendon transection alone ($n = 2$) or dorsal burn with tendon transection ($n = 2$) as previously described.[14] For all comparisons, the experimental group was the leg that underwent tendon transection, and the control group was the contralateral leg. For the purposes of this study, tendon transection and dorsal burn with tendon transection were combined ($n = 4$) as to compare injured *versus* uninjured tissue. Spectroscopy measurements were taken at five prespecified sites along the injured and uninjured posterior hindlimb of each mouse (Fig. 1A). Measurements were obtained immediately before surgery as baseline (T0), immediately after surgery (T1), 1 wk after surgery (T2), 3 wk after surgery (T3), 6 wk after surgery (T4), or 9 wk after surgery (T5). microCT imaging was then performed to confirm the presence or absence of HO 9 wk after injury. microCT imaging showed characteristic presence of radiographically evident HO corresponding to sites 1, 2, and 3 (Fig. 1B).

Spectra were collected with a hand-held filtered N-around-1 fiber optic probe (EMVision, Loxahatchee, FL; interrogating a tissue volume <1 mm^3 connected to a portable Raman spectroscopy system [Rxn 1, Kaiser Optical Systems; 785 nm laser]) operated with 6-8 cm^{-1} resolution. The reflectance measurements were conducted using an unfiltered 1:1 probe connected to a portable spectrometer (USB2000 + coated for visible/near-infrared wavelengths, Ocean Optics; HL-2000-FHSA, Ocean Optics) operated with <2 nm resolution between 350 and 800 nm.

To produce high-quality spectra, the fiber-probe system was operated with 60 s integration time for Raman spectra and 5 s integration time for reflectance spectra. In principle, measurement time could be reduced to 1-3 s for Raman and <1 s for reflectance collections. Such collection times could enable more thorough mapping, but scanning a relatively large region of interest remains difficult with a fiber-probe system. Spectra were preprocessed as previously described.[17] Before analysis, Raman spectra were normalized to the 1001 cm^{-1} phenylalanine band, and reflectance spectra were normalized to their peak. Principal components analysis was performed with built-in MATLAB functions. Data were

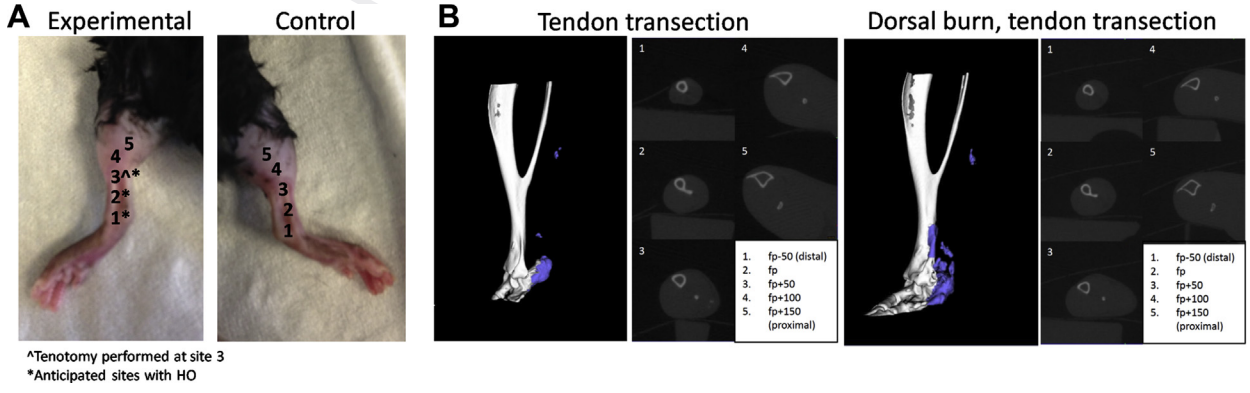

Fig. 1 – Study design, including (A) measurement sites and (B) microCT results. Note that the blue indicates HO, corresponding to anticipated sites of HO in (A). (Color version of figure is available online.)