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ANTIBACTERIAL ACTIVITY OF BONE ALLOGRAFTS: COMPARISON OF A NEW VANCOMYCIN-TETHERED ALLOGRAFT WITH ALLOGRAFT LOADED WITH ADSORBED VANCOMYCIN

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Abstract

Bacterial contamination of bone allograft is a significant complication of orthopaedic surgery. To address this issue, we have engineered a method for covalently modifying bone allograft tissue with the antibiotic vancomycin. The goal of this investigation was to compare the biocidal properties of this new allograft material with those of vancomycin physisorbed onto graft material. The duration of antibiotic release from the vancomycin-modified allograft matrix was determined and no elution was observed. In contrast, the adsorbed antibiotic showed a peak elution at 24 h that then decreased over several days. We next used an *S. aureus* disk diffusion assay to measure the activity of the eluted vancomycin. Again we found that no active antibiotic was eluted from the covalently-modified allograft. Similarly, when the vancomycin-modified allograft morsel was used in the assay, no measurable elution was observed; amounts of antibiotic released from the adsorbed samples inhibited *S. aureus* growth for 4–7 days. Probably the most telling property of the allograft was that after two weeks, the tethered-allograft was able to resist bacterial colonization. Unlike the elution system in which vancomycin was depleted over the course of

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Ethical Board Review statement: No animals or human subjects

Conflict of Interest: CK, CSA, IMS, JP, and NJH have filed an invention disclosure on this process.

RESEARCH HIGHLIGHTS

- Vancomycin that is covalently tethered to allograft bone retains its anti-bacterial activity for times far longer than classical elution systems.
- Allograft bearing covalently-bonded vancomycin retains active antibiotic for times approximating bone allograft replacement (3–6 months)
- Osteoblastic-cell morphology and expression of maturation markers are indistinguishable between control allograft and allograft bearing covalently-bonded vancomycin.

days-weeks, the antibiotic on the allograft was stably bound even after 300 days, while its biocidal activity remained undiminished for 60 days. This finding was in stark contrast to the antibiotic impregnated allograft which was readily colonized by bacteria. Finally we chose to evaluate three indicators of cell function: expression of a key transcription factor, expression of selected transcripts, and assessment of cell morphology. Since the tethered antibiotic appeared to have little or no effect on any of these activities, it was concluded that the stable, tethered antibiotic prevented bacterial infection while not modifying bone cell function.

Keywords

Bone allograft; *S. aureus*; vancomycin; bone biocompatibility; infection

INTRODUCTION

The use of bone grafts is well established in revision total joint replacement surgery, spine fusion and orthopaedic oncology [1-8]. One of the most serious complications of graft implantation is infection which often leads to poor patient outcomes including increased morbidity, repeated operations and lengthy antibiotic treatments [9-11]. After allograft placement, infection rates can be as high as ~16% [11-12] so that post-operative administration of antibiotics is recommended [13]. However, as bone grafts are avascular foreign bodies in which penetration of systemic antibiotics is poor, antibiotic treatment often fails [14].

Although the decellularized allograft bone is limited in its access to systemic antibiotic therapy, the porous tissue is well-suited for the physisorption of antibiotics [15-16]. The rate of initial antibiotic release can be very high, however upon elution, levels quickly wane leaving the graft unprotected. In fact, independent of the concentration and time allowed for antibiotic impregnation, ~75 % of allograft-adsorbed vancomycin and ~99% of netilmicin is eluted within 120 h [17]. The overall duration of this elution and more importantly the time it takes to drop below the minimum inhibitory concentration (MIC, the lowest concentration of an antibiotic that visibly and reproducibly inhibits bacterial growth,) appears, at the very least, to depend on the immediate pH of the bathing solution, the porosity of the bone, and the size of the bone granules [17]. Additional variability in release kinetics is dependent on the mineral content and the overall surface area of the bone morsels. [17-20]. Finally, as with any controlled release system, the high doses of antibiotic may cause osteoblast toxicity [21-22], leading to repair failure with devastating outcomes.

To address the problem associated with high release kinetics, we have covalently attached vancomycin (VAN) to bone allograft. Moreover, we have shown that the tethered antibiotic resists bacterial colonization [23-24]. The goal of the current study, is to compare this VAN-modified allograft with an allograft containing the physisorbed antibiotic. For both materials, we have determined the kinetics of elution and measured the activity of both the eluted and allograft-associated VAN as a function of time. We show that the modified allograft resists bacterial colonization without apparent changes in osteoblast function.

METHODS

Experimental design

The main goals of this study are to compare the activity and longevity of allograft impregnated with VAN (Ads-VAN-allograft) with an allograft that displaying covalently-bonded VAN (VAN-allograft). In all experiments, morselized cortical and cancellous bone chips were partially demineralized and thoroughly washed before use. Fluorescent,

dansylated VAN (dVAN) was either adsorbed to the bone chips or chemically coupled to the graft material using our previously published protocol [23]. This dVAN (a gift of Dr. Chang-po Chan, Thomas Jefferson University) was prepared so as to allow measurement of eluting VAN by fluorescence; note that the site of this modification is stereochemically removed from the carboxylic group that is used for its attachment to the allograft (Fig. 1, inset). dVAN elution from the two preparations was determined by incubation in phosphate-buffered saline (PBS), 37°C, with collection of the supernatant daily and replacement with fresh PBS. The fluorescence of dVAN in the supernatant medium was measured to determine elution kinetics. These experiments profiled the elution of total VAN.

To determine the antibiotic activity profile during elution, VAN was either adsorbed or chemically coupled to the bone chips using the above method. The samples were collected daily and an aliquot of the supernatant (dried onto a disk) as well as a bone chip were placed on a lawn of *Staphylococcus aureus* (*S. aureus*, *Xen36* derived from ATCC® 49525; Caliper Life Sciences, Hopkington, MA) to measure eluted activity. In this assay, antibiotic diffusing from the sample onto the lawn causes a clearing around the sample—this “zone of inhibition” was compared to that of a standardized antibiotic disk.

The goal of the next set of experiments was to examine the ability of the grafts to resist bacterial colonization after 15 days elution. At this time, antibiotic elution from the Ads-VAN-allograft is too small to be measured by the disk diffusion assay. After a 10 h challenge with *S. aureus*, bacterial colonization was visualized through staining with the Live/Dead BacLight™ bacterial viability kit (Invitrogen, Carlsbad, CA) or determined by direct bacterial counting. To further determine the stability of VAN-allograft, it was incubated in PBS for 300 days and VAN coverage was visualized by immunofluorescence. Alternatively, the fluorescent antibodies were released and the levels of the tethered VAN were measured as a function of the incubation time. To assess the activity of the VAN-allograft long after its modification, samples were either incubated in PBS or kept dry for up to 60 days; their ability to prevent colonization was assessed throughout this period. Finally, the effects of the VAN-allograft on the morphology of an immortalized human fetal osteoblastic cell line (hFOB) were assessed by SEM. The maturation status of osteoblast-like cells (MLO-A5) cultured on the VAN-allograft surface for 6 days was determined by measurement of the expression of a number of key transcripts (Runx2, alkaline phosphatase, osteopontin, osterix and osteocalcin).

Allograft preparation

Bone granules that are a mixture of cancellous and cortical human bone (0.5-2 mm³, morselized) or bone squares cut from femoral shafts (2 × 2cm, both gifts of Musculoskeletal Transplant Foundation, MTF, Edison, NJ) were washed and sonicated with distilled, deionized H₂O (dH₂O) until the washings were clear. This allograft was then partially demineralized with 12.5% disodium ethylenediaminetetraacetic acid (EDTA), pH 7, room temperature (RT) for 3 days with shaking and daily replacement of the EDTA. The partially demineralized allograft will be referred to as “allograft” and was used for all experiments. Prior to synthesis, all samples were washed with dH₂O and sonicated twice for 30 min in dimethylformamide (DMF, Acros Organics, Morris Plains, NJ).

Allograft modification [23]

As previously described, washed allograft was coupled with 10mg/ml Fmoc-[2-(2-aminoethoxy)-ethoxy]-acetic acid (Fmoc-AEEA, Peptides International, Louisville, KY) in dimethylformamide (DMF)/diisopropylethylamine (DIEA, 100:1, Fisher Scientific, Pittsburgh, PA) in the presence of O-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexa-fluorophosphate (HATU, 2 mg/ml, Sigma-Aldrich, St. Louis, MO), 2 h, RT, followed

by washing 10X with DMF. The linker was deprotected by treatment with 20% piperidine (Alfa Aesar, Ward Hill, MA) in DMF, 30 min, washed 10X with DMF and a second linker coupled and deprotected as above. After the final Fmoc deprotection, samples were coupled with 10 mg/ml clinical grade VAN (American Pharmaceutical Partners, Inc., Schaumburg, IL) in DMF/DIEA, in the presence of HATU, for 12-16 h. After synthesis, the modified bone was washed extensively with DMF and dH₂O to remove any adsorbed vancomycin.

Elution Kinetics

dVAN was either (1) coupled to 30 mg dry weight of allograft as described above (dVAN-allograft) or (2) adsorbed onto control, unmodified allograft by incubation with 10 mg/ml dVAN in PBS, RT, 12-16 h (Ads-dVAN-allograft). Samples were rinsed 3X and incubated in PBS, 37°C. Supernatants were collected daily for measurement of eluted antibiotic and replaced with fresh PBS for times out to 3 weeks. Fluorescence intensity ($\lambda_{\text{Excitation}}=337\text{nm}$, $\lambda_{\text{Emission}}=520\text{nm}$) of the eluted dVAN was measured spectrophotometrically using a plate reader (Photon Technology International PTI 814, Birmingham, NJ).

Bacterial Culture and Challenge

Ten (10) mg dry weight of control, Ads-VAN-allograft or VAN-allograft were sterilized with 70% ethanol for 15 min, washed 3X with PBS and 3X with BBL™ Trypticase™ soy broth (TSB, Fisher Scientific, Pittsburgh, PA). *Xen36 S. aureus* were cultured in TSB, 250 rpm, 37°C, 12-14 h (overnight culture). Using a 0.5 McFarland standard (a turbidity standard; when $A_{600} = 0.10$, *S. aureus* numbers are $\sim 1 \times 10^8$ colony forming units/ml (cfu/ml)), 1×10^4 cfu/ml bacteria were incubated with the sterilized samples in TSB, 37°C under static conditions. Upon harvesting, samples were washed 3X with PBS to remove non-adherent bacteria. Half of the samples were stained with the Live/Dead BacLight™ Kit and fluorescent, adherent bacteria visualized by epifluorescence. Sample epifluorescence was visualized using Z-sectioning and reconstruction with confocal laser microscopy (Olympus Fluoview 300). The other half of the samples were washed 3 more times, sonicated 5 min in 0.3% Tween-80 to suspend the adherent bacteria, and the suspended bacteria diluted and plated on 3M® Petrifilms. Films were scanned and the colonies were counted using a macro in Adobe Photoshop CS3.

Activity of eluted VAN

To create Ads-VAN-allograft, VAN (10 mg/ml in PBS) was incubated for 12-16 h, RT, with 100 mg dry morselized bone. VAN-allograft, as well as Ads-VAN-allograft (immediately following the incubation with VAN) were washed 3X with vortexing and incubated in PBS, 37°C. Supernatants were collected daily and replaced with fresh PBS; at the same time, one bone morsel was collected. 5 μ l of the collected supernatant was dried onto a sterile, standard filter disk and the disks or sampled bone morsels were placed onto agar plates that were pre-seeded with uniform lawns of *S. aureus*. Plates were incubated, 37°C, overnight and zones of inhibition radiating from the filter papers or morsels measured to determine the amount of eluted VAN as a function of time. At the end of the elution period (15 days), the remaining Ads-VAN-allograft or VAN-allograft morsels were challenged with *Xen36 S. aureus* for 10 h and number of adherent bacteria was determined by direct counts and fluorescent staining.

Immunohistochemical detection and quantification of VAN

VAN-allograft was incubated in PBS, 37°C for up to 300 days. Samples were washed 3X with phosphate-buffered saline (PBS), incubated with 10% fetal bovine serum in PBS (blocking buffer), 1 h, and incubated with rabbit anti-VAN IgG (1:500, US Biological, Swampscott, MA) in blocking buffer, 4°C, 12 h. Samples were washed 3X with PBS and incubated with AlexaFluor 488-goat anti-rabbit IgG (1:1000, Invitrogen) in blocking buffer,

RT, 1 h. Two sets of samples were analyzed. In the first set, samples were washed with PBS 3X, and fluorescence visualized using confocal laser scanning microscopy. In the second set, 30 mg aliquots of the VAN-allograft were incubated with 1 ml of 7 M urea with slow rocking at 4°C, overnight to disrupt the antigen-antibody complexes. Released fluorescent antibody in the supernatant was measured in a plate reader ($\lambda_{\text{Excitation}}=494\text{nm}$; $\lambda_{\text{Emission}}=520\text{nm}$) and expressed as fold increase of VAN-allograft fluorescence over that of controls.

Long-term antibiotic stability

VAN-allograft and control were incubated in PBS, 37°C or maintained dry at RT for up to 60 days. At various times, antibiotic activity was determined by challenge with *S. aureus* ($C_i=10^4$ cfu) for 10 h. Briefly, 10 mg dry weight of sterilized control or VAN-allograft were incubated with 1×10^4 cfu/ml *S. aureus* in TSB, 37°C under static conditions for 6 h. The samples were washed 6X with PBS to remove non-adherent bacteria and reincubated in TSB for another 4 h. Samples were washed 6X with PBS, sonicated 5 min in 0.3% Tween-80 to suspend the adherent bacteria, and the suspended bacteria diluted and plated on 3M® Petrifilms. Films were scanned and the colonies were counted using a macro in Adobe Photoshop CS3.

Scanning Electron Microscopy (SEM)

Samples were washed with dH₂O, fixed with 4% paraformaldehyde, 1h, dehydrated with ethanol, and vacuum dried overnight. Samples were sputter-coated with gold and visualized using a Hitachi TM-1000 SEM.

PCR analysis

Osteoblast-like cells (MLO-A5) were seeded on 2x2 cm bone squares of sterilized cortical control or VAN-allograft. At 6 days, cells were lysed, RNA was harvested, purified (RNAeasy kit, Qiagen) and quantified. The Illustra Ready-To-Go kit (GE Healthcare) was used to synthesize cDNA and RT-PCR was performed to amplify Lamin, Runx2, Osteopontin, Osteocalcin, Alkaline Phosphatase and Osterix, using the primer pairs detailed in Table 1. PCR products were fractionated by electrophoresis on a 2% agarose gel. Bands were quantified by densitometry using Scion Image and normalized to lamin.

Statistical analysis

All experiments were performed at least 3 times. Data is presented as means $\pm$ standard errors. The statistical analyses were performed on normal, equally variant data unless indicated otherwise in the legend. Statistical test used was a one or two way analysis of variance (ANOVA) with a Tukey multiple comparison procedure. An alpha of 0.05 was considered significant.

RESULTS

No antibiotic elution from VAN-allograft

Using a fluorescently-labeled dansylated-VAN, the kinetics of elution of Ads-dVAN-allograft was compared with that of dVAN-allograft (**dVAN, inset Fig. 1**). Eluted fluorescence from the Ads-dVAN-allograft showed an initially high antibiotic release followed by a rapid decrease in elution that approached steady state levels by 7-10 days (Fig. 1). In contrast, the fluorescence in the dVAN-allograft eluent remained at background levels as would be expected for any moiety covalently bound to the bone matrix.

In order to measure the activity of vancomycin associated with the allograft, we used a disk diffusion assay in which diffusion of an antibiotic from a central disk onto agar plates pre-seeded with a lawn of *S. aureus* is detected by a clear zone of inhibition. Antibiotic was eluted from Ads-VAN-allograft at both 1 h and 1 day as evidenced by formation of a clear zone around the filter paper (Fig. 2a). In parallel, a bone morsel retrieved from the Ads-VAN-allograft incubation medium eluted sufficient antibiotic to cause inhibition of *S. aureus* growth, as evidenced by a clear zone in the *S. aureus* lawn (Fig. 2b). Neither the eluent from the VAN-allograft (Fig. 2a) nor the VAN-allograft morsel inhibited *S. aureus* growth, as would be expected for the non-eluting covalently-bonded VAN (Fig. 2b). Elution from the Ads-VAN-allograft decreased with time, until by 5-6 days, only a halo could be seen around disk containing the aliquot of the supernatant or the bone morsel. Throughout this time period, no elution was measured from the VAN-allograft. The clearing zones produced from three independent repeat experiments were measured as a function of time (Fig. 2c). At all times, the zone of inhibition was greater around the Ads-VAN-allograft chips than that measured from its eluent; neither the eluent nor the bone chips from the VAN-allograft showed a zone of inhibition. Thus whereas Ads-VAN-allograft rapidly eluted the adsorbed antibiotic, the covalently-modified VAN-allograft showed no antibiotic elution confirming the stable attachment of VAN to allograft in this time frame.

VAN-allograft but not Ads-VAN-allograft retains its ability to inhibit bacterial colonization

We next determined if the allograft preparations were able to retard bacterial adhesion after measurable VAN elution was completed. After incubation for 15 days in PBS, Ads-VAN-allograft was readily colonized by *S. aureus* as evidenced by abundant green fluorescence on the allograft surface with both small and larger colonies present (see inset for higher magnification, Fig. 3a). In contrast, the VAN-allograft only exhibited the normal background fluorescence (gold) of the allograft, with more intense staining in topographical niches (Fig. 3b). To better characterize the relative colonization of the two surfaces, adherent bacteria, after suspension by sonication, were counted and expressed as a percentage of the bacteria recovered from the Ads-VAN-allograft (Fig. 3c). The VAN-allograft was colonized by significantly fewer bacteria than the Ads-VAN-allograft. Thus while the Ads-VAN-allograft was susceptible to bacterial colonization after a two week elution, the covalently-modified VAN-allograft retained its ability to robustly resist bacterial colonization.

VAN-allograft is stable for at least 60 days

Ideally the VAN-allograft should remain active over the time required for bone ingrowth and incorporation (6 wks–1 yr) [25]. By immunofluorescence, anti-VAN staining of the VAN-allograft appeared undiminished over a 300 day incubation period in PBS at 37°C; the control allograft showed only background staining characteristic of the autofluorescence of bone (Fig. 4a). In a parallel study, the amount of VAN determined by measurement of released fluorescent antibody was 1.5-2 times greater than fluorescent antibody release from control bone. This range of values was consistent over 60 days suggesting that the VAN-allograft linkage does not breakdown over time (Fig. 4b).

VAN-allograft remains active for at least 45 days

We then asked if the VAN-allograft retained activity over at least 60 days. After a 10 h challenge with *S. aureus*, VAN-allograft that had been stored dry for 20, 40 or 60 days showed a significant decrease in the number of adherent bacteria when compared to the control allograft (Fig. 5a); these levels were similar to those measured at shorter times (compare to Fig. 3). Similarly, the VAN-allograft that had been stored in PBS for 1, 15 or 45 days showed significantly fewer adherent *S. aureus* than the control allograft under the same

conditions (Fig. 5b). Together, these studies suggest that the VAN-allograft is stable to storage.

Cells cultured on VAN-allograft show normal morphology

A major function of allograft is to serve as a site for new bone formation. Therefore, we determined if adherent osteoblast-like cells retained their normal morphology on the VAN-allograft surface. By SEM, hFOB cells appeared fibroblastic in shape on both control (Fig. 6A) and VAN-allograft surfaces (Fig. 6B). Thick cytoplasmic processes addressed not only the bone matrix but appeared to serve as bridges between cells. Cells were well-spread and populated the surface as well as the pores and crevasses of the allograft surface.

Cells cultured on VAN-allograft express osteoblast-maturation markers

Finally, we used committed osteoblast-like cells (MLO-A5) to determine if the VAN-allograft surface supported osteoblastic maturation, a process necessary for bone ingrowth to occur. Both by densitometry of three separate RT-PCR experiments (Fig. 7, top panel) or by examination of the representative amplification products (Fig. 7, bottom panel), expression patterns of different markers of osteoblastic maturation appeared similar on the VAN-allograft and control surfaces after 6 days. After normalization to the nuclear protein lamin, alkaline phosphatase (AP) and osteocalcin expression appeared to be slightly higher on control surfaces, equivalent expression was seen with osteopontin and osterix, whereas Runx2 expression appeared higher on VAN-allograft. Even with this small degree of variability, no clear trends towards a less differentiated phenotype were obvious. Therefore, overall the control and the VAN-allograft surfaces appeared to support osteoblastic-cell maturation to the same extent.

DISCUSSION

Infection associated with implantation of allograft bone remains a serious problem which is conventionally treated by pre-loading the graft with antibiotics. The goal of this investigation was to compare the properties of such an allograft with that of an allograft that had been covalently modified with vancomycin [23,26-28]. When fluorescent dVAN was used to assess the duration of antibiotic release from the dVAN-allograft matrix, no elution was observed at any time. In contrast, the adsorbed dVAN showed a peak elution within the first 24 h which then decreased to a steady base level by 7-10 days. Because the dansyl modification could affect antibiotic activity, we used a disk diffusion assay to measure elution of unmodified VAN. Again we found that no active antibiotic was eluted from the covalently-linked allograft whereas active antibiotic was eluted from the Ads-VAN-allograft over the first week. When the VAN-modified allograft morsel was placed on an agar plate coated with *S. aureus*, no measurable elution was observed. Probably the most telling property of the VAN-allograft was that after two weeks the tethered-allograft was able to resist bacterial colonization. This finding was in stark contrast to the antibiotic impregnated ads-VAN-allograft which was readily colonized by bacteria. In addition, this study evaluated both the impact of the bound antibiotic on bone cells as well as the stability of the tethered antibiotic. Unlike the elution system in which VAN was depleted over the course of days-weeks, the VAN on the VAN-allograft was stably bound even after a 300 day incubation; its biocidal activity remained undiminished for at least 60 days. With respect to toxicity which can cause degeneration or even death of bone cells, the tethered antibiotic did not influence osteoblast adhesion or expression of critical osteogenic transcripts.

The most critical aspect of the modified allograft was that it retained the covalently-attached antibiotic in conditions where the adsorbed antibiotic was rapidly depleted. Both the fluorescence and activity measurements indicated that the vancomycin was stably attached

to the bone allograft and that it retained its antibiotic-properties long after elution has ceased from the vancomycin-adsorbed allograft. While the elution kinetics described here may not model the *in vivo* situation, the very large differences in retained activity suggest that the tethered allograft would be vastly superior to the more conventional antibiotic loaded grafting system. In our own elution studies, both the labeling and activity measurements indicated that the impregnated morselized bone eluted most of its vancomycin within the first week of incubation as measured by assessment of activity; although a small amounts of vancomycin could still be detected for as long as 2 weeks after loading. This finding is in agreement with studies of the *in vitro* release characteristics of antibiotics from cancellous bone [20]; only rifampicin showed a concentration higher than the MIC for longer than 21 days. At that time, levels of the other antibiotics were already at sub-inhibitory concentrations. Given that three-quarters of infections associated with allograft occur within four months of surgical transplantation [9,29], it is unlikely that impregnation/physisorption techniques would be able to protect the allograft throughout this initial critical period. In contrast, the covalently bonded antibiotic would maintain the biocidal properties of the allograft.

Importantly, while the elution systems may achieve their desired antibiotic levels over the short term, once antibiotic depots have been depleted, the “empty” allografts become an attractive surface for bacteria. Indeed after a 15 day elution of the adsorbed antibiotic on morselized human allograft, it was readily colonized by *S. aureus*; in contrast, when the antibiotic was tethered to the allograft, it resisted bacterial colonization. It is worth noting that the initial inoculum of bacteria, *i.e.*, 10^4 cfu, expands to over 10^7 cfu within 24 h; this number of organisms would be a challenge for any anti-bacterial surface. Nevertheless, the observation that despite the high level of organisms, the tethered antibiotic does prevent bacterial proliferation suggests that it would be especially useful *in vivo* when there is a gross level of infection. That the implanted allograft would be inactivated by deposition of blood or bacterial proteins is unlikely. In a previous study, we showed that despite the presence of a protein coating and even multiple rounds of infection, the antibiotic-surface resisted bacterial colonization [27].

A very worrisome complication of any antibiotic delivery system is the specter of antibiotic resistance. With elution, concentration gradients are created in which the antibiotic concentration falls below the MIC. Exposure of bacteria to sub-inhibitory concentrations of antibiotics at the tail-end of elution has been suggested to increase the risk of selection and potentiation of resistant strains [30-31]. Using our fluorescent elution system, small amounts of vancomycin eluting from the Ads-VAN-allograft can still be detected for as long as 2 weeks; the functional disk diffusion assays suggest that the trace amounts eluted in the second week are sub-inhibitory to *S. aureus* thus maintaining the concern about resistance. In contrast, the tethered VAN-allograft showed no significant antibiotic elution for up to ~3 weeks, with readings consistently at background levels. Functional assays confirmed the lack of elution from the VAN-allograft. Based on these findings, it would be unlikely that bacteria are exposed to sub-inhibitory concentrations of antibiotic from the VAN-allograft, a key condition for fostering of resistance. Nevertheless, based on our earlier studies of antibiotic-grafted metals [27] this concern cannot be realistically assessed until *in vivo* assessments have been performed.

Aside from problems of resistance, it is imperative that the presence of the antibiotic does not interfere with bone cell functional activities. Indeed the literature is replete with studies of the effects of antibiotics on osteoblasts, osteoclasts and chondrocytes, cells that are required for callus formation, endochondral ossification and bone turnover. We chose to evaluate three indicators of cell function: expression of a key transcription factor, expression of selected transcripts, and assessment of cell morphology. Since the tethered antibiotic

appeared to have little or no effect on either hFOB's or MLO–A5 osteoblast-like cells, it was concluded that the tethered antibiotic did not block cell function. Of course, these studies provided a limited overview of the biocompatibility of the modified graft material. *In vivo* studies are now underway that should provide a more detailed assessment of the allograft in terms of bone healing in a compromised environment.

In summary, the high risk of infections [8-9,32] calls for the creation of a stable, long-lived biocidal grafting material. While a number of eluting antibiotic agents are currently available to the surgeon, the anti-bacterial protection remains relatively short-lived. In this report, we have developed and tested a new allograft in which the antibiotic is not released from the surface of the bone. In keeping with the stable bonding, there is no apparent decrease in antibiotic coverage or vancomycin activity over prolonged incubation times with high bacterial titers. Finally, the results of the investigation suggest that this engineered construct has a long shelf life with predictable biocompatibility.

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Figure 1. Fluorescent dansylated VAN does not elute from VAN-allograft

(Inset) A fluorescent dansyl-glycine molecule (green) was attached on the sugar residue of vancomycin to allow its detection by fluorescence (dVAN). Note that the site of this modification is stereochemically removed from the carboxylic group (red) that is used for its attachment on the linkers. **(Graph)** Elution profile of Ads-dVAN-allograft and dVAN-allograft over an 11-day period. Eluted fluorescence was measured daily using allograft bone that had been either soaked or modified with dVAN. Note that eluents from dVAN-bone remain at background levels throughout the study.

Figure 2. Elution of VAN from Ads-VAN-allograft wanes with time

Antibacterial activity of eluted VAN was measured as a function of time. Ads-VAN-allograft and VAN-allograft were incubated in PBS with daily changes. Eluting antibiotic was measured in the eluent and antibiotic remaining in the morsels by testing for production of a zone of inhibition. **(a)** Eluent spotted onto filter papers and tested for production of zones of growth inhibition on *S. aureus* plates: Ads-VAN-allograft (ads) and VAN-allograft (VAN). Zones of inhibition smaller than those observed at day 6 were not measurable. **(b)** Activity of the remaining adsorbed VAN in the bone morsels as measured by production of a zone of inhibition in a uniform lawn of *S. aureus*. **(c)** Graphical representation of the average of three experiments measuring the clearing zones produced as in (b) and (c) above.

Figure 3. VAN-allograft retains anti-bacterial activity after 15 day incubation

(a) Fluorescent visualization of viable (green) and dead (gold due to superposition of the red dead bacteria onto the live green image) adherent *S. aureus* on the Ads-VAN-allograft. The Ads-VAN-allograft is populated by green colonies of bacteria, punctuated by larger yellow areas thought to represent stain retention in bone features (for both micrographs, inset shows the size of individual colonies and their organization on allograft). (b) The VAN-allograft after incubation in PBS for 15 days. VAN-allograft appears devoid of green colonies, with similar large gold features. *Magnification*: Scale bar=200 μ m, inset=50 μ m; (c) Following 15 days in PBS, Ads-VAN-allograft was readily colonized by *S. aureus* as judged by recovery of adherent *S. aureus* ($C_i = 10^4$ cfu; 10 h); many fewer *S. aureus* were recovered from the VAN-allograft. Data presented represent cfu represented as a percentage of adherent bacteria on Ads-VAN-allograft; * $p < 0.005$.

Figure 4. VAN remains stably attached to the allograft over time

(a) VAN and Control allografts were incubated in PBS for 15, 75, 150 and 300 days, and samples stained for immunoreactive VAN. The stained Control allograft only exhibited some non-specific background staining. Note that the VAN-allograft generated a robust fluorescent signal that was undiminished over time. *Magnification:* Scale bar: 400 μ m. (b) Equal dry weights of VAN and control allografts were incubated in PBS for up to 60 days. At 15, 30, 45 and 60 days, samples were removed and immunostained for VAN. The fluorescent antibodies were released by incubation with 7M urea and measured using a plate reader as an indication of the amount of tethered VAN. Data represented as fold increase of fluorescent reading from VAN-allograft as compared to control allograft. Note that retrieved fluorescence remained between 1.5-2 times higher than that retrieved from controls over the whole 60 day period.

Figure 5. VAN-allograft retains its activity after long time periods in aqueous media and air Following storage in air for 20, 40, or 60 days **(a)** or incubation in PBS for 1, 15 or 45 days **(b)** control or VAN-allograft were challenged with *S. aureus* for 10 hours and adherent bacteria were re-suspended by sonication, serially diluted and plated. Note that the VAN allograft retained its activity in aqueous medium or in the dried state, successfully resisting colonization throughout the challenge period. * $p<0.05$.

Figure 6. Morphology of hFOBs on control and VAN-allograft

Human Fetal Osteoblast like cells (hFOBs) were visualized by SEM after culturing on cortical control (**a**) or VAN-allograft (**b**) for 48 hours. Both control and VAN-allograft allowed normal attachment of cells. Note the fibroblastic shape and cell spreading across natural topographical features of bone with thick protrusions addressing the underlying matrix and the neighboring cells. *Magnification:* Scale bars: 20 μ m.

Figure 7. MLO cells on VAN-allograft show normal expression profiles of markers of osteoblastic phenotype

After 6-day culture on control or VAN-allograft, murine osteoblast-like cells (MLO-A5) were assessed for mRNA levels for proteins that are indicative of normal osteoblast maturation. As determined by densitometry, expression levels from three independent experiments were averaged, and normalized to lamin levels. Control and VAN-allograft gave similar expression levels for each of the assayed mRNAs (top panel), with an indicative experiment shown in the bottom panel. While there was variability in the expression levels of some mRNAs between the two surfaces, no clear trend indicating reduced maturation could be seen. The error bars indicate standard error from 3 densitometry determinations. Alkaline phosphatase=AP.

TABLE 1

PCR conditions

Primer	Forward	Reverse	T_{Annealing} (°C)	T_{Extension} (°C)	Cycles
OCN	CTC TCT GAC CTC ACA GAT GC	TGC TT GAC ATC CAT ACT TG	51	94	30
OPN	TCC TCT GAA GAA AAG GAT GA	CTT CAT GTG AGA GGT GAG GT	51	94	30
Runx2	GCG TAT TTC AGA TGA TGA CA	TAC CAT TGG GAA CTG ATA GG	50	94	30
collα	TTG ATC TGT ATC TGC CAC AA	GCT GAT TTT TCA TCA TAG CC	47	94	30
BSP	GAA AAT GGA GAC TC GAT AG	TTT CAT CCA CTT CTG CTT CT	50	94	30
Osterix	GCT ATC TCC TTG CAT GTC TC	GGG GCA ATA AAT TAT CAT CA	47	94	30
Alk Phose	GCT TTA AAC CCA GAC ACA AG	GCA GTA ACC ACA GTC AAG GT	50	94	25
Lamin A	AGC TAG AGC TGA GCA AAG TG	GTT CCT CGC TGT AAA TGT TC	51	94	20

* Cycles and annealing times are 1 min