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FUNCTIONALIZING HYDROXYAPATITE BIOMATERIALS WITH BIOMIMETIC PEPTIDES OF INTEGRIN LIGANDS

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CELLULAR AND MOLECULAR PHYSIOLOGY

ABSTRACT

Hydroxyapatite (HA) coatings of hard tissue implants have been shown to increase osseointegration, but the mechanisms by which HA improves implant integration are not well understood. Numerous studies have shown that modifying HA with adhesive peptides, including RGD, and the collagen I mimetics, DGEA and P15, stimulates the adhesion of mesenchymal stem cells (MSCs), a cell type that differentiates along the osteoblast lineage. However, HA is a highly adsorptive biomaterial, and therefore it is unlikely that cells at the implant site would ever encounter a peptide-modified HA surface in the absence of an adsorbed protein layer. In fact, our laboratory hypothesizes that the ability of HA to rapidly adsorb proadhesive proteins such as fibronectin and vitronectin from blood plays a key role in promoting MSC attachment. To better understand the role of adsorbed proteins in regulating MSC behavior, cell adhesion was evaluated on peptide-modified HA disks that were either overcoated with serum or implanted briefly into rat tibiae to allow deposition of native adhesive proteins. Surprisingly, these studies indicated that RGD inhibited MSC adhesion. Conversely, collagen mimetics were not inhibitory, although they did not enhance cell adhesion either. We hypothesize that RGD peptides, but not collagen mimetics, compete with adsorbed adhesive proteins for binding to cell surface integrins, given that blood adhesive proteins bind through an RGD-dependent mechanism. Interestingly, although collagen mimetics did not improve cell adhesion, they did increase osteoblastic differentiation,

presumably due to activation of collagen-selective integrins that are known to stimulate MSC differentiation along the osteoblast lineage. Importantly, our *in vitro* studies of the effects of adhesive peptides, when presented in combination with adsorbed blood proteins, were very predictive of the effects of these peptides on bone repair. RGD peptides inhibited, while collagen I mimetics enhanced, bone formation and bone-implant contact on HA disks implanted into rat tibiae. In sum, our collective studies highlight a potential role for collagen mimetic peptides in enhancing the performance of HA biomaterials, and further suggest that *in vitro* studies incorporating a protein modeling step provide a reliable indicator of the efficacy of biomimetic peptides in promoting implant osseointegration.

DEDICATION

I would like to dedicate my thesis to the most important people in my life. To my mother, who was always willing to listen when science hadn't worked that day, and to my father who always made me strive for my best, and never let me settle for less than that. And to my soon-to-be husband, who has encouraged me in my science, and in my life. I would not have made it through this process without all of them.

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positive results. They also helped me with my editing skills, as I spent the summers reading everything they had to turn in to the REU program.

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Joey Goodwin, our resident IT guy and coffee maker, has been the best help a girl could ask for. Joey never hesitated to drop what he was doing and help me with computer problems, usually consisting of my computer not wanting to talk with another piece of equiptment, such as the printer or the projector. Joey also made us coffee every morning in his office, which was sometimes the only way I stayed awake through those months of paper and dissertation writing.

Graduate school is a journey, and one which is not taken alone. There are many people who took the journey with me, and helped me along the way. There was specifically a group of women without whom I would not have maintained my sanity. To Alencia Grice, Portia McCoy, Valerie Bomben, and Elizabeth Staley, you all were my harbors in the storm when I needed them. Alencia and Portia listened to every talk I gave multiple times, and were always there for laboratory related catastrophes. Val and Liz, two-thirds of "the girl's house," helped me to relax when I came home. These women are so important to me that all of them are in my wedding. I could keep going on and on about the ways each of these wonderful women have helped me, but I think the best way

to put it is to quote my favorite musical. “Who can say if I’ve been changed for the better, but because I knew you, I have been changed for good.”

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INTRODUCTION

Tissue Engineering and Regenerative Medicine

Tissue engineering, introduced more than 20 years ago, is defined as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ [1]." In the case of bone tissue engineering, the biological process which is recapitulated is natural bone repair. In order to facilitate bone formation, scaffolds created from a variety of biomaterials, and in some cases functionalized with cells and bioactive molecules, are placed within the existing bone matrix, with the goal of inducing bone growth on the surface of the biomaterial.

Prior to 1891, the treatment for joint disease was joint excision or amputation, which did not allow for the return of normal function to the affected area [2]. Then, in 1891, Professor Themistocles Glück, from Berlin, Germany, created the first known joint replacement, an ivory ball and socket joint, which he fixed to the existing bone matrix with nickel-plated screws [2]. However, it was not until the 1960s that the modern stainless steel hip and polymer socket were implemented by Dr. John Charnley in the UK [3, 4]. Today, bone and joint disease account for more than 50% of all chronic disease in people over 50 [5]. Modern bone tissue engineering uses a combination of biomaterials, and bioactive molecules to induce bone cell adhesion and differentiation to induce osseointegration of the implant into the existing bone matrix.

Natural Process of Bone Formation

Bone formation in response to damage occurs in a predictable pattern. First, there is a short inflammatory response. Next, cells with the potential to form bone, notably mesenchymal stem cells (MSCs), a bone marrow derived stem cell with the ability to differentiate along the osteoblast lineage, and osteoblasts from the surrounding environment migrate into the affected area. Once the MSCs have differentiated along the osteoblast lineage, the cells begin to lay down an extracellular matrix (ECM) known as osteoid. This protein precursor to bone consists primarily of collagen I fibers, with the rest of the osteoid consisting of a conglomerate of proteins, including but not limited to bone sialoprotein (BSP), osteocalcin (OCN) and osteopontin (OPN). Then, there is the formation of a callus of new bone around the fracture site. The mineral crystals of this matrix are deposited in the gaps between the collagen fibers. Finally, the bone which is formed is remodeled into a structure similar to that of the bone prior to the break (Figure 1).

Cells

Prior to 1965, it was thought that osteoblasts were the only cells critical to healing of a bone fracture [6]. However, since then, biologists have come to realize that the response to wounds within the bone requires multiple cell types, and that this response occurs in multiple phases [6, 7]. The first phase, sometimes referred to as the reactive phase or the inflammatory phase, is the phase in which a blood clot forms and inflammatory cells respond to the wound. During this phase, the cells within hematoma die, along with cells in the adjacent tissue. This phase is also when new blood vessels

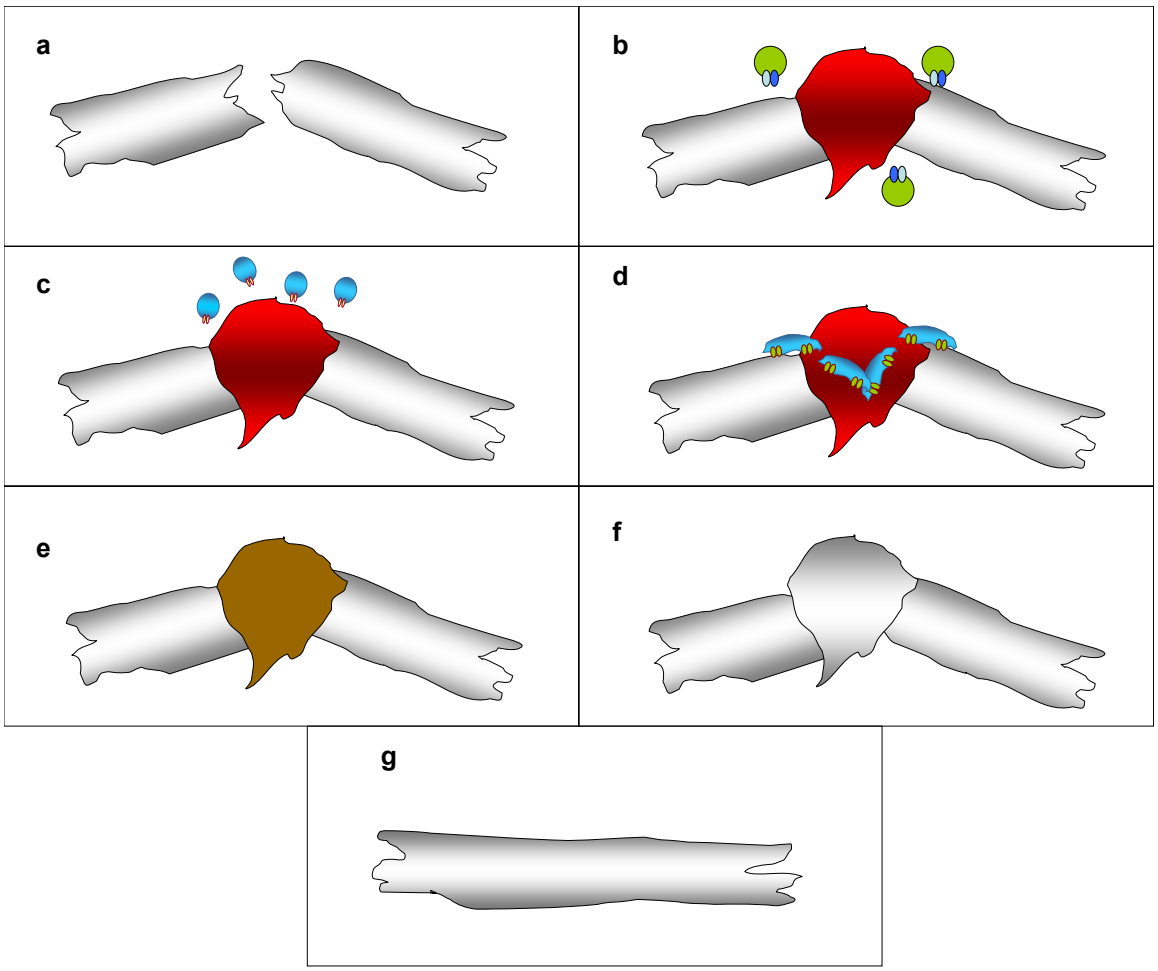

Figure 1: Bone formation in response to injury a) an injury to the bone occurs. b) a large hematoma forms at the site of injury, and immune cells, including macrophages, migrate into the injured area. c) bone forming cells, including osteoblasts and mesenchymal stem cells, migrate into the defect. d) undifferentiated mesenchymal stem cells differentiate along the osteoblast lineage. d) the differentiated osteoblasts begin to secrete osteoid, the protein precursor to bone, consisting of a conglomerate of proteins, including osteopontin, osteocalcin and collagen I. e) crystals of new bone form along the osteoid fibers. g) the injury site is healed, and resembles the bone prior to breakage.

form, allowing for the flow of nutrients into the area [6, 8]. In addition, inflammatory cells, including macrophages and giant cells, migrate into the area, remove the clot, and release cytokines to induce migration of bone forming cells into the area. In humans, this phase lasts about two weeks [6]. The second phase, also referred to as a reparative phase, is the phase during which new bone is formed. First cells within the area differentiate into chondrocytes and osteoblasts and form the organic extracellular matrices of cartilage and osteoid respectively [9]. A week or so later, mineralized bone begins to form along the organic matrix, finally forming into a fracture callus [6]. This process can take anywhere from four to sixteen weeks [6]. The third phase, also referred to as the remodeling phase, is the phase in which the newly formed callus is remodeled. Osteoclasts, or bone resorbing cells, are necessary for the resorption of the callus [6]. This process can take up to a year from the initial fracture event [6].

Integrins

Integrin-mediated cell adhesion is the predominant mechanism by which MSCs bind to implant surfaces. Integrins are heterodimeric transmembrane glycoproteins, consisting of an α and a β subunit. To date, there have been 24 distinct mammalian integrins described, each with its own unique function [10]. Various α and β subunits associate to form an ECM protein selective receptor. For example, the β1 integrin can associate with α1, α2, α10, or α11 to form a collagen selective receptor, while the association of β1 with α5 forms a fibronectin (FN) selective receptor. Integrins bind short amino acid sequences, known as the integrin binding site, within various ECM proteins. While these short amino acid sequences form the integrin binding domain,

integrins recognize full length proteins through synergy sites, giving integrin pairs specificity for various ECM molecules [11]. Once integrins have engaged their respective ligands, they associate with cytosolic proteins, such as talin, focal adhesion kinase (FAK) and paxillin, forming aggregates called focal adhesions. The formation of these focal adhesions leads to the recruitment of many other intracellular signaling proteins, including extracellular signal regulated kinase 1/2 (ERK 1/2) and src. These molecules have been shown to lead to an increase in cytoskeletal tension and cause MSC commitment along the osteoblast lineage [12]. Conversely, previous researchers have shown that insufficient integrin activation, due to a lack of ligand binding or binding to an inappropriate ligand, can lead to recruitment of caspase-8 to the cytoplasmic tail of the β subunit, causing to an apoptotic event termed "integrin mediated death" [13]. Thus, integrin binding events appear to be necessary to induce downstream signaling cascades that regulate many fundamental cell behaviors including survival, proliferation, motility and differentiation.

MSCs carry a wide range of integrins on their surface. Analyses of MSCs have identified the presence of α1, α2, α3, α5, α6, αv, β1, β3, β4, and β5 integrin subunits [14-19]. It has been suggested that activation of several integrin receptors, including αvβ3, α5β1 and α2β1, play a role in MSC survival and differentiation. Our laboratory found that the interaction between αv containing integrin receptors and proadhesive proteins adsorbed from serum was an important mediator of MSC adhesion on hydroxyapatite (HA) biomaterials [15].

Mesenchymal stem cell differentiation

Studies have shown that MSCs are one of the major cell types recruited to the surface of bone implants [20, 21]. These cells, when subjected to the correct external cues, have the ability to differentiate along the osteoblast lineage. *In vitro*, Jaiswal et al found that MSCs can be cultured in osteogenic (OS) media, containing ascorbic acid-2-phosphate (AsAP), β-glycerolphosphate (β-GP), and dexamethasone (Dex), to, in part, recapitulate *in vivo* osteoblastic differentiation [22]. In the initial stages of differentiation, cells change morphology from their extended, fibroblast-like shape to assume a cuboidal morphology. At the same time, alkaline phosphatase (ALP) activity increases, and early marker genes such as cbfa1 (runx2), a transcription factor which promotes osteoblast gene expression, are upregulated. The cells then begin laying down a matrix of osteoid, the protein precursor to calcified bone. The osteoid consists of a conglomerate of proteins, including OPN, OCN, BSP, and collagen I (Figure 2). At this stage of differentiation from osteoblast precursor cells to mature bone forming osteoblasts, the fos related transcription factor, fra-1, is activated [23]. Fra-1 is known to dimerize with Jun proteins, such as c-Jun, JunB, or JunD, to form the heterodimeric transcription factor AP1 [24] which is thought to control late stage osteogenesis, including transcriptional induction of many osteoid ECM proteins [23]. Fra-1 knockout mice develop osteopenia due to a lack of late stage osteoblast protein transcription [23].

Bone Formation

There are two types of bone formation essential to fetal bone development and bone repair. Endochondral ossification is the process of mineralization of cartilage formed

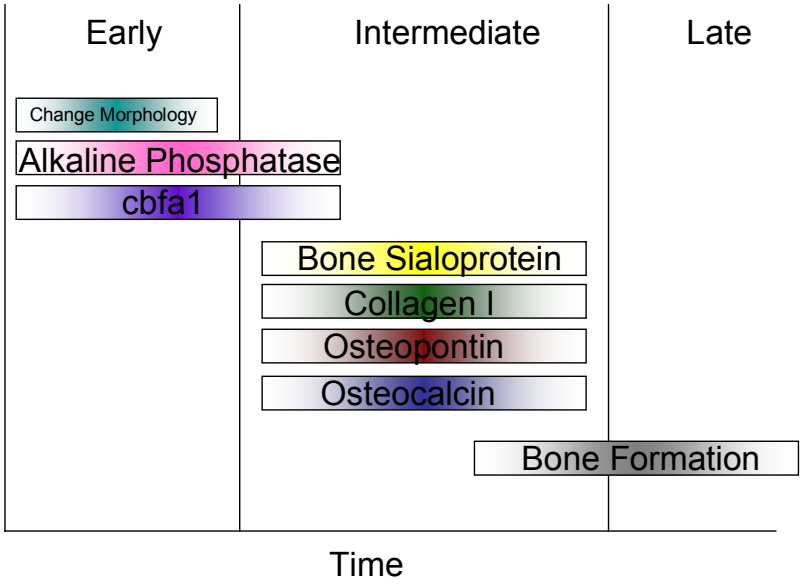

Figure 2: Genetic and Protein Markers of Osteoblast Differentiation The markers of mesenchymal stem cell differentiation along the osteoblast lineage can be separated into three distinct phases. Early markers include a change in morphology from an extended fibroblast like morphology into a more cuboidal shape. In addition, alkaline phosphatase activity and cbfa1 transcription are upregulated. In the intermediate stages, production of the proteins which make up osteoid, including bone sialoprotein, osteopontin, osteocalcin and collagen I, are increased. In the late stage of differentiation, the cells begin to create mineralized bone along the fibers of the osteoid, to create a new bone matrix.

prior to bone formation. This process is essential in fetal bone development, and growth of long bones prior to growth plate fusion [25]. Intramembranous ossification is the process of bone formation which, unlike endochondral ossification, does not require the presence of cartilage prior to bone formation. This process is also crucial in fetal bone development, and additionally necessary for fracture healing [9]. In the case of cellular response to implants, it is typically intramembranous ossification which occurs at the implant interface. Once MSCs have differentiated into osteoblasts, the cells begin the process of mineralizing the osteoid ("matrix mineralization") into a calcium phosphate bone matrix, in the form of carbonate-substituted HA. At this point, the bulk of the final mineral content is deposited [26]. It is believed that small vesicles within the osteoblasts contain the phosphate and calcium ions, which bud from the osteoblasts, and associate with the organic matrix [26]. The apatite crystals which form the inorganic matrix are deposited along the fibers of the osteoid, allowing the organic matrix to determine the organization of the new bone tissue [27].

Implant Biomaterials

Implant biomaterials, in the case of bone repair, are materials which are placed within the bone environment, with the hope of facilitating new bone formation. These biomaterials must be reasonably biocompatible, in addition to allowing bone formation. Bone implants can be characterized as either osseoconductive, osseoinductive or both. Osseoconductive materials have the ability to support bone growth when they are placed in the bone environment, while osseoinductive materials can actually induce bone growth, even in non-bone environments. When assessing the efficacy of each

biomaterial, the biomaterial is compared with the gold standard of bone implants, autografts. It has been widely shown that autografts, which have been performed since 1911, are both osseoconductive and osseoinductive, as they contain all of the matrix proteins and cells necessary to induce bone regeneration at the implant site. However, it is necessary to harvest autografts, most commonly at the iliac crest, and this harvest has been shown to induce donor site morbidity. It is hoped that artifical biomaterials can replace autografts, completely eliminating the donor site pain.

The evolution of implant biomaterials over the past 60 years has been divided into three generations [28]. The first generation was categorized as bioinert materials [5, 28]. These materials included metals, ceramics and polymers [5]. First generation metal implants were initially created out of stainless steel and cobalt-chrome [5]. However, these metals exhibit relatively poor wear resistance, which leads to the formation of wear debris and implant loosening [5]. Then, in the 1960s, it was found that titanium (Ti) and Ti alloys were able to integrate into the bone [29]. First generation ceramics were most commonly made of alumnia and zirconia [5], and used at articulating surfaces, due to their low friction and wear coefficients [5]. Polymer materials used in the first generation included materials created from silicone, acrylic resins, and polymethyl-methacrylate (PMMA) [5]. These materials were used as bone cements [4] and as liners and spacers within articulating surfaces [5]. One of the major drawbacks to the first generation of biomaterials was their inert nature. These materials, as they were bioinert, were regularly encapsulated in a layer of fibrous tissue [5]. In order to avoid this, the second generation of biomaterials was created to interact with the *in vivo* environment.

The second generation of implant biomaterials, which appeared in the mid-1980s, was comprised of "bioactive materials" [5, 28]. These materials have been designed to interact with cells in the *in vivo* environment to induce cell adhesion [5, 28, 30]. In the case of bone biomaterials, these materials were created with the intent to induce cellular responses which would cause a bone layer to form at the material interface. It was at this point that synthetic calcium phosphate type ceramics, including HA ($Ca_{10}(PO_4)_6(OH)_2$), began to be used as bone substitutes and bone fillers [31, 32]. These calcium phosphate materials can be used in dental, and other non-load bearing applications. However, in the case of orthopaedic implants, it was still necessary to have the tensile strength of metals. While it was not possible to change the bioactivity of the base metals and polymers, these materials were modified to induce more favorable cellular responses. For example, metal implants began to be coated with calcium phosphate ceramics, such as HA, to allow for better implant integration, while still maintaining the favorable properties of the metal itself [5, 28]. Alternatively, metal and polymer implants underwent other surface modifications, including tethering short amino acid chains to the surface, to increase cell adhesion [5].

Third generation biomaterials have been characterized as both bioactive and bioresorbable. These materials, not only have the ability to interact with the environment, but they are also able to be degraded by the body, allowing for native tissue to eventually replace the biomaterial scaffold. The hope is that these biomaterials will stimulate specific cellular responses at the molecular level [5, 28]. These materials, which are just beginning to be examined, are designed, not only to allow for cell adhesion, but also to activate signaling cascades, leading to controlled differentiation of

cells along a specific lineage, and eventual replacement with host tissue. These materials will combine the abilities of the second generation biomaterials with further modifications, such as, in the case of bone, delivery of osteogenic factors, to stimulate cells to differentiate along the osteoblast lineage and form a mineralized matrix [28].

Hydroxyapatite Biomaterials

Some types of materials used in tissue repair, such as certain metals and polymers, do not readily support osteoblast adhesion, differentiation, and matrix mineralization. The ability of an implant to adsorb proadhesive molecules from the blood, and present them in conformations which allow for receptor binding is known to contribute to the osseoconductive properties of the implant [33-35] (Figure 3). Synthetic HA has been studied as a potential coating for hard tissue implants to increase implant osseoconductivity, due to the fact that it readily adsorbs proadhesive proteins. In addition, it is suggested by some that HA coatings would also be osseoinductive [36]. The use of calcium phosphate ceramics, including HA, began in the 1970s [5]. Coating the surface of metal implants with calcium-phosphate biomaterials, such as HA, has been shown to increase implant osseointegration [37-43]. Studies have shown that within 24 hours of adhering to HA biomaterials, osteoblast precursor cells exhibit significant changes in the expression pattern of multiple key genes [44], including genes regulating both proliferative and ECM proteins. For example, the protein tenascin C, which is thought to be involved in osteogenesis due to its stimulation of ALP activity, is found to be upregulated when MSCs are seeded on HA [45-47]. At the same time, other osteoblastic genes, such as OCN, are upregulated [48, 49]. *In vitro*, HA biomaterials

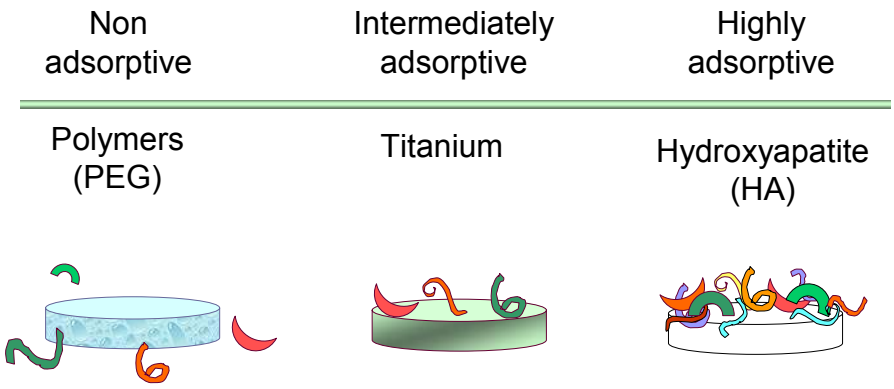

Figure 3: Types of Bone Implant Biomaterials Biomaterials can be divided into three categories. The first type are the non-adsorptive biomaterials such as the polymer PEG. These materials, when placed in serum or blood, do not adsorb proadhesive proteins. The second type, the intermediately adsorptive materials, such as titanium and stainless steel, adsorb some proadhesive proteins from blood or serum. The third type, highly adsorptive materials, such as hydroxyapatite, are able to adsorb abundant amounts of proadhesive proteins from the serum or blood.

have been extensively studied as a substrate for MSC differentiation and matrix mineralization.

In order to study the properties of HA *in vitro*, our laboratory presses commercially available, synthetic HA into disk form and then sinters the material. These HA disks are subsequently functionalized with cell attachment peptides/proteins, and MSCs are attached to the surface. Studies have shown that not only does HA have the ability to increase osteoblastic lineage genes in MSCs, but it also causes an increase in matrix mineralization [50, 51]. In one study, when compared to cells seeded on tissue culture plastic, differentiation markers and matrix mineralization increased two-fold in cells seeded on HA [48].

HA coatings on hard tissue implants have been shown to increase osseointegration in both animal models and human clinical studies [37-43, 52-55]. Hard tissue implants, when coated with HA, are shown to increase bone deposition directly onto the implant surface, thereby decreasing the amount of fibrous encapsulation [37-42, 52]. On HA biomaterials implanted *in vivo*, there is a rapid bone formation on the implant surface. It was found that when HA disks were implanted into rat tibiae, it was virtually impossible to detach the implant from the bone surface, with the surrounding tissue fracturing before the implant/bone interface [55]. In animal models, HA coatings have been shown to promote greater osseointegration than uncoated titanium or titanium alloys [56-62]. In humans, a plasma spray coating of HA onto titanium implants was shown to significantly increase bone deposition, with around 70-75% of the surface area of the implant covered with bone, compared to 24-38% on the controls [43]. Many studies in patients have linked this increase in osseoconductivity of HA coated implants

to an increase in longevity of the implants. For example, a study examining external-fixation pins used for leg lengthening found that after an average of 530 days, titanium pins had loosened 80%, while HA coated pins had only loosened 4% [53]. Knee implants coated with HA showed less inducible displacement at 1 year and had migrated less after 5 years, as measured by clinical, radiographic, and radiostereometric analysis, than uncoated titanium implants [54]. All of these data show that HA coatings on hard tissue implants are beneficial to the lifespan of the implant.

In an additional application to hard tissue implant coatings, porous, resorbable HA scaffolds are being used to fill non-load bearing bone defects. ProOsteon has created a porous, degradable HA scaffold, which begins as a calcium carbonate structure derived from coral. The coralline scaffold undergoes a chemical conversion, creating a surface calcium phosphate HA layer. These porous scaffolds have been shown to increase the rate of bone formation at the site of implantation above that of unfilled defects [63-66]. In a clinical trial, the use of ProOsteon scaffolds and iliac crest autografts for cervical interbody fusion were compared, and it was found that there was no significant difference in the fusion rates between the two grafts [67]. Therefore, these scaffolds are advantageous because, not only is there no donor site morbidity, but like autografts, they are eventually resorbed and replaced with new, native bone tissue.

Protein Functionalization of Biomaterials

In order for cells to attach to a biomaterial surface, there must be a matrix which the cells will recognize, and which will activate cell survival cascades. MSCs recognize multiple cell adhesion molecules which allow for this downstream activation. In the case

of non-fouling, or non-adsorptive, biomaterials, which include many synthetic polymers and some types of metals, the addition of synthetic cell attachment factors to their surfaces is necessary in order for MSCs to have an ECM to bind. It is has been found that functionalizing various biomaterial surfaces, including the HA surface [68-72], with FN or VN significantly increases cell attachment. Thus, it was thought that pre-loading HA implants with these proteins would increase osseointegration. However, data from our laboratory show that proadhesive proteins, including FN, VN, and fibrinogen (Fbg), are adsorbed to the HA surface within minutes following implantation [73]. The primary source of these proadhesive proteins *in vivo* is most likely blood, as blood contains high concentrations of FN, VN and Fbg. Our laboratory also found that these proteins were adsorbed, from serum [74, 75] and the tibial microenvironment [73], in conformations that support MSC integrin recognition and activation. Thus, our laboratory hypothesizes that, while functionalizing non-fouling materials with FN, VN and/or Fbg would provide a benefit, functionalizing HA with these proteins would be redundant, given that HA implants would adsorb these proteins in sufficient concentrations to induce MSC attachment and survival on the HA surface.

While attachment of MSCs to the material surface is an important factor in regulating osseointegration, it is also critical that MSCs are able to differentiate into osteoblasts following the initial adhesion event. Thus, some researchers are examining the effects of modifying HA implants with proteins which induce MSC differentiation in addition to initial adhesion protein modification.

Collagen I

Collagen I is the major protein found in the organic phase of bone, but is not present in abundance in the blood. While this in itself makes collagen I a promising target, it is also known that signaling through a collagen-selective integrin receptor increases osteoblast specific gene expression through the FAK/ERK signaling pathway [76]. MSC adhesion to collagen I is known to induce OPN mRNA expression [77]. In addition, it has been found that the activation of the collagen-selective $\alpha 2\beta 1$ integrin receptor plays a key role in downstream signaling leading to the differentiation of MSCs into mature, bone forming osteoblasts [78] (Figure 4). Adhesion to collagen I, in the absence of other differentiation factors, induces matrix mineralization by MSCs [16]. The addition of collagen I to an HA scaffold material shows a statistically significant increase in the amount of bony ingrowth after 3 months, when compared with bony ingrowth on an HA scaffold alone [79]. Therefore, adsorbing collagen I, or collagen mimetic peptides, to the surface of HA may, in addition to promoting cell adhesion, have the benefit of increasing the rate of differentiation and matrix mineralization of the MSCs bound to the surface.

Bone Morphogenetic Protein 2

Bone Morphogenic Protein 2 (BMP-2), a known factor in the osteoblastic differentiation of MSCs, has been used to increase the rate of new bone synthesis in animal and human models. In a mouse model of osteopenia, systemic injections of BMP-2 caused an increase in the activity of MSCs, and an increase in bone mass [80]. MSCs transformed with an adenoviral vector containing constitutively active BMP-2 showed

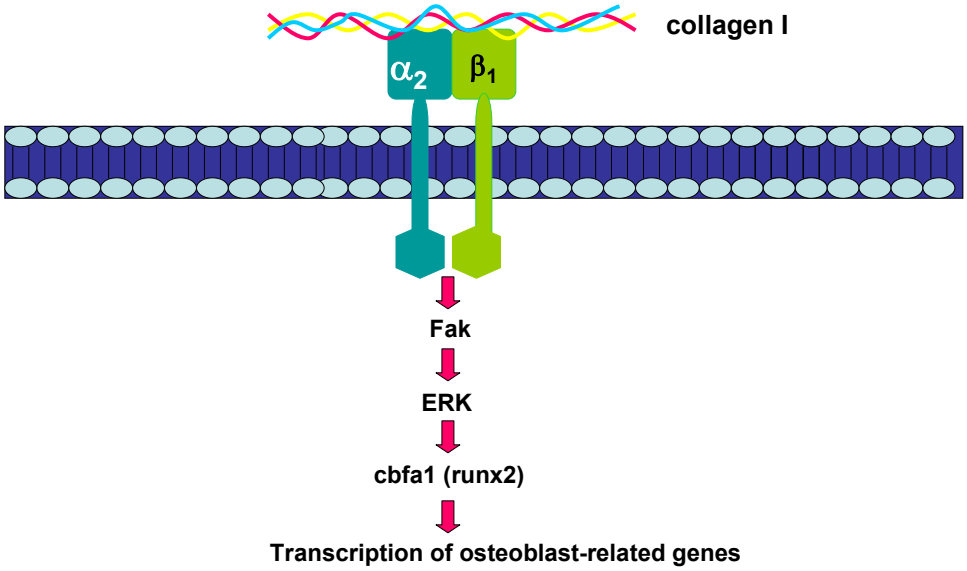

Figure 4: Integrin activation by Collagen I α2β1 integrin binding to the ligand collagen I has been shown to lead to increases in multiple osteoblastic genes, in the absence of any additional differentiation factors. Based on this, it is thought that functionalizing hydroxyapatite implants with collagen I would increase bone formation at the implant interface, due to the increased rate of MSC differentiation along the osteoblast lineage.

increased ALP activity, and increased mRNA expression of collagen I, OPN, and OCN [81].

While systemic injections of BMP-2 have been shown to increase bone mass in mice, the cost of the necessary dosage of BMP-2 in humans brings into question the economic viability of the systemic use of BMP-2. One way to address this concern is to adsorb BMP-2 to the HA biomaterials. This would allow for BMP-2 delivery directly to the implant site, vastly decreasing the cost of BMP-2 delivery. In fact, the addition of BMP-2 to HA scaffolds, either through passive adsorption or the use of BMP-2 producing cells, caused an increase in the osseoinductive capabilities of HA biomaterials [82-85].

Recent evidence suggests that, in addition to mediating differentiation, BMP-2 could also act as a chemotactic factor for osteoprogenitor cells. It was found that BMP-2, vascular endothelial growth factor (VEGF), transforming growth factor beta 1 (TGF-β1) and pleiotropin (PTN) all mediate chemotaxis for osteoblastic cells in a dose dependent manner [86-89], suggesting that treating HA with BMP-2 could induce both MSC recruitment and differentiation. Thus, delivery of BMP-2 proteins directly to the implant site, through the adsorption of proteins to the surface of HA biomaterials will not only significantly decrease the cost of BMP-2 delivery, but it will also have the added benefit of causing a BMP-2 chemotactic gradient to attract MSCs.

Peptide Functionalization of Biomaterials

While full length proteins make the best ligands for receptor recognition, there are issues associated with functionalizing some biomaterials with full length proteins. In

addition to the cost associated with functionalizing a biomaterial with full length proteins, some proteins will not survive the sterilization techniques used on biomaterial implants. One of the strategies used by implant researchers to increase cell adhesion to implants is to functionalize implant surfaces with small integrin peptides, referred to as mimetic peptides, derived from the integrin binding sequences of cell adhesion proteins, rather than full length proteins.

RGD

The RGD sequence is widely known to be the integrin recognition site of many cell attachment proteins including FN, VN, and Fbg, proteins which are abundant within blood [74, 90, reviewed in 91, 92, 93] (Figure 5). RGD was one of the first biomimetic peptides used in functionalizing biomaterial surfaces, and this peptide has been used with much success to improve osseointegration of non-fouling biomaterials [92, 94, 95, reviewed in 96]. In these instances, RGD is typically covalently attached to the surface of the implant to allow for the adhesion of osteoblast precursor cells.

In addition to inducing increased cell adhesion on non-fouling biomaterials, RGD has been shown to increase cell adhesion on metal and calcium-phosphate surfaces, as compared with uncoated surfaces [97, 98]. The standard method for testing peptide efficacy is to compare a peptide-modified material with the unmodified material, however, this does not necessarily recapitulate the biomaterial surface *in vivo*. In contrast to many biomaterials, we and others have shown that HA is very efficient in adsorbing proadhesive proteins [74, reviewed in 91]. More specifically, we reported that HA adsorbed 6-10 fold more FN and VN from serum than titanium or stainless steel surfaces,

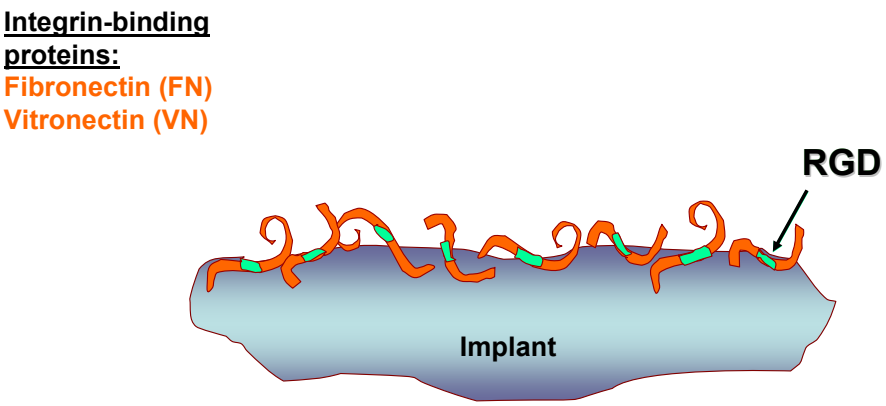

Figure 5: Integrin recognition of fibronectin and vitronectin One of the goals of biomaterials research is to functionalize integrin ligands to implant surfaces. αvβ3 integrins on the surface of mesenchymal stem cells recognize the RGD domain within full length fibronectin and vitronectin. It is thought that, rather than functionalizing implants with full length ligands, which can be costly and technically challenging, implants can be functionalized with this integrin binding domain.

and moreover, these proteins were adsorbed in conformations that allowed for the adhesion of both purified integrins and intact MSCs [74]. Thus, it is unlikely that cells which would bind to the biomaterial surface would encounter peptide coated HA in the absence of adsorbed proadhesive proteins. In light of these data, we questioned whether there was any benefit to linking RGD peptides to the surface of HA implants. Our initial hypothesis was that the RGD peptides would have little to no effect on cell adhesion in the presence of adsorbed proadhesive proteins. To address this question, we assayed cell adhesion to HA surfaces coated with varying concentrations of RGD, followed by an overcoat of fetal bovine serum (FBS) to simulate the *in vivo* environment where the patient's blood would coat an RGD-functionalized HA implant. Results from these studies showed that high concentrations of RGD, in combination with FBS, supported significantly less cell adhesion and spreading than FBS alone, suggesting that RGD was inhibiting MSC adhesion in some fashion [75]. Interestingly, this decrease was not due to a lack of proadhesive protein adsorption. Levels of adsorbed FN and VN on the surfaces of HA coated with a combination of RGD and FBS were equivalent to surfaces coated with FBS alone [75]. While the mechanisms mediating decreased cell adhesion and spreading are not currently understood, one possibility is that, in the presence of RGD, not enough integrins are engaged with native, full-length integrin ligands, and therefore the cell does not receive the type of stimuli required for full integrin activation (Figure 6). In previous studies from our laboratory, it was found that MSCs use αv-containing integrin heterodimers (αvβ3 and/or αvβ5) to bind both FN and VN which are adsorbed from serum onto HA [74]. Studies have shown that RGD elicits significantly less integrin activation than full-length FN or VN [99-103]. It is thought that co-

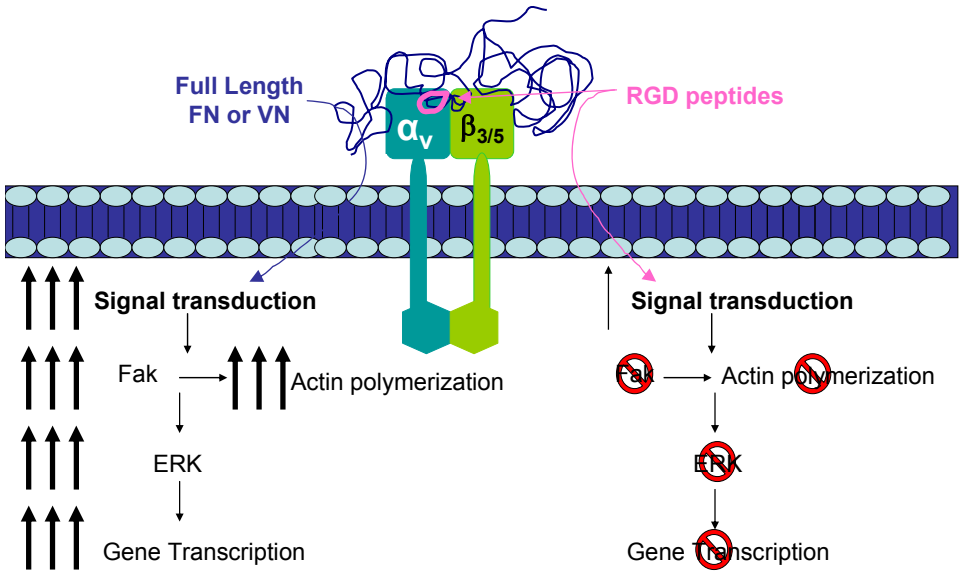

Figure 6: Integrin activation When a full length ligand binds to an integrin, downstream signaling cascades induce actin polymerization and gene transcription. However, when RGD binds to that same integrin, the signal transduction is significantly reduced, leading to a lack of actin polymerization and gene transcription. Thus, when RGD is present on the surface of the HA disks, there is a competition between full length ligands and RGD peptides, causing a decrease in downstream signaling, leading to a lack of cell adhesion and actin cytoskeletal rearrangement

stimulatory domains, such as the PHSRN synergy site of FN, cooperate with integrin binding sites, such as RGD, and are necessary to fully induce the cell spreading events seen with full length proteins [104-106]. Since FN and VN bind to integrins through their RGD domains, it is possible that RGD competes with FN and VN for αvβ3/β5 integrins, leading to attenuated cytoskeletal activity and signaling events.

FN fragments

As RGD has been shown to be less effective at activating integrins, due at least in part, to the lack of synergy sites found on FN and VN, some researchers have begun using longer FN fragments which contain these sites, with the belief that the addition of these synergy sites to the short peptides will be able to activate integrins and induce cell adhesion and bone formation more effectively. Many of these peptides combine the RGD site and the PHSRN synergy site with a linker between [107-110]. These longer peptides are also thought to be more selective for the α5β1 integrin [111], as this is the major FN binding integrin. Most notably is the FNIII 7-10, a polypeptide which integrates the RGD site with the PHSRN synergy site in such a way that they are structurally similar to that of intact FN [109]. This motif has been shown to increase adhesion, and FAK phosphorylation above that of RGD coated surfaces [109]. Cells adherent to these surfaces were shown to have focal adhesions containing talin and vinculin [110]. It has been found to significantly improve titanium implant function compared with both uncoated titanium and RGD coated titanium [112].

Collagen I mimetic peptides

Given the expense and technical challenges associated with functionalizing implants with intact collagen I, many investigators are exploring the use of collagen derived peptides. Linear peptides, such as Asp-Gly-Glu-Ala (DGEA) [113], and GTPGPQGIAGQRGVV (P15) [114], two sequences derived from the α1 helix of collagen I, have shown some efficacy. However, it has recently been hypothesized that the triple-helical structure of the collagen I molecule is necessary for maximal integrin activation, therefore a small triple-helical peptide has also been evaluated for potential use in functionalizing implant surfaces. This peptide, comprised of the amino acid sequence GFOGER, spontaneously assumes a triple-helical structure due to the presence of Gly-Pro-Pro (GPP) repeats engineered onto the ends of the peptide [115, 116]. The collagen I mimetic peptides have shown some degree of efficacy in directing cell attachment to selected biomaterials. However a side-by-side comparison of the peptides, when adsorbed to calcium phosphate biomaterials, has never been performed. It is our belief that, unlike RGD, which we hypothesize competes with the adsorbed proadhesive proteins on the surface of HA for integrins on the surface of MSCs, collagen-derived peptides will not inhibit MSC attachment or spreading in the presence of adsorbed serum/blood proteins, due to the fact that these peptides interact with a different integrin than the receptor involved in binding to adsorbed endogenous FN or VN. We also hypothesize that the peptides will accelerate osteoblastic differentiation, as compared with surfaces coated with blood/serum alone (Figure 7).

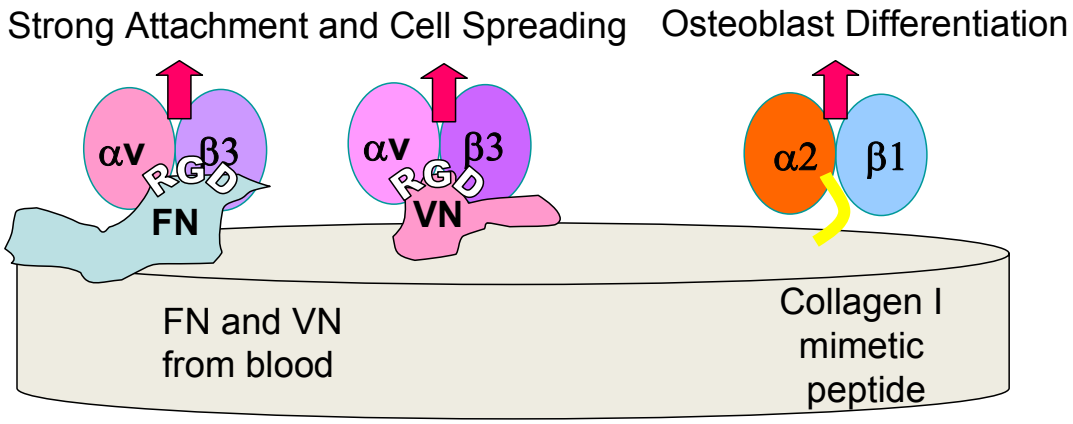

Figure 7: Integrin activation by collagen I mimetic peptides Upon implantation of hydroxyapatite implants, the full length integrin ligands fibronectin (FN) and vitronectin (VN) are adsorbed to the surface of the biomaterial from blood. αvβ3 integrins on the surface of bone forming cells are activated by these full length integrin ligands, leading to bone forming cell survival, differentiation, and implant integration. In the presence of collagen I mimetic peptides, αvβ3 integrins on the surface of these bone forming cells are allowed to be activated by the adsorbed full length integrin ligands. In addition, α2β1 integrins could be activated by the collagen I mimetic peptides, further activating differentiation of mesenchymal stem cells along the osteoblast lineage, possibly increasing the rate of implant osseointegration.

DGEA. The αI helix of collagen I contains a DGEA sequence which binds the α2β1 integrin receptor [113]. In fact, DGEA peptides in solution inhibit MSC binding to collagen I, and block collagen I induced increases in MSC differentiation [78]. DGEA adsorbed to the surface of HA has been shown to increase the levels of the activated form of two kinases, p-FAK and p-ERK, in the murine MC3T3-E1 preosteoblast cell line [117]. This is important because the MAPK pathway has been linked to the expression of osteoblast specific genes, such as cbfa1 [118].

P15. In 1997, Bhatnagar et al. reported that the cell binding domain of collagen I rested in the exposed β bend on the αI helix. They found that an analogous 15-amino acid sequence, which they termed P-15, caused an increase in fibroblast attachment to an anorganic bovine mineral (ABM) [114, 119]. Furthermore, ABM coated with P-15 had the ability to increase bone regeneration in dental implants in humans [120, 121]. However, much of the *in vitro* work with P-15 has been performed with human osteosarcoma cell lines (HOS) [122]. Our laboratory has shown that HOS cells utilize different integrin receptors than MSCs when binding HA [15, 123], and therefore these cells may not be a good model system for predicting which factors will optimally promote the adhesion of MSCs. Further, no attempt has been made to determine which integrin receptor P-15 utilizes to cause this increase in cell attachment. In light of the fact that the α2β1 integrin receptor plays a key role in MSC differentiation, further studies on this peptide are necessary.

GFOGER. Knight et al. in 1998 identified an α2β1 integrin recognition site in collagen I on the αI chain corresponding to the sequence 502GFOGERGVEGPOGPA516 (O=hydroxyproline) [115]. They further determined that both α1β1 and α2β1 utilized the same recognition sequence, which was confined to the GFOGER sequence of the previous peptide [116]. However, this sequence was only recognizable to the integrins when in a triple-helical conformation, similar to that of native collagen I. In order to accomplish spontaneous helix formation, GPP flanking sequences were added to the peptide. GFOGER was found to bind not only α1β1 and α2β1, but also α11β1, another collagen selective receptor [124]. When adsorbed to tissue culture plastic, GFOGER increased the rate of osteoblastic precursor differentiation [125], however, the use of the GFOGER peptide has not been studied on HA.

Research Objectives

The goal of the current research was, first, to examine the effects of RGD peptide coatings on cell adhesion to HA biomaterials which had been implanted in the *in vivo* environment. Our laboratory had previously shown that high concentrations of RGD peptides inhibited cell adhesion to serum proteins adsorbed to HA biomaterials [75]. While the concentrations of FN and VN are high in both serum and blood, other proteins in blood might have an effect on the reaction of MSCs to HA biomaterials. Therefore, in order to more closely mimic the surface a pre-osteoblast would come into contact with in a patient, we implanted HA disks, either uncoated or coated with RGD in rat tibial osteotomies. We found that RGD inhibited cell adhesion to adsorbed proadhesive proteins from the *in vivo* environment. In addition, RGD was found to significantly

inhibit new bone formation on the surface of HA implants, as compared with uncoated HA. Our data suggest that RGD peptides inhibit the interaction between proadhesive proteins adsorbed to the HA surface and MSC integrins. We hypothesize that the RGD peptides are competing with full length integrin ligands for integrins on the surface of MSCs coming into contact with the HA biomaterials (Figure 8). Our findings additionally suggest that our *in vitro* studies using serum as a model for blood overcoatings are a good predictor of biomaterial performance *in vivo*.

We examined a number of different possible mechanisms by which RGD could be inhibiting cell adhesion and osseointegration. We first hypothesized that the RGD was either inhibiting proadhesive protein adsorption, or changing the conformation of the adsorbed proteins. However, we found that the RGD peptide allowed for abundant FN, VN, and Fbg adsorption, and that the FN and VN were adsorbed in conformations which allowed for integrin binding. Further, when we examined whether the concentrations of RGD released into solution were enough to block cell adhesions we found that the concentrations released from the HA surfaces were significantly less than those necessary to block cell adhesion to adsorbed proadhesive proteins. Rather, we found that sheer forces created by washing were removing the loosely bound cells from the RGD coated disks. In addition, we found that those cells which remained on the RGD coated HA surfaces were more likely to undergo apoptosis than those on serum proteins alone, suggesting that those cells on the RGD coated surfaces were not receiving the essential survival signals the MSCs were receiving from serum molecules. These two mechanisms together seem to contribute to the lack of cell adhesion and bone formation seen on RGD coated HA biomaterials. Collectively, these results suggest that there is no therapeutic

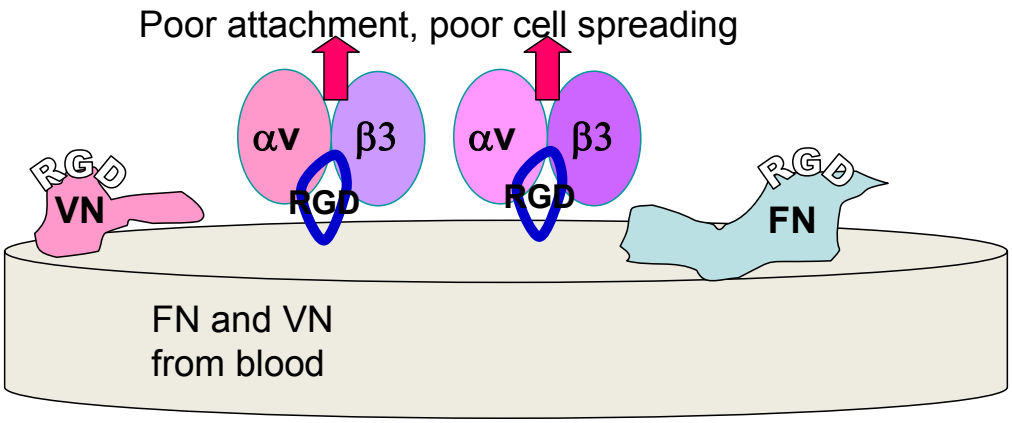

Figure 8: Effects of RGD on integrin activation by full length ligands Integrin activation has been shown to be crucial to long term implant performance. When RGD peptides are coated onto on the surface of HA biomaterials, prior to implantation, full length fibronectin (FN) and vitronectin (VN) are absorbed to the surface of the biomaterial following implantation. However, the RGD peptides compete with these full length integrin ligands for αvβ3 integrins on the surface of the bone forming cells which migrate into the area of the implant. αvβ3 integrins bound to RGD elicit significantly less downstream activity, leading to a lack of osseointegration of the implant biomaterial.

benefit to functionalizing HA biomaterials with RGD peptides, but rather, that RGD peptides will cause a decrease in osseointegration of HA.

Based on our finding that RGD biomimetic peptides inhibit MSC adhesion and bone formation, our laboratory went on to examine the effects of peptides which are not present in high quantities in the blood and body fluids, and which might enhance MSC adhesion and differentiation on HA biomaterials. We hypothesized that, as RGD seems to inhibit the interaction between MSCs and proteins adsorbed to the HA surface, most likely due to a competition mechanism, that proteins which did not compete with FN, VN and Fbg for integrin binding would not inhibit MSC adhesion to those adsorbed blood/serum molecules. The fibrillar protein collagen I is not present in high quantities in the blood/serum, thus it would not be one of the major cell attachment proteins which would adsorb to the HA surface. In addition, binding of a collagen I selective receptor, the $\alpha 2\beta 1$ integrin, has been shown to play a role in osteoblast differentiation. Our laboratory chose to examine three collagen I mimetics. DGEA [113] and P15 [114] are two linear peptides derived from the $\alpha 1$ helix of collagen I, while GFOGER [115, 116] is a peptide created to mimic the triple helical structure of collagen I. Our laboratory found that DGEA and P15 were able to induce cell adhesion and spreading in the absence of adsorbed proadhesive proteins, unlike what we found previously with RGD [75], suggesting that DGEA and P15 induce higher integrin activation than RGD. In addition, DGEA and P15 did not inhibit cell adhesion to adsorbed proadhesive proteins, either from serum *in vitro* or the tibial milieu *in vivo*. This suggests that these peptides are not competing with adsorbed proadhesive proteins for integrins on the surface of MSCs.

While DGEA and P15 did not inhibit cell adhesion in the presence of serum/tibial proteins, they also did not seem to have any benefit as initial adhesion molecules, as they did not enhance initial cell adhesion in the presence of adsorbed serum molecules. However, the peptides did appear to stimulate osteoblastic differentiation. Not only were DGEA and P15 able to enhance both ALP activity and OCN secretion, even in the absence of additional differentiation molecules, but both were also able to increase new bone formation around HA implants. Our findings suggest that these short amino acid sequences are able to, at least partially, activate integrins on the surface of MSCs to allow for downstream activation of differentiation pathways. This finding is markedly different than what we found with RGD peptides, which inhibited bone formation on HA implants.

THE EFFECT OF RGD PEPTIDES ON OSSEOINTEGRATION OF HYDROXYAPATITE BIOMATERIALS

by

KRISTIN M HENNESSY, WILLIAM C CLEM, MATTHEW C PHIPPS, AMBER A SAWYER, FAHEEM M SHAIKH, AND SUSAN L BELLIS

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Abstract

Given that hydroxyapatite (HA) biomaterials are highly efficient at adsorbing proadhesive proteins, we questioned whether functionalizing HA with RGD peptides would have any benefit. In this study, we implanted uncoated or RGD-coated HA disks into rat tibiae for 30 minutes to allow endogenous protein adsorption, and then evaluated mesenchymal stem cell (MSC) interactions with the retrieved disks. These experiments revealed that RGD, when presented in combination with adsorbed tibial proteins (including fibronectin, vitronectin and fibrinogen), has a markedly detrimental effect on MSC adhesion and survival. Moreover, analyses of HA disks implanted for 5 days showed that RGD significantly inhibits total bone formation as well as the amount of new bone directly contacting the implant perimeter. Thus, RGD, which is widely believed to promote cell/biomaterial interactions, has a negative effect on HA implant performance. Collectively these results suggest that, for biomaterials that are highly interactive with the tissue microenvironment, the ultimate effects of RGD will depend upon how signaling from this peptide integrates with endogenous processes such as protein adsorption.

Introduction

Following surgical placement, hard tissue implants are exposed to blood and other body fluids. An implant's ability to adsorb proteins from these fluids, and present them in conformations which engage osteogenic cell receptors is an important factor in implant osseointegration [1-3]. It has been suggested that hydroxyapatite (HA) biomaterials are particularly efficient at adsorbing pro-adhesive proteins [4-6], which may contribute to HA's high degree of osseoconductivity. To model in vivo events, we previously coated

HA disks with serum to mimic blood, and evaluated protein adsorption and adhesion of human mesenchymal stem cells (MSCs) [7], a cell type that can differentiate along the osteoblast lineage. These studies indicated that HA adsorbs abundant vitronectin (VN) and fibronectin (FN) from serum [4, 7], and that these proteins are adsorbed in conformations that promote the binding of purified integrins and MSCs [4]. Moreover, MSC adhesion to serum-coated HA is mediated by an αv-containing integrin heterodimer [8], a subtype that binds both VN and FN.

Given the importance of osteogenic cell attachment, a common strategy for improving cell/biomaterial interactions is to functionalize material surfaces with biomimetic peptides such as RGD. RGD is the known integrin recognition site within many cell attachment proteins, including FN, VN and Fibrinogen (Fbg) [9-11]. Numerous studies have shown that RGD peptides promote increased binding of osteogenic cells, including MSCs, to many types of biomaterials [3, 12, 13]. For example, we and others have reported that RGD-modified HA stimulates better cell adhesion as compared with naive HA [7, 14-19]. However, in vivo, any biomimetic peptide tethered to the HA surface would be presented to MSCs within the context of an adsorbed protein layer. To model this process in vitro, we previously monitored MSC attachment to HA surfaces coated sequentially with RGD and serum [7]. Surprisingly, we found that disks coated with high concentrations of RGD, followed by serum, supported less cell adhesion and spreading than disks coated with serum alone [7], suggesting that the presence of RGD had some inhibitory effect on MSC interactions with HA. Importantly, this effect was observed with three variants of RGD; a linear peptide (GRGDdSP) [7], a cyclic peptide (GPenGRGDSPCA) [7], and a peptide

expressing an HA-binding domain (EEEEEEEGPenGRGDSPCA) [19].

The pro-adhesive proteins FN and VN are known to be abundant within both serum and blood, however, there are significant differences in the concentration of other molecules within these fluids. Thus, the use of serum as an in vitro model for the blood overcoating that occurs during implantation requires validation. To address this issue, we monitored the adhesion of MSCs to uncoated or RGD-coated HA disks that had been briefly implanted into tibial osteotomies, to allow for protein adsorption from within the bone milieu. In addition, disks were implanted into tibiae for longer time intervals to evaluate bone growth at the implant interface. Our results indicate that, when presented within the context of an adsorbed protein layer, RGD has a detrimental effect on both MSC adhesion and new bone synthesis at the implant site.

Materials and Methods

Peptide preparation

RGD peptides (GPenGRGDSPCA, 948.1g/mol, American Peptide) were reconstituted in ddH2O at 1mg/mL, aliquotted and stored at -20°C

Disk preparation

Clinical grade HA powder (Fisher Scientific) was pressed into disks as previously described for in vitro studies [7], or using a 3mm steel hardened die, under 1000 psi for in vivo studies. Pressed disks were coated with RGD peptide as previously described [7]. The disks were subsequently washed with phosphate-buffered saline (PBS) to remove unbound peptide, and warmed to 37°C prior to incubation with cells, or insertion into

tibial osteotomies.

Cell culture

As previously described [4], MSCs were isolated from human bone marrow samples with approval from the University of Alabama Institutional Review Board. Cells from passages 3-13 were used for all experiments.

Animal surgeries and histology

Bone formation on HA implants was evaluated using a rat tibial implant model due, in part, to the relative ease and inexpensive of this system, as well as the comparability of the model to humans. Rat tibial implantation has been extensively employed in investigations of implant integration, including those focused on RGD-modified biomaterials. For our studies, 6-8 month-old male Sprague-Dawley rats were anesthetized with isoflourane, and a 3.25mm x 2.1mm osteotomy was created in the proximal tibia using a Vetroson dental drill fitted with a size 8 burr. HA disks were inserted into the osteotomies (without additional fixation) and left in place for either 30 minutes or 5 days. Only one implant was placed per animal. Implants were placed into the intramedullary region of the bone, although variability in parameters such as the size of individual tibiae and surgical technique did sometimes influence the exact location of disk placement. All experiments were executed in accordance with guidelines established by the University of Alabama Institutional Animal Care and Use Committee.

HA disks implanted for 30 minutes were retrieved from the osteotomies and then washed extensively in PBS with agitation. The disks were subsequently subjected to cell

adhesion assays as described below. At least 5 disks were implanted and analyzed for each of the three treatment groups (uncoated HA, 1 μg/ml RGD coated HA, and 1000 μg/ml coated RGD).

For the 5-day implants, tibiae were retrieved (with disks in place), and embedded in either paraffin for hematoxylin and eosin (H&E) staining, or in poly(methyl methacrylate) for Goldner's trichrome staining. For H&E staining, three implants were evaluated per treatment group (9 animals total). For Goldner's trichrome, which stains mineralized tissue green, 5 implants were analyzed for each of the three treatment groups (15 animals total), with at least two tissue sections per implant evaluated.

The amount of total new bone surrounding 5-day implants, as well as the amount of bone in direct contact with the implant perimeter, were quantified from Goldner's stained sections using Bioquant imaging software. Briefly, images of the tibiae, with the implant centered in the field, were taken at a 4X magnification. The area of the tissue in the field, with the area of the implant removed, was quantified to determine total tissue area. The area of new bone formation, as evidenced by the green staining (excluding the preexisting cortical bone), was then measured, and quantified in relation to the total tissue area. For perimeter contact measurements, the perimeter of the implant was quantified. The areas of contact between the implant and the new bone were then measured and quantified in relation to the total perimeter of the implant.

Adhesion and morphology of MSCs seeded onto implanted HA disks

Disks retrieved from tibial osteotomies after a 30 minute implantation were washed to remove debris and loosely-bound proteins. Human MSCs were seeded onto

the disks in serum-free media and allowed to adhere for 1 hr. Following this incubation, unbound cells were removed with three PBS washes with agitation unless otherwise indicated. The adherent cells were subsequently fixed in 3.7% formaldehyde, permeabilized with 0.2% Triton-X-100, and stained with phalloidin-Alexa 488 and DAPI (Molecular Probes). The samples were mounted with 4.7mM n-propyl-gallate, and visualized using a Nikon fluorescent microscope. Cell adhesion was quantified by counting the number of cells per microscopic field.

Western blotting of desorbed tibial proteins

Retrieved disks were washed, and proteins remaining on the surface were solubilized in boiling SDS-buffer (50mM Tris buffer, 2% SDS, 5% β-mercaptoethanol) for 30 minutes with agitation. Desorbed proteins were resolved by SDS-PAGE, transferred to PVDF membranes, and then blotted with antibodies against fibronectin (Chemicon), vitronectin (Santa Cruz), or fibrinogen (Abcam). An HRP-conjugated secondary antibody was subsequently added and proteins were detected by enhanced chemiluminescence (Amersham Life Sciences).

Blockade of cell adhesion by soluble RGD peptides

RGD release from the HA surface was monitored through multiple reaction monitoring – liquid chromatography mass spectrometry (MRM-LCMS). Briefly, disks were coated with RGD peptide, washed, and incubated for 1 hour in serum-free media to reproduce conditions of a cell adhesion assay. The media was then retrieved, and the amount of peptide in solution was determined by comparing readings to a standard curve.

To determine the amount of RGD peptide required for blockade of cell attachment, MSCs (pre-labeled with a fluorescent dye, CMFDA, Molecular Probes) were seeded onto FBS-coated HA disks in serum-free media containing varying concentrations of soluble RGD peptide. After 1 hr, cells were lysed in 1% TX-100 in 50mM Tris to release the fluorescent dye into solution, and fluorescence was quantified on a fluorometer.

ELISA

HA disks were coated with RGD, FBS or sequentially-coated RGD/FBS as previously described [7]. Following the coatings, the disks were washed and blocked with denatured BSA. Disks were incubated with purified human α5β1 or αvβ3 (Chemicon) for 1 hr. Disks were then washed and exposed to antibodies for α5β1 or αvβ3 (Chemicon); followed by an HRP-conjugated secondary antibody. A colorimetric substrate was added, and the absorbance read at 450 nm.

Caspase 3 activation

MSCs were seeded onto HA disks previously coated with RGD, FBS or RGD/FBS. After 24 hours at 37°C, disks were washed and treated with boiling SDS-buffer to solubilize the adherent cells. Cellular proteins were resolved on a 17% polyacrylamide gel, transferred to PVDF membranes, and active (cleaved) caspase 3 was detected using an antibody from Cell Signaling.

Statistical analysis

For cell adhesion assays performed on retrieved disks, at least 5 implants per

treatment group were evaluated. For measurements of bone formation, 5 implants per treatment group were subjected to Bioquant software analyses of Goldner's trichrome-stained sections, with at least 2 sections per implant analyzed. For all other graphical data, at least 3 independent experiments were performed, with each experiment performed in triplicate. Data were plotted as mean + s.e.m., and a One-Way ANOVA parametric analysis was used to calculate statistics. A confidence level of 95% ($p<0.05$) was considered significant.

Results

MSC adhesion to HA disks coated with proteins from the tibial microenvironment

Our prior studies indicated that MSCs adhere and spread better on HA disks coated with serum as compared with uncoated or RGD-coated surfaces [7], presumably due to the presence of adsorbed serum FN and/or VN. In addition, we found that when disks were sequentially coated with RGD/serum, RGD inhibited cell adhesion to adsorbed serum proteins [7]. To determine whether similar cell responses were elicited by endogenous proteins, HA disks were implanted into rat tibial osteotomies for 30 minutes to allow protein adsorption; the disks were then retrieved, washed, and MSCs were seeded onto the disks and allowed to adhere. Prior to implantation, disks were pre-coated with either low or high concentrations of RGD (1 μg/ml or 1000 μg/ml, respectively), or alternately left uncoated. A comparison of MSC adhesion on the retrieved disks indicated that disks initially left uncoated, then overcoated with endogenous tibial proteins, promoted significantly greater MSC adhesion than either of the disks that had been pre-coated with RGD prior to implantation (Figure 1a and b). As

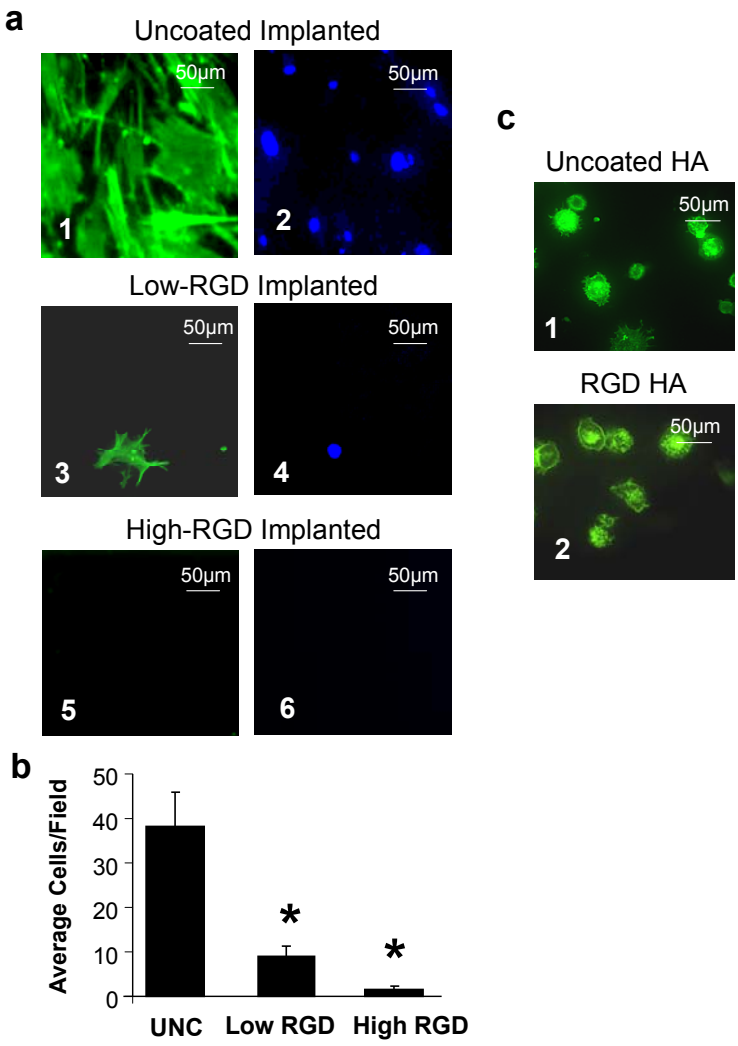

Figure 1: RGD peptides inhibit cell adhesion to HA disks coated with proteins from the tibial microenvironment. a) Representative images of MSCs adherent to HA disks retrieved from tibial osteotomies. Prior to implantation, disks were left uncoated (panels 1,2), or coated with either 1μg/mL RGD ("low RGD", panels 3,4), or 1000 μg/mL RGD ("high RGD", panels 5,6). Cells were double-labeled with phalloidin-Alexa 488 (green stain, panels 1, 3 and 5) and DAPI (blue stain, panels 2, 4, 6) b) Cells adherent to the retrieved disks were quantified by counting the average number of cells per field. * denotes significant difference from uncoated samples. c) Phalloidin-stained cells adherent to uncoated (panel 1), or RGD-coated (panel 2), HA disks in the absence of implantation.

Note: From "The effect of RGD peptides on osseointegration of hydroxyapatite biomaterials" KM Hennessy, WC Clem, MC Phipps, AA Sawyer, FM Shaikh, and SL Bellis, 2008, *Biomaterials, 29*, p. 3075. Copyright 2008 by Elsevier Ltd. Reprinted with permission.

a control, we also evaluated MSC adhesion on uncoated or RGD-coated disks that had not been placed into tibiae. As shown (Figure 1c), RGD alone was not able to induce cell spreading, a response that reflects full integrin activation and also contributes to strong cell adhesion [20, 21]. Taken together the results in Figure 1 suggest that MSCs adhere and spread better on adsorbed endogenous proteins than on RGD alone, and importantly, when RGD is combined with endogenous proteins, RGD appears to have a strong inhibitory effect on MSC attachment.

Effect of RGD on the adsorption of proadhesive proteins

We speculated that poor cell attachment to retrieved HA disks pre-coated with RGD might have resulted from RGD blockade of protein binding sites on HA, thus reducing the amount of adsorbed endogenous proteins. To evaluate protein adsorption, HA disks retrieved from tibiae were incubated in SDS buffer to desorb proteins, and the amounts of FN, VN and Fbg were assessed by Western blotting. As shown (Figure 2), low concentrations of RGD pre-coatings did not have any inhibitory effect on the adsorption of FN, VN or Fbg, although the high RGD coatings did slightly diminish VN and Fbg deposition. Thus, MSC adhesion to disks retrieved from tibiae was inhibited by the presence of RGD peptides despite an abundance of proadhesive proteins on the HA surface. As well, the marked inhibition of cell adhesion by low concentrations of RGD (see Figure 1a and b), which do not block protein adsorption (Figure 2), suggests that diminished protein adsorption is not the major mechanism by which RGD attenuates MSC binding to implanted HA disks.

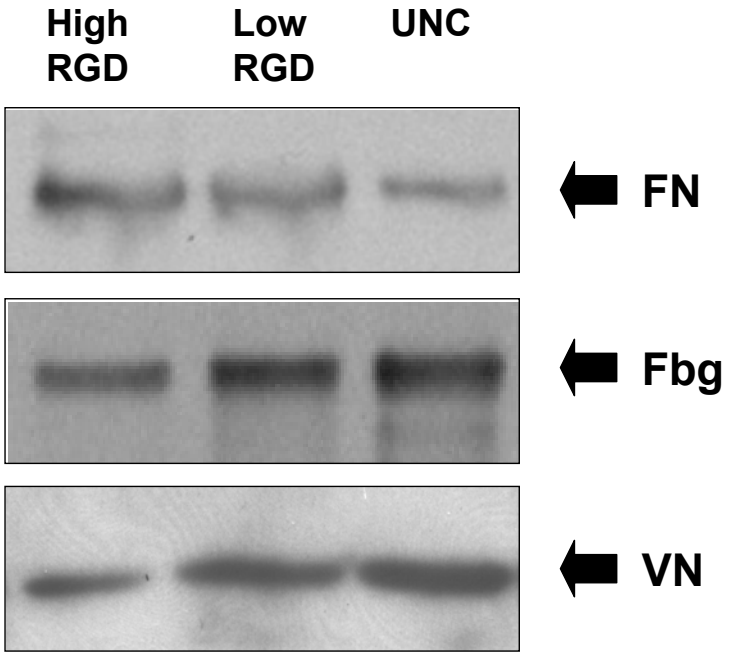

Figure 2: Pre-coating HA with RGD has a minimal effect on the adsorption of pro-adhesive proteins from the tibial microenvironment. Western blots of fibronectin (FN), fibrinogen (Fbg) and vitronectin (VN) following desorption from HA disks that were implanted into tibiae for 30 minutes.

Note: From "The effect of RGD peptides on osseointegration of hydroxyapatite biomaterials" KM Hennessy, WC Clem, MC Phipps, AA Sawyer, FM Shaikh, and SL Bellis, 2008, *Biomaterials, 29*, p. 3075.

Strength of cell attachment on RGD-coated HA

We hypothesized that RGD peptides on HA might compete with adsorbed proteins for integrins on the MSC surface. RGD peptides are known to promote weaker integrin activation than full-length adhesive proteins [20, 21], therefore it follows that if a majority of integrin receptors was bound with RGD rather than FN or VN, this might result in attenuated integrin signaling and weaker cell attachment. Consistent with standard methods for monitoring cell adhesion, our protocol includes a wash step at the end of the attachment interval to remove unbound cells. It was possible that, in the case of RGD-modified HA, loosely-bound cells were also removed during this step. To test this, MSCs were allowed to adhere to RGD-modified retrieved disks, and then disks were washed very gently. This experiment was performed side-by-side with our standard protocol, which includes several washes with agitation. As shown in Figure 3, more MSCs were present on the gently-washed retrieved disks ("low stringency wash") as compared with disks subjected to a standard wash protocol. Importantly, even after a gentle wash, there were fewer cells, and these were significantly less spread, than cells adherent to disks coated with endogenous proteins only (compare Figure 3 with Figure 1a, panel 1). These data suggest that disks coated with endogenous proteins only (i.e., no RGD) stimulate greater integrin activation and stronger cell adhesion than disks coated with RGD prior to implantation.

Effect of RGD on new bone synthesis and bone/implant contact

The adhesion of osteogenic cells to orthopaedic and dental biomaterials is a significant factor in implant osseointegration. To test whether the weak cell adhesion

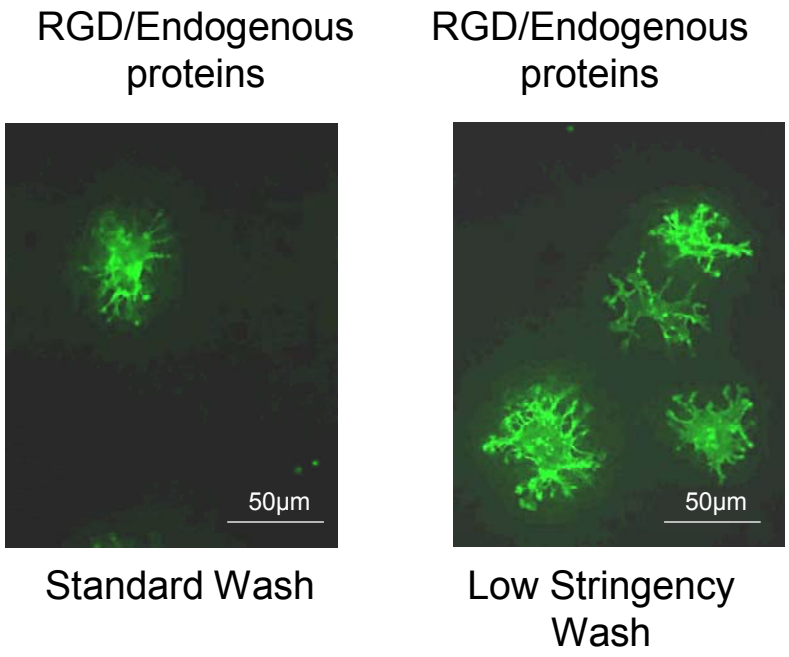

Figure 3: The presence of RGD weakens cell attachment to retrieved HA disks. Representative images of cells adherent to retrieved disks following exposure to either a standard or low stringency wash protocol.

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associated with RGD pre-coatings (Figure 3) had any effect on implant integration, uncoated and RGD-coated HA disks were placed in tibial osteotomies for 5 days. The tibiae, with implants in place, were then retrieved, and new bone deposition on the HA surface was measured by either H & E staining or Goldner's Trichrome. Images of H & E-stained sections (Figure 4a) showed trabecular-like bone (pink staining) in apposition to the perimeter of HA disks that were left uncoated prior to implantation. In contrast, there was a marked dearth of bone-like tissue surrounding RGD-coated implants. To more definitively assess bone formation, sections stained with Goldner's trichrome, which is highly specific for mineralized tissue (green staining), were subjected to Bioquant imaging analysis. Specifically, Bioquant software was used to quantify the total amount of newly-synthesized bone in the vicinity of the implant, as well as the percentage of the implant surface that was in direct contact with bone. As shown in Figs.4b and c, both the low and high RGD peptide coatings significantly inhibited the total amount of new bone formed, as well the amount of bone directly contacting the HA surface.

Influence of RGD on integrin binding sites within adsorbed proteins

There are multiple mechanisms by which the presence of RGD in combination with adsorbed endogenous proteins might contribute to diminished cell attachment. We next tested the hypothesis that RGD peptides on the HA surface cause a disruption in conformation of adsorbed FN and VN, thus diminishing the accessibility of the integrin binding site within these proteins. Because of the large number of samples required for mechanistic studies, we used serum as an in vitro model for the overcoating of blood that

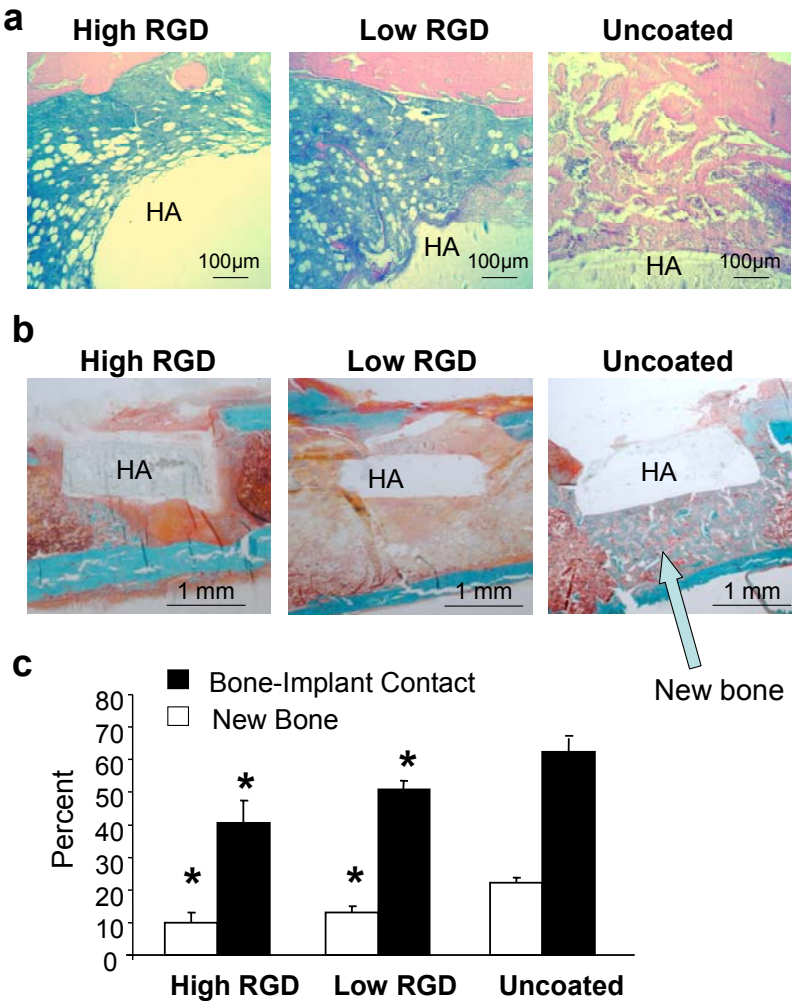

Figure 4: RGD peptides inhibit osseointegration of HA implants. a) Representative images of tibiae with embedded HA disks following a 5-day implantation. Sections were stained with hematoxylin and eosin. b) Representative images of 5-day implants stained with Goldner's trichrome, which stains mineralized tissue green. c) The amount of total new bone surrounding the implant (white bars), and the amount of bone directly contacting the perimeter of the implant (black bars) were quantified using Bioquant software. * denotes significant difference from uncoated samples.

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happens in vivo on the implant surface. To this end, disks were pre-coated with RGD, serum (FBS), or a sequential RGD/FBS coating, and then the binding of purified integrin receptors to the disks was quantified by ELISA. We evaluated the binding of two integrins, αvβ3 which binds to VN (in addition to other matrix molecules including FN), and α5β1, which binds to FN. Results from these experiments revealed that both αvβ3 and α5β1 integrins bound significantly better to FBS-coated surfaces than to RGD-coated surfaces (Figure 5a and b), consistent with the fact that full-length FN and VN are known to promote stronger integrin binding than the isolated RGD sequence [20, 21]. However, there was no significant decrease in integrin binding to RGD/FBS sequential-coatings as compared to FBS alone (Figure 5a and b), suggesting that the presence of RGD on the HA surface does not disrupt the availability of the integrin binding site on adsorbed proadhesive proteins.

RGD release from the HA surface

We next questioned whether RGD peptides might be released from the HA surface in sufficient quantities to bind MSCs in solution and block cell attachment. To examine this possibility, HA disks were pre-coated with RGD peptide, and then incubated in serum-free media to reproduce the conditions of a cell adhesion assay. At the end of this incubation, the solution was collected and the concentration of released RGD peptide was determined by MRM-LCMS, through comparison with a standard curve. It was found that approximately 100-200 ng/mL of peptide were released into solution (data not shown). To determine if this amount of soluble RGD was sufficient to block cell adhesion to protein-coated HA, MSCs were seeded onto FBS-coated HA disks

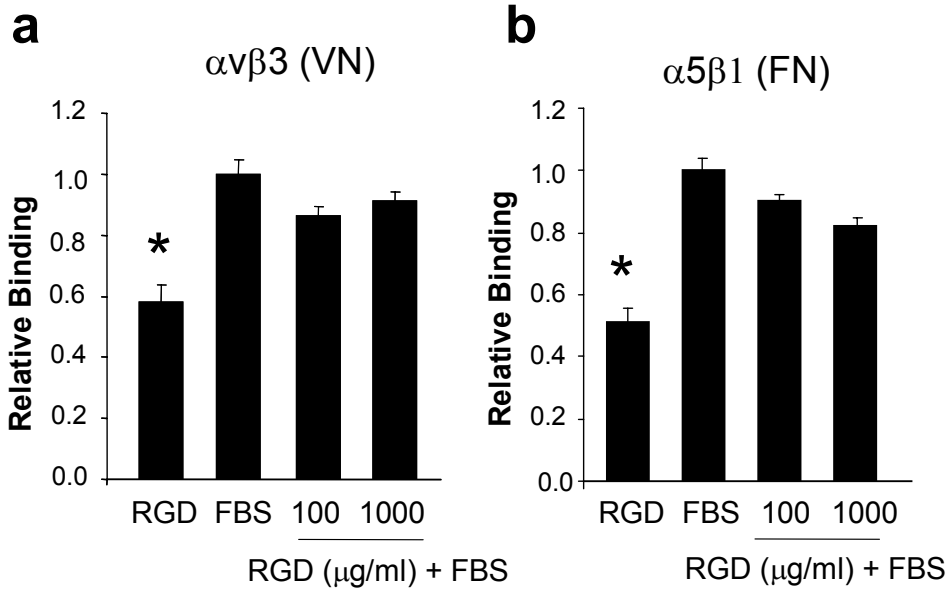

Figure 5: RGD peptides do not disrupt accessibility of integrin binding sites on adsorbed proadhesive proteins. a) Purified αvβ3 integrin binding to HA disks coated with RGD, FBS, or sequential RGD/FBS. b) Purified α5β1 integrin binding to disks coated with RGD, FBS, or sequential RGD/FBS. * in panels a and b denotes difference from FBS-coated samples.

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in media containing varying concentrations of soluble RGD to allow blockade of integrin receptors. MSC adhesion was then quantified as previously described [7, 19]. Results from these experiments showed that RGD concentrations up to, and including, 1 μg/ml had no significant effect on cell adhesion (Figure 6). Thus, the amount of RGD released from the HA surface under the conditions of our adhesion assays is many-fold less than the amount required to significantly diminish MSC attachment to protein-coated HA.

Cell apoptosis on RGD-coated HA

While the collective results described above suggested that RGD inhibits implant integration through inducing weak cell attachment, we also questioned whether RGD might affect cell survival. Interestingly, it was reported that adherent cells that either have unliganded integrins, or integrins bound to inappropriate ligands, undergo apoptosis [22]. Accordingly, we speculated that cells adherent to RGD for extended intervals might perceive RGD as an "inappropriate" signaling ligand. To test this hypothesis, MSCs were seeded onto HA disks coated with either FBS or sequentially coated with RGD/FBS, and apoptosis was evaluated by monitoring caspase 3 activation. Pre-coating HA disks with RGD induced significantly greater caspase 3 activation (Figure 7), indicating that the presence of RGD on the HA surface, when presented in the context of adsorbed proteins, induces apoptosis.

Discussion

HA is highly osseoconductive, and we hypothesize that this is partially due to the fact that calcium-phosphate biomaterials adsorb proteins from the microenvironment that

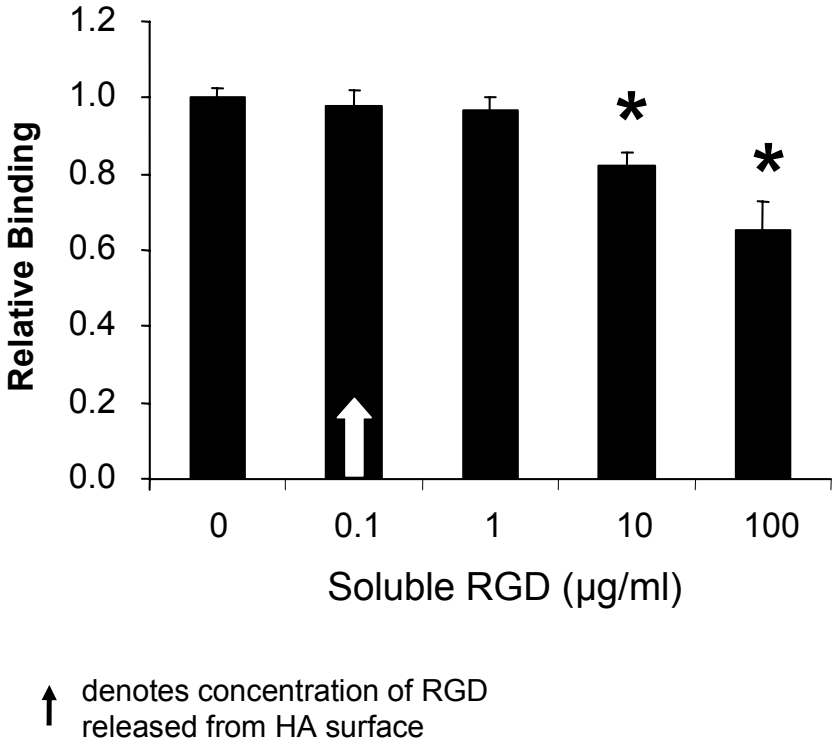

Figure 6: RGD peptides released from the surface of HA do not significantly inhibit cell adhesion. Cell adhesion to FBS-coated HA disks in the presence of varying concentrations of soluble RGD. * denotes significant difference from cell adhesion in the absence of soluble RGD peptide.

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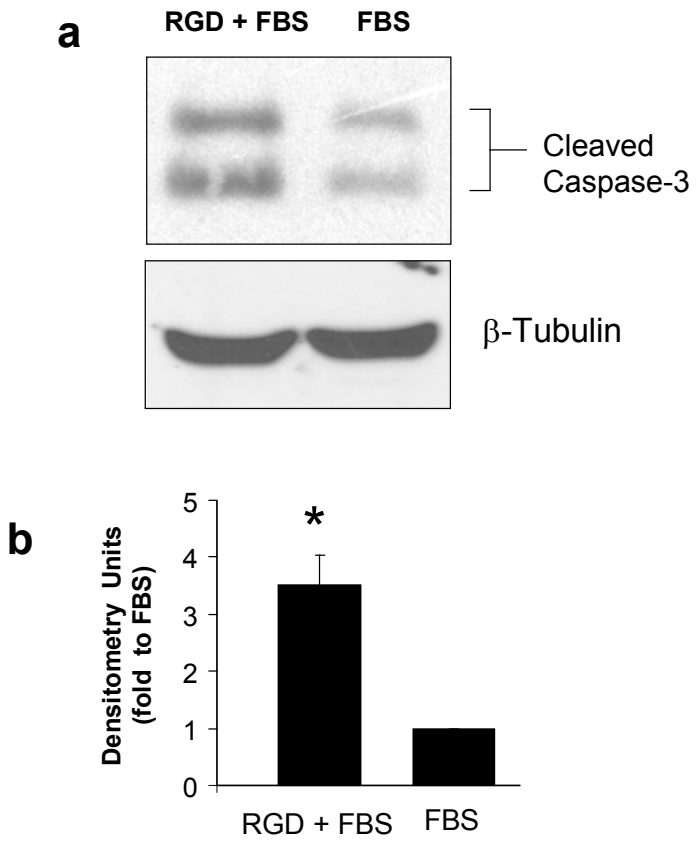

Figure 7: RGD peptides initiate apoptotic signaling cascades. a) Representative western blot of active (cleaved) caspase 3 in cells grown for 24 hours on HA disks coated with either FBS or sequentially coated with RGD/FBS. b) Densitometric analysis of western blots. * denotes significant difference from FBS

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assist in bone regeneration. To model protein adsorption from body fluids, many investigators have characterized protein adsorption from serum. We and others have shown that HA adsorbs more FN and VN from serum than materials such as titanium, stainless steel, or poly(l-lactic acid) [4-6], and preincubation of HA with either protein significantly enhances osteogenic cell attachment [7, 23, 24] Moreover, adsorption of serum proteins protects cells from apoptosis [6], presumably through induction of cell survival signals elicited by engaged and activated integrins. Cell adhesion to serum-coated HA surfaces is RGD-dependent [25], and inhibited by function-blocking antibodies against the αv integrin subunit [8], suggesting that cells adhere via adsorbed FN and/or VN. Our current results show that HA adsorbs abundant FN, VN, and Fbg within the first 30 minutes of implantation in the tibial environment, and that adsorption of endogenous proteins is required for optimal MSC adhesion and spreading.

FN, VN and Fbg, representing the most abundant adhesion-promoting proteins in blood [26-28] bind to integrins through an RGD-dependent mechanism [9, 10]. However, in addition to the requisite RGD sequence, there are multiple other domains within these proteins that bind to integrins and either synergistically or additively stimulate integrin signaling [11, 29, 30]. Hence, the RGD sequence by itself elicits weaker integrin activation than full length adhesion proteins [20, 21]. For example, integrin binding to full length FN and VN activates the downstream signaling molecules FAK and ERK [31], leading to the induction of osteogenic gene expression [32], alkaline phosphatase activity, calcium deposition [33, 34], and runx2 activation [33]. In contrast, cell adhesion to RGD was shown to activate FAK, but not ERK [35]. In light of these observations, our initial prediction was that RGD peptides would have little effect on cell

adhesion to HA implants, given that HA would adsorb adhesion proteins in vivo, and that molecular cues from these adsorbed proteins would likely over-ride signaling from RGD. To test this hypothesis, we implanted uncoated or RGD-coated HA disks in tibiae to allow endogenous protein adsorption, retrieved the disks and then monitored MSC attachment. Surprisingly, we found that RGD peptides negatively impacted cell adhesion. The mechanisms underlying this finding are not currently understood, however our results appear to argue against several possibilities. First, the presence of RGD coatings on the HA disks had little effect on the adsorption of FN, VN or Fbg from the tibial microenvironment. Thus, cell adhesion was attenuated despite the presence of abundant adhesion proteins on the HA surface. Secondly, in vitro ELISA-type assays using purified α5β1 and αvβ3 receptors indicated that RGD pre-coatings did not significantly disrupt the accessibility of integrin binding sites on the full-length proteins, suggesting that loss of cell adhesion was not due to conformational-disruption of adsorbed proteins. Finally, the blockade in cell attachment did not appear to be due to release of soluble RGD peptides from the HA surface, a process which could theoretically block cell attachment to HA by saturating the integrins of cells still in suspension.

Our working hypothesis is that there is competition between RGD and adsorbed proteins for cell surface integrins, and that, as the concentration of RGD increases, more integrins become bound with RGD rather than full-length proteins. In turn, this causes attenuated integrin signaling, leading to a lack of full cell spreading and weaker overall cell attachment (see Figure 8 for model). This concept is supported by current results showing that wash steps more readily removed cells from disks coated sequentially with RGD/endogenous proteins as compared with endogenous proteins alone. Additionally,

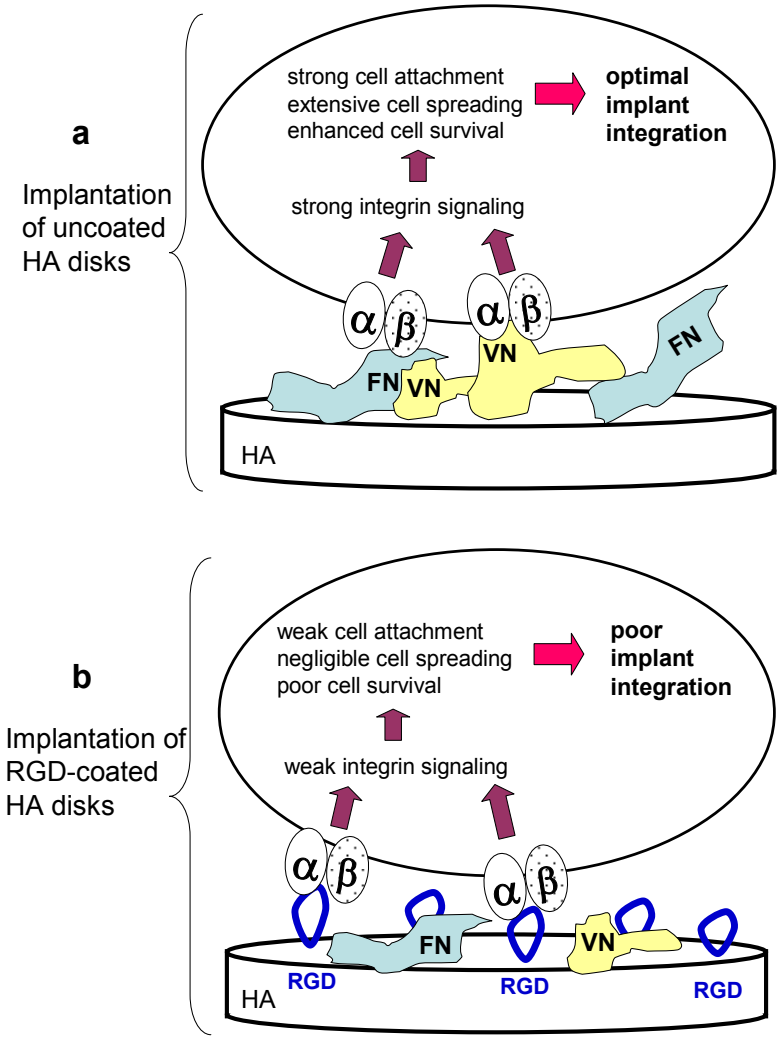

Figure 8: Model describing negative effects of RGD on implant osseointegration. a) When adsorbed native matrix proteins, such as FN and VN, bind to integrin receptors on the MSC surface, this induces robust integrin-dependent signaling, leading to strong cell adhesion, cell spreading and initiation of survival signals. These events are crucial for osteoblastic differentiation of MSCs and deposition of a bone matrix on the implant surface. b) When RGD peptides are coupled to the biomaterial surface, there is competition between RGD and native adsorbed proteins for binding to integrin receptors. If a high proportion of integrins are bound with RGD rather than native proteins, then weak integrin signaling will ensue, resulting in poor cell adhesion and spreading, increased cell apoptosis, and ultimately, poor osseointegration.

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the cells that did remain bound to RGD-modified surfaces following wash steps were more poorly spread than those attached to HA disks coated with endogenous proteins only.

Initial cell attachment is an essential first step in osseointegration, however there are many other factors that ultimately influence implant fixation. To test the effects of RGD on implant integration, we placed uncoated and RGD-coated HA disks in tibiae, and then monitored bone formation 5 days later. These experiments revealed that RGD peptides had a strong inhibitory effect on both the total amount of new bone formed, and the amount of bone directly contacting the implant perimeter. These data are consistent with the hypothesis that RGD inhibits osteogenic cell attachment, however we speculated that other events may also play a role in this process. We were particularly intrigued by work from Stupack et al. which described a phenomenon known as "integrin-mediated death", a process whereby adherent cells with unliganded integrins or integrins bound with "inappropriate" ligands undergo apoptosis [22]. Based on this work, we questioned whether RGD peptides might be saturating integrin receptors, preventing binding to full-length FN, VN or Fbg, and that in turn, cells might perceive RGD as an inappropriate signaling ligand. Indeed, we found that the presence of RGD peptides caused greater activation of the apoptotic marker, caspase 3. Thus, our collective results suggest that RGD peptides, by competing with adsorbed proteins for integrin receptors, have a negative effect on implant integration by reducing both the initial attachment and survival of osteogenic cells on HA surfaces.

Interestingly, despite extensive in vitro results describing a beneficial effect for RGD, the number of animal studies aimed at assessing the performance of RGD-

modified biomaterials is limited. In general, these studies support the view that RGD increases implant integration [36-42]. However, in some instances, RGD peptides either had no effect on new bone synthesis [43], or were actually detrimental [44]. For example, RGD peptides were reported to inhibit peri-implant bone formation on polymer-coated titanium surfaces [44]. Clearly there are multiple factors that could influence the bioactivity of RGD in vivo including peptide density, the amino acid sequences flanking the RGD domain, and the stability of peptide bonding to the material surface. However, in addition to these factors, we hypothesize that interactive processes between the material surface and host tissue may have contributed to some of the variable results previously reported for in vivo studies using RGD.

Conclusions

The broad implication of the current investigation is that the potential benefits of RGD with regard to implant osseointegration will likely be context-dependent. For biomaterials that are highly interactive with the tissue microenvironment, the effects of RGD will depend upon how signaling from these peptides integrates with endogenous processes such as protein adsorption. Accordingly, there is a compelling need to study and characterize these endogenous processes in order to gain meaningful predictive information about biomaterials performance. This concept is strikingly illustrated by the fact that, in the absence of adsorbed proteins, RGD consistently improves cell adhesion to HA, whereas in contrast, RGD is markedly detrimental when presented in combination with adsorbed proteins.

Acknowledgments

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THE EFFECT OF COLLAGEN I MIMETIC PEPTIDES ON MESENCHYMAL STEM CELL ADHESION, AND OSTEOBLASTIC DIFFERENTIATION AS WELL AS BONE FORMATION ON HYDROXYAPATITE BIOMATERIALS

by

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Abstract

Integrin-binding peptides increase cell adhesion to naive hydroxyapatite (HA), however, in the body, HA becomes rapidly modified by protein adsorption. Previously we reported that, when combined with an adsorbed protein layer, RGD peptides interfered with cell adhesion to HA. In the current study we evaluated mesenchymal stem cell (MSC) interactions with HA disks coated with the collagen-mimetic peptides, DGEA, P15 and GFOGER. MSCs adhered equally well to disks coated with DGEA, P15, or collagen I, and all three substrates, but not GFOGER, supported greater cell adhesion than uncoated HA. When peptide-coated disks were overcoated with proteins from serum or the tibial microenvironment, collagen mimetics did not inhibit MSC adhesion, as was observed with RGD, however neither did they enhance adhesion. Given that activation of collagen-selective integrins stimulates osteoblastic differentiation, we next monitored osteocalcin secretion and alkaline phosphatase activity from MSCs adherent to DGEA or P15-coated disks. Both of these osteoblastic markers were uregulated by DGEA and P15, in the presence and absence of differentiation-inducing media. Finally, bone formation on HA tibial implants was increased by the collagen-mimetics. Collectively these results suggest that collagen-mimetic peptides improve osseointegration of HA, most probably by stimulating osteoblastic differentiation, rather than adhesion, of MSCs.

Introduction

In order for hard tissue implants to integrate into existing bone, osteoblast precursor cells must bind to the implant surface, differentiate, and form a new bone

matrix to tether the implant in place. One common strategy for improving implant integration is to functionalize biomaterial surfaces with peptides that mimic the native extracellular matrix, with the goal of providing attachment sites for adhesion receptors present on osteogenic cells. One of the most widely-studied adhesion-promoting peptides is Arg-Gly-Asp (RGD), a tri-amino acid sequence found within matrix proteins such as fibronectin (FN) and vitronectin (VN). RGD is a principal ligand for the integrin family of adhesion receptors, although it is known that other domains within FN and VN act synergistically with RGD to more strongly activate integrins [1-8]. Many types of biomaterials have been modified with RGD, and *in vitro* studies consistently suggest that RGD-modified surfaces promote better cell attachment than unmodified surfaces [9-15].

However, some types of biomaterials, including hydroxyapatite (HA), are very efficient at adsorbing adhesive proteins present within body fluids at the surgical site, and therefore it isn't clear that functionalizing HA with RGD would be beneficial *in vivo*. We and others have shown that FN, VN and fibrinogen (Fbg) from the surgical environment become adsorbed to the HA surface within minutes following implantation [16-19]. Thus, an RGD-modified HA surface would be presented to cells within the context of an adsorbed layer of endogenous integrin-binding proteins. To model the role of protein adsorption in influencing cell attachment to RGD-modified HA biomaterials, we previously studied mesenchymal stem cell (MSC) adhesion to RGD-modified HA disks that were either over-coated with fetal bovine serum (FBS) [15], or implanted for 30 minutes into rat tibiae to allow endogenous protein adsorption [19]. Surprisingly, these studies revealed that RGD was detrimental to MSC attachment; more cells adhered to the FBS-coated [15] or tibial-implanted [19] HA disks that lacked RGD pre-coatings.

Moreover, the combined RGD/FBS-coated HA surfaces elicited greater activation of the cell apoptotic marker, caspase 3, than FBS-coated HA [19], suggesting that RGD had a negative effect on cell survival. Finally, a comparison of uncoated and RGD-coated HA disks implanted for 5 days into rat tibiae showed that the RGD coatings significantly inhibited the amount of new bone formed on the implant surface, as well as the degree of direct bone-implant contact [19]. Taken together, these results suggested that RGD inhibits the osseointegration of HA biomaterials, most probably through diminished attachment and survival of MSCs.

The mechanisms underlying the inhibitory effects of RGD on cytocompatibility and implant integration are not currently understood, however we hypothesize that synthetic RGD peptides on the HA surface compete with adsorbed proteins such as FN, VN or Fbg for binding to cell surface integrin receptors. FN, VN and Fbg are among the most abundant adhesion molecules in blood [20-22], and all of these proteins bind to integrins through an RGD-dependent mechanism [16, 23, 24]. It is possible that synthetic RGD peptides divert integrin receptors away from binding adsorbed FN, VN or Fbg proteins, and thereby elicit diminished integrin signaling, given that RGD peptides are weaker integrin ligands than native full-length adhesion proteins [7, 25]. We further speculate that RGD will be detrimental for biomaterials that have high affinity for adsorbing integrin ligands from the tissue environment, but alternately beneficial for biomaterials that lack any other integrin ligand (e.g., nonfouling types of materials).

Regardless of mechanism, our prior results clearly showed that RGD peptides were not beneficial for HA biomaterials, and we therefore questioned whether there were any other adhesive peptides that would improve cell and/or tissue responses to HA. As

an alternative to RGD, many investigators have evaluated osteogenic cell attachment to materials functionalized with collagen-derived peptides. Collagen mimetic peptides are attractive for a number of reasons. First, collagen I binds to different integrin receptors than FN, VN and Fbg, and the integrin/collagen I interaction is thought to be RGD-independent. Hence, collagen mimetic peptides would not be expected to compete with adsorbed FN, VN or Fbg for binding to cell surface integrins. Secondly, it is unlikely that collagen I would adsorb to the HA surface in substantial amounts upon surgical placement, given that collagen I is not highly abundant within the blood. Finally, activation of the collagen-selective integrin, $\alpha 2\beta 1$, induces osteoblastic differentiation [26-29], and therefore it is possible that collagen-derived peptides could serve as both cell attachment and differentiation factors. In light of these considerations, the focus of the current study was to monitor the cytocompatibility and osseointegration of HA biomaterials modified with collagen I-derived peptides. Three collagen I mimetics were evaluated; DGEA [30] and GTPGPQGIAGQRGVV ("P15") [31], which are linear peptides derived from the $\alpha 1$ helix of collagen I, and the GFOGER peptide [32, 33], which spontaneously assumes a triple-helical structure due to the presence of GPP repeats engineered onto the ends of the peptide. All of these peptides have shown some degree of efficacy in directing osteogenic cell attachment, however, to our knowledge, a side-by-side comparison of the peptides, when adsorbed to calcium phosphate biomaterials, has never been performed.

Materials and Methods

Peptide preparation

DGEA (370.4 g/mol, American Peptide), P15 (GTPGPQIAGQAGVV, 1393.5 g/mol, American Peptide) and GFOGER (a generous gift from Dr. Richard Farndale, Cambridge University [32, 33]) were reconstituted in ddH_2O at 1mg/ml, aliquoted and stored at -20°C.

Disk preparation

For *in vitro* studies, clinical grade HA powder (Fisher Scientific) was pressed into disks using a 5/8" die, under 3000 psi. For *in vivo* studies, clinical grade HA powder (Fisher Scientific) was pressed into disks using a 3mm die, under 1000 psi. Pressed disks were sintered at 1000˚C for 3 hours and allowed to cool in the furnace at decreasing intervals. Disks were then stored under sterile conditions. Peptides (1mg/ml) were coated onto sintered HA disks as previously described [15]. Briefly, for peptide-only coatings, disks were incubated at 4°C overnight in peptide solution. For sequential coatings, disks were incubated in peptide solution at 37°C for 1 hour, and then overcoated with serum overnight at 4°C. The disks were washed with PBS to remove unbound peptide, and warmed to 37°C prior to cell seeding or *in vivo* implantation.

Cell culture

MSCs were isolated from human bone marrow as previously described [34]. Briefly, cells were collected using low speed centrifugation, and resuspended in Dulbecco's modified Eagle's Medium (DMEM). The cell suspension was applied to a

histopaque-1077 column, and centrifuged to establish a density gradient. The MSC layer was extracted, and the cells grown in DMEM supplemented with 10% FBS (standard growth media). Cells from passage 3-13 were used for our experiments. Human bone marrow samples were obtained with prior approval from the University of Alabama Institutional Review Board.

For differentiation experiments, osteogenic media (OS media), consisting of DMEM supplemented with PenStrep, Amphotericin B, 10% FBS, 100nM dexamethasone, 10mM sodium β-glycerolphosphate, and 0.05mM L-ascorbic acid-2-phosphate [35], was used.

Cell adhesion studies

As previously described [36], MSCs were incubated in a solution of 2 μM CMFDA, a fluorescent dye ("Cell Tracker Green", Molecular Probes), as recommended by the vendor. Cells were detached from tissue culture flasks by trypsinization, followed by incubation in trypsin inhibitor. $1x10^5$ labeled cells were re-suspended in serum-free media, seeded onto HA substrates, and allowed to adhere for 1 hour. After PBS washes with agitation to remove loosely bound cells, the remaining adherent cells were lysed (1% TX-100 in 50mM Tris) to release the fluorescent marker into solution. Fluorescence was quantified by reading samples on a fluorometer.

Cell morphology studies

$5x10^4$ MSCs were seeded in serum-free media onto disks for 1 hour. Unbound cells were washed away with PBS, while the adherent cells were fixed in 3.7%

formaldehyde, permeabilized with 0.2% Triton-X-100, and then stained with Alexa-488 phalloidin (Molecular Probes). These cells were then mounted with 4.7mM n-propyl-gallate mounting fluid, and visualized on a Nikon fluorescent microscope.

Western blot analysis of adsorbed serum proteins

Disks which had been coated with FBS or sequentially coated with peptide/FBS overnight were washed in PBS to remove loosely bound proteins. Proteins remaining on the surface were solubilized in boiling SDS-buffer (50mM Tris, 2% SDS, 5% β-mercaptoethanol). Briefly, the disks were submerged in the buffer for 30 minutes, with constant agitation. The supernatant was collected and stored at -80°C. Desorbed proteins were run on a 7% polyacrylamide gel. Proteins were transferred to a PVDF membrane, and exposed to antibodies for FN (Chemicon), or VN (Abcam); followed by an HRP-conjugated secondary antibody (Amersham Life Sciences). Proteins were detected using chemiluminescence reagents (Amersham Life Sciences or Millipore).

MSC differentiation studies

Disks which had been coated with FBS or sequentially coated with peptide/FBS overnight were washed with PBS to remove loosely bound proteins. $2x10^5$ MSCs were then seeded onto the surface in serum-free media and allowed to attach for 24 hours. At this time, cells were fed growth media, and allowed to grow for 72 hours in order to reach confluence. Media was then changed to OS media, and cells were incubated for 2 weeks, with media replaced every two days. Following the two-week incubation in OS media,

samples were stained for alkaline phosphatase (ALP) activity, or alternately, the supernatants were collected for osteocalcin (OCN) ELISA.

ALP activity

Two weeks post-differentiation induction, disks were washed 3 times in PBS. At this time, a colorimetric ALP activity kit (Sigma) was used to detect ALP on each sample. Briefly, cells were fixed, and then a colorimetric substrate was added to the disks. Following incubation, disks were washed to remove any remaining substrate. Images of the relative activity were taken.

OCN ELISA

Supernatants from the differentiation samples were tested with a commercially available kit (Biomedical Technologies, Inc.). Briefly, samples were incubated on a capture antibody coated plate in the presence of antiserum. Plates were washed, and detection reagent was then added. The colorimetric product was analyzed at 450nm, and readings were compared with a standard curve.

Tibial implantation

Animal studies were conducted as previously described [19]. Briefly, male Sprague-Dawley rats were anesthetized. An osteotomy was created in the right proximal tibia. 3mm HA disks either uncoated or coated with collagen I mimetic peptides were then placed into the defect, and extracted at either 30 minutes or 5 days. All protocols

were performed in accordance with guidelines established by the University of Alabama Institutional Animal Care and Use Committee.

Cell adhesion on retrieved disks

HA disks retrieved from tibial osteotomies after 30 minutes and used for cell adhesion assays were treated as previously described [19]. Briefly, following PBS washes, MSCs were seeded onto the HA surfaces. After 1 hour, cells were stained with Alexa-488 phalloidin (Cell Signaling) and DAPI (Cell Signaling) for the actin cytoskeleton and nucleus, respectively.

Protein adsorption from retrieved tibial implants

HA disks were retrieved and proteins from the surface were desorbed as previously described [19]. Briefly, upon removal, disks were washed with PBS. Disks were then incubated in boiling-SDS buffer as described above. Samples were resolved by SDS-PAGE and transferred to a PVDF membrane. Membranes were subjected to FN (Chemicon), VN (Chemicon), or Fbg (Abcam) primary antibodies. Secondary antibodies to each of the primaries were then added (Amersham), and signal was detected using chemiluminescence (Amersham Life Science or Millipore).

Bone formation around implanted HA disks

Following 5 days of implantation, tibiae, with disks in place, were retrieved, and embedded in poly(methyl methacrylate) (PMMA) for Goldner's trichrome staining, which stains mineralized tissue green [19]. 5 implants were analyzed for each of the

three treatment groups (15 animals total), with at least two tissue sections per implant evaluated.

The amount of total new bone surrounding 5 day implants, as well as the amount of bone in direct contact with the implant perimeter, were quantified from Goldner's stained sections using Bioquant imaging software as previously described [19].

Statistics

I*n vitro* assays were performed at least three independent times, with each trial performed in triplicate. I*n vivo* experiments were performed with at least five animals per treatment group, with at least two fields or sections analyzed for quantification. Data sets were assessed using students t-test parametric analysis. A confidence level of 95% ($p<0.05$) was considered significant.

Results

MSC adhesion to HA disks coated with collagen I mimetic peptides

To determine the efficacy of collagen-derived peptides in promoting cell adhesion, we monitored MSC attachment to HA disks coated with DGEA, P15, GFOGER or collagen I. These experiments revealed that DGEA and P15 were able to support a level of cell adhesion equivalent to that of collagen I, and all three of these integrin ligands stimulated greater binding than uncoated HA disks (Figure 1a). However, in contrast to DGEA and P15, the GFOGER peptide failed to stimulate cell adhesion above that of uncoated HA.

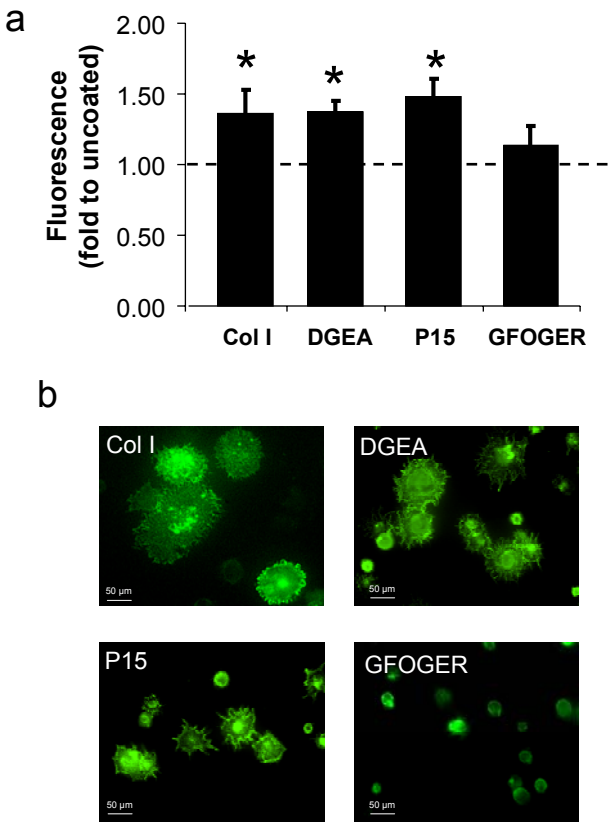

Figure 1: DGEA and P15 increase cell adhesion and cell spreading on HA a) MSCs labeled with Cell Tracker dye were allowed to adhere for 1 hour to HA disks pre-coated with collagen I (Col I), DGEA, P15 or GFOGER. The adherent cells were subsequently lysed and adhesion was quantified by measuring fluorescence. Values were folded to uncoated HA (dotted line) (*=$p<0.05$ to uncoated HA). b) Representative images of MSCs allowed to adhere for 1 hour to collagen I (Col I), DGEA, P15 or GFOGER-coated HA. Cells were labeled with Alexa-488 phalloidin (Cell Signaling Technologies).

Cell spreading on HA disks coated with DGEA, P15, GFOGER or collagen I

To evaluate cell morphology, MSCs were seeded onto HA disks that had been pre-coated with DGEA, P15, GFOGER, or collagen I. Following a 1 hour attachment interval, cells were stained with phalloidin to visualize the actin cytoskeleton. As shown in Figure 1b, DGEA, P15 and collagen I were all able to induce some degree of cell spreading, whereas GFOGER was completely ineffective. These results suggest that DGEA and P15 engaged integrin receptors and elicited sufficient integrin activation to promote some restructuring of the actin cytoskeleton.

It was surprising that the GFOGER peptide was ineffective in promoting cell adhesion and spreading, given prior reports in the literature suggesting that this triple helical peptide promotes robust integrin activation [32, 33, 37-39]. This result could be due to a number of factors including; poor GFOGER adsorption to HA, a change in the GFOGER tertiary structure upon HA adsorption, or inactivity of the peptide preparation. To insure that there were no problems with our peptide preparation, we examined cell attachment to GFOGER-coated tissue culture plastic, the material substrate on which the peptide had originally been tested [38]. We found that, when adsorbed to tissue culture plastic, the GFOGER peptide induced cell adhesion and spreading equivalent to that stimulated by intact collagen I, indicating that the peptide was active (data not shown). However, because of the poor performance of the GFOGER peptide when adsorbed to HA, this peptide was not studied further in the current investigation.

MSC adhesion to HA disks coated sequentially with peptide and FBS

Because HA is known to rapidly adsorb significant quantities of proadhesive proteins such as FN, VN and Fbg from the *in vivo* environment [16-19], it is unlikely that cells within a patient would ever encounter peptide-coated HA in the absence of an adsorbed protein layer. In order to mimic the process of endogenous protein adsorption, peptide-coated HA disks were overcoated with FBS, which we have previously shown deposits abundant FN and VN onto HA surfaces [15]. MSCs were then seeded onto these sequentially-coated surfaces and evaluated for attachment. As shown in Figure 2a, MSC adhesion to the DGEA/FBS and P15/FBS-coated surfaces was equivalent to that of adhesion observed on HA coated with FBS alone. In contrast, RGD peptides significantly inhibited cell adhesion when presented in combination with serum proteins. This result was highly specific to the RGD sequence, because the RGE control peptide was not inhibitory.

Cell spreading on HA disks coated sequentially with peptide and FBS

To determine whether DGEA or P15 peptides had any effect on cell spreading when presented in combination with adsorbed serum proteins, cells were seeded onto disks coated with DGEA, P15, collagen I, RGD or RGE, followed by an FBS-coating (Figure 2b). After a 1 hour incubation, cell morphology was evaluated by staining cells with phalloidin. These experiments revealed extensive cell spreading on disks coated with DGEA/FBS, P15/FBS, collagen I/FBS, RGE/FBS or FBS alone, whereas markedly different results were noted with RGD/FBS surfaces. The RGD/FBS-coated samples did

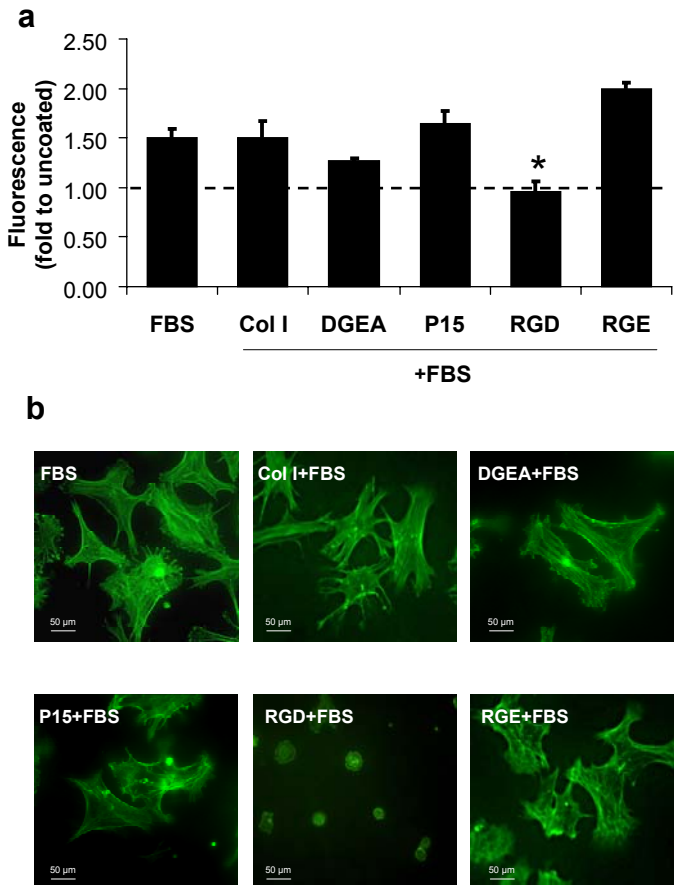

Figure 2: DGEA and P15 do not inhibit cell adhesion when presented in the context of adsorbed serum proteins a) HA disks were coated with collagen I (Col I), DGEA, P15, RGD or RGE followed by an over-coating with serum (FBS) to allow protein adsorption. As a control, some disks were coated with FBS only. Fluorescently-labeled MSCs were then seeded onto the disks and monitored for cell adhesion as previously described. Values were folded to uncoated HA (dotted line) (*=$p<0.05$ to FBS). b) Representative images of Alexa-488 phalloidin stained MSCs adherent to HA disks coated with FBS alone or to disks coated first with either collagen I (Col I), DGEA, P15, RGD or RGE, followed by an FBS over-coating.

not support any degree of cell spreading, suggesting that the presence of RGD in combination with adsorbed serum proteins blocks integrin-mediated signaling.

MSC adhesion to HA disks adsorbed with proteins from the tibial microenvironment

To better model the process of protein adsorption on a biomaterial surface, we placed uncoated or peptide-coated disks into tibial osteotomies for 30 minutes to allow the adsorption of native proteins present within the bone microenvironment. The disks were then retrieved, washed extensively with PBS, and MSCs were subsequently seeded onto the disks and evaluated for degree of cell attachment (Figure 3). Similar to results generated from disks overcoated with FBS, neither DGEA nor P15 inhibited cell adhesion to disks coated with endogenous proteins. Notably, we previously reported that RGD inhibits at least 75% of cell adhesion when presented in the context of adsorbed tibial proteins [19].

The effect of collagen I mimetic peptides on protein adsorption from serum and the tibial microenvironment

We next tested whether DGEA or P15 had any effect on the adsorption of proadhesive proteins. To this end, disks were coated with DGEA/FBS, P15/FBS, or FBS alone. The disks were then washed to remove loosely-bound proteins, and the adsorbed proteins were subsequently desorbed by incubation in boiling SDS buffer. Desorbed samples were immunoblotted for FN and VN. As shown in Figure 4a, all of the surfaces adsorbed similar amounts of FN and VN, suggesting that the DGEA and P15 peptides did not significantly alter serum protein adsorption to the HA disks.

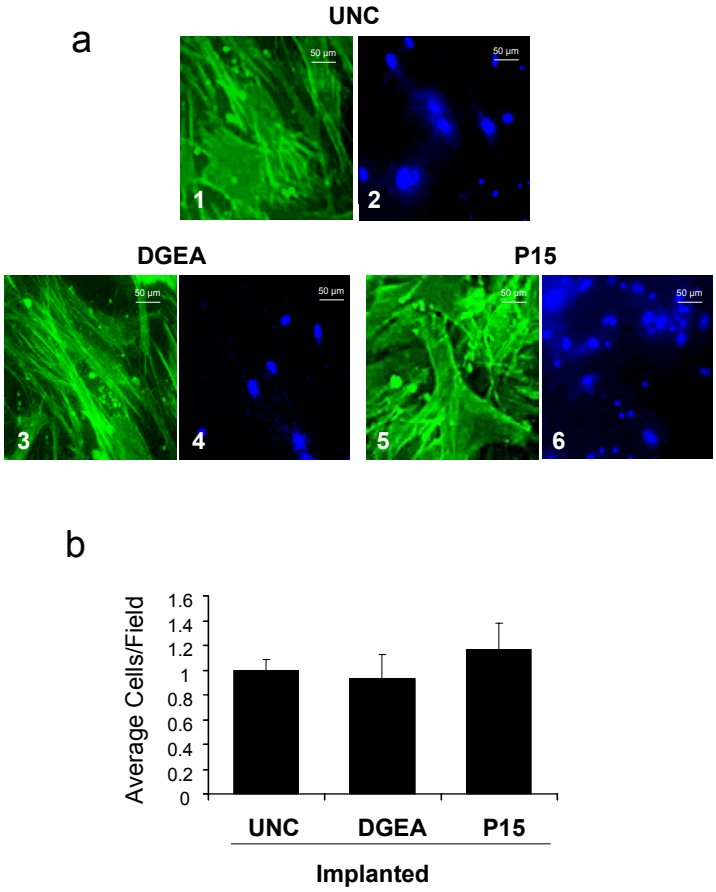

Figure 3: DGEA and P15 do not inhibit cell adhesion when presented in the context of adsorbed proteins from the tibial microenvironment a) HA disks were either left uncoated (UNC, panels 1-2) or coated with DGEA (panels 3-4) or P15 (panels 5-6). The disks were then implanted into the rat tibiae for 30 minutes to allow protein adsorption, retrieved, and washed extensively with PBS. MSCs were seeded onto the surfaces, and allowed to adhere for 1 hour. Cells were then fixed and labeled with Alexa-488 phalloidin (1,3,5) and DAPI (2,4,6). Representative images are shown. b) Adherent cells were quantified by counting cells from multiple fields. No significant difference was observed in the number of adherent cells for the 3 surface treatments.

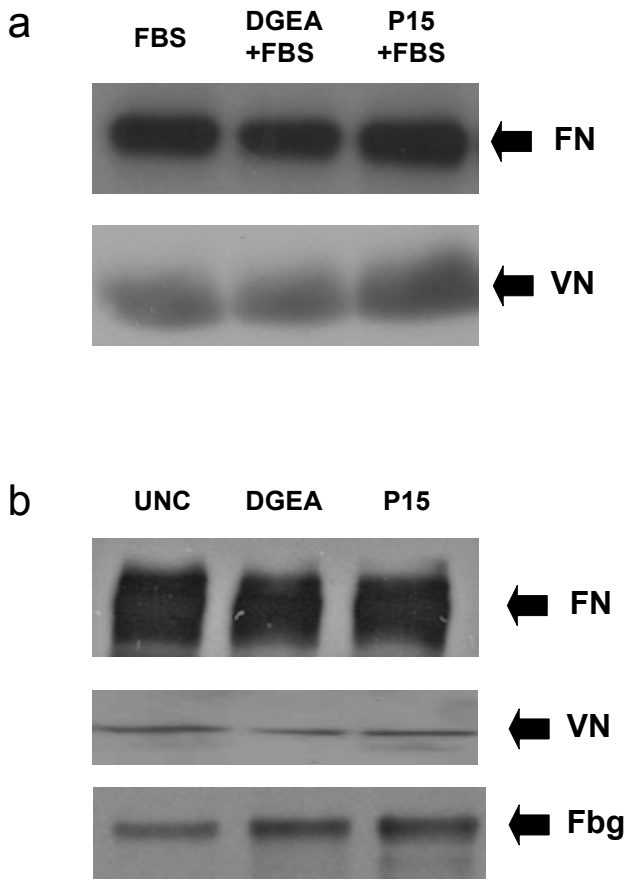

Figure 4: DGEA and P15 pre-coatings do not affect protein adsorption from serum or the tibial microenvironment a) Representative western blots of FN and VN desorbed from disks coated with either FBS alone, or with DGEA or P15, followed by FBS. b) Western blots of FN, VN and Fbg desorbed from uncoated, DGEA-coated or P15-coated, HA disks implanted for 30 minutes into rat tibiae.

We also evaluated protein adsorption to peptide-coated disks placed into tibial osteotomies. Specifically, uncoated, DGEA-coated, or P15-coated HA disks were implanted for 30 minutes; the disks were then retrieved, proteins desorbed and analyzed by immunoblotting. As shown in Figure 4b, similar amounts of FN, VN and Fbg were adsorbed to the uncoated and peptide-coated surfaces, indicating that DGEA and P15 coatings did not interfere with protein adsorption from the tibial microenvironment.

The influence of collagen-mimetic peptides on alkaline phosphatase activity

Results presented in Figures 1-3 indicated that, unlike RGD, the DGEA and P15 peptides did not inhibit cell attachment or spreading on protein-coated HA. However, DGEA and P15 did not enhance cell adhesion either, suggesting that there is little benefit to using these peptides as cell attachment factors. However, there is a growing literature suggesting that activation of a collagen-selective integrin, the $\alpha 2\beta 1$ species, induces MSC differentiation along the osteoblastic lineage [26-29]. Accordingly, we were interested in whether collagen-derived peptides might serve as differentiation factors for MSCs. To test this hypothesis, cells were seeded onto peptide-coated disks overcoated with FBS, or disks coated with FBS alone as a control. The cells were then grown for 2 weeks in osteogenic media, and stained for ALP activity. As shown in Figure 5a, the disks coated with DGEA/FBS and P15/FBS supported greater levels of ALP activation than FBS-coated disks, indicating a greater degree of osteoblastic differentiation.

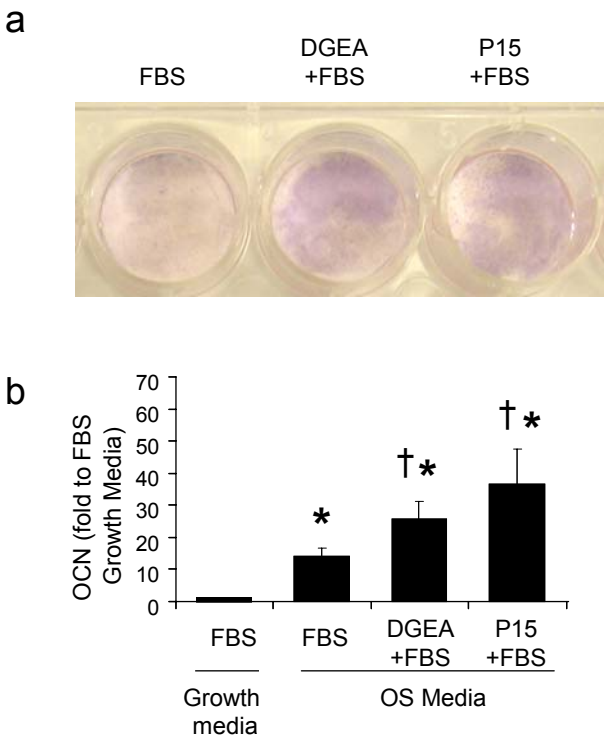

Figure 5: DGEA and P15 increase osteoblastic markers in the presence of osteogenic media a) MSCs were seeded onto disks coated with FBS alone, or on disks sequentially coated with either DGEA/FBS or P15/FBS. Cells were allowed to grow in osteogenic media (OS media) for 2 weeks. A representative image of an alkaline phosphatase (ALP) activity assay is shown. b) MSCs adherent to peptide/protein-coated disks were grown in OS media for 2 weeks as previously described. As a control, some cells were grown on FBS-coated disks in growth media rather than OS media. Following a 2-week incubation in either OS or growth media, culture supernatants were collected and evaluated for osteocalcin (OCN) secretion by ELISA (*=$p<0.05$ to FBS (growth media))(†=$p<0.05$ to FBS (OS media))

The influence of collagen-mimetic peptides on osteocalcin secretion

To confirm that DGEA and P15 stimulated osteoblastic differentiation of MSCs, we examined secretion of OCN using an ELISA assay (Figure 5b). All of the cells grown in osteogenic media secreted more OCN than cells grown in standard growth media, indicating that the osteogenic media was effective in inducing osteoblastic differentiation. However, cells grown on the DGEA/FBS and P15/FBS-coated surfaces had increased levels of secreted OCN, as compared with FBS-coated disks. These results, combined with the measurements of ALP activity, suggest that DGEA and P15 are able to enhance MSC differentiation along the osteoblast lineage.

The influence of collagen-mimetic peptides on ALP activity and OCN secretion in the absence of osteogenic media

Results shown in Figure 5 indicated that DGEA and P15 were able to upregulate the expression of osteoblastic markers when presented to cells in the presence of osteogenic media. However, some studies have suggested that activation of $\alpha 2\beta 1$ integrins can stimulate osteoblastic differentiation even in the absence of other differentiation-inducers [26-29]. Hence, we tested whether DGEA and P15 were able to stimulate osteoblastic differentiation in the absence of osteogenic media. To this end, cells adherent to peptide-coated disks were incubated in standard growth media for two weeks and then ALP activity and OCN levels were measured. We found that cells grown on HA disks coated with DGEA/FBS or P15/FBS exhibited greater ALP activity and OCN secretion than cells grown on disks coated with FBS alone (Figure 6a and b). These results suggest that DGEA and P15 are able to activate collagen-binding integrins

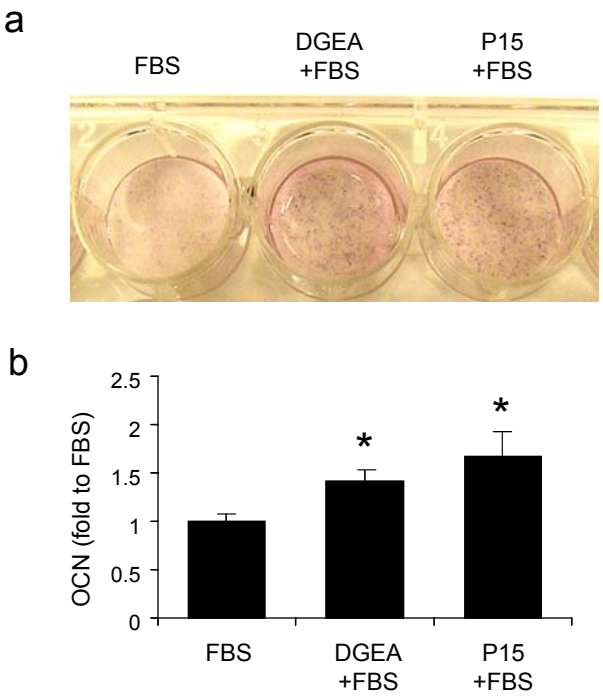

Figure 6: DGEA and P15 increase osteogenic markers in the absence of osteogenic media a) Representative image of an alkaline phosphatase activity assay for MSCs grown in growth media on HA disks pre-coated with FBS, DGEA/FBS, or P15/FBS. b) Quantification of osteocalcin (OCN) secretion from samples incubated in standard growth media for two weeks. (*=$p<0.05$ to FBS).

enough to induce some degree of osteoblastic differentiation in the absence of standard differentiation factors.

The effect of collagen-mimetic peptides on bone/implant contact and new bone synthesis surrounding tibial implants

In order to examine if the differentiation-inducing features of collagen-mimetic peptides had any effect on implant osseointegration, uncoated, DGEA-coated, or P15-coated HA disks were placed into rat tibial osteotomies and monitored for bone formation. Specifically, implants were left in place for 5 days; the tibiae were then removed (with implants in place), and embedded in PMMA. Sections of the embedded tibiae were stained with Goldner's Trichrome, a stain which labels mineralized tissue green (Figure 7a). When the amount of new bone was quantified (Figure 7b), we found that both DGEA and P15 stimulated greater bone formation around the HA implants. In addition, DGEA was able to increase the amount of bone directly contacting the perimeter of the implants. P15 showed a trend toward increased bone-implant contact, but this was not statistically significant. Taken together these results suggest that collagen-mimetic peptides improve bone tissue responses to HA biomaterials.

Discussion

Previous studies have shown that the addition of adhesive proteins, such as FN [40-43], VN [41], and Fbg [44], to the surface of HA biomaterials increases cell adhesion. Furthermore, we and others have reported that HA adsorbs abundant adhesive proteins, including FN, and VN, from serum [15, 34, 45] as well as from the tibial

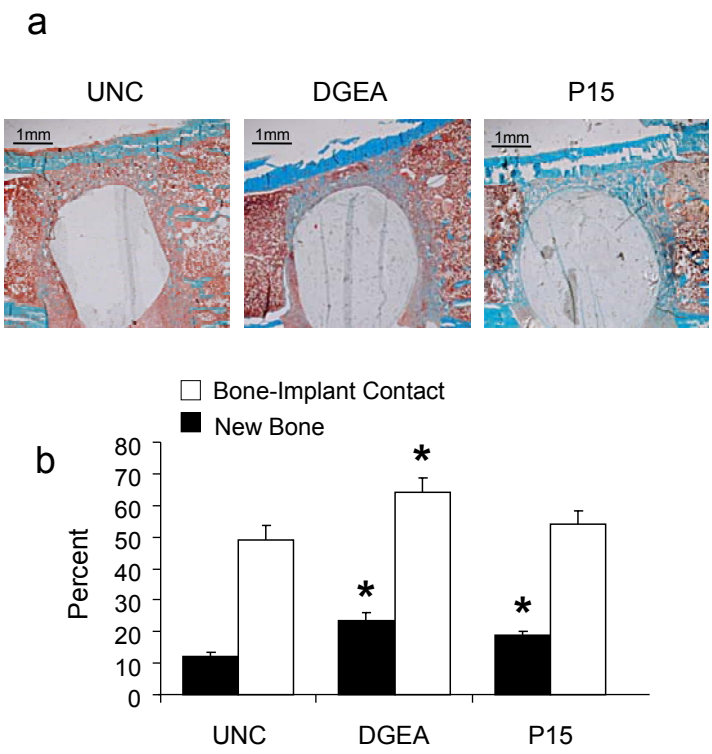

Figure 7: DGEA and P15 increase bone formation around HA implants a) Representative images of Goldner's Trichrome-stained sections from tibiae implanted with uncoated (UNC), DGEA-coated, or P15-coated, HA disks. b) Quantification of the amount of new bone formed (black bars) and the amount of bone directly contacting the implant perimeter (white bars) around uncoated (UNC), DGEA-coated or P15-coated implants (*=$p<0.05$ to UNC)

microenvironment [19]. We also found that HA adsorbs significantly more VN and FN from serum than titanium or stainless steel, and these adsorbed proteins are present on the HA surface in conformations appropriate for binding purified integrin receptors and MSCs [34]. The importance of proadhesive blood proteins in regulating cell attachment to HA has been confirmed by other studies. For example, Zreiqat et al. [46], showed that human bone-derived (HBD) cells do not adhere to hydroxyapatite if vitronectin is depleted from serum.

In light of these results, we questioned the utility of synthetic adhesive peptides, and proposed instead that adsorbed endogenous integrin-binding proteins may be sufficient for promoting optimal osteogenic cell attachment and survival. Indeed we previously reported that cell adhesion to serum-coated HA is significantly better than cell adhesion to RGD-coated HA [15], and this result was observed with 5 different species of serum [1] and both linear and cyclic RGD peptides [15]. Moreover, when RGD was combined with adsorbed serum [15] or tibial proteins [19], it was unexpectedly found that RGD peptides were strongly detrimental to cell attachment and survival. RGD also significantly inhibited the osseointegration of HA disks implanted into rat tibiae [19]. The unfavorable cell and tissue responses to RGD-coated HA biomaterials prompted an evaluation of whether other adhesive peptides would be beneficial for HA. In the current study, three peptides derived from collagen I were examined; DGEA, P15, and GFOGER. These peptides are $\alpha 2\beta 1$ integrin-binding motifs, [30-33, 47], and all have been shown to increase MSC adhesion to a variety of biomaterials [31-33, 48, 49]. Interestingly, we found that GFOGER was unable to promote cell adhesion or spreading on HA surfaces, although the mechanism underlying this negative result is not currently

understood. In contrast, DGEA and P15 coatings supported a level of cell adhesion equivalent to that of HA coated with full-length collagen I. In addition, the DGEA and P15 peptides did not inhibit cell adhesion when presented in combination with adsorbed adhesive proteins from either serum or the tibial microenvironment. This striking disparity between the effects of RGD vs. collagen-derived peptides on MSC adhesion suggests that collagen-derived peptides interact with MSCs through different mechanistic pathways. We hypothesize that the binding of DGEA and P15 to collagen-specific integrins, including the $\alpha 2\beta 1$ receptor, would not compete with adsorbed blood proteins such as FN, VN and Fbg for cell surface integrins, given that these latter proteins bind distinct integrin species including $\alpha 5\beta 1$, $\alpha v\beta 3$ and $\alpha IIb\beta 3$. As well, FN, VN and Fbg all bind integrins through RGD-dependent mechanisms, whereas the interaction between $\alpha 2\beta 1$ and collagen I is RGD-independent.

While it was encouraging that DGEA and P15 did not inhibit cell adhesion when presented within the context of an adsorbed protein layer, it was noted that these peptides did not enhance cell adhesion either. Adsorbed serum or tibial proteins (including FN, VN and Fbg) appeared to promote maximal cell adhesion and spreading, suggesting that the use of collagen-derived peptides as attachment factors is of limited value. However, our results alternately suggested that DGEA and P15 may serve as effective differentiation factors. Activation of the $\alpha 2\beta 1$ integrin receptor has been reported to upregulate many markers of osteoblastic differentiation including osteopontin mRNA [29] and protein [26, 50], ALP activity [26, 50], OCN protein [26, 50] and matrix mineralization [26, 27, 50]. In our studies, MSCs grown on HA disks coated with DGEA or P15 exhibited higher ALP activity and greater secretion of OCN, as compared MSCs

grown on serum-coated HA. This enhanced osteoblastic activity was observed both in the presence and absence of osteogenic media, indicating that collagen-derived peptides can stimulate some degree of osteoblastic differentiation even in the absence of other differentiation-inducing agents. Consistent with these results, DGEA and P15-coated HA disks implanted into rat tibiae promoted better bone in-growth and DGEA stimulated greater bone-implant direct contact than unmodified HA. Collectively these results are in excellent agreement with many other studies implicating collagen and collagen-derived peptides in osteogenesis. In particular, the P15 peptide has been extensively studied, and has been used for many clinical applications. P15-coated anorganic bovine mineral (ABM), used for periodontal defects, has been shown to increase bone regeneration at dental implant sites in humans [51-58]. In addition, in a side-by-side comparison study, P15-coated ABM performed better than open flab debridement [56, 57], Puros, a form of allograft, [58] and C-Graft 228, a calcified biomaterial derived from algae [58]. Finally, P15-coated ABM has been successfully used in maxillary sinus augmentation to induce bone growth [59].

Collagen I and HA are the two principal components of native bone, and therefore HA biomaterials modified with collagen-derived peptides represent a matrix that mimics the endogenous surface that MSCs would likely encounter *in vivo*. Both HA [60-62] and collagen I [26, 29, 50] have been reported to enhance osteoblastic differentiation of MSCs, and composite biomaterials encompassing collagen I and calcium phosphates including HA and TCP have been shown to significantly increase osteoblastic differentiation [63, 64] and bony ingrowth [63, 65]. In the aggregate, these studies indicate a promising role for collagen-related biomaterials in bone regeneration.

However there are some concerns regarding the use of collagen I. Collagen I can be immunogenic in some instances, and has the potential to transmit pathogens when xenografted. These obstacles could theoretically be circumvented by using collagen-derived peptides such as DGEA and P15. As well, synthetic peptides are significantly less expensive to produce than native collagen I, providing a more cost-effective strategy for optimizing biomaterials used for bone repair.

Beyond identifying collagen-derived peptides as a promising substrate for enhancing HA bioactivity, our study is noteworthy because it highlights the importance of considering endogenous processes such as protein adsorption when evaluating cell responses to biomaterials. In our studies, we modeled protein adsorption by coating HA with either serum, or implanting disks for 30 minutes into tibial osteotomies to allow protein adsorption from the bone microenvironment. We recognize that this is an approximation of initial events at the implant site, and that other factors undoubtedly influence the characteristics of material surfaces presented to endogenous MSCs. For example, it is possible that protein-adsorbed material surfaces might be remodeled by other cells within the wound site (e.g., blood cells) prior to the arrival of MSCs. Nonetheless, it is important to note that our *in vitro* studies incorporating a protein adsorption modeling step are far more predictive of *in vivo* biomaterial performance than studies comparing peptide-modified HA with uncoated HA (the latter being the standard approach for evaluating adhesive peptides in vitro). For instance, when compared with unmodified HA (i.e., no protein adsorption), HA substrates coated with RGD, DGEA or P15 significantly increase MSC adhesion [14, 31, 49, 66, 67], suggesting that all of these integrin-binding peptides would enhance implant integration. However, our studies of

HA disks implanted into rat tibiae clearly show that RGD inhibits, whereas DGEA and P15 stimulate, the amount of new bone deposited around HA implants. These *in vivo* results are, in fact, very consistent with *in vitro* studies incorporating a protein adsorption step. Our current working model (Figure 8) is that RGD competes with adsorbed proteins for integrin receptors, and thereby elicits diminished overall integrin signaling, whereas DGEA and P15 enhance integrin signaling from the cell surface by binding integrin receptors that are distinct from those that would be bound by endogenous adsorbed proteins.

Conclusions

When compared with unmodified HA substrates, MSCs adhere significantly better to HA surfaces coated with RGD, DGEA and P15, consistent with the known role of these peptides as integrin-binding attachment factors. However, as HA is a highly adsorptive biomaterial, it is unlikely that cells would ever encounter an HA surface in the absence of an adsorbed protein layer. To model the process of protein adsorption, our laboratory evaluated cell adhesion to peptide-coated HA disks that were over-coated with serum, or alternately implanted for 30 minutes in rat tibial osteotomies to allow endogenous protein adsorption. Our prior and current studies show that, when presented in the context of adsorbed proteins, the collagen-derived peptides, DGEA and P15, enhance the osseointegration of HA implants, whereas RGD is strongly detrimental. Our results further suggest that the beneficial effects of DGEA and P15 are due to the role of these peptides as differentiation, rather than adhesive, factors.

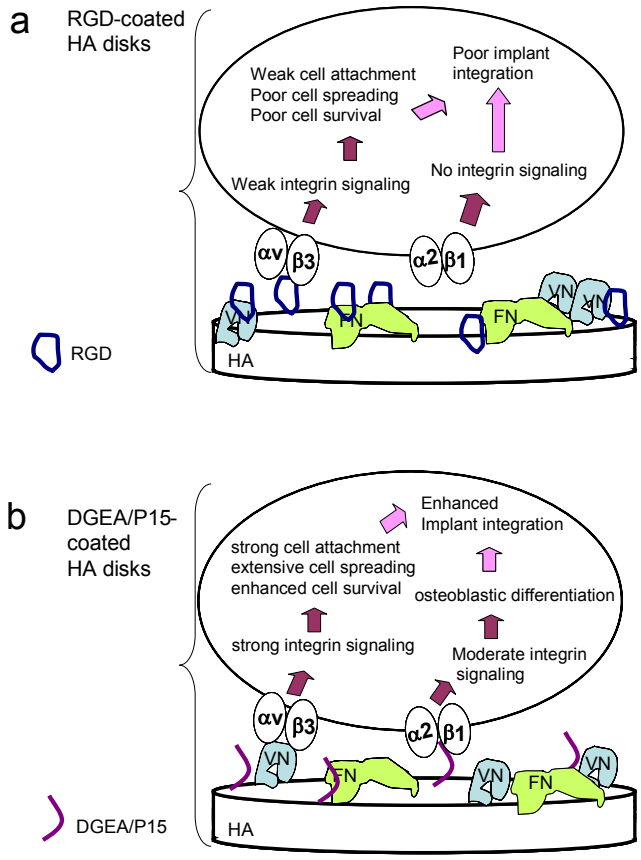

Figure 8: Model describing the effects of integrin-binding peptides on osseointegration of HA implants a) Integrin activation by adsorbed proteins, such as FN and VN, plays a key role in MSC adhesion, survival and osteoblastic differentiation. When RGD is present on the HA surface, we hypothesize that integrins such as αvβ3 bind the RGD rather than full-length FN or VN, leading to poor cell adhesion and survival. Collagen-binding integrins such as α2β1 would not likely be engaged with ligand, given that minimal amounts of collagen I would adsorb to the HA surface from blood (given that fibrillar collagen I is not abundant in blood). The combination of weak signaling from RGD-dependent integrins (e.g. αvβ3, α5β1, αIIbβ3), and a lack of signaling from collagen-selective integrins, is proposed to contribute to poor implant integration. b) Conversely, the presence of either DGEA or P15 on the HA surface provides a ligand for collagen-selective integrins that, upon activation, initiate signaling mechanisms promoting osteoblastic differentiation. As well, RGD-dependent integrins would engage the native FN, VN or Fbg adsorbed from blood, resulting in strong adhesive and survival signaling. Collectively, signaling from these multiple integrin species is hypothesized to enhance osseointegration of HA biomaterials.

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CONCLUSIONS

Calcium-phosphate biomaterials, such as HA, have been shown to be highly osseoconductive [126, 127]. These materials mimic the natural structure of bone, making them also highly biocompatible [128, 129]. Hydroxyapatite biomaterials have been successfully used as coatings for prosthetic and dental implants and scaffolds to facilitate bone repair [43, 53, 54, 130, 131]. While these biomaterials have been shown to increase osseointegration [130, 131], there have been few studies examining how cells interact with the biomaterial in the body.

Previous studies have examined cell adhesion to HA *in vitro*, and have found that HA alone does not readily support cell adhesion [73, 75]. Based upon these findings, it was believed that modification of the HA surface with bioadhesive molecules was necessary to induce initial cell adhesion. Multiple peptide sequences have been used to functionalize biomaterials, with the hope of increasing cell adhesion. The peptide RGD, which is the integrin binding site of a number of cell adhesion molecules, has been widely used to functionalize other biomaterials such as PMMA [95, 132, 133] and titanium [134, 135]. In fact, RGD peptides have been shown to increase bone formation around polymer and titanium implants [136]. In addition, DGEA, P15 and GFOGER, three peptides derived from collagen I [113-116], have been used to functionalize multiple biomaterials [103, 114-117, 119, 125, 137]. It was thought that the addition of RGD peptides to HA biomaterials would enhance the initial cell adhesion events necessary for osseointegration [33]. We and others have shown that, when compared

with uncoated HA, RGD, DGEA and P15 coatings on HA do, in fact, increase cell adhesion [75, 114, 117, 138-142].

Multiple researchers have shown that pre-incubating HA implants with full length integrin ligands such as FN [68-71], VN [69], and Fbg [72] *in vitro* increases cell adhesion, most likely due to the fact that these proteins are integrin ligands. Our laboratory questioned the efficacy of coating HA with these proteins, as we have hypothesized that one of the reasons that HA biomaterials are more osseoconductive than other materials such as titanium and steel is due to the fact that HA has the ability to adsorb cell attachment factors such as FN and VN from the blood [73] and serum [74, 75] in conformations which support the binding of both purified integrins and MSCs [74]. We and others have shown that HA adsorbs proadhesive proteins from the surgical microenvironment within the first 90 seconds to 30 minutes of implantation [73, 91, 143, 144]. As it is unlikely that cells, especially MSCs, would encounter HA in the absence of adsorbed proadhesive proteins, we believe that any peptide modifications made to HA biomaterials would be presented to MSCs in the context of these adsorbed cell adhesion molecules. However, most studies aimed at elucidating the effects of biomimetic peptides, including RGD [75, 138-142, 145], DGEA [117] and P15 [114, 119] peptides, on HA biomaterials were performed without the addition of this critical component.

Previous research from our laboratory focused on the effects RGD peptides had on MSC adhesion to HA biomaterials, in the presence of adsorbed proadhesive proteins. When compared with MSC responses to serum coated HA, cells on RGD coated HA exhibited significantly less adhesion and spreading [75]. This is not surprising, given that there are synergy sites within the full length integrin ligands, including FN and VN,

adsorbed to the HA surface from serum [74] which, together with the RGD domain, allow for full integrin activation and downstream signaling [99-103]. We compared RGD/serum sequentially coated HA disks with serum coated disks, and found that high concentrations of RGD peptides inhibited cell adhesion and spreading on adsorbed serum proteins on the HA surfaces [75]. While the mechanism was unclear, we hypothesized that RGD was somehow inhibiting the interaction between adsorbed serum molecules and MSC integrins.

Based on our prior findings, the primary focus of this dissertation has been to observe the effects of various biomimetic peptide coatings on cell adhesion to HA biomaterials in the context of the *in vivo* environment. Studies from our laboratory have compared serum coated HA with peptide/serum sequentially coated HA disks. We have shown that cell adhesion increases on FBS coatings as compared with uncoated HA [74, 75]. In fact, previous research from our laboratory has shown that adsorption of proteins from serum of five different species, including rat, human and goat, increases cell adhesion above that of uncoated HA [104]. While we believed that these studies were more representative of the surface MSCs would encounter *in vivo* than uncoated HA, these studies still only examined serum coatings. We wished to further examine whether serum was a good model for the *in vivo* environment, as there are other factors within blood which could play a role *in vivo* which cannot be accounted for with serum. It was possible that, while FN and VN were present in high concentrations in both serum and blood [146-148], other mediators within blood which were not present in serum would induce different interactions between MSCs and the protein coated HA than what we observed *in vitro* using serum as a model. Thus, we set out to examine the effects of

adhesion peptides in the presence of proteins adsorbed from the rat tibial microenvironment, to more closely mimic the surface MSCs would see in a human patient.

The main focus of our first study was to examine the role of the RGD peptide on cell adhesion and bone formation on HA which had been placed into the rat tibial microenvironment. We found that RGD peptides inhibited cell adhesion on adsorbed proadhesive proteins, further suggesting that the RGD peptide might inhibit integrin interaction with these adsorbed molecules. As soluble RGD peptides have been used to block cell adhesion to FN and VN [149-151], it is possible that the attached RGD peptides are performing a similar function, binding to the integrins, and effectively blocking integrin activation by these full length integrin ligands. This finding is supported by the recent data from our laboratory, showing that the control peptide RGE, which carries the same charge as RGD but does not bind integrins [152] does not block cell adhesion or spreading on HA biomaterials in the presence of adsorbed proadhesive proteins (paper 2).

In addition to RGD inhibiting cell adhesion, RGD significantly decreased bone formation on HA biomaterials, as compared with uncoated HA. Following these findings, we began to study the mechanisms by which RGD might be causing this inhibition. We found that the RGD peptide did not inhibit protein adsorption. We further found that the presence of RGD does not change the conformation of adsorbed proteins in such a way as to block the accessibility of the integrin binding site. Rather, the presence of RGD peptides on the surface of HA caused a reduction in MSC integrin signaling, leading to poor cell adhesion and ultimately cellular apoptosis. We hypothesize that, as

FN and VN bind to integrins through their RGD domain [91, 93, 153], the synthetic RGD peptides are competing for integrins on the surface of MSCs. As more integrins bind to the RGD, rather than their full length ligands, less downstream signaling occurs to strongly attach the cells to the surface and to induce cell survival.

Our findings suggest that RGD peptides inhibit cell adhesion to proteins adsorbed to the HA surface [73, 75, 138]. Based on these findings, we began to study peptides derived from proteins which would not be adsorbed to the HA surface, such as collagen I. Previous studies have suggested that the addition of collagen I to HA biomaterials would increase bone formation [77, 78, 154]. However, xenogenic collagen I [155, 156] and the MMP degradation products of collagen I [157] have been shown to induce a prolonged immune response. It has been suggested that a prolonged immune response causes fibrous encapsulation of implants, leading to implant failure. Further, there exists the possibility that xenografted collagen I could transmit pathogens. Thus, functionalizing HA with intact collagen I could induce a negative response, rather than the increases in osseointegration for which it was added. Therefore, we began to study collagen I mimetic peptides, as these peptides are thought to be non-immunogenic, due to the fact that they are not subject to degradation by collagenases. The studies included in our second paper examined the effects of the collagen I mimetic peptides DGEA, and P15. These peptides have each been shown to increase cell adhesion on various biomaterials [114-117, 119], including HA [117]. We, in fact, found that DGEA and P15 were able to induce cell adhesion on HA equivalent to that of native collagen I (paper 2). This finding is interesting, as RGD peptides were not found to induce cell adhesion equal to that of FN or VN [75]. However, again, these peptides have never been examined on HA in the

presence of adsorbed proadhesive proteins. We found that, unlike RGD, DGEA and P15 did not inhibit cell adhesion to adsorbed proadhesive proteins. This finding supports the idea that RGD is inhibiting cell adhesion because it binds to the same receptors as FN and VN [91, 93, 153] , as DGEA and P15 bind the $\alpha 2\beta 1$ integrin rather than the $\alpha v\beta 3$ or $\alpha 5\beta 1$ [113-116, 158] and would therefore not compete with these full length ligands for receptor binding.

Interestingly, we found that the short mimetic peptides DGEA and P15 were able to induce increases in MSC differentiation along the osteoblast lineage above that of serum coated HA. Unlike RGD, which is unable to activate integrins enough to induce cell survival, these peptides are able to active integrins to the extent that they cause upregulation of OCN and ALP (paper 2). These findings carried over to increases in bone formation at the HA surface when DGEA and P15 coated implants were placed in rat tibial osteotomies. While P15 has previously been shown to increase bone growth on other calcium-phosphate materials, such as anorganic bovine mineral [120, 121, 159-164], the effects of neither DGEA nor P15 on osteoblast differentiation and bone formation on HA surfaces have been studied. This finding suggests that peptide functionalization of HA biomaterials has a benefit, as long as the peptide does not compete with full length ligands on the implant surface.

As there have been few studies performed examining the role of protein adsorption on cell interactions with HA biomaterials, one of our major findings within this body of work has been that the studies we have performed previously with serum overcoats correlate well with our findings using proadhesive proteins adsorbed from the tibial microenvironment. We found that FN and VN from the tibial microenvironment

adsorb to the HA surface [73], as we have previously shown with serum [75]. In addition, we also found that the adsorption of proadhesive proteins from the tibial microenvironment allow for increases in cell adhesion above that of uncoated HA [73], as was previously shown with serum, suggesting that these proteins mediate initial cell attachment to HA biomaterials [75].

Collectively, this dissertation provides a further understanding of the mechanisms which regulate MSC adhesion to modified and unmodified HA surfaces. Our studies have suggested that proteins adsorbed to the HA surface mediate initial cell adhesion to the biomaterial. In addition, we found that our *in vitro* studies using FBS as a model for blood coatings correlate well with our findings using proteins adsorbed from the tibial microenvironment. The major focus of this dissertation was to examine the role of biomimetic peptides on cell adhesion to HA biomaterials in the context of the *in vivo* environment. Our results suggest that the incorporation of protein adsorption, whether it be serum or blood proteins, prior to cellular studies on HA biomaterials, are more indicative of implant function than the classic method of comparing peptide coated HA with uncoated HA. These findings have allowed us to better understand the relationship between protein adsorption and MSC adhesion on HA biomaterials, and will allow us, in the future, to better understand the benefits of peptide and protein functionalization of HA surfaces.

Future Studies

The current standard of care in the biomaterials field is to coat metal hard tissue implants with a thin plasma sprayed layer of HA, due to the fact that the addition of this

coating is known to enhance implant osseointegration, in addition to lowering the immune response to the metal of the implant. A major goal of future research will be to modify the HA surface layer in order to promote even better integration of HA-based implants. In addition to adding bioactive factors to the implant surface, there are methods which can be used to modify the HA topography in a manner that regulates cell behavior. For example, studies have shown that the roughness of a biomaterial has a direct effect on the rate of MSC differentiation along the osteoblast lineage. Thus, increasing the roughness of the HA layer on hard tissue implants could increase the rate of bone tissue deposition. In addition, the bone tissue which is created on the implant biomaterial can integrate into the implant more closely by creating a porous metal implant, and coating that porous surface with HA. This will allow for the new bone to be formed within the porous cavities of the implant, to more closely integrate the new bone with the implant itself, lessening the possibility of implant loosening.

In addition to modifying HA topographical features, methods for coupling integrin-binding peptides to the HA surface are being developed, with the goal of improving MSC attachment. The standard method of testing the efficacy of biomimetic peptides is to compare a functionalized implant with an uncoated implant. In these cases, biomimetic peptides are considered to be advantageous if the peptide coated biomaterial induces cell adhesion as compared with the uncoated biomaterial. Based on this method, many biomimetic peptides, including RGD, FNIII 7-10, DGEA and P15, would be believed to increase cell adhesion and osseointegration on multiple biomaterials, including HA. In the case of non-fouling materials such as PEG, this method should mimic what will happen *in vivo*, given that the implant, upon placement, will not adsorb

cell attachment factors from the blood and biomaterials. However, in the case of adsorptive materials such as HA, this method does not accurately portray the implant surface an MSC will encounter in a patient. Upon implantation, full length integrin ligands are adsorbed to the HA surface, drastically changing the surface of the biomaterial. Thus, the efficacy of biomimetic peptides on HA biomaterials should be studied in the presence of adsorbed proadhesive proteins. Based on our studies, it is likely that the addition of biomimetic peptide αvβ3 integrin ligands on the surface of HA biomaterials is redundant, even possibly detrimental, given that full length αvβ3 integrin ligands will be adsorbed to the HA surface upon implantation. Instead, the addition of biomimetic peptides and proteins which induce MSC migration towards the implant site or induce MSC differentiation along the osteoblast lineage should induce increases in osseointegration of HA biomaterials.

Based on our findings, our laboratory believes that DGEA and P15 both have the potential to increase the rate of osseointegration of HA biomaterials. However, one drawback of these studies is that the absorption of peptides to the HA surface is unregulated. When peptides are attached to polymer and titanium surfaces, the peptides are covalently coupled to the biomaterials surfaces. However, many of the coupling methods used to covalently attach peptides to the material surfaces would block or destroy the HA, possibly altering the protein adsorption capabilities of the surface. Multiple bone-binding proteins contain a string of polar or negatively charged amino acids, which are used to bind to the minerals in bone. Based on this, there are some studies which have used chains of amino acids, including the seventeen asparagine (N17) domain of statherin [117, 145], and a seven glutamate (E7) domain derived from bone

sialoprotein [140, 142] to form an ionic bond between the peptides and the HA. Previous studies from our laboratory and others have shown that the addition of an E7 domain on the N terminus of RGD peptides significantly increased peptide retention on HA biomaterials as compared with RGD peptides alone [138, 142]. In addition, preliminary data from our laboratory has shown that, when placed subcutaneously for seven days, significant amounts of E7-RGD peptide remained on HA surfaces (data not shown). When we examined the effects of the E7-RGD on cell adhesion to serum coated HA, we found that this peptide, as with RGD, inhibited cell adhesion to adsorbed serum molecules [138]. While the E7-RGD peptide did not induce a favorable cell response in the presence of proadhesive proteins, the data suggested that the E7 domain would increase peptide retention on HA surfaces both *in vitro* and *in vivo*. Thus, to improve differentiation peptide adsorption, our laboratory is beginning to examine whether the addition of an E7 domain to the N terminus of the DGEA and P15 peptides will allow for more stable binding and increases in differentiation. While it is ideal to covalently link the peptides to biomaterial surfaces, the E7 domain will allow for an ionic link between the peptides and the HA surface, while still maintaining the integrity of the HA itself. To study whether the E7 domain will increase peptide binding, MSC differentiation and matrix mineralization, we will examine the E7-conjugated peptides, and compare the effects of these peptides with the collagen I mimetic peptides lacking the E7 domain.

Our studies suggest that adhesion molecules, such as RGD, which bind to the same integrins as FN, VN, and Fbg would not have a benefit on HA biomaterials, given that they will most likely compete for integrins on the surface of MSCs. While there are peptides which are emerging to induce a greater degree of integrin signaling than RGD,

including the FNIII peptide, which has been shown to increase bone formation on titanium implants [112], full length protein ligands seem to induce the greatest degree of integrin activation. Given the fact that the proteins FN, VN and Fbg are present in the blood of the patient, and they readily adsorb to the HA surface, we believe that cell adhesion molecules, including RGD and FNIII, do not need to be engineered to the surface of HA biomaterials. Further, full length proteins also contain domains which bind to receptors distinct from integrins on the MSC surface, while the mimetic peptides do not. Thus, our research has expanded to include the efficacy of non-integrin binding factors, which may work synergistically, rather than competitively, with the proadhesive proteins adsorbed to the HA surface. The proteins which we are beginning to examine are thought to increase cell survival and bone formation around implants, by binding cellular receptors other than integrins.

Our laboratory has begun to examine whether vascular endothelial growth factor (VEGF) and/or BMP-2 will increase bone formation around HA implants. It has been hypothesized that adsorbing VEGF to the HA surface prior to implantation could induce neovascularization in and around bone implants. In fact, there is some literature suggesting that pre-adsorption of VEGF to HA implants would improve blood flow to the implant site [165] . This is vital, as a lack of oxygen and nutrients at the implant site would decrease cell survival and possibly implant integration. In addition, we are also examining the effects of adsorbing the differentiation factor BMP-2 to the HA surface. The BMP-2 protein has long been shown to activate MSC differentiation along the osteoblast lineage, both *in vitro* [166] and *in vivo* [167, 168]. BMP-2 systemic injections have been shown to increase bone wound healing [80]. Adsorption of BMP-2 to HA

biomaterials would allow the BMP-2 to be delivered directly at the damage site, rather than systemically, decreasing the concentrations of BMP-2 necessary for optimal activity. Further, recent literature suggests that, not only is BMP-2 a differentiation factor, but it could also be acting as a chemoattractant for osteoblasts and MSCs to migrate into the area of new bone formation [86-89]. If this is the case, then passive adsorption of BMP-2 would not only deliver the protein to the wound site, but would also allow for the formation of a chemotactic gradient along which the bone forming cells could migrate. To examine the effects of adsorption of these proteins to the HA surface, we will first examine whether VEGF and BMP-2, once adsorbed to the HA surface, maintain their activity. Once it is determined that adsorption of these proteins does not negate their activity, we will then examine what effect adsorption of each of these peptides has on MSC adhesion and differentiation to HA biomaterials. Finally, we will examine whether adsorption of one or both of these molecules increases bone formation around HA implants.

Our studies to this point have focused on the efficacy of biomimetic peptide coatings on HA biomaterials. HA biomaterials themselves have been shown to be less immunogenic than stainless steel or cobalt chrome [169, 170], both of which are commonly used for hard tissue implants. While there is a wealth of evidence to suggest that HA biomaterials are non-immunogenic, there has been little research done into the immune response of peptide coated HA biomaterials. The belief is that these peptides are too small to initiate the immune cascade, however, it is possible that some of these biomimetic peptides might induce a prolonged immune response, which would have a negative effect on implant integration. In order to examine this, early time point implants

(1-3 days post implantation) should be examined for immune cell markers. Changes in these markers would show whether or not these peptides have any effect on the immune response to HA biomaterials.

In sum, the research presented in this dissertation has focused on the role of adhesive peptides in promoting MSC adhesion to HA, with the goal of improving implant integration. Our studies collectively suggest that biomimetic peptide strategies aimed at increasing MSC adhesion to HA biomaterials will have little, if any, benefit, given that significant amounts of proadhesive proteins will be adsorbed to the surface. Further, our work suggests that biomimetic peptides which bind to the $\alpha v\beta 3$ integrin might, in fact, be detrimental to implant integration, as they will inhibit MSC interaction with full length ligands. In light of these results, we believe that the most promising new application for HA biomaterials, in addition to acting as the scaffold upon which bone is formed, is the potential use of this material as a delivery system for bioactive factors, such as proteins that regulate cell growth and/or differentiation. We believe that collagen mimetic peptides adsorbed to the HA surface can be used as effective differentiation factors, and that a more stable linkage between the mimetic and the biomaterial surface will increase the efficacy of these peptides. In addition, the highly adsorptive properties of HA, as compared with other biomaterials, suggest proteins can be adsorbed to the HA surface in high concentrations through noncovalent linkage, which may be important for delivering differentiation factors that are bioactive in soluble form, such as BMP2 or VEGF. Future studies should be directed at optimizing HA coatings with these factors, rather than initial adhesion molecules, to increase the rate of bone deposition on the biomaterial surface, and take advantage of the natural properties of HA implants.

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APPENDIX

INSTITUTIONAL REVIEW ANIMAL CARE AND USE COMMITTEE APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: October 24, 2007

TO: Susan L. Bellis, Ph.D.
MCLM 904 0005
FAX: 975-9028

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: Functionalizing Hydroxyapatite with Proadhesive Peptides
Sponsor: NIH
Animal Project Number: 071007667

On October 31, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Rats	B	200

Animal use is scheduled for review one year from October 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 071007667 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
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