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A multifunctional streptococcal collagen-mimetic protein coating prevents bacterial adhesion and promotes osteoid formation on titanium

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ABSTRACT

The major barriers to the clinical success of orthopedic and dental implants are poor integration of fixtures with bone tissue and biomaterial-associated infections. Although multifunctional device coatings have long been considered a promising strategy, their development is hindered by difficulties in integrating biocompatibility, anti-infective activity and antithrombotic properties within a single grafting agent. In this study, we used cell adhesion assays and confocal microscopy of primary murine osteoblasts and human osteoblast cell lines MG-63 and Saos-2 to demonstrate that a streptococcal collagen-like protein engineered to display the α1 and α2 integrin recognition sequences enhances osteoblast adhesion and spreading on titanium fixtures. By measuring calcium deposition and alkaline phosphatase activity, we also showed that selective activation of α2β1 integrin induces osteoblast differentiation, osteoid formation and mineralization. Moreover, cell adhesion assays and scanning electron microscopy of clinical isolates *Staphylococcus aureus* Philips and *Staphylococcus epidermidis* 9491 indicated that streptococcal collagen-mimetic proteins inhibit bacterial colonization and biofilm formation irrespective of their interaction with integrins. Given that streptococcal collagenous substrates neither interact with platelets nor trigger a strong immune response, this novel bioactive coating appears to have desirable multifaceted properties with promising translational applications.

1. Introduction

Implantable devices are often used to restore function or regenerate tissue. Subsets of these devices, such as orthopedic and dental fixtures, require integration into the host tissue [1]. Extensive epidemiological studies have revealed that failures with long-term implants can be largely attributed to aseptic loosening and infections associated with the surgically embedded fixture [2]. In more than half of implant failures, the lack of rapid bone deposition on the implant surface followed by suboptimal osseointegration results in detachment and prolongs the length of patient recovery [2,3]. Although not as frequent, implant-associated infections result in devastating functional and financial consequences [4]. Failure to eliminate infection often requires a revision surgery, which triples the initial procedure cost and may increase the risk of infection due to improper elimination of bacteria residing in the peri-implant tissue, bacteremia or osteomyelitis [4]. Over the past couple of decades, therapeutic management of implant-associated infections has been further complicated by the emergence of antibiotic-resistant bacteria [5,6].

Staphylococcus epidermidis and *Staphylococcus aureus* are the most common causes of infections associated with prosthetic implants, likely because of their innate ability to rapidly attach to abiotic surfaces or host tissues [4,7]. Upon binding to a substrate, staphylococci proliferate and develop dense communities encased in a protective matrix [8]. These structures, or biofilms, enable bacteria to avoid antimicrobial activity and host immune responses. Biofilms are a source of persistent

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bacteremia as well as localized tissue destruction, which can have devastating consequences, with high morbidity and healthcare costs [9]. Thus, it is clear that development of new grafting agents for implants that selectively promote eukaryotic cell adhesion over microbial attachment is an unmet medical need [10].

Given their unique tensile strength and chemical stability, titanium alloys are used extensively for orthopedic and dental applications [11]. However, their modest osteoinductive and osteoconductive properties often lead to poor integration into the host tissue [1]. Attempts to augment Ti bioactivity have been centered chiefly on modifying the surface with biomolecules that promote adhesion of cells to the fixture. Fibronectin, bone morphogenetic proteins and Arg-Gly-Asp (RGD)-containing peptides have been exploited to enhance cell attachment to the implant both in vitro and in experimental animal models [12–15]. However, the efficacy of these bioactive coatings is limited by their lack of specificity. For example, RGD peptides promote broad mammalian cell adhesion rather than targeted osteoblast attachment and the subsequent activation of signaling pathways required for cell differentiation and new bone formation [16]. Reports suggest that collagen I (CnI) has desirable osteoinductive properties because of its interaction with $\alpha2\beta1$, an integrin highly expressed by osteoblasts and critical for their differentiation [17–19]. Furthermore, grafted biomaterials that were based on the CnI GFOGER (glycine–phenylalanine–hydroxyproline–glycine–glutamic acid–arginine) integrin recognition sequence enhanced periimplant bone formation [20]. Importantly, this sequence must be located within the collagen triple helical backbone for integrin recognition [21]. Similar to other coatings that are based on biomolecules, the potential of CnI as a valuable biomaterial is limited by its high susceptibility to bacterial colonization and interaction with platelets [22,23]. For example, staphylococci express both Fn- and Cn-binding proteins on their surface, which allows these bacteria to rapidly colonize the implant [24,25].

Proteins containing collagen-mimetic domains have been identified in a subset of prokaryotic organisms [26,27]. Scl2 from group A *Streptococcus* contains a 73 amino acid N-terminal noncollagenous variable domain and a 79 residue typical collagen GXY repeat region (where X and Y positions are often occupied by hydroxyproline and proline in mammalian collagens), but lacks hydroxyproline residues that stabilize the mammalian collagenous triple helix [26]. Despite the absence of proline posttranslational modification, these subdomains still behave in biochemical analysis as triple helical collagens, likely stabilized by electrostatic interactions and interchained hydrogen bonds [28]. Scl2 does not support eukaryotic cell adhesion, but acquires cell adhesive properties while maintaining the triple helical backbone when engineered to contain the GFOGER-like $\alpha1$ and $\alpha2$ integrin binding sites GFPGER (Scl2-2) or the $\alpha1$ integrin recognition sequence GFPGEN (Scl2-3) via site-directed mutagenesis of the Scl2-1 parent protein at positions 126–130 [29]. Endothelial cells, fibroblasts, and smooth muscle cells adhere to modified Scl2 immobilized on polystyrene or incorporated into bioactive hydrogels [30–32].

In this study, we sought to assess whether osteoblasts also adhere to Scl2-2 and Scl2-3 proteins. As a proof-of-concept, we first examined the adherence of primary osteoblasts and osteoblast-like cell lines to polystyrene coated with collagenous substrates. Next, we determined the resistance of these coatings to staphylococcal colonization. Moreover, we determined whether engineered Scl2 proteins maintain their properties when grafted onto Ti. Finally, we determined the effect of Scl2 proteins on osteoblast differentiation and matrix mineralization.

2. Materials and methods

2.1. Bacterial culture

Staphylococci were cultured in tryptic soy broth (TSB) at 37 °C and were shaken at 250 rpm. Biofilms were grown in TSB supplemented with 1% glucose (TSBG) at 37 °C without shaking.

For recombinant protein expression, pASK-IBA2 Scl2-1 (unmodified), pASK-IBA2 Scl2-2 (GFPGER) and pASK-IBA2 Scl2-3 (GFPGEN) were transformed into *Escherichia coli* TOPP3 (Stratagene). Overnight starter cultures were diluted at 1:50 in Luria–Bertani medium (LB) containing ampicillin (100 μg ml^{-1}) and were incubated with shaking until reaching exponential phase (OD_{600} 0.6–0.8). Protein expression was induced by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration), and growth was continued for 4 h, after which bacteria were harvested by centrifugation, resuspended in phosphate-buffered saline (PBS) and frozen at −80 °C [26].

2.2. Primary murine osteoblast isolation and cell culture

Animal protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas M.D. Anderson Cancer Center, and all experiments were completed in accordance with institutional guidelines. Primary osteoblasts were isolated as described [33]. Neonatal mice (30–36 h old) were sedated by hypothermia and then decapitated. After the calvariae had been removed and the surrounding fibrous tissue wiped off, they were washed in PBS. The bone was then subjected to five successive collagenase digestions (2 mg ml^{-1} collagenase, 0.25% trypsin) in Dulbecco's minimum modified Eagle's medium (DMEM) at 37 °C in an oscillating water bath (10 min each). The first two digestions were discarded. After the enzymatic activity was neutralized with 30% fetal bovine serum (FBS), the last three digestions were combined and filtered through a 200 μm sterile polypropylene mesh. Primary osteoblasts were recovered by centrifugation for 6 min at 1500 rpm. Cells were enumerated via hemocytometer, using trypan blue to exclude nonviable cells, and were cultured in DMEM supplemented with 10% FBS, 100 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin sulfate, 50 μg ml^{-1} gentamicin, 1.25 μg ml^{-1} Fungizone and 100 μg ml^{-1} ascorbate.

MG-63 cells were maintained in Eagle's minimum essential medium containing 10% FBS (ATCC) at 37 °C in a humidified incubation chamber with 5% CO_2. McCoy's 5a modified medium supplemented with 15% FBS (ATCC) was used to culture Saos-2 osteoblasts.

2.3. Protein expression and purification

Recombinant proteins were generated with use of the Strep-tag II expression and purification system (IBA-GmbH). DNA fragments were ligated to the vector pASK-IBA2, and clones were confirmed by sequencing. Recombinant proteins were expressed in *E. coli* and purified by affinity chromatography on a Strep-Tactin-Sepharose column [26]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was used to verify the size (~35 kDa) and purity of the proteins. The endotoxin was removed by two-phase extraction with Triton X-114, as described [34]. Triton X-114 was added to the protein preparation to a final concentration of 1% and incubated at 4 °C for 30 min with constant stirring. The mixture was then incubated for 10 min at 37 °C in a water bath, and the detergent–endotoxin complex was separated from the protein preparation by centrifugation (20,000*g* for 10 min) at 25 °C. Detergent traces were eliminated with