**Intracellular Staphylococcus aureus Infection: In Vivo Evidence for Chronic Osteomyelitis Disease**

**INTRODUCTION:** A broad range of bacterial species have been isolated in many cases of human osteomyelitis. *Staphylococcus aureus* (*S. aureus*) has been identified as the principle causative pathogen in 80% of these cases. Staphylococcal bone infections can originate from direct penetration of the microorganism into bones after trauma or surgery. Several groups, including ours, have shown that *S. aureus* can be internalized by different types of cells (such as osteoblasts). *S. aureus* can survive within osteoblasts and ultimately escape out of the cells. This internalization provides a means by which the bacteria can evade the host immune system and most conventional antibiotic therapies which may contribute to the recurrence of bone infection. The main objective of this study was to determine whether intracellular *S. aureus* can induce bone infection in vivo.

**HYPOTHESIS:** We hypothesize that intracellular *S. aureus* within osteoblasts contributes to the pathogenesis of *S. aureus* infection.

**SPECIFIC AIMS:** (i) Develop an in vitro co-culture model of rat osteoblasts and *S. aureus*. (ii) Determine whether intracellular *S. aureus* can induce bone infection in an open fracture rat model.

**METHODS:** An osteoblast- *S. aureus* co-culture model was established to obtain infected osteoblasts (Fig. 1). Rat osteoblasts were infected with *S. aureus* at 500:1 multiplicity of infection (MOI) for 2 hours. Infected osteoblasts were obtained after washing off non-adherent bacteria and incubating with gentamicin to kill extracellular *S. aureus*. Gentamicin is known not to affect the intracellular bacteria. Next, an open femur fracture model was created using Sprague-Dawley rats. The animal's femur was fractured, fixed using a stainless steel Kirschner wire (K-wire), and injected, at the fracture site, with rat osteoblasts (un-infected as control) or infected osteoblasts of 10^6 colony forming unit (CFU)/0.1 ml and 10^7 CFU/0.1 ml. Rats were euthanized on post-operative day 21. Samples of blood, K-wire, lymph node, bone (femur), and muscle were collected to determine the infection rate and local inflammatory response. Explanted K-wires were rolled over blood agar plates for bacterial culturing. Complete blood count was carried out (data not shown). X-ray radiographs were also taken on post-operative day 0 and day 21.

**RESULTS & DISCUSSION:** Quantitative culturing of bone and muscle tissue homogenates was conducted to determine infection (Fig. 2). The two animal groups injected with infected osteoblasts had high infection outcomes, and higher CFUs were observed on their bone samples compared to their muscle samples. By contrast, the animal group injected with un-infected osteoblasts (control) had no colony growth. Meanwhile, gross observation reports documented the presence of pus at the sites of fractures in the animal groups injected with infected osteoblasts while no pus in the control group. These infection outcomes were in consistent with the K-wire experiments where heavy growth of *S. aureus* was observed in the animal groups injected with infected osteoblasts and no growth in the control group (Fig. 3). In addition, no signs of septicemia were detected because no bacterial growth was observed in all blood samples cultured for 24 hours on blood agar plates. This suggests that the infection in the animals injected with infected osteoblasts was localized to the fracture sites.

The host immune responses to intracellular *S. aureus* bone infection are not fully understood. In this study, isolated lymphocytes were labeled with different fluorochrome labeled antibodies specific to CD4^+^ T-cells, CD8^+^ T-cell, dendritic cells, B-cells, natural killer cells, and macrophages. Dominating macrophages, B-cells, and CD4^+^ T-cells (38%, 22%, and 12%, respectively, out of 80% live cells) were found in the lymph nodes of animals infected with infected osteoblasts. These data confirmed the observations made by other research groups showing that macrophages and other polymorphonuclear cells are actively recruited to the site of infection and often are the first line of defense against the invading microbes. Activation of macrophages and CD4^+^ T-cells may have led to the priming of the cell-mediated immune response that is specific against intracellular pathogens.

**CONCLUSIONS:** This work provided evidence that intracellular *S. aureus* can induce bone infection in vivo and may be responsible for chronic infection. *S. aureus* was internalized and survived within human cells like osteoblasts. Dominating macrophages, B-cells, and CD4^+^ T-cells were observed in the lymph nodes in infected animals; this may indicate that cell-mediated immune response plays a major role against intracellular pathogens. In upcoming studies, we will develop advanced therapies targeting intracellular *S. aureus* thereby reducing bone infection.

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