Chapter III. Comparative cell response to artificial extracellular matrix proteins containing the RGD and CS5 cell-binding domains

Abstract

This study addresses endothelial cell adhesion and spreading on a family of artificial extracellular matrix (aECM) proteins designed for application in small-diameter vascular grafts. The aECM proteins contain domains derived from elastin and from fibronectin. aECM **1** contains the RGD sequence from the tenth type III domain of fibronectin; aECM **3** contains the fibronectin CS5 cell-binding domain. Negative control proteins aECM **2** and **4** are scrambled versions of aECM **1** and **3**, respectively. Competitive peptide inhibition studies and comparisons of positive and negative control proteins confirm that adhesion of HUVEC to aECM proteins **1** and **3** is sequence specific. When subjected to a normal detachment force of 780 pN, 3-fold more HUVEC remained adherent to aECM **1** than to aECM **3**. HUVEC also spread more rapidly on aECM **1** than on aECM **3**. These results: i) indicate that cellular responses to aECM proteins can be modulated through choice of cell-binding domain, and ii) recommend the RGD sequence for applications that require rapid endothelial cell spreading and matrix adhesion.

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1. Introduction

Cardiovascular disease afflicts more than 61 million Americans[1] and causes 4 million deaths each year in Europe.[2] Severe atherosclerosis often requires surgical removal of the affected tissue and implantation of an autologous or synthetic vascular graft. The most widely used materials in synthetic vascular grafts are poly(ethylene terephthalate) (PET) and expanded poly(tetrafluoroethylene) (ePTFE); when used in small-diameter grafts, both materials are characterized by high failure rates due to thrombosis and intimal hyperplasia.[3-5] Autologous saphenous vein yields higher patency rates than synthetic materials, particularly when used to reconstruct the infrapopliteal artery,[6,7] but autologous vein is limited in supply and patients often suffer from coexisting disease that makes these vessels unsuitable as grafts.[5,8]

A family of artificial proteins that exhibit some of the essential characteristics of the extracellular matrix has been developed for application in small-diameter vascular grafts.[9-13] Artificial matrices that incorporate functional protein domains have been produced for a variety of applications.[14-17] The artificial extracellular matrix (aECM) proteins in this study consist of domains derived from elastin and fibronectin (Figure 1). The elastin-based repeats are designed to yield the needed mechanical properties while cell-binding domains from fibronectin are incorporated to support the growth of an endothelial monolayer. Urry and coworkers have investigated the physical properties of related elastin-based polymers,[18,19] demonstrated their biocompatibility,[16,20] and shown that the GRGDSP cell adhesion sequence in synthetic elastomeric matrices increases cell adhesion.[21]

An important criterion in the design of aECM proteins is the tensile modulus; compliance mismatch between the graft and tissue has been strongly implicated in graft failure. It is believed that flow patterns caused by disparities in mechanical properties contribute to intimal hyperplasia[22-25] and thrombosis.[26] In attempts to address these issues, several laboratories have developed compliant polyurethane composites.[27-29] In the approach presented here, we focus on elastin, which forms a crosslinked network in the

arterial wall[30] and, which, along with collagen, imparts elasticity and resiliency to the vessel. By crosslinking at reactive residues interspersed within the elastin-like domains, the modulus of a crosslinked, freestanding aECM film can be tuned into the range characteristic of elastins (0.3-0.6 MPa).[31] The extent of crosslinking can be varied to control the compliance.[10,11,13]

A second cause of graft failure is the absence of a confluent endothelial monolayer. Endothelial cells play an important role in maintaining homeostasis of the vasculature. They secrete anticoagulants and procoagulants; control the trafficking of leukocytes, platelets and red blood cells; and regulate the growth and migration of smooth muscle cells.[32-34] Deutsch and coworkers found that pre-seeding ePTFE grafts with endothelial cells resulted in a 65% patency rate after nine years, versus 16% for non-endothelialized grafts.[35] Collagen,[36] fibronectin,[37] laminin,[38] gelatin,[38] pre-clotted blood,[39] RGD peptides,[37] and lectins[40] have all been used as coatings to enhance cell retention in synthetic grafts. To promote endothelialization of grafts derived from aECM proteins, cell-binding domains have been incorporated at regular intervals. In this work, aECM **1** contains the RGD sequence derived from the tenth type III domain of fibronectin; [41-43] this sequence serves as a ligand for the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins.[44,45] aECM **2** is a negative control for aECM **1,** in which the sequence of the RGD cell-binding domain has been scrambled. aECM **3** has been previously characterized[12] and contains the CS5 cell-binding domain from the alternatively spliced type III connecting segment of fibronectin.[46,47] The CS5 cell-binding domain is recognized by the $\alpha_4\beta_1$ integrin.[48] When the peptide GREDVY, which includes the minimal binding sequence from the CS5 cell-binding domain, was immobilized on glass surfaces, endothelial cells adhered while fibroblasts, vascular smooth muscle cells, and human blood platelets did not.[49] The CS5 cell-binding domain has been scrambled in aECM **4** to provide a negative control for aECM **3**. The goal of this study is to compare cell adhesion and spreading on aECM proteins containing the RGD and CS5 cell-binding domains.

aECM **1**:
M-MASMTGGQQMG-HHHHHHH-DDDDK-{LD-YAVTGRGDSPASSKPIA-G[(VPGIG)$_2$VPGKG(VPGIG)$_2$]$_4$VP}$_3$-LE
T7 tag | His tag | Cleavage site | RGD binding domain | Elastin-like domain

aECM **2**:
M-MASMTGGQQMG-HHHHHHH-DDDDK-{LD-YAVTGRDGSPASSKPIA-G[(VPGIG)$_2$VPGKG(VPGIG)$_2$]$_4$VP}$_3$-LE
T7 tag | His tag | Cleavage site | Scrambled RGD binding domain | Elastin-like domain

aECM **3**:
M-MASMTGGQQMG-HHHHHHH-DDDDK-{LD-GEEIQIGHIPREDVDYHLYP-G[(VPGIG)$_2$VPGKG(VPGIG)$_2$]$_4$VP}$_3$-LE
T7 tag | His tag | Cleavage site | CS5 binding domain | Elastin-like domain

aECM **4**:
M-MASMTGGQQMG-HHHHHHH-DDDDK-{LD-GEEIQIGHIPREVDDYHLYP-G[(VPGIG)$_2$VPGKG(VPGIG)$_2$]$_4$VP}$_3$-LE
T7 tag | His tag | Cleavage site | Scrambled CS5 binding domain | Elastin-like domain

Figure 1. Amino acid sequences of aECM proteins **1-4**. Each protein contains a T7 tag, a hexahistidine tag, an enterokinase cleavage site, and elastin-like domains containing lysine residues for crosslinking. The RGD cell-binding domain is found in aECM **1**, while the minimal recognition sequence in the RGD cell-binding domain has been scrambled in aECM **2** to provide a negative control. aECM **3** includes the CS5 cell-binding domain while aECM **4**, the negative control, contains a scrambled version of the CS5 cell-binding domain.

2. Materials and methods

2.1. Protein expression and purification

Standard methods for cloning, bacterial growth, protein expression, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blotting were performed to produce **1** and **2**.[50,51] The genes for **1** and **2** were placed under control of a T7 bacteriophage promoter in the pET28 expression vector (Novagen, Madison, WI) and transformed into the protein expression host, BL21(DE3)pLysS (Novagen). Protein expression was performed as described previously[11] except that cells were harvested after 1.5-2 hours after induction with isopropyl-1-□-D-thiogalactosidase (IPTG) (Calbiochem, Inc., San Diego, CA). The wet cell mass averaged 230 g per 10 L fermentation for **1** and **2**. The cells were resuspended in TEN buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100

mM NaCl) at a concentration of 0.5 g/mL and frozen at –20°C. The cells were defrosted at 4°C with 10 μg/mL of deoxyribonuclease I (Sigma, St. Louis, MO), 10 μg/mL of ribonuclease A (Sigma), and 50 μg/mL of phenylmethylsulfonyl fluoride (Sigma). Water was added to bring the total volume of the solution to 1.3 L. Because the lower critical solution temperature (LCST) of **1** is 35°C (10 mg/mL in PBS, pH 7.3), proteins **1** and **2** were readily purified via a series of three temperature cycles. The pH of the solution was adjusted to 9 and the solution was centrifuged (2 h, 39,750 *g*, 4°C). The resulting supernatant was adjusted to 1 M NaCl at 4°C, warmed to 37°C, and centrifuged (2 h, 39,750 *g*, 37°C). The pellet was then redispersed in water at a concentration of 100 mg/mL. This process was repeated twice. The solution was dialyzed at 4°C for 3 days and lyophilized. The purity and molecular weights of the proteins were verified by SDS-PAGE gels, Western blots, amino acid analysis, and matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). Average yields were 580 mg of protein per 10 L fermentation for **1** and **2**. The expression and purification of **3** and **4** were similar and have been reported previously.[11,12]

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) (BioWhittaker, Inc., Walkersville, MD) were maintained in a 37°C, 5% CO_2 humidified environmental chamber. The cells were grown in Endothelial Growth Medium-2 (EGM-2, 2% serum) (BioWhittaker), which was replaced every two days. Near confluent HUVEC cultures were passaged non-enzymatically by treatment with 0.61 mM EDTA (Gibco, Grand Island, NY). Passages 2-10 were used; no differences in cell behavior due to passage number were observed.

2.3. Surface preparation

Solutions of **1-4** in PBS (1 mg/mL) were adsorbed onto tissue culture polystyrene at 4°C overnight. A fibronectin solution (10 μg/mL) was adsorbed under similar conditions. The surfaces were rinsed with PBS, blocked with a 0.2% solution of heat-

inactivated bovine serum albumin (BSA) (fraction V, Sigma) for 30 minutes at room temperature, and rinsed with PBS. To ensure that surfaces coated with aECM **1-4** presented similar numbers of cell-binding domains, protein adsorption was quantified by using a modified protocol for the QuantiPro BCA Assay Kit (Sigma). aECM **1** presented 4.6 ± 0.6 x 10^{11} cell-binding domains per well; aECM **3** had 4.3 ± 0.6 x 10^{11} cell-binding domains per well. Three independent experiments in triplicate were performed.

Cell viability on adsorbed aECM proteins was measured by monitoring the cleavage of WST-1 (Boehringer Mannheim, Mannheim, Germany). Three independent experiments in triplicate demonstrated that up to six hours, there were no differences in viability between cells grown on **1-4** and those grown on fibronectin in basal medium.

2.4. Peptide inhibition

A colorimetric binding assay described in previous studies was used to examine inhibition of cell adhesion by soluble peptides.[12] Briefly, the wells of a 96-well plate were prepared as described above and 40,000 HUVEC in serum-free EBM-2 were added to each well. A solution of the peptide [GRGDSP (Calbiochem), GRDGSP (Biopolymer Synthesis and Analysis Facility, California Institute of Technology, Pasadena, CA), GREDVDY (Commonwealth Biotechnologies, Inc., Richmond, VA), or GREVDDY (Commonwealth Biotechnologies, Inc.)] in EBM-2 was added. After 30 minutes of incubation at 37°C and 5% CO_2, non-adherent cells were removed by inversion of the plate and rinsing with PBS. Cells were fixed with 70% ethanol, stained with 0.1% crystal violet (Sigma), and thoroughly rinsed with water. The dye was solubilized with a 0.2% Triton X-100 (Sigma) solution. The absorbance was measured at 595 nm on a Molecular Devices SPECTRAmax Plus^{384} microplate spectrophotometer (Sunnyvale, CA). At least three independent experiments were carried out in triplicate.

2.5. Cell adhesion

Cell adhesion experiments were adapted from a previously described method.[52] Detached HUVEC cells were labeled with a 5 μM solution of calcein acetoxymethyl ester

(Molecular Probes, Eugene, OR) in serum-free EBM-2 at room temperature for 30 minutes. The cells were rinsed with and resuspended in PBS^+ (PBS containing 1.8 mM $CaCl_2$ and 10 mM $MgSO_4$). After the cells were counted, 0.15 mL of a cell suspension (2.67 x 10^5 cells/mL in PBS^+) was added to each well of a 96-well plate and incubated for 30 min at 37°C and 5% CO_2. Each well was filled with 0.2 mL of a solution of Percoll™ (Sigma) (21% w/w in PBS). The plates were centrifuged for 10 minutes at 1, 100, 1000, 2000, or 3000 *g*. The non-adherent cells were wicked away using a harvesting frame (Molecular Devices) with the filters removed. PBS was added to each well and a fluorescence reading was taken on a Perkin Elmer HTS 7000 Bio Assay Reader (Wellesley, MA) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm.

The fluorescence measured in this way is linearly proportional to cell number in a given labeling experiment (data not shown). But because the amount of dye taken up by each cell varies from experiment to experiment, the fluorescence readings could not be used to determine absolute cell numbers. Instead, a cell adhesion index (CAI) was defined as the fluorescence reading of the test well divided by the fluorescence reading of HUVEC attached to fibronectin subjected to a force of 1 *g*. Error bars represent the standard deviations of three or more independent experiments, each of which evaluated cell adhesion in 6 wells.

To estimate the force applied to each cell, Archimedes' theorem was employed: $F = (\rho_c - \rho_m) \cdot V_c \cdot RCF$, where F is the force, ρ_c is the density of the cell (~1.07 g/mL),[53] ρ_m is the density of the medium (1.123 g/mL),[52] V_c is the volume of the cell (~0.5 pL),[53] and RCF is the relative centrifugal force. Estimated normal detachment forces ranged from 26 to 780 pN.

2.6. Cell spreading

HUVEC in serum-free EBM-2 were added to each well of a 6-well plate at a concentration of 48,000 cells per well. At 15 minute intervals, the plates were removed from

the environmental chamber and cells were imaged using a 10x phase contrast objective on a Nikon Eclipse TE 300 inverted microscope (Tokyo, Japan). Images were captured on a Sony CCD color video camera (Model DXC-151A, Tokyo, Japan) equipped with Studio DC10 Plus software, v. 1.06.4 (Pinnacle Systems, Mountain View, CA) and were density-sliced to determine the number of spread (i.e., dark) versus non-spread (i.e., bright and refractive) cells using Scion Image for Windows, release beta 4.0.2 (Scion Corporation, Frederick, MD). Three independent experiments were performed.

2.7. Immunofluorescence microscopy

Cells in serum-free EBM-2 were added to an 8-well Lab-Tek II Chamber Slide (Nalge Nunc International, Rochester, NY) at a density of 30,000 cells per well and grown for 4 hours at 37°C and 5% CO_2. The cells were rinsed twice with warm PBS, fixed with ice-cold acetone for one minute, and rinsed twice with PBS. Cells were blocked with a 10% BSA solution for 30 minutes and then rinsed twice with PBS. The primary antibody solution was incubated in the well for one hour at room temperature. All primary antibodies were obtained from Chemicon International, Inc. (Temecula, CA). Antibody clones LM609, JBS5, and V284 were used to detect $\alpha_v\beta_3$, $\alpha_5\beta_1$, and vinculin at dilutions of 1:80, 1:40, and 1:80, respectively. The wells were then rinsed three times with PBS. The secondary antibody solution contained 0.76 units/mL of rhodamine-phalloidin (Molecular Probes), 3% BSA, and a Cy2 conjugated affinity-purified goat anti-mouse secondary antibody (Chemicon) (at concentrations of 12.5, 6.25, and 12.5 μg/mL for the anti-$\alpha_v\beta_3$, -$\alpha_5\beta_1$, and -vinculin antibodies, respectively). The wells were thoroughly rinsed with PBS and incubated for five minutes at room temperature in the dark with a 3 x 10^{-7} M 4',6-diamidino-2-phenylindole (DAPI) (Molecular Probes) solution for staining cell nuclei. After rinsing the wells three times with PBS, the chambers were removed. A mounting solution of 50% glycerol and 50% PBS was used. Images were examined by using a 40x objective on a Zeiss Axioplan II fluorescence microscope (Oberkochen, Germany) equipped with monochrome Axiocam and AxioVision 3.1 software.

3. Results and discussion

3.1. Competitive peptide inhibition

To test the hypothesis that HUVEC adhere to aECM **1** specifically through the RGD cell-binding domain, competitive peptides were used to inhibit adhesion. When HUVEC were incubated on aECM **1** with 1.3 mM of the competitive GRGDSP peptide, the number of adherent cells was reduced 6-fold (Figure 2a). The numbers of adherent HUVEC in Figure 2a are expressed relative to the number of cells adherent to fibronectin in the absence of peptide to normalize for passage-to-passage variations. Furthermore, increasing the concentration of the competitive peptide GRGDSP from 0 to 1.7 mM decreased the numbers of adherent HUVEC to aECM **1** with a half-inhibition concentration (IC_{50}) of ~0.58 mM (Figure 2c). The negative control peptide GRDGSP had no significant effect on the number of adherent cells to aECM **1**. Neither of the peptides inhibited adhesion to fibronectin. These results demonstrate that HUVEC specifically adhere to the RGD cell-binding domain in aECM **1** and that this binding can be disrupted in a concentration dependent manner by a competitive peptide presenting the same cell-binding domain.

In the absence of peptide, the number of adherent HUVEC on aECM **3** was 33.3 ± 6.1% of that on fibronectin. Incubating HUVEC with 1.8 mM of the competitive GREDVDY peptide decreased the number of adherent HUVEC approximately 10-fold (Figure 2b). The non-competitive peptide GREVDDY did not decrease cell adhesion to aECM **3**, and neither GREDVDY nor GREVDDY inhibited adhesion to fibronectin. These results show that HUVEC adhere to aECM **3** in a sequence-specific manner and that cell adhesion can be inhibited by soluble peptides that contain the authentic REDV sequence.

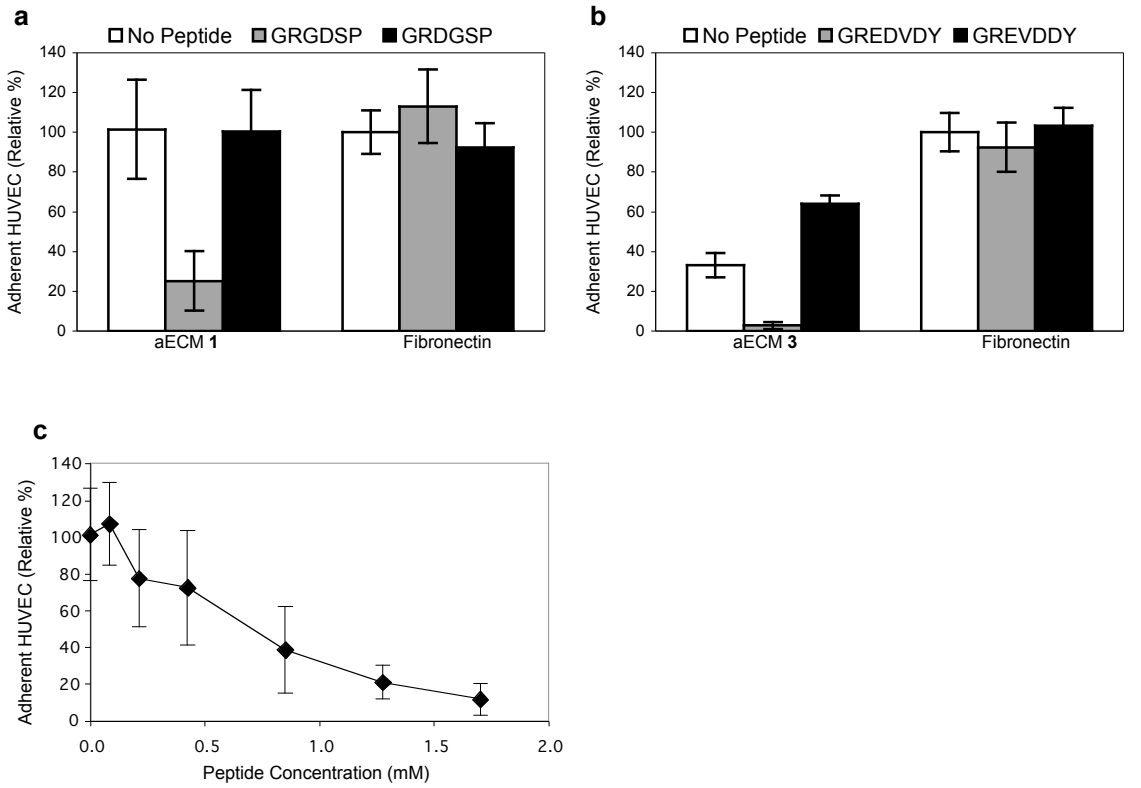

Figure 2. Competitive peptide inhibition. (a) Percentage of adherent HUVEC on aECM **1** and fibronectin relative to fibronectin in the absence of peptide. The competitive peptide, GRGDSP, and the non-competitive peptide, GRDGSP, were added at 1.3 mM. (b) Percentage of adherent HUVEC on aECM **3** and fibronectin in the presence of 1.8 mM of GREDVDY, the competitive peptide, and GREVDDY, the non-competitive peptide. (c) Increasing the concentration of competitive peptide GRGDSP from 0 to 1.7 mM decreased HUVEC adhesion on aECM **1**. Data represent three experiments, each performed in triplicate; error bars represent one standard deviation.

3.2. HUVEC resistance to detachment forces

To probe further the specificity of HUVEC adhesion, comparisons were made between aECM proteins **1** and **3**, and the corresponding negative control proteins that contain scrambled cell-binding domains. After HUVEC were incubated for 30 minutes on

each test substrate, they were subjected to a normal detachment force for 10 minutes. The remaining HUVEC were quantified in terms of a cell adhesion index (CAI) as described in Materials and Methods. At a detachment force of 780 pN, HUVEC on aECM **1** had a CAI of 100 ± 11.2% while those on aECM **2** had a CAI of 32.5 ± 11.8% (Figure 3a). The same detachment force resulted in a CAI of 34.6 ± 11.0% on aECM **3** and a CAI of 16.9 ± 14.4% on aECM **4** (Figure 3b). In each case, adhesion to the protein bearing the authentic cell adhesion ligand is more robust than attachment to the negative control protein containing the sequence-scrambled ligand.

Furthermore, under all of the conditions examined in this work (i.e., for detachment forces ranging from 26 to 780 pN), a larger number of HUVEC remained adherent to aECM **1** than to aECM **3** (Figure 3). Because aECM **1** and **3** present similar numbers of cell-binding domains and do not differentially affect cell viability, we believe that this result reflects a difference in the robustness of adhesion mediated by RGD as compared to that mediated by CS5.

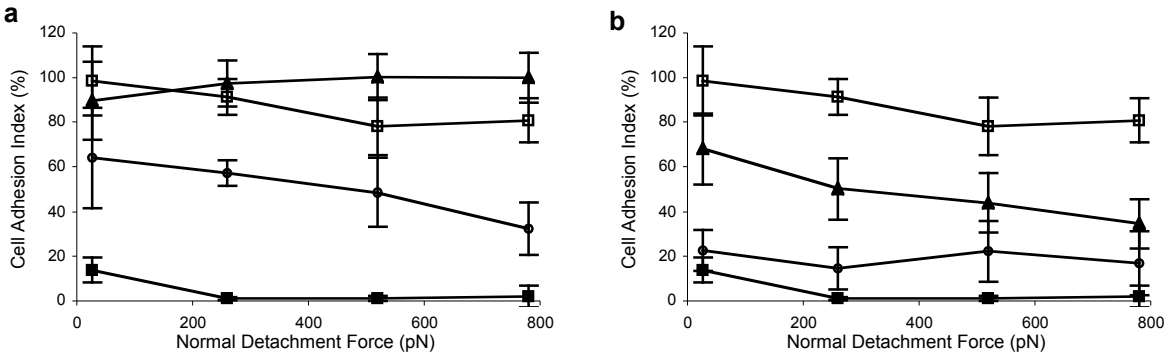

Figure 3. HUVEC resistance to detachment forces. (a) Percentages of cells that remain adherent to aECM **1** (▲), aECM **2** (●), fibronectin (□), and BSA (■) after being subjected to detachment forces. (b) Percentages of cells that remain adherent to aECM **3** (▲), aECM **4** (●), fibronectin (□), and BSA (■) after being subjected to normal detachment forces. Data represent three independent experiments in which six wells were tested; error bars represent one standard deviation.

3.3. Time course of HUVEC spreading

Cell spreading on aECM substrates was examined at 15 minute intervals by phase contrast microscopy. Images were analyzed (as described in Materials and Methods) to distinguish between dark, spread cells and bright, rounded cells. After 15 minutes, 57.0 ± 2.7% of HUVEC were spread on aECM **1** while no cells were spread on aECM **2** (Figure 4a)**.** These differences persisted after 60 minutes of adhesion (data not shown). After 75 minutes, 18.2 ± 10.3% of HUVEC were well-spread on aECM **3** while only 3.8 ± 5.0% were well-spread on aECM **4** (Figure 4b). Nearly all of the HUVEC were well-spread on fibronectin at all time points tested while fewer than 1% of cells spread on BSA at all time points (data not shown).

After 60 minutes of adhesion, essentially all the HUVEC were well-spread on aECM **1** (92.3 ± 1.5%) while very few had spread on aECM **3** (7.3 ± 3.7%). These results indicate that HUVEC spread more rapidly on proteins containing the RGD cell-binding domain and do not spread well at short times on proteins containing the CS5 cell-binding domain. If HUVEC adhere to the CS5 cell-binding domain through the $\alpha_4\beta_1$ integrin as has been previously reported,[54] the results shown in Figure 4 are consistent with previous studies showing that the α_4 cytoplasmic tail reduces cell spreading. K562 erythroleukemic cells containing chimeric forms of the α_4 integrin subunit in which α_4 extracellular and transmembrane domains were joined to β_2 and β_5 cytoplasmic tails spread more rapidly than cells containing the wild-type α_4 integrin subunit.[55] Furthermore, when the α_4 cytoplasmic tail was joined to the α_{IIb} extracellular and transmembrane domains, $\alpha_{IIb}\beta_3$-dependent cell spreading of CHO cells was reduced.[56]

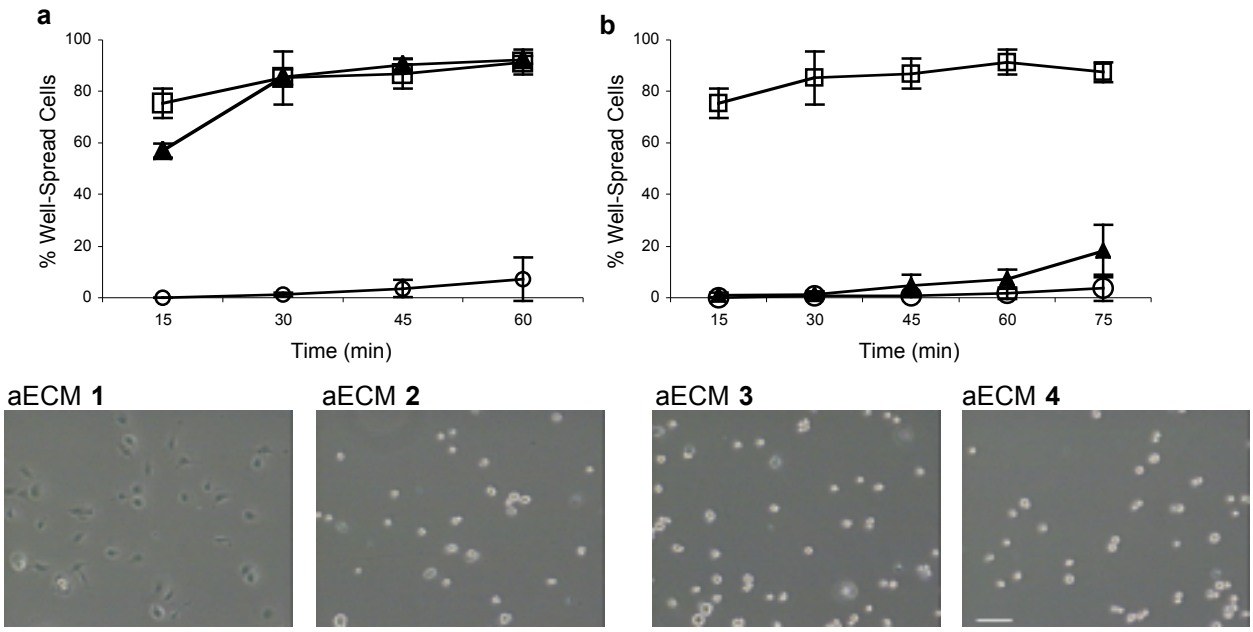

Figure 4. Cell spreading on aECM substrates. (a) Percentage of well-spread cells on aECM **1** (▲), aECM **2** (○), and fibronectin (□) from 15 to 60 minutes after seeding. The phase contrast images show dark, well-spread cells on aECM **1** and bright, rounded cells on aECM **2** after 60 minutes of incubation. (b) Percentage of well-spread cells on aECM **3** (▲), aECM **4** (○), and fibronectin (□). The phase contrast images show that the HUVEC are not well-spread on either aECM **3** or aECM **4** after 60 minutes. Fewer than 1% of the cells on BSA were well-spread. The phase contrast images were analyzed using Scion Image to determine the number of spread (i.e., dark) versus non-spread (i.e., bright) cells. Three independent experiments were performed and the error bars represent one standard deviation. The scale bar represents 100 μm.

3.4. Visualization of focal adhesions and integrin clusters

Since HUVEC spread well on aECM **1**, spreading on this substrate was investigated more thoroughly by labeling cells for F-actin and vinculin, a protein found at focal adhesions. Figure 5 shows cells on aECM **1** with long, well-formed stress fibers and focal adhesions localized at the ends of these fibers. Similarly, cells on fibronectin had well-formed actin networks with vinculin localized in clusters at the ends of these

filaments. Cells on BSA were small and rounded while cells on aECM **2** did not have well-formed actin networks (data not shown). Vinculin was non-specifically distributed throughout the cells when cells were examined on BSA or aECM **2**. These results further support the observation that cells are well-spread on aECM proteins bearing the RGD cell-binding domain and indicate that HUVEC form distinct focal adhesions when adhering to aECM **1**.

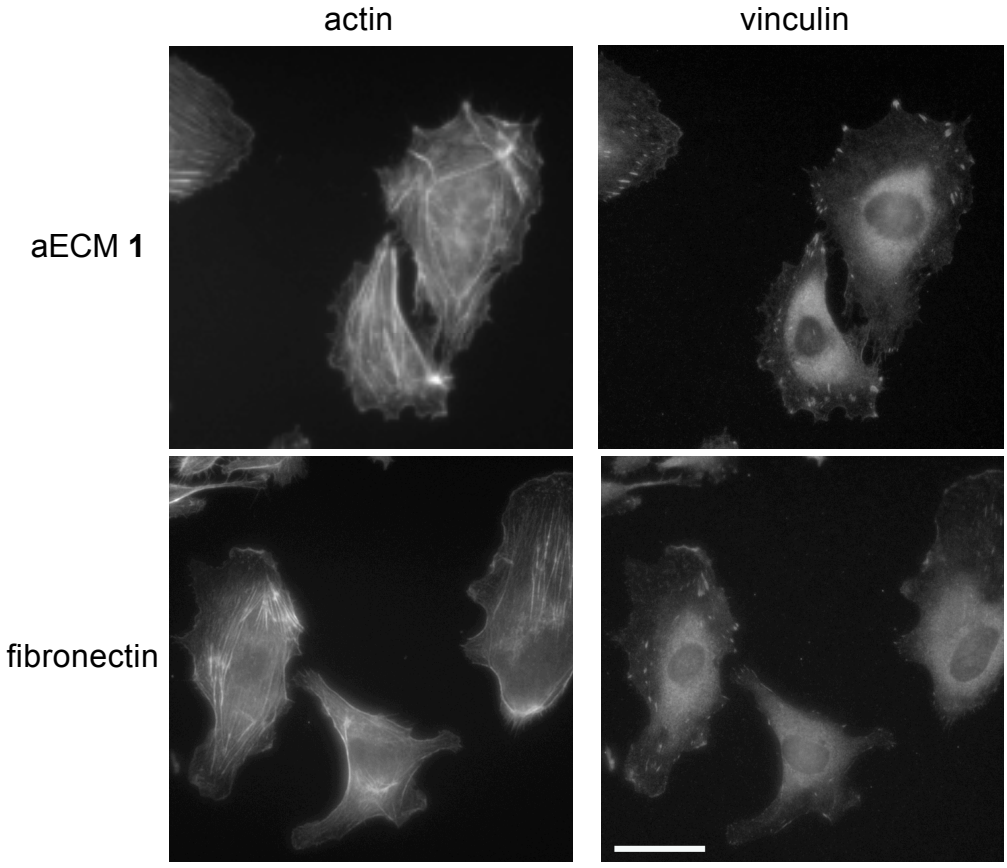

Figure 5. Fluorescence micrographs of actin filaments and focal adhesions in HUVEC. Cells incubated on aECM **1** or fibronectin for 4 hours were labeled with rhodamine-phalloidin and an anti-vinculin IgG_1 antibody and detected with a Cy2-conjugated secondary antibody. The scale bar represents 25 □m.

To investigate the mechanism by which HUVEC bind to and spread on aECM **1,** immunofluorescence microscopy was used to visualize the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. When HUVEC adhered to aECM **1**, the $\alpha_v\beta_3$ integrin was localized in small clusters found at the ends of actin filaments (Figure 6a) while the $\alpha_5\beta_1$ integrin was found non-specifically throughout the cell (Figure 6b). This result suggests that the $\alpha_v\beta_3$ integrin is found in focal adhesions and that it is involved in HUVEC adhesion to the RGD cell-binding domain in aECM **1**. In HUVEC attached to fibronectin, the $\alpha_v\beta_3$ integrin was found non-specifically throughout the cell (Figure 6a) while the $\alpha_5\beta_1$ integrin was found throughout the cell in large structures corresponding to the ends of actin filaments (Figure 6b). This result shows that the $\alpha_5\beta_1$ integrin is found in focal adhesions when cells adhere to fibronectin and suggests that HUVEC bind to this substrate through the $\alpha_5\beta_1$ integrin. The finding that HUVEC bind to aECM **1** through the $\alpha_v\beta_3$ integrin and to fibronectin through the $\alpha_5\beta_1$ integrin is not surprising given that the $\alpha_5\beta_1$ integrin binds to the RGD cell-binding domain in conjunction with the PHSRN synergy site found in fibronectin[57,58] while the $\alpha_v\beta_3$ integrin has been found to be less stringent in its requirements and binds a variety of proteins with the RGD sequence, including vitronectin,[44] fibronectin,[59] fibrinogen, von Willebrand factor,[60] and thrombospondin.[61]

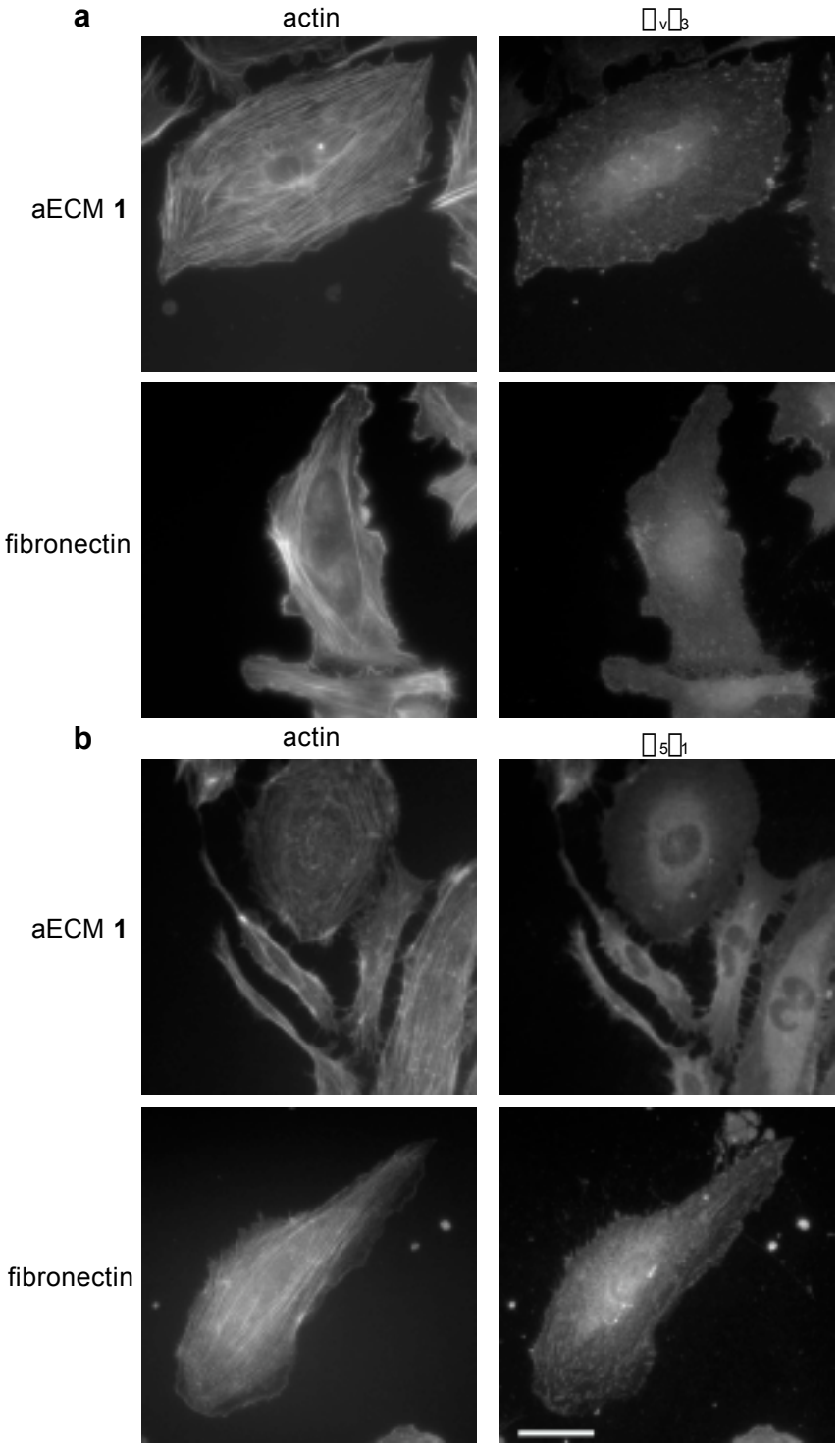

Figure 6. Fluorescence micrographs of actin filaments and integrins. (a) Cells cultured for 4 hours on aECM **1** or on fibronectin were labeled with rhodamine-phalloidin and an anti-$\alpha_v\beta_3$ IgG_1 antibody and detected with a Cy2-conjugated secondary antibody. (b) Cells cultured for 4 hours on aECM **1** or on fibronectin were labeled with rhodamine-phalloidin and an anti-$\alpha_5\beta_1$ IgG antibody and detected with a Cy2-conjugated secondary antibody. The scale bar represents 25 μm.

4. Conclusion

Sequence-specific cell adhesion to aECM proteins containing the RGD and CS5 cell-binding domains has been demonstrated. In the context of the same aECM protein backbone, the RGD cell-binding domain binds endothelial cells more strongly and elicits faster cell spreading than the CS5 cell-binding domain. Cell response to the aECM proteins can thus be altered by judicious choice of cell-binding domains. Further studies will determine the degree to which endothelial cell responses can be modulated by mixing cell-binding domains.

In addition to varying the cell response through the cell-binding domain, it has been previously shown that the mechanical properties of crosslinked, freestanding films can be controlled through factors such as the extent of crosslinking.[10,11,13] The ability to control both cell response and mechanical properties in a single material is highly desirable in a vascular graft. Current and future studies will examine cell responses to crosslinked, freestanding aECM films and continue to assess the suitability of aECM proteins for use in vascular implants.

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