The American Journal of Pathology, Vol. ■, No. ■, ■ 2016

ELSEVIER

The American Journal of PATHOLOGY

ajp.amjpathol.org

Near-Infrared Optical Imaging Noninvasively Detects Acutely Damaged Muscle

Q37 Stephen M. Chrzanowski,* Abhinandan Batra,† Brittany A. Lee-McMullen,‡ Ravneet S. Vohra,* Sean C. Forbes,† Huabei Jiang,§ Q1 Krista Vandenborne,† and Glenn A. Walter*

From the Departments of Physiology and Functional Genomics, Physical Therapy,† Biochemistry,‡ and Biomedical Engineering,§ University of Florida, Gainesville, Florida*

Accepted for publication June 8, 2016.

Address correspondence to Glenn A. Walter, 1600 S.W. Archer Rd., M552 P.O. Box 100274, Gainesville, FL 32610-0274. E-mail: glennw@ufl.edu.

Muscle damage is currently assessed through methods such as muscle biopsy, serum biomarkers, functional testing, and imaging procedures, each with its own inherent limitations, and a pressing need for a safe, repeatable, inexpensive, and noninvasive modality to assess the state of muscle health remains. Our aim was to develop and assess near-infrared (NIR) optical imaging as a novel noninvasive method of detecting and quantifying muscle damage. An immobilization—reambulation model was used for inducing muscle damage and recovery in the lower hindlimbs in mice. Confirmation of muscle damage was obtained using *in vivo* indocyanine green—enhanced NIR optical imaging, magnetic resonance imaging, and *ex vivo* tissue analysis. The soleus of the immobilized—reambulated hindlimb was found to have a greater amount of muscle damage compared to that in the contralateral non-immobilized limb, confirmed by *in vivo* indocyanine green—enhanced NIR optical imaging (3.86-fold increase in radiant efficiency), magnetic resonance imaging (1.41-fold increase in T_2), and an *ex vivo* spectrophotometric assay of indocyanine green uptake (1.87-fold increase in normalized absorbance). Contrast-enhanced NIR optical imaging provides a safe, sensitive, rapid, and noninvasive screening method that can be used for imaging and quantifying muscle damage and recovery *in vivo*. *(Am J Pathol 2016, ■: 1—9; http://dx.doi.org/10.1016/j.ajpath.2016.06.019)*

Q9 Techniques of Assessing Muscle Damage

Q10 Muscle damage is an important and unavoidable outcome of many pathologic states, such as muscular dystrophies, inflammatory myopathies, and physical trauma. Several preclinical models have been developed to induce acute muscle damage, including eccentric loading,[1—5] immobilization-reloading,[6] and myotoxin injection.[7—10] In particular, reloading of muscle following unloading has demonstrated an ability to robustly induce eccentric loading muscle damage.[4—6,11] Reloading, in the form of reambulation following immobilization, has demonstrated ultrastructural perturbations to muscle consistent with muscle damage.[6,11—13] Compromised sarcolemmal membranes release muscle enzymes such as creatine kinase while passively taking up large serum proteins and markers such as Evans blue dye (EBD).[14]

Muscle pathology has been measured by a number of techniques, all of which possess their own inherent limitations. These techniques include muscle biopsy, serology, functional measures, and imaging methods. Muscle biopsy, although the most direct measure of pathology, has a capacity too limited to be considered a longitudinal measure of muscle pathology due to the necessity of repeated sample collections. Although serology and functional testing provide a proxy to the overall state of muscle health, they fail to sensitively localize pathology, instead providing information regarding the general health of all muscles in the body, and are complicated by the changes in lean body mass typically associated with myopathy. Magnetic resonance imaging (MRI) has evolved Q11

Supported by the US Department of Defense project W81XWH-12-1-0387, T32 Neuromuscular Plasticity Training Program project HD043730 (K.V.), and T32 Hypertension Center project HL083810 (Wood). Q2 Q3 Q4 Q5

Research was performed at the University of Florida, Gainesville, FL.

Disclosures: None declared.

http://dx.doi.org/10.1016/j.ajpath.2016.06.019

as a noninvasive method of detecting and quantifying muscle pathology[6,15–19] but has several limitations, such as cost; speed of operations; and contraindications in subjects with metallic implants, claustrophobia, and compliance issues.[20] An attractive possible alternative would be the use of clinically approved fluorescent optical contrast agents for imaging muscle damage *in vivo*, similar to those currently used for conventional histologic measurements.[21–23]

Near-Infrared Imaging and Indocyanine Green

In preclinical models of disease, fluorescent optical imaging is a technique widely used for detecting pathology by fluorescent dyes, proteins, and conjugates.[24,25] By using optical imaging in the near-infrared (NIR) range (700 to 1000 nm), two primary advantages exist over conventional fluorophores that operate at shorter wavelengths: deeper photon penetration within tissues and minimal tissue autofluorescence.[24,26,27] When imaging in the NIR range, penetration of signal can overcome some of the scattering encountered with other fluorescent imaging techniques at shorter wavelengths.[28]

The first, and still the only, NIR fluorescent contrast agent approved by the US Food and Drug Administration (FDA) is indocyanine green (ICG).[29] ICG is rapidly bound to albumin within the circulation and thus acts as a blood-pooling NIR fluorescent agent, highlighting vasculature in several systems.[30,31] Furthermore, through the enhanced permeation and retention effect, ICG passively accumulates in tumors, in a manner parallel to that of the MR contrast agent gadolinium.[32,33] Despite its widespread use in imaging other organs, only conjugates of ICG have demonstrated the capability of imaging compromised muscle.[22] With this in mind, we hypothesized that clinical-grade ICG will behave similarly to EBD,[14] and accumulate in damaged muscle fibers, allowing for the quantification of muscle damage in a longitudinal and *in vivo* manner. Importantly, because access to preclinical fluorescent imaging is more ubiquitous than is access to MRI, ICG-enhanced optical imaging may be a suitable complement to more expensive and time-consuming MRI scanners in preclinical muscle pathology studies. In this article we assessed whether ICG-enhanced NIR optical imaging is capable of measuring acute muscle damage and recovery in a targeted deep hindlimb muscle in mice. To validate the NIR optical imaging findings, direct comparisons to MR measures of muscle damage and *ex vivo* tissue assessments were performed.

Materials and Methods

Animal Experimental Protocol

All studies were approved by the University of Florida's institutional animal care and use committee. Male C57BL/6J mice ($n = 60$) were bred in-house through the University of Florida's Animal Care Services and were 6 to 8 weeks of age during experimentation. Mice were housed in a facility regulated by the Association for Assessment and Accreditation of Laboratory Animal Care (12 hours light/dark, 40°C, 42% humidity) and provided food *ad libitum*. Additionally, a transgenic dough diet (BioServ, Flemington, NJ) was provided for the mice at the base of the cages during the entire procedure to ensure that dietary needs were met during and after hindlimb immobilization. Right hindlimbs were immobilized in a plantar-flexed position, first by medical-grade paper tape, followed by plaster of Paris (OrthoTape, Blufton, SC), and finally an encompassing single layer of casting material (Patterson Medical, Warrenville, IL), as previously described.[6,16] The contralateral leg (nonimmobilized) served as each mouse's own control. Mice were checked daily for abrasion wounds as a result of the casting procedure, and animal weight was monitored. After 2 weeks of immobilization, casts were removed, and the animals were allowed to undergo free-cage ambulation. Data (MRI, NIR optical imaging, and tissue assessment) were acquired at 0, 1, 2, 3, 5, and 7 days following the removal of casts ($n = 10$ per time point). Eighteen hours before sacrifice, 1% filter-sterilized EBD (Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (0.1 g/mL per mg i.p.) was administered to the mice as previously described.[14]

Near-Infrared Optical Imaging

One hour before NIR optical imaging, NirawaveC ICG (Miltenyi Biotech Inc., San Diego, CA) was administered to the mice according to the package insert (1 mg ICG/kg body weight i.v.). It was determined that a steady signal was maintained between 30 minutes and 3 hours after injection (data not shown); thus, NIR optical imaging data were collected during this window of time following injections of ICG contrast dye. Mice were anesthetized using an oxygen and isoflurane mixture (3% induction; 0.75% to 1% maintenance) and two-dimensional NIR optical imaging was performed using an *In Vivo* Fluorescence Imager (PerkinElmer, Waltham, MA). Image capture took, on average, <1 minute/mouse. Acquired images were analyzed using Living Image software (PerkinElmer) on the same *In Vivo* Fluorescence Imager. Data acquisition was controlled for binning, F/stop, subject height, and field of view. Additionally, normalization to the exposure time of the camera was manually performed for each image. Excitation and emission wavelengths were 745 and 820 nm, respectively. Regions of interest were carefully drawn over both the immobilized and nonimmobilized lower hindlimbs, and total radiant efficiency $(p/cm^2/sr)/(\mu W/cm^2)$ within the designated regions of interest was recorded by the *In Vivo* Fluorescence Imager.

Magnetic Resonance Imaging and Spectroscopy

MRI was performed in a 4.7 T horizontal 22.5-cm bore magnet (Agilent, Santa Clara, CA) immediately following

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