Published in final edited form as:

J Biomed Mater Res A. 2010 November ; 95(2): 574–579. doi:10.1002/jbm.a.32890.

UV- Killed *Staphylococcus aureus* Enhances Adhesion and Differentiation of Osteoblasts on Bone-associated Biomaterials

Shankari N. Somayaji^a, Yvette M. Huet^a, Helen E. Gruber^b, and Michael C. Hudson^a

^a University of North Carolina at Charlotte

^b Carolinas Medical Center, Charlotte

Abstract

Titanium alloys (Ti) are the preferred material for orthopaedic applications. However, very often, these metallic implants loosen over a long period and mandate revision surgery. For implant success, osteoblasts must adhere to the implant surface and deposit a mineralized extracellular matrix. Here, we utilized UV-killed *Staphylococcus aureus* as a novel osteoconductive coating for Ti surfaces. *S. aureus* expresses surface adhesins capable of binding to bone and biomaterials directly. Furthermore, interaction of *S. aureus* with osteoblasts activates growth factor-related pathways that potentiate osteogenesis. While UV-killed *S. aureus* cells retain their bone-adhesive ability, they do not stimulate significant immune modulator expression. All of the above properties were utilized for a novel implant coating so as to promote osteoblast recruitment and subsequent cell functions on the bone-implant interface. In the present study, osteoblast adhesion, proliferation, and mineralized extracellular matrix synthesis were measured on Ti surfaces coated with fibronectin with and without UV-killed bacteria. Osteoblast adhesion was enhanced on Ti alloy surfaces coated with bacteria compared to uncoated surfaces while cell proliferation was sustained comparably on both surfaces. Osteoblast markers such as collagen, osteocalcin, alkaline phosphatase activity and mineralized nodule formation were increased on Ti alloy coated with bacteria compared to uncoated surfaces.

Keywords

Titanium implant surfaces; Osseointegration; *Staphylococcus aureus*; Calvarial osteoblasts; Osteoblast differentiation

Introduction

Artificial joints and orthopedic implants are used to repair or restore function to damaged and diseased tissue. Conventionally, such devices are constructed of metals such as titanium and cobalt-chromium which are relatively non-immunogenic, and corrode slowly when placed in contact with host tissues [1]. Despite the successful application of the metallic implants, one of the significant problems encountered with such biomaterials is inadequate intimate apposition of bone to the implant surface (osseointegration) [2–4]. This is often due to the lack of ability of bone to bind to the implant material or as a result of a fibrous layer being deposited at the interface as opposed to an osseous layer. This may result in slow fixation of implant and subsequent loosening over a long period. As a result, the functional lifespan of artificial prostheses is estimated at about 15 years, which makes them unsuitable

Correspondence name and address: Dr. Helen E. Grube, Director, Biology Division, Orthopaedic Research Laboratory, Cannon Research Center, Room 304, Carolinas Medical Center, PO Box 32861, Charlotte, N.C. 28232, Telephone and fax numbers: 704-355-5665, 704-355-5620 (fax), helen.gruber@carolinashealthcare.org.

for active and younger patients [1]. Enhancement of biomaterials is therefore necessary in order to increase the implant success rate and extend their functional lifespan.

Osseointegration is desirable for the clinical success of implants because it provides a functional connection between the implant and bone by strong bonding of the surrounding bone tissue on implant surfaces. Formation of a proper interface requires immediate immobilization of the implant since any implant motion can also cause release of particle debris which can initiate a deleterious inflammatory response [5]. At the cellular level, proper interface formation requires osteoblast adhesion and subsequent functions pertinent to osteogenesis [6].

Primary mouse osteoblasts undergo an ordered sequence of differentiation which results in the formation of a mineralized extracellular matrix (ECM) that consists of type I collagen, alkaline phosphatase and specialized noncollagenous proteins such as osteocalcin [7]. Successful communication between osteoblasts and the ECM is mediated by a family of cell surface receptors known as integrins and these receptors stimulate signal transduction pathways and mediate osteoblast adhesion, differentiation, and bone morphogenesis [8–10].

Staphylococcus aureus is a gram-positive bacterium capable of binding to and colonizing tissues via a collection of cell-surface adhesins referred to as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). MSCRAMMs recognizing collagen, fibronectin mediate adherence of *S. aureus* to bone and biomaterials coated with host proteins [8]. There is evidence that *S. aureus* can bind to cells [9] and biomaterials [10] directly. Interaction of *S. aureus* with host cells via MSCRAMMs can induce signal transduction [9]. UV-killed *S. aureus* can attach efficiently to osteoblasts and are not potent inducers of immune modulator expression by osteoblasts, unlike viable *S. aureus* cells [11].

In the present work, we investigated the effects of coating Ti surfaces with dead bacteria on osteoblast functions pertinent to new bone formation. Cell adhesion, proliferation as well as synthesis and mineralization of the extracellular matrix were examined using mouse calvarial osteoblasts, a well-characterized, cell culture model. Osteoblast differentiation on Ti-6Al-4V alloy surfaces coated with fibronectin (Ti-Fn) surfaces was compared to Ti-Fn surfaces coated with UV-killed *S. aureus* (Ti-Fn-SA). Fibronectin was chosen to pre-coat the alloy surface as it is pre-adsorbed on all biomaterials following in vivo insertion and is considered to be the model host protein that promotes *S. aureus* attachment to biomaterial surfaces [6].

Methods

Normal mouse osteoblast cell culture

Primary osteoblasts were isolated from mouse neonatal calvariae by sequential digestion with collagenase and protease as described previously [12]. Osteoblasts were pooled in mouse osteoblast growth medium (OBGM), seeded in 25 cm² flasks and incubated at 37°C in a 5% CO₂ atmosphere until they reached confluence (6–7 days). Cells were then washed with 10 ml of Hank's balanced salt solution (HBSS), treated with 5 ml of trypsin (Sigma) for 5 min at 37°C. Following centrifugation, osteoblasts were seeded on to the biomaterial surfaces as described below.

Biomaterial preparation

12- well polystyrene tissue culture dishes were coated with the titanium alloy Ti-6Al-4V (hereby abbreviated as Ti) (Teledyne Allvac/Vasco, NC) using electron beam deposition (Varian 3125 electron beam evaporator). Ti-coated dishes were then sprayed with isopropanol and sterilized by exposure to ultraviolet radiation for 1 hr. The dishes were then

treated with osteoblast growth medium (OBGM) supplemented with mouse Fibronectin (hereby abbreviated as Fn) (Affiland Company, Belgium) at a concentration of 2 µg/ml for 24 hr at 37° C. Following incubation, plates were rinsed with HBSS to remove unbound Fn. Selected plates were incubated an additional 24 hr with different doses of UV-killed *S. aureus* – 1×10^6 , 10^7 and 10^8 numbers of bacteria per well. For all experiments, Ti-Fn coated plates with no bacteria (hereby abbreviated as Ti-Fn) served as controls for comparison with bacteria-coated surfaces (hereby abbreviated as Ti-Fn-SA). Following incubation, plates were rinsed with HBSS to remove unbound bacteria. Osteoblasts subcultured from tissue culture flasks (as described above) were seeded into Ti-Fn +/- dead *S. aureus*-coated 12 well dishes at a density of 2×10^4 cells/cm² and incubated at 37°C at 5% CO₂ for various time points.

Bacterial strain, growth conditions and UV-killing

S. aureus strain UAMS-4 is a co-isogenic mutant of osteomyelitis-clinical isolate UAMS-1 (ATCC 49230) with an inactivated *agr* (accessory gene regulator) locus. The *agr* locus is responsible for expression of cell surface and secreted virulence factors and is inactive during exponential growth where cell surface adhesins are maximally expressed. Entry into stationary phase results in activation of *agr* and down-regulation of cell surface adhesins. Therefore, *agr* mutants retain the ability to colonize host tissue, but demonstrate decreased virulence [13,14]. UAMS-4 was grown overnight in 15 ml of tryptic soy broth (TSB) supplemented with erythromycin (5 µg/ml) (Em5) at 37°C with aeration. Ten ml of this overnight culture was then transferred to 1 liter of TSB-Em5 and grown for 3 hr in conditions as described above. Bacteria were harvested in the resulting exponential phase to ensure maximal expression of cell surface adhesions. Cells were harvested by centrifugation at $4300 \times g$ at 4°C for 10 min and washed in HBSS. Bacteria were killed by exposure to short-wave (254 nm) ultraviolet radiation for 5 min and centrifuged at conditions described above. Subsequently, bacteria were suspended in OBGM to give the final concentration of 1 to 5×10^8 colony-forming units (CFU/ml). To verify successful killing, aliquots of the cell suspension were plated on tryptic soy agar (TSA) supplemented with erythromycin (10 µg/ml) (Em10), followed by incubation for 24 h at 37°C.

Osteoblast attachment

Attachment of osteoblasts to Ti in the presence and absence of UV-killed *S. aureus* was analyzed using phosphor-screen autoradiography [15]. Before subculturing onto Ti-Fn +/- *S. aureus* surfaces, osteoblasts were incubated with methionine-free OBGM supplemented with 5 µCi/ml ³⁵S-methionine. Following 18 h incubation with the labeling medium, cells were removed from flasks using 0.05% trypsin/0.01% EDTA, washed in OBGM, and seeded into culture dishes prepared as described above. Osteoblasts suspended in the labeling medium were seeded onto Ti-Fn and Ti-Fn-SA coated plates and cell attachment was assessed at 1 and 36 h. At the indicated time points, culture plates were rinsed 3 times with HBSS, air dried, and exposed to a phosphor screen for 2 hrs. Following exposure, the screen was analyzed using a Typhoon 8600 Variable Mode Phosphor Imager and the pixel intensities, indicative of the relative number of attached osteoblasts, were measured using appropriate software (Molecular Dynamics, CA).

Osteoblast adhesion

Cell adhesion was assessed by reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to the formazan product (Promega, WI) [16]. Osteoblasts were incubated on Ti-Fn and Ti-Fn-SA surfaces for 1, 3, 7 and 10 days. At the indicated time points, culture medium was removed; the wells were washed with HBSS and replaced with OBGM without phenol red (Hyclone labs, UT). Twenty µl of MTS reagent was added to each well and incubated overnight at 37 °C in a

humidified atmosphere with 5% CO₂. The extent of osteoblast adhesion was determined by measuring the absorbance of the culture media at 540 nm in a plate reader.

Osteoblast proliferation

Osteoblast proliferation was assessed by measuring incorporation of ³H-thymidine into osteoblasts as previously described [17].

Insoluble matrix collagen synthesis assay

To determine the bone-matrix forming ability of osteoblasts, production of insoluble collagen was measured to assess the bone matrix-forming ability of osteoblasts as previously described [18]. The absorbance of the released dye was measured spectrophotometrically at 540 nm.

Osteocalcin production

Osteoblasts were cultured in growth medium (OBGM) on Ti-Fn and Ti-Fn-SA-coated dishes for 7, 14 and 21 days. Cell culture media was collected at the indicated time points and osteocalcin production was measured in the culture media using an ELISA kit (Biomedical Technologies, Inc., MA) as described previously [19]. The measurement sensitivity was 1 ng/ml.

Measurement of alkaline phosphatase activity

Alkaline phosphatase activity was measured using an assay based on the hydrolysis of p-nitrophenyl phosphate (p-NPP) to p-nitrophenol (p-NP) previously described [20]. Alkaline phosphatase specific activity was expressed as nanomoles of product (p-nitrophenol) produced per milligram of protein.

Osteoblast mineralization

The degree of mineralization was determined using Alizarin Red S staining as described [21].

Statistical analysis

Data are reported as mean $\pm$ SEM of triplicate values from a single experiment. All experiments were repeated three times, except for the osteocalcin assay which was repeated twice, and yielded similar results. The statistical significance of all the results, except for the osteocalcin assay, was analyzed using a one way analysis of variance (ANOVA) with one repeated measure, followed by a Dunnett's test for a *post hoc* comparison (Sigmastat, SPSS Inc.). A *p* value of less than 0.05 was considered significant.

Results

Osteoblast attachment, adhesion and proliferation

Results from these studies demonstrate that using dead *S. aureus* enhances osteoblast attachment to thin films of titanium alloy in a dose dependent manner following 1 or 36 hours of incubation. The magnitude of attachment is dependent on the numbers of bacteria added. When pixel intensity was quantified by the phosphor imager, a significant increase in attachment was noted in osteoblasts cultured in the presence of the 10⁷ and 10⁸ doses of bacteria, following 36 hours of incubation (Fig. 1). Osteoblast adhesion on both surfaces was indirectly estimated by measuring the absorbance of the end product of MTS dye degradation, formazan. Figure 2 shows a dose-dependent, as well as kinetic, increase in the number of osteoblasts that adhere on to Ti-Fn surfaces coated with dead *S. aureus* compared

to those incubated on Ti-Fn surfaces. There is a significant increase in osteoblast adhesion in the presence of highest dose of bacteria following 1, 3 and 10 days of incubation. There were no significant differences in the proliferation rate of osteoblasts on Ti-Fn surfaces compared to Ti-Fn surfaces coated with different numbers of dead *S. aureus* bacteria at any time point studied (Fig. 3).

Extracellular matrix synthesis and mineralization

As shown in Figure 4, the kinetics of total collagen synthesis, as measured by staining with a dye specific for collagen, Sirius red F3B, is similar when osteoblasts were cultured for time periods of 3 and 6 days. After 9 days of incubation, however, collagen production was markedly increased in the presence of 10^7 and 10^8 doses of bacteria. As shown in Table 1, there is an at least 25-fold increase in the amount of osteocalcin produced from osteoblasts grown on Ti-Fn coated with the highest dose of *S. aureus* versus osteoblasts grown on Ti-Fn coated dishes. Osteocalcin secretion from osteoblasts incubated on Ti-Fn alone and Ti-Fn surface with 10^6 or 10^7 bacteria remained below the detection limits of the assay.

We next examined the ability of dead bacteria to induce successful mineralization activity from the osteoblasts. As shown in Figure 5, ALP activity of osteoblasts added on both Ti-Fn and Ti-Fn-SA-coated dishes increased in a time-dependent manner. However, significantly greater enzyme activity was observed in osteoblasts incubated on the Ti-Fn surface coated with 10^7 numbers of bacteria at day 6 compared with the Ti-Fn surface.

As shown in Figure 6, there is a dose-dependent increase in mineralization activity in osteoblasts grown on Ti-Fn-SA-coated surfaces compared to Ti-Fn group following 7, 14 and 21 days of incubation in the culture media. At all time periods, the highest dose of bacteria induces a significant increase in mineralization from osteoblasts grown on Ti-Fn-SA surface.

Discussion

Previous studies from our lab have demonstrated that UV-killed *S. aureus* retain the ability to attach efficiently to osteoblasts but, in contrast to live bacteria, do not induce the expression of immune modulators such as interleukin (IL)-6 and IL-12 [11]. Therefore, as *S. aureus* can tenaciously bind biomaterials, effectively colonize bone, and stimulate signal transduction in host cells, we examined the efficacy of UV-killed *S. aureus* as an osteoconductive coating for titanium alloy in the current study. For all experiments, the ability of dead *S. aureus* to enhance osteoblast adhesion, proliferation and matrix synthesis was compared to fibronectin (Fn)-coated Ti surfaces (Ti-Fn). Fn is a component of plasma that coats virtually any device implanted within the body and more importantly, osteoblasts have been demonstrated to bind Fn preferentially when compared to other serum proteins [6].

Osteoblast attachment and adhesion to the implant interface is a crucial pre-requisite for successful osseointegration of the implant [22–25]. Our results show that osteoblasts effectively attach to Ti-Fn surfaces, both coated and uncoated, within 1 h in vitro. However, osteoblasts attach significantly more on Ti surfaces coated with dead bacteria especially after 36 h of incubation (Fig. 1). Osteoblast adhesion, in contrast to osteoblast attachment, involves activation of transcription factors and formation of focal adhesion points which involves the cell membrane as well as proteins associated with the cytoskeleton (vinculin, talin) and ECM (fibronectin, type I collagen) [6]. As indicated in Fig. 2, we have demonstrated that osteoblasts adhere better on Ti-Fn-SA surfaces compared to Ti-Fn surfaces alone and sustain increased adherence up to 10 days in culture. This increase in osteoblast attachment and adherence can be explained by enhanced hydrophilicity of the Ti-

Fn surface coated with *S. aureus* as it has been established that surface hydrophilicity of a biomaterial surface greatly facilitates cell adhesion [6]. Metals, such as Ti alloy, have an oxide layer that is spontaneously deposited on all their surfaces and are therefore hydrophilic due to presence of hydroxyl groups present on the oxide layer [26]. It is possible that the surface hydrophilicity is compromised by coating with fibronectin as would be the case in both Ti-Fn as well as Ti-Fn-SA substrates. However, coating the Ti-Fn alloy with bacteria renders the surface more hydrophilic because bacterial surfaces in aqueous solutions are always ionized [27]. Moreover, the cell wall of *S. aureus* is slightly more negatively charged because of the presence of teichoic acids in their cell walls [28]. As a result, Ti-Fn-SA surfaces by being considerably more hydrophilic than Ti-Fn alone facilitate enhanced binding of osteoblasts.

The strength of the ECM and osteoblast interactions in the presence and absence of dead *S. aureus* can also be a contributing factor for observed differences in cell adhesion on both substrates. Successful formation of bone requires integrin-mediated communication between osteoblasts and components of the extracellular matrix (ECM), such as fibronectin and type I collagen [29]. It is known that Fn binding to the $\alpha_5\beta_1$ - integrin receptor, present on the Osteoblast surface, is associated with a dissociation constant of $K_d = 8 \times 10^{-7}$ M. The strength of this interaction is much lower than that of fibronectin-binding receptor (FnBP) - mediated binding of *S. aureus* with fibronectin, which is irreversible with a dissociation constant of $K_d = 1.8 \times 10^{-9}$ M [9]. Also, in addition to FnBP, other MSCRAMMs present on the *S. aureus* surface may subsequently interact with other ligands of the extracellular matrix as the matrix production ensues, further 'cementing' the osteoblasts in a cohort of multivalent ECM-cell interactions. Therefore, single process interaction of fibronectin (Fn) with integrin on the osteoblast surface is weaker in strength than the array of processes possible between osteoblasts and ECM potentiated by the surface receptors of the interlaying bacteria.

Osteoblast differentiation involves a temporal sequence of events characterized by a) active proliferation of osteoblasts, which is accompanied by the onset of expression of the enzyme alkaline phosphatase b) synthesis of high levels of several collagens (type I primarily) and other non collagenous proteins such as osteocalcin and c) deposition of the mineral component, hydroxyapatite (CaPO_4) in the extracellular matrix [7]. We measured rates of proliferation of osteoblasts cultured on both Ti-Fn+/- *S. aureus* by liquid scintillation analysis. In spite of increase in cell attachment and adhesion on Ti-Fn-SA surfaces compared to Ti-Fn alone, osteoblast proliferation occurred equally well on both substrates, revealing no significant differences at all times of incubation. One possible explanation is the existence of a restriction point in the developmental sequence of an osteoblast where a decrease in osteoblast proliferation is coupled with the subsequent induction of genes associated with matrix maturation and mineralization [7]. Therefore, proliferation of osteoblasts grown on Ti-Fn-SA may be down-regulated following the onset of a more differentiated phenotype on the alloy surface (Figs. 4–6).

We next examined the synthesis of collagen and osteocalcin which are the key proteinaceous constituents of the extracellular matrix synthesized by osteoblasts. In this study, osteoblasts grown on Ti-Fn surfaces coated with dead *S. aureus* were found to express insoluble collagen at similar levels when compared to the uncoated levels up to 6 days in culture, followed by a significant increase at later time points (Fig. 4). Osteocalcin production is also significantly upregulated on bacteria-coated alloy compared to Ti-Fn surface up to 3 weeks of culture (Table 1). Given that cell adhesion, a necessary pre-requisite for subsequent cell functions on biomaterials was enhanced in the presence of the bacteria, it is corroborative that synthesis of an extracellular matrix on bone-biomaterial interface is increased on the Ti-Fn-SA surface.

Another aspect of osteoblast differentiation is the mineralization of bone matrix which occurs by deposition of apatitic calcium phosphates along the collagen framework aided by osteocalcin and ALP. Our findings demonstrate that the difference in ALP activity between Ti-Fn-SA and Ti-Fn alloy was maximal on day 6 and is insignificant at later time points (Fig. 5). As shown in Fig. 6, osteoblasts also showed a dose-dependent and kinetic increase of apatite deposition on surfaces coated with dead bacteria as early as day 7, with sustained mineralization activity demonstrated by osteoblasts on both surfaces at day 14 and 21. One possible explanation for enhanced mineral deposition in the presence of dead bacteria is that the presence of hydroxyl groups on *S. aureus* cell walls facilitates the deposition of calcium and phosphate ions on Ti-Fn-SA surfaces.

It is well documented that increasing surface roughness of implants is greatly beneficial to their biological performance as osteoblast differentiation is enhanced on rougher biomaterial surfaces [30]. The findings from our study suggest that coating the Ti surfaces with dead bacteria could render the surface 'rougher' compared to Ti alloy alone which can explain the enhanced differentiation of osteoblasts on bacterial-coated surfaces versus uncoated surfaces.

In conclusion, Ti-Fn alloy coated with dead *S. aureus* has been demonstrated to promote osteoblast adhesion, sustain cell proliferation and enhance the expression of a mineralized extracellular matrix. Current studies in the laboratory are utilizing purified *S. aureus* cell wall components as a biomaterial coating instead of UV-killed bacteria. Further investigations are also warranted to determine the osseointegration of Ti-Fn-SA alloy in animal models.

Acknowledgments

This work was supported by grant R21AR049263 from the National Institutes of Health (M.C.H.)

References

1. Long M, Rack HJ. Titanium alloys in total joint replacement--a materials science perspective. *Biomaterials*. 1998; 19(18):1621–39. [PubMed: 9839998]
2. Davies JE, Lowenberg B, Shiga A. The bone-titanium interface in vitro. *Journal of biomedical materials research*. 1990; 24(10):1289–306. [PubMed: 2283350]
3. Berube P, Yang Y, Carnes DL, Stover RE, Boland EJ, Ong JL. The effect of sputtered calcium phosphate coatings of different crystallinity on osteoblast differentiation. *Journal of periodontology*. 2005; 76(10):1697–709. [PubMed: 16253092]
4. Arola DD, McCain ML. Abrasive waterjet peening: a new method of surface preparation for metal orthopedic implants. *Journal of biomedical materials research*. 2000; 53(5):536–46. [PubMed: 10984702]
5. Galante JO, Jacobs J. Clinical performances of ingrowth surfaces. *Clin Orthop Relat Res*. 1992; 276:41–9. [PubMed: 1537173]
6. Anselme K. Osteoblast adhesion on biomaterials. *Biomaterials*. 2000; 21(7):667–81. [PubMed: 10711964]
7. Stein GS, Lian JB, Owen TA. Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. *Faseb J*. 1990; 4(13):3111–23. [PubMed: 2210157]
8. Hudson MC, Ramp WK, Frankenburg KP. Staphylococcus aureus adhesion to bone matrix and bone-associated biomaterials. *FEMS microbiology letters*. 1999; 173(2):279–84. [PubMed: 10227156]
9. Dziewanowska K, Patti JM, Deobald CF, Bayles KW, Trumble WR, Bohach GA. Fibronectin binding protein and host cell tyrosine kinase are required for internalization of Staphylococcus aureus by epithelial cells. *Infect Immun*. 1999; 67(9):4673–8. [PubMed: 10456915]

10. Harris LG, Richards RG. Staphylococcus aureus adhesion to different treated titanium surfaces. *Journal of materials science*. 2004; 15(4):311–4. [PubMed: 15332591]
11. Bost KL, Ramp WK, Nicholson NC, Bento JL, Marriott I, Hudson MC. Staphylococcus aureus infection of mouse or human osteoblasts induces high levels of interleukin-6 and interleukin-12 production. *J Infect Dis*. 1999; 180(6):1912–20. [PubMed: 10558948]
12. Ramp WK, Lenz LG, Kaysinger KK. Medium pH modulates matrix, mineral, and energy metabolism in cultured chick bones and osteoblast-like cells. *Bone Miner*. 1994; 24(1):59–73. [PubMed: 8186734]
13. Papakyriacou H, Vaz D, Simor A, Louie M, McGavin MJ. Molecular analysis of the accessory gene regulator (agr) locus and balance of virulence factor expression in epidemic methicillin-resistant Staphylococcus aureus. *J Infect Dis*. 2000; 181(3):990–1000. [PubMed: 10720522]
14. Gillaspay AF, Hickmon SG, Skinner RA, Thomas JR, Nelson CL, Smeltzer MS. Role of the accessory gene regulator (agr) in pathogenesis of staphylococcal osteomyelitis. *Infect Immun*. 1995; 63(9):3373–80. [PubMed: 7642265]
15. Dalton BA, Dziegielewski M, Johnson G, Underwood PA, Steele JG. Measurement of cell adhesion and migration using phosphor-screen autoradiography. *Biotechniques*. 1996; 21(2):298–303. [PubMed: 8862816]
16. Foppiano S, Marshall SJ, Marshall GW, Saiz E, Tomsia AP. The influence of novel bioactive glasses on in vitro osteoblast behavior. *Journal of biomedical materials research*. 2004; 71(2):242–9. [PubMed: 15372470]
17. Degasne I, Basle MF, Demais V, Hure G, Lesourd M, Grolleau B, Mercier L, Chappard D. Effects of roughness, fibronectin and vitronectin on attachment, spreading, and proliferation of human osteoblast-like cells (Saos-2) on titanium surfaces. *Calcif Tissue Int*. 1999; 64(6):499–507. [PubMed: 10341022]
18. Sheng MH, Lau KH, Mohan S, Baylink DJ, Wergedal JE. High osteoblastic activity in C3H/HeJ mice compared to C57BL/6J mice is associated with low apoptosis in C3H/HeJ osteoblasts. *Calcif Tissue Int*. 2006; 78(5):293–301. [PubMed: 16604280]
19. Wenstrup RJ, Fowlkes JL, Witte DP, Florer JB. Discordant expression of osteoblast markers in MC3T3-E1 cells that synthesize a high turnover matrix. *J Biol Chem*. 1996; 271(17):10271–6. [PubMed: 8626594]
20. Effah Kaufmann EA, Ducheyne P, Shapiro IM. Evaluation of osteoblast response to porous bioactive glass (45S5) substrates by RT-PCR analysis. *Tissue Eng*. 2000; 6(1):19–28. [PubMed: 10941197]
21. Abe T, Nomura S, Nakagawa R, Fujimoto M, Kawase I, Naka T. Osteoblast differentiation is impaired in SOCS-1-deficient mice. *J Bone Miner Metab*. 2006; 24(4):283–90. [PubMed: 16816922]
22. Schneider G, Burrige K. Formation of focal adhesions by osteoblasts adhering to different substrata. *Experimental cell research*. 1994; 214(1):264–9. [PubMed: 7521844]
23. Puleo DA, Holleran LA, Doremus RH, Bizios R. Osteoblast responses to orthopedic implant materials in vitro. *J Biomed Mater Res*. 1991; 25(6):711–23. [PubMed: 1874756]
24. Kornu R, Maloney WJ, Kelly MA, Smith RL. Osteoblast adhesion to orthopaedic implant alloys: effects of cell adhesion molecules and diamond-like carbon coating. *J Orthop Res*. 1996; 14(6):871–7. [PubMed: 8982128]
25. Shah AK, Sinha RK, Hickok NJ, Tuan RS. High-resolution morphometric analysis of human osteoblastic cell adhesion on clinically relevant orthopedic alloys. *Bone*. 1999; 24(5):499–506. [PubMed: 10321910]
26. Zhao G, Schwartz Z, Wieland M, Rupp F, Geis-Gerstorfer J, Cochran DL, Boyan BD. High surface energy enhances cell response to titanium substrate microstructure. *Journal of biomedical materials research*. 2005; 74(1):49–58. [PubMed: 15924300]
27. Hogt AH, Dankert J, Feijen J. Adhesion of Staphylococcus epidermidis and Staphylococcus saprophyticus to a hydrophobic biomaterial. *Journal of general microbiology*. 1985; 131(9):2485–91. [PubMed: 4067581]

28. Gross M, Cramton SE, Gotz F, Peschel A. Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. *Infection and immunity*. 2001; 69(5):3423–6. [PubMed: 11292767]
29. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell*. 1996; 84(3):345–57. [PubMed: 8608588]
30. Schneider GB, Perinpanayagam H, Clegg M, Zaharias R, Seabold D, Keller J, Stanford C. Implant surface roughness affects osteoblast gene expression. *Journal of dental research*. 2003; 82(5):372–6. [PubMed: 12709504]

Fig. 1.

Average pixel intensity, an index of osteoblast attachment, as measured from Ti-Fn and Ti-Fn-*S. aureus*-coated culture dishes following 1 and 36 hours of incubation. Pixel intensity was calculated using the appropriate image phosphor software. Results are expressed as means $\pm$ SEM. The data are representative of three separate experiments performed in triplicate. * $p < 0.05$ compared to Ti-Fn at 36 hr.

Fig. 2.

Osteoblast adhesion to Ti-Fn and Ti-Fn-*S. aureus*-coated culture dishes at 1, 3, 7 and 10 days of incubation. Cells were treated with MTS dye and formation of formazan product, proportional to the number of adherent cells, was spectrophotometrically measured at 540 nm. Results are expressed as means $\pm$ SEM. The data are representative of three separate experiments performed in triplicate. * $p < 0.05$ compared to Ti-Fn at all time points.

Fig. 3.

Osteoblast proliferation rate as measured from Ti-Fn and Ti-Fn-*S. aureus*-coated culture dishes following 24, 48 and 72 hours of incubation. Cells were cultured in standard Osteoblast growth medium supplemented with 0.5 μ Ci/ml tritiated thymidine (³H). Disintegrations per minute were determined by measuring ³H incorporation. Results are expressed as means $\pm$ SEM. The data are representative of three separate experiments performed in triplicate.

Fig. 4.

Insoluble collagen matrix synthesis activity by osteoblasts on Ti-Fn and Ti-Fn-*S. aureus*-coated culture dishes following 3, 6 and 9 days of incubation.. Cells were cultured and stained with Sirius red F3B to quantify total collagen synthesis. After elution with NaOH, total absorbance of the released dye was measured spectrophotometrically at 540 nm. Results are expressed as means $\pm$ SEM. The data are representative of three separate experiments performed in triplicate. * $p < 0.05$ compared to Ti-Fn at day 9.

Fig. 5.

Alkaline phosphatase specific activity, on Ti-Fn and Ti-Fn-*S. aureus*-coated culture dishes following 3, 6 and 9 days of incubation. Cells were cultured and enzyme activity was measured by conversion of a p-Nitrophenol Phosphate (p-NPP) substrate to p-Nitrophenol (p-NP). Specific activities are expressed as nmol of p-Nitrophenol produced per minute per milligram of protein. Results are expressed as means $\pm$ SEM. The data are representative of three separate experiments performed in triplicate. * $p < 0.05$ compared to Ti-Fn at day 6.

Fig. 6.

Osteoblast mineralization on Ti-Fn and Ti-Fn-*S. aureus*-coated culture dishes following 7, 14 and 21 days of incubation. Cells were cultured and stained with alizarin Red S to quantify the extent of mineralization. Alizarin red S was then eluted and measured spectrophotometrically at 570 nm. Results are expressed as means $\pm$ SEM. The data are representative of three separate experiments performed in triplicate. * $p < 0.05$ compared to Ti-Fn at the respective time points.

Table 1

Osteocalcin synthesis from osteoblasts on Ti-Fn and Ti-Fn-*S. aureus*-coated culture dishes following 7, 14 and 21 days of incubation^a.

Amount of osteocalcin secreted (ng/ml)	Ti-Fn	Ti-Fn-10 ⁶ S.a	Ti-Fn-10 ⁷ S.a	Ti-Fn-10 ⁸ S.a
7 days	< 1ng/ml	< 1ng/ml	< 1ng/ml	> 25 ng/ml
14 days	< 1ng/ml	< 1ng/ml	< 1ng/ml	> 25 ng/ml
21 days	< 1ng/ml	< 1ng/ml	< 1ng/ml	> 25 ng/ml

^aConcentration of osteocalcin in the cell culture supernatants was measured by ELISA. The data are representative of two separate experiments performed in triplicate. The measured values were either below or above the minimal and maximal detection limits of the assay.