Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biomaterials 24 (2003) 4245–4252
Biomaterials
www.elsevier.com/locate/biomaterials

Endothelial cell adhesion to the fibronectin CS5 domain in artificial extracellular matrix proteins

Sarah C. Heilshorn[a], Kathleen A. DiZio[b,1], Eric R. Welsh[b], David A. Tirrell[a,*]

[a] Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125, USA
[b] Department of Polymer Science and Engineering, University of Massachusetts, Amherst, Amherst, MA 01003, USA

Received 16 December 2002; accepted 8 April 2003

Abstract

This study examines the spreading and adhesion of human umbilical vein endothelial cells (HUVEC) on artificial extracellular matrix (aECM) proteins containing sequences derived from elastin and fibronectin. Three aECM variants were studied: aECM **1** contains lysine residues periodically spaced within the protein sequence and three repeats of the CS5 domain of fibronectin, aECM **2** contains periodically spaced lysines and three repeats of a scrambled CS5 sequence, and aECM **3** contains lysines at the protein termini and five CS5 repeats. Comparative cell binding and peptide inhibition assays confirm that the tetrapeptide sequence REDV is responsible for HUVEC adhesion to aECM proteins that contain the CS5 domain. Furthermore, more than 60% of adherent HUVEC were retained on aECM **1** after exposure to physiologically relevant shear stresses ($\leqslant 100$ dynes/cm^2). Finally, the levels of thrombogenic markers (tissue plasminogen activator and plasminogen activator inhibitor–1) secreted by HUVEC monolayers on aECM **1** were found to be similar to those secreted by HUVEC monolayers cultured on fibronectin. These characteristics, along with the physical strength and elasticity of crosslinked films prepared from these materials, make aECM proteins promising candidates for application in small-diameter vascular grafts.

Keywords: Cell adhesion; Endothelial cell; Extracellular matrix; Protein design; Vascular graft

1. Introduction

A promising new approach to engineered biomaterials involves the design and synthesis of polypeptides that mimic the essential properties of naturally occurring proteins. The fidelity and efficiency of bacterial production systems allow highly specialized artificial proteins to be obtained in good yield [1–3]. By this approach, we have prepared several artificial extracellular matrix (aECM) proteins with the objective of achieving the mechanical and biological properties needed for use in small-diameter vascular grafts [4,5]. Current polymeric materials used for small-diameter (i.e. smaller than 5 mm) vascular grafts fail rapidly due to occlusion upon implantation [6–8].

The aECM proteins examined in this work are shown in Fig. 1. Each includes elastin-like repeats comprising the pentapeptide VPGIG, interspersed with the CS5 domain of fibronectin (the CS5 sequence in aECM **2** is scrambled to provide a negative control substrate for cell-binding studies). The elastin-like sequences give these materials elasticity and mechanical integrity [5,9] while the CS5 domains provide cell adhesion signals [4]. Lysine residues were incorporated into each sequence, either within the elastin-like domains (aECM **1** and **2**) or near the C-and N-termini (aECM **3**), to allow site-specific crosslinking without interruption of the CS5 binding domains. A T7-tag leader sequence is included to increase expression levels and to aid in protein detection. A hexahistidine tag was incorporated into aECM **1** and **2** as an alternate method of purification along with an enzymatic cleavage site to remove the fusion sequences.

Mechanical integrity is an obvious design criterion for a successful vascular graft material. Crosslinked films of aECM **1**–**3** have been shown to exhibit Young's moduli

*Corresponding author. Tel.: +1-626-395-3140; fax: +1-626-793-8472.
E-mail address: tirrell@caltech.edu (D.A. Tirrell).
[1] Current address: Center for Bio/Molecular Science and Engineering, Naval Research Laboratory, Washington, DC 20375, USA.

0142-9612/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0142-9612(03)00294-1

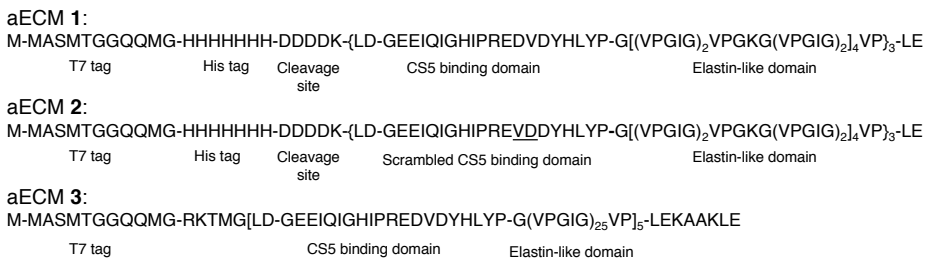

Fig. 1. Amino acid sequences of the aECM proteins. Each sequence contains a T7 tag to aid in protein expression and detection. aECM **1** and **2** contain a hexahistidine tag, an enzymatic cleavage site, and three cassettes of the CS5 binding domain interspersed with elastin-like repeats that contain lysine sites for crosslinking. In aECM **2**, the minimal recognition sequence within the CS5 binding domain has been scrambled to provide a negative control. aECM **3** contains five cassettes of the CS5 binding domain interspersed with twenty-five elastin-like repeats. These cassettes are flanked by lysine residues for site-specific crosslinking.

comparable to those of native elastin (0.3–0.6 MPa) ([5,9,10] and K. A. DiZio, P. J. Nowatzki, D. A. Tirrell, unpublished results). Furthermore, the mechanical properties can be controlled by manipulating the molecular weight between lysine residues [5], the extent of crosslinking, and the choice of crosslinking chemistry. These tuning parameters could be used to fabricate freestanding, implantable grafts of crosslinked aECM proteins with mechanical properties similar to those of the surrounding tissue. Current synthetic graft technology utilizes expanded poly(tetrafluoroethylene) and poly(ethylene terephthalate), both of which yield grafts characterized by low mechanical compliance. The mismatch in mechanical properties between these synthetic grafts and the host blood vessel is thought to contribute to smooth muscle cell migration into the graft and eventual graft failure [11].

A second design criterion for vascular graft materials is appropriate biological interaction with the host tissue. Endothelial cell adhesion remains an elusive goal in the engineering of all synthetic vascular grafts [12,13]. In healthy blood vessels, endothelial cells form a monolayer along the luminal surface of the tunica intima. The endothelium provides a non-thrombogenic surface for blood contact, and secretes several autocrine and paracrine molecules that regulate the vascular environment [14]. Current synthetic graft materials do not support re-establishment of an endothelial cell layer subsequent to implantation, and are subject to eventual thrombosis and loss of graft patency [6–8].

To encourage endothelial cell binding, the CS5 segment of fibronectin has been incorporated into the aECM proteins examined in this work. Previous studies have identified the CS5 segment, located within the alternatively spliced IIICS region of fibronectin, as a ligand for the $\alpha_4\beta_1$ integrin [15]. Integrin binding is attributed to the internal REDV peptide sequence [16]. The peptide GREDVY, when grafted to a glass substrate was shown to promote endothelial cell attachment while remaining non-adhesive to smooth muscle cells and platelets [17]. Although initial attempts to incorporate the REDV sequence into elastin-like polypeptides did not yield adhesive substrates [18], inclusion of the longer CS5 domain has afforded more positive results. Previous work on a related series of aECM proteins has shown that increasing the density of CS5 domains increases endothelial cell binding to the protein [4]. The goal of this work is to characterize the spreading and adhesion of endothelial cells on aECM proteins containing periodic CS5 domains to determine the suitability of these materials for use in small-diameter vascular grafts.

2. Materials and methods

2.1. Protein expression and purification

For aECM **1** and **2**, standard protocols for DNA manipulation, bacterial growth, protein expression, SDS-PAGE and Western blotting were used [19]. The requisite coding sequences were cloned into pET28 (Novagen, Madison, WI) and protein synthesis was induced under T7 promoter control in the expression host BL21(DE3)pLysS (Novagen). Expression was carried out in a 10 l Bioflow 3000 (New Brunswick, Edison, NJ) fermenter with Terrific Broth as the expression medium. A 400 ml overnight culture was used for inoculation of the fermentation medium. The pH was maintained at 7.2 and the temperature at 37°C. The culture was induced at an OD_{600} of 5–6 with 2.5 mM β-isopropyl thiogalactoside (CalBiochem, La Jolla, CA) and expression was allowed to continue for 2–3 h (final $OD_{600} = 13–18$). Cells were harvested by centrifugation to yield an average of 200 g wet cell mass per fermentation run. The wet cell mass was re-dispersed in TEN buffer at a concentration of 1 g/ml, frozen, and then defrosted at 4°C with addition of 1.0 ng/ml DNAse, 1.0 ng/ml RNAse, and 50 ng/ml PMSF. The solution was centrifuged (2 h, 35,000g, 4°C) to separate soluble and insoluble fractions. The supernatant was brought to pH 9 and 1 M NaCl at 4°C, and then allowed

to equilibrate to 37°C before another centrifugation (2 h, 35,000*g*, 37°C). This pellet was re-dispersed in distilled water, brought to pH 9, 1 M NaCl at 4°C and centrifuged (2 h, 35,000*g*, 4°C). The supernatant was then warmed to 37°C and centrifuged (2 h, 35,000*g*, 37°C). This process was repeated twice with the pellet from this step. The final pellet was re-dispersed in distilled water, dialyzed for 3 days at 4°C, and lyophilized to retain the purified product. Purity was assessed by SDS-PAGE, western blotting, amino acid analysis, and mass spectrometry. The expression and purification of aECM **1** were reported previously [5].

2.2. Lipopolysaccharide quantitation

The quantity of lipopolysaccharide (LPS), a gram-negative bacterial endotoxin, contaminating the final preparation of the aECM protein was determined using a *limulus* amebocyte lysate gel-clot assay (LAL, Associates of Cape Cod, Falmouth, MA).

2.3. Cell maintenance

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics and maintained in endothelial growth medium-2 (EGM-2, 2% serum, Clonetics, Walkersville, MD). Cells were kept in a humidified, 5% CO_2 environment at 37°C and passaged non-enzymatically using a 0.61 mM EDTA solution (Gibco, Grand Island, NY). Cells between passages 3 and 8 were used for all experiments.

2.4. Substrate preparation and characterization

An aECM protein solution, 1 mg/ml in phosphate buffered saline (PBS), was spread evenly on the experimental surface (i.e., glass slides, 96-well plates, or tissue culture-grade polystyrene Petri dishes). The protein was allowed to adsorb for 1 h at 4°C. The surfaces were then rinsed three times with PBS at 4°C. An anti-T7-tag antibody conjugated to horseradish peroxidase was utilized to confirm the homogeneity of protein adsorption. Fibronectin films were prepared in a similar fashion from a 10 μg/ml solution in PBS. Blocking of non-specific interactions was achieved by immersing substrates in a 0.2% bovine serum albumin (BSA) solution (in PBS), which had been heat treated at 85°C for 10 min. The BSA was allowed to adsorb for 30 min at room temperature before the surface was rinsed three times with PBS. BSA alone was used as a negative control substrate.

2.5. Phase contrast density slicing

Freshly harvested HUVEC at a cell density of 1×10^4 cells/cm^2 were plated on microscope slides coated with fibronectin or with one of the aECM proteins. Samples were incubated with serum-free EGM-2 at 37°C and 5% CO_2 for one hour. Samples were viewed using phase contrast microscopy and imaged at five locations using a CCD video camera (Sony, Model DXC-151A). Digital images were analyzed using NIH Image (public domain software available at http://rsb.info.nih.gov.nih-image/). Images were density sliced twice to differentiate cells that were refractive (i.e., not spread) from those that were dark in color (i.e., adherent). Five samples of each substrate were analyzed and each sample contained at least 100 cells.

2.6. Comparison and inhibition of endothelial cell binding to aECM proteins

All binding assays were carried out with HUVEC suspensions of 4×10^5 cells/ml in serum-free EGM-2. A colorimetric microassay on 96-well plates was adapted from previous studies [20]. Wells were coated as described above, and 0.1 ml of cell suspension was added to each well along with 0.1 ml serum-free EGM-2. For peptide inhibition assays, peptides were added directly to the wells at a final concentration of 1.7–2.9 mM. REDV, GREDVY, PREDVDY, GREDVDY, and GREVDDY peptides (>95% purity) were purchased from Commonwealth Biotechnologies (Richmond, VA). Following incubation at 37°C and 5% CO_2 for 30 min, the supernatant was removed, and the wells were rinsed three times with PBS at 37°C to remove non-adherent cells. Following fixation of the adherent cells with 70% ethanol for 10 min, the ethanol was aspirated and a 0.1% solution of crystal violet in distilled water was added to the cells. After staining for 25 min, the cells were rinsed five times with distilled water, and the crystal violet that was adsorbed on the adherent cells was solubilized with 0.2% Triton X-100. After 5 h of stain extraction, the optical density at 595 nm was measured on a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA). The method was validated by a control study in which cell-seeding density was linearly related to optical density. In each experiment, <5% of seeded HUVEC were adherent to the BSA negative control.

2.7. Cell detachment by shear stress

In order to examine the attachment strength of HUVEC to various substrates, a flow chamber was built based on the design by Usami and coworkers [21]. The induced flow field is based upon the low-Reynolds number, Newtonian-gap flow associated with Hele–Shaw cells. Based on this flow field, a linear shear stress

(τ_w) profile is created according to

$$\tau_w = \frac{6\mu Q}{h^2 w}\left(1 - \frac{z}{L}\right), \quad (1)$$

where Q is the flow rate, μ is the fluid viscosity, h is the gap width between the two plates, and the dimensions z, L, and w are the lengths along the z-axis, the total chamber, and the entrance width, respectively. The chamber geometry was validated by measuring the observed total pressure drop across the chamber using a manometer and relating to the theoretical pressure drop, Δp, as calculated from the experimental flow rate:

$$\Delta p = \frac{6\mu Q L}{h^3 w}. \quad (2)$$

The flow chamber was incorporated into an autoclavable, non-cytotoxic, and non-pyrogenic flow system. The system flow rate was controlled using a variable, multi-channel peristaltic pump, and the entire system was contained within a constant temperature environmental chamber at 37°C. The maximum flow rate employed was 7 ml/min, corresponding to a maximum shear stress of 200 dynes/cm^2. The flow chamber was mounted on the stage of an inverted microscope while a CCD video camera allowed recording of all events within the video field.

Substrates were prepared and HUVEC were harvested and seeded as described above. After incubation for 1 h, HUVEC-seeded microscope slides were mounted and secured in the flow chamber. At time zero, images were taken at 5-mm intervals along the z-axis of the chamber. HUVEC were then exposed to shear flow for 2 min. This interval was chosen on the basis of previously reported observations showing that the majority of detachment takes place in the first 30 s [22]. This observation was confirmed by monitoring HUVEC detachment levels during a period of 15 min of flow. After the flow was stopped, the z-axis was again imaged at 5-mm intervals. The initial attachment value is an absolute number obtained by counting the number of cells visible after loading the slide and prior to shear stress exposure. The percent detachment was found by subtracting the number of cells visible after flow from the initial attachment value and then normalizing to the initial attachment value. All flow conditions were tested in quadruplicate.

2.8. Secretion of tPA and PAI-1

Freshly harvested HUVEC were plated on clean polystyrene dishes and dishes coated with fibronectin or with one of the aECM proteins at a cell density of 1×10^4 cells/cm^2 and grown to confluence in EGM-2. Once the cells reached confluence, half of the dishes were challenged by the addition of LPS (serotype O55:B5, Sigma) to a final concentration of 10 μg/ml. Samples of media were taken from each dish at 1, 6, 12, 24, and 72 h. The levels of tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI-1) secreted by HUVEC were determined using ELISAs (American Bioproducts, Parsippany, NJ). Each culture condition was repeated in triplicate. Measurements are reported as mass/10^5 cells/min assuming a confluent monolayer contains 1×10^5 HUVEC/cm^2.

3. Results

3.1. Synthesis and analysis of aECM proteins

aECM **1** and **2** were both expressed at 0.3 g/l, while expression of aECM **3** consistently yielded about 0.6 g/l of protein. The purity of each protein was confirmed by SDS-PAGE, Western blotting, and mass spectrometry. Furthermore, the amino acid analysis of each sequence was within 1% of the expected value for each residue. Using LAL assays of several fermentation batches, LPS contamination was determined to be in the range of 0.065–0.115 EU/mg protein.

3.2. HUVEC adhesion to aECM proteins

Initial endothelial cell response to aECM **3** was probed using phase contrast density slicing as described in Section 2.5 After incubation on aECM **3** for 1 h, $91 \pm 5\%$ of HUVEC displayed spread morphologies as judged by phase contrast microscopy. This result was nearly identical to the fraction of cells ($94 \pm 1\%$) found to spread on fibronectin.

In order to test the hypothesis that cell adhesion to aECM **1** and aECM **3** is dependent on the CS5 domain, two comparative cell-binding assays were performed. First, we compared cell adhesion to aECM **1** and **2**, which contain authentic and scrambled CS5 sequences, respectively. After 30 min of incubation, the number of HUVEC adherent to aECM **1** was ca. seven-fold greater than the number adherent to aECM **2** (Fig. 2), confirming the role of the authentic cell-binding domain. Second, we examined inhibition of endothelial cell binding by soluble peptides containing the REDV sequence. Although several peptides were used as potential binding inhibitors to aECM **3**, only one, GREDVDY, resulted in significant inhibition (see Section 4). Because of passage-to-passage variability in overall cell adhesion, the data are presented as the number of cells adherent under a given set of conditions relative to the number of cells adherent to fibronectin from that same passage. After treatment with 1.8 mM GREDVDY, HUVEC adhesion to aECM **3** was reduced from $77 \pm 10\%$ to $2 \pm 1\%$, which corresponds to $97 \pm 1\%$ inhibition. No inhibition of adhesion to fibronectin was observed (Fig. 3). No inhibition of

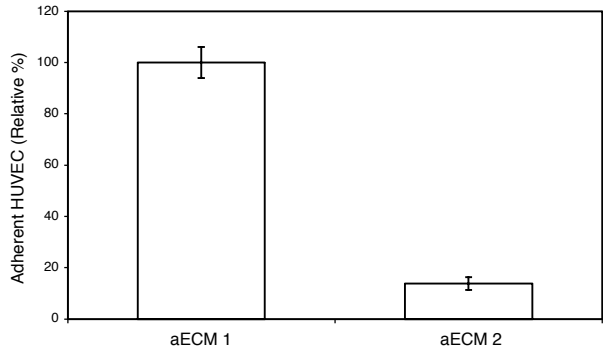

Fig. 2. Percentage of adherent HUVEC on aECM **1**, containing the REDV sequence, and aECM **2**, containing the REVD sequence, normalized to aECM **1** after blocking with BSA, incubation for 30 min, and three washes. Data represent one of four similar experiments. Error bars represent one standard deviation. Fewer than 5% of HUVEC adhered to the BSA negative control substrate in all experiments.

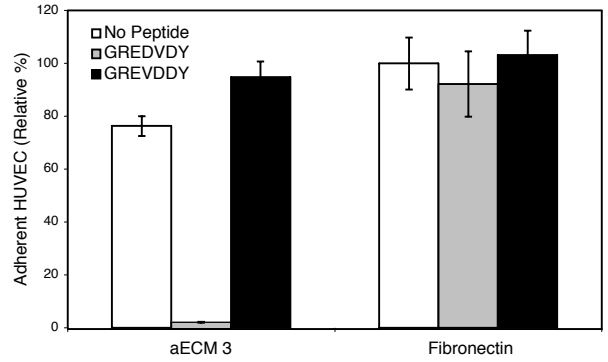

Fig. 3. Percentage of adherent HUVEC on fibronectin and aECM **3** relative to fibronectin in the absence and presence of competitive peptides (1.8 mM) after blocking with BSA, incubation for 30 min, and three washes. Data represent one of four similar experiments. Error bars represent one standard deviation. Fewer than 5% of HUVEC adhered to the BSA negative control substrate in all experiments.

adhesion on either substrate was observed upon treatment with the negative control peptide, GREVDDY.

3.3. HUVEC adhesion under physiologically relevant shear stresses

Parallel plate flow chamber studies were conducted to determine the shear stresses required to detach HUVEC cultured on various substrates. At shear stresses above 75 dynes/cm^2, more than 90% of HUVEC adherent to BSA were detached (Fig. 4). Below a shear stress of 100 dynes/cm^2, approximately 60% of HUVEC remained adherent to aECM **3**. Almost no HUVEC detachment from fibronectin was observed at shear stresses up to 150 dynes/cm^2.

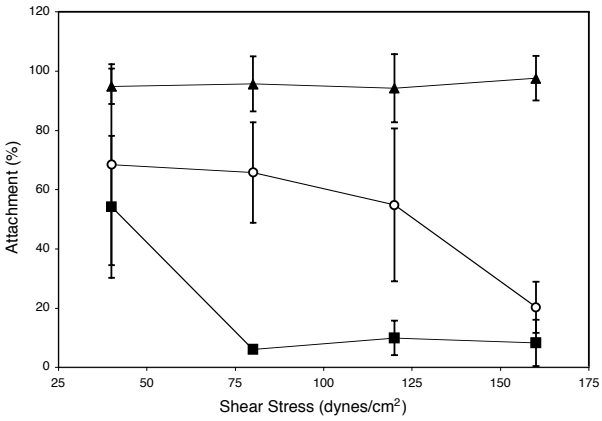

Fig. 4. Percentages of HUVEC on fibronectin (▲), aECM **3** (○), and BSA (■) that remain adherent after shear stress exposure for 2 min. Data represent averages of three experiments. Error bars represent one standard deviation.

3.4. Secretion of fibrinolytic regulators

HUVEC seeded on polystyrene, fibronectin, and aECM **3** at 1×10^4 cells/cm^2 required 4 days of proliferation to reach confluence; no differences in proliferation rates were observed for these substrates. The basal levels of tPA and PAI-1 secreted from HUVEC monolayers were determined by collecting medium samples over a span of 3 days. PAI-1 secretion by HUVEC monolayers on aECM **3** was found to be 0.17–0.90 ng/10^5 cells/min while the tPA range was 3.6–12.3 pg/10^5 cells/min. These secretion levels were similar to those observed for HUVEC cultured on polystyrene and on fibronectin (Table 1). Furthermore, these values are very similar to previously published basal secretion levels of HUVEC on collagen also reported in Table 1 [23]. In order to test the possibility of HUVEC activation due to LPS contamination of the aECM proteins, HUVEC monolayers were challenged with an amount of LPS known to increase PAI-1 secretion [24]. In all LPS challenge experiments, HUVEC monolayers on polystyrene in the absence of LPS were included as a control, and the secretion level in response to LPS is quantified as a fraction of that observed for the control sample. HUVEC monolayers on aECM **1** and on polystyrene showed approximately three-fold increases in PAI-1 secretion 72 h after LPS activation (Fig. 5). PAI-1 secretion by LPS-activated monolayers on fibronectin was similar (data not shown). No increase in PAI-1 secretion was detected at any time from HUVEC cultured on aECM substrates in the absence of added LPS (Fig. 5). As expected, secretion levels of tPA were not affected by LPS activation on any of the substrates [24] (data not shown).

Table 1
Secretion levels of tPA and PAI-1 by HUVEC monolayers on various substrates[a]

	tPA (pg/10^5 cells/min)	PAI-1 (ng/10^5 cells/min)
Tissue-culture polystyrene	4.3–8.5	0.20–0.94
Fibronectin	10.7–12.8	0.72–0.77
aECM **3**	3.6–12.3	0.17–0.90
Collagen	3.5–11.8[b]	0.21–0.56[b]

[a] Secretion of tPA and plasminogen activator inhibitor (PAI-1) by HUVEC monolayers on tissue-culture polystyrene, fibronectin, and aECM **3** were measured using ELISAs. Ranges represent the averages of several experiments using media collected from different passages of HUVEC and at various times between 1 and 72 h.

[b] Data for collagen substrates were taken from Iba et al. [23].

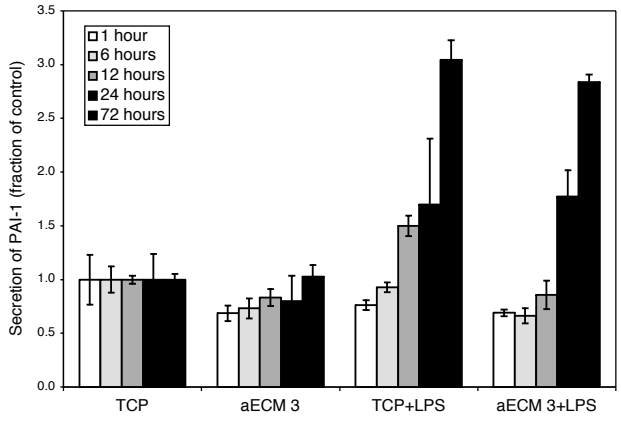

Fig. 5. PAI-1 secretion at 1, 6, 12, 24, and 72 h by HUVEC monolayers on tissue-culture polystyrene (TCP) and on aECM **3** in the absence and presence of LPS (10 μg/ml). Data are represented as fractions of the control, i.e., secretion from monolayers cultured on polystyrene without LPS activation, and are averages of three experiments. Error bars represent one standard deviation.

4. Discussion

The results of HUVEC culture on aECM proteins suggest that these materials exhibit several properties required for application in small-diameter vascular grafts. To examine the sequence-specific nature of HUVEC adhesion to aECM proteins, a series of competitive binding experiments was performed. Although four peptides were evaluated (see Section 2), only one, GREDVDY, was found to inhibit binding. These results suggest that short peptides containing the REDV minimal binding sequence may not be effectively recognized by endothelial cells, owing perhaps to their conformational preferences. This may explain why previous attempts to insert the REDV minimal binding sequence into elastin domains resulted in non-adhesive substrates [18]. Incorporation of the entire CS5 region into aECM proteins appears to allow the binding sequence to adopt a recognizable conformation. The specific nature of the cell binding is indicated by the fact that scrambling the REDV sequence (either within the aECM protein or in the putative inhibitory peptide) eliminates the observed effects of integrin recognition.

Considerable research has been devoted to the technology of seeding vascular grafts with endothelial cells prior to implantation [25–27]. However, the majority of seeded cells are washed away once blood flow is restored to the graft. Therefore, the ability of endothelial cells to withstand exposure to shear stress might enhance the viability of pre-seeded vascular grafts. Endothelial cell attachment strength to aECM **3** was sufficient at physiological shear stresses ($\leqslant 100$ dynes/cm^2) to retain more than 60% of cells adherent at zero shear. The mean wall shear stress in large arteries of uniform geometry is generally 20–40 dynes/cm^2 [28]. However, in regions of non-uniform geometry (branches and arches) transient shear stresses in excess of 100 dynes/cm^2 have been reported during episodes of increased blood flow [28–30]. The results shown here suggest that aECM proteins may enhance endothelial cell retention if used in pre-seeded vascular grafts. Ongoing studies address the ability of aECM proteins to support endothelial cell migration. If aECM proteins can effectively support migration, pre-seeding of grafts may not be required. Properly engineered materials might also recruit endothelial cells circulating within the vascular system [31,32].

Endothelial cells are important regulators of the vascular environment, affecting fibrinolysis, leukocyte migration, and smooth muscle cell contraction [33,34]. The fibrinolysis pathway controls the kinetics of blood clot formation and dissolution through the activation of plasmin, a potent protease with wide substrate specificity. Plasmin activity must be strictly controlled by plasminogen activators and inhibitors, including tPA and PAI-1 [35]. The primary source of tPA in vivo is secretion by endothelial cells. Because the basal and activated levels of tPA and PAI-1 secretion by HUVEC on aECM proteins are similar to those on fibronectin, it is reasonable to anticipate that endothelial cells cultured on such substrates will retain their fibrinolytic characteristics. Furthermore, HUVEC monolayers cultured on aECM proteins do not exhibit an LPS-activated phenotype, as evidenced by their failure to show the increase in PAI-1 secretion characteristic of LPS-challenged endothelial cells [21]. This is further evidence that the low levels of LPS in aECM proteins do not prohibitively affect HUVEC phenotype.

Direct determination of LPS in purified aECM proteins revealed residual low levels of endotoxin in the range 0.065–0.115 EU/mg. Because the FDA limit on LPS for vascular grafts is specified in terms of the amount of LPS in a water-rinse of the device, it is difficult to determine the maximum implantable mass based on measurements on the soluble protein.

However, a conservative estimate can be made on the basis of the FDA limit (5 EU/kg/min) for drugs, which allows administration of 350 EU of LPS, assuming a body mass of 70 kg [36]. Given the measured levels of LPS in purified samples of aECM **1**–**3**, the FDA limit would allow implantation of gram quantities of such polymers, quantities adequate for fabrication of small-diameter vascular grafts composed entirely of free-standing, crosslinked aECM proteins. Further analysis of crosslinked aECM films and devices will be required to verify this assertion.

Elastin-based materials containing GVGVP repeats have been subjected to extensive in vivo testing and have performed well in assays of mutagenicity, toxicity, antigenicity, pyrogenicity, and thrombogenicity [37]. Furthermore, GVGVP coatings on silicone rubber have been shown to elicit low cytokine secretion from monocytes and to reduce fibrous encapsulation by 33% compared to silicone rubber alone [38]. These results suggest that aECM proteins may exhibit desirable biocompatibility characteristics in vivo. This hypothesis is being explored in ongoing in vivo studies.

5. Conclusion

A modular design approach to artificial ECM proteins has been successfully demonstrated. The proteins examined here exhibit properties of elastin and fibronectin, the two natural ECM proteins upon which the sequences were based. This approach to protein engineering should yield not only novel biomaterials for use in implantable devices as demonstrated here, but also tissue engineering scaffolds and well characterized substrates for basic research in cell biology.

Acknowledgements

We thank Paul Nowatzki for expression and purification of aECM **3**, Krystle Wang and Summer Zhang for help with the lipopolysaccharide assays, Sarah Wilhoit and Michelle Giron for help with the competitive inhibition binding assays, and Julie Liu for helpful discussions. Funding was provided by the National Institutes of Health and the National Science Foundation.

References

[1] Krejchi MT, Atkins EDT, Waddon AJ, Fournier MJ, Mason TL, Tirrell DA. Chemical sequence control of β-sheet assembly in macromolecular crystals of periodic polypeptides. Science 1994;265:1427–32.

[2] Petka WA, Harden JL, McGrath KP, Wirtz D, Tirrell DA. Reversible hydrogels from self-assembling artificial proteins. Science 1998;281:389–92.

[3] Yu SJM, Conticello VP, Zhang GH, Kayser C, Fournier MJ, Mason TL, Tirrell DA. Smectic ordering in solutions and films of a rod-like polymer owing to monodispersity of chain length. Nature 1997;389:167–70.

[4] Panitch A, Yamaoka T, Fournier MJ, Mason TL, Tirrell DA. Design and biosynthesis of elastin-like artificial extracellular matrix proteins containing periodically spaced fibronectin CS5 domains. Macromolecules 1999;32:1701–3.

[5] Welsh ER, Tirrell DA. Engineering the extracellular matrix: a novel approach to polymeric biomaterials. I. Control of the physical properties of artificial protein matrices designed to support adhesion of vascular endothelial cells. Biomacromolecules 2000;1:23–30.

[6] Bos GW, Poot AA, Beugeling T, van Aken WG, Feijen J. Small-diameter vascular graft prostheses: current status. Arch Physiol Biochem 1998;106:100–15.

[7] Conte MS. The ideal small arterial substitute: a search for the Holy Grail? FASEB J 1998;12:43–5.

[8] Nerem RM, Seliktar D. Vascular tissue engineering. Annu Rev Biomed Eng 2001;3:225–43.

[9] Urry DW, Prasad KU, Long MH, Harris RD. Elastomeric polypeptides as potential vascular prosthetic materials. Abstr Pap Am Chem Soc 1988;196:143–PMSE.

[10] Abbott WM, Cambria RP. Biologic and synthetic vascular prostheses. New York: Gurne & Stratton, 1982. p. 189–220.

[11] Kobashi T, Matsuda T. Fabrication of compliant hybrid grafts supported with elastomeric meshes. Cell Transplant 1999;8:477–88.

[12] Eberhart A, Zhang Z, Guidoin R, Laroche G, Guay L, De la Faye D, Batt M, King MW. A new generation of polyurethane vascular prostheses: Rara avis or ignis fatuus? J Biomed Mater Res 1999;48:546–58.

[13] Meinhart JG, Deutsch M, Fischlein T, Howanietz N, Froschl A, Zilla P. Clinical autologous in vitro endothelialization of 153 infrainguinal ePTFE grafts. Ann Thorac Surg 2001;71:S327–31.

[14] Ross R. The pathogenesis of atherosclerosis: perspective for the 1990s. Science 1993;362:801–9.

[15] Humphries MJ, Akiyama SK, Komoriya A, Olden K, Yamada KM. Identification of an alternatively spliced site in human-plasma fibronectin that mediates cell type-specific adhesion. J Cell Biol 1986;103:2637–47.

[16] Mould AP, Komoriya A, Yamada KM, Humphries MJ. The CS5 peptide is a 2nd site in the IIICS region of fibronectin recognized by the integrin $\alpha_4\beta_1$-inhibition of $\alpha_4\beta_1$ function by RGD peptide homologs. J Biol Chem 1991;266:3579–85.

[17] Massia SP, Hubbell JA. Vascular endothelial-cell adhesion and spreading promoted by the peptide REDV of the IIICS region of plasma fibronectin is mediated by integrin $\alpha_4\beta_1$. J Biol Chem 1992;267:14019–26.

[18] Nicol A, Gowda DC, Parker TM, Urry DW. Cell adhesive properties of bioelastic materials containing cell attachment sequences. In: Gebelein CG, Carraher CE, editors. Biotechnology and bioactive polymers. New York: Plenum Press; 1994.

[19] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Laboratory Press; 1989.

[20] Aumailley M, Mann K, von der Mark H, Timple R. Cell attachment properties of collagen type-VI and Arg–Gly–Asp dependent binding to its alpha-2(VI) and alpha-3(VI) chains. Exp Cell Res 1989;181:463–74.

[21] Usami S, Chen HH, Zhao YH, Chien S, Skalak R. Design and construction of a linear shear-stress flow chamber. Ann Biomed Eng 1993;21:77–83.

[22] Powers MJ, Rodriguez RE, Griffith LG. Cell-substratum adhesion strength as a determinant of hepatocyte aggregate morphology. Biotechnol Bioeng 1997;53:415–26.
[23] Iba T, Shin T, Sonoda T, Rosales O, Sumpio BE. Stimulation of endothelial secretion of tissue-type plasminogen-activator by repetitive stretch. J Surg Res 1992;50:457–60.
[24] Orbe J, Chorda C, Montes R, Paramo JA. Changes in the fibrinolytic components of cultured human umbilical vein endothelial cells induced by endotoxin, tumor necrosis factor-alpha and interleukin-1 alpha. Haematologica 1999;84: 306–11.
[25] Bordenave L, Remy-Zolghadri M, Fernandez PH, Bareille R, Midy D. Clinical performance of vascular grafts lined with endothelial cells. Endothelium 1999;6:267–75.
[26] Vinard E, Leseche G, Andreassian B, Costagliola D. In vitro endothelialization of PTFE vascular grafts: a comparison of various substrates, cell densities, and incubation times. Ann Vasc Surg 1999;13:141–50.
[27] Fernandez P, Bareille R, Conrad V, Midy D, Bordenave L. Evaluation of an in vitro endothelialized vascular graft under pulsatile shear stress with a novel radiolabeling procedure. Biomaterials 2001;22:649–58.
[28] Davies PF. Flow-mediated endothelial mechanotransduction. Physiol Rev 1995;75:519–60.
[29] Dewey CF. Dynamics of arterial flow. Adv Exp Med Biol 1979;115:55–89.
[30] Nerem RM, Harrison DG, Taylor WR, Alexander RW. Hemodynamics and vascular endothelial biology. J Cardiovasc Pharmacol 1993;21:S6–10.
[31] Dignat-George F, Sampol J. Circulating endothelial cells in vascular disorders: new insights into an old concept. Eur J Haematol 2000;65:215–20.
[32] Kaushal S, Amiel G, Guleserian KJ, Tjorvi PE, Soker S, Roth SJ, Atala A, Bischoff J, Mayer JE. Circulating endothelial cells for tissue-engineered small diameter vascular grafts. Circulation 2000;102:II-766,3698.
[33] Carmeliet P, Collen D. Molecular analysis of blood vessel formation and disease. Am J Physiol-Heart Circul Physiol 1997;42:H2091–104.
[34] Purcell C, Tennant M, McGeachie J. Neo-intimal hyperplasia in vascular grafts and its implications for autologous arterial grafting. Ann R Coll Surg Engl 1997;79:164–8.
[35] Irigoyen JP, Munoz-Canoves P, Montero L, Koziczak M, Nagamine Y. The plasminogen activator system: biology and regulation. Cell Mol Life Sci 1999;56:104–32.
[36] Food and Drug Administration (1987). Guideline on validation of the limulus amebocyte lysate test as an end-product test for human, animal parenteral drugs, biologcal products, and medical devices. Rockville, MD: US Department of Health and Human Services.
[37] Urry DW, Parker TM, Reid MC, Gowda DC. Biocompatibility of the bioelastic materials, poly(GVGVP) and its γ-irradiation cross-linked matrix: summary of generic biological test results. J Bioac Comp Poly 1992;6:263–82.
[38] DeFife KM, Shive MS, Hagen KM, Clapper DL, Anderson JM. Effects of photochemically immobilized polymer coatings on protein adsorption, cell adhesion, and the foreign body reaction to silicone rubber. J Biomed Mater Res 1999;44:298–307.