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Original Full Length Article

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ABSTRACT

Over 60% of our severely combat-injured patient population develops radiographically apparent heterotopic ossification. Nearly a third of these require surgical excision of symptomatic lesions, a procedure that is fraught with complications, and delays or regresses functional rehabilitation in many cases. Unfortunately, for the combat injured, medical contraindications and logistical limitations limit widespread use of conventional means of primary prophylaxis. Better means of risk stratification are needed to both mitigate the risk of current means of primary prophylaxis as well as to evaluate novel preventive strategies currently in development. We asked whether Raman spectral changes, measured ex vivo, correlated with histologic evidence of the earliest signs of HO formation using tissue biopsies from the wounds of combat casualties. In doing so, we compared normal muscle tissue to injured muscle tissue, unmineralized HO tissue, and mineralized HO tissue. The Raman spectra of these tissues demonstrate clear differences in the amide I and amide III spectral regions of HO tissue compared to normal tissue, denoted by changes in the 1640/1445 cm^{-1} ($p < 0.01$), and 1340/1270 cm^{-1} ($p < 0.01$) band area ratios (BARs). Additionally, analysis of the bone mineral in HO by Raman spectroscopy appears capable of determining bone maturity by measuring both the 945/960 cm^{-1} and the 1070/1445 cm^{-1} BARs. Raman may therefore prove a useful, non-invasive, and early diagnostic modality to detect HO formation prior to it becoming evident clinically or radiographically. This technique could ostensibly be utilized as a non-invasive means to risk stratify individual wounds at a time thought to be amenable to various means of primary prophylaxis.

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Introduction

Heterotopic ossification (HO) is defined as the formation of mature lamellar bone in soft tissues. Recently, our group has shown that the prevalence of HO in combat-wounded service members approaches 65% [1,2]. Both the frequency and severity of this HO are far greater than that observed following civilian trauma. Clinically, the impact of this disease process can be devastating in some cases, and spans all phases of treatment and rehabilitation. In fact, a recent analysis identified HO as the single most important barrier to functional mobility and return to duty in active military amputees [3].

Conventional means of primary prophylaxis, such as external beam radiotherapy (XRT) and non-steroidal anti-inflammatory drugs

(NSAIDs) exist, and, while they may be useful in some settings, they are often impractical for combat casualties. For the combat casualty, these options are logistically infeasible during the intercontinental aeromedical evacuation process (XRT) and may be medically contraindicated (NSAIDs) due to the high proportion of concomitant injuries [4,5]. As such, these modalities cannot be recommended for widespread use in all patients sustaining blast, or otherwise high-energy, combat related injuries. Better means of early risk stratification and novel alternative means of prophylaxis are needed.

The typical combat wound requires a series of operative debridement procedures due to severe contamination and slowly evolving tissue declaration of viability. The debridements begin immediately after injury and are generally continued three times per week until definitive wound closure using local tissue, flaps or skin grafts can be attempted [6]. General regions where HO will eventually form almost always become clinically evident during the first three to four weeks. Experienced surgeons have learned to recognize changes in the physical properties of tissue that are indicative of HO, most notably a palpable thickening and stiffening of tissues, which are evident during debridement procedures. The physical effects of ossification of these tissues can be felt when debriding with a scalpel; however, once mineralization

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occurs (usually weeks after injury), the process cannot be reversed, and conventional means of primary prophylaxis are typically ineffective [7]. Furthermore, areas of mineralization are often ill-defined, and efforts to preserve residual limb length and/or to provide durable muscular coverage over fractures preclude excision of all tissues exhibiting early signs of ectopic bone formation. Thus, in general, lesions continue to develop until the ectopic bone is mature.

If wound-specific changes portending eventual HO formation could be identified prior to the point at which lesions become palpable and/or radiographically apparent, prophylactic measures could be brought to bear systemically or locally. Ideally, this process of detection and prophylaxis should occur at a time early enough to influence osteoblastic differentiation (the differentiation of progenitor cells into a bone-specific lineage) and other requisite signaling processes necessary for the development of HO. Similarly, if the exact location of the pre-HO tissue could be more precisely defined, focused early debridement could be performed, which may help prevent subsequent formation, and also, theoretically, decrease the prevalence of symptomatic lesions.

Raman vibrational spectroscopy is a modality that offers the capability to accurately detect and identify various molecules that comprise the wound environment in their native state. It is a spectroscopic technique in which the precise biochemical composition of biologic samples can be obtained via noninvasive and nondestructive means. Over the past two decades, Raman spectroscopy has proven effective in assessing tissues at the molecular level with diverse clinical and diagnostic applications to include the analysis of cellular structure and the determination of tumor grade and type [8–12]. Accordingly, fundamental changes in the molecular environment of wounds that occur as a result of pathologic alterations can be analyzed by vibrational spectroscopy [13–16]. These identified changes can provide objective markers that correlate with the various phases of wound healing, which can, with other clinical observations, be used to guide surgical decision-making. For instance, we previously demonstrated that changes in collagen vibrational bands can be correlated with alterations in collagen deposition associated with normal re-epithelialization of the wound bed, while perturbations of the normal collagen ratios portended impaired or delayed wound healing [14]. Prior studies have also demonstrated the ability of Raman to effectively identify and analyze bone and other mineralized tissues [17–20]. Because clinical evidence and histological evidence point to mature HO tissue closely resembling woven bone with a cortical shell, we postulate that the Raman spectral profile of HO tissue resembles that of normal bone.

We ultimately seek to identify which wounds will develop HO as early as possible – at a time thought to be amenable to primary prophylaxis. As such, the purpose of this proof of concept study was to determine whether Raman spectral changes, measured *ex vivo*, correlate with histologic evidence of the earliest signs of HO formation using tissue biopsies from the wounds of combat casualties.

Materials and methods

Clinical studies

The clinical studies were approved by the institutional review boards of our institutions. Informed consent was obtained from all study participants including those patients who donated untraumatized muscle tissue that served as our control group. All injured muscle specimens were obtained from U.S. service members evacuated following high-energy combat-related extremity injuries sustained abroad. Eventually, radiographic, formation of HO was subsequently confirmed at least 30 days post-injury for specimens in the injured/HO cohort.

Sample collection

“Normal” muscle samples ($n = 10$) were collected from excess, discarded muscle tissue harvested during hamstring autograft preparation following routine, elective, anterior cruciate ligament reconstruction.

Injured muscle ($n = 10$) was collected from combat-injured patients sustaining high-energy extremity injuries, during the initial debridement upon arrival to our institution, prior to definitive wound closure or coverage. All patients were treated with negative pressure wound therapy. Tissue biopsies, approximately 1 cm^3 , were obtained during surgical procedures and immediately snap frozen. HO tissue biopsies were collected during the surgical removal of symptomatic lesions from twenty different injured patients and were subdivided into “early” (palpable, but not radiographically apparent, $n = 10$) and “mature” ($n = 10$) lesions. Additionally, “normal bone” control samples were collected from transfemoral and ulnar amputations, an ACL reconstruction, and a non-union fracture callus ($n = 4$). Samples were stored at -80°C until Raman spectroscopic analysis. Prior to Raman spectral acquisition, samples were thawed in 0.9% NaCl saline solution. Samples were unfixed at room temperature for no more than 15 min, to thaw the sample and collect a Raman spectrum. After Raman spectral collection, the sample was immediately refrozen and stored at -70°C . Prior to histopathological examination, all muscle and early HO tissue biopsies were paraffin-embedded and stained with hematoxylin and eosin (H&E), Masson's trichrome, and Alcian blue. Mature HO tissue was embedded in glycolmethacrylate (GMA) and stained with H&E and von Kossa stains. Stained tissue sections were examined for standard microscopic evaluation.

Raman spectroscopy

Tissue samples were placed on an aluminum foil-covered weighing dish prior to spectral acquisition. A 785 nm Raman PhAT system (Kaiser Optical Systems, Inc., Ann Arbor, MI) was used to collect spectra of the tissue biopsies. Final spectra were the accumulation of forty 5-second spectra, acquired using the 3 mm spot size. At least three dark-subtracted, illumination-corrected spectra were obtained for each biopsy/sample. All spectral preprocessing was performed in GRAMS/AI software (Thermo Fisher Scientific, Madison, WI). Raman spectra were truncated to $1800\text{--}400 \text{ cm}^{-1}$ and baseline corrected with a sixth degree polynomial. Spectral subtraction of blood was performed if spectral interference of blood was noted. All spectra were intensity normalized to the CH_2 scissoring band at 1445 cm^{-1} . Subsequently, curve fitting was performed over three spectral regions, $1730\text{--}1500 \text{ cm}^{-1}$, $1525\text{--}1185 \text{ cm}^{-1}$, and $1150\text{--}900 \text{ cm}^{-1}$. All Raman bands were fit with mixed Gaussian/Lorentzian bands. The fit was considered good when the R^2 value reached at least 0.99.

Band area ratios (BARs), a pseudoquantitative measure, were calculated by dividing the band area of a Raman band of interest (for example 1660 cm^{-1}) by another band area (for example 1445 cm^{-1}). Compositional trends in related samples can be explored using BARs, such as an increase in mineral carbonation ($1070/960 \text{ cm}^{-1}$) or a decrease in reducible collagen crosslinks ($1680/1660 \text{ cm}^{-1}$); absolute Raman band intensities and band areas can be greatly affected by optical effects [18]. BARs calculated in this study include mineral carbonation ($1070/960 \text{ cm}^{-1}$ and $1070/1445 \text{ cm}^{-1}$), mineral maturity ($945/960 \text{ cm}^{-1}$), protein order/disorder ($1240/1270 \text{ cm}^{-1}$), and α -helical structure ($1340/1270 \text{ cm}^{-1}$). Mineral crystallinity is determined by the full width at half maximum of the $960 \text{ cm}^{-1} \nu_1$ phosphate band.

Statistical analysis

Differences in band area ratios and band centers between groups (normal muscle control, injured muscle, early HO, mature HO and normal bone) were assessed using independent-samples of Kruskal–Wallis tests. Because four independent statistical tests were performed for each parameter, a Bonferroni-adjusted significance level of 0.0125 was calculated to account for the increased possibility of type-I error. Differences were considered statistically significant by a two-tailed $\alpha \leq 0.0125$. Analyses were performed using SPSS software (SPSS 18.0, SPSS Inc., Chicago, IL).

Results

Histopathologic characterization of tissue

Stained tissue sections of normal muscle and injured muscle (Fig. 1) exhibit typical skeletal muscle histological attributes. Normal myofibers are uniform while fibrous tissue infiltrates the injured muscle myofibers. Heterotopic ossification is generally an intensely vascularized lesion, appearing disorganized and hypercellular [21]. In all HO specimens, lesion development begins adjacent to injured muscle and is surrounded by fibrous connective tissue with long standing collagen fibrils. Towards the periphery of the lesion, trabeculae become more clearly defined. Immature and mature HO osteoids (Figs. 2 and 3, respectively) contain large, immature osteocytes surrounded by primitive woven bone. Unlike the immature HO tissue in Fig. 2, mature HO tissue more closely resembles lamellar bone where cement lines are obvious and bone marrow elements are prevalent throughout the lesion. The presence of osteoclasts in the mature HO, indicative of active remodeling, is not evident; this is supported by the lack of scalloped edges along the trabeculae, which appear smooth.

Raman spectroscopy of tissue biopsies

Raman spectroscopic examination was conducted for 44 ex vivo biopsies collected from 44 patients, including control or “normal” muscle ($n = 10$), injured muscle ($n = 10$), and HO tissue ($n = 20$). Additionally, Raman spectra of collagen (types I, II and IV) were procured as reference standards. The Raman spectral profiles of types I, II, and IV collagen and normal muscle (Fig. 4A), along with common Raman spectral

bands and their assignments for collagen, muscle and bone are presented to highlight differences between their spectral profiles (Table 1). Raman spectra of ex vivo samples of uninjured (or control) muscle, injured muscle, and surgically excised heterotopic ossification were also compared (Fig. 4B). Spectral differences are prominent in the amide I, amide III, and fingerprint regions of the Raman spectra. These changes reflect variation in the composition of the tissue itself, both for the matrix and mineral components of tissue.

A more in depth characterization of both the matrix and mineral components of tissue are presented in Figs. 5 and 6, respectively. Fig. 5 displays calculated band area ratios for the Raman spectra of control muscle, injured muscle, early HO tissue, and mature HO tissue for matrix bands. In general, when comparing muscle (normal or injured) to HO tissue (early or mature), we noted a decrease in the $1660/1445\text{ cm}^{-1}$ ($p < 0.03$), $1680/1445\text{ cm}^{-1}$ ($p = 0.03$), and $1340/1270\text{ cm}^{-1}$ (α -helical structure, $p < 0.01$) band area ratios (BARs). There is also an increase in the $1640/1445\text{ cm}^{-1}$ BAR ($p < 0.01$) and the $1240/1270\text{ cm}^{-1}$ (protein order/disorder) BAR ($p < 0.10$). These changes in BARs can also be examined as a progression of normal tissue to diseased tissue.

Initially, after muscle injury, we observe an increase in $1660/1445\text{ cm}^{-1}$, $1640/1445\text{ cm}^{-1}$, and protein order/disorder BARs. The transition from injured muscle to early HO tissue demonstrates a decrease in the $1660/1445\text{ cm}^{-1}$ and α -helical structure BARs, and an increase in the protein order/disorder BAR. Finally, the mineralization of HO tissue shows a significant decrease in the α -helical structure BAR.

Fig. 6 compares mineral band area ratios for normal bone and for HO tissue. Both the $945/960\text{ cm}^{-1}$ band area ratio (mineral maturity) and the $1070/1445\text{ cm}^{-1}$ band area ratio (mineral carbonation) provide a

Fig. 1. Muscle histopathology. Normal muscle (A, B — H&E and C — Masson's trichrome) is compared to injured muscle (D, E — H&E and F — Masson's trichrome). Upon microscopic examination, injured muscle myofibers are surrounded by fibrous connective tissue while normal muscle myofibers are relatively uniform. Closer examination demonstrates inflammation (evidenced by lymphocytes), myofiber degradation, necrosis, and hemorrhage in injured muscle tissue.

Fig. 2. Osteoid histopathology. Immature (A–D) and mature osteoid (E–H) development of HO tissue is compared. Both tissue specimens were collected from a patient approximately 60 days post-injury. Sections are stained with H&E (A, B, E, F), Masson's trichrome (C & G), and von Kossa (D & H). Immature osteoid on H&E stained slides appears more eosinophilic than mature bone (B) and only stains blue with Masson's trichrome (C). As the osteoid matures, Masson's trichrome reveals osteoid that stains both blue and red (G). Von Kossa staining corroborates these results by demonstrating a greater degree of mineralization (darker stain) in the mature osteoid (D & H).

measure of mineral maturity; as bone matures, the $945/960\text{ cm}^{-1}$ BAR decreases ($p = 0.012$) and the $1070/1445\text{ cm}^{-1}$ BAR increases ($p = 0.011$). Early HO tissue was surgically removed within 165 days post-injury (mean value) while mature HO was excised on average of over 600 days post-injury; this is reflected in the Raman spectral band area ratios. Early HO has the highest mineral maturity BARs (mean = 0.44 ± 0.10) and the lowest mineral carbonation BARs (mean = 0.18 ± 0.04). This trend is reversed for mature HO, where the mineral maturity band area ratios are lower than early HO (mean = 0.24 ± 0.10) and the mineral carbonation band area ratios are higher than early HO tissue (mean = 1.23 ± 0.52).

Discussion

Combat related HO formation is theorized to follow a common sequence of events [22]. First, an induction signal must occur [22] which, in our patient population, is the creation of a high-energy penetrating wound, usually from a blast. This leads to a pronounced and

prolonged systemic and local inflammatory response [23]. Second, a population of progenitor cells, previously identified in our patients [24], expands and is primed to undergo osteogenic differentiation in an environment conducive to osteogenesis. Adipocytes play a key role in creating this milieu conducive to osteogenesis by providing an environment of low oxygen tension in the tissue [25]. Ossification then proceeds by either an endochondral bone formation (as with fibrodysplasia ossificans progressiva) or intramembraneous bone formation (as with progressive osseous heteroplasia) or both (as with myositis ossificans circumscripta) [26]. In the case of endochondral ossification, a hypoxic environment is conducive to brown fat development [25] and chondrocyte differentiation from mesenchymal cells [27]. Newly differentiated chondrocytes become hypertrophic and express factors that promote vascular ingrowth to include vascular endothelial growth factor (VEGF) [28]. Finally, the subsequent neoangiogenesis allows for the matrix mineralization and eventual ossification.

In this preliminary study, we used Raman spectroscopy to discern molecular changes that occur prior to and during the formation of HO.

Fig. 3. Mature HO histopathology. The figure displays stained tissue sections of mature HO tissue (H&E and Masson's trichrome – A & B and C & D, respectively). In this particular patient, the HO developed directly adjacent to the femur, beneath a skin grafted area which had been treated with INTEGRA® bioartificial dermal replacement (Integra Life Sciences; Plainsboro, NJ) application pre-skin graft. The HO lesion was excised over 160 days post-injury, after ulcerating and becoming persistently symptomatic.

Fig. 4. Representative Raman spectral profiles. A) Raman spectra of normal muscle (—), type I collagen (---), type II collagen (....), and type IV collagen (— · —). Obvious differences are apparent in the amide I, the amide III envelope, and the C–C backbone stretching bands (denoted by the shaded boxes). The Raman spectrum of muscle contains an amide I band at $\sim 1655 \text{ cm}^{-1}$ while the collagen amide I band is shifted to $\sim 1665 \text{ cm}^{-1}$. Additionally, the amide III 1340 and 1320 cm^{-1} bands are much more prominent in the muscle spectrum but the collagen spectra display increased amide III 1270 and 1245 cm^{-1} spectral bands. Finally, the 876 and 855 cm^{-1} Raman spectral bands of the collagen spectra are more intense than those exhibited in the muscle spectrum. B) Raman spectra of normal muscle (—), early HO tissue (---), and mature HO tissue (....). Shaded boxes indicate regions where vibrational bands differ significantly. The mean band center for the amide I band of uninjured muscle is 1655 cm^{-1} . For the HO tissue, whether early or mature, the amide I band shifts to a higher frequency and is centered at 1660 cm^{-1} . Differences are also apparent in the amide III envelope of the spectra. The intensity of the 1340 cm^{-1} Raman vibrational band is decreased in the spectra of the HO tissue compared to the uninjured muscle tissue. The 1270 cm^{-1} and 1240 cm^{-1} Raman vibrational bands are increased in the spectra of the HO tissue compared to the uninjured muscle. The most notable difference in the spectrum of the mineralized HO tissue is the presence of the 960 cm^{-1} band, a ν_1 P–O stretching mode. This is a typical Raman vibrational band observed for hydroxyapatite, and in this case, for the carbonated hydroxyapatite in bone mineral. Finally, the intensities of the 921 cm^{-1} , 876 cm^{-1} , and 855 cm^{-1} bands are more intense in the spectra of the HO tissue than in the spectrum of the uninjured or injured muscle.

Table 1
Raman vibrational band assignments [29–31,34,49–53].

ν (cm ⁻¹)	Band assignment	Component
593	ν_4 PO ₄ ³⁻ bending	Hydroxyapatite
821	ν (CC) of backbone	Collagen; muscle
856	ν (CC) of hydroxyproline ring	Collagen; muscle
873	ν_3 P–OH stretching	Bone
876	ν (CC) of hydroxyproline ring	Collagen; protein
921	ν (CC) of proline ring	Collagen; protein
938	ν (CC) of protein backbone	Collagen; muscle; protein
945–952	ν_1 PO ₄ ³⁻ stretch	Amorphous calcium phosphate
959	ν_1 PO ₄ ³⁻ stretch	Hydroxyapatite
1004	ν (CC) aromatic ring	Phe; collagen; muscle
1032	ν_3 PO ₄ ³⁻ ; ν (CC) skeletal; C–O stretch	Bone; collagen; muscle
1071	ν_1 (CO ₃ ²⁻)	Bone
1075	ν_3 PO ₄ ³⁻ stretch	Hydroxyapatite
1080	ν (CC) and ν (CN) skeletal	Collagen; muscle
1159	ν (CC) and ν (CN) skeletal	Carotenoid
1178	ν (CC) and ν (CN) skeletal	Collagen; muscle
1244	δ (CH ₂) wagging; ν (CN) amide III disordered/ β -sheet	Collagen; muscle
1274	ν (CN) and δ (NH) amide III α -helix	Collagen; muscle
1297	δ (CH ₂) twisting	Collagen; muscle
1343	γ (CH ₂ , CH ₃) wagging	Collagen; muscle
1385	δ (CH ₃) symmetric	Collagen
1448	δ (CH ₂) scissoring	Collagen; muscle
1524	Carotenoid	Collagen; muscle
1552	ν (CC) ring stretch	Collagen; muscle; Trp
1665	ν (CO) amide I	Collagen; muscle

We compared normal muscle tissue to injured muscle tissue, early HO tissue, and mature HO tissue. While mature HO tissue is generally apparent upon physical examination and/or radiologic examination, immature and largely unmineralized HO tissue is not as clinically obvious. The Raman spectra of various tissues demonstrate that there are clear differences in the amide I and amide III spectral regions of HO tissue compared to normal muscle tissue, which may indicate whether or not muscle tissue will develop HO. These differences include a significant shift in the location of the amide I band and an increase in some of the amide III bands.

As injured muscle transitions to mineralized tissue, significant changes in many matrix bands (Fig. 4B) are apparent. First, there is a

Fig. 6. Comparison of Raman vibrational band area ratios for mineral components of tissue. Statistically significant differences ($p < 0.0125$) are indicated by an asterisk (*).

shift in the amide I band in the Raman spectra from 1655 cm⁻¹ to 1660 cm⁻¹ – this is likely due to increased collagen content ($p < 0.01$). Second, the bandwidth of amide I band increases, evidenced by a decrease in the 1660/1445 cm⁻¹ BAR, a decrease in the 1680/1445 cm⁻¹ BAR, and an increase in 1640/1445 cm⁻¹ BAR (Fig. 5). Bands at 1640 and 1660 cm⁻¹ are assigned to α -helix protein secondary structure while the band at 1680 cm⁻¹ is assigned to β -sheet protein secondary structure [29–31]. Muscle is composed largely of actin and myosin, both which are more alpha helical in structure than collagen [32]. As collagen content increases and muscle myofibers degenerate, the band center and width more closely resemble that of collagen, specifically type I collagen. Additionally, an increase in the 1680/1445 cm⁻¹ could be attributed to increased collagen crosslinking [33]. Third, the intensity of amide III bands changes, most notably with an increase in the protein order/disorder BAR and a decrease in the α -helical structure BAR (Fig. 5). The Raman band at 1240 cm⁻¹ is attributed to more disordered protein structures, such as those containing a large number of β -pleats or random coils, while the 1270 cm⁻¹ Raman band is assigned to more ordered protein structures, such as those

Fig. 5. Comparison of Raman vibrational band area ratios for matrix components of tissue. Statistically significant differences ($p < 0.0125$) are indicated by an asterisk (*).

containing a large number of helical coils [34,35]. In fact, Raman bands below 1270 cm^{-1} are very weak in protein with a large proportion of helical structure and the absence of a Raman band at 1240 cm^{-1} is indicative of helical structure [34], such as myosin in muscle.

The Raman spectrum of unmineralized HO tissue closely resembles the Raman spectrum of type I collagen. Type I collagen plays an important role not only in the process of wound healing, but also in the formation of osseous tissue, such as HO. Osteoblasts secrete and deposit type I collagen, which comprises 90% of bone matrix prior to mineralization [36]. In some cases, the collagen that serves as an initiator of wound healing may also act as the scaffold for the deposition of bone mineral.

Raman spectroscopy also provides insight into the actual mineralization of the soft tissue. When symptoms persist and cannot be mitigated via conservative modalities, HO is typically removed after surgeons are confident that the HO has fully matured. This, in theory, decreases the likelihood of local recurrence. Historically, scintigraphy in addition to serum alkaline phosphatase has been used to confirm maturity of lesions [37–39], which was thought to occur approximately one year from injury. We have, however, observed combat-related lesions to be mature at approximately six months post-injury, when cortical margins appear well-defined and remain unchanged on serial radiographs taken at least one month apart. Raman spectroscopy can evaluate the maturity of lesions directly [18]. The 960 cm^{-1} ν_1 phosphate band in the Raman spectrum of bone can be deconvoluted into more than one Raman spectral band, depending on the species of mineral present in the tissue. For instance, the presence of a shoulder at 945 cm^{-1} has been attributed to an amorphous calcium phosphate [40,41], thought to be a precursor to mature bone's carbonated hydroxyapatite. As bone matures, this shoulder will decrease; thus, the $945/960\text{ cm}^{-1}$ BAR can be used as a measure of mineral maturity. This is evidenced by early HO in Fig. 6, in which the least mature HO, had the highest $945/960\text{ cm}^{-1}$ BAR (corresponding histology shown in Fig. 2). Furthermore, the 1070 cm^{-1} Raman spectral band is attributed to a ν_1 carbonate vibration. As bone matures, the incorporation of carbonate into the mineral lattice increases. Increased carbonate content has been associated with increased crystallinity of bone mineral and bone maturity; thus, as increase in the $1070/1445\text{ cm}^{-1}$ BAR is indicative of increased mineral maturity. Again, early HO has the lowest mineral carbonation BAR and histologically, exhibits the least mature HO. The relationship between the mineral maturity BAR and maturity is further illustrated by comparing early HO to the mineral carbonation BAR of both mature HO and normal, mature, lamellar bone (histology not shown). These band area ratios have been correlated with bone mechanical properties such as modulus, yield stress, and fracture stress [20,42–47].

Based on these results, Raman spectroscopy may be useful to identify early wound specific changes that portend eventual HO formation. As an intraoperative modality, Raman spectroscopy may have distinct advantages over other techniques of assessing tissue during surgery such as histology or inspection by the surgeon. Though, not widely used, frozen section and/or permanent pathologic analysis can be used to identify early stages associated with eventual HO formation. This technique requires multiple wound biopsies, is time and labor intensive and may not be sufficiently precise with regard to overall HO precursor location and potential severity to guide surgical decision-making. Also, the ability of a surgeon to identify early HO tissue during a debridement is subjective and relies heavily on personal experience. Conversely, the Raman technique described above could be adapted as an objective, intraoperative, non-invasive means by which to risk stratify wounds, without significant preparation or bias on the part of the interpreter. If Raman spectroscopy demonstrates that a wound has Raman spectral features associated with the formation of HO, prophylaxis could be employed in select cases. Alternatively, in areas that appear prone to HO development, which can be mapped with vibrational spectroscopy more precisely than gross clinical assessment, the clinician can consider early preferential excision of pre-HO tissue while the patient is undergoing debridement and/or amputation revision for final closure.

Additionally, Raman may also be useful for monitoring the development and progression of HO non-invasively and without radiation. For example, tomography techniques currently in development are able to model bone mineral density in three-dimensional space [48]. With such a model, the clinician may also be able to visualize areas of HO formation as well as monitor the maturation of each portion of the lesion(s), over time.

Conclusions

Though the number of samples examined herein was small and our findings preliminary, the results are encouraging and certainly deserving of further study. Furthermore, all data acquisition and analysis in this study were performed *ex vivo* and outside of the surgical suite. Nevertheless, these results suggest that Raman spectroscopy correlates with histologic appearance of tissues within combat wounds, and can identify the earliest forms of mineralization in patients who are actively forming HO. We believe it is possible to optimize existing Raman spectroscopic equipment, such as fiber-probe coupled systems, for use in the operating room during surgical debridements. This could provide treating surgeons and researchers with an accurate, non-invasive means by which to risk stratify individual wounds to receive systemic and local means of primary prophylaxis currently in development.

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content: NC, EAE, BKP and JAF. Approving final version of manuscript: NC, EP, EAE, BKP, and JAF. JAF takes responsibility for the integrity of the data analysis.

Disclosures

All authors state they have no conflicts of interest. Specifically, in the 36 months prior to submission of the manuscript, none of the authors have any potential conflicts of interest or financial disclosures pertaining to any organization or other entity that might be perceived to have influenced the objectivity or integrity of the work being reported. This includes employment, research funding, income (e.g., fees for consulting, expert testimony, or speaking), or ownership interests (e.g., stock, patents) in or from an organization that may gain or lose financially from the work being submitted for publication.

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