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Advantages of RGD peptides for directing cell association with biomaterials

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Abstract

Despite many years of in vitro research confirming the effectiveness of RGD in promoting cell attachment to a wide variety of biomaterials, animal studies evaluating tissue responses to implanted RGD-functionalized substrates have yielded more variable results The goals of this report are to present some of the reasons why cell culture studies may not always reliably predict in vivo responses, and more importantly, to highlight potential applications that may benefit from the use of RGD peptides.

1. Introduction

In recent years, the biomaterials community has increasingly embraced the concept that implanted substrates should not only provide structural support for damaged tissues, but also integrate with these tissues and, ideally, promote regeneration. While it was previously thought that materials should present a relatively inert (or “biotolerant”) surface in order to minimize immune and fibrotic responses, a wealth of evidence now suggests that interactive, biomimetic types of materials often exhibit enhanced performance even though the mechanisms regulating tissue responses to these materials are not well-understood. This evolution in thinking has prompted a burgeoning effort to modify synthetic surfaces with biologic elements to promote integration with surrounding tissues, or alternately, to engineer substrates directly from biologic molecules.

A key starting point for designing interactive materials is to functionalize surfaces with factors that promote the adhesion and survival of selected cell types involved in the wound healing or tissue regenerative process (although it should be noted there are still many clinical applications for which biotolerant materials are optimal). The capacity of a material to support cell adhesion is not only critical for stimulating proper tissue development at implant/tissue interfaces, but also necessary for materials that serve as carriers for delivery of reparative cells to wound sites. Furthermore, cell attachment to a biomaterial scaffold is an important early step in the generation of in vitro-engineered tissue substitutes. Given the crucial role of cell/material association, it is not surprising that modifying material surfaces with pro-adhesive factors represents a major subdiscipline in the biomaterials field. There are several molecular interactions that can mediate cell attachment, however much of the

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research in this area has centered on utilizing adhesive peptides that engage and activate integrin adhesion receptors on the cell surface. Integrins are heterodimeric transmembrane receptors that bind to proteins within the extracellular matrix (ECM) including fibronectin, laminin, various collagens, and many other molecules [1, 2]. Integrins are composed of one α and one β subunit, and the pairing of these subunits dictates specificity for ligand. For example, α2β1 binds to selected collagen family members, α5β1 binds to fibronectin, and αvβ3 binds to a number of ligands including fibronectin, vitronectin and fibrinogen.

2. Advantages of RGD peptides for enhancing cell/biomaterial interaction

The most widely studied adhesive peptide in the biomaterials field is the tri-amino acid sequence, arginine-glycine-aspartate, or “RGD”. An exhaustive literature has established that RGD is highly effective at promoting the attachment of numerous cell types to a plethora of diverse materials. RGD is the principal integrin-binding domain present within ECM proteins such as fibronectin, vitronectin, fibrinogen, osteopontin, and bone sialoprotein [3]. RGD is also present in some laminins and collagens, however RGD may be inaccessible within these molecules (depending upon conformation), and other amino acid motifs are known to serve as alternative binding modules for laminin and collagen-selective receptors [4–6]. The RGD sequence can bind to multiple integrin species, and synthetic RGD peptides offer several advantages for biomaterials applications. Because integrin receptors recognize RGD as a primary sequence (although conformation of the peptide can modulate affinity), the functionality of RGD is usually maintained throughout the processing and sterilization steps required for biomaterials synthesis, many of which cause protein denaturation. The use of RGD, as compared with native ECM proteins, also minimizes the risk of immune reactivity or pathogen transfer, particularly when xenograft or cadaveric protein sources are utilized. Another benefit is that the synthesis of RGD peptides is relatively simple and inexpensive, which facilitates translation into the clinic. Finally, RGD peptides can be coupled to material surfaces in controlled densities and orientations. These advantages of straightforward synthesis, minimal cost, and tight control over ligand presentation cannot readily be achieved when using full-length native matrix proteins to functionalize material surfaces.

3. Factors that influence RGD performance in vivo

Despite the technical feasibility and proven adhesive potency of RGD peptides, the question of whether RGD is the optimal strategy for regulating cell adhesion to biomaterials is now being debated. In fact the answer to this question is quite complex, and arguably best-answered with a “maybe” or “sometimes”. The ultimate efficacy of RGD will likely be influenced by many different processes occurring during the in vitro loading of cells onto material carriers, as well as molecular events transpiring in vivo following implantation of both cell-loaded and acellular scaffolds. One critical factor that has not been sufficiently addressed is that, for most materials functionalized with RGD, the RGD domain will not act in isolation. Cells secrete integrin-binding proteins both in culture and in vivo. Integrin-binding proteins are present in serum, which is a fundamental component of the growth media for most cell culture protocols. As well, blood and other body fluids contain high concentrations of integrin-binding proteins including fibronectin, vitronectin and fibrinogen. The vast majority of biomaterials will adsorb these proteins to some extent, depending upon the physicochemical properties of the specific substrate, and the material surface properties will coordinately control the three-dimensional conformation adopted by adsorbed proteins [7–13]. In consequence, cells exposed to material surfaces will perceive the synthetic RGD within a background of native integrin-binding proteins. Why is this important? Because it has long been known that native integrin-binding proteins stimulate much more robust integrin signaling than the isolated RGD domain [14, 15], as evidenced by the 1000-fold

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lower potency of the GRGDSP hexapeptide derived from fibronectin when compared with native fibronectin itself [16]. The reason for this phenomenon is that integrin-binding proteins contain additional domains that cooperate with RGD in enhancing the activation of integrin-depending signaling. For instance, the PHSRN sequence within fibronectin synergizes with RGD in activating the α5 β1 receptor [17–20]. Further highlighting the limited information encoded within RGD, it is well-established that all RGD-containing matrix proteins do not elicit the same cellular response. Although the RGD domain is required for the binding of both fibronectin and vitronectin to integrins, fibronectin and vitronectin do not bind the same subset of integrin species, nor do they stimulate identical downstream signaling cascades or cellular behaviors. Vitronectin associates with αv β3, but not α5 β1, even though the RGD domain is required for the vitronectin/αv β3 interaction [21]. Simply stated, integrin-mediated signaling mechanisms induced by native RGD-containing matrix proteins cannot be completely recapitulated by the isolated RGD motif. Accordingly, RGD-functionalized materials that subsequently adsorb serum or blood proteins will present a complex matrix of integrin-binding domains to attaching cells, which in turn will alter the effects of the synthetic RGD. Moreover, the adsorption of molecules other than integrin-binding proteins could potentially modulate the strength and specificity of integrin signaling. Growth factor receptors are known to cooperate with integrins in the regulation of adhesion-related signaling networks [22, 23], therefore adsorbed growth factors (also present in body fluids) would be expected to impact RGD potency. Conversely, adsorbed albumin usually inhibits cell adhesion.

Given this complexity, is it possible to predict physiologic responses to implanted RGD-modified biomaterials? While there is no simple answer to this question, it is becoming apparent that the traditional in vitro methods used to evaluate RGD are sometimes unreliable reporters of in vivo activity, particularly for highly adsorptive or interactive types of biomaterials. For years, the standard in vitro approach has been to perform quantitative cell adhesion assays on a pristine vs RGD-modified material (usually executed in serum-depleted media). These studies nearly always show improved cell attachment to the RGD-coupled material, primarily because surfaces presenting some type of integrin activator are preferable to those with no integrin ligand. However, unless the material carrier of RGD is nonfouling, this same substrate will rapidly adsorb proteins from the tissue microenvironment upon implantation, and the protein-rich surface will then be remodeled over time. This surface remodeling almost certainly underlies, at least in part, the variable effectiveness of RGD that has been observed in vivo, with some studies reporting enhanced tissue responses to RGD-modified biomaterials [24–29] and others showing no benefit [30–33].

Studies from our laboratory have attempted to address the issue of protein adsorption by comparing cell adhesion to hydroxyapatite (HA) biomaterials coated with serum proteins, RGD, or a combination of RGD and adsorbed serum proteins. HA is known to be highly adsorptive, and fibronectin and vitronectin from serum are rapidly deposited onto HA in conformations recognized by integrin receptors [34]. Furthermore, mesenchymal stem cell adhesion to HA with adsorbed serum proteins is significantly greater than adhesion to RGD-modified HA [35, 36], consistent with the stronger integrin signaling elicited by native integrin-binding proteins. More recently we compared mesenchymal stem cell adhesion to RGD-modified vs unmodified HA substrates that were subsequently overcoated with serum, or implanted in vivo for 30 minutes, to allow protein adsorption. While this protocol clearly represents an oversimplification of processes occurring in vivo following implant placement, the results generated from such experiments were very intriguing. We speculated that the synthetic RGD peptide, when presented within a context of adsorbed integrin-binding proteins, would have a negligible effect on cell adhesion due to the more substantive signaling elicited by the native proteins. In fact we found that, although there was a modest

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enhancement in cell adhesion at low RGD concentrations, higher concentrations of RGD were strongly inhibitory [33, 37]. Specifically, HA surfaces with combined RGD/adsorbed proteins, as compared with surfaces with adsorbed proteins alone, supported significantly less cell adhesion and spreading, and induced greater caspase activation, reflecting diminished cell survival [33]. This inhibitory effect was specific to the RGD sequence because another adhesive peptide, DGEA (derived from collagen I), was not inhibitory when presented in combination with adsorbed serum proteins, and interestingly, DGEA peptides stimulated osteoblastic differentiation of mesenchymal stem cells [38]. Although the mechanism underlying this observation remains undefined, we hypothesize that synthetic RGD may compete with adsorbed native proteins for binding to integrin receptors, thereby attenuating the overall strength of integrin signaling. But regardless of mechanism, the most striking finding emerging from this series of experiments was that the in vivo performance of RGD or DGEA-modified HA biomaterials implanted into rat tibiae mirrored the results obtained from in vitro studies incorporating a protein adsorption step. Modifying HA implants with RGD inhibited the amount of newly-synthesized bone and direct bone-implant contact, whereas DGEA peptides alternately enhanced bone formation, when compared with HA implants with no adhesive peptide coatings [33, 38]. These results are in marked contrast to in vitro studies performed in the absence of an adsorbed protein layer, where both RGD and DGEA-modified HA materials stimulated significantly greater cell adhesion than pristine HA [36–38]. The broader implication suggested is that the bioactivity of RGD may be context-dependent. The stimulatory, lack of, or even inhibitory, effect of RGD on cell and tissue responses to implanted biomaterials may be regulated, at least in part, by the availability of other integrin-binding species present within the microenvironment. Hence there is a compelling need for more studies aimed at defining the molecular and cellular events that occur at the tissue/implant interface in the early hours and first few days following implantation of the substrate.

Another factor that has confounded our ability to leverage information gained from in vitro studies to a better understanding of RGD activity in vivo is that many different RGD-containing peptides, with variable structures and flanking amino acids, have been examined. The identity of flanking sequences is known to alter both the adhesive strength of RGD as well as selectivity for distinct integrin heterodimers [39–47]. The three-dimensional structure of RGD also influences activity, with cyclic peptides eliciting a different biologic response than linear peptides. Interestingly, the linear RGD sequences, GRGDSP, GRGDNP, and RGDSPASSKP are selective for α5 β1, whereas cyclic RGD peptides including GPenGRGDSPCA and cyclo(RGDf(NMe)V) bind preferentially to αv β3 [48]. RGD isoforms with variant sequence and structure will likely evoke divergent signaling pathways in vivo, and may also confer some selectivity on which cell types adhere to the material surface (due to the specific complement of integrin receptors expressed by selected cell types). Furthermore, the cyclic RGD peptide is more stable in vivo [48]. In addition to these parameters, the type of biomaterial to which RGD is attached can impact cell behavior. Recent studies have revealed that the stiffness of the matrix is a critical regulator of cell adhesion, spreading, differentiation status, and survival [49, 50]. Thus, when cells encounter an RGD-modified material, the biomechanical properties of the substrate will cooperate with RGD in regulating the cell adhesive response. All of these diverse variables inherent in prior investigations pose a challenge with regard to formulating generic conclusions concerning the therapeutic use of RGD (although it is important to recognize that such studies have provided invaluable insight into the basic molecular mechanisms regulating integrin activity).

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4. Presentation of RGD peptides on nonfouling backgrounds

One strategy adopted by many investigators to minimize uncontrolled variables such as protein adsorption is to pattern RGD onto a material surface in combination with a nonfouling polymer such as poly (ethylene glycol) (PEG). With RGD/PEG types of composites, or related material surface coatings, in vitro studies of RGD are likely to be better predictors of RGD activity in vivo, given that they more closely approximate a model in which RGD acts in isolation. Such an approach also facilitates analyses focused on the effects of RGD density, orientation and patterning on integrin activation and integrin-mediated cellular responses, due to the minimization of background influences such as integrin-independent cell attachment, protein adsorption, and biomaterial topographical features. It is now well-accepted that the mode of RGD presentation can significantly impact cell attachment strength, cytoskeletal reorganization (cell spreading) and migration [51–61]. RGD density regulates the efficiency of cell attachment, and can also control the clustering of integrin receptors, which is a critical feature of integrin activation [51, 59, 60]. Similarly, distinct patterns of RGD, commonly produced by varying the degree of spacing between clusters of RGD peptides, can influence the integrin-dependent formation of cytoskeletal structures such as focal adhesions and actin stress fibers [57, 61]. The obvious advantage of engineering RGD onto nonfouling surfaces from a clinical perspective is the potential for tight control over cell behavior, presumably leading to more uniform and predictable tissue repair. The disadvantage of this approach is that all of the biological information needed to evoke a given physiologic response must be engineered directly onto the material surface, which poses a formidable technical challenge. One anticipates that optimal tissue regeneration will require presentation of adhesive factors like RGD in combination with many other types of molecules including growth and differentiation factors, which in turn must be delivered with appropriate temporal kinetics. The goal of engineering a multi-functional and well-controlled substrate that induces a complex tissue response upon implantation is of considerable merit, however current technologies for synthesizing intricate and multi-component patterns of biologic molecules on nonfouling backgrounds are still quite limited. At the opposite end of the spectrum from substrates presenting bioactive molecules on a nonadsorptive background lies the family of materials composed of, or incorporating, native matrix molecules such as collagen, fibrin or fibronectin. By definition, these are highly interactive with the tissue microenvironment. Such substrates will: (1) provide natural integrin-binding proteins for cell attachment and signaling, (2) adsorb other proteins (e.g. growth factors), (3) be degraded by endogenous proteases, and (4) release peptide fragments or other molecules. Thus, materials composed of native matrix molecules represent a counterpoint to patterned biologic/nonfouling materials; they are rich in biologic information, but are not as readily manipulated to achieve tight control of cellular behavior and tissue maturation. Another concern is that there is currently a very limited understanding at the molecular level of the in vivo response to materials incorporating native molecules. The presumption is that the body responds to ECM-derived substrates as if they are native matrix, and then initiates a relatively normal wound healing response. Indeed the therapeutic use of natural materials, including several FDA-approved fibrin and collagen-based products, has been associated with some very favorable clinical outcomes, despite the lack of mechanistic insight. The overarching question emerging is whether the optimal material for tissue regeneration is represented by a substrate composed of native biologic molecules, which performs well but has limited tunability, or one that presents biologic motifs or modules (e.g. RGD) patterned onto an inert background, which allows greater control but is more difficult to engineer with sufficient biologic information. This is yet another dilemma for which there is no straightforward answer; each type of approach will likely have benefit for certain types of therapeutic applications. Efforts to improve both of these strategies are needed, and will likely be advanced by studies focused on: (1) developing technologies that foster generation of more complex combinations of bioactive factors on nonfouling surfaces,

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(2) expanding capabilities for the fine-tuning of mechanical and architectural features of natural matrix-derived materials, and engineering of additional biologic modifiers onto these surfaces, (3) refining in vitro protocols to better model the implant/tissue interface and (4) defining the early in vivo cell and molecular events occurring in response to interactive biomaterials in order to establish the mechanistic foundation needed for further optimization.

5. Therapeutic applications that may benefit from the use of RGD peptides

Despite the issues raised above, there are some clinical applications for which the use of RGD peptides may have clear advantages. There are numerous synthetic materials that exhibit unique structural or mechanical properties that are highly useful for tissue repair, but do not support strong cell adhesion without the addition of some exogenous adhesive factor (certain metals and polymers fall into this category). In many orthopaedic and dental treatments, for instance, there is still a need for implant metals that integrate into bone. Other types of materials that adsorb adhesive proteins in a denatured state may also benefit from modification by RGD. However, whenever possible, better results are likely to be achieved by combining RGD with additional functional domains, as illustrated by recent reports of enhanced cell adhesion on surfaces presenting both RGD and the PHSRN synergy domain [17, 62–65]. Another relatively under-investigated area of research is to combine RGD with other types of adhesive peptides, such as the YIGSR sequence, which binds laminin-selective receptors, or the triple-helical GFOGER peptide, which binds to collagen-specific integrins. Surfaces presenting RGD in association with either YIGSR or GFOGER would be expected to activate more subtypes of adhesive receptors than RGD alone, and may therefore regulate cell behavior in more complex ways, and also better model natural matrices, which are comprised of multiple ECM proteins. In addition, RGD could be combined with peptides that engage receptors other than integrins that are known to modulate cell adhesiveness and signaling, such as growth factor receptors [22, 23] or the syndecan family of proteoglycans [66, 67]. Several investigators have explored combining RGD with proteoglycan-binding peptides, and in some (but not all) instances this strategy has facilitated cell/material association [36, 68–72]. The fundamental concept is that RGD may be beneficial for materials that are relatively inert biologically, but have other advantageous features such as injectability, mechanical strength, or 3-dimensional architecture, however future efforts should be aimed at combining RGD with other sequences to better control the cell response.

The ability to tightly control the density, conformation, and patterning of synthetic RGD may also be useful for directing cell responses that would not normally be elicited by native matrix molecules. For example, the density of RGD domains accessible to cells within native matrices is limited by the relatively large size and tertiary structure of most ECM glycoproteins. However, through various patterning technologies, synthetic RGD domains can be concentrated to very high densities that would not normally be found within the native matrix. There is a rapidly growing literature describing the effects of high density RGD clusters and RGD multimers on integrin signaling [61, 63, 73–78]. Wangler et al. reported that a cyclic RGD hexadecimer exhibited 131-fold greater avidity for αv β3 than monomeric cyclic RGD [79]. Manipulation of RGD can also be employed to achieve unusually high specificity for distinct integrin heterodimers. Through altering the conformation and flanking amino acids of conformationally-restrained cyclic RGD peptides, a peptide was generated with a greater than 10,000-fold increased affinity for the platelet receptor, αIIbβ3, as compared with the original RGD sequence derived from fibronectin [80, 81]. This peptide has recently been conjugated to liposomes in order to develop an injectable product that can bind to activated platelets and induce thrombolysis [82]. Nonetheless, there is still minimal information regarding the effects of RGD density or patterning on in vivo

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bioactivity, despite the many impressive in vitro studies elucidating the role of these parameters in regulating integrin signaling. However, recent animal studies have provided proof of concept regarding the potential for isolated adhesive domains to elicit better tissue repair than the cognate parent protein. Garcia's group observed more substantial bone regeneration in response to polycaprolactone scaffolds modified with the GFOGER peptide, as compared with scaffolds coated with full-length collagen-I [83]. Similarly, greater osteointegration of titanium implants was elicited by a recombinant fragment of fibronectin containing the cell binding domain than by full-length fibronectin [84]. While further studies are needed to decipher the molecular mechanisms regulating these tissue responses, the underlying hypothesis is that adhesive domains within native proteins are often conformationally-masked and/or available only at low densities, therefore presentation of the isolated adhesive sequence in controlled densities or patterns holds great potential for amplifying certain cell behaviors.

A final, and intriguing, new direction that may benefit from the use of RGD is the development of materials composed of artificial engineered proteins, which are built from selected functional domains from multiple diverse native proteins [85–91]. These various combinations of modules are encoded within a cDNA plasmid, and then the protein is expressed recombinantly via bacterial, insect or mammalian expression systems. This protocol yields an engineered protein containing novel groupings of signaling motifs that would not normally be encountered by cells. RGD has been combined with several different domains, including structural modules derived from elastin, which allowed tailoring of both the adhesive surface and matrix elasticity to selectively regulate endothelial cell responses [89]. One envisions that very complex proteins could be engineered that integrate adhesive sequences, protease cleavage sites (to control degradation kinetics), structural modules that can be cross-linked to control matrix stiffness, and many other types of functional motifs. The advantage of this system is that both the chemical and biomechanical properties of the matrix can be simultaneously manipulated, which is obviously more difficult to achieve when using native proteins as basic structural elements. These highly tunable systems with multiple functionalities are particularly promising candidates for cell-supportive matrices used for in vitro-engineered tissues. In addition, substrates composed of artificial engineered proteins represent an excellent platform for elucidating mechanisms regulating cell-microenvironment interactions. However one concern is that, as with native proteins, engineered proteins will be highly interactive with the implant environment. Artificial proteins will present unique antigenic epitopes, and also release non-natural peptide fragments, both of which could elicit significant immune reactivity. As well, engineered proteins may adsorb native proteins from body fluids, and also engage in unconventional types of protein-protein interactions with cell surface and native ECM proteins. The binding of engineered proteins to cell surface receptors via artificial combinations of protein-binding modules could evoke cell responses that are difficult to predict or control. Thus, while the concept of biomaterials created from engineered proteins is elegant and potentially very useful, extensive animal and other types of preclinical studies will be required prior to translation of this technology into the clinic.

6. Conclusion

Although extensive in vitro research has confirmed the effectiveness of synthetic RGD peptides in directing cell attachment to a myriad of biomaterials, results from animal studies have been less consistent, with some, but not all, investigations reporting a benefit. The underlying reasons for these discrepant results are likely complex, and may relate to several factors including the variable densities, spacing and structures of the many RGD peptides studied, as well as the specific physicochemical features of the carrier material including biomechanical properties. Moreover, it must be considered that, following implantation, the

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surface of an RGD-functionalized biomaterial may be highly remodeled. In particular, adsorptive types of substrates will acquire a layer of endogenous proteins, including integrin ligands, that will correspondingly alter cell reactivity toward the synthetic RGD. These multifaceted issues highlight a critical need for more definitive studies of the cell and molecular processes regulating early events at the tissue/implant interface, especially as the field moves increasingly toward the use of natural and mimetic, rather than biotolerant, biomaterials. Nevertheless, there are some applications for which the use of synthetic RGD peptides may be warranted. RGD peptides hold good potential for enhancing cell responses to materials that have limited bioactivity, but possess architectural, mechanical or other features that are advantageous for the repair of specific tissues. In addition, the ability to tightly control the density, patterning, structure and orientation of synthetic RGD peptides provides rich opportunities for inducing cell responses that would not normally be evoked by native matrix molecules, and that may facilitate the generation of tissue-engineered constructs. Ultimately, however, one anticipates that a more favorable clinical outcome is likely to be achieved by combining RGD with other functional domains in order to better recapitulate the multiplicity of biologic information encoded within the natural extracellular matrix.

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