Using avidin-mediated binding to enhance initial endothelial cell attachment and spreading

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Abstract: Binding between the protein avidin and the vitamin biotin was used as an extrinsic, high affinity receptor–ligand system to augment the intrinsic integrin-dependent cellular adhesion mechanism. Glass substrates were coupled with avidin receptors through an adsorbed film of biotinylated bovine serum albumin (b-BSA). The avidin-treated slides then were seeded with biotinylated bovine aortic endothelial cells (BAEC). A 3:1 ratio of BSA:b-BSA provided the best results in terms of specific cellular attachment, growth, and spreading. Control surfaces consisted of bare glass or glass with adsorbed BSA. Attachment of unmodified BAEC to glass decreased in the presence of anti-β1 integrin antibody. Adhesion of biotinylated BAEC to avidin-treated slides was not affected by anti-β1 integrin antibody, consistent with integrin-independent avidin-mediated adhesion. The initial rate of cell spreading was greatest for avidin–biotin-mediated adhesion (80.0 ± 25.6 $\mu m^2/h$), followed by integrin-dependent cellular adhesion on plain glass (35.7 ± 7.7 $\mu m^2/h$) and, finally, by adhesion on BSA-coated protein surfaces (10.2 ± 0.3 $\mu m^2/h$). Biotinylated and unmodified BAEC, cultured for 1 h in serum-containing media, were subjected to laminar flow in a variable-height flow chamber that provided a range of shear stresses from 0.2 to 75 dynes/cm^2. The critical shear stress required to detach 50% of the cells in serum-containing media increased from 4.6 ± 0.8 dynes/cm^2 for integrin-dependent adhesion to 12.6 ± 1.2 dynes/cm^2 for avidin–biotin-mediated adhesion. Avidin-mediated attachment for biotinylated BAEC increased initial cellular spreading rates and strength of attachment (i.e., at 1 h) by a factor of two and three, respectively. These results support the hypothesis that integrin-mediated cell attachment and spreading can be enhanced using high affinity integrin-independent binding. © 1998 John Wiley & Sons, Inc. *J Biomed Mater Res,* **40,** 57–65, 1998.

Key words: cell adhesion; avidin–biotin; endothelialization; vascular grafts

INTRODUCTION

Synthetic vascular graft endothelialization is recognized as one of the most important unsolved problems in the use of cardiovascular biomaterials.[1,2] Spontaneous endothelialization of synthetic graft surfaces does not occur in humans;[3] hence, seeding of grafts with host endothelial cells (EC) prior to implantation is critical to increasing the patency of small-diameter vascular grafts. EC seeding has increased graft patency in various *in vitro* studies,[4–6] animal models,[7,8] and clinical trials.[9,10] EC seeding also confers greater resistance to bacterial adherence and graft infection.[11]

The majority of work on promoting EC attachment and growth to synthetic materials has involved the modification of surfaces with a single ligand. Table I summarizes the recent work on surface modification with ligands for the enhancement of EC adhesion. Preadsorption of grafts with cell adhesion proteins, such as fibronectin, vitronectin, and fibrinogen, or the incubating of a graft with a recipient's plasma improves cellular adhesion on biomaterial surfaces.[4,12–15] The cell attachment activity of these proteins is due largely to the amino acid sequence Arg-Gly-Asp (RGD), which serves as a binding site for specific integrins, a family of transmembrane heterodimers located on the EC surface.[16,17] Peptide sequences such as RGD (of fibronectin), YIGSR (of laminin), and RGDV (of vitronectin) also have been successfully grafted onto polymer surfaces and shown to increase cell adhesion and cell spreading.[18–23] Other nonligand-based techniques, such as carbon deposition and plasma dis-

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TABLE I
Relevant Studies of EC Adhesion and Growth Enhancement By Surface Modifications

Surface Modification (Experimental Condition)	Surface	Effect	Ref.
Single Ligand Treatment			
FN adsorption (*in vitro*)	TCPS, PS, FEP, PMMA, HEMA/EMA, ePTFE, PET	Increased attachment kinetics and cell adhesion; no significant increase in attachment kinetics on PET; did not prevent initial detachment on ePTFE but cell adhesion better than with blood	[4–6, 37, 47]
FN adsorbed ePTFE & gelatin impregnated-Dacron (*in vitro*)	ePTFE, PET	EC layer resists physiologically relevant shear	[48]
FN adsorption & preclot (*in vitro*)	ePTFE	Increased shear resistance (preclot > FN)	[31]
COL I/III adsorption (*in vitro*)	PU	Faster initial adhesion but comparable proliferation to uncoated surface	[49]
Covalently attached peptide sequences (RGD and YIGSR, (*in vitro*)	PET, PTFE	Increased cell adhesion	[19]
Covalently attached peptide sequence (GREDVY, *in vitro*)	Glycophase glass, PEG-modified PET	Selective endothelialization; peptide does not support other cells and platelets	[50]
Covalent peptide (GRGDSY) coupling (*in vitro*)	PU	Increased cell attachment	[44]
Covalently attached cyclic RGD, linear RGD, and adsorbed FN (*in vitro*)	Glass	Increased strength of attachment, cyclic RGD > linear RGD	[51]
Multiple ligand Treatment			
Pressure perfusion of modified fibrin glue (*in vivo*)	ePTFE	Endothelialized lumina and capillary ingrowth	[7]
Coating with FN and ECM (*in vitro*)	ePTFE	Enhanced cellular adhesion and coverage	[52]
Coating with Transglutine a biological glue (*in vitro*)	PET, ePTFE	Increase in adhesion, doubling time, and density	[27]
COL I + FN/laminin	ePTFE	Better cell adhesion on multiple proteins	[26]
Photochemical covalent attachment of FN + IV COL (*in vivo*)	PU, ePTFE	Increased cell adhesion and growth as compared to FN or IV COL separately	[8, 28]

COL: collagen; ePTFE: expanded polytetrafluroethylene; FEP: fluorinated ethylene propylene copolymer; FN: fibronectin; HEMA/EMA: copolymer of polyhydroxyethylmethacrylate and hydroxyethylmethacrylate; PET: polyethyleneterephthalate; PMMA: polymethyl methacrylate; PTFE: polytetrafluroethylene; PU: polyurethane; SPU: segmented polyurethane; TCPS: tissue culture polystyrene.

charge on polymer surfaces, also have been investigated for improving cellular adhesion.[5,24,25]

Cell attachment *in vivo* involves the interplay of multiple receptors, and a few studies have investigated a multicomponent approach (Table I). Combinations of proteins and peptide sequences have been used for promoting rapid and stable cell adhesion. Kaehler et al.[26] sequentially coated 6 mm expanded polytetrafluoroethylene (ePFTE) grafts with collagen type I and then with either fibronectin or laminin followed by seeding with human umbilical vein endothelial cells (HUVEC). EC adhesion was better on grafts treated with multiple proteins than on grafts treated with fibronectin or laminin alone. Others have attempted to enhance EC adhesion through the use of ''fibrin glues,'' for example Transglutine®, which is a mixture of fibrinogen with fibronectin and von Willebrand factor.[27] Cell adhesion to fibrin glue-coated ePTFE and tissue culture polystyrene produced comparable confluent cultures in terms of high cell density.[27]

Unitl now the majority of multiple ligand-treated surfaces, in spite of consisting of a mixture of different proteins, used integrin-mediated cell adhesion systems. Combining integrin-dependent and integrin-independent adhesion mechanisms to enhance cell adhesion first was put to practice by Amos et al.,[28] who photoimmobilized polyurethane and ePTFE vascular grafts with a mixture of fibronectin and type IV collagen. This treatment produced surfaces that contained both the integrin-dependent RGD sequence in fibronectin and the integrin-independent CB 3 fragment of collagen type IV,[29] both of which promote EC adhesion. Compared to grafts coated with fibronectin and

type IV collagen alone, grafts coated with multiple proteins showed two to three times greater patency rates and luminal endothelial coverage when implanted in dogs for 30 days.[28] From results of *in vitro* EC cultures on glass, Basson et al.[30] hypothesized that integrin-independent interactions of cells with laminin create higher affinity contacts ($K_d \approx nM$) that provide stability for long-term attachment whereas lower affinity integrin-dependent contacts ($K_d \approx \mu M$) are important for transient interactions, such as initial cell adhesion, spreading, and cell motility.

Ligand-based surface modifications have greatly improved cell attachment and spreading *in vitro*. However, under *in vivo* conditions, restoration of blood flow in treated grafts leads to a significant amount of initial cell detachment, although less than untreated grafts. For example, Ramalanjaona et al.[4] seeded ePFTE vascular grafts with and without fibronectin precoating and then implanted them in the carotid arteries of dogs. In both cases, 70% of the seeded cells were lost within the first 30 min. At 24 h postimplantation, 21% of cells had been retained on the fibronectin-coated graft as compared to 4% of cells on the uncoated grafts. Vohra et al.[31] and Miyata et al.[32] examined *in vitro* EC retention to fibronectin coated and then seeded ePTFE grafts under flow conditions. After 2 h of exposure to flow, with maximum shear stresses of 2.6 dynes/cm^2, 60% of the seeded EC was retained on both fibronectin precoated and preclotted grafts whereas the preclotted grafts exhibited better EC retention than did the fibronectin surface at lower shear stresses.[31] Miyata et al.[32] observed that EC seeded to fibronectin precoated grafts for 24 h and then exposed to pulsatile flow were retained at 92%. However, for a shorter seeding time of 1.5 h, only 42% of the EC were retained.

To ensure rapid and strong initial attachment within a short time period of 1–1.5 h, it may not be sufficient to rely on a single integrin-mediated protein or peptide sequence. A more practical approach to promoting cellular adhesion would be to address separately the two steps of cell adhesion: cell attachment and cell spreading. The approach described in this paper was to (1) introduce high affinity, integrin-independent binding for specific, rapid, and firm initial attachment of the cell membrane to the substrate that (2) augments the existing lower affinity integrin-dependent binding necessary for cell spreading and actin filament assembly. The avidin–biotin complex offered an excellent model for the extrinsic ligand owing to its being highly characterized, highly specific, and one of the strongest binding affinities ever observed for a noncovalent bioreceptor–ligand interaction ($K_d = 10^{-15}\ M^{-1}$).[33]

Avidin-mediated binding of biotinylated cells has been shown to convert nonadhesive mouse ascite carcinoma cells to anchorage-dependent cells.[34,35] These biotinylated cells attached and began spreading within 30 min, attaining epithelial-like morphology after 1.5–2 h with wide lamella, focal contacts, and circular actin bundles. Because the process of spreading could be blocked by metabolic inhibitors and low temperature, it thus was determined to be energy dependent. These papers showed that simple, high affinity interaction between a biotinylated plasma membrane and an avidin-coated substrate can simulate the role played by integrins or other adhesion proteins in cell attachment and cytoskeleton rearrangement.

The current paper investigates the initial cell attachment and spreading of biotinylated bovine aortic endothelial cells (BAEC) on avidin-treated glass surfaces. Compared to integrin-mediated attachment of unmodified BAEC, results show initial cellular spreading rates and strength of attachment (i.e., at 1 h) for biotinylated BAEC were increased by a factor of two and three, respectively.

MATERIALS AND METHODS

Biotinylation of EC and glass slides

Flasks of confluent BAEC were washed three times with phosphate-buffered saline (PBS), pH 7.2, treated with 1 m*M* sulfosuccinimidyl-6-(biotinamido)hexanoate (NHS-LC-biotin) solution (Pierce, Rockford, IL), 0.557 mg/mL in PBS, pH 7.2) and incubated for 30 min,[36] washed again with PBS, and detached using trypsin.

Standard 1'' × 3'' × 1 mm soda lime glass microscope slides (Goldseal, Clay Adams) were sonicated in 5% PCC-54 cleaning solution (Pierce, Rockford, IL) for 2 h, rinsed with copious amounts of distilled–deionized water (Milli Q, Millipore, Inc.), and dried overnight at 150°C. The slide was treated with ethyl alcohol for 0.5 h, washed clean, and treated with 2 mg/mL of biotinylated bovine serum albumin (BSA-b) in PBS for 1 h.

Avidin–biotin-mediated attachment

A flask of confluent BAEC was biotinylated, washed with PBS, and then trypsinized. Clean glass slides were treated with a mixture of BSA:BSA-biotin (final concentration 2 mg/mL) with ratios of 1:0 (all BSA), 1:3, 1:1, 3:1, or 0:1 (all BSA–biotin) and then with 0.5 mg/mL of avidin in PBS for 40 min. The biotinylated cells were suspended in DMEM with 10% fetal bovine serum (FBS) and seeded on the treated glass slides.

Antisera adhesion-blocking assay

A protocol described by Massia and Hubbell[21] was used, with minor modifications, for this assay. Clean 4-well glass

slides were treated with DMEM (+ serum) for 1 h at 4°C. Biotinylated BAEC from confluent flasks were detached using 2 m*M* of EDTA in Ca^{2+}- and Mg^{2+}-free PBS. These cells were resuspended in DMEM (+ serum) with different concentrations of anti-human integrin β1 (Upstate Biotechnology Inc., NY) for 30 min at 4°C. The cells in each well were counted and then incubated for 2 h at 37°C in 5% CO_2. The cells then were washed gently with PBS, unattached cells aspirated, and the remaining attached and spread cells (flattened and polygonal or flattened with pseudopodia) were counted. As a control, BAECs were seeded on DMEM (+ serum) treated glass slides with different concentrations of anti-integrin antibody. The fractions of both attached and spread cells were calculated and compared using a one way analysis of variance (Instat 2.0, GraphPad Software).

Cellular spreading measurements

Biotinylated glass slides treated with avidin were seeded with biotinylated BAEC in DMEM with 10% serum, as described above. A plain clean glass slide and a BSA-treated slide then were seeded with BAEC in DMEM with 10% serum and incubated.

The cells were examined using phase contrast microscopy with a 20× objective. A camera attached to the microscope was connected to a Macintosh IIx computer, and images were taken at regular intervals of 2, 5, 16, and 24 h. A total of 100 cells were followed at each time point for each of the three experiments. The projected area from the images of the cells was determined using the software Image 1.43 (NIH, Washington, DC).

Flow experiments

BAEC were biotinylated and seeded on avidin–biotin-treated glass slides, as described above. BAEC seeded on a plain glass slide served as a control. All experiments were carried out separately in media with 10% FBS and in media without serum. Cells were maintained in an incubator at 37°C with an atmosphere of 5% CO_2 and 95% air for 1 h for both sets of experiments.

The slides were placed in a variable height flow chamber and cell detachment was measured as described elsewhere.[37] The flow cell consisted of a lucite upper plate (7 × 2 × 3/4 in) with inlet and outlet headers and aluminum lower plate (7 × 2 × 3/16 in). The flow path was formed by medical-grade silicone rubber gaskets (0.02 in Dow–Corning Corp.) inserted between the two plates. Glass slides seeded with cells were placed on a 0.02 in thick silicone rubber gasket located in a recess within the lower plate. A 2.75 × 0.75 in recess was milled out beneath the spot where the glass slide would rest on the lower plate. The flow chamber was held together by 10 wing-nut screws. The flow chamber was mounted on the stage of an inverted phase contrast microscope (Nikon Diaphot). Cells were observed by a video camera (Series 70, Dage–MTI Inc.) attached to the microscope and connected to a Macintosh IIx computer and analyzed using NIH Image Software.

Prior to exposure of flow, five fields in the horizontal direction were counted per specified location along the slide at an objective magnification of 20×. A 100 mL syringe mounted on a calibrated pump was attached to the inlet port of the chamber and flow started. At the end of the experiment, five fields of cells were counted in the horizontal direction per the same specified location along the slide. Finally, the percentage of cells attached was calculated per applied shear stress.

For one-dimensional laminar flow, the wall shear stress τ_w (dyne/cm^2) is related to the volumetric flow rate Q (cm^3/s):

$$\tau_w = \frac{6\mu Q}{wH(x)^2}$$

where μ is the media viscosity, w is the width of the flow channel, and H(x) is the height of the flow chamber as a function of position along the microscope slide.[38] The applied τ_w ranged from 0.2 dynes/cm^2 to 75 dynes/cm^2.

Statistical analysis of flow experiments

Analysis of the data was performed as described before.[39] Briefly, the variation in attachment strength among the cell population was modeled assuming that the adhesive stresses fit a log-normal distribution.[40] The fraction of population detached per unit area at a given shear stress τ_w and exposure time equals the integral of the probability distribution of adhesive stresses. The model was fit to the data using nonlinear regression.[41]

RESULTS

Avidin-Biotin-mediated adhesion

BAEC were biotinylated using water-soluble chain-extended anhydroxy succinimide ester-derivatized biotin. The ester binds to the amino group of an accessible lysine or arginine in the plasma membrane by forming amide bonds. The membrane attachment of biotin moiety was detected by treating the modified cells with fluoroscently labeled FITC–avidin and observing the labeled cell membrane under epifluorescence microscopy. Biotinylated BAEC took up the fluoroscently labeled dye whereas unmodified cells did not. Biotinylated glass slides also were treated with FITC–avidin and observed under epifluorescence microscopy to confirm biotinylation.

Biotinylated cells attached and spread on the avidin-treated biotinylated glass slides. Involvement of avidin–biotin interactions in BAEC attachment was indicated in control experiments with nonmodified BAEC. Glass slides coated with biotinylated BSA, with

and without avidin treatment, were seeded with nonmodified BAEC. The nonmodified cells showed very little or no attachment and growth on either of these surfaces. In addition, biotinylated BAEC did not attach on BSA-biotinylated surfaces in the absence of avidin.

An avidin-mediated attachment sequence is illustrated in the inset of Figure 1. Cell attachment and spreading as a function of biotinylated-BSA density (Fig. 1) shows that the maximum cellular spread area and highest density of spread cells ($p < 0.001$) were obtained with cells plated on the 3:1 mixture of BSA:BSA–biotin. The density of spread cells was 4–6-fold higher than that obtained with slides coated with BSA–biotin. This suggests that high densities of avidin binding sites inhibited cell attachment and spreading. Elsewhere it was shown that high densities of avidin receptors at surfaces inhibit the binding of biotin.[42] The use of a mixed monolayer provided sufficient spacing of avidin receptors to promote specific cell attachment and to prevent nonspecific adhesion of biotinylated cells as BSA provides a nonadhesive protein surface.[43]

Blocking integrin-mediated attachment

The role of integrin-mediated adhesion was tested by titrating antisera against the fibronectin receptor (anti-integrin β_1) of biotinylated cells (Fig. 2). Unmodified BAEC were used as controls. With increasing concentration of the antibody, unmodified cells showed a statistically significant decrease in both cell attachment ($p < 0.03$) and cell spreading [$p < 0.001$, Fig. 2(a)]. In contrast, biotinylated BAEC titrated with increasing concentration of integrin-blocking antibody showed no significant decrease in cell attachment but showed considerable decrease in cell spreading [$p < 0.01$, Fig. 2(b)]. Cell attachment of biotinylated BAEC with their integrins blocked suggested avidin–biotin interactions were responsible for the attachment mechanism.

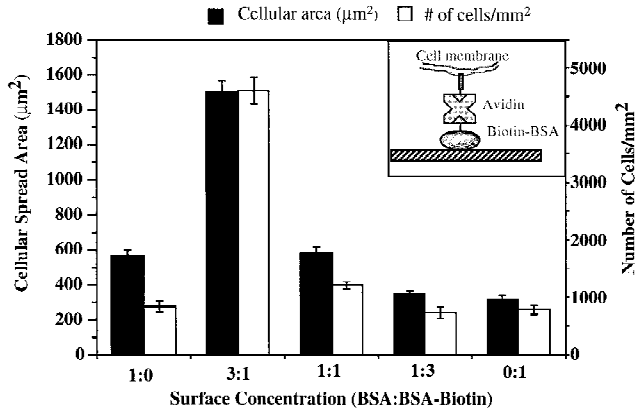

Figure 1. Effect of BSA:BSA–biotin ratio on the attachment of biotinylated BAEC in media with 10% FBS. Cell area and density of spread cells increased significantly ($p < 0.001$) for surfaces coated with 3:1 BSA:BSA–biotin. Data from three experiments were pooled and cell area was measured for a total of 100 cells. The error bars represent the SEM. Inset is an illustration of avidin–biotin-mediated adhesion scheme.

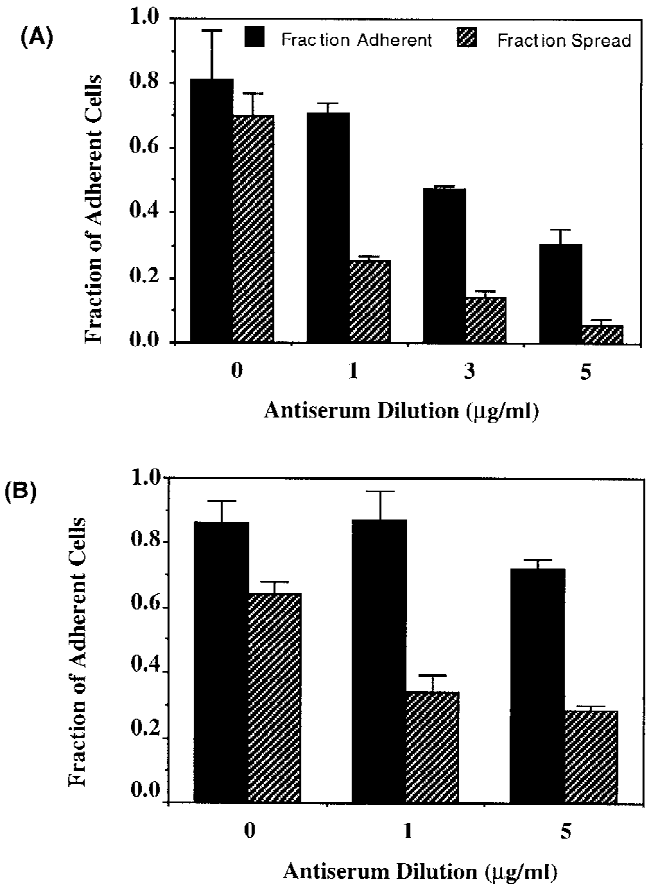

Figure 2. Antisera adhesion blocking assay on cells seeded for 1 h in media with 10% FBS: (A) initial cell attachment ($p < 0.03$) and spreading ($p < 0.001$) of BAEC on plain glass shows a statistically significant decrease with increasing concentration of anti-integrin antibody; (B) cell attachment mediated by avidin–biotin complex shows no statistically significant change, but cell spreading shows a significant decrease ($p < 0.01$).

Cellular spreading rates

The spreading of unmodified BAEC on plain glass and on BSA-coated glass, and biotinylated BAEC on avidin-treated, biotinylated BSA-coated glass were used to assess the effect of avidin-mediated adhesion on cell spreading. After 24 h, the mean cell size of unmodified cells on plain glass and biotinylated cells on avidin were not significantly different (Fig. 3). This meant that biotinylated cells could reach their fully spread states and attain confluency, as observed, with their counterpart unmodified cells. In contrast, nonad-

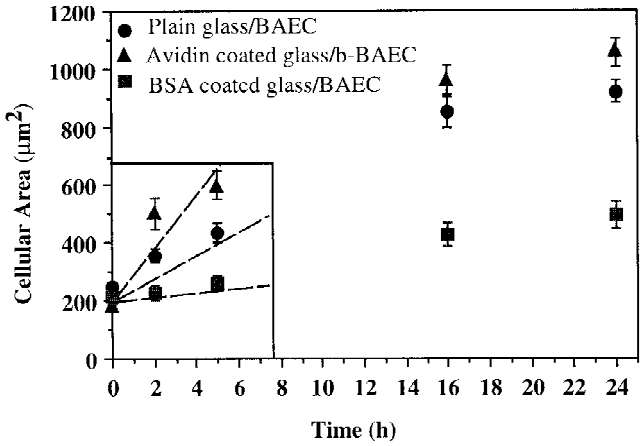

Figure 3. Spreading of biotinylated BAEC on avidin–biotin-treated glass slide and nonmodified BAEC on plain and BSA-coated glass slides in media with 10% FBS. The slope of the dotted lines give the initial spreading rates for avidin–biotin-mediated adhesion (80.0 ± 25.6 $\mu m^2/h$) and integrin-mediated adhesion on plain glass (35.7 ± 7.7 $\mu m^2/h$) and on a BSA-coated glass slide (10.2 ± 0.3 $\mu m^2/h$).

hesive BSA-coated surfaces did not support cell attachment and spreading. The initial spreading rate (dotted lines in box of Fig. 3) was significantly higher ($p < 0.05$) for avidin-mediated adhesion (80.0 ± 25.6 $\mu m^2/h$), followed by integrin-dependent cellular adhesion on plain glass (35.7 ± 7.7 $\mu m^2/h$), and, finally, by adhesion on BSA-coated protein surfaces (10.2 ± 0.3 $\mu m^2/h$).

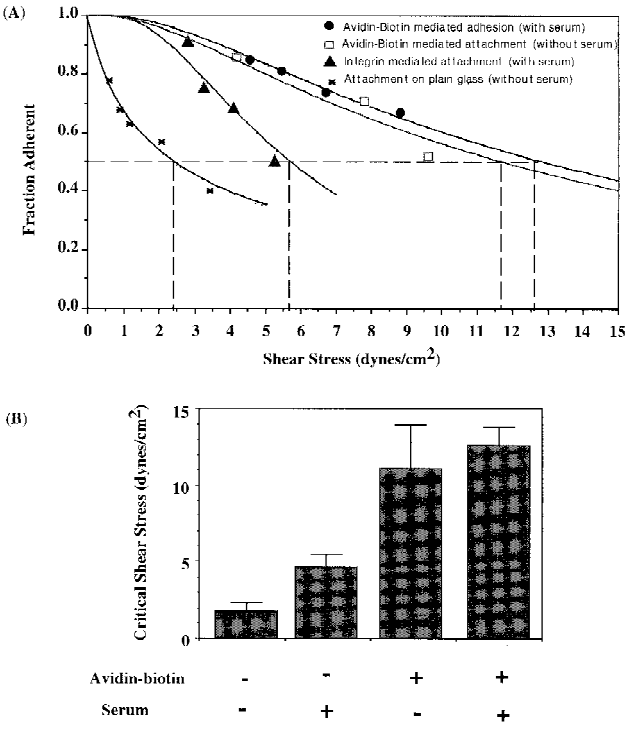

Figure 4. (A) Typical detachment profile of BAEC attached through integrin-dependent and avidin–biotin-mediated adhesion mechanism after 1 h of seeding in media with 10% FBS serum and in media without serum. Critical shear stress was defined as the shear stress under which 50% of the initially seeded cells would detach; (B) mean critical shear stresses for integrin-mediated and avidin–biotin-mediated adhesion mechanism for cells seeded with and without serum.

Strength of avidin–biotin-mediated adhesion

The strength of avidin–biotin-mediated initial attachment of EC was investigated by subjecting adherent cells to a brief exposure of laminar flow in a variable height flow chamber. Under static conditions, the cells were plated for 1 h in two conditions, one with media containing 10% serum and the other with media containing no serum. The cell population was exposed to a range of shear stresses. A total of three experiments was performed on nonmodified BAEC and biotinylated BAEC in each condition of serum-containing and serum-free media, and critical shear stresses were calculated.

The effect of wall shear stress on avidin–biotin-mediated adhesion and integrin-mediated adhesion is illustrated in Figure 4. Due to variations in attachment strength among the cell population, a log-normal distribution reproduced the shape of the detachment curve [solid lines in Fig. 4(a)]. The critical shear stress (τ_c) was defined as the applied wall shear stress for which 50% of the cells remained adherent [dashed lines in Fig. 4(a)]. The mean critical shear stress for nonmodified BAEC on plain glass without serum was 1.7 ± 0.6 dynes/cm^2 and 4.6 ± 0.8 dynes/cm^2 for cells on plain glass with serum [Fig. 4(b)]. Biotinylated BAEC on avidin–biotin-treated slides without serum was 11.1 ± 2.8 dynes/cm^2 and with serum it was 12.6 ± 1.2 dynes/cm^2 [Fig. 4(b)]. The initial strength of adhesion of biotinylated BAEC for avidin-mediated adhesion was stronger than integrin-mediated adhesion of unmodified BAEC.

DISCUSSION

Several methods have been used to enhance cellular adhesion to polymer surfaces, primarily adsorbing or covalently attaching cell adhesion proteins; immobilizing peptide sequences (Table I), carbon deposition, and plasma discharge treatments.[5,18,44] The technique presented in this paper uses avidin–biotin as an extrinsically introduced, integrin-independent, high-affinity receptor–ligand complex to enhance initial cell attachment and spreading.

Biotinylated cells attached, spread, and attained confluency on a mixed monolayer of BSA:BSA–biotin

treated with avidin. The results from control experiments showed that BSA–biotin-coated glass in the absence of avidin–mediated binding are nonadhesive to both nonmodified BAEC and biotinylated BAEC. The role of integrins was investigated by treating unmodified and biotinylated BAEC with anti-integrin antibody. With increasing concentration of the antibody, cell attachment and spreading decreased significantly for unmodified BAEC, but biotinylated BAEC experienced no significant change in cell attachment. The biotinylated BAEC attached in spite of integrins being blocked, which suggests that cell attachment occurred through the avidin–biotin interactions. With the integrins blocked, biotinylated BAEC experienced a decrease in cellular spreading, showing the need for integrin-mediated attachment for cell spreading. However, the decrease in cell spreading for biotinylated BAEC, with their integrins blocked, was not as great as that for nonmodified cells. This suggests that avidin–biotin interactions in conjunction with integrins can help to enhance the rate of spreading.

The initial spreading rate for avidin-mediated attachment was significantly higher compared to integrin-mediated attachment on plain glass and BSA-coated glass slides. These experiments were conducted in serum-containing media. Therefore, the modified cells had access to serum proteins and could make integrin-dependent attachments and initiate spreading. This explains the higher spreading rates for modified cells as they were anchored by a combination of avidin–biotin interactions and integrin-mediated attachment, which, in turn, initiated cell spreading. This result supports our hypothesis that avidin–biotin complex not only helps cell attachment but also brings the membrane-bound integrins in close proximity to surface-adsorbed serum proteins, hastening the integrin-mediated linkages required for cell spreading. The model for this attachment mechanism is illustrated in Figure 5.

Initial strength of attachment for unmodified and biotinylated BAEC was investigated by exposing the cells to a range of shear stresses within 1 h of seeding in serum-containing media (Fig. 4). The critical shear stress for avidin-mediated attachment (12.6 ± 1.2 dynes/cm^2) was significantly higher than for nonmodified BAEC adhesion (4.6 ± 0.8 dynes/cm^2). Because cell adhesion strength is related to the log of the affinity constant, Kuo and Lauffenberger[45] speculated that the $10^{15}M^{-1}$ affinity of avidin for biotin would increase cell adhesion strength on a per bond basis by a factor of 2–3 over a typical substrate with bond affinities of integrin receptors with serum proteins on the order of $10^{8}M^{-1}$. The mean critical shear stress of 12.6 ± 1.2 dynes/cm^2 for avidin-mediated attachment over 4.6 ± 0.8 dynes/cm^2 for integrin-mediated attachment supports this speculation made by Kuo and Lauffenberger.[45] Further, the mean critical shear stress

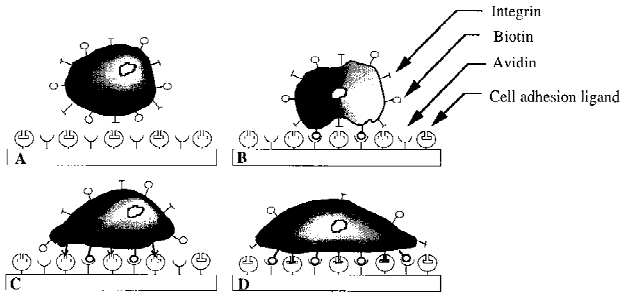

Figure 5. (A) Rounded, biotinylated cell approaches heterogenous surface; (B) cell binds rapidly first with avidin ligands, thus stabilizing the cell at the substrate surface; (C) binding through avidin–biotin coupling brings integrins in close proximity with integrin receptors, thus facilitating the formation of integrin-mediated bonds; (D) cell spreads rapidly as avidin–biotin coupling continues to bring cell membrane in close proximity to the surface. Although biotin–avidin coupling secures the cell at the surface, the cell will not spread without integrin-mediated binding.

for unmodified BAEC attachment on plain glass in serum-free media was 1.7 ± 0.6 dynes/cm^2. This cell attachment in the absence of serum is to a large extent nonspecific attachment although the cells may have secreted their own extracellular matrix proteins, which adsorbed and initiated the integrin-dependent attachment. The mean critical shear stress for biotinylated cells on avidin–biotin-treated glass in serum-free media, however, was comparable to its strength in serum-containing media, and was 11.1 ± 2.8 dynes/cm^2. This suggests that the increased strength of attachment of biotinylated cells was largely due to the avidin–biotin interactions. The increased strength of attachment of EC under shear stress conditions can be very helpful in solving the problem of cell detachment on seeded vascular grafts in the initial period after reestablishment of blood flow, during which the cells are most susceptible to detachment.

As seen from Table I, a multiple ligand approach has been more successful in endothelialization of polymer surfaces, but there is still a need for preventing initial cell detachment. The introduction of an external high-affinity receptor–ligand system would help in strong and rapid initial cell attachment, following which the cell can form integrin-mediated links to initiate spreading. The results summarized in Table II show the feasibility of introducing an external receptor–ligand complex to the cell adhesion system. In the current paper, the combination of integrin-independent attachment (avidin–biotin complex) with integrin-dependent (serum proteins) attachment and spreading showed an overall increase in strength of attachment and spreading. It is also reasonable that the cellular attachment and spreading rates could be further ''tuned'' by optimizing the avidin and integrin ligands (like fibronectin and peptide sequences) on the surface.

TABLE II
Cell Attachment and Spreading Results for Nonmodified and Biotinylated BAEC

Cell and Surface Modifications	Initial Spreading Rates (With Serum) ($\mu m^2/h$)	Critical Shear Stress (With Serum) (dynes/cm^2)	Critical Shear Stress (Without Serum) (dynes/cm^2)
BAEC + glass slide	35.7 ± 7.7	4.6 ± 0.8	1.7 ± 0.6
Biotin–BAEC + avidin + BSA–Biotin + glass slide	80.0 ± 25.6	12.6 ± 1.2	11.1 ± 2.8

Saterak and Lauffenburger[46] have shown that the specific adhesion strength of the cell/substrate contacts comprised of two receptor/ligand bonds in series is less than the specific adhesion strength of the cell substrate contacts comprised of either of the bonds separately. The present technique of avidin-mediated adhesion consisting of two protein/protein interactions in a series (biotinylated surface–avidin–biotinylated BAEC) therefore may not be the best method of increasing strength of adhesion. Further, the adsorption of BSA–biotin onto the surface may be the weakest link in this attachment sequence. Covalently attaching biotin on the surface or reducing the protein series to one protein/protein interaction (avidin–biotinylated BAEC) by covalently attaching the avidin directly to the surface can further improve the initial strength of cell attachment. Future work should include optimizing heterogeneous receptor surface modifications, controlling cell membrane biotinylation, and investigating the focal contact formations using various microscopic techniques such as confocal microscopy and total internal reflection fluorescence microscopy.

CONCLUSIONS

The results of the study suggest that an externally introduced high-affinity system of avidin–biotin interactions increases the initial strength of attachment and also helps in cellular spreading. By incorporating an extrinsic membrane receptor and optimizing the heterogeneous composition of ligands for maximum cell adhesion, we more powerfully and rapidly can address the desired end of establishing a secure, intact biochemically viable endothelial cell layer on polymeric vascular grafts.

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