Published in final edited form as:

Biomaterials. 2010 December ; 31(36): 9586–9594. doi:10.1016/j.biomaterials.2010.08.020.

Enhancement of peptide coupling to hydroxyapatite and implant osseointegration through collagen mimetic peptide modified with a polyglutamate domain

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Abstract

Hydroxyapatite (HA) is a widely-used biomaterial for bone repair due to its high degree of osteoconductivity. However, strategies for improving HA performance by functionalizing surfaces with bioactive factors are limited. In this study, we explored the use of an HA-binding domain (heptaglutamate, “E7”) to facilitate coupling of the collagen mimetic peptide, DGEA, to two types of HA-containing materials, solid HA disks and electrospun polycaprolactone matrices incorporating nanoparticulate HA. We found that the E7 domain directed significantly more peptide to the surface of HA and enhanced peptide retention on both materials *in vitro*. Moreover, E7-modified peptides were retained *in vivo* for at least two months, highlighting the potential of this mechanism as a sustained delivery system for bioactive peptides. Most importantly, E7-DGEA-coupled HA, as compared with DGEA-HA, enhanced the adhesion and osteoblastic differentiation of mesenchymal stem cells, and also increased new bone formation and direct bone-implant contact on HA disks implanted into rat tibiae. Collectively, these results support the use of E7-DGEA peptides to promote osteogenesis on HA substrates, and further suggest that the E7 domain can serve as a universal tool for anchoring a wide variety of bone regenerative molecules to any type of HA-containing material.

INTRODUCTION

Hydroxyapatite (HA) is a calcium phosphate crystal that makes up the principal constituent of bone mineral. Synthetic HA has been used for decades as a coating for metal prosthetics and other devices, and it has been extensively reported that HA-coated implants promote more robust bone formation than uncoated metal implants [1,2]. More recently, calcium phosphates including HA have shown promise as a scaffolding material for synthetic bone grafts [2], and as a bone-mimetic component within composite degradable biomaterials [3,4]. HA is known to be highly osteoconductive; however, it has limited osteoinductive

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capacity. Thus, there is significant interest in developing methods for coupling osteogenic factors, including bioactive peptides, to the HA surface.

Covalent linkage of peptides to material surfaces is commonly employed to achieve tight peptide anchoring [5]. However, HA presents few functional groups that can be utilized for covalent bonding. As an alternative, Durrieu et al. immobilized peptides on HA by attaching silane-derived spacer arms to the HA surface [6]. In another approach, peptide immobilization was accomplished via chemisorptive-coupling techniques involving water-soluble carbodiimides to direct carboxyl-amine conjugation of peptides to HA [7]. While these protocols were successful in anchoring peptides to HA, one concern is that both of these methods significantly alter the chemistry and topography of the HA surface, which could affect the natural osteoconductivity of HA. HA is a highly adsorptive type of biomaterial, and it has been suggested that the adsorption of bioactive proteins from the bone microenvironment contributes to the favorable tissue responses observed with this substrate [8,9]. As an example, adhesion proteins such as fibronectin, vitronectin and fibrinogen, become rapidly adsorbed to HA following implantation into bone, and these adsorbed proteins can provide a provisional matrix for the attachment and survival of mesenchymal stem cells (MSCs) [9-11]. MSCs play a key role in bone regeneration because these cells migrate into sites of bone injury, differentiate along the osteoblastic lineage, and then lay down an osteoid matrix that is subsequently mineralized and remodeled to form bone [12-14].

As a substitute for covalent attachment, we and others have explored the use of an acidic amino acid domain to direct ionic coupling of peptides to HA [15-23]. This strategy recapitulates the mechanism employed by endogenous bone-matrix proteins to achieve bone localization. Many bone-matrix proteins, including osteocalcin (OCN) [24] and bone sialoprotein (BSP) [25], contain stretches of negatively-charged amino acids that bind to the calcium within the biologic HA of bone. Modeling this process, we previously engineered the integrin-binding adhesive peptide, RGD, with a heptaglutamate domain (E7), and found that this increased peptide anchoring to synthetic HA disks [21]. However, some studies have suggested that RGD peptides have limited efficacy *in vivo* [26,27], and in particular, RGD actually inhibits bone formation and bone-implant contact on implanted HA [11]. Therefore, there is a need to identify other peptides that can enhance tissue responses to HA.

Native bone consists primarily of collagen I and HA; therefore, a biomaterial that incorporates both of these components would closely model the environment MSCs encounter *in vivo*. Studies from our group have shown that biomimetic peptides derived from collagen I, including DGEA and P15, stimulate MSC attachment to HA disks and induce osteoblastic differentiation of MSCs adherent to HA [11]. Furthermore, these peptides enhanced bone formation on HA disks implanted into rat tibiae [11]. Accordingly, in the current study we engineered the DGEA peptide with an E7 domain and compared the tethering capacity of DGEA and E7-DGEA on two HA-containing materials, sintered HA disks and electrospun degradable polycaprolactone matrices incorporating nano-HA particles (PCL/HA). Additionally, the bioactivity of DGEA-coated and E7-DGEA-coated HA disks was evaluated by monitoring MSC adhesion, spreading and osteoblastic differentiation *in vitro*, and bone formation *in vivo*.

MATERIALS AND METHODS

Peptide preparation

Collagen mimetic peptide DGEA (390.3g/mol) was obtained from American Peptide Co., Inc. (Sunnyvale, CA). Synthesized modifications of the above peptide sequence were also prepared by American Peptide Co., Inc and included E7-DGEA (EEEEEEEDGEA, 1294.2

g/mol), DGEA-FITC (DGEAK-FITC, 906.9 g/mol), and E7-DGEA-FITC (EEEEEEEDGEAK-FITC, 1811.7 g/mol). All peptides were reconstituted in ddH₂O at 1 mg/ml, aliquoted and stored at -20°C. FITC-tagged peptides were used for studies of peptide retention, whereas untagged peptides were used for the studies of MSC adhesion, cell spreading, osteoblastic differentiation and bone formation.

Disk Preparation

For both *in vitro* and *in vivo* studies of peptide retention and bioactivity, HA powder (MP Biomedicals, Solon, Ohio) was pressed into disks using a 15.875 mm die, under 3000 psi. For *in vivo* bone formation studies, HA powder (MP Biomedicals, Solon, Ohio) was pressed into disks using a 3mm die at 1000 psi. Both disk sizes were sintered at 1000°C for 3 hours and allowed to cool in a furnace at decreasing intervals. Disks were stored under sterile conditions.

Scaffold preparation

Polycaprolactone (PCL) (MW = 110,000 Da) was purchased from Birmingham Polymers (Birmingham, AL), and HA nanoparticles (20 -70 nm particle size) from Berkeley Advanced Biomaterials (Berkeley, CA). Two types of scaffolds, (1) 100% PCL, (2) 80wt% PCL + 20wt% HA, were produced by electrospinning as described previously [28]. Briefly, solutions were made with hexafluoroisopropanol (HFP, Sigma-Aldrich, St. Louis, MO), such that the solid weight was 7.5% of the total solution weight. Solutions were magnetically stirred at room temperature for 1h before being electrospun using 15kV voltage (Gamma High Voltage Research, Ormond Beach, FL). The scaffold was collected on a grounded aluminum foil located 12cm from the tip of the electrically charged 27- gauge needle. A syringe pump was used to feed polymer solution into the needle at a rate of 2mL/h. No chemical or radiation-induced cross-linking of PCL was performed. Scaffolds were sterilized with ethanol, rinsed in sterile phosphate buffered saline (PBS), and stored under sterile conditions.

Peptide coating

Peptides were coated onto materials overnight at 4°C at concentrations of 50μM, 100μM, or 200μM. For differentiation studies, after overnight coating with appropriate peptide, disks were coated for 2 hours with fetal bovine serum (FBS) at 37°. The disks and scaffolds were washed with PBS to remove unbound peptides and proteins, and warmed in PBS to 37°C prior to cell seeding or *in vivo* implantation.

Visualization of immobilized peptides *in vitro*

The fluorescein conjugate, FITC, allows direct visualization of immobilized peptide using fluorescent microscopy. Materials coated with 50μM, 100μM or 200μM DGEA-FITC or E7-DGEA-FITC peptides were washed in PBS with agitation for 7 days, changing PBS every 1-2 days. Upon completion of washes, relative retention of peptides was visualized using a fluorescent Nikon microscope. Additionally 100μM E7-DGEA-FITC was coated onto materials and washed for 1 hour with agitation. Using a fluorescent microscope, side by side analysis of peptide retention was performed on HA-disks, titanium alloy (Ti-64), and stainless steel (SS).

Peptide quantification using enzyme- linked immunosorbent assay (ELISA)

Conjugation of peptides to a FITC tag also allows detection of immobilized peptides using a modified ELISA assay, described previously [21]. HA disks were placed in 24-well low attachment plates and coated with 100μM peptide, as described above. Disks were then washed for 1 or 7 days, before blocking with 2% heat-denatured BSA for 2 hours at 4°C.

Additionally, non-coated disks were blocked to provide a control for non-specific adsorption of ELISA reagents. After peptide-coating and blocking, disks were exposed to an anti-FITC antibody conjugated to horseradish peroxidase (1:5000 dilution) (A21253, Molecular Probes, Carlsbad, CA) for 1 hour at 37°C. The disks were subsequently washed and incubated with a horseradish peroxidase chromogenic substrate (Biosource International, Camarillo, CA) for 30 minutes at room temperature, with the reaction being stopped by incubation with the stop solution (SS02, Invitrogen, Carlsbad, CA) for 15 minutes at room temperature. Enzyme activity in the solution was quantified by absorbance spectroscopy (450 nm). The absorbance of uncoated disks was also measured to account for relative amounts of background fluorescence.

Depletion of fluorescence from solution

Equimolar peptide solutions were made (100 μ M DGEA-FITC or E7-DGEA-FITC) and used to coat HA disks. These solutions were confirmed to have equivalent fluorescent values (3533 ± 226 for DGEA-FITC, and 3262 ± 54 for E7-DGEA-FITC). Samples were taken from the supernatant initially, after 2 hours, and after 24 hours of incubation with HA disks, diluted with PBS, and solution fluorescence measured using a fluorometer. These fluorescence values were plotted to show depletion of fluorescence from solution (indicating amount of peptide directed to the surface of scaffolds or disks).

In vivo peptide retention

HA disks or scaffolds were coated with 100 μ M DGEA-FITC or E7-DGEA-FITC as described above and implanted into dorsal subcutaneous sites of male Sprague--Dawley rats. After 7 - 60 days, animals were sacrificed and implants were retrieved. Qualitative assessments of relative peptide retention were made by fluorescently visualizing the explanted materials in side-by-side comparisons under a Nikon Fluorescent microscope.

Cell isolation and culture

Isolation and culture of MSCs was performed as previously described [9]. Human bone marrow cells were centrifuged at low speeds and resuspended in Dulbecco's modified Eagle's Medium (DMEM). A density gradient was established by applying the cell suspension to a histopaque-1077 column. The MSCs were extracted from the column and grown in DMEM with 10% FBS (growth media).

Osteogenic media (OS media) was used for all differentiation studies and consisting of DMEM supplemented with PenStrep, Amphotericin B, 10% FBS, 100 nM dexamethasone, 10 mM sodium β -glycerolphosphate, and 0.05 m μ L-ascorbic acid-2-phosphate, a well known formulation for differentiation media [29]. For all experiments, cells used were between passages 3 and 10. All cell culture work was performed with the prior approval of the University of Alabama at Birmingham Institutional Review Board.

Cell adhesion

Adhesion of MSCs to peptide-coated disks was evaluated using a standard fluorescence based assay adopted from a previous source [30]. Briefly, MSCs were incubated with 2 μ M CMFDA ("Cell Tracker Green" Molecular Probes) in serum free/thiol free media to label cells. Cells were detached from the tissue culture flasks by trypsinization and the reaction stopped using trypsin inhibitor (Sigma Aldrich). Aliquoted suspensions of 1×10^5 cells, in serum free/thiol free media, were seeded onto peptide-coated HA substrates and allowed to adhere for 90 minutes. Disks were then washed with PBS to remove loosely-bound cells. To release fluorescent marker into solution, cells were lysed (1% Triton-X-100 in 50m μ Tris). Relative cell adhesion was assessed by reading fluorescence of samples on a fluorometer.

Cell spreading

Spreading of MSCs was evaluated using morphology assays described previously [11]. MSCs (5×10^4 cells/disk) in serum free media were seeded onto peptide-coated, collagen-coated, or uncoated disks for 90 minutes or 4 hours. Loosely bound cells were removed by washing with PBS. Remaining adherent cells were fixed with 3.7% formaldehyde, permeabilized (0.2% Triton-X-100), and stained with Alexa-488 phalloidin (Molecular Probes) to visualize the actin cytoskeleton. Slides were mounted with 4.7 mM *n*-propyl-gallate mounting fluid and imaged using a Nikon fluorescent microscope.

MSC differentiation

Solid HA disks were coated sequentially with peptide and FBS as described above. Cells were seeded at 1×10^5 cells per disk in serum free media and allowed to attach for 24 hours (under these conditions the degree of cell attachment was equivalent on all of the various HA surface treatments, due to the presence of adsorbed adhesion-promoting serum proteins). In order to establish confluence, cells were allowed to grow in standard growth media for 3 days before replacing growth media with OS media and culturing the samples for an additional 2 weeks (media replaced every 2-3 days). After 2 weeks, supernatants were collected for measurement of osteocalcin (OCN) production and secretion using an ELISA kit (Biomedical Technologies Inc). Simultaneously the disks were stained for alkaline phosphatase activity (ALP) activity using a kit from Sigma Aldrich.

Bone formation on HA disks

DGEA-coated, E7-DGEA-coated, or uncoated disks were implanted into the intermedullary region of tibiae of male Sprague Dawley rats as previously described (1 implant per rat) [10]. After 7 days, implants were retrieved, embedded in poly(methyl methacrylate), and stained with Goldner's trichrome staining. Images were analyzed for all three groups, 4 animals per group, with two sections per implant evaluated. The amount of new bone surrounding each implant and the amount of bone in direct contact with each implant's perimeter were both quantified from Goldner's trichrome stained sections using Bioquant imaging software as previously described [10].

Statistics

In vitro peptide binding and bioactivity assays were performed at least three independent times, with each experiment being performed in triplicate. Subcutaneous implantations were performed in two independent runs, with each experiment being performed in duplicate. Tibial implantations were performed with 4 animals per treatment group, and at least two sections were analyzed per sample for quantification. Data sets were assessed using Student's t-test parametric analysis. A confidence level of at least 95% ($p < 0.05$) was considered significant and denoted by ("**"). Additionally, ($p < 0.01$) was denoted ("***"), ($p < 0.005$) denoted ("##"), and ($p < 0.001$) was denoted ("†").

RESULTS

E7-peptide immobilization to solid HA disks

To study peptide immobilization on solid HA disks, both DGEA and E7-DGEA were engineered to express a FITC tag on the C-terminus. This tag allowed peptide quantities to be measured by fluorescent microscopy, ELISA, and solution fluorescence. HA disks were coated with concentrations ranging from $50 \mu\text{M}$ to $200 \mu\text{M}$ of DGEA or E7-DGEA. Disks were then washed with agitation for 7 days and visualized using fluorescent microscopy. As shown in FIG 1A, at all coating concentrations E7-DGEA was retained in much higher concentrations than unmodified DGEA on HA disks, highlighting the increased strength of

the E7-HA bond compared to passively adsorbed DGEA. There appeared to be a greater amount of peptide on disks coated with 100 μ M E7-DGEA as compared with 50 μ M. However the fluorescence values for 100 μ M and 200 μ M were approximately equal, suggesting that a saturation point may have been reached at 100 μ M of E7-DGEA.

To quantitatively measure peptide retention, we employed an anti-FITC ELISA assay to detect immobilized FITC-tagged peptides on HA disks. As an experimental control, we also included a group of uncoated disks to account for non-specific adsorption of ELISA reagents to the HA disks. At both 1 and 7 days, significantly greater amounts of E7-DGEA were observed on HA disks as compared with DGEA (FIG 1B). In fact, the levels of DGEA on the surface of disks were not statistically different from the detected background signal obtained from uncoated disks. While this phenomenon can be partially attributed to DGEA being retained at low concentrations, it is also likely due to high background noise associated with the nonspecific adsorption of ELISA reagents to HA. Numerous studies from our group and others have shown that HA has a very high capacity for nonspecifically adsorbing proteins (including antibodies), which renders biochemical assays such as ELISAs challenging to perform. Nonetheless, the quantitative results in FIG 1B unequivocally show that E7-DGEA is retained on HA disks at higher concentrations than DGEA, thus confirming the semi-quantitative results obtained from microscopic visualization of peptide-modified HA surfaces (FIG 1A).

We next assessed whether the enhanced coupling of E7-DGEA to HA could be attributed, at least in part, to more efficient initial binding (vs. peptide retention after washing). To this end, we monitored the ability of the E7 domain to direct more peptide to the surface by incubating HA disks with either DGEA-FITC or E7-DGEA-FITC, and then measuring depletion of fluorescence from solution (indicating peptide deposition onto the HA surface). As shown in FIG 1C, significant depletion of E7-DGEA-FITC, but not DGEA-FITC, from solution was observed at both 2 and 24 hours, suggesting that markedly more peptide had been directed to the surface of the HA disk by the E7 domain compared with unmodified DGEA. This heightened peptide attachment with E7-DGEA coating was especially evident after 24 hours. E7-DGEA solution fluorescence levels were 65% of initial fluorescence readings, while unmodified DGEA fluorescence levels remained around 96% of initial fluorescence readings, consistent with much greater initial loading of E7-DGEA peptide onto the HA surface.

To confirm the specificity of the E7 sequence for HA compared with other types of biomaterials, we coated equimolar amounts (100 μ M) of E7-DGEA-FITC onto HA, SS, or Ti-64. The substrates were then washed to remove unbound peptide and visualized by fluorescent microscopy. As shown in FIG 1D, there was no detectable binding of the peptide to either SS or Ti-64, in contrast to the strong binding observed on HA disks. Similarly, no detectable fluorescence was observed on E7-DGEA-FITC-coated tissue culture plastic, indicating negligible peptide association (not shown).

E7-peptide immobilization to HA-containing electrospun scaffolds

We next investigated whether the affinity of E7 for HA could be exploited to functionalize composite materials containing HA, including resorbable materials designed for tissue engineering applications. As a model for functionalizing degradable materials, we investigated the binding of DGEA and E7-DGEA to electrospun polycaprolactone scaffolds containing nano-HA particles (PCL/HA). Increasing concentrations of DGEA-FITC or E7-DGEA-FITC (50, 100, and 200 μ M) were coated onto PCL/HA and then the substrates were washed with agitation for 7 days and visualized using fluorescent microscopy. At all concentrations investigated, E7-DGEA was retained at much higher concentrations than unmodified DGEA on HA-containing scaffolds (FIG 2A). We then monitored the binding of

E7-DGEA to PCL scaffolds lacking nano-HA particles (PCL), and at every concentration evaluated, only negligible E7-DGEA was observed (FIG 2B). Thus, HA is necessary to facilitate increased binding of E7-DGEA to electrospun scaffolds. These results not only confirm the specificity of the E7 domain for HA, but also suggest that the E7 domain can serve as a useful tool for anchoring peptides to composite materials incorporating HA.

Retention of E7-DGEA peptides *in vivo*

A strong peptide/biomaterial bond is vital because without an efficient linkage of peptides to the underlying substrate, cells can remove peptides by internalization [31,32]. Consequently, the ability of E7-HA bonds to withstand *in vivo* forces is important to the applicability of the E7 domain to serve as a tool for delivering bioactive peptides and proteins. To assess peptide retention *in vivo*, HA disks and PCL/HA scaffolds were coated with FITC-modified peptides, implanted into rat dorsal subcutaneous pouches, and then retrieved and imaged by fluorescent microscopy. At both 7 and 21 days following retrieval, E7-DGEA-FITC-modified HA disks exhibited markedly greater fluorescence than substrates with unmodified DGEA-FITC, indicating much higher rates of retention of E7-DGEA on the HA surface (FIG 3A). Similarly, greater retention of E7-DGEA peptide was observed on retrieved electrospun PCL/HA at 21 days following implantation (FIG 3B). We also implanted E7-modified PCL scaffolds (lacking HA) for 21 days and found that there was no detectable fluorescence, which supports *in vitro* data suggesting that HA is necessary for high affinity binding of E7 modified peptides (FIG 3B). We next extended these studies to evaluate samples implanted for 2 months. Upon retrieval and visualization, it was clear that a substantial amount of E7-DGEA was retained on the surface of both HA disks and PCL/HA electrospun scaffolds. These results underscore the significant potential of the E7 domain to serve as a vehicle for long-term delivery of bioactive factors to sites of bone regeneration.

Adhesion of MSCs to E7-DGEA

One issue that must be addressed when modifying peptides with additional domains is whether the domain alters the bioactivity of the peptide [5]. In prior studies, we determined that the DGEA peptide stimulates MSC adhesion when passively adsorbed to HA surfaces [11]. Accordingly, we performed a side-by-side comparison of MSC adhesion to HA disks coated with either DGEA or E7-DGEA, or with collagen I as a positive control (note that peptides used for cell studies were not tagged with FITC). As shown in FIG 4A, both of the peptides, as well as collagen I, induced significantly greater MSC adhesion than uncoated disks ($p < 0.05$). However, MSC adhesion was significantly increased on E7-DGEA-coated HA disks compared to DGEA-coated disks.

We next examined cell spreading on peptide-coated surfaces, given that our prior studies have indicated that DGEA can stimulate this cell response when coupled to HA [11]. MSCs were seeded onto disks coated with peptides or collagen I and then cell morphology was evaluated at 90 minutes (FIG 4B) and 4 hours (FIG 4C) by labeling the actin cytoskeleton with Alexa-conjugated phalloidin. On uncoated disks, cells retained a very rounded morphology at both time points, whereas DGEA-coated, E7-DGEA-coated, and collagen-coated disks were able to induce cell spreading at both 90 minutes and 4 hours. Taken together, results in FIG 4 clearly show that the addition of an E7 domain to the DGEA peptide does not interfere with early cell responses known to be elicited by unmodified DGEA and in fact it enhances cell adhesion, presumably due to greater peptide density on the surface of the disks.

Osteoblastic differentiation induced by E7-DGEA

To further assess the effect of adding an E7-domain to DGEA, we studied osteoblastic differentiation of MSCs. Using an early marker (alkaline phosphatase activity) and late

marker (osteocalcin production) of osteoblastic differentiation, we monitored differentiation of MSCs grown on peptide-coated, or collagen-coated, HA disks in standard osteogenic media, described previously [29]. After a 2-week incubation in osteogenic media, substrates were stained for ALP activity (FIG 5A). As shown, disks coated with DGEA, E7-DGEA and collagen I all induced greater ALP activity than uncoated disks, and the greatest staining was observed with MSC cultures adherent to E7-DGEA-coated disks. In tandem with these studies, we collected conditioned media from differentiating cultures, and monitored production and secretion of osteocalcin (OCN) into the media using an ELISA assay. To control for basal levels of OCN in undifferentiated cells, cells were grown for 2 weeks in standard growth media (lacking osteogenic supplements). As expected, all groups grown in osteogenic media elicited increased OCN production above that of MSCs in growth media (indicated by a horizontal line in FIG 5B). However, for the samples grown in osteogenic media, significantly greater OCN was produced by MSCs adherent to E7-DGEA-coupled HA substrates as compared with uncoated or DGEA-coated samples.

Bone formation on E7-DGEA-modified HA implants

Given the greater anchoring and activity of E7-modified DGEA as compared with DGEA, we anticipated that E7-DGEA would enhance bone formation and deposition on implanted HA substrates. To test this hypothesis, we implanted uncoated or peptide-coated disks (DGEA or E7-DGEA) into a rat tibial model for 7 days. Using Goldner's trichrome staining and Bioquant software we quantified percent new bone formation and percent direct bone-implant contact. FIG 6 shows that both DGEA and E7-DGEA induced significantly more bone integration than uncoated disks. However, substantially greater percent new bone synthesis and percent bone-implant contact were elicited by the disks coated with E7-DGEA. These results strongly support the utility of the E7-modified DGEA peptide to enhance osteoconductivity of HA implants.

DISCUSSION

Composite biomaterials incorporating collagen I and calcium phosphates such as HA have been shown to stimulate substantial bone formation [33,34] and bony ingrowth [35] *in vivo*. While these studies indicate a promising role for collagen/HA composite materials in bone regenerative applications, there are some concerns regarding the implantation of native collagen I. Collagen I is typically xenografted, which poses a risk of both immune response and pathogen transfer. Accordingly, collagen-derived peptides, including DGEA [11,18,36], P15 [7,11,37-39], and GFOGER [40-45], have been investigated as a substitute for native collagen I. Collagen I binds to several cell surface integrin receptors, including $\alpha_2\beta_1$, and the activation of collagen-selective integrins plays a critical role in stimulating osteoblastic differentiation of MSCs and other osteoprogenitor cell types [46-51]. The triple-helical GFOGER peptide is a well-established ligand for the $\alpha_2\beta_1$ integrin; this peptide directs cell attachment [40,41] and also stimulates integrin-dependent signaling cascades [43]. The linear peptide DGEA was initially proposed as an $\alpha_2\beta_1$ ligand [51,52], however more recent structural analyses suggest that triple-helical collagen mimetics are significantly better ligands for $\alpha_2\beta_1$ than linear peptides [40,41]. Nonetheless, the DGEA peptide has been shown to support cell adhesion [11,18,36] and osteoblastic differentiation of MSCs [11,36], and interestingly it was suggested that DGEA may associate with the β_1 integrin subunit through a non-traditional binding mechanism [53,54]. In a prior study we did not observe any activity of the GFOGER peptide when coupled to HA substrates, although this peptide was active when coated onto tissue culture plastic [11]. Contrarily, GFOGER was highly effective in inducing bone formation when coupled to PCL matrices implanted into rat femoral defects [45]. While the reasons for the conflicting results obtained with GFOGER are currently unclear, it is possible that GFOGER activity may be influenced by the

efficiency of peptide adsorption to particular substrates such as HA, and/or by the conformation of the adsorbed peptide on diverse surfaces.

In light of our prior results indicating that DGEA stimulates MSC adhesion and osteoblastic differentiation, as well bone formation on HA disks implanted into rat tibiae [11], the focus of this study was on developing methods for better coupling of DGEA to HA. Given that HA surfaces are not readily amenable to covalent linkage of peptides, we and others have investigated the use of negatively-charged amino acid domains to promote ionic attachment of bioactive peptides to HA [15-22]. For example, Murphy et. al. used a sequence that mimicked the acidic bone-binding region of OCN to immobilize a bone morphogenetic protein-2 (BMP-2) peptide to an HA-coated polymer film; the BMP-2 peptide sequence modified with an HA-binding domain proved to be biologically active, as it promoted osteogenic differentiation of MSCs [20]. In addition to the BMP-2 peptide, several groups have utilized acidic domains to improve coupling of the RGD adhesive peptide, with the goal of promoting osteogenic cell attachment [16-19,21]. These studies uniformly demonstrated that the addition of an acidic domain does not interfere with the capacity of the RGD sequence to direct cell adhesion. In fact, we reported that E7RGD stimulated greater MSC adhesion than RGD across a range of concentrations [21]. Although further studies are needed, we speculate that enhanced cell adhesion resulted from either increased peptide density on the HA surface due to the E7 domain, or a change in peptide conformation that presented the cell binding motif to integrin receptors in a more favorable orientation [5]. Specifically, the tethering of the peptide to HA via the E7 domain could potentially lift the RGD sequence off of the HA surface, making this sequence more accessible to cells, as suggested by LeBaron et al. [5]. Supporting this concept, cell binding has been shown to increase on immobilized RGD peptides that have been synthesized with spacer arms of additional amino acids; this increase was suggested to occur because the lifted RGD motif was more apt to reach the binding site on the integrin cell surface receptor [55].

Although RGD and E7RGD peptides on HA surfaces promote cell adhesion under *in vitro* conditions [16,17,21], emerging evidence suggests that RGD-modified HA biomaterials are not effective in stimulating bone formation *in vivo* (although RGD can enhance osteogenesis on some materials other than HA). In fact, we reported that RGD coatings inhibit new bone synthesis and bone-implant contact on HA disks implanted into rat tibiae [10]. The discrepancy between *in vitro* and *in vivo* results likely relates to the fact that when implanted in the body, many biomaterials, including HA, rapidly acquire an adsorbed protein layer [10,56-58], which subsequently alters the biological context within which RGD is presented to cells. Specifically, blood and other body fluids contain high levels of fibronectin, vitronectin and fibrinogen [59,60], all of which bind to cell surface integrins via an RGD domain [57,61,62]. We previously hypothesized that synthetic RGD peptides compete with these native adsorbed proteins for binding to integrin receptors [10], which attenuates cell signaling mechanisms, given that native integrin ligands are more potent inducers of integrin activation than isolated RGD domains. Importantly, DGEA and P15 peptides do not inhibit cell adhesion to HA disks when presented in combination with an adsorbed protein layer, and moreover these peptides stimulate greater bone formation on HA disks implanted into tibiae [11].

In the current study, our objective was to improve the efficacy of the DGEA peptide through better anchoring of the peptide to the HA surface. We found that modification of DGEA with an E7 domain increased the amount of peptide loaded onto the HA surface, and also enhanced peptide retention after extensive washing *in vitro*, as well as following implantation into rat skin pouches. Remarkably, a substantial amount of E7-DGEA was retained on HA-containing substrates retrieved after a 2-month implantation, underscoring the significant potential of the E7 domain to facilitate sustained delivery of bioactive

peptides via HA biomaterials *in vivo*. We also validated the specificity of the E7/HA interaction by demonstrating that the E7-modified DGEA peptide exhibited negligible binding to SS, Ti-64, PCL, and tissue culture plastic. Consistent with these studies, Fujisawa et. al confirmed that tethering of E7-RGD to HA was mediated specifically by the E7 domain because soluble E7, but not RGD, peptides competed for binding of E7-RGD to HA [17]. Most importantly, E7-DGEA-modified HA disks stimulated significantly greater bone formation and direct bone-implant contact than either uncoated or DGEA-coated HA. To our knowledge this is the first report showing that osteogenesis can be enhanced by utilizing an acidic domain to anchor bioactive peptides to HA surfaces. Notably, the E7-DGEA peptide is an 11-amino acid sequence that is technically straightforward and inexpensive to synthesize, suggesting the potential utility of this peptide for clinical applications.

Finally, the results from this investigation demonstrate the ability of the E7 domain to facilitate tight peptide anchoring to electrospun PCL scaffolds incorporating nano-HA particles. Degradable biomaterials, including electrospun fibrous matrices, are emerging as excellent platforms for the delivery of tissue regenerative factors, and as carriers for stem cells [63,64]. However, methods for coupling peptides and proteins to the surface of electrospun matrices are limited. The inclusion of nanoparticulate HA within degradable substrates could provide unique anchoring sites for E7-modified peptides/proteins, thus facilitating tightly localized and sustained delivery of many types of bioactive molecules including osteoinductive or angiogenic factors.

CONCLUSIONS

The important finding of this study is that the E7 domain facilitated retention of DGEA peptides for at least two months *in vivo* on both dense HA and nano-HA containing degradable matrices. Furthermore, enhanced loading and retention of E7DGEA resulted in increased cell adhesion and osteoblastic differentiation *in vitro* as well as increased bone formation and bone-implant contact *in vivo*. Collectively these results demonstrate that the E7 domain serves as an excellent tool for directed coupling of peptides to HA-containing materials, while concurrently facilitating enhanced cell and tissue responses to DGEA. The broader implication from these studies is that negatively-charged amino acid domains, including E7, have the potential to be used as a universal tool for anchoring a wide range of peptides to any kind of solid HA substrate or composite material incorporating HA.

Acknowledgments

This research was supported by NIH/NIAMS grant R01AR51539 (SLB). The authors gratefully acknowledge Dr. Jack Lemons (University of Alabama, Birmingham) for providing the stainless steel and titanium alloy disks, and the Bone Histomorphometry Core Facility for their assistance with tissue processing and staining.

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FIG 1. E7 domain enhances binding and retention on HA disks

A) HA disks were coated with increasing equimolar concentrations of DGEA-FITC or E7-DGEA-FITC and then washed with PBS for 7 days. Representative images are shown of relative peptide retention. B) Disks were coated with 100 μ M DGEA-FITC or E7-DGEA-FITC or left uncoated and washed for 1 day or 7 days. A modified ELISA was employed to quantify relative amounts of peptide retention. Absorbance values of uncoated disks are shown to account for non-specific binding of ELISA reagents (* p <0.05 and ** p <0.01). C) HA disks were incubated in solutions containing 100 μ M E7-DGEA-FITC or DGEA-FITC. Samples were taken from the initial solutions, or at 2 and 24 hours after incubation, and plotted as percent fluorescence remaining ($\dagger$ p <0.001). D) HA, titanium alloy (Ti-64), and stainless steel (SS) were coated with 100 μ M E7DGEA-FITC and washed for 1 hour. Top panel represents phase contrast image; lower panel depicts fluorescent image.

FIG 2. E7 domain facilitates greater retention of DGEA peptides on HA-containing electrospun scaffolds

Polycaprolactone (PCL) scaffolds and PCL/HA scaffolds were coated with 50μM, 100μM, or 200μM DGEA-FITC or E7-DGEA-FITC as previously described. Scaffolds were washed extensively in PBS for 7 days. A) Representative images are shown of direct comparisons of retention of DGEA-FITC and E7-DGEA-FITC on HA-containing scaffolds (PCL/HA). B) PCL or PCL/HA scaffolds coated with equimolar concentrations of E7-DGEA-FITC were washed for 7 days and imaged. Representative images are shown.

FIG 3. E7-DGEA is retained on HA-containing materials for at least 2 months *in vivo*

A) HA disks coated with 100 μ M DGEA-FITC or E7-DGEA FITC were implanted subcutaneously into the backs of male Sprague Dawley rats for 7 or 21 days. Upon retrieval disks were washed in PBS and imaged. B) PCL and PCL/HA electrospun scaffolds were coated with 100 μ M DGEA-FITC or E7-DGEA FITC and implanted subcutaneously for 21 days. No detectable E7-DGEA-FITC was observed on PCL substrates. A comparison of PCL/HA scaffolds with E7-DGEA-FITC and DGEA-FITC is depicted, which shows greater retention of the E7-DGEA-FITC peptide. Of note, the same E7-DGEA-coated PCL/HA scaffold was used as a reference point (i.e., we were unable to fit 3 samples in the same microscopic field). C) HA disks and PCL/HA electrospun scaffolds were coated with 100 μ M DGEA-FITC or E7-DGEA FITC and implanted subcutaneously for 2 months. E7-DGEA was retained on both materials at much higher densities than DGEA-FITC. Representative images are shown for all experiments.

FIG 4. DGEA and E7-DGEA increase cell adhesion and spreading on HA

A) MSCs labeled with Cell Tracker dye were allowed to adhere for 90 minutes to HA disks coated with DGEA, E7-DGEA, or collagen I (peptides used for cell experiments lacked FITC tags). Loosely bound cells were removed by washing, and adherent cells were lysed and relative adhesion quantified by measuring fluorescence. Values were folded to uncoated HA disks (* $p < 0.05$).

Representative images were taken of MSCs allowed to adhere for 90 minutes (B) or 4 hours (C) to DGEA, E7-DGEA, or collagen I-coated HA disks. Cells were stained with Alexa-488 phalloidin (Cell Signaling Technologies).

FIG 5. E7-DGEA increases markers of osteoblastic differentiation

MSCs were seeded onto DGEA- or E7-DGEA-coated HA disks, collagen I-coated disks, or alternately, disks lacking peptide (“UNC”) (all disks were also overcoated briefly with FBS to facilitate strong cell adhesion). Cells were subsequently grown for 2 weeks in OS media and monitored for expression of osteoblastic markers. A) Representative image of cells stained for alkaline phosphatase activity (ALP). B) Conditioned media was collected from cells grown in osteogenic media and evaluated for osteocalcin (OCN) production by ELISA. In some trials, cells were grown for 2 weeks in standard media to measure basal OCN production in the absence of osteogenic supplements (reflecting undifferentiated cells). Values obtained from cells in osteogenic media were folded to values from cells in standard growth media (# $p < 0.005$).

FIG 6. E7-DGEA enhances bone formation and bone integration to a greater degree than DGEA DGEA-coated, E7-DGEA-coated, or uncoated disks were implanted into rat tibiae for 7 days. A) Representative images of Goldner's trichrome stained sections of HA disks and surrounding tissue (newly-synthesized bone is represented by the light blue staining immediately surrounding the disk, denoted by white arrow) B) Quantification of amounts of new bone formed (black bars) and the amount of bone directly contacting the implant perimeter (gray bars) of uncoated, DGEA-coated, and E7-DGEA-coated HA disks normalized to tissue area and presented as percentages (* $p < 0.05$).