Biomaterials 29 (2008) 3882–3887

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The effect of surface demineralization of cortical bone allograft on the properties of recombinant adeno-associated virus coatings

Cemal Yazici [a], Laura Yanoso [a,b], Chao Xie [a], David G. Reynolds [a,b], R. Jude Samulski [c,d], Jade Samulski [d], Judith Yannariello-Brown [e], Arthur A. Gertzman [e], Xinping Zhang [a], Hani A. Awad [a,b], Edward M. Schwarz [a,*]

[a] The Center for Musculoskeletal Research, University of Rochester, Rochester, NY, United States
[b] Department of Biomedical Engineering, University of Rochester, Rochester, NY, United States
[c] Gene Therapy Center, University of North Carolina, Chapel Hill, NC, United States
[d] Asklepios BioPharmaceutical, Inc., Chapel Hill, NC, United States
[e] Musculoskeletal Transplant Foundation, Edison, NJ, United States

ARTICLE INFO

Article history:
Received 8 April 2008
Accepted 11 June 2008
Available online 30 June 2008

Keywords:
Allograft
Bone
Recombinant adeno-associated virus (rAAV)
Freeze-dried
Bioluminescence imaging (BLI)

ABSTRACT

Freeze-dried recombinant adeno-associated virus (rAAV) coated structural allografts have emerged as an approach to engender necrotic cortical bone with host factors that will persist for weeks following surgery to facilitate revascularization, osteointegration, and remodeling. However, one major limitation is the nonporous cortical surface that prohibits uniform distribution of the rAAV coating prior to freeze-drying. To overcome this we have developed a demineralization method to increase surface absorbance while retaining the structural integrity of the allograft. Demineralized bone wafers (DBW) made from human femoral allograft rings demonstrated a significant 21.1% ($73.6 \pm 3.9\%$ versus $52.5 \pm 2.6\%$; $p < 0.001$) increase in percent surface area coating versus mineralized controls. Co-incubation of rAAV-luciferase (rAAV-Luc) coated DBW with a monolayer of C3H10T1/2 cells in culture led to peak luciferase levels that were not significantly different from soluble rAAV-Luc controls ($p > 0.05$), although the peaks occurred at 60 h and 12 h, respectively. To assess the transduction efficiency of rAAV-Luc coated DBW *in vivo*, we first performed a dose response with allografts containing 10^7, 10^9 or 10^{10} particles that were surgically implanted into the quadriceps of mice, and assayed by *in vivo* bioluminescence imaging (BLI) on days 1, 3, 5, 7, 10, 14, and 21. The results demonstrated a dose response in which the DBW coated with 10^{10} rAAV-Luc particles achieved peak gene expression levels on day 3, which persisted until day 21, and was significantly greater than the 10^7 dose throughout this time period ($p < 0.01$). A direct comparison of mineralized versus DBW coated with 10^{10} rAAV-Luc particles failed to demonstrate any significant differences in transduction kinetics or efficiency *in vivo*. Thus, surface demineralization of human cortical bone allograft increases its absorbance for uniform rAAV coating, without affecting vector transduction efficiency.

1. Introduction

Although massive allografts are widely used during reconstructive surgery of critical defects in long bones, their limited osteogenic and remodeling potentials are directly associated with the 25–35% failure rate within 3 years due to fracture and nonunion [1,2]. For those massive allografts that survive, the failure rate at 10 years has been documented to be as high as 60% [3–5]. The fractures at this late stage are the result of the accumulation of microcracks that cannot be repaired by the necrotic bone. As a result of this poor clinical success, the use of structural allografts has been restricted to repair segmental defects following tumor resection in cancer patients in an attempt to salvage the limb and eliminate the need for prostheses. To the end of a revitalizing structural allograft, we have developed a combination of gene therapy–tissue engineering approach that introduces angiogenic, osteoclastogenic and osteogenic signals on the cortical surface via immobilized recombinant adeno-associate virus (rAAV) [6,7]. The utility of this rAAV coating approach has subsequently been realized for soft tissue allografts [8] and stents [9], and the subject has been reviewed [10,11].

Despite the aforementioned problems with massive allograft and concerns regarding transmission of infectious agents [12,13], cortical allografts have remain the most popular biomaterial used

* Corresponding author. The Center for Musculoskeletal Research, University of Rochester Medical Center, 601 Elmwood Avenue, Box 665, Rochester, NY 14642, United States. Tel.: +1 585 275 3063; fax: +1 585 275 1121.
E-mail address: edward_schwarz@urmc.rochester.edu (E.M. Schwarz).

0142-9612/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2008.06.007

for limb sparing reconstructive surgery due to their biocompatibility and biomechanical properties that have yet to be matched by synthetic biomaterials [14]. However, one major limitation of cortical allograft as a biomaterial is its poor porosity, which compromises its ability to be uniformly and reproducibly coated with therapeutic molecules. In efforts to overcome these shortcomings, investigators have evaluated partially demineralized cortical bone coated with hydrophilic coatings including biodegradable foams of polycaprolactone (PCL), poly(propylene fumarate) (PPF) and poly(D,L-lactic-*co*-glycolic acid) (PLGA) with some success [15–17]. In our studies of rAAV-coated allografts, in which a sorbitol solution of the vector is directly applied to the cortical surface, we have consistently found that gravitational pooling of the vector prior to freeze-drying leads to nonuniform distribution [6]. As a consequence, the *in vivo* gene therapy induced bone formation is asymmetric and in most cases limited to one side of the allograft [7]. Thus, if the results of new bone formation and connectivity with the host are to be improved, we must develop an approach that increases the surface distribution of the rAAV coating by increasing surface absorption, without decreasing transduction efficiency. To this end, we investigated the effects of acid surface demineralization of human femur cortical allografts on the release and transduction efficiency of rAAV coatings.

2. Materials and methods

2.1. Preparation of mineralized and surface demineralized cortical bone graft wafers

Human cadaver femurs, previously debrieded and soaked in gentamycin solution, were cut into cross-sectional rings ~5 mm in width. Surface demineralization of the cortical bone was performed by the Musculoskeletal Transplant Foundation and achieved by Tween/Triton treatment for 30 min, soaked in H_2O_2 for 15 min, sonicated in purified H_2O for 5 min, air dried for 15 min, soaked in HCl for 5 min, sonicated 3 times in purified H_2O for 15 min each, and finally soaked in EtOH for 60 min. Acid penetration depth was determined to be 30–80 μm. Experimental bone wafers were prepared from both mineralized and surface demineralized cortical bone rings by cutting them into 10 mm × 5 mm × 3 mm samples using a diamond saw as we have previously described [18]. The wafers were sterilized under UV light prior to their use *in vitro* and *in vivo* experiments.

2.2. Preparation of rAAV-Luc

The rAAV vector expressing the firefly luciferase gene (rAAV-Luc) was purchased from the Gene Core Facility of the University of North Carolina, Chapel Hill, North Carolina, USA. The vector was prepared using the help virus free transfection method [19], and the titter of purified stock was determined to be 10^{12} particles/ml.

2.3. Coating of cortical bone grafts

An aliquot of the rAAV-Luc stock solution containing 10^7, 10^9 or 10^{10} particles was suspended in 20 μl of a 1% sorbitol-PBS solution, and directly pipetted onto the cortical surface of bone grafts. The coated wafers were frozen on dry ice and placed at −80 °C for 1 h before lyophilization as we have previously described [6]. Then the rAAV-Luc freeze-dried coated wafers were stored at −80 °C until they were used.

2.4. Scanning electron microscopy (SEM) imaging of mineralized and surface demineralized bone wafers

Both coated and uncoated, nondemineralized bone (NDB) wafers and demineralized bone wafers (DBW) were mounted on a stainless steel stage with conductive tape and a drop of conductive graphite adhesive was used to provide a pathway for electrons from the conductive tape to the nonconductive bone grafts. All samples were freeze-dried and sputter coated to apply a thin and completely continuous layer (~10 nm) of gold. A LEO 982 FE-SEM was then used with an accelerating voltage of 10 kV to view the surface microarchitecture of the grafts. To analyze the coating of the bone grafts and surface microarchitecture, micrographs were taken 9× and 3000× magnification of the specimens.

2.5. Quantification of surface coating on cortical bone wafers

A 30 μl of a 1% sorbitol solution of PBS with red food coloring was pipetted onto NDB or DBW ($n = 4$) and freeze-dried. A digital photograph was taken for the wafers and the percent surface coating was quantified using NIH Image by manually tracing and dividing the red surface area by the total wafer surface area.

2.6. In vitro *rAAV-Luc transduction assays*

C3H10T1/2 cells (10^5 per well in 12-well plates) were grown to confluence in BME supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 10% FCS (Hyclone, Logan, UT). To characterize rAAV-Luc transduction with DBW, four groups of C3H10T1/2 monolayers were studied: Group 1 untreated cells; Group 2 cells incubated with soluble rAAV-Luc only; Group 3 cells incubated with soluble rAAV-Luc in the presence of DBW; and Group 4 cell incubated with rAAV-Luc coated DBW. After 12 h, 24 h, 36 h, 48 h, 60 h, or 72 h of culture at 37 °C, the cells were harvested and assayed for firefly luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Cat. # E1980) and a single-sample luminometer as we have previously described [8].

2.7. In vivo *rAAV-Luc transduction assays*

The transduction efficiency of rAAV-Luc coated NDB and DBW *in vivo* was determined via longitudinal bioluminescent imaging (BLI) as previously described

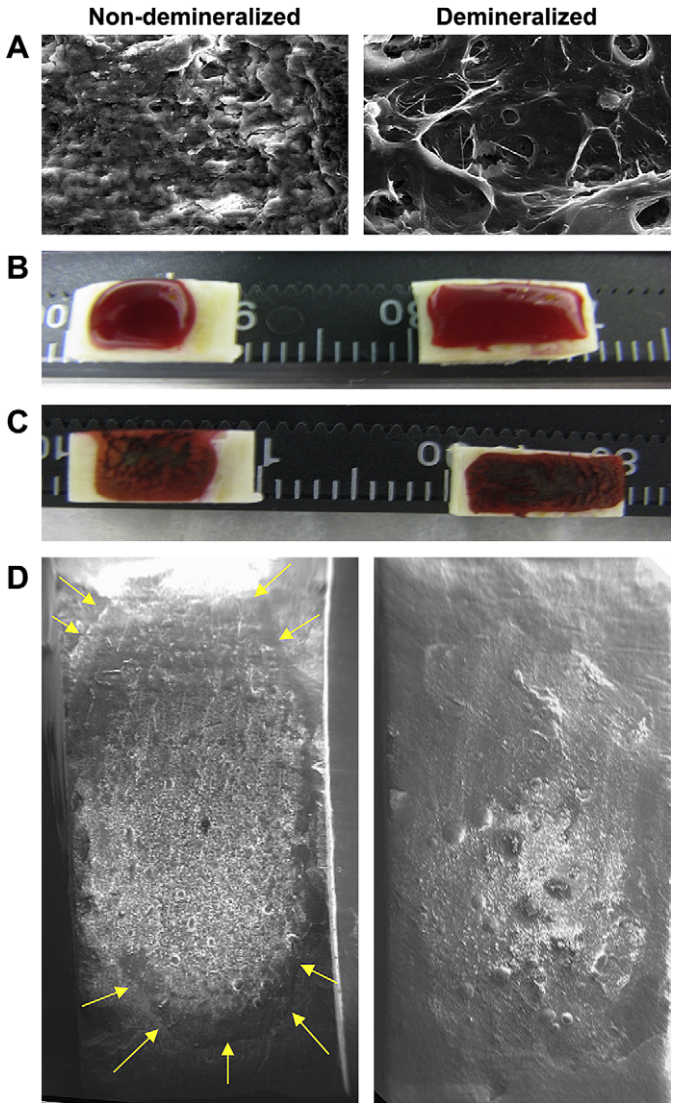

Fig. 1. Surface demineralization of cortical allograft bone increases its porosity and absorptive distribution of rAAV-Luc. Scanning electron microscopy (SEM) images of nondemineralized bone (NDB) (left) and demineralized cortical bone wafers (DBW) (right) prior to rAAV coating are shown at 3000× magnification to demonstrate the marked increase in porosity of the demineralized bone (A). A 30 μl of a 1% sorbitol solution of PBS with red food coloring containing 10^8 particles of rAAV-Luc was pipetted onto a 10 mm NDB (left) or DBW (right), and photographed before (B) and after (C) freeze-drying. Note that the solution pools in the center of the mineralized bone leading to poor coating after freeze-drying, while the solution is more evenly distributed on the demineralized bone leading to a significant increase in surface coating after freeze-drying (52.5 ± 2.6% versus 73.6 ± 3.9%; $p < 0.001$). Further evidence of improved rAAV coating uniformity is provided by 9× SEM images of the freeze-dried wafers (D) in which the border of the rAAV coating is clearly visible on the NDB (arrows), while the even distribution on the DBW renders this boarder more transparent.