Intracellular *Staphylococcus aureus*

A MECHANISM FOR THE INDOLENCE OF OSTEOMYELITIS

J. K. Ellington, M. Harris, L. Webb, B. Smith, T. Smith, K. Tan, M. Hudson

From Wake Forest University School of Medicine, Winston Salem, USA

Staphylococcus aureus is the bacterial pathogen which is responsible for approximately 80% of all cases of human osteomyelitis. It can invade and remain within osteoblasts. The fate of intracellular *Staph. aureus* after the death of the osteoblast has not been documented.

We exposed human osteoblasts to *Staph. aureus*. After infection, the osteoblasts were either lysed with Triton X-100 or trypsinised. The bacteria released from both the trypsinised and lysed osteoblasts were cultured and counted. Colonies of the recovered bacteria were then introduced to additional cultures of human osteoblasts.

The number of intracellular *Staph. aureus* recovered from the two techniques was equivalent. *Staph. aureus* recovered from time zero and 24 hours after infection, followed by lysis/trypsinisation, were capable of invading a second culture of human osteoblasts.

Our findings indicate that dead or dying osteoblasts are capable of releasing viable *Staph. aureus* and that *Staph. aureus* released from dying or dead osteoblasts is capable of reinfecting human osteoblasts in culture.

Osteomyelitis is an infection of bone which may result from haematogenous seeding, surgical contamination leading to the inoculation of bacteria into bone, the spread of infection from an adjacent area or injury to a limb with the associated loss of the protective soft-tissue envelope.[1] Early diagnosis and aggressive treatment in its early stages will reduce the risks of chronic infection. This is particularly relevant to the patient with compromised host defences, as occurs in diabetes mellitus, peripheral vascular disease, AIDS, etc.

Chronic osteomyelitis is difficult to eradicate without aggressive surgical debridement in combination with the appropriate antibiotic therapy. The chronic form of osteomyelitis is generally associated with abnormal local bone morphology and a locally compromised blood supply. Surgical debridement associated with the implantation of antibiotic beads[2] and the administration of long-term intravenous antibiotics have become accepted methods of treatment, albeit without consistent success. Rarely is a therapeutic level of antibiotics achieved locally owing to the compromise of local blood supply and bone-tissue perfusion. Additionally, 'therapeutic levels' of antibiotic may be insufficient to arrest bacterial growth in its nascent adherent state.[3-5]

Staphylococcus aureus has several characteristics which facilitate its role as a common pathogen in human osteomyelitis. It has micro-organism-specific cell-surface adhesion molecules which assist its binding to bone.[6-13] It also secretes toxins leading to local bone resorption.[14] *Staph. aureus* not only colonises the matrix of bone, but also incorporates itself within the osteoblasts (Fig. 1) both in vitro[15-17] and in vivo.[18] This process of intracellular incorporation may be one of the critical mechanisms of its resistance to intravenous antibiotics, and its resistance to host defences including engulfment by white cells.

Although there has been extensive work detailing the mechanisms of invasion of normal mouse and human osteoblasts by *Staph. aureus*,[15-19] and the immune response of the host,[20,21] the fate of intracellular *Staph. aureus* after the death of the osteoblast has, to date, not been described. In previous studies, the membrane of the osteoblast was lysed with detergent and intracellular pathogens were removed. This method, however, does not mimic the natural process of the death of osteoblasts. Our aim therefore was to document the fate of the intracellular bacteria after a more natural cause of death of osteoblasts, creating a process which reflected the clinical situation more closely. Furthermore, once the intracellular *Staph. aureus* has been released, its

J. K. Ellington, MS
M. Harris, MD, FACS, Professor
L. Webb, MD, Professor
B. Smith, PhD
T. Smith, PhD
Department of Orthopaedic Surgery, Wake Forest University School of Medicine, Medical Centre Boulevard, Winston Salem, North Carolina 27103, USA.

K. Tan, PhD
Department of Life Science, Winston Salem State University, 601 Martin Luther King Drive, Winston Salem, North Carolina 27110, USA.

M. Hudson, PhD
Department of Biology, University of North Carolina at Charlotte, 9201 University City Boulevard, Charlotte, North Carolina 28223, USA.

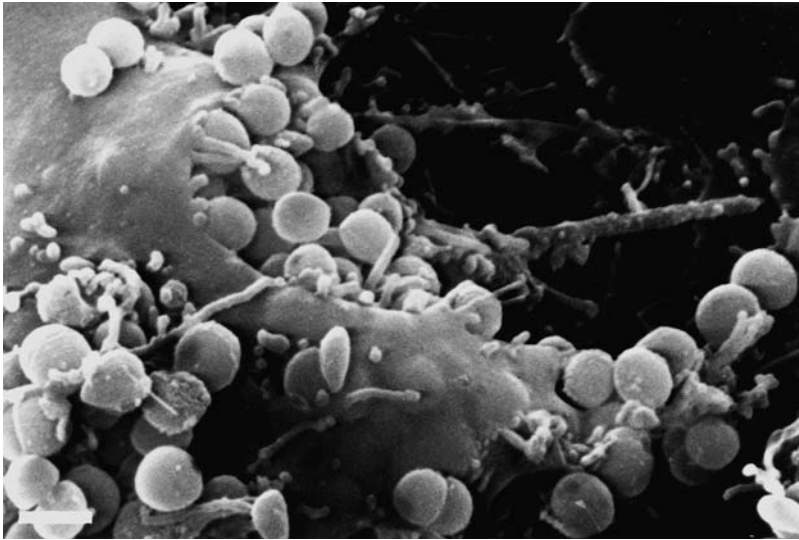Correspondence should be sent to Professor M. Harris.

Fig. 1

Scanning electron micrograph of an osteoblast engulfing *Staph. aureus*.

ability to infect other osteoblasts has not been described. The method chosen to induce death of osteoblasts was nutrient starvation, a process similar to that observed in necrosis. By placing osteoblasts directly on to bacterial media, the necessary nutrients and environment required by osteoblasts such as serum, vitamins and buffer are absent. On the death of the osteoblast, if *Staph. aureus* survives, the bacteria are released directly on to bacterial media and can be quantified. These data may help to explain the observations in the clinical setting, whereby there are symptom-free intervals between episodes of activity, which is a characteristic of chronic osteomyelitis.

Materials and methods

Bacterial strain. We used *Staph. aureus* strain UAMS-1 (ATCC 49230) which is a human osteomyelitis clinical isolate.[22]

Normal human osteoblast cultures. Normal human osteoblasts (Clonetics, San Diego, California) were purchased and propagated according to the guidelines provided by the manufacturer. Cells were seeded in 25 cm^2 flasks and incubated in a humidified incubator at 37˚C in a 5% CO_2 atmosphere in growth medium containing 10% fetal calf serum, ascorbic acid and gentamicin. Between five and nine days, after they had reached approximately 80% confluency, they were removed from the flasks by the use of 0.025% trypsin-0.01% EDTA, washed in growth medium, and seeded into six-well plates. The osteoblasts were then maintained in growth medium (OBGM) and grown as above until they reached confluency, between six and seven days. The growth medium was changed every 48 hours after seeding.

Invasion assay. *Staph. aureus* were grown overnight (16 hours) in 5 ml of tryptic soy broth (TSB) in a shaking water bath at 37˚C. The bacteria were harvested by centrifugation for ten minutes at 4300 *g* at 4˚C, and washed twice in 5 ml of Hank's balanced salt solution (HBSS; pH 7.4; Sigma

Chemical Co, St Louis, Missouri). The pellet was then resuspended in 5 ml of OBGM without antibiotics or antimycotics (Abx/Amx). Confluent cell layers of osteoblasts were washed three times with 4 ml of HBSS to remove growth media containing Abx/Amx. This is the accepted method to ensure removal of Abx/Amx since all bacterial invasion assays use this technique. Cultures of osteoblasts were then infected at a multiplicity of infection (MOI) of 250:1 with *Staph. aureus*. After infection for 45 minutes at 37˚C, cell cultures were washed and then incubated with OBGM containing 25 µg/ml of gentamicin to kill the remaining extracellular *Staph. aureus.* Gentamicin cannot penetrate eukaryotic cells.[23] The cultures were then washed as described and subsequently lysed by the addition of 1.2 ml of 0.1% Triton X-100 (Fisher Biotech, Fair Lawn, New Jersey) followed by incubation for five minutes at 37˚C. In order to quantify the number of intracellular bacteria, suspension dilutions of the lysates were plated in triplicate on tryptic soy agar (TSA) plates followed by incubation at 37˚C overnight.

Assay to determine the fate of intracellular bacteria after the death of osteoblasts. Following the previously described infection protocol, instead of detergent lysis, infected osteoblast cultures were trypsinised. We added 0.025% trypsin-0.01% EDTA to the culture wells, and after digestion of the matrix, approximately five minutes, osteoblasts were in suspension. The trypsinised cultures were then transferred to TSA plates, allowed to die because of lack of nutrients, and placed in an incubator at 37˚ overnight. The following day, viable, previously intracellular bacteria, were quantified.

Assay to determine the ability of *Staph. aureus* to re-invade osteoblasts. After either detergent lysis or death of osteoblasts via trypsinisation, previously intracellular bacteria were harvested and grown overnight to achieve equivalent numbers for infection and then used to infect other sterile osteoblast cultures. After infection, including incubation in gentamicin to kill extracellular bacteria, the osteob-

Table I. Comparison of techniques to recover intracellular *Staph. aureus*

Time course of infection (hrs)	Number of intracellular *Staph. aureus* (cfu/well)	
	Lysed (control)	Trypsinised
0	1.49×10^5	1.5×10^5
24	1.72×10^4	1.68×10^4
48	7.16×10^3	7.49×10^3

Table II. The ability of *Staph. aureus* (cfu/well) to re-invade osteoblasts after trypsinisation.

Time zero (control)	Re-infection from time zero	Re-infection from infection at 24 hrs
1.49×10^5	9.79×10^4	9.98×10^4

last cultures were lysed or trypsinised, and plated on TSA plates for quantification of intracellular bacteria.

Statistical analysis. The results were analysed by one-way analysis of variance, with $p < 0.05$ considered to be significant, using mean values with the standard deviation.

Results

Viable *Staph. aureus* is released from dead human osteoblasts. Data generated utilising a single osteoblast culture are presented and are representative of the results obtained with three osteoblast cultures. In order to assess whether dead osteoblasts released viable *Staph. aureus* cells, osteoblast cultures and all subsequent cultures were infected with *Staph. aureus* at an MOI of 250:1 and quantified either by lysis or trypsin treatment. Lysed and trypsinised cultures released 1.49×10^5 cfu/well and 1.5×10^5 cfu/well of intracellular *Staph. aureus* cells, respectively, at time zero, 1.72×10^4 cfu/well and 1.68×10^4 cfu/well at infection at 24 hours, respectively, and 7.16×10^3 cfu/well and 7.49×10^3 cfu/well at infection at 48 hours, respectively (Table I). Differences owing to osteoblast treatment were not statistically significant. These data indicate that dead or dying osteoblast cultures release viable *Staph. aureus* cells, equivalent to those osteoblast cultures lysed with detergent.

In addition, subsequent to either culture lysis or trypsinisation, small colony variants (SCV) were observed but there was no predictable pattern of their appearance. Previously intracellular *Staph. aureus* cells were capable of re-invading sterile human osteoblast cultures.

Cultures infected with previously intracellular *Staph. aureus* from infections at 45 minutes and 24 hours contained 9.79×10^4 cfu/well and 9.98×10^4 cfu/well, respectively. This number for the control osteoblast cultures exposed to triton was 1.49×10^5 cfu/well (Table II), which is the number of *Staph. aureus* cells recovered after infection of osteoblasts with bacteria which had not been in contact with osteoblasts. There was no statistically significant difference between the control numbers of recovered intracellular *Staph. aureus* and those obtained after re-infection.

These data indicate that the ability of *Staph. aureus* to invade human osteoblasts is not compromised after being previously exposed to the intracellular environment of osteoblasts. In addition, there was no difference between the post-infection recovery methods of *Staph. aureus*.

Discussion

Our study suggests that two possible mechanisms are responsible for the relapsing and chronic clinical presentation of osteomyelitis. First, osteoblasts, killed both by lysis or nutrient starvation, are capable of releasing viable *Staph. aureus* cells, and furthermore, these newly-released cells are able to re-infect additional cultures of normal human osteoblasts. The method used to re-infect osteoblasts required overnight growth of *Staph. aureus*. Although this is not physiological, in that released bacteria are not in immediate contact with other osteoblasts, the fact that previously intracellular *Staph. aureus* are capable of re-infection is nonetheless intriguing. In the in vivo setting, *Staph. aureus* would not need to re-invade instantly but could subsequently.

It is generally believed that the recalcitrant pathological changes found with persistent osteomyelitis is located within areas of dead bone in which the osteocytes and osteoblasts are no longer present. Our data support this clinical finding in that osteoblasts which are infected with *Staph. aureus* harbour the bacterium and when the osteoblasts die, release viable *Staph. aureus* cells. This would perpetuate the infection and, in addition, those previously intracellular *Staph. aureus* cells would then be capable of re-infecting healthy bone.

Applying these findings to the clinical setting gives us a better understanding of the challenges of antibiotic treatment for osteomyelitis. Specifically, most antibiotics currently used in primary treatment, such as vancomycin, cefazolin and gentamicin, do not penetrate eukaryotic cells. Also, the extent to which the intracellular bacteria remain metabolically active is unknown, (i.e. in the SCV state). If they are dormant, they may be further protected from the action of many antibiotics even if they could be delivered to the intracellular compartment.

Additionally, if viable micro-organisms are released from dead osteoblasts and these are capable of re-infecting in vivo, these findings suggest the mechanism for reactivating osteomyelitis.

Our study presents support for a mechanism whereby infection with *Staph. aureus* persists in bone in a state of quiescence. Once *Staph. aureus* invades the osteoblast, its intracellular location is a relative sanctuary against standard chemotherapeutic regimens and the humoral immune response of the host. In addition, these intracellular bacteria may be released by processes which result in the death of host osteoblasts. This could be in the form of local or systemic trauma, a surgical intervention leading to reactivation of the clinically dormant but previously infected bone. This mechanism gives new insight into the latency and recrudes-

cence of clinical disease after apparent 'cure'. 'Remission' may be a more appropriate description of the state of clinically quiescent osteomyelitis.

References

1. **Waldvogel FA, Medoff G, Swartz MN**. Osteomyelitis: a review of clinical features, therapeutic considerations and unusual aspects. *N Eng J Med* 1970;282:198-206.

2. **Calhoun MD, Mader JT**. Treatment of osteomyelitis with a biodegradable antibiotic implant. *Clin Orthop* 1997;341:206-14.

3. **Gristina A G, Jennings RA, Naylor PT, Myrvik QN, Webb LX**. Comparative in vitro antibiotic resistance of surface colonizing coagulase negative staphylococci. *Antimicrob Agents Chemother* 1989;33:813-6.

4. **Webb LX, Holman J, de Araujo B, Zaccaro DJ, Gordon ES**. Antibiotic resistance in staphylococci adherent to cortical bone. *J Orthop Trauma* 1994;8:28-33.

5. **Gristina AG, Habgood CD, Webb LX, Myrvik QN**. Adhesive colonisation of biomaterials and antibiotic resistance. *Biomaterials* 1987;8:423-6.

6. **Boden MK, Flock JI**. Cloning and characterisation of a gene for a 19 kDa fibrinogen-binding protein from *Staphylococcus aureus*. *Mol Microbiol* 1994;12:599-606.

7. **Cheung AI, Projan SJ, Edelstein RE, Fischetti VA**. Cloning expression and nucleotide sequence of a *Staphylococcus aureus* gene (*fbpA*) encoding a fibrinogen-binding protein. *Infect Immun* 1995;63:1914-20.

8. **Flock JI, Froman G, Jonsson K, et al**. Cloning and expression of the gene for a fibronectin-binding protein from *Staphylococcus aureus*. *EMBO J* 1987;6:2351-7.

9. **Jonsson K, Signas C, Muller HP, Lindberg M**. Two different genes encode fibronectin binding proteins in *S. aureus*: the complete nucleotide sequence and characterization of the second gene. *Eur J Biochem* 1991;202:1041-8.

10. **McDevitt D, Francois P, Vaudaux P, Foster TJ**. Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*. *Mol Microbiol* 1994;11:237-48.

11. **McGavin MH, Krajewska-Pietrasik D, Ryden C, Hook M**. Identification of a *Staphylococcus aureus* extracellular matrix-binding protein with broad specificity. *Infect Immun* 1993;61:2479-85.

12. **Park PW, Rosenbloom J, Abrams WR, Mecham RP**. Molecular cloning and expression of the gene for elastin-binding protein (ebpS) in *Staphylococcus aureus*. *J Biol Chem* 1996;271:15803-9.

13. **Patti JM, Jonsson H, Guss B, et al**. Molecular characterization and expression of a gene encoding a *Staphylococcus aureus* collagen adhesion. *J Biol Chem* 1992;267:4766-72.

14. **Nair S, Song Y, Mehgji S, et al**. Surface-associated proteins from *Staphylococcus aureus* demonstrate potent bone resorbing activity. *J Bone Miner Res* 1995;10:726-34.

15. **Ellington JK, Reilly SS, Ramp WK, et al**. Mechanisms of Staphylococcus aureus invasion of cultured osteoblasts. *Microb Pathog* 1999;26:317-23.

16. **Hudson MC, Ramp WK, Nicholson NC, Williams AS, Nousiainen MT**. Internalization of *Staphylococcus aureus* by cultured osteoblasts. *Microb Pathog* 1995;19:409-19.

17. **Jevon M, Guo C, Ma B, et al**. Mechanisms of internalization of *Staphylococcus aureus* by cultured human osteoblasts. *Infect Immun* 1999;67:2677-81.

18. **Reilly SS, Hudson MC, Kellam JF, Ramp WK**. In vivo internalization of *Staphylococcus aureus* by embryonic chick osteoblasts. *Bone* 2000;26:63-70.

19. **Ellington JK, Elhofy A, Hudson MC**. Involvement of mitogen-activated protein kinase pathways in *Staphylococcus aureus* invasion of normal osteoblasts. *Infect Immun* 2001;69:5235-42.

20. **Bost KL, Bento JL, Ellington JK, Marriott I, Hudson MC**. Induction of colony-stimulating factor expression following *Staphylococcus* or *Salmonella* interaction with mouse or human osteoblasts. *Infect Immun* 2000;68:5075-83.

21. **Bost KL, Ramp WK, Nicholson NC, et al**. *Staphylococcus aureus* infection of mouse or human osteoblasts induces high levels of interleukin-6 and interleukin-12 production. *J Infect Dis* 1999;180:1912-20.

22. **Gillaspy AF, Hickmon SB, Skinner RA, et al**. Role of the accessory gene regulator (agr) in pathogenesis of staphylococcal osteomyelitis. *Infect Immun* 1995;63:3373-80.

23. **Wilson CB, Jacobs RF, Smith AL**. Gentamicin is inactive against bacteria sequestered in the eucaryotic intracellular environment. Cellular antibiotic pharmacology. In *Seminars in perinatology* 1982;6:205-13.