Alterations to Bone Mineral Composition as an Early Indication of Osteomyelitis in the Diabetic Foot

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OBJECTIVE—Osteomyelitis in the diabetic foot is a major risk factor for amputation, but there is a limited understanding of early-stage infection, impeding limb-preserving diagnoses. We hypothesized that bone composition measurements provide insight into the early pathophysiology of diabetic osteomyelitis.

RESEARCH DESIGN AND METHODS—Compositional analysis by Raman spectroscopy was performed on bone specimens from patients with a clinical diagnosis of osteomyelitis in the foot requiring surgical intervention as either a biopsy (n = 6) or an amputation (n = 11).

RESULTS—An unexpected result was the discovery of pathological calcium phosphate minerals in addition to normal bone mineral. Dicalcium phosphate dihydrate, also called brushite, and uncarbonated apatite were found to be exclusively associated with infected bone.

CONCLUSIONS—Compositional measurements provided a unique insight into the pathophysiology of osteomyelitis in diabetic foot ulcers. At-patient identification of pathological minerals by Raman spectroscopy may serve as an early-stage diagnostic approach.

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Osteomyelitis of the diabetic foot, herein called diabetic osteomyelitis, is a major cause of lower-extremity amputation, yet an understanding of the pathophysiology and technologies enabling early diagnosis of this serious infection are lacking. Clinical and imaging tests show that whole-tissue properties of bone, including hardness and mineralization, are directly affected by diabetic osteomyelitis (1,2). We hypothesized that compositional changes to bone mineral and collagen matrix accompany clinically observable alterations in bone hardness and mineralization. However, no studies to our knowledge have reported on the chemical composition of bone in diabetic osteomyelitis. The objective of the present study was to measure bone composition in diabetic osteomyelitis with the use of Raman spectroscopy.

RESEARCH DESIGN AND METHODS

Clinical study

This is an ongoing translational study performed at the University of Michigan Health System (UMHS) and the Ann Arbor Veterans Affairs (AAVA) Hospital and has been reviewed and approved by their respective institutional review boards. Bone was obtained from 17 patients with a clinical diagnosis of diabetic osteomyelitis requiring surgical intervention to collect a bone biopsy specimen (n = 6) or to amputate (n = 11). No patients were treated with bone cements. Bone fragments were prepared separately for microbiological and histopathological analyses. All patients had bone cultures performed, and some had additional soft tissue and exudates cultured. For microbiology analysis, bone fragments were stored in an ESwab Collection and Transport system (Becton Dickinson, Sparks, MD) and analyzed through standard hospital procedure. Fragments for histopathology were prepared by the UMHS Tissue Procurement Core or the AAVA pathology laboratory. Only otherwise-to-be-discarded bone fragments were used for research purposes.

Bone fragment preparation

Bone fragments for Raman spectroscopic analysis were transported and stored in gauze soaked with PBS enriched with protease inhibitor (0.1% volume for volume) and sodium azide (0.005% weight for volume) to prevent enzymatic or bacterial digestion of bone collagen and stored at −20°C until examination. Most specimens were examined by Raman spectroscopy within 24 h of the biopsy or amputation surgery and thawed at room temperature immediately before analysis. The average size of the biopsy specimens was <5 mm^3, and the average size of the amputation specimens was >1 cm^3. Raman spectra were collected with microscopy instrumentation adapted for Raman microspectroscopy as described elsewhere (3).

RESULTS—Table 1 shows the clinical imaging, pathology, microbiology, and Raman spectroscopy data for all study participants. In most cases, multiple clinical imaging modalities (magnetic resonance imaging, X ray, ultrasound, or bone scan) were used for preoperative identification of osteomyelitis. Pathology data on a range of pathophysiological states were reactive, active remodeling, necrotic, or osteomyelitic bone. Additional histopathological findings of acute inflammation or fibrosis were found in a few participants in the amputation group. As expected, bone cultures revealed a mixed population of gram-positive bacteria, with *Staphylococcus*, *Streptococcus*, or *Enterococcus* as the dominant species. Raman spectroscopy of the bone fragments revealed the presence of pathological minerals in addition to normal bone

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Table 1—*Summary of clinical data (imaging, histopathology, and microbiology) and experimental Raman spectroscopy data for bone specimens obtained from biopsy specimens or amputations*

ID	Anatomic location	Imaging	Pathology	Microbiology	Raman
B01	Great toe	+	+	*Staphylococcus*	+
B02	Medial arch	+	+	*Staphylococcus aureus*	+
B03	2nd toe	+/−	−	*Serratia marcescens, Enterococcus faecalis*	+
B04	Calcaneus	+	−	*E. faecalis, Stenotrophomonas maltophilia*	+
B05		+	+	*S. aureus*	+
B06		+	−	*Group G streptococcus*	+
A01		+	−	*Group B streptococcus*	+
A02	Great toe	+	−	*E. faecalis*	−
A03	2nd toe	+	−	*Escherichia coli, S. aureus*	−
A04	2nd toe	NA	−	*S. aureus*	−
A05	2nd toe	NA	+	*S. aureus*	−
A06	2nd toe	+/−	+	*Staphylococcus*	+
A07	Great toe	+	−	*Staphylococcus*	−
A08	3rd toe	+	−	*Staphylococcus*	−
A09	2nd toe	+	+	*S. aureus, Group B streptococcus*	−
A10	BKA	+	+	*E. faecalis, Pseudomonas putida*	−
A11	2nd toe	+	−	*Candida sp.*	−

Clinical evaluation of study participants also included age, sex, height, weight, disease duration, and history of foot ulcers. Study participants were 41–87 years old. The biopsy cohort comprised two women and four men, and the amputation cohort comprised 11 men. In most cases, the affected foot was assessed by X ray, magnetic resonance imaging, bone scan, or ultrasound imaging within 1 month of the biopsy or amputation. If known, the anatomic location of the surgery or biopsy is included. In several cases, multiple clinical imaging modalities were used to ascertain the presence of osteomyelitis, and any diagnostic radiology report is identified with a +. In a few cases, multiple clinical imaging tests did not yield consistent or unambiguous preoperative identification of osteomyelitis. For those cases, the results are reported from the positive test. Inconclusive or ambiguous diagnostic radiology reports are identified with a +/−. A + value for pathology results was reported only if the histopathological diagnosis was either acute or chronic osteomyelitis. Positive histopathology reports included evidence of bone remodeling, inflammation, necrosis, the presence of reactive bone, and osteolysis. As expected, *Staphylococcus*, *Streptococcus*, and *Enterococcus* were the primary bacterial species recovered from bone cultures. Raman identification of abnormal minerals, either brushite or uncarbonated apatite, are also denoted with a +. Hypercalcemia and chronic metabolic acidosis were ruled out as a possible cause of pathological mineralization because all participants had normal-to-low serum calcium levels and normal serum bicarbonate levels. A, amputation; B, biopsy; BKA, below-the-knee amputation; NA, not available.

mineral. Two pathological minerals were identified: brushite and uncarbonated apatite. A + for Raman spectroscopic results was reported if brushite or uncarbonated apatite was detected. Raman spectra of control bone specimens were consistent with normal bone composition and did not show evidence of pathological mineralization. Storage in enriched PBS did not affect induced compositional changes in a control study of healthy bone fragments.

CONCLUSIONS—In this study, we applied Raman spectroscopy to measuring compositional changes in bone infected by osteomyelitis of the diabetic foot. Bone fragments were examined from patients who underwent either surgical biopsy/debridement or amputation. An unexpected finding was Raman spectral patterns corresponding to dicalcium phosphate dihydrate, also called brushite, and uncarbonated apatite. Compositional changes in bone currently cannot be identified by standard clinical imaging or histopathology but are easily measured by Raman spectroscopy. This study provides insight into the pathophysiology of diabetic osteomyelitis and identified a possible early-stage marker of clinical disease.

Many mechanisms of bone loss in osteomyelitis have been proposed in the literature (4–6). Even though bacterial biofilms are known to form in osteomyelitis, direct bacterial attack on bone is believed to be a negligible mechanism (7–9). The present results suggest that pathological mineralization accompanies bacterial infection, providing insight into the pathophysiology of osteomyelitis. The presence of pathological minerals may also serve as a compositional marker of early-stage bone infection. Brushite is only found in vivo under chronically acidic conditions, such as dental calculus, urinary stones, and chondrocalcinosis. To the best of our knowledge, this is the second report of brushite in mature human bone. Brushite was identified by X-ray absorption and infrared spectroscopy in fibrous dysplasia of the jaw (10). However, this finding has not been reproduced in other studies, and results from only one patient were reported. Poorly carbonated apatite can be found in woven, or immature, bone and is less crystalline than mature bone mineral (11). By contrast, the uncarbonated apatite found in infected bone was more crystalline than immature bone mineral and suggests deposition of a pathological mineral.

Normal serum calcium values in all the participants argue against the possibility that we were observing brushite and uncarbonated apatite as a precursor in normal bone formation or as a nonbone precipitate resulting from systemic hypercalcemia. The likelihood that pathological minerals were formed by an inflammatory response, immune response, or excessive bone remodeling is not supported by our observations and previous studies (12,13). Thus, we hypothesize that a bacteria biofilm is responsible for generating the acidic environment necessary to form brushite. If the localized microenvironment cannot be adequately buffered, then acidic calcium phosphate minerals such as uncarbonated apatite and brushite may precipitate onto the bone surface. This mechanism, although new in its application to diabetic osteomyelitis, is the accepted pathway in microbial degradation of bone postmortem (14).

Associating Raman spectroscopy data with anatomic location was an issue in the measurements and may have had an impact on the rate of identifying pathological minerals. Biopsy specimens were small (<5 mm^3) and taken directly from the wound bed, so there was a greater association between the spectroscopy data and the anatomic location of the active infection. Thus, we were able to identify pathological minerals in 100% of the biopsy specimens. However, the amputated tissue was large relative to the recovered fragments. Although we worked closely with the pathology laboratory to obtain bone specimens near the site of suspected infection, obtaining precise anatomic information was a challenge. This challenge was also apparent when we examined the imaging and histopathology data. The lack of correlation between imaging and

histopathology data in the amputation cohort underscores the difficulty in identifying osteomyelitis across a large anatomical unit, such as a digit or limb. We suspect that incomplete sampling was primarily responsible for inconsistent Raman spectroscopic identification of pathological minerals in amputated bone. Future translational studies will address developing enhanced anatomic precision with respect to geographic analysis of diabetic wounds.

It is intriguing to conceptualize an at-patient Raman spectroscopic measurement of pathological mineralization. Intraoperative or transcutaneous Raman spectroscopic identification of pathological minerals during biopsy or amputation surgeries may distinguish bone infections from noninfectious bone lesions. Point-of-care measurements are feasible because Raman spectroscopy is amenable to fiber-optic–based instrumentation. Our laboratory has developed portable fiber-optic instrumentation for transcutaneous bone measurements at bedside or in a surgical suite, and our ongoing human studies demonstrate in vivo feasibility and establish a basis for future translational Raman studies of diabetic foot wounds (15).

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