Chapter 1

INTRODUCTION

1.1 Artificial Proteins in Tissue Engineering

Materials used in many therapeutic applications (e.g., titanium hip replacements, Dacron vascular grafts, polyetherurethane artificial hearts) were designed originally for non-medical purposes and often were implemented with only minor modifications.[1] When these biomaterials were first utilized, the predominant thought was that they should interact minimally with the rest of the body. In other words, replacement materials should be biologically and chemically inert. For example, it was desired that vascular grafts resist protein adsorption and act simply as a conduit for blood flow.[2] The inability of these traditional biomaterials to integrate successfully with the surrounding tissue and restore complete function has led to a paradigm shift in biomaterial sciences. Researchers are now designing materials that guide cellular behavior and function. To this end, biochemical and biophysical cues from the natural extracellular matrix (ECM) are being incorporated into new biomaterials.[3]

One strategy to meet this goal is to graft cell-adhesive peptides such as the RGD sequence to polymers.[4] Bulk ligand density and nanoscale clustering have been shown to govern cellular functions such as proliferation, adhesion, motility, and differentiation.[5-10] Purified components of the ECM such as fibrin or collagen also have been used for tissue engineering (e.g., skin and cartilage repair),[11-14] but these products raise concerns regarding immunogenicity and disease transmission.

Genetic engineering of artificial polypeptides offers a novel method of developing materials for tissue regeneration. Proteins can be designed using any combination of the twenty canonical amino acids and can be produced through a host organism such as *E. coli*. In contrast to synthetic polymers, artificial proteins have a well-defined sequence and molecular weight. Furthermore, functionality that is not possible in naturally-occurring proteins can be incorporated through introduction of non-natural amino acids.[15] Some possible disadvantages include low protein expression or purification yields and potential immunogenicity concerns.

One significant advantage of artificial proteins is their modular nature—structural and bioactive domains can be incorporated easily into engineered constructs to form materials with multiple functionality. Polypeptides can be designed to form diverse structures ranging from β-sheets[16] to smectic liquid crystalline phases[17] to self-assembling hydrogels.[18] Furthermore, certain protein architectures can be recapitulated through the use of peptide repeats such as the VPGXG (where X is any amino acid) sequence from elastin[19,20] and AG-rich consensus repeats from silk.[21] Examples of bioactive sequences that have been incorporated successfully into artificial proteins to promote cell interactions include cadherin-like domains,[22] growth factors,[23] heparin-binding domains,[24] protease degradation sites,[24-26] and cell-binding domains.[24,25,27-32]

1.2 Small-Diameter Vascular Graft Application

The artificial extracellular matrix (aECM) protein family described in this thesis was designed originally for application in small-diameter vascular grafts. The preferred

graft materials are autologous vein and artery, but they are limited in supply and patients often suffer from coexisting disease that results in poor quality vessels. The most widely used materials in synthetic vascular grafts are poly(ethylene terephthalate) (PET) and expanded poly(tetrafluoroethylene) (ePTFE). These materials have been successful in large-diameter grafts but are characterized by high failure rates when used in small-diameter vessels (<6 mm) or in low-flow areas such as coronary arteries and peripheral arteries below the knee.[33-35] In 2003, over 460,000 coronary bypass surgeries were performed in the United States alone.[36] Furthermore, ~8 million Americans are afflicted with peripheral arterial disease.[36] Given these statistics, there is a vital need for better synthetic materials.

The most common ways in which grafts fail are thrombosis, a process in which blood clots occlude the vessel, and intimal hyperplasia, a phenomenon in which excessive smooth muscle cell proliferation leads to luminal narrowing. It is widely believed that synthetic materials do not perform well due to (i) a compliance mismatch between the synthetic graft and the surrounding host tissue and (ii) the inability of grafts to support an endothelial monolayer.

PET and ePTFE are two to five times stiffer than native vessels.[37-39] Compliance differences, vessel diameter mismatches, and suture site stresses can result in low shear stress and recirculation zones.[37-39] These flow disturbances can lead to vascular remodeling and intimal hyperplasia. In addition, zones of recirculation allow blood to contact the thrombogenic surface of a synthetic graft for prolonged time periods, which can cause clots and vessel occlusion. Because of the importance of mechanical

properties, compliant materials such as polyurethane grafts are being investigated for use in small-diameter vascular grafts.[40,41]

Native vessels are lined with monolayers of endothelial cells that present a non-thrombogenic surface to circulating blood. Endothelial cells regulate vascular permeability, secrete anticoagulants and procoagulants, and control trafficking of leukocytes, platelets, and red blood cells.[42-44] In addition, they govern vascular tone and smooth muscle cell migration and proliferation. Because of their importance in maintaining vascular homeostasis, endothelial cells have been pre-seeded on ePTFE grafts. Deutsch and coworkers conducted a human clinical study that compared endothelialized versus untreated ePTFE femoropopliteal grafts.[45] In the first phase of their study, 65% of pre-seeded grafts (n=27) remained patent after nine years versus 16% of non-endothelialized grafts (n=17). The second phase of their study demonstrated that the five-year primary patency rate was 68% for endothelialized grafts (n=113). To enhance cell retention, synthetic grafts have been treated with a number of coatings including collagen,[46] fibronectin,[47] gelatin,[48] laminin,[48] RGD peptides,[47] pre-clotted blood,[49] and fibrin glue.[50]

1.3 Artificial Extracellular Matrix Proteins: Design and Previous Results

Our approach to synthetic graft design has been to create artificial proteins that uniquely combine properties from natural proteins. The criteria for our proteins are: (i) match the elastic modulus of the graft to that of the native vessel and (ii) enhance endothelial cell adhesion to the graft surface. To meet these goals, aECM proteins are

composed of elastin-like repeats that confer the desired elastomeric properties and fibronectin cell-binding domains that support endothelialization. The current proteins also include a T7 tag, a heptahistidine (His) tag, and an enterokinase cleavage site at their N-terminus. The T7 and His tags aid in antibody detection, and the enterokinase cleavage site allows for these tags to be removed. Interspersed lysine residues serve as specific crosslinking sites that allow water insoluble, free-standing films to be formed.

1.3.1 Elastin-like Repeats

Crosslinked elastin networks are found in the arterial wall and impart elasticity and resiliency to the vessel.[51] Urry and coworkers have worked extensively with the VPGVG peptide derived from bovine elastin and have shown that polymers based on this peptide mimic elastin's mechanical behavior.[52] Because elastin-based polymers have tunable mechanical properties and can be cast easily into tubes, these materials have been recommended for use in vascular prostheses.[53] Work in the Tirrell laboratory confirms that elastin-based polymers can be crosslinked via glutaraldehyde,[29] bifunctional N-hydroxysuccinimide esters,[30] or hexamethylene diisocyanate[20] to form free-standing films whose Young's moduli lie within the range characteristic of native elastin (0.3–0.6 MPa).[54] Furthermore, the moduli can be tuned easily by varying the protein molecular weight, the stoichiometric ratio of crosslinker, and protein weight fraction.

Polymerized VPGVG is non-mutagenic, non-toxic, non-antigenic, non-sensitizing, non-pyrogenic, and non-hemolytic *in vitro*.[55] Furthermore, *in vivo* tests indicate that these proteins do not provoke a significant inflammatory response.[27,55,56] Another advantage of elastin-based polymers is that they are purified easily due to their

lower critical solution temperature (LCST). In contrast to most bacterial proteins, elastin-based polymers are soluble at temperatures below the LCST but aggregate and become insoluble at temperatures above the LCST. To increase ease of handling, we have lowered the LCST from 25 °C to 10 °C by replacing valine residues with isoleucine.[57] We thus have chosen the pentapeptide VPGIG as the basis for the elastin-like repeats in our aECM proteins.

1.3.2 Fibronectin Cell-Binding Domains

The CS1, CS5, and RGD cell-binding domains and the PHSRN synergy site from fibronectin all have been incorporated into separate aECM proteins. The CS1 and CS5 domains are derived from the alternatively spliced type III connecting segment (IIICS) of fibronectin; their minimum active sequences are LDV and REDV, respectively.[58,59] Both adhesive peptides bind the $\alpha_4\beta_1$ integrin.[60-62] Humphries and coworkers showed that the CS1 and CS5 peptides both supported melanoma cell spreading, but 100-fold greater concentration of CS5 peptide was needed to achieve the same percent of well-spread cells.[63] Hubbell and coworkers demonstrated that the $\alpha_4\beta_1$ receptor mediated specific interactions of human umbilical vein endothelial cells with GREDVY-grafted surfaces;[64] however, other vascular cell types such as fibroblasts, vascular smooth muscle cells, and platelets did not spread on GREDVY-grafted substrates.[65]

Significant endothelial adhesion was not seen when short REDV peptides (5–6 amino acids) were incorporated into VPGVG polymers.[66] Early work in the Tirrell laboratory demonstrated that an elastin-based protein containing 20 amino acids of the CS5 domain was successful in binding nearly all the seeded endothelial cells.[28] We

believe that these longer CS5 sequences may have allowed for a conformation more conducive to endothelial attachment. Later work showed that endothelial cell adhesion to the CS5 sequence in elastin-based proteins was sequence-specific.[67] Furthermore, more than 60% of cells remained adherent to these proteins at physiologically relevant shear stresses ($\leq$100 dynes/cm^2).

The RGD and PHSRN sequences are from the tenth and ninth type III fibronectin domains, respectively.[68-71] The RGD sequence is recognized by the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins.[72,73] The $\alpha_5\beta_1$ integrin, or fibronectin receptor, often binds to the RGD sequence in conjunction with the PHSRN synergy site.[71] Urry and coworkers demonstrated that GRGDSP copolymerized with VPGVG at ratios of 1:60 exhibited robust endothelial adhesion while polymerized VPGVG alone did not.[74]

1.4 Thesis Outline

The goal of this thesis was to characterize cell response to aECM proteins designed to serve as materials for small-diameter vascular grafts. Chapter 2 describes the cloning of aECM constructs containing the RGD and PHSRN bioactive domains and the corresponding sequence-scrambled negative control proteins. A protein containing a scrambled version of the CS1 domain was also cloned. The protein design was done in collaboration with Kathleen Di Zio and Sarah Heilshorn, but I completed all of the subsequent work.

Chapter 3 compares endothelial cell response to proteins containing the RGD and CS5 sequences. Sequence-specific adhesion to these aECM proteins was demonstrated.

Furthermore, proteins containing the RGD sequence elicited stronger adhesion and faster spreading of endothelial cells compared to proteins containing the CS5 sequence. I conducted the majority of the experiments and wrote this chapter. Sarah Heilshorn provided valuable technical advice and performed the peptide inhibition experiments with the CS5-containing protein.

Chapter 4 explores the importance of amino acids distant from the cell-binding domain and their influence on cell behavior. aECM proteins containing the CS5 sequence demonstrated that proteins with lysine residues confined to the termini resulted in more robust adhesion and spreading compared to variants with lysine residues interspersed within the elastin cassettes. Sarah Heilshorn and I contributed equally to the experimental design, data collection, and writing of this chapter.

Chapter 5 characterizes endothelial response to RGD density in crosslinked protein films. Poly(ethylene glycol) was bound covalently to the films to decrease nonspecific cell interactions. Cells were able to recognize specifically the cell-binding domain even when aECM proteins were crosslinked. Increasing the RGD density led to more robust adhesion and spreading but did not affect significantly the migration speeds. I developed and performed the experiments and wrote this chapter. Paul Nowatzki kindly made the AFM height measurements on dry crosslinked films.

Chapter 6 focuses on aECM proteins containing the non-canonical amino acid, *para*-azidophenylalanine (*p*N_3Phe). These proteins can be crosslinked at 365 nm through the azide moiety, and a patterned mask was used to produce two-dimensional protein patterns. Isaac Carrico, Sarah Heilshorn, and Marissa Mock developed the original photopatterning methods using aECM proteins containing the CS5 sequence. They also

demonstrated that the underlying protein template directed the formation of cell patterns. Marissa Mock and I demonstrated sequence-specific recognition of RGD variants of this protein. Stacey Maskarinec and Marissa Mock cloned the proteins containing the RGD and RDG sequences and Stacey Maskarinec now is using these proteins to form cell patterns. Paul Nowatzki assisted Isaac Carrico with mechanical characterization and he and Christian Franck contributed to the AFM measurements.

Chapter 7 relates the development of a new fluorescence imaging method that could be used to study cells on aECM substrates. Homopropargylglycine (Hpg) was incorporated into newly synthesized proteins within a wide variety of mammalian cells. The alkyne-containing amino acid was ligated to an azide-containing dye. Newly synthesized proteins were fluorescently imaged, and a subset of these new proteins was shown to be localized in the nucleoli. Kimberly Beatty and I contributed equally to developing the method for use in mammalian cells, performing the experiments, and writing the chapter.

1.5 References

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