Adhesive protein interactions with chitosan: Consequences for valve endothelial cell growth on tissue-engineering materials

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Abstract: Stable endothelialization of a tissue-engineered heart valve is essential for proper valve function, although adhesive characteristics of the native valve endothelial cell (VEC) have rarely been explored. This research evaluated VEC adhesive qualities and attempted to enhance VEC growth on the biopolymer chitosan, a novel tissue-engineering scaffold material with promising biological and chemical properties. Aortic VEC cultures were isolated and found to preferentially adhere to fibronectin, collagen types IV and I over laminin and osteopontin in a dose-dependent manner. Seeding of VEC onto comparison substrates revealed VEC growth and morphology to be preferential in the order: tissue culture polystyrene > gelatin, poly(DL-lactide-*co*-glycolide), chitosan > poly(hydroxy alkanoate). Adhesive protein precoating of chitosan did not significantly enhance VEC growth, despite equivalent protein adsorption as to polystyrene. Initial cell adhesion to protein-precoated chitosan, however, was higher than for polystyrene. Composite chitosan/collagen type IV films were investigated as an alternative to simple protein precoatings, and were shown to improve VEC growth and morphology over chitosan alone. These findings suggest potential manipulation of chitosan properties to improve amenability to valve tissue-engineering applications. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 67A: 538–547, 2003

Key words: chitosan; valve endothelial cell; tissue-engineering; protein adsorption; cell adhesion and growth

INTRODUCTION

Heart valve replacement is extensively performed in the United States, with >96,000 substitute valves implanted each year.[1] The concept of a tissue-engineered valve has been explored in recent years[2] as a possible solution to the thrombosis, infection, structural failure, and calcification still experienced by current mechanical and tissue valve replacements. The implanted endothelium is an important feature of a tissue-engineered valve, as endothelialized surfaces are likely integral in determining the antithrombotic, dynamic, immunogenic, and calcific properties of implanted valves.[3] Unlike other animal species,[4] conflu-

ent endothelial linings have not been shown to naturally develop upon implanted vascular grafts in humans, thus necessitating the effective seeding of tissue-engineered constructs *in vitro* before implantation.

Relatively few groups have isolated and characterized valvular cells, but some studies have suggested that valve endothelial cells (VECs) may have certain unique attributes that distinguish them from blood vessel–derived endothelial cells (ECs).[5,6] For the purposes of heart valve tissue-engineering (TE), knowledge of the adhesive characteristics of VECs becomes particularly important because it is desirable to optimize cell–scaffold interaction and adhesion, and to ensure that endothelium integrity and function are ultimately maintained *in vivo*.

To develop an effective tissue-engineered construct, both the cell and scaffold components must be carefully selected and optimized. Chitosan, the deacetylated derivative of chitin (a polysaccharide found in crustaceans) has been suggested in recent years to be a possible TE scaffold material, including for cartilage and hepatic applications.[7,8] Some promising properties of this biopolymer include minimal foreign body reaction, mild processing conditions, controllable deg-

radation, structural similarity to glycosaminoglycans, and availability of hydroxyl and amino functional groups for conjugation to molecules that may be used to produce a bioactive scaffold.[7]

The goals of the present studies were to evaluate chitosan for use in valve TE applications and identify specific adhesive properties of VECs to enhance endothelialization of such a construct. This research has addressed protein precoatings and composites to improve VEC attachment, proliferation, and morphology on chitosan. Ultimately, this understanding may be used to develop new cell systems for valve TE or to enhance repopulation of a tissue-engineered valve construct by native cells, potentially improving implant function, durability, and healing *in vivo*.

MATERIALS AND METHODS

Materials

Waymouth's medium with L-glutamine, antibiotic/antimycotic, trypsin, ethylenediaminetetraacetic acid, penicillin/streptomycin, bovine plasma fibronectin (FN) and mouse anti-human β_1 (in mouse ascites) were obtained from Gibco/Life Technologies (Rockville, MD). Soybean trypsin inhibitor, mouse immunoglobulin (Ig) G, and porcine gelatin (type A, 300 bloom) were purchased from Sigma-Aldrich (St. Louis, MO). Mouse collagen IV (COL IV), mouse laminin (LM), and rat-tail collagen I (COL I) were obtained from Becton Dickinson/Collaborative Biomedical Products (Bedford, MA). IodoBeads, D-Salt dextran plastic desalting columns, and bicinchoninic acid (BCA) assay reagents were from Pierce (Rockford, IL). Horseradish peroxidase (HRP)-conjugated rabbit anti-goat and goat anti-mouse antibodies were from Jackson ImmunoResearch Labs (West Grove, PA). Mouse anti-human $\alpha_9\beta_1$ (clone Y9A2) and $\alpha_v\beta_3$ (clone LM609) were from Chemicon International (Temecula, CA). Chitosan (high-purity from crab shells, deacetylation >80%, MW $\approx$ 430,000) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHB 93/PHV 7) (natural origin) were purchased from CarboMer (Westborough, MA), and 75:25 poly(DL-lactide-*co*-glycolide) (PLGA) (i.v. = 0.69 dL/g) was from Birmingham Polymers (Birmingham, AL). Maxisorp polystyrene (PS) was from Nalge Nunc International (Rochester, NY), and tissue culture polystyrene (TCPS) was from Becton Dickinson/Falcon (Franklin Lakes, NJ). Other reagents included [125]I (PerkinElmer Life Science, Boston, MA), collagenase type I (Worthington Biochemical, Freehold, NJ), DiI-acetylated (Ac)-LDL (Biomedical Technologies, Stoughton, MA), phycoerythrin (PE)-conjugated goat anti-mouse IgG (Biomeda, Foster City, CA), alamarBlue™ (Biosource, Camarillo, CA), goat anti-human VE-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), and calcein AM (Molecular Probes, Eugene, OR). Mouse anti-bovine photoreceptor (RPEMIC) was the gift of John Saari's lab [University of Washington (UW) Department of Opthalmology], mouse anti-human α-smooth muscle actin (clone HHF-35) was donated by Allen Gown's lab (UW Department of Pathology),

and rat-HIS-osteopontin (OPN) was synthesized as previously described.[9]

Valve cell isolation and culture identification

Bovine aortic VECs were obtained from fresh aortic valve leaflets incubated at 37°C for 25 min in an enzymatic mixture of collagenase type I (165 u/mL), soybean trypsin inhibitor (0.375 mg/mL), and plating media (Waymouth's medium, 20% fetal bovine serum, 1% antibiotic/antimycotic). Cells were centrifuged for 10 min at 1000 rpm, and seeded into six-well plates for culture in a 37°C humidified atmosphere with 5% CO_2. Media was changed every $\sim$2–3 days, and cells were detached using 0.05% trypsin-ethylenediaminetetraacetic acid for passage. Subsequent culture was performed in T75 vented culture flasks using growth media containing Waymouth's medium, 5% fetal bovine serum, and 1% penicillin/streptomycin.

The endothelial cell nature of isolated cultures was confirmed by DiI-Ac-LDL uptake, in accordance with manufacturer's instructions. Western blotting for VE-cadherin was conducted with a 1:500 dilution of 1° antibody (Ab) (PAb goat-anti-human VE-cadherin) and a 1:10,000 dilution of 2° Ab (HRP-rabbit-anti-goat IgG). Western blotting for α-smooth muscle actin was conducted using a 1:1000 dilution of 1° Ab (MAb mouse-anti-human α,γ muscle actin, clone HHF-35) and a 1:10,000 dilution of 2° Ab (HRP-goat-anti-mouse).

Flow cytometry

VECs underwent flow cytometric analysis for culture purity and integrin expression in a FACScan equipped with CellQuest version 3.3 analysis software (Becton Dickinson, Bedford, MA). Gates for positive results included <1% of the negative control peak. Culture purity was assessed via DiI-Ac-LDL uptake from three sets of VECs, each read twice, and the percent gated for all readings averaged for an approximate purity. Integrin 1° MAbs used were mouse anti-human $\alpha_9\beta_1$ (1 μg/mL, clone Y9A2), mouse anti-human $\alpha_v\beta_3$ (1:100, clone LM609), and mouse anti-human β_1 (1:100, in mouse ascites). A 1:50 dilution of PE-conjugated goat anti-mouse IgG (1 mg/mL stock) was used as a 2° Ab for fluorescent tagging. Negative controls included mouse IgG, mouse anti-bovine photoreceptor MAb in mouse ascites, and 2° Ab alone.

Adhesion experiments

Assays for cell adhesion to substrates using toluidine blue staining were performed as previously described.[10] Briefly, test proteins were coated onto 96-well plates overnight at 4°C. Wells were washed with phosphate-buffered saline (PBS) and blocked with 10 mg/mL bovine serum albumin (BSA) in PBS at 37°C for an hour. Cells were resuspended in Waymouth's medium with 1 mg/mL BSA, plated at 30,000

cells/well, and allowed to attach to substrates for 1 h at 37°C. Adherent cells were fixed with 4% paraformaldehyde, stained with 0.5% toluidine blue in 4% paraformaldehyde, solubilized with 1 or 2% sodium dodecyl sulfate (SDS), and read at 630 nm. Adhesion is reported as net absorbance (control subtracted).

Polymer substrates

Four biodegradable polymer substrates were used in this study: chitosan, gelatin, 75:25 PLGA, and PHB 93/PHV 7, a member of the poly(hydroxy alkanoate) (PHA) family of bacterial polyesters that will henceforth be referred to by this more general term.

Chitosan films were created by casting a 1% (w/v) solution of chitosan in $0.1N$ acetic acid (0.8 μm filtered) into PS multiwell plates (500 μL/well for 24-well, 80 μL/well for 96-well). Films were air-dried in a sterile hood for 1–2 days, then neutralized with $0.1M$ NaOH for 1 h. For composite chitosan/COL IV films, COL IV was added to dissolved chitosan for a final concentration of 5 or 25 μg/mL (0.05 and 0.25 weight percent COL IV in chitosan, respectively). The chitosan/COL IV solution was then cast as above. Gelatin films were created by casting a 5% (w/v) solution of gelatin in deionized/distilled water into PS multiwell plates. Casting volumes and drying protocol were the same as for chitosan. PLGA films were formed by casting a 10% (w/v) solution of 75:25 PLGA in $MeCl_2$ into 5-mL Teflon beakers at 500 μL/beaker. PHA films were formed by casting a 10% (w/v) solution of PHA in chloroform into 20-mL glass scintillation vials at 1.5 mL/vial. Both PLGA and PHA films were air-dried in a chemical hood for ~1 week to constant weight. Uniform samples of PLGA and PHA were obtained using an 8-gauge (15-mm diameter) metal biopsy punch.

All films were stored at room temperature until use, and sterilized by immersion in 70% ethanol for 1 h (except chitosan/COL IV composites). Before protein adsorption or cell seeding, films were rinsed overnight with either sterile PBS or Waymouth's media with 1% antibiotic/antimycotic, respectively. For growth assays involving protein-precoated substrates, protein solutions were adsorbed overnight at 4°C and briefly rinsed with PBS before cell seeding.

Microscopy and image analysis

All microscopy was performed using a Nikon Eclipse TE200 (Melville, NY) inverted microscope. Image analysis was performed using MetaMorph version 4.6 (Universal Imaging, Downingtown, PA). To determine VEC substrate coverage areas, live cell cytoplasm was fluorescently labeled with calcein AM (2 μm in PBS, 15 min at 37°C) after 6 days in culture. Three low-power (4× objective) images of VECs on each substrate were taken using a fluorescein isothiocyanate (ex 492/em 520 nm) optical filter. Area of cell coverage was quantified by thresholding areas above a minimum fluorescent intensity set for each substrate, to compensate for material-specific background fluorescence. Percent thresholded of the total image area was calculated by Meta-Morph.

VEC growth assays

VEC growth on substrates over time was conducted using alamarBlue™, in accordance with manufacturer's instructions. For each timepoint, 10% alamarBlue™ in 500 μL of culture media was added to each test well of a 24-well plate and incubated for 4 h at 37°C. To calibrate cell number to percent reduction of alamarBlue™ on comparison substrates, VECs seeded at known densities were cultured, assayed for percent reduction, trypsinized off of culture substrates, and quantified by Coulter counter (model Z1; Beckman Coulter, Miami, FL).

Protein adsorption studies

Radioiodination

Solutions of FN and LM were radiolabeled with ^{125}I using the IodoBeads iodination reagent and dialyzed of unincorporated iodide using D-Salt dextran plastic desalting columns according to manufacturer's instructions. Spiked protein solutions were adsorbed onto 96-well PS or chitosan-coated plates at 50 μL/well for 1 h at room temperature, followed by solution removal and a PBS rinse. Protein was desorbed in 1% SDS for 30 min at room temperature. SDS/desorbed protein samples were read by a 1185 GammaTrac Gamma Counting System (TmAnalytic) for 0.5 min per count × 2 counts each using MicroPhone version 1.0.1 data recording software.

BCA assay

Nonradioactive solutions of FN and LM were adsorbed onto 96-well PS or chitosan-coated plates, rinsed, and desorbed as for radioiodination studies. Protein samples were reacted with the MicroBCA Protein Assay Reagent according to manufacturer's protocol. Protein concentrations reported are based on a calibration curve of BSA standards relating OD_{560} to protein concentrations between 0–45 μg/mL.

Statistical analysis

Significant differences were determined utilizing analysis of variance and Tukey-Cramer pairwise comparisons, calculated using JMP-IN version 3.2.6 (SAS Institute, Cary, NC) software. Results are reported as mean ± SD. Statistical significance was set at $p < 0.05$.
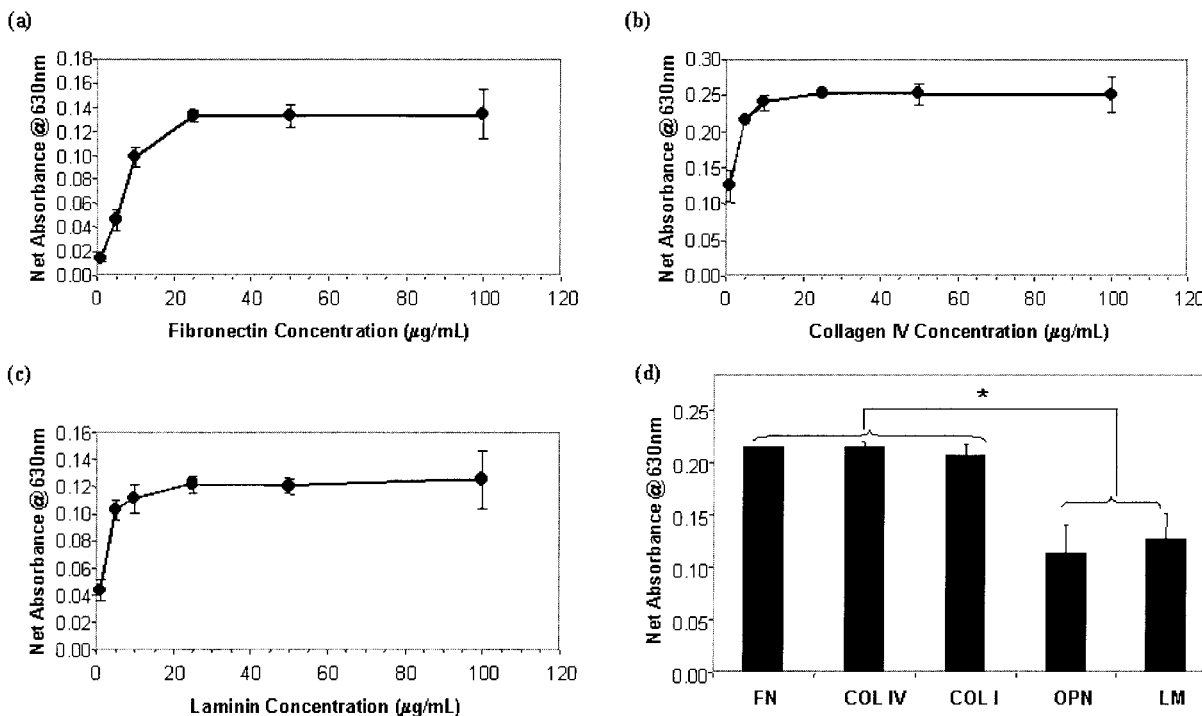
Figure 1. VEC adhesion to protein-precoated PS expressed in terms of toluidine blue net absorbance (control subtracted) at 630 nm. VECs were seeded at 30,000 cells/well in a 96-well plate and allowed to adhere for 1 h ($n = 3$). (a) FN dose response; (b) COL IV dose response; (c) LM dose response; (d) protein comparison at 25 μg/mL (*$p < 0.05$).

RESULTS

VEC cultures are positive for VE-cadherin expression and Ac-LDL uptake

VECs were successfully isolated from bovine aortic valve leaflets. Cells displayed EC-typical cobblestone morphology and monolayer coverage of TCPS. VECs were positive for EC/macrophage-specific Ac-LDL uptake, and were typically $\approx$90% (88.4 $\pm$ 1.5%) pure by flow cytometry for DiI-Ac-LDL. By Western blot analyses, VECs displayed strongly positive expression of EC-specific VE-cadherin, and little to no expression of smooth muscle cell (SMC)-specific α-smooth muscle actin, likely because of some contaminating cell types (e.g., valve interstitial cells) (data not shown).

### VECs adhere preferentially to FN-, COL IV-, and COL I-coated PS

Integrin profiling by flow cytometry revealed expression of $\alpha_9\beta_1$, $\alpha_v\beta_3$, and subunit β_1 on the surface of bovine aortic VECs (data not shown). Based on these findings, selected adhesive proteins were targeted as precoating factors to enhance VEC adhesion to TE materials. As shown in Figure 1(a–c), VECs adhered in a dose-dependent manner to FN, COL IV, and LM

plated onto PS. Similar results were also seen for OPN and COL I (data not shown). All proteins appeared to plateau in VEC adhesion efficacy by 25 μg/mL, where VECs had statistically higher adhesion to FN, COL IV, and COL I compared with LM and OPN [Fig. 1(d)].

VEC growth on unmodified chitosan and PLGA is superior to PHA

VEC growth on chitosan was compared with other commonly used TE scaffolds over a 7-day period. The percent reduction of alamarBlue™ was seen to correlate linearly to the number of (presumably live) cells present on the test substrates over a range of cell concentrations, as exemplified by chitosan [Fig. 2(a)]. The slope of correlation was observed to vary only slightly between VECs at the same passage number seeded onto different substrates, indicating a relative lack of impact of substrate type on VEC metabolic activity/alamarBlue™ reduction capability (data not shown).

VEC proliferation was monitored upon TCPS and films of gelatin, PLGA, chitosan, and PHA [Fig. 2(b)]. TCPS and gelatin were used as positive controls for cell growth, because cell behavior on both has previously been extensively characterized. VEC growth appeared to be preferential in the order: TCPS > (gelatin,
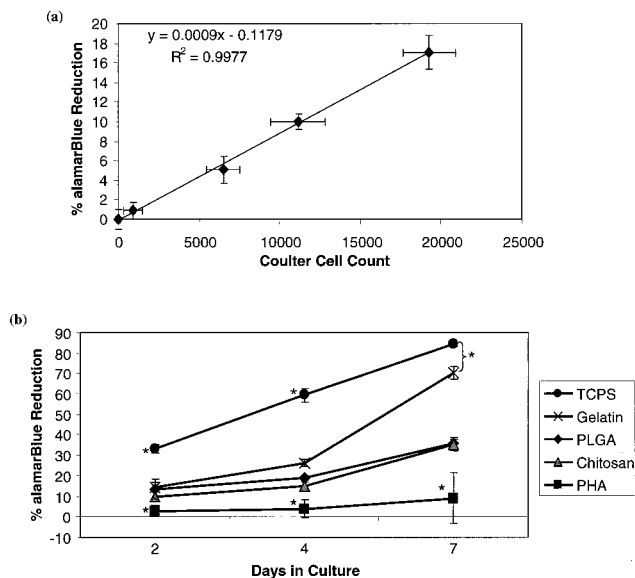
Figure 2. VEC growth on seeded substrates as monitored by alamarBlue™ reduction ($n = 3$). (a) Correlation of percent alamarBlue™ reduction to cell number on chitosan as assessed by Coulter counter; (b) cell growth on comparison substrates initially seeded at 60,000 cells/well in a 24-well plate (*$p < 0.05$, individual * = significance from all other data in timepoint).

PLGA, chitosan) > PHA. For all timepoints, TCPS supported a significantly higher cell number than all other substrates, except gelatin at day 7. In contrast, PHA had a significantly lower cell number than all other substrates at all timepoints. Notably, PLGA and chitosan were comparable in growth, and were not statistically different from gelatin until day 7 in culture.

VEC morphology at 6 days in culture varied somewhat between comparison substrates as revealed by fluorescence (calcein AM) imaging (Fig. 3). VECs on TCPS and gelatin displayed cobblestone morphology, spreading, and confluent monolayer formation typical of ECs, although confluency did not occur at the same timepoint for both. VECs on PLGA and chitosan tended to be less spread and more elongated (more typical of fibroblast/SMC morphology), and lacking cell–cell junction formation. VECs on PLGA were slightly more spread than on chitosan and tended to be more evenly distributed, whereas VECs on chitosan were often observed to clump into stellate clusters. Cells on PHA displayed the least amount of spreading of all comparison substrates, with many cells remaining spherical and unspread, even at 6 days in culture.

VEC coverage area per low power ($4\times$ objective) field was evaluated to quantify both the extent of cell spreading and confluent monolayer formation upon each substrate at day 6 in culture (Table I). The percent substrate area covered by VECs generally correlated with the overall quality of growth behavior observed

for each substrate with respect to both proliferation and cell morphology, that is, TCPS > (gelatin, PLGA, chitosan) > PHA.

Adhesive protein precoating of chitosan minimally enhances VEC growth

Precoating with adhesive proteins was hypothesized to be a simple method of enhancing VEC adhesion and growth upon chitosan. TCPS and chitosan, either left uncoated or precoated with 25 µg/mL FN or LM adsorption solutions, were used as substrates for VEC proliferation over time [Fig. 4(a)]. Although both COL I and IV enhanced VEC adhesion more than LM, simple precoating is prohibited because both chitosan and COL are acid soluble. At all timepoints, VEC number on TCPS with precoat was not significantly different from TCPS alone. LM-coated TCPS, however, did appear to slightly decrease VEC growth, as has been noted previously for EC on PS.[11] VEC growth on FN-coated chitosan was not statistically different from chitosan control. However, VEC number on chitosan was significantly improved by LM-precoating at day 4. VEC growth under all chitosan precoating conditions was significantly lower than growth on TPCS for essentially the entire observation period. Cells on chitosan also displayed low amounts of spreading and altered elongated morphology under all precoating conditions [Fig. 4(b)].

Protein adsorption to chitosan and PS is comparable

It was hypothesized that poor protein adsorption to chitosan might explain the observations of reduced cell growth (relative to TCPS) and minimal improvement with protein precoating. FN and LM adsorption to chitosan and PS were quantified by both ^{125}I-radiolabeling and BCA assay. As shown in Figure 5(a,b), no significant difference in the amounts of protein adsorbing to chitosan versus highly adsorbant PS was observed. In addition, dose-dependent protein adsorption was not observed (except for LM on PS by BCA assay), indicating that saturating levels of FN and LM were likely adsorbed to both chitosan and PS at the concentrations used.

Initial cell adhesion to protein-precoated chitosan is enhanced over TCPS

An alternative hypothesis for the differences in growth of VECs on chitosan compared with TCPS was
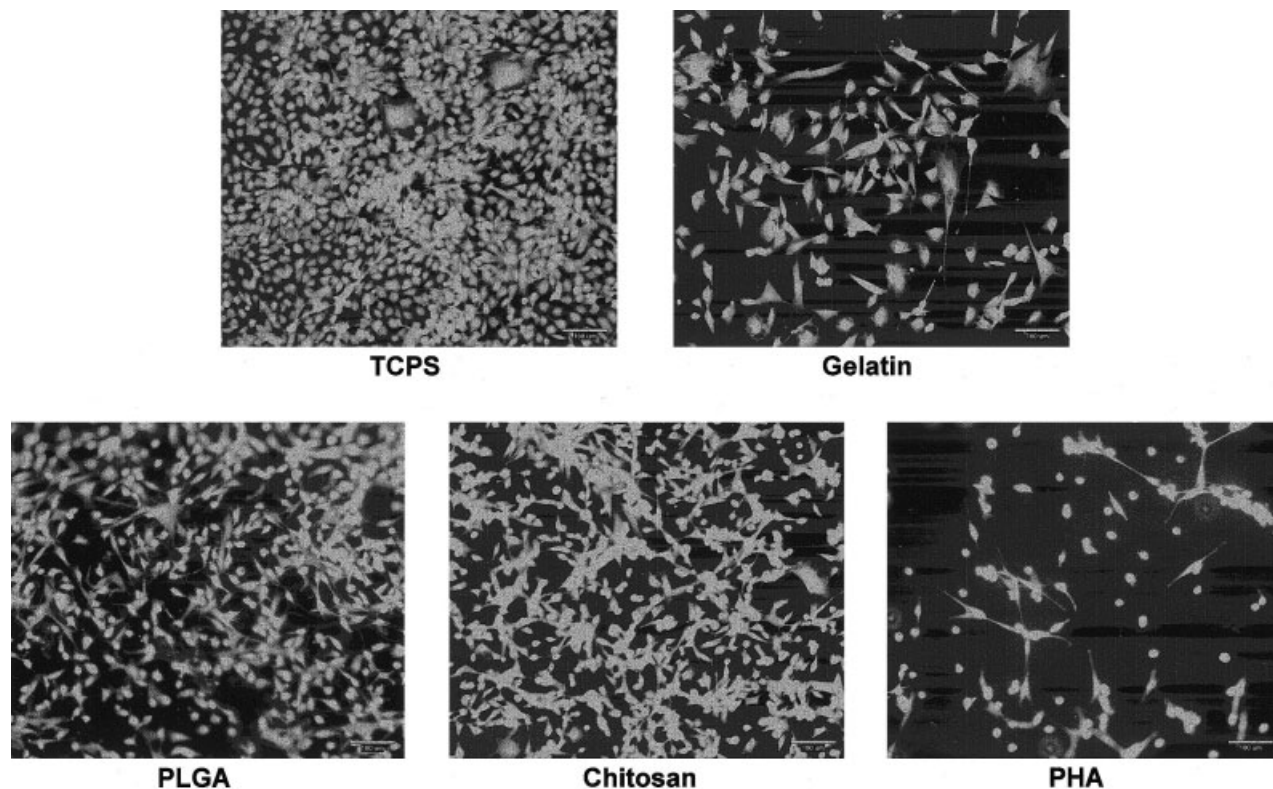
Figure 3. Fluorescence micrographs of VECs seeded onto comparison substrates. Substrates were initially seeded at 60,000 cells/well in a 24-well plate and fluorescently imaged at 6 days in culture following cytosolic viability labeling with 2 μM calcein AM. Bar = 100 μm.

that differences in initial VEC adhesion occurred depending on substrate. To test this, VEC adhesion to protein-precoated chitosan at different precoat concentrations was examined. Maximum VEC adhesion to precoated chitosan occurred at $\geq$25 μg/mL FN or LM [Fig. 6(a,b)], similar to results using precoated PS [Fig. 1(a,b)]. Indeed, when compared directly, precoated chitosan was significantly superior to TCPS in terms of initial cell adhesion [Fig. 6(c)], indicating that deficient adhesion to precoated chitosan did not account for the difference in VEC growth compared with TCPS.

Composite chitosan/COL IV films enhance VEC growth

The synthesis of composite chitosan/COL IV films was proposed as an alternative method for improving VEC attachment and growth on chitosan. Growth studies comparing pure chitosan films to 5 and 25 μg/mL COL IV composite films [Fig. 7(a)], suggested that VEC growth was improved by the presence of COL IV, although this was only significant for chitosan/25 μg/mL COL IV at day 4 in culture. VECs on chitosan/COL IV films at both protein concentrations

also displayed more spreading and a less elongated morphology than on chitosan alone [Fig. 7(b)].

DISCUSSION

This study investigated VECs with regard to their adhesive properties and interaction with chitosan as a potential TE valve substrate. Dose-dependent adhesion of VECs to FN, COL IV, COL I, LM, and OPN was observed. VEC behavior on uncoated chitosan, al-

TABLE I
Coverage Area of VECs Seeded on Comparison Substrates

| Substrate	VEC Coverage Area per 4× Field 60,000 Cells Seeded/6 Days in Culture (Mean % ± SD, n = 3)
TCPS	88.87 ± 5.52*
Gelatin	32.38 ± 9.02
PLGA	45.08 ± 14.12[a]
Chitosan	39.85 ± 5.13[b]
PHA	16.82 ± 4.33[a,b]

[a,b]Significance between matching letters, $p < 0.05$.
*Significance from all other conditions, $p < 0.05$.
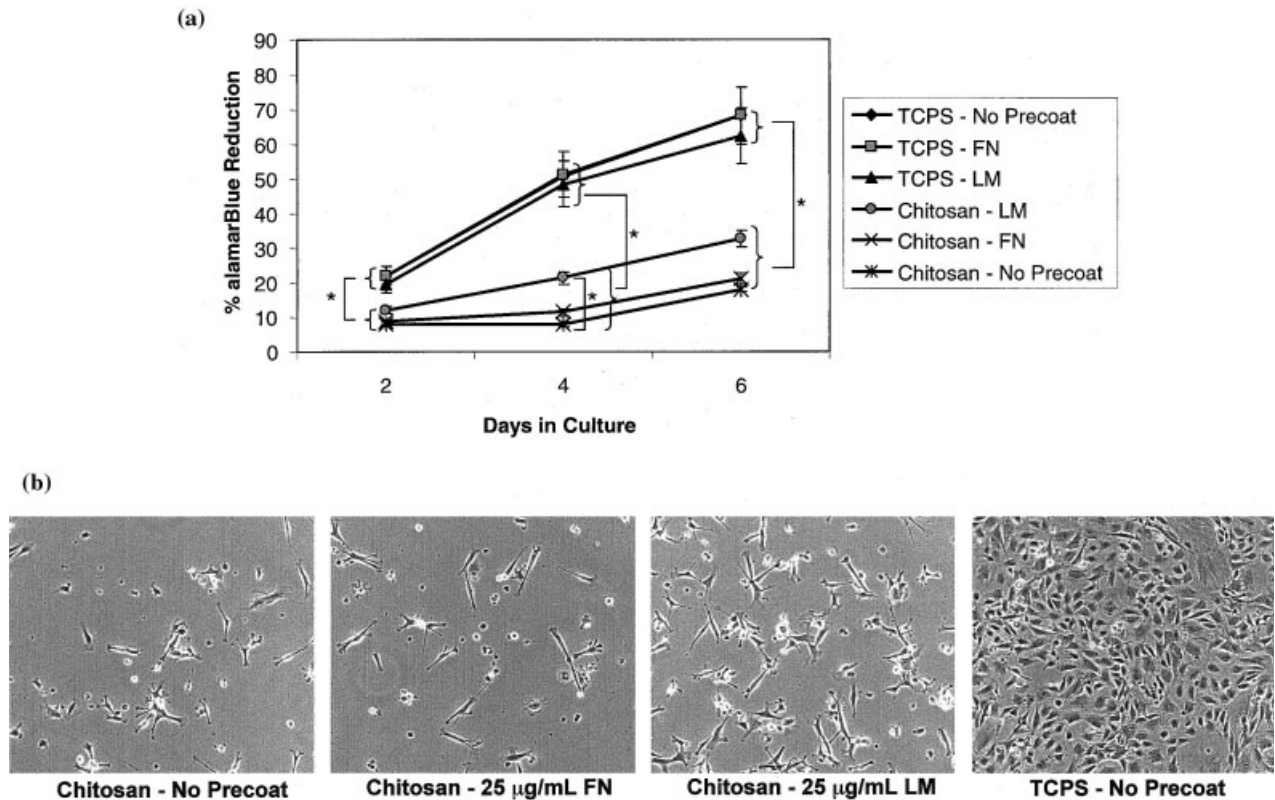
Figure 4. VEC growth on control, FN-, or LM-precoated chitosan and TCPS as monitored by alamarBlue[TM] reduction. (a) Cell growth on substrates initially seeded at 30,000 cells/well in a 24-well plate ($n = 3$, *$p < 0.05$). Note that the growth curves for non-precoated and FN-precoated TCPS overlap; (b) phase micrographs of the cells monitored in (a) at 4 days in culture, displaying typical VEC morphology on chitosan and TCPS.

though comparable to PLGA and superior to PHA, was still found to be reduced compared with TCPS and gelatin controls. FN or LM precoating of chitosan showed slight enhancement of cell growth and morphology. The marginal enhancement of VEC growth

on precoated chitosan did not appear to be caused by lack of protein adsorption, because both chitosan and highly adsorbant PS displayed similar adsorption by protein iodination and BCA assay. Likewise, differences in cell growth on precoated chitosan compared
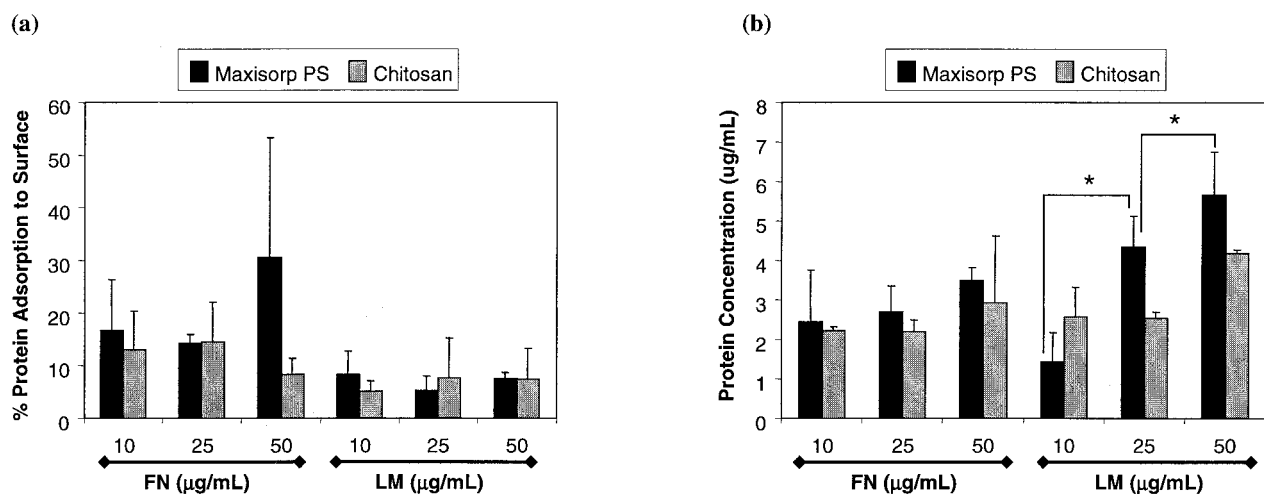
Figure 5. FN and LM adsorption to chitosan and PS. Proteins were adsorbed to substrates for 1 h at room temperature, followed by rinsing in PBS and desorption in 1% SDS for 30 min ($n = 3$). (a) Percent protein adsorption to surface from solution as determined by ^{125}I-radiolabeling of adsorbed proteins; (b) concentration of protein in desorbed samples as determined by BCA assay (*$p < 0.05$).
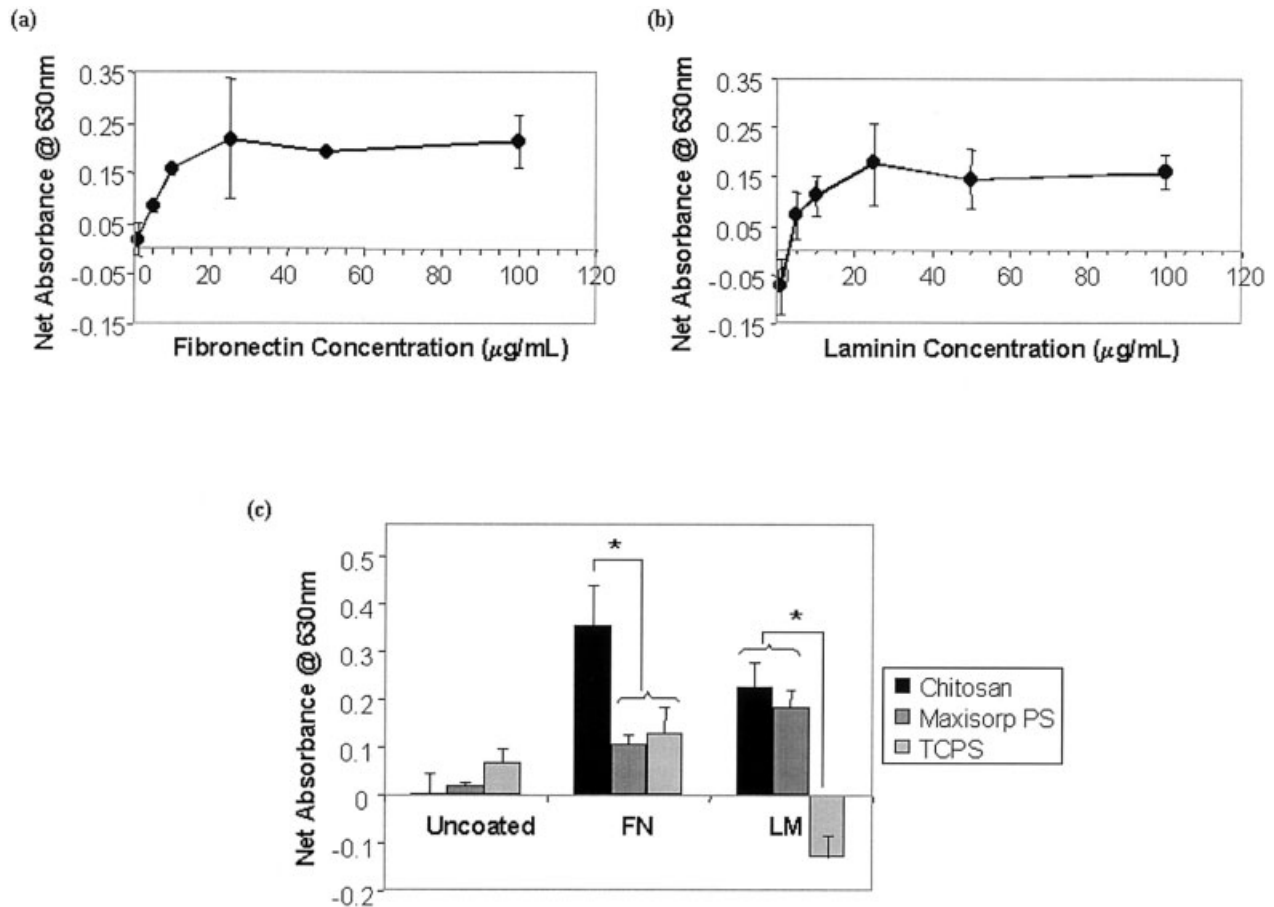
Figure 6. VEC adhesion to protein-precoated substrates expressed in terms of toluidine blue net absorbance (control subtracted) at 630 nm. VECs were seeded at 30,000 cells/well in a 96-well plate and allowed to adhere for 1 h ($n = 3$). (a) FN dose response on chitosan; (b) LM dose response on chitosan; (c) comparison on uncoated, FN-, and LM-precoated chitosan, PS, and TCPS. Note the negative net absorbance for VEC adhesion to LM-precoated TCPS because of the decrease in adhesion relative to a BSA-blocked TCPS control ($*p < 0.05$).

with TCPS did not appear to be caused by deficient initial cell adhesion to protein-treated chitosan. Instead, VEC adhesion to LM-and FN-precoated chitosan was significantly higher than adhesion to TCPS. Finally, the chitosan/COL IV composite film, a precoating alternative, was observed to improve VEC morphology and growth over chitosan alone.

Nonvalvular, blood vessel–derived vascular ECs have typically been used for the seeding of valve TE constructs to date.[2] However, growing evidence suggests that native VECs have attributes distinct from other vascular ECs that may prove to be important for function and should be taken into account when selecting cells for tissue-engineered valve constructs. Notable findings include lack of FN biosynthesis by porcine VECs,[5] and lack of ABH blood group antigen expression on human valve endothelium.[6] Likewise, our studies indicate that VEC adhesive properties may be distinct from other vascular ECs. We found that VECs expressed $\alpha_9\beta_1$ integrin, whereas vascular ECs have not been reported to express this previously.[10] Such adhesive characterization is significant in the TE

field in that integrins are involved not only in cell adhesion and growth, but also cell signaling. Since appropriate function of VECs is likely to depend on appropriate matrix–receptor interactions, knowledge of their integrin profile provides valuable guidance in the design of bioactive surfaces.

VECs have not previously been examined with regard to behavior on polymers and biomaterials of interest in TE applications. Chitosan, the focus of our research, has become the subject of recent TE studies because of its documented biocompatibility, easily manipulated solubility and porosity, availability of functional groups for modification/conjugation, and possible biological activity as a glycosaminoglycan analog.[7] Our observations revealed similar trends in VEC growth upon chitosan and the synthetic polymer PLGA. This resemblance is promising, considering that PLGA has been used extensively in previous TE applications because of its low toxicity, good cell adhesion capabilities, and easily manipulated biodegradation and mechanical properties.[12] Our studies found PHA to be inferior to both PLGA and chitosan
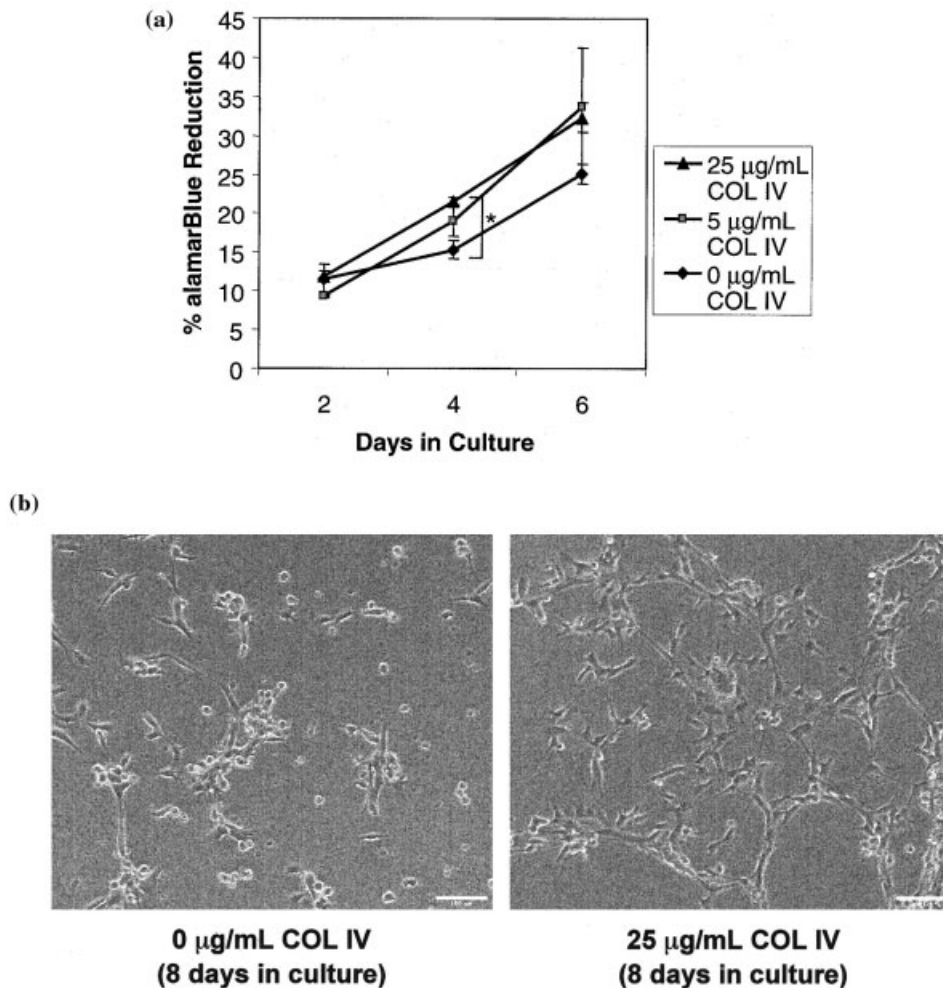
Figure 7. VEC growth on composite chitosan/collagen IV films. (a) Cell growth as monitored by alamarBlue™ reduction. VECs were initially seeded at 30,000 cells/well in a 24-well plate ($n = 3$, *$p < 0.05$); (b) phase micrographs of the cells monitored in (a) at 8 days in culture.

in terms of VEC growth potential and spreading. Similarly, vascular cell adhesion and growth on PHA has previously been found to be inferior to PGA in a porous construct.[13]

Cell–chitosan interactions have been previously studied with mixed results. Chupa et al.[14] observed coronary artery ECs and SMCs to have comparable morphology on chitosan compared with control PS, yet ECs displayed increased spreading and decreased proliferation, whereas SMCs exhibited decreases in both. However, Kawase et al.[15] found that hepatocytes grown on chitosan spread less than those grown on collagen, and thus maintained hepatocyte-specific behavior longer in culture. Whereas reduced cell spreading may be advantageous in the hepatic setting, this same quality is undesirable in valve endothelialization, where continuity of the cell monolayer is essential for antithrombogenicity. Such variations in cell behavior upon chitosan make it necessary to view this as an application-specific characteristic, and both ma-

terial properties and cell type must be appropriately chosen and optimized for each particular TE case.

Precoating of biomaterials with adhesive proteins has long been investigated as a means of enhancing cell attachment and promoting proliferation.[16] There has, however, been a surprising lack of research on matrix protein adsorption to chitosan. Some very recent studies have documented the adsorption of blood proteins onto chitosan and their role in complement activation.[17] More relevant to the current studies, antibody-detectable LM and FN adsorption to chitosan was investigated by enzyme-linked immunosorbent assay.[17] Compared with a gelatin surface, chitosan adsorbed higher levels of LM and lower levels of FN.[17] We found only modest improvement in VEC growth induced by FN or LM precoating of chitosan, leading us to investigate the possibility that chitosan may have reduced protein adsorption properties. However, deficient protein adsorption to chitosan did not appear to account for the minimal stimulation of VEC growth,

because adsorption to chitosan did not differ significantly from the highly adsorbant substrate, PS. However, the finding that initial cell adhesion to protein-precoated chitosan was significantly higher than on TCPS suggests that decreased VEC proliferation on chitosan may be attributed, in part, to excessive strength of attachment.[18] Alternate contributors to poor VEC behavior on chitosan compared with gelatin or TCPS might include instability of protein adsorption over time in culture, or decreased matrix synthesis/inhibited proliferation of cells seeded onto the biopolymer, because of unknown signaling mechanisms.

Finally, composite films of chitosan/COL IV were developed to take advantage of the acid solubilities of the components, and avoid the potential complications associated with simple protein adsorption. Our results indicated that a chitosan/COL IV composite supported enhanced growth of VECs compared with chitosan alone. Moreover, morphology of VECs on the composite was superior to chitosan with or without adhesive protein precoating. Others have also observed improvement in cell growth on chitosan composite materials. Enhanced hepatocyte growth on albumin, gelatin, or collagen-blended chitosan membranes compared with chitosan alone has been observed, with preferred protein blends differing between species.[8] In contrast, collagen-chitosan gels have been observed to inhibit hematopoietic cell proliferation with increasing proportions of chitosan, possibly because of excess cell–matrix contact,[18] thus indicating a need for optimization of chitosan/protein ratio.

In conclusion, our research represents a novel effort to isolate, characterize, and utilize VECs and chitosan for valve TE purposes. Knowledge of VEC adhesive characteristics provides new insight into distinct valve cell biology, and suggests molecular targets for improved VEC growth on surfaces. Chitosan has shown varied compatibility as a TE material, depending on cell source, but has displayed promise as a potential valve scaffold. Our studies suggest that chitosan combined with appropriate protein treatment is a promising substrate for valve TE.

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