A new generation of bone grafts with tethered antibiotics

INTRODUCTION:
Bone allograft is commonly used in orthopedic procedures to help restore defects and provide structural stability. Of the many complications of allograft use, infection remains the most devastating to the patient. One strategy to minimize infection following the use of allograft bone is to incorporate antibiotic into the allograft by “soaking” the graft in antibiotic solution. These antibiotic-loaded bone allografts, however, are far from ideal as they exhibit variable elution kinetics and have the potential for toxicity, and induction of resistance. We describe a new method of allograft modification whereby an antibiotic is covalently tethered to the bone rendering it resistant to infection. The allograft displays stable and long-term antibacterial activity.

METHODS:
Allograft modification: Morselized human bone was washed extensively and sequentially coupled: 2X with excess Fmoc-aminothoxyethoxycetate (Fmoc-AEEA), deprotected with 20% piperidine in Dimethylformamide (DMF); and coupled with vancomycin (VAN) for 12-16 hours. The modified bone was washed extensively with DMF and PBS for times out to 24 h. Fluorescamine staining: Fresh morselized bone was washed with acetone and incubated in a 1mg/ml solution of fluorescamine in acetone for 40mins in the dark. After washing, bound fluorescence was visualized by confocal laser microscopy. VAN immunofluorescence: Control or VAN-derivatized allograft (VAN-bone) was washed 5X with PBS, blocked with 10% FBS (1hr), incubated with rabbit anti-VAN IgG (4°C, 12h) followed by an AlexaFluor 488-coupled goat anti-rabbit IgG (1hr), and visualized by confocal laser microscopy. Elution Kinetics: Fluorescent dansyl-glycine modified VAN (dVAN) was coupled to allograft or adsorbed onto control un-modified allograft. The adsorbed dVAN and dVAN-bone were incubated in PBS, 37°C for up to 3 weeks and eluted dVAN measured daily. Kirby Bauer: 10µl of eluted dVAN were spotted onto standard filter circles, placed on a uniform lawn of S.aureus and incubated at 37°C overnight. Cleared zones around the filter papers were measured to determine antibiotic amounts. Antibiotic Activity: At the above incubation, dVAN or adsorbed dVAN samples were sterilized with 70% ethanol, rinsed with PBS, and incubated with S. aureus (C=10⁵ cfu) in trypticase soy broth (TSB), 37°C, for 4 hrs. Bacterial visualization: Non-adherent bacteria were removed by washing with PBS and adherent bacteria stained with the Live/Dead BacLight Kit (20mins, RT) to cause viable bacteria to fluoresce green. Samples were visualized by confocal microscopy. Cell toxicity: Human Fetal Osteoblasts (hFOBs) were prestained with SybrGreen for 1h and seeded on sterile bone chips. The cells were allowed to attach for 2 hours and potential toxicity was assessed with confocal microscopy

RESULTS:

Surface amines: Staining of primary amines with fluorescamine revealed a bright uniform signal suggesting that there is an abundance of free amines on the bone surface. Vancomycin coverage: These primary amines were used to couple two linkers and VAN to the allograft. Following derivatization, staining with anti-VAN antibodies showed intense, diffuse staining of VAN-bone whereas no specific fluorescence was detected on controls, indicating extensive vancomycin coverage.

DISCUSSION:
Infection is the most devastating complication associated with use of allograft bone. Currently used techniques that attempt to address this problem, such as antibiotic impregnation, fail to provide long-term protection due to rapid elution. We have described a novel allograft modification that renders them bactericidal over long periods of time to remedy some of the shortcomings of antibiotic incorporation such as unpredictable elution kinetics, lack of long-term activity, and potential systemic and local toxicity. Despite its potent activity against bacteria, VAN-modified bone remains biocompatible allowing similar attachment of bone cells with minimal toxicity. Thus, our proposed modification in surface design serves as a starting point for the development of a new generation of bone grafts that are biologically active at sites of physiological importance.

ACKNOWLEDGEMENTS:
Supported by a Grant from the Musculoskeletal Transplant Foundation.

Fig 1: Synthesis of VAN-Bone, AEEA linkers are tethered to the naturally occurring amines followed by coupling with VAN.

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Fig 2: Amine and VAN coverage of the bone. (A) Fluorescamine staining indicates the bone is uniformly covered with amines (B) Staining with anti-VAN antibodies reveals a bright signal on the modified bone (right) which is absent from controls (left) indicating abundant attached antibiotic over the bone surface.

Elution Kinetics: Dansyl-modified VAN allowed us to track the movement of the antibiotic out of the bone and into solution. The bone with adsorbed dVAN lost most of the antibiotic to the surrounding media within the first week whereas the dVAN on the modified bone remained stably attached with only traces found in the solution. This eluted dVAN was adsorbed onto filter discs and placed on a lawn of S. aureus. By 3 days, the dVAN eluted from the VAN-adsorbed bone dropped below the MIC and failed to inhibit bacterial growth by the Kirby-Bauer assay (Fig. 3). Antibiotic activity: By the end of 3 weeks, all adsorbed dVAN had eluted from the sample. At that time, we challenged the sample that had been loaded with dVAN and the permanently coupled dVAN-bone with S. aureus and stained. The bone that had contained adsorbed dVAN was readily colonized by bacteria, with areas of biofilm development apparent as large, intensely stained areas; the dVAN-bone exhibited few bacteria, with staining mainly due to background fluorescence. Cell Toxicity: To assess whether VAN-bone supported mammalian cell attachment, we seeded hFOBS on control and VAN-bone. No apparent differences in morphology or cell numbers colonizing the VAN and control bone were seen.

Fig 3: Stability and antibiotic activity of VAN modified bone. (A) Adsorbed VAN is released within the first week; dVAN-bone appears to be stable by these measurements (B) dVAN eluted after adsorption to allograft falls below MIC within the first 4 days (C) VAN-bone retains its VAN and prevents bacterial attachment even after 3 weeks; the bone that had contained adsorbed VAN was readily colonized

ACKNOWLEDGEMENTS:
Supported by a Grant from the Musculoskeletal Transplant Foundation.

Poster No. 549 • 55th Annual Meeting of the Orthopaedic Research Society