Evidence of *S. aureus* Deformation, Proliferation and Migration in Canaliculi of Cortical Bone Using a Murine Model of Osteomyelitis

Karen L. de Mesy Bentley1,2, Ryan Tombetta1,3, Kohei Nishitani1, Sheila N. Bello-frizarry1, Stephen L. Kates4, Hani A. Awdal5 and Edward M. Schwarz1,2,3,4

1Center for Musculoskeletal Research, University of Rochester Medical Center, Rochester, New York, USA.
2Pathology and Laboratory Medicine, University of Rochester Medical Center, Rochester, New York, USA.
3Department of Biomedical Engineering, University of Rochester Medical Center, Rochester, New York, USA.
4Department of Orthopedics and Rehabilitation, University of Rochester Medical Center, Rochester, New York, USA.

DISCUSSION: The authors have nothing to disclose.

INTRODUCTION: Chronic osteomyelitis caused by *Staphylococcus aureus* infections is a major challenge in orthopedics. Moreover, this condition is considered to be incurable due to biofilm bacteria persisting deep within cortical bone. Previously, the mechanisms by which *S. aureus* establishes these deep infections in bone were unknown. Using transmission electron microscopy (TEM), we recently discovered malformed rod-shaped *S. aureus* deep within microcracks of infected cortical bone. However, how rigid cell-walled cocci with a diameter larger than sub-micron microcracks and canaliculi can colonize these regions of cortical bone without flagella, cilia or pseudopodia mediated motility remains enigmatic. To investigate the rod-shaped bacteria’s viability we performed a series of immuno-electron microscopy (IEM) studies using our murine chronic implant associated osteomyelitis models to test the hypothesis that *S. aureus* colonizes and establishes biofilm in canaliculi by: 1) structural deformation into sub-micron spaces in cortical bone; and 2) proliferation at the leading edge of the colonizing bacteria propelling biofilm formation further into canaliculi and vacant osteocyte lacunae.

METHODS: Two protein A deficient strains of *S. aureus* (UAMS-1 deltaSPA and USA300 deltaSPA) were used in two different murine models of implant-associated osteomyelitis (transplant pin and plate-fixed femoral osteotomy). For metabolic labeling studies, the mice were given water containing 0.5% BrdU throughout day 1-14 of infection (eutanized day14). The tibias and femurs were immersion fixed in 4% paraformaldehyde/0.2% glutaraldehyde decalcified and embedded into LR White resin. Thin sections on formvar grids were treated with 5N HCl to denature the DNA, incubated in 1/100 anti-BrdU sheep primary antibody (Fitzgerald) and anti-sheep 12nm gold-tagged secondary (Jackson ImmunoResearch). Negative controls used 1% bovine serum albumin substituted for the primary antibody. The grids were stained and digitally imaged using a Hitachi 7650 TEM and an 11 megapixel camera. RESULTS: In the initial study of infected tibiae, both strains of *S. aureus* effectively colonized cortical bone fragments to establish Staphylococci abscess communities (SACs) within narrow spaces. However, exhaustive evaluation of these sections (>100 ROIs) failed to identify significant evidence of intracellular bacteria, demonstrating chronic *S. aureus* osteomyelitis in these models is primarily caused by extracellular biofilm in soft tissue and bone. Remarkably, we observed *S. aureus* within very narrow canaliculi (<0.4 microns) morphologically transformed into rod shape bacteria facilitating colonization deep (125 microns) within cortical bone (Figure 1A). The outer walls of expanded canaliculii and osteocyte lacunae were scalloped, providing evidence of cortical bone resorption by colonizing bacteria. IEM with anti-BrdU antibodies tagged with a 12nm gold tagged secondary, confirmed the metabolic status of both UAMS-1 in canaliculi (Figure 1B, 1C, 1D), supporting the theory of bacteria proliferation at the leading edge of the invading biofilm.

DISCUSSION: Our understanding of biofilm infiltration into cortical bone during the establishment of chronic osteomyelitis has been limited due to lack of *in vivo* TEM-based imaging studies of *S. aureus* in infected bone. Interestingly, a leading theory to explain chronic osteomyelitis has identified the osteoblast and/or the osteocyte as the bone cells responsible for phagocytic uptake of *S. aureus*, leading to persistence of infection within bone tissue. However, our TEM studies of mice with chronic (>8-months) osteomyelitis have clearly demonstrated that the vast majority of *S. aureus* persists as biofilm on the implant, in *S. aureus* communities (SACs) and in bone. While we have made the very rare observation of intracellular *S. aureus* within osteoblasts, these infected cells displayed features of apoptosis. Thus, we conclude that intracellular infection is a minor mechanism of *S. aureus* persistence. Our most interesting and novel observations address the mechanism by which *S. aureus*, a non-motile cocci larger than submicron canaliculi, colonizes the deep 0.125nm regions of cortical bone during osteomyelitis via three distinct mechanisms. The first is the unexplained deformation of its rigid cell wall, which allows the bacteria to assume a bacillus/rod-shaped form, and squeeze through the submicron (<0.4 microns) bone space. The second is proliferation of the bacteria at the leading edge of the biofilm, which provides a postulated propulsion force to drive the invading *S. aureus* through the vacated canaliculi network. The third mechanism is consumption of the cortical bone, via lactic acid demineralization and enzymatic digestion of the matrix, which provides *S. aureus* an inexhaustible food supply extending its life beyond that of the host. Further genetic and biochemical studies are needed to validate these theories and identify novel drug targets to prevent and treat these inoperable abscesses that occur in chronic osteomyelitis.

SIGNIFICANCE: *S. aureus* accounts for 80% of chronic osteomyelitis via unknown mechanisms of deep bone infection. We demonstrated *S. aureus* (UAMS-1 and USA300) invasion into the lacuno-canicular system, which has not been previously described in the literature. Our *in vivo* BrdU uptake and IEM studies provide the first evidence of *S. aureus* proliferation at the leading edge of biofilm formation in deep cortical bone. These findings may lead to elucidation of drug targets to prevent and treat *S. aureus* chronic osteomyelitis.

ACKNOWLEDGEMENTS: Gayle Schneider .This work was funded by grants from AOTrauma & NIH (T32AR053459, P30 AR061307, P50 AR054041).

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<th>Figure 1A</th>
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<td>Toluidine blue stained 1s section of <em>S. aureus</em> (arrow) infiltrating deep into cortical bone (IEM labeling on this area).</td>
<td>Low magnification IEM image of BrdU positive deformed UAMS-1 cocci x 15,000. Note scalloping (arrows) of bone matrix by <em>S. aureus</em>.</td>
<td>IEM digital micrograph of rod-shaped bacteria with a diameter of 0.36μ x the leading edge of bacterial infiltration x 40,000.</td>
<td>High magnification digital micrograph of 1C (boxed area) identifies BrdU labeled chromosome tagged with 12 nm gold (dark round) particles (red arrows) x 200,000.</td>
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Figure 1A: Toluidine blue stained 1s section of *S. aureus* (arrow) infiltrating deep into cortical bone (IEM labeling on this area).