THE EFFECT OF SCAFFOLD PHYSICAL PROPERTIES ON ENDOTHELIAL CELL FUNCTION

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ABSTRACT

Endothelial cells (EC) are ubiquitous – as vascular epithelial cells they line the inner surface of all vessels and are the contact surface with flowing blood. Macrovascular EC are the first line barrier between flowing blood and mural structures. The microvasculature includes EC-lined vessels that contact virtually every cell in the body. These EC are potent bioregulatory cells, modulating thrombosis, inflammation and control over mural smooth muscle cells and vascular health.

The biochemical roles of EC can be retained when cells are embedded within three-dimensional matrices without recapitulation of the full vessel architecture. Within these matrices, surface and structural properties impose a set of forces on the embedded EC. Indeed, substrata pore size and modulus have profound effects on phenotype and function of a range of cell types. In the first part of this work, we examined the effect of pore size, matrix relative density and modulus on matrix-embedded EC growth and secretion and found a greater biological dependence on modulus than pore size or density. In the second part of this work, we examined the effect of isolated changes in modulus on BC growth, secretion of growth regulators, and modulation of smooth muscle cell growth.

EC growth is maximal at intermediate moduli over a range from 50 Pa-1500 Pa. Secretion of heparan sulfate proteoglycans (HSPGs), which inhibit smooth muscle cell growth, is maximal at low moduli and flat at high moduli. Secretion of growth factors such as FGF2 and PDGF-BB were also modulus responsive. Inhibition of smooth muscle cell growth rose as modulus decreased from 510 Pa to 50 Pa and was the result of a balance between increased HSPG secretion and reduced secretion of vasoactive growth factors. Changes in endothelial function correlated with extracellular matrix gene and integrin α,β3 and α5β1 expression. Changes in the forces experienced by the cell - a change in substrate modulus - cause the cell to alter its ECM and integrin expression in an effort to return the force balance to normal, leading to downstream effects on cell function. While growth stimulatory function largely conserved, growth inhibitory function was altered to a much larger degree.

In the final part of this work, we examined the effect of scaffold modulus on EC response to inflammatory stimuli, and attempted to correlate it to changes in smooth muscle cell regulation and integrin expression. While cytokine secretion was independent of modulus, surface expression of ICAM-1 and VCAM-1, and induction of CD4+ T cell proliferation followed a similar pattern to smooth muscle cell inhibition, suggesting that similar mechanisms may be involved in their regulation by substrate modulus.

Alteration of scaffold modulus has a profound impact on EC function including growth regulation and inflammatory response. The model offered in this thesis – wherein EC attempt to neutralize changes in environmental force balance by altering ECM and integrin expression, leading to changes in downstream function – offers insight into how environmental changes effect functional changes in ECs.

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I would also like to thank my thesis committee, who helped shape my work from a collection of scattered experiments into a coherent, scientific work. Lorna Gibson, by providing me with the materials science tools and ideas I needed to accomplish my research goals. Ioannis Yannas, by forcing me to think about the big picture and how things fit together. And Sangeeta Bhatia, by keeping me focused on the biology.

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CHAPTER 1: INTRODUCTION AND BACKGROUND

INTRODUCTION

Endothelial cells, the cells which line all blood vessels in the body, have a wide range of important functions, not only acting as a barrier between the blood and tissues, but also playing roles in coagulation, fibrinolysis, regulation of vessel tone, regulation of smooth muscle cell growth, response to inflammation and the formation of new blood vessels. In the last several years, tissue engineering techniques have been used to develop endothelial cell constructs, using commercially available surgical sponges, which can regulate these same functions in the absence of an intact endothelium. By utilizing these constructs it has been possible to prevent neointimal hyperplasia in animal models of angioplasty and arterio-venous fistula.

One of the initial motivations for this work was to determine if altering scaffold physical properties could enhance embedded cell functionality. It has long been known that cells’ properties are affected by the physical properties of the substrate on which they are grown, including, topography, roughness and rigidity. Changes in these properties can lead to alterations in a wide range of cellular functions, ranging from proliferation and migration to cell specific functions such as cytokine secretion.

Significant work has been performed to date to examine cell-substrata interaction and yet the majority of these studies focus on cells grown in 2D cultures. To understand how cells function in 3D, it is not always possible to extrapolate from 2D. Many cell types, including fibroblasts, smooth muscle cells, and endothelial cells act significantly differently in three dimensional culture. Additionally, few of the studies which examine how changes in three dimensional physical properties affect cells focus on endothelial cells.
This thesis sought to examine the effects of several parameters, including pore size, matrix density and matrix modulus, on endothelial cells cultured in three dimensional structures. We found that endothelial cells do respond to these factors by altered growth and secretion, but that modulus seemed to have a dominant effect over pore size or relative density. Based on the results of the first part of this work, the remainder of the thesis examined the effect of isolated changes in modulus on endothelial cell function, still in three dimensional culture.

The bulk of this work examines the hypothesis that endothelial cell function is altered by substrate mechanical properties in a manner which is mediated by changes in the cell-substrate interface, namely the extracellular matrix and integrins. This was accomplished by carrying out the following specific aims:

1. To develop a system of tissue culture scaffolds which vary in modulus while retaining the same physical structure.

2. To examine the effect of changes in scaffold modulus on aspects of endothelial cell biology including regulation of growth, secretion, smooth muscle cell inhibition and response to inflammatory stimuli.

3. To correlate changes in cell biology to changes in the cell-substrate interface.

4. And, finally, to develop a model which takes into account the data from aims 1-3 to explain how changes in substrate modulus lead to modulation of endothelial cell function.

BACKGROUND

Substrate Mechanics and Cell Function

Substrate mechanics have long been known to affect both cellular morphology and function. Many cell types have been shown to spread more on stiffer substrates\textsuperscript{8-11} or migrate
towards regions of stiffer substratum\textsuperscript{9,12-14}. Properties of fibroblasts, smooth muscle cells and chondrocytes, including focal adhesion formation\textsuperscript{8,15}, tyrosine signaling\textsuperscript{15,16}, and proliferation\textsuperscript{17-19} are all affected by substrate mechanics across both 2D and 3D culture. Cell type specific functions are affected by substrate rigidity. Hepatocytes grown on polyacrylamide gels with different mechanical properties show differences in albumin production and p450 gene expression.\textsuperscript{20} Mammary epithelial cells grown in stiff 3D collagen gels did not form acini, and had increased FAK (focal adhesion kinase) phosphorylation along with more focal adhesions containing vinculin, both associated with a malignant phenotype.\textsuperscript{21} Spinal neurons exhibit more branching on softer substrates.\textsuperscript{22}

Less is known about the effect of substrate rigidity on endothelial cells than on other cell types. To date, most studies using endothelial cells have looked at the effect of rigidity on the physical state of the cells, rather than their functionality. For example, Gray et. al showed that endothelial cells will migrate towards, and accumulate on, regions of increased stiffness when cultured in 2D.\textsuperscript{12} Several studies have found that like other cell types, when cultured in 2D, endothelial cells will show increased spreading and actin stress fiber formation on stiffer substrates than on soft ones.\textsuperscript{23,8,24}

Much of the work examining the effect of stiffness on endothelial cells has taken place in 2D. There have, however, also been a significant number of studies examining the effect of stiffness on the morphology of endothelial cells in 3D culture. Tubulogenesis (or the formation of tube-like structures) by endothelial cells is significantly affected by stiffness of the substrate in which they are cultured. Studies have consistently found that endothelial cell tubulogenesis is increased in softer gels compared to stiffer ones. Additionally, the morphology of the tubes formed also varied with the substrate’s mechanical properties.\textsuperscript{25-28}
Beyond morphology, many studies examining alterations in endothelial cell function due to changes in substrate rigidity have focused on integrins and focal adhesions. Wallace et. al showed that focal adhesion structure in 2D culture was affected by stiffness, with smaller adhesions found on softer materials. Yamamura et. al found a similar effect in 3D culture, with increased expression of vinculin, a focal adhesion associated protein, in stiff gels compared to softer ones. However, beyond adhesion, there is very little in the literature on the effect of substrate stiffness on the function of endothelial cells.

The question of how substrate rigidity affects endothelial cell function is an important one. In vivo, many diseases, such as atherosclerosis and hypertension, are associated with altered vessel mechanical properties. In addition to clinical effects such as increased systolic blood pressure and an association with increased cardiac death, these changes in mechanical properties have also been linked to alterations in endothelial cell gene expression associated with altered extracellular matrix deposition, integrin expression and cell signaling.

**Tissue Engineered Endothelial Cell Constructs: Motivation**

Knowing how endothelial cell function is affected by substrate stiffness, especially in three dimensions, is important for tissue engineering applications as well. Endothelial cells embedded in Gelfoam, a porous, 3D, gelatin surgical sponge, have been examined for over 10 years as a way to replace the functionality of a damaged endothelium without recapitulating its structure. In this system, endothelial cells are engrafted within the Gelfoam and allowed to grow to confluence. At confluence, the cells produce the same mix of growth factors and heparan sulfate proteoglycans as endothelial cells cultured in two dimensions. Conditioned media from
these cells is able to inhibit smooth muscle cell growth in much the same way as conditioned media from cells cultured on TCPS.

When implanted perivascaularly, these constructs show great promise in the prevention of restenosis after balloon angioplasty.\textsuperscript{32-34} Early studies examined the ability of bovine endothelial cells on Gelfoam to inhibit hyperplasia in rat carotid arteries denuded with balloon angioplasty. Intimal hyperplasia 14 days after endothelial denudation was reduced by 88.2\% versus controls, and versus only 61.8\% by the local delivery of heparin.\textsuperscript{35} The mechanism behind this reduction was related to heparan sulfate proteoglycan secretion by the endothelial cells.\textsuperscript{36} Similar results were seen in a porcine model of carotid artery angioplasty. Intimal hyperplasia was reduced at both 4 weeks (54\% with allogenic cells, and 46\% with xenogenic [bovine] endothelial cells)\textsuperscript{33} and 3 months (56\% and 31\% for porcine and bovine endothelial cells, respectively).\textsuperscript{32} It should be noted that this later time point was taken approximately 2 months after the implant itself had degraded, indicating that the implant’s function lasts long beyond its physical presence.

Endothelial cell implants have promise in the setting of arterio-venous fistula creation for dialysis access. Only 60\% of these are fistulae are patent at one year, due to intimal hyperplasia of the venous side of the fistula caused by increased vascular pressures. Allogenic endothelial cell implants reduce intimal hyperplasia by 35\% at one month and 68\% at two months compared to a sham implant.\textsuperscript{34} A-V grafts similarly benefit from endothelial cell implants, with a reduction of stenosis of 81\% at 28 days.\textsuperscript{37} Use of Gelfoam implants for A-V fistulae has moved to clinical trials, and has recently completed Phase 2 testing.\textsuperscript{38}

In addition to their clinical use, these constructs have the elicited the interesting observation that they cause only a weak immune response after implantation, even when using
xenogenic cells. Endothelial cells embedded in Gelfoam express significantly lower levels of surface adhesion molecules such as ICAM, VCAM, selectins, and MHCs in response to inflammatory stimuli. Cytokine secretion and CD4+ T cell proliferation are also markedly reduced. Other effects seen in vitro include decreased induction of dendritic cell maturation, decreased immune response of splenocytes on exposure to endothelial cells, and alterations in the NF-κB pathway. These results are translated in vivo as well. Allogenic and xenogenic endothelial cell implants elicit a much dampened immune response to embedded endothelial cells than to free endothelial cells, in both naive and pre-sensitized mice.

The original choice of Gelfoam as the scaffold used for supporting endothelial cell growth was based on availability. Although these constructs have been very effective, and display an interesting ability to modulate the immune response, it may be possible to improve their performance. Knowing how the scaffold’s physical and mechanical properties affect endothelial cell function may allow for further optimization of this system in addition to offering insights into endothelial cell biology.
CHAPTER 2: EFFECT OF SCAFFOLD PORE SIZE, DENSITY AND MODULUS ON ENDOTHELIAL CELL GROWTH AND SECRETION

MATERIALS AND METHODS

Production of Gelatin Scaffolds

Gelatin scaffolds with varying structures and mechanical properties were created using a foaming method based on the procedure outlined in the original patent for Gelfoam, a porous gelatin surgical sponge. Type A porcine skin gelatin was dissolved in distilled water to a concentration of 3-6% (wt/vol) and heated to 37°C. 200mL of gelatin solution was transferred to a beaker and placed in a 34°C water bath (to keep solution from hardening without causing foam to melt). An overhead mixer (VWR) was fitted with either a whisk-like Squirrel mixing head (Smooth-on) or a standard propeller. The gelatin solution was mixed at high speed for 5 minutes, creating a thick, white foam which was spread into polypropylene molds. Foams dried at room temperature for 2 days in a laminar flow hood. After trimming to approximately 0.5cm thick, foams were crosslinked and sterilized by placing in sealed heatproof bags and heating in an oven at 145°C for 3 hours. The end-result was a porous, insoluble, sterile gelatin sponge which could be used for cell culture. Two commercially available gelatin sponges, Gelfoam and Surgifoam, were used in addition to the scaffolds made as described above. Scaffold nomenclature is as follows: for lab made scaffolds, percentage of gelatin followed by the mixed type (i.e. a 5% gelatin solution scaffold made with a Squirrel mixing head is referred to as 5S, while a 3%, propeller mixed scaffold is 3P). Gelfoam is referred to as GF, and Surgifoam as SF.
Characterization of Scaffold Density, Pore size and Mechanical Properties

Dry scaffolds were cut into 1cm x 1cm or 1x2x0.5cm pieces and dimensions confirmed using calipers. The mass of each sample was then weighed using a balance with an accuracy of 0.1mg. Density was calculated as mg mass per cm$^3$. Scaffolds were visualized using a Hitachi S3400 scanning electron microscope in low vacuum mode at a magnification of 40x, under 100Pa of pressure, in backscatter mode with a 15kV beam. After imaging, each visible pore was outlined by hand using a tablet PC with ImageJ software. (Figure 1) The same software calculated the diameter and circularity of each pore. Scaffold stiffness was characterized by uniaxial compression. An Instron 8848 Microtester with a 20N load cell compressed the scaffolds at a rate of 0.02mm/s. Young’s modulus was calculated from the initial slope of the resulting stress-strain curve.
Figure 1: Scaffold SEM Images for Pore Size Measurement. Scaffolds were imaged with SEM (Above). The contrast was adjusted make pores easier to see and then individual pores were outlined and diameter measured using ImageJ software. (Below) The scaffold shown here is 6S (6% gelatin made with a Squirrel mixer).
**Cell Isolation and Culture**

Endothelial cells (PAEC) were isolated from porcine aortas using collagenase and cultured in phenol red free Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 2% glutamine. Media was changed three times per week, and cells were used between passages four and eight. For engraftment onto scaffolds, PAEC were trypsinized, resuspended at 1x10^6 cells/mL in culture media and 100μl of cell suspension pipetted evenly onto 1x2cm scaffolds. Scaffolds were incubated for 2 hours at 37°C, transferred to 30mL polypropylene tubes with 10mL of culture media and maintained at 37°C, 10% CO2 with regular media changes. For experiments requiring free cells, cells were recovered by digestion in collagenase IV in PBS at 37°C. After gelatin scaffolds were completely digested, cells were recovered by centrifuging.

**Cell Visualization Using Environmental SEM**

PAEC seeded on Gelfoam were visualized with environmental scanning electron microscopy using an FEI/Philips XL30 FEG ESEM with a cooled stage. Samples were fixed with 4% paraformaldehyde overnight at 4°C and washed thoroughly with distilled water. Wet samples were placed directly on the microscope stage and imaged using a 15kV beam in secondary electron mode. The microscope stage was kept at 1°C, and the relative humidity in the sample chamber was kept above 50% (water vapor pressure above 2 torr) to ensure that the sample did not dry out during imaging.
Cell Visualization Using Quantomix WetSEM Capsules

PAEC seeded on Gelfoam were imaged inside Quantomix WetSEM tissue capsules, which allow visualization of tissues under high vacuum in the hydrated state. Samples were fixed with 4% paraformaldehyde overnight at 4°C and washed thoroughly with PBS. A 2mm biopsy punch was used to cut small samples from the Gelfoam block, and these were further washed with distilled water and stained with 0.5% uranyl acetate for 20 minutes a room temperature. Stained samples were washed three times with distilled water. Some samples were immuno-stained for CD31 (PCAM, an endothelial cell specific cell adhesion molecule) prior to uranyl acetate staining. Samples were blocked 15 minutes in 1% bovine serum albumin (BSA) in PBS followed by incubation with primary antibody (1:10 dilution of mouse anti-CD31 in 1%BSA/PBS) overnight at 4°C with shaking. Samples were washed extensively with 1% BSA/PBS then incubated with goat anti-mouse IgG antibody conjugated with 0.8nm gold beads (1:50, Aurion) overnight at 4°C. Samples were then washed extensively with PBS, post-fixed for eight minutes with 2% gluteraldehyde at room temperature, and washed extensively with distilled water. Gold staining was silver enhanced using an Aurion SE-EM silver enhancement kit and then stained with uranyl acetate as described above. Stained samples were placed in a Quantomix tissue caspsule and imaged using a Hitachi S3400 SEM in backscatter mode with a 10-18kV beam voltage.
Cell Growth

Scaffold were cut to 1x1cm, seeded with 4.5x10^4 PAEC and cultured for up to 19 days under normal culture conditions. On days 0, 2, 5, 9, 14 and 19, cells were recovered from 3 scaffolds of each type and counted using a Coulter Counter.

Preparation of Conditioned Media

Endothelial cell conditioned media was prepared by incubating confluent 1x2cm scaffolds (n=3 for each scaffold type) in 10mL serum free DMEM + PSG for 24 hours. Conditioned medium was then collected, centrifuged to remove any particulates, and the supernatant frozen at -80°C until use. Cells were recovered from scaffolds and counted using a Coulter Counter.

Total Protein, TGF-β1, Prostacyclin Assays

Total protein in conditioned medium was measured using a bicinchoninic acid (BCA) protein assay (Pierce) according to kit directions. TGF-β1 in conditioned medium was quantified using a TGF-β1 ELISA (R&D) according to kit directions. Prostacyclin has a very short half life, therefore its breakdown product 6-keto-prostaglandin F_1α was measured in conditioned medium using an EIA kit (Amersham) according to assay directions. All measured values were normalized to cell number.

Glycosaminoglycan Quantification

Conditioned media was concentrated 2x using 3000 MWCO centrifugal concentrators (Millipore). Total glycosaminoglycan and heparin sulfate proteoglycan in the concentrated media
was measured using a dimethylmethylene blue (DMB) assay. DMB assay solution was prepared according to the method of Farndale et al. To determine the total amount of GAG in samples, 700µl DMB was added to a 500µl sample and absorbance was immediately read at 523 nm using a spectrophotometer (Perkin-Elmer) and compared to a standard curve prepared using chondroitin sulfate in serum free DMEM. To determine the amount of heparan sulfate (HSPG) in the samples, conditioned media was digested with 0.036U/mL protease-free chondroitinase ABC for 3 h at 37°C prior to measurement of GAG with DMB. The GAG remaining after digestion was considered to be the HSPG fraction. All samples were measured with and without chondroitinase digestion, in duplicate.

Statistics

Data are presented as mean +/- standard error (SEM) unless noted. Groups were compared using one way ANOVA. A value of \( p < 0.05 \) was considered significant.

RESULTS

Mechanical Characterization of Gelatin Scaffolds

The Young's modulus of bulk gelatin scaffolds was measured by compression using an Instron mechanical tester. (Figure 2) Scaffold modulus increased with the concentration of gelatin used in the foaming solutions. Additionally, modulus increased at each gelatin concentration when matrices were formed with a propeller rather than the Squirrel mixing attachment. Moduli of the prepared scaffolds ranged from 20.4kPa for scaffold 3S up to 278.5kPa for scaffold 5P. Gelfoam and Surgifoam, the commercial gelatin foams, bracketed the gelatin scaffolds produced in the lab, with a modulus respectively at the low (47.6kPa) and high
(291.4 kPa) ends of the measured range. Statistics were not performed, as only one sample of each type was measured. (Nomenclature, as outlined in Methods, holds here: # = % gelatin solution, S = Squirrel Mixer, P = Propeller Mixer, GF = Gelfoam, SF = Surgifoam)

**Figure 2: Bulk Young’s (Compressive) Modulus of Gelatin Scaffolds.** Strain rate = 0.02 mm/s. Alteration of gelatin concentration and mixer type led to different scaffold mechanical properties. Nomenclature: # = % gelatin solution, S = Squirrel Mixer, P = Propeller Mixer, GF = Gelfoam, SF = Surgifoam.
**Physical Characterization of Gelatin Scaffolds**

Scaffold density (mass per volume), calculated from dimensions and mass, increased with gelatin content. Scaffolds made with the propeller mixer were denser. (Figure 3)

Accordingly, the least dense sample was 3S (7.73 +/- 0.18 mg/cm$^3$), and the most dense 5P (17.99 +/- 2.40 mg/cm$^3$). As with modulus, Gelfoam and Surgifoam fell towards the low and high ends of the density range, respectively. Unlike with true, porous, isotropic structures, density was linearly related to scaffold modulus. (Figure 4) Only Surgifoam did not fit this relationship, possibly due to a difference in the base material. (Surgifoam is made of gelatin, but the exact type and method of production/cross-linking is not known.)
Figure 3: Scaffold Density. Alteration of gelatin concentration and mixer type led to changes in scaffold density. In general, increased gelatin concentration and use of a propeller mixer led to increased density. Nomenclature: # = % gelatin solution, S = Squirrel Mixer, P = Propeller Mixer, GF = Gelfoam (uncompressed), SF = Surgifoam (uncompressed). Data expressed as mean +/- SEM, n=3.

Figure 4: Modulus vs. Scaffold Density. Scaffold density was mostly linearly related to modulus. However, Surgifoam did not fit this linear relationship.
Pore size was measured by imaging scaffolds (Figures 5-6) and using ImageJ software to outline individual pores and measure their diameter. Scaffold pore size decreased with increased gelatin content, and scaffolds made with the propeller mixer had smaller pore sizes. (Figure 7-8) Pore size was not normally distributed, with many more small pores present than large ones in all scaffolds. Maximal average pore diameter was measured in scaffold 3S (263 μm) and minimum average pore diameter in scaffold 6S (133 μm). Gelfoam and Surgifoam had average pore diameters of 212 μm and 157 μm respectively. Although these were the average pore sizes measured, pores as large as 600 μm were seen in all scaffold types. Qualitative differences could be seen in scaffold structure as well. The commercial scaffolds (Gelfoam and Surgifoam) appeared to have thinner pore walls than the scaffolds produced in the lab, and a higher degree of pore interconnectedness. The lab made scaffolds, especially those made using the propeller mixer, clearly had pores which were walled off from their neighbors. These isolated pores were excluded from analysis.
Figure 5: Macroscopic Images of Selected Scaffolds. a) Gelfoam, b) Surgifoam, c) 4S, d) 3P, e) 5P. Scale bar is 1.25cm.
Figure 6: SEM Images of Scaffolds. Scaffolds were imaged with SEM for pore size analysis. A) Gelfoam, B) Surgifoam, C) 3S, D) 4S, E) 5S, F) 6S, G) 3P, H) 5P. Commercial scaffolds (Gelfoam, Surgifoam) appear to have thinner pore wall than those made in the lab. Additionally, the scaffolds made with a propeller mixer do not appear to have fully interconnected pores (some pores are clearly walled off and were not used in analysis).
Figure 7: Pore Diameter. Points represent the average diameter of at least 250 individual pores across 3 fields. Bars represent the standard deviation of pore size within this sample group. (Larger bars = more heterogeneity)
Figure 8: Change in Pore Size Distribution With Gelatin Content Depends on the Type of Mixing Head Used. Top: Change in pore size distribution when using Squirrel mixing attachment. As gelatin content decreases (from 6% to 3%), the mean pore size not only increases (the peak of pore size vs. number fraction moves to the right), but the distribution of pore sizes becomes flatter, with more very large pores. Bottom: Change in pore size distribution when using a propeller mixing attachment. As gelatin content decreases (from 5% to 3%), mean pore size increases, mainly due to a shift in the entire curve to the right. The width of the number fraction peak remains approximately the same.
Visualization of Cells on Gelatin Scaffolds Using SEM

Two methods were used image endothelial cells within the gelatin scaffolds: environmental SEM and conventional SEM paired with Quantomix WetSEM capsules. Gelfoam containing endothelial cells were imaged by environmental SEM was in a cold (1C) chamber with a humid atmosphere in secondary electron mode. Cells were extremely difficult to see using this method. (Figure 9) There was essentially no contrast between the cells and the scaffold, so cells were only able to be identified by their nuclei and edges. Cell-cell boundaries were even more difficult to discern. However, it was still possible to appreciate the way in which cells were wrapped around individual struts of the scaffold, and to see that cells were not completely confluent. That is, areas of scaffold were visible between individual cells. Overall, however, this method was not ideal for in situ imaging. Several images of cells taken using this method are shown below, with a cell free scaffold for comparison.
Figure 9: Environmental SEM Images of Endothelial Cells on Gelfoam. Top: Cell free Gelfoam. Note the smooth walls. Middle: EC on Gelfoam. Note more textured walls, nuclei (white arrows), and cell projections and cell-scaffold boundaries (black arrow). Bottom: High magnification (1500x) image of EC on Gelfoam. Note that the cell-cell boundary is not continuous and that the scaffold is visible between the cells.
The second method used to visualize cells directly in the scaffold used Quantomix WetSEM tissue capsules to visualize uranyl acetate stained endothelial cells on Gelfoam. (Figure 10) Cells imaged using this method were much more clearly visible than those imaged with environmental SEM. Cell-scaffold boundaries and nuclei were extremely clear, though cell-cell boundaries were still not clearly visible. This technique also had the advantage that uranyl acetate staining allowed fine details of the plasma membrane or cytosol to be visible as well (as can be seen in the image below). However, all structural information about the scaffold itself was lost with this technique. Scaffolds placed in the imaging capsules were compressed, masking any information about the scaffold’s pores, and the way the cells interacted with them.

To gain more information about cell interaction, immuno-staining for PCAM was attempted. (Figure 11) Samples were immunostained, labeled with a gold conjugated secondary antibody and silver enhanced before imaging within the WetSEM capsules. While the staining was clearly visible, the process of labeling and silver enhancement appeared to destroy some of the benefits created by imaging in the capsules. Although the cell-scaffold boundary was still clear, the detail of the cell membrane was lost. Additionally, charging artifacts, which were already a problem when using the capsules, became much worse and made it nearly impossible to capture high quality, high magnification images. Finally, although it was possible to easily stain for surface molecules like PCAM, staining for intracellular or subcellular molecules (such as vinculin or integrins) was not successful. WetSEM proved useful for imaging of overall cell morphology. However, it was not ideal for immuno-staining or imaging cell interaction with the pore structure of the scaffold.
Figure 10: WetSEM images of endothelial cells on Gelfoam. Top: Low magnification (85x) image of uranyl acetate stained EC. Note that scaffold is compressed and pore structure is lost. Middle: 500X. Bottom: 900x. Note that cell-scaffold boundary is clear, but cell-cell boundaries are not. Fine details of the cell membrane are visible at this magnification.
Figure 11: PCAM Stained Endothelial Cells Imaged with WetSEM. Top: 500x. Note that cell-cell boundaries (white arrow) are now clear, but at a loss of fine cell detail. Bottom: PCAM stained EC, 2100X. Again, cell-cell boundaries (white arrow) are clear, but fine detail is lost, and charging artifacts (black arrow) are severe.
**Endothelial Cell Growth**

PAEC were seeded onto scaffolds and allowed to grow for up to 19 days. Cells were recovered from scaffolds and counted on days 0, 2, 5, 9, 14 and 19. Cell number increased rapidly between days 0 and 5 for all scaffold types. (Figure 12) After day 5, cell number either plateaued or increased much more slowly until day 19. Final cell number, at day 19, was significantly affected (p < 0.008 by ANOVA) by scaffolds produced in the lab, ranging from a low of 257.4 +/- 16.9 x 10^3 cells for scaffold 5S to a high of 389.6 +/- 24.8 x 10^3 cells for scaffold 5P. (Figure 13) Final cell number was independent of gelatin concentration but did correlate with modulus and density. A parabolic curve can be fit to these data with an r^2 ~ 0.8.

Interestingly, both the commercial sponges included in the study (Gelfoam and Surgifoam), supported significantly higher cell numbers than any of the lab made scaffolds, and do not fall on the cell number/density best fit curve, although they fall on opposite ends of the measured ranges of all measured physical parameters. The reason for this is not clear, but it is possible that the commercial scaffolds have a different chemistry or are cross-linked differently than the scaffolds made in the lab.
Figure 12: Cell Number vs. Time. Cell number increases at a rapid rate until day 5, and then more slowly until day 19.
Figure 13: Final Cell Number (Day 19) vs. Scaffold Modulus. Cell number on lab-made scaffolds is fit by a parabolic curve with an $r^2 = 0.78$. Although the correlation is fairly good, the changes in absolute cell number are very small. Gelfoam and Surgifoam (the commercial scaffolds) are outliers to this curve.
Secretion of Regulatory Factors is Related to Scaffold Properties.

Total secreted protein in conditioned media was measured using a BCA assay and normalized to cell number. (Figure 14) Measured values ranged from $5.34 \pm 0.35 \text{ mg/10}^6 \text{ cells}$ for Gelfoam to $9.12 \pm 0.88 \text{ mg/10}^6 \text{ cells}$ for scaffold 6S. Although ANOVA analysis showed that scaffold type has a significant effect on total secreted protein ($p < 0.0006$), there was no real correlation between protein and any of the measured scaffold parameters. (Figure 15). Total protein secreted per scaffold (irrespective of cell number) was also dependent on scaffold type ($p < 0.04$ by ANOVA), and linearly related to scaffold density ($r^2 \approx 0.7$). (Figure 16) This suggests that the scaffold itself may be interfering in the measurement of total protein, perhaps through degradation or retention of serum containing medium.
Figure 14: Total Secreted Protein (Normalized to Cell Number)  Top: Versus scaffold pore size. Middle: Versus scaffold density. Bottom: Versus scaffold modulus. Note that distribution seems random in all three cases.
Figure 15: Total Protein per Scaffold Versus Scaffold Density. Total protein per scaffold was linearly related to scaffold density, suggesting that the scaffold itself may be interfering in the protein measurement.
Conditioned media was assayed for total TGF-β1, a molecule which regulates cell proliferation and extracellular matrix deposition, by ELISA and normalized to cell number. (Figure 16) TGF-β1 levels were significantly dependent on scaffold type (p < 0.0009 by ANOVA), and range from 1.10 +/- 0.28 ng/10^6 cells for scaffold 3S up to 4.08 +/- 1.14 ng/10^6 cells for scaffold 3P. This difference in TGF-β1 levels is large enough to be functionally significant. TGF-β1 levels did not correspond to scaffold pore size. However, TGF-β1 was related to scaffold modulus by a parabolic fit with a maximum at mid-range moduli, yielding an r^2 ~ 0.6. Although there is a linear relationship between the modulus and density of most scaffolds, Surgifoam does not follow the E ∝ ρ relationship, and does not allow for good correlation between density and TGF-β1 (r^2 ~ 0.3).
Figure 16: Total TGF-β1 (Normalized to Cell Number) Top: Versus pore size. Middle: Versus density. Bottom: Versus modulus. There is no clear relationship between TGF-β1 and pore size or density. However, TGF-β1 is related to scaffold modulus by a parabolic curve which has a maximum at mid range moduli, with an $r^2 = 0.61$. 
The level of prostacyclin, a vasodilator, in conditioned media could not be measured directly due to its short half life. Instead, 6-keto-prostaglandin F\textsubscript{1α}, a breakdown product of prostacyclin, was measured using an EIA kit. (Figure 17) The level of prostaglandin varied significantly with scaffold type (p = 0.001 by ANOVA), ranging from 8.21 ± 0.61 ng/10\textsuperscript{6} cells for Surgifoam to 17.48 ± 0.41 ng/10\textsuperscript{6} cells for scaffold 6S. However, prostaglandin level did not strongly correlate with any of the measured scaffold parameters. The closest match, modulus, correlated only slightly with prostaglandin levels (r\textsuperscript{2} = 0.49 for an inverse parabolic fit). (Similarly to TGF-b1, although there is a linear relationship between the modulus and density of most scaffolds, Surgifoam does not follow the E \propto \rho relationship, and does not allow for good correlation between density and prostacyclin [r\textsuperscript{2}<0.1].) The reason for this is not clear. It is possible that more than one parameter plays an important role in prostacyclin levels, confounding the data, or that some non-measured parameter is playing a role.
Figure 17: Prostacyclin (Normalized to Cell Number). Top: Versus pore diameter. Middle: Versus scaffold density. Bottom: Versus scaffold modulus. Although levels of prostacyclin vary significantly between scaffolds ($p = 0.001$ by ANOVA), its levels do not correlate well with any the measured parameters. The best fit is a parabolic curve with $r^2=0.49$ fit to the data of the modulus vs. prostacyclin graph.
Total sulfated glycosaminoglycan and heparan sulfate proteoglycan were measured in conditioned media using a DMB assay. These are molecules secreted by confluent endothelial cells, which play an important role in the endothelial cell mediated regulation of smooth muscle cell inhibition. Total GAG ranged from a low of 4.99 +/- 0.36 µg/10^6 cells for scaffold 3S to a high of 13.73 +/- 1.81 µg/10^6 cells for scaffold 6S. (Figure 18) HSPG ranged from 3.47 +/- 0.16 µg/10^6 cells for scaffold 3S to 9.74 +/- 0.96 µg/10^6 cells for scaffold 3P. (Figure 19) Although these differences are large secretion of both total GAG and HSPG did not significantly affected by scaffold type given the large errors on the data (p > 0.2 for both by ANOVA). (However, when the scaffold type with the largest error bars is removed from the analysis (scaffold 6S), the change in HSPG with scaffold type does become significant. This suggests that the changes seen may be real, but are being masked by the large error bars generated using this method.)

Total GAG did not correlate with either pore size or scaffold density. However, scaffold modulus and total GAG were able to be related using an inverse parabolic curve (r^2 ~ 0.74). Similarly, HSPG did not correlate well with either pore size or density, but did somewhat correlate with modulus. Like with total GAG, an inverse parabolic curve provided an acceptable fit (r^2 ~ 0.72) between HSPG and scaffold modulus. (Similarly to TGF-b1 and prostacyclin, although there is a linear relationship between the modulus and density of most scaffolds, Surgifoam does not follow the E ~ ð relationship, and does not allow for good correlation between density and either total GAG [r^2 ~ 0.5] or HSPG [r^2 ~ 0.5].)
Figure 18: Total GAG Secretion by Endothelial Cells Top: Versus pore size. Middle: Versus scaffold density. Bottom: Versus scaffold modulus. It was seen that GAG secretion was not correlated to either pore size or density, although there was a fairly good correlation between sulfated GAG and scaffold modulus (r² = 0.744 for a parabolic fit).
Figure 19: HSPG Secretion by Endothelial Cells  
Top: Versus pore size. Middle: Versus scaffold density. Bottom: Versus scaffold modulus. It was seen that HSPG secretion was not correlated to either pore size or density, although there was a fairly good correlation between HSPG and scaffold modulus ($r^2 = 0.717$ for a parabolic fit).
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Table 1: Summary of Chapter 2 Secretion Data. Peak refers to the peak of the *curve* fit to the relevant data.
DISCUSSION

Previous studies utilizing a variety of cell types have shown that three-dimensional structure and mechanical properties can have a significant effect on cells grown in scaffolds. Many different cell types are sensitive to aspects of structure such as pore size or porosity. Pore size has been shown to affect the attachment and proliferation of a variety of cell types, including fibroblasts\cite{46}, vascular smooth muscle cells\cite{47,48} and MC3T3 osteogenic cells\cite{49}. It has additionally been shown to affect cell type specific functions, including cartilage production by chondrocytes\cite{50}, the differentiation of mesenchymal\cite{51} and embryonic\cite{52} stem cells, and bone formation by bone marrow stromal cells\cite{53}. Similarly many types of cells are sensitive to scaffold mechanical properties. Mammary epithelial cells\cite{21}, chondrocytes\cite{54} and endothelial cells\cite{25-28} all have cell type specific functions (formation of acini, cartilage production, and tubulogenesis respectively) which are altered by substrate stiffness in three dimensions.

The aim of the first section of this thesis was to elucidate the effect of scaffold physical properties, including pore size, density and modulus on endothelial cell functions other than tubulogenesis. To achieve this aim, gelatin scaffolds with different physical properties were prepared using a foaming method based on the procedure outlined in the original patent for Gelfoam, and utilizing different percentages of collagen and different mixing heads. Using this technique, and with the addition of two commercial gelatin sponges, it was possible to obtain scaffolds with average pore diameter ranging from 133\textmu m up to 263\textmu m, a 2.3x change in density, and a 14x change in modulus.

In general, all scaffolds appeared to be able to support “normal” endothelial cells. Cells on all scaffolds were able to proliferate, form cobblestone structures (though they did not cover
the entire available area) and secrete regulatory factors such as TGF-β1, prostacyclin, glycosaminoglycans and heparan sulfate proteoglycans. However, the alterations in scaffold structure did appear to make a difference in the level at which many of these functions occurred.

For example, although all cells supported growth, cell number at confluence depended significantly on scaffold type. When taken as a complete group, there was no correlation between cell number and any of the measured scaffold properties. However, when only the lab made scaffolds were taken into account, there was some correlation of cell number with modulus, though not with pore size or density. (The lack of correlation with scaffold density despite the largely linear relationship of density with modulus is due to Surgifoam not following the $E \propto \rho$ curve and reducing the $r^2$ values of correlation fits to below 0.5.) Overall cell function seemed to correlate more strongly with scaffold modulus than with scaffold structure. In the range examined, TGF-β1, GAGs, HPSGs and, to some extent, prostacyclin, were all were nonlinearly related to modulus, while no correlation was seen with pore size or density. (Summarized in Table 1.)

Interestingly, TGF-β1 and HSPG secretion were linearly related to each other, as well as correlating with scaffold modulus. The relationship of these molecules to scaffold modulus, and each other, is not completely unexpected. It has been previously reported in the literature that mechanical stimuli upregulate both TGF-β and HSPGs in endothelial cells through a common pathway.\textsuperscript{55} In our system, cells are not mechanically stressed, but the scaffolds do provide different mechanical environments, which may act in a similar way.

The data gathered in this part of the thesis strongly suggested that modulus plays a larger role in affecting endothelial cell function than pore size or scaffold density, at least in the ranges of those properties examined here. It is still possible that pore size is important for endothelial
cell function. The range of moduli examined in this work was significantly larger than the range of pore sizes. It is entirely possible that the range of pore sizes at which endothelial cell function is altered may not have been hit upon by this scaffold preparation method. Zeltinger et. al. showed that a transition in endothelial cell growth structure (from disconnected webs to confluent multi-layers) takes place at a pore size of ~38μm, while a range of larger pore sizes has no effect.\textsuperscript{48} If this pore size, which is much smaller than the average pore size of any of the scaffolds used in this study, is indicative of the range at which endothelial cell function is affected, the effect of pore size would not necessarily been seen here. Additionally, the very large pore size distribution within each scaffold could have masked any effects of pore diameter as well, as large pores would be offset by small pores, and vice versa.

Based on these data, it was decided that future research efforts would focus on examining the effect of only modulus on endothelial cell function, rather than the effect of scaffold physical properties as a whole. The current system of scaffold production makes it impossible to change only one scaffold property at a time. In our system, scaffold modulus and pore size are related, albeit in a complex rather than linear manner.

In addition to adding the confounding factor of pore size to changes in modulus, the interaction between mechanical properties and structure means that it is difficult to know what the modulus of the material under the cell is (the modulus of the scaffold strut itself). Scaffold bulk modulus is linearly related to strut modulus, but only if the ratio of scaffold density to the density of the scaffold material (in this case gelatin) is unchanged. In our system, bulk modulus changes, but, assuming that the density of gelatin is constant as all solutions were prepared and cured in a similar manner, so does the ratio of bulk density to material density. This means that the modulus the cell sees may not be changing in the same way as the bulk scaffolds'. For
example, it is possible that, because they are made from the same percentage gelatin solution, scaffolds 5S and 5P have the same strut modulus, and that their bulk modulus only differs due to changes in structure. In fact, there is some evidence that this is occurring. When data for TGF-
\[\beta1,\] GAG, HSPG, and prostacyclin secretion are compared with gelatin concentration, the points for 4% gelatin and 5% gelatin fall almost exactly on top of one another, irrespective of mixer type. For prostacyclin, the same holds true for the 3% gelatin scaffolds as well.

This means that to study modulus in isolation, a new system of scaffolds would need to be devised in which the modulus of the scaffold material was altered while leaving structure unchanged.
CONCLUSIONS

Scaffold physical properties can significantly impact the secretory function of endothelial cells cultured in three dimensions. Scaffold modulus plays a larger role in this impact than either pore size or scaffold density. Thus, the remainder of the work in thesis will focus on examining the effect of an isolated change in scaffold mechanical properties on endothelial cell function.
CHAPTER 3: EFFECT OF SCAFFOLD MODULUS ON ENDOTHELIAL CELL REGULATION OF GROWTH AND SMOOTH MUSCLE CELL INHIBITION

INTRODUCTION

In Chapter 2 we found that the effect of scaffold modulus was dominant over that of pore size or relative density on the function of endothelial cells. Based on those results, it was decided to focus on the effect of isolated changes in scaffold modulus.

The first aim of this chapter was to develop a system of scaffolds in which only one physical parameter, modulus, was altered. Scaffold bulk modulus is related to the relative density of the scaffold, the modulus of the material from which the scaffold is made, and the density of the material from which the scaffold was made. By choosing one scaffold structure and then adding chemical crosslinks, it was possible to alter the modulus of the base material, and therefore of the scaffold, even while keeping the other two parameters the same. Gelfoam was chosen as the base scaffold material because the biology of endothelial cells grown within it has already been well characterized.7,32-34,37

The second aim of this chapter was to determine how changes in substrate modulus affect endothelial cell growth and secretion of several growth regulatory factors found in healthy endothelial cells including TGF-β1, FGF2, PDGF-BB and heparan sulfate proteoglycans. The third aim was to correlate any changes in secretion of growth regulators to changes in endothelial cell functionality, namely smooth muscle cell growth inhibition.

The next aim was to determine if changes in secretion and functionality were related to changes in the interface between the cells and their substrate – the extracellular matrix and integrins. Finally, we attempted to create a model which could explain how the endothelial cell
growth regulatory system is mediated by substrate modulus and changes in the cell/substrate interface..

METHODS

*Alteration of Gelfoam Modulus*

Gelatin scaffolds of varying modulus were created by modifying a commercially available gelatin surgical sponge (Gelfoam). Three different methods were examined to alter the mechanical properties of the sponge. First, scaffolds were heat treated at 145°C for 0, 6 or 24 hours to increase gelatin crosslink density and increase scaffold modulus. Second, scaffold modulus was altered through carbodiimide chemistry. 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC, EMD Biosciences) and N-hydroxysuccinimide (NHS, Thermo Scientific) created amide bonds between the amine and carboxyl groups of gelatin.\(^{54,56}\) Scaffolds were hydrated and incubated in a sterile solution of EDAC/NHS in PBS twice for 1.5 hours at room temperature with shaking, followed by 4, 15 minute washes with sterile PBS. The concentrations of EDAC and NHS were varied (54.0/22.0, 36.1/14.6, 9.0/3.6, 3.6/1.4 EDAC/NHS mM) to create different degrees of cross-linking, and therefore different degrees of stiffness. Finally, scaffolds of reduced stiffness were created by autoclaving Gelfoam in PBS for either 10 or 20 minutes at 121°C. Only scaffolds created by EDAC/NHS crosslinking and autoclaving, in addition to unmodified Gelfoam, were used for further experiments. All scaffolds were stored in sterile PBS at 4°C until use.
Scaffold Mechanical Property Characterization

Scaffold stiffness was characterized by uniaxial compression using a Zwick mechanical tester with a 20N load cell. 12mm disks of hydrated scaffolds were cut using a biopsy punch. Scaffolds were immersed in a PBS bath and compressed at a rate of 0.01mm/s, and the Young’s modulus calculated from the initial slope of the resulting stress-strain curve.

Endothelial Cell Culture and Scaffold Engraftment

Human aortic endothelial cells (HAEC) from three healthy donors were purchased from Promocell, pooled and used between passages 5 and 8. Prior to scaffold engraftment, cells were cultured on dishes coated with 0.1% type A porcine gelatin for 30 minutes. Cells were maintained in endothelial cell growth medium 2 (ECGM2, Promocell) supplemented with 7% FBS and 1% penicillin-streptomycin at 37°C, 5% CO2 with 3 media changes per week. For culture on scaffolds, HAEC were trypsinized, resuspended at 1x10^6 cells/mL in culture media and pipetted evenly onto scaffolds at a density of ~3.6x10^4 cells/cm^2. Scaffolds were incubated for 3 hours at 37°C, transferred to 30mL polypropylene tubes with 6mL of culture media and incubated at 37°C, 5% CO2. For experiments requiring free cells, cells were recovered by digestion in collagenase IV in 1:1 ECGM2:PBS at 37C. After gelatin scaffolds were completely digested, cells were recovered by centrifuging.

Preparation of Scaffolds for Staining

Scaffolds were cultured for the desired period of time, rinsed with PBS and fixed with 4% paraformaldehyde overnight at 4°C. All subsequent steps were carried out on ice or at 4°C. Following fixation, scaffolds were washed 3x 5 minutes with PBS followed by a 10 minute
incubation with 200mM glycine in PBS to quench remaining free aldehyde groups. Scaffolds were again washed 3x 5 minutes with PBS and then transferred to ice cold 18% sucrose in PBS for 3 hours, followed by ice cold 30% sucrose for an additional 3 hours. Scaffolds were then washed thoroughly with PBS and flash frozen with liquid nitrogen. Sections with a thickness of 20-60μm were cut using a cryotome and captured on positively charged slides. (SuperFrost Plus, VWR) Sections were stored at -80°C for up to 3 weeks before staining.

**Actin and Immuno-staining**

Slides were allowed to reach room temperature and a PAP pen (Electron Microscopy Sciences) used to create a hydrophobic barrier around each section. Sections were washed twice with PBS and then permeabilized with 0.2% Triton X-100 in PBS for 10 minutes. Sections were blocked for 1 hour in 1% BSA + 20% goat serum in PBS. Cells were immediately incubated in primary antibody to PCAM (1:50, mouse anti-porcine CD31, Serotec) in 1%BSA/PBS overnight at 4°C in a humidified chamber. Sections were then washed 3x five minutes with 1%BSA/PBS and incubated 1 hour in the dark at room temperature with a fluorescent secondary antibody (Alexa 488 conjugated goat anti-mouse IgG, 1:75, Invitrogen) in 1%BSA/PBS. In some cases a 1:200 dilution of rhodamine phalloidin was added to the secondary antibody solution. Finally, sections were washed 3x five minutes with PBS and coverslipped using a fade resistant mounting medium containing DAPI, a blue fluorescent nuclear stain (Vectashield with DAPI, Vector). Stained samples were stored in the dark at 4°C until imaged.
**Imaging and Actin Quantification**

Sections were imaged using a Perkin-Elmer spinning disk confocal microscope at 63X magnification with an oil immersion lens. Samples were imaged at z-intervals of 1 μm. The resulting z-stack was turned into a maximal intensity z-projection image using ImageJ software. For quantification of actin staining intensity only, samples were imaged using a Zeiss LSM 510 confocal microscope at 40X magnification with a water immersion lens. After the creation of a z-projection image, ImageJ was used to select only those areas of the image representing cells, and the average pixel intensity of image quantified. The average of 10, 40x fields was used to calculate actin intensity for each scaffold type.

**Cell Growth**

8mm diameter scaffold discs of each type were prepared and seeded with 2x10⁴ HAEC. Scaffolds were cultured for up to 28 days under normal conditions. On days 0, 2, 5, 8, 12, 16, 21 and 28, cells were recovered from 3 scaffolds of each type and counted using a Coulter Counter. Cell number was plotted as a function of both time and modulus. Growth rate for each scaffold type was determined to be the slope of the linear regression of the cell number vs. time curve during the growth phase (days 5-21).

**Conditioned Media Preparation**

Endothelial cell conditioned media was prepared for use in assays of secreted factors and for smooth muscle cell inhibition experiments. For non-immune assays, a low serum conditioned media was prepared to reduce interference from factors contained in FBS. This conditioned media was prepared by washing confluent, 12mm scaffolds with serum free medium for 3x 10
minutes, followed by incubation in 3.5mL endothelial cell basal medium 2 (ECBM2, Promocell) supplemented with 0.5% FBS and antibiotics for 24 hours. Conditioned medium was then collected, centrifuged to remove any particulates, and the supernantant frozen at -80C until use. Cells were recovered from scaffolds and counted using a Coulter Counter.

Assays of Growth Regulatory Factors

Endothelial cell secretion of several growth regulatory factors was examined. Low serum conditioned media was assayed for TGF-β1, FGF2, and PDGF. All were measured using colorometric ELISAs from R&D according to kit directions.

Glycosaminoglycan and Heparan Sulfate Proteoglycan Assay

The concentrations of sulfated glycosaminoglycans and heparan sulfate proteoglycans (which have been implicated in the smooth muscle cell growth inhibition function of endothelial cells) were measured using a DMB assay as described in Chapter 2.

Smooth Muscle Cell Inhibition

The effect of EC conditioned media on smooth muscle cell proliferation was measured using a radioactive thymidine incorporation assay. Human aortic smooth muscle cells (HASMC) were purchased from Promocell and cultured at 37C, 5% CO₂ in DMEM supplemented with 5% calf serum (CS), penicillin-streptomycin (PS) and glutamine.

For inhibition experiments, HASMCs were sparsely seeded in 48 well plates, allowed to attach overnight, washed twice with PBS, and then starved for 24 hours with DMEM supplemented with 0.1% CS and PS. Following starvation, culture medium was replaced with
either low serum endothelial cell conditioned medium (n = 3, in duplicate) or fresh ECBM2+0.5% FBS+PS (n = 3, in duplicate) and all wells then adjusted to 5% total serum. After 24 hours, to measure proliferation, $^3$H-thymidine was added to each well to a concentration of 1μCi/mL and the cells incubated an additional 6 hours. Cells were then washed 3x with ice cold PBS followed by incubation with 10% trichloroacteic acid in PBS for 30 minutes at 4C. Cells were washed 2x with 95% ethanol and solubilized with 0.1% SDS in 0.25N NaOH. The lysate was transferred to scintillation vials with UltimaGold LSC cocktail and $^3$H-thymidine incorporation measured using a liquid scintillation counter and QuantaSmart software. Results were expressed as a percent decrease in $^3$H-thymidine incorporation vs. control medium, either per scaffold or normalized to endothelial cell number.

**Integrin and Extracellular Matrix Gene Expression**

The effect of the substrate modulus on cell attachment, gene expression of various adhesions molecules (integrins) and extracellular matrix proteins was examined. After 12 or 21 days of culture, 49.9Pa, 173Pa and 1345Pa scaffolds were rinsed with PBS, flash frozen with liquid nitrogen and cut into thin sections using a cryotome. Total RNA was extracted from the cut scaffolds using an RNeasy Mini Kit with on column DNase treatment (Qiagen, Valencia, CA) according to kit directions, except that the volume of lysis buffer was increased to 1mL to accommodate the volume of the scaffold. Complementary DNA was synthesized by reverse transcription using the TaqMan reverse transcription reagents from Applied Biosystems (Foster City, CA). Real-time PCR analysis was performed with an Opticon Real Time PCR Machine (MJ Research, see Table 2 below for settings) using the SYBR Green PCR Master Mix Reagent Kit (Applied Biosystems) and primers purchased from Invitrogen. (Table 3) Melting curves were
done and examined to ensure that only the transcript of interest was amplified during the PCR reaction, and only those genes with good melting curves were subsequently analyzed. Transcript copy numbers were calculated from the C(t) value measured by the Opticon software.

<table>
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Table 2: PCR Settings. These settings were used with the MJ Opticon PCR machine for RT-PCR analysis of endothelial cell gene expression.
Table 3: PCR Primers. All primers were designed with Primer3 and purchased from Invitrogen.

Integrin Flow Cytometry

Two important endothelial cell integrins, $\alpha_5\beta_3$ and $\alpha_5$ (found as $\alpha_5\beta_1$ in endothelial cells) were measured using flow cytometry to see how substrate modulus affects their expression.

Endothelial cells were grown on the full range of scaffolds until confluence and the cells recovered by collagenase treatment. These cells were washed 2x with ice cold PBS + 2% heat inactivated FBS + 0.09% sodium azide (Cell Staining Buffer, BD). $3 \times 10^5$ cells from each sample were resuspended in 100ul of staining buffer and the appropriate volume of FITC conjugated antibody (mouse anti-human integrin $\alpha_5$ or mouse anti-human integrin $\alpha_5\beta_3$). After briefly vortexing to mix, cells were incubated on ice for 45 minutes in the dark with shaking. Cells were then centrifuged and washed 2x with ice cold staining buffer to remove excess antibody. Finally,
cells were resuspended in 500µl of 1% paraformaldehyde in PBS. 10⁴ cells were analyzed by flow cytometry using a FACS caliber instrument and CellQuest software (Becton Dickinson, San Diego, CA). All samples were compared to similarly prepared cells stained with the appropriate FITC conjugated isotype controls.

Statistics

Statistical analyses were performed with Prism (GraphPad) or Excel (Microsoft) software. Data are expressed as mean +/- SEM unless noted. Comparisons between groups were made by ANOVA followed by Tukey’s multiple comparison test. A value of p < 0.05 was considered statistically significant.

RESULTS

Scaffold Mechanical Properties are Related to Treatment

Three different methods were examined to alter the mechanical properties of Gelfoam. The first method, heat treatment, yielded only small changes in compressive modulus of Gelfoam. (Figure 20) Non-heat treated Gelfoam had a modulus of 297.1 +/- 21.9 Pa. Gelfoam treated for 6 hours at 145C had a modulus of 348.7 +/- 6.9 Pa. Gelfoam treated for 24 hours at 145C had a modulus of 358.9 +/- 16.9 Pa. There was a maximal difference of only ~20% with 24 hours of heat treatment, likely due to the fact that the heat used to cross-link the Gelfoam was already close to saturation prior to treatment.

The second method used to alter the mechanical properties of Gelfoam was crosslinking using a carbodiimide solution. (Figure 21) With this treatment method, scaffold modulus was dependent on crosslinker concentration (p < 0.0001 by ANOVA). Unmodified Gelfoam had a
hydrated modulus of 173 +/- 5.9Pa which could increased to 1345 +/- 34.5Pa by treatment with 54.0/22.0 EDAC/NHS mM solution. This is a nearly eight fold increase in modulus over the base scaffold – a much larger range than provided by heat treatment. Additionally, the increase in scaffold modulus with EDAC/NHS treatment was linearly related (r²=0.98) to the concentration of cross-linker used, allowing easy creation of scaffolds with a desired modulus. (Figure 22)

The final method used to alter the modulus of Gelfoam involving autoclaving matrices to break protein-protein bonds and reduce scaffold stiffness. (Figure 21) Ten minutes of autoclaving was sufficient to reduce the modulus of Gelfoam from 173 +/- 5.9Pa to 68.7 +/- 12.9 Pa. Twenty minutes of autoclaving further reduced the modulus to 49.9 +/- 16.7 Pa - a ~71% reduction in modulus from the base material. It should be noted that scaffolds autoclaved for 20 minutes are extremely fragile and easily damaged even with normal handling.

By using the carbodiimide and autoclaving methods, it was possible to create a set of scaffolds with a 26 fold difference in modulus between the softest and stiffest scaffolds.
Figure 20: Effect of Heat Treatment on Gelfoam Scaffold Modulus. Although heat treatment offered tight control over scaffold modulus, only small absolute (and relative) changes were able to be achieved.
Figure 21: Effect of Autoclaving and Carbodiimide Crosslinking on Gelfoam Scaffold Modulus. By combining autoclaving and crosslinking methods, a 26x difference in modulus between softest and stiffest scaffolds was obtained. Deformation rate = 0.01 mm/s, p < 0.0001 by ANOVA.

Figure 22: Scaffold Modulus is Linearly Related to Crosslinker Concentration. EDAC concentration is directly related to scaffold modulus ($r^2 = 0.98$), allowing the easy creation of scaffolds of any modulus within this range. (Note that the ratio EDAC:NHS stays constant through all treatments.)
Endothelial cells on scaffolds have normal morphology and can be visualized using confocal imaging techniques.

Endothelial cells on scaffolds were prepared for confocal imaging by fixing, cryoprotecting, flash freezing, sectioning and immunostaining. Using this multi-step technique, it was possible to directly image cells within the scaffold. PECAM antibody staining revealed colonies of endothelial cells in cobblestone-like patterns within scaffolds. (Figure 23) Although cells reached a quiescent state, as seen by the cessation of cell proliferation after day 21, they did not populate all available surfaces within the scaffold, instead forming islands of cells, not all of which were interconnected. These islands were found throughout the scaffold, but were much more extensive along the periphery of the scaffold, where cell networks tended to be much larger than in the interior of the scaffold. Cells grew on both the flat surfaces of pore walls and wrapped around thinner struts. Images below show cells on 49.9Pa and 1345Pa scaffolds.
Figure 23: Endothelial Cells in Scaffolds Grow in a Normal Cobblestone Pattern.

Endothelial cells in 49.9Pa (A,B) and 1345Pa (C,D) scaffolds expressed a normal cobblestone morphology with PCAM expressed at their edges. Cells can be seen growing in relatively flat islands (B,C) as well as wrapped around pore walls and struts (A,D). Stress fibers are visible in most cells, regardless of the scaffold modulus.
Actin cytoskeleton is similar across scaffolds.

Actin intensity was quantified in 10 40x fields for 49.9Pa and 1345Pa scaffolds. Some qualitative differences were seen in endothelial cells on different matrices. Some cells on 1345Pa scaffolds appeared to have more stress fibers than cells on 49.9Pa scaffolds, although both cell types had prominent stress fibers. (Figure 23) However, when total actin was quantified, average pixel intensity per area within each rhodamine phalloidin stained cell, the overall level of actin was identical between the two types of scaffolds. (Figure 24) Rhodamine phalloidin stains filamentous actin only, and staining is brighter when larger stress fibers are present. That the intensity of staining was the same on both scaffold moduli suggests that their cytoskeletons were similar.

Figure 24: Average Brightness of Rhodamine Phalloidin Stained Cells is Independent of Scaffold Type. The average pixel intensity of actin staining with cells was measured and unchanged between 49.9Pa and 1345Pa scaffolds. (p = 0.985)
**Endothelial Cell Growth Rate and Cell Number is Affected by Scaffold Modulus**

Endothelial cells proliferated and reached confluence in all scaffolds by ~21 days. (p = NS for difference between cell numbers on day 21 and day 28 by t-test for all scaffold types.) In all scaffolds, cell number began to increase slowly between days 2-5. (Figure 25) Cell number at confluence (day 21) was dependent on modulus (p < 0.0004 by ANOVA). Cell number was highest on scaffolds of 508Pa, with a 831.9 +/- 0.116 x10^3 cells. Cell number decreased at both higher and lower scaffold moduli, reaching 387.5 +/- 1.194 x10^3 cells on 49.9Pa scaffolds, and 518.0 +/- 0.127 x10^3 cells on 1345Pa scaffolds. (Figure 26) The large error bars on the 49.9Pa scaffold cell number can likely be attributed to the fragility of the scaffolds – just the act of removing the scaffold from media to count cells was enough to damage the scaffolds in some cases, causing error in the measurements.

By day 5, growth rate (Figure 27) became nearly linear, and could be approximated by a straight line for all scaffolds. Growth rate was dependent on scaffold modulus (p < 0.0001 by ANOVA) and followed a similar pattern to cell number, with 0.297 +/- 0.014 x10^3 cells per day, 0.469 +/- 0.033 x10^3 cells per day, and 0.264 +/- 0.051 x10^3 cells per day on scaffolds of 1345Pa, 508Pa, and 49.9Pa respectively. All pairs of cell growth rate were statistically different (p < 0.05 by Tukey’s post test) except for 173Pa vs. 68.7Pa and 508Pa vs. 376.3Pa.
Figure 25: Cell Number vs. Days Growth. Cell number begins to increase between days 2-5 for all scaffolds. Rate of cell growth increases around day 5 and becomes nearly linear through day 21 for most scaffold types. Cell number plateaus around day 21 for all scaffolds.

Figure 26: Cell Number Versus Modulus. Each line represents a different time point (labeled with days of culture next to each line). Cell numbers at day 21 and 28 are not statistically different. Maximum cell number is reached on 508Pa scaffolds.
Figure 27: Growth Rate is Dependent on Scaffold Modulus. (p < 0.0001) Growth rate, calculated as the slope of the growth curve between days 5-21, peaked at 508Pa. All pairs of rates are statistically different (p < 0.05 by Tukey’s post test) except 173Pa vs. 68.7Pa and 508Pa vs. 376.3Pa.
Secretion of Growth Factors

TGF-β1 in conditioned media was measured using a colorometric ELISA kit and normalized to cell number. (Figure 28) TGF-β1 levels ranged from 2289 +/- 123 pg/10^6 cells on 1345Pa scaffolds up to 2826 +/- 184 pg/10^6 cells on 376Pa scaffolds. The differences were relatively small, and the differences between scaffolds were not significant. (p = 0.795 by ANOVA), indicating that scaffold modulus did not effect its secretion. In fact, the total TGF-β1 secreted by each scaffold was linearly related to cell number in the scaffold (r^2 = 0.948). (Figure 28)
Figure 28: TGF-β1 versus modulus and cell number. Top: TGF-β1 secretion is not affected by scaffold modulus. Differences in TGF-β1 secretion were small and not significant (p = 0.878 by ANOVA). Bottom: Total TGF-β1 secreted by each scaffold is linearly related to cell number. (r² = 0.948)
FGF2 in conditioned media was measured using a colorometric ELISA kit and normalized to cell number. (Figure 29) FGF2 levels ranged from 228.0 +/- 34.9 pg/10^6 cells on 68.7Pa scaffolds up to 363.9 +/- 7.6 pg/10^6 cells on 1062Pa scaffolds. The differences were relatively small but were statistically significant. (p = 0.005 by ANOVA), indicating that scaffold modulus does effect its secretion. A parabolic curve was able to be fit to the data with an r^2 = 0.77.

**Figure 29: FGF2 Secretion is Dependent on Scaffold Modulus.** FGF2 significantly varies with modulus (p = 0.005 by ANOVA). A parabolic curve can be fit to the data with r^2=0.767.
PDGF-BB in conditioned media was measured using a colorometric ELISA kit and normalized to cell number. (Figure 30) PDGF-BB levels ranged from 122.3 +/- 8.6 pg/10^6 cells on 173Pa scaffolds up to 229.7 +/- 12.0 pg/10^6 cells on 1062Pa scaffolds. The differences were relatively small, but the differences between scaffolds were significant. (p = 0.01 by ANOVA), indicating that scaffold modulus does affect its secretion. Although there were differences in secretion between groups, there was no apparent correlation with scaffold modulus, and only one pair of scaffolds is statistically different by Tukey’s post test (173Pa and 1062Pa). In fact, the dependence of PDGF-BB secretion on scaffold modulus is completely due to one point: 173Pa. When this point is removed, ANOVA gives a value of p = 0.174 for differences between groups.
**Figure 30: PDGF-BB Secretion.** PDGF-BB secretion is dependent on modulus \((p = 0.01 \text{ by ANOVA})\). However, there does not appear to be any correlation between modulus and secretion, and the dependence on modulus disappears if one point (*) is removed from the analysis.
**Heparan Sulfate Proteoglycan Secretion is Dependent on Modulus**

Total sulfated glycosaminoglycan (GAG) and heparan sulfate proteoglycan (HSPG), a sulfated glycosaminoglycan secreted by confluent endothelial cells, which inhibits the growth of smooth muscle cells, was measured using a DMB assay. Both total GAG and HSPG secretion were dependent on scaffold modulus (p < 0.00001 for GAG and p = 0.03 for HSPG, by ANOVA). (Figure 31) Both GAG and HSPG followed the same general pattern, with the highest levels found in the softest scaffolds (68.7Pa and 49.9Pa), with levels then decreasing and plateauing. The maximum level of total GAG measured was in the 49.9Pa scaffold, at 10.6 +/- 1.2 μg/10⁶ cells, and the minimum level was 4.4 +/- 0.2 μg/10⁶ cells at a modulus of 508Pa. The maximum level of HSPG measured was 8.8 +/- 1.6 μg/10⁶ cells at 49.9Pa, and the minimum level of HSPG was 4.6 +/- 0.4 μg/10⁶ cells at 508Pa.
Figure 31: GAG and HSPG Versus Modulus. Top: Total GAG was dependent on modulus (p < 0.0001 by ANOVA). Secretion was highest at low moduli, and then dropped to a plateau.
Bottom: HSPG was also dependent on modulus (p = 0.03 by ANOVA). Again, secretion was highest at low moduli, with a plateau starting around 173Pa.
Smooth Muscle Cell Inhibition is Dependent on Modulus

Inhibition of smooth muscle cell proliferation by endothelial cell conditioned media was measured using a 3H thymidine incorporation assay. The inhibition of smooth muscle cell inhibition by endothelial cell conditioned medium (vs. control medium) was strongly dependent on scaffold type, both when normalized to endothelial cell number (p < 0.0004) and when expressed as inhibition per scaffold (p < 0.0006). (Figure 32)

When normalized to cell number (as a measure of the inhibitory potential of each cell within the scaffold), inhibition is highest for the softest scaffold (97.5 +/- 22.1 % per 10^6 cells) and drops with increased modulus, until a plateau is reached at ~376Pa (32.2 +/- 2.8 % per 10^6 cells). The minimum inhibition is seen at 508Pa, with inhibition of 20.0 +/- 0.6 % per 10^6 cells. The inhibition curve can be approximated by a power curve with an r^2 = 0.79. These data indicate that the inhibitory potential of cells is highest at low moduli, and then decreases until a plateau is reached at higher moduli.

These data correlate well with the HSPG secretion data presented earlier. There is a linear relationship between HSPG secretion and smooth muscle cell proliferation inhibition normalized to cell number (r^2 = 0.799). (Figure 33) This correlation becomes even stronger (r^2 = 0.933) if the common “plateau” region of inhibition and HSPG secretion (1062Pa and 1345Pa) is removed from the analysis, suggesting that at high moduli, factors other than HSPG may play a role in the inhibitory potential of endothelial cells.

When data are expressed as decrease in smooth muscle 3H-thymidine incorporation per scaffold, inhibition peaks at an intermediate modulus of 173Pa, with 51.8% +/- 3.6% inhibition. Inhibition then dropped off at both higher and lower moduli, reaching a low of 21.2 +/- 0.8% at a modulus of 1345Pa.
Figure 32: Smooth Muscle Cell Inhibition Versus Modulus. Top: Inhibition normalized to endothelial cell number. Inhibition at low modulus appears to be modulus dependent, while it is modulus independent at higher moduli. Bottom: Inhibition per scaffold (not normalized to cell number). Maximal inhibition occurs at an intermediate modulus, 173Pa, and drops off at both lower and higher moduli.
Figure 33: Smooth Muscle Cell Inhibition (Normalized to Cell Number) Versus HSPG Secretion. Top: Inhibition is linearly related to HSPG secretion, with $r^2 = 0.799$. This correlation improves to $r^2 = 0.933$ (Bottom) when “plateau” points are removed, indicating other factors may be involved in this region.
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<td>Non-Normalized SMC Inhibition</td>
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Table 4: Summary of Cell Growth and Secretion Data From Chapter 3. Peak refers to the peak of the curve fit to the relevant data unless it occurs at the end of the range of modulus (i.e. 50Pa or 1345Pa).
Extracellular Matrix Protein Gene Expression is a Function of Modulus and Changes with Confluence.

To determine if the mechanism by which functional changes are induced in endothelial cells is related to changes in the cell-substrate interface, expression of extracellular matrix genes was examined using RT-PCR. Gene expression was examined at 12 days, before cells reached confluence, and at 21 days, after confluence was reached. At both time points some extracellular matrix genes were differentially expressed on different scaffold types. Only three scaffold moduli were used in this set of experiments: 49.9Pa, 173Pa and 1345Pa. All data for each gene were normalized to the average 49.9Pa scaffold expression for that gene. N = 6 for all. Data was analyzed by ANOVA followed by Tukey’s post test. A value of p < 0.05 was considered significant. Versican, decorin, MMP9 and tenascin data were not used in analysis, as the melting curves for these primers showed multiple peaks.

Analysis of day 12 RT-PCR data showed that there were small (generally a change of less than 50% of the value of 173Pa scaffold expression) but statistically significant differences in the expression of some extracellular matrix genes in endothelial cells grown on scaffold of different moduli. The data are summarized in the graphs below. Collagen, one of the major structural proteins of the endothelial extracellular matrix, was differentially expressed on different scaffolds. Collagen IV, α1 (the most abundant collagen in endothelial cell matrix) was expressed significantly more on 173Pa scaffolds than on 49.9Pa or 1345Pa scaffolds. Collagen IV, α5 was expressed statistically significantly more on 173Pa scaffolds than on 49.9Pa scaffolds. Collagen III, α1 was expressed statically significantly more on 173Pa scaffolds than on 1345Pa scaffolds. In
general, collagens seem to be upregulated on 173Pa scaffolds compared to other scaffold moduli. (Figure 34)

Other matrix protein genes were also differentially expressed. Expression of the non-heparan sulfate proteoglycan core protein biglycan was statistically lower on 1345Pa scaffolds than on 173Pa or 49.9Pa scaffolds. Fibrillin expression was statistically higher on 1345Pa scaffolds than on 49.9Pa ones. Elastin, which would be expected to decrease the stiffness (and therefore modulus) of a substrate, was expressed statistically significantly more on 1345Pa scaffolds than either 49.9Pa scaffolds or 173Pa scaffolds. This was the largest difference seen, with over a two-fold change between the two softer scaffolds and the 1345Pa one. (Figure 35)

In addition to the matrix protein genes themselves, genes for enzymes which control remodeling of the matrix were also affected. MMP2 expression was significantly higher on 173Pa scaffolds than on 1345Pa scaffolds. TIMP2 expression was significantly higher on 173Pa scaffolds than on either 49.9Pa or 1345Pa scaffolds. And MT1-MMP expression was higher on 173Pa scaffolds than on 49.9Pa scaffolds. These data suggest that extracellular matrix turnover was occurring more on 173Pa scaffolds than on the 49.9Pa or 1345Pa scaffolds, which corresponds to the growth rates measured for these scaffold types. (Matrix turnover would be expected to increase as cell proliferation increased.) (Figure 36)
Figure 34: Collagen Gene Expression Is Altered With Scaffold Stiffness at Day 12. Expression of collagen IV $\alpha_1$, collagen IV $\alpha_5$, and collagen III $\alpha_1$ were all affected by scaffold modulus. Expression was measured by RT-PCR. $n = 6$ for all. Data analyzed by ANOVA followed by Tukey’s post test.

![Collagen Gene Expression Graph](image)

Figure 35: Other Extracellular Matrix Genes Were Affected By Scaffold Stiffness, As Well. Biglycan was down regulated on 1345Pa scaffolds, while elastin was up regulated. Fibrillin was higher on 1345Pa scaffolds compared to on 49.9Pa ones. Expression was measured by RT-PCR. $n = 6$ for all. Data analyzed by ANOVA followed by Tukey’s post test.

![Other Extracellular Matrix Genes Graph](image)
Figure 36: Genes Controlling Matrix Remodeling Are Affected By Scaffold Modulus. MT1-MMP, MMP2, and TIMP2 were all upregulated on 173Pa scaffolds. This follows the pattern of cell growth rate. Expression was measured by RT-PCR. n = 6 for all. Data analyzed by ANOVA followed by Tukey’s post test.
Analysis of day 21 (confluence) RT-PCR data showed that there were small but statistically significant differences in the expression of some extracellular matrix genes in endothelial cells grown on scaffolds of different moduli (Figures 37-39 below). Some collagens were differentially expressed on different scaffolds. Collagen IV, α1 and collagen IV, α5 were expressed statistically significantly more on 49.9Pa scaffolds than on 1345Pa scaffolds. (Figure 37) Fibronectin expression was statistically higher on 49.9Pa scaffolds than on either 173Pa or 1345Pa scaffolds. Perlecan, a proteoglycan normally attached to heparan sulfate, was expressed at higher levels on 49.9Pa scaffolds than on 173Pa or 1345Pa scaffolds. Biglycan was statistically significantly upregulated on 49.9Pa scaffolds compared to 134Pa scaffolds. Elastin expression was statistically significantly higher on 1345Pa scaffolds compared to on 173Pa. (Like with subconfluent scaffolds, this was the largest fold change seen.) Finally, fibrillin was down regulated statistically significantly on 1345Pa scaffolds compared to 49.9Pa ones. (Figure 38)

Genes for proteins involved in matrix remodeling were also affected by scaffold modulus. TIMP1 and TIMP2 were both upregulated statistically significantly on 1345Pa scaffolds compared to 173Pa scaffolds, and TIMP2 was also upregulated on 1345Pa scaffolds compared to 49.9Pa ones. (Figure 39)
Figure 37: Collagen Gene Expression Is Altered With Scaffold Stiffness at Day 21. Expression of collagen IV α1 and collagen IV α5 were up regulated on 49.9Pa scaffolds compared to 1345Pa ones. Expression was measured by RT-PCR. n = 6 for all. Data analyzed by ANOVA followed by Tukey’s post test.

Figure 38: Other Extracellular Matrix Genes Were Affected By Scaffold Stiffness, As Well. Fibronectin, perlecan and biglycan were down regulated on stiffer (173Pa, 1345Pa) scaffolds, while elastin was up regulated on 1345Pa scaffolds. Fibrillin was higher on 49.9Pa scaffolds when compared to on 1345Pa ones. Expression was measured by RT-PCR. n = 6 for all. Data analyzed by ANOVA followed by Tukey’s post test.
Figure 39: TIMP Expression Is Affected By Scaffold Modulus. TIMP1 and TIMP2 were upregulated on 1345Pa scaffolds compared to softer ones. MT1-MMP and MMP2 were not affected. Expression was measured by RT-PCR. n = 6 for all. Data analyzed by ANOVA followed by Tukey’s post test.

Although there are statistically significant differences in expression of several major extracellular matrix proteins, including collagen IV, fibronectin and perlecan, between matrices of different moduli, the ratios of these proteins are largely unchanged. (Figures 40-41 below) The ratios of the various collagen IV chains are not statistically different. Similarly, the ratio of collagen IV α1 to other common ECM proteins - including fibronectin, laminin, biglycan and perlecan - is unchanged with scaffold modulus. The ratios of fibronectin to biglycan, and collagen IV α1 to elastin and fibrillin do change with scaffold modulus. Still, the ratios of the major constituents of the extracellular matrix appear to be fairly consistent among scaffolds, meaning that while
the *amount* of ECM is changing, its *composition* is similar. None of the scaffolds are causing the cells to produce a grossly changed or pathologic ECM.

Matrix remodeling gene (MMP2, TIMP1, TIMP2, MT1-MMP) ratios are largely statistically unaffected by scaffold modulus. However, the ratio of MMP2:TIMP2 was statistically significantly lower on 1345Pa scaffolds than on 49.9Pa scaffolds. (Figure 42) TIMP2 is capable of potentiating the activation of MMP2 by MT1-MMP as well as inhibiting the active molecule. The effect of the change in ratio of the two seen here on MMP2 activation is unknown and depends on the actual concentrations of the molecules around the cells. *This data, together with the ECM gene ratio data, suggests that the cell and its ECM secretory/remodeling mechanism is not being changed at a fundamental level, but that, rather, the changes are a matter of degree and level of stimulation.*
Figure 40: Ratio of Collagen IV Gene Expression to Other Major ECM Genes Is Largely Unchanged With Scaffold Modulus. The expression ratios of the various collagen IV chains is unchanged with scaffold modulus. The ratio of collagen IV α1 to other major extracellular matrix proteins is generally not statistically significant. The exceptions are the ratio of Collagen IV α1 to fibrillin (173Pa vs. 1345Pa, p < 0.05) and elastin (49.9Pa vs. 1345Pa, p < 0.01).

Figure 41: Ratios of Other ECM Protein Genes is Also Largely Unchanged by Scaffold Modulus. The ratio of laminin gene expression to other ECM proteins is not statistically significantly different with different scaffold moduli. The ratio of fibronectin to perlecan is not statistically different on different moduli, but the ratio of fibronectin to biglycan is statistically higher on 1345Pa scaffolds than on 49.9Pa scaffolds.
Figure 42: Ratios of Matrix Remodeling Genes Are Largely Unaffected By Scaffold Modulus. However, the ratio of MMP2:TIMP2 is statistically higher on 49.9Pa scaffolds than on 1345Pa scaffolds. Depending on the concentration, TIMP2 can either inhibit MMP2 or potentiate its activation by MT1-MMP.58
Integrin Expression is Dependent on Scaffold Modulus

Integrin gene expression at confluence was studied using RT-PCR. Cell surface expression was studied using flow cytometry. Some types of integrins are down regulated as substrate modulus increases, while others are not affected. RT-PCR was used to examine gene expression in confluent endothelial cells of integrin subunits, $\alpha_5$, $\alpha_6$, $\alpha_v$, $\beta_1$ and $\beta_3$. (These are normally found complexed as $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_6\beta_1$ in endothelial cells.) (Figure 43) Subunit $\alpha_5$ was up regulated on 49.9Pa scaffolds compared to either 173Pa or 1345Pa scaffolds. Subunit $\beta_1$ was also upregulated on 49.9Pa scaffolds compared to 1345Pa ones. Gene expression of other integrin subunits were not affected by scaffold modulus.

In addition to RT-PCR, cell surface expression of integrins $\alpha_5$ and $\alpha_v\beta_3$ was examined using flow cytometry. Like with RT-PCR, integrin expression generally decreases as substrate modulus increases. $\alpha_5$ was expressed by 100% of cells, but the level of expression decreased exponentially with increasing modulus ($r^2 = 0.93$) from a high of 547 +/- 87.6 fluorescence units for 173Pa scaffolds to 252 +/- 1.4 fluorescence units on 1345Pa scaffolds. (Figure 44) This drop was statistically significant ($p < 0.001$ by ANOVA). Expression of $\alpha_v\beta_3$ also decreased with increasing modulus, but followed a different pattern. (Figure 45) All cells which expressed $\alpha_v\beta_3$ did so at approximately the same level, but the number of cells which expressed this integrin decreased slightly with increased modulus. A maximum of 96.3 +/- 0.1% of cells expressed $\alpha_v\beta_3$ at 68.7Pa, and dropped to 79.3 +/- 1.6% of cells at 1345Pa. This drop was statistically significant by ANOVA ($p < 0.001$). Tukey post analysis shows that the scaffolds can be divided into two groups: scaffolds with moduli of 49.9-376Pa and scaffolds with moduli 508-1345Pa.
Within each group, the percentage of $\alpha_\beta_3$ positive cells is not statistically different, but every pair between the two groups is ($p < 0.05$ by Tukey).

Figure 43: Integrin Gene Expression Is Affected By Scaffold Modulus: Integrin $\alpha_5$ expression is statistically higher on 49.9Pa scaffolds than on either 173Pa or 1345Pa scaffolds. Integrin $\beta_1$ expression is higher on 49.9Pa scaffolds than on 1345Pa scaffolds. Other integrins were not significantly affected.
Figure 44: Integrin α5 Cell Surface Expression is Dependent on Modulus. All cells express α5 integrin on their surface (Top), but the geometric mean of fluorescence (Bottom) decreases with increased modulus (p < 0.001 by ANOVA, exponential fit $r^2 = 0.935$).
Figure 45: αvβ3 Cell Surface Expression Depends on Modulus. The percentage of cells which express αvβ3 (Top) can be divided into two groups: one at lower moduli, and one at higher moduli. Fewer cells on scaffolds in the higher modulus group express αvβ3 compared to the lower modulus group. (p < 0.001 by ANOVA) Expression within each group is not statistically different by Tukey. The average fluorescence of cells expressing this integrin is the same regardless of scaffold modulus.
DISCUSSION

Previous studies examining the effect of substrate rigidity on endothelial cell function in three dimensional culture have focused mainly on cell morphology and attachments. Tubulogenesis and focal adhesion formation are both affected. However, the effect on cell functionality has not been closely examined. In this work, we have begun to examine the effect of substrate modulus on the functionality of endothelial cells in 3D culture, including such aspects as proliferation, integrin presentation, extracellular matrix production and inhibition of smooth muscle cells.

The Young’s modulus of an isotropic, porous structure (E*) depends on three variables: the density of the scaffold (ρ*, a factor related to the physical structure of the scaffold), the density of material from which the material is made (ρs), and the modulus of the material from which the scaffold is made (Es). These variables are related by the equation\[^{59}\]

\[
\frac{E^*}{E_s} = \left( \frac{\rho^*}{\rho_s} \right)^2
\]

If ρ* and ρs are unchanged, the Young’s modulus E* is linearly related to the modulus of the scaffold material Es. Thus, the Young’s modulus can be used as an easy to measure surrogate for the modulus of the material on which the cells actually sit.

In this work, established techniques utilizing a carbodiimide cross-linker\[^{60,56}\] were used modify a “base” scaffold (Gelfoam, a commercially available gelatin surgical sponge) to have an increased, predictable modulus by adding crosslinks to the gelatin substrate while retaining the same physical structure. Autoclaving broke down some of
the protein crosslinks inherent in the base material, creating scaffolds with the same physical structure which were softer than the base material. Between these two techniques, it was possible to create a series of scaffolds with a 27-fold difference in modulus and the same chemical and physical structure.

The modulus of the material on which the cells are sitting can also be estimated. An AFM cantilever technique was used to measure the modulus of a single strut of a hydrated “base” scaffold (173Pa bulk modulus) to be 4387 +/- 1072Pa. Due to technical issues, only one strut was able to be measured - and only with a large degree of error - and therefore was not included in this thesis, but this value allows us to estimate the substrate material modulus for the complete range of Young’s moduli used in this work, since $\rho^*$ and $\rho_s$ were kept constant by the modification techniques used. Es for the range of Young’s moduli used in this work range from 1.27kPa to 34.1kPa (for the 49.9Pa and 1345Pa scaffold respectively).

The range of substrate material moduli corresponds well with published in vivo vessel values. Most of the reported mechanical values for blood vessels look at the vessel as a whole, and the modulus of the endothelial cell extracellular matrix has not been reported in the literature. However, the modulus of the vessel media (smooth muscle cell layer), on top of which endothelial cells sit, as measured by AFM nanoindentation is in the 5-8kPa range – close to the substrate material modulus for the 173Pa scaffold used in this work.

Many diseases, including atherosclerosis, diabetes and hypertension, can increase the stiffness (and modulus) of the vessel wall. Although most mechanical values reported in the literature for these conditions are not directly comparable to those measured in this
work, the high end of the range of the substrate material moduli approaches the moduli of atherosclerotic plaques. Reported atherosclerotic plaque moduli span a huge range, from 33kPa up to 2.3MPa\textsuperscript{62}, depending on many factors including the level of fibrosis of the plaque and the method of measurement used.

The techniques used in this work to alter scaffold modulus allowed us to isolate the effect of substrate modulus on cellular function. Additionally, the range of substrate material moduli were estimated to be in a physiologically relevant range, with the 173Pa scaffold in the same range as a normal artery, and the stiffest scaffolds approaching the modulus of an atherosclerotic plaque.

In this study, proliferation of endothelial cells was strongly dependent \( (p<0.0001) \) on scaffold modulus. Growth rate was highest on scaffolds of intermediate modulus (508Pa), and dropped off significantly at high or low moduli. Total cell number at confluence (21 days) follows the same general pattern as growth rate. It should be noted that the softest scaffolds studied (49.9Pa) were extremely difficult to handle without damage, possibly adding to the sharp drop off in growth rate and cell number between the two softest substrates. These results suggest that there is an optimal stiffness for the proliferation of endothelial cells in 3D culture. This is somewhat different than what has been reported in the literature for other cell types. In 2D, many cell types, including fibroblasts\textsuperscript{18} and smooth muscle cells\textsuperscript{19}, proliferate more quickly on stiffer substrates compared to softer ones, although adult neural stem cells proliferate best on substrates of intermediate modulus.\textsuperscript{63} Less has been reported about proliferation in 3D, but chondrocytes are either not affected by modulus\textsuperscript{54}, or proliferate better on stiff scaffolds\textsuperscript{60,17}. Similarly, fibroblasts in 3D culture proliferate better on stiff substrates\textsuperscript{64}. 

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This suggests that endothelial cells may respond differently to modulus than other cell types, that their "ideal" range of substrate stiffness is narrower, or that determination of "ideal" scaffold modulus is strongly dependent on the range of moduli investigated.

In addition to cell proliferation, the secretory function of endothelial cells was also examined. Endothelial cells normally secrete a wide variety of growth and regulatory factors. These include the growth factors TGF-β1, FGF2, and PDGF-BB, which were examined here. All three growth factors are affected by scaffold modulus in a slightly different way. PDGF-BB and FGF2 were dependent on modulus. For PDGF-BB, however, only one pair of scaffolds was statistically different for PDGF-BB (173Pa of 1062Pa), and there was no correlation between scaffold modulus and PDGF-BB. FGF2 was also dependent on scaffold modulus by ANOVA. However, in this case, there was a fairly good correlation between FGF2 and modulus, which followed the same general pattern as growth rate and cell number (an inverse parabolic curve, with a maximum at intermediate moduli). TGF-β1 was independent of scaffold modulus. It should be noted, however, that only total TGF-β1 was measured, not activated TGF-β1. It is possible that even when total protein is not affected, TGF-β activation is. It is noted in the literature that mechanical strain, which can be thought of as analogous to the changes in the cells mechanical environment in our system, can change TGF-β activation without changing total levels.\textsuperscript{55}

In addition to examining growth factor secretion, the secretion of heparan sulfate proteoglycans was also measured. HSPGs, which are a subset of total sulfated proteoglycans, are necessary for endothelial cell mediated inhibition of vascular smooth muscle cell proliferation.\textsuperscript{2} HSPG secretion was dependent on scaffold modulus.
Secretion was highest at 49.9Pa, and then decreased until a plateau was reached between approximately 173Pa and 376Pa. The maximum HSPG secretion (per million cells) was over twice that of the minimum, a difference which could be expected to be functionally significant.

When used in vivo, matrix embedded endothelial cells exert their effect by secretion of factors including HSPGs, into the vessel wall, which inhibits the growth of smooth muscle cells, preventing neointimal hyperplasia. To test whether the differences in growth factor and HSPG secretion with scaffold modulus are functionally significant, endothelial cell conditioned media ability to inhibit smooth muscle cell growth was measured using a $^3$H thymidine incorporation assay. $^3$H thymidine incorporation was normalized to endothelial cell number, yielding a measure of the inhibitory potential of individual cells. Inhibition was seen to decrease linearly. As with growth rate, the error induced by the fragility of the $E=49.9$Pa scaffolds must be taken into account when analyzing its cell number normalized inhibition, but $r^2 = 0.98$ between 69-508Pa, with an increase in modulus, until a plateau was reached with the stiffest scaffolds ($E >= 508$Pa).

It is interesting to note that the pattern of smooth muscle cell inhibition closely follows the pattern of HSPG secretion. However, HSPG secretion plateaus at lower modulus than smooth muscle cell inhibition. This difference may be explained by looking at FGF secretion as a promoter in addition to HSPG secretion as an inhibitor of smooth muscle cell proliferation. FGF2 secretion is increased at high moduli compared to very low moduli. ($<=69$Pa) This means that at high scaffold modulus, endothelial cells are secreting not only less inhibitory HSPGs, but also more stimulatory FGF2, further decreasing their inhibitory potential.
Clinically, the inhibitory ability of individual cells is less important than the inhibition caused by the scaffold as a whole. When analyzing the inhibition caused by the scaffolds as a whole, inhibition increases gradually as stiffness decreases, then plateaus and then finally drops sharply with the softest scaffolds. This pattern is similar to, but offset from, the pattern of growth rate.

These data suggest that modulus affects the inhibitory potential of the embedded endothelial cells in two distinct ways. The first is simply a mass effect. The modulus controls the number of endothelial cells able that can colonize each scaffold, and therefore the mass of inhibitory HSPGs produced by the construct as a whole. The second is an effect on the individual cells. Cells on softer scaffolds have an inhibitory potential, which is clearly higher than that of cells on stiff scaffolds, caused by both increased HSPGs and decreased FGF2. Together, these two effects combine to produce a range of moduli where actual inhibition plateaus. It is interesting to note that Gelfoam, the base material here that has been used for previous in vivo experiments\textsuperscript{32-34}, falls in the middle of this plateau, suggesting that no optimization of the scaffold may be needed for maximal effectiveness in vivo.

Even though the underlying substrate provides the physical support for cell growth, the cells do not interact directly with it. Instead, cells sit on extracellular matrix that they themselves produce. To determine if changes in this cell-matrix-substrate interface correlate with our functional data, extracellular matrix production was examined. Different ECM gene expression patterns were found in subconfluent cells compared to confluent cells. In subconfluent cells only a few ECM protein genes were affected by scaffold modulus. Specifically, collagen IV expression was higher on 173Pa
scaffolds than on either 50Pa or 1345Pa scaffolds. Similarly, matrix remodeling genes MT1-MMP, MMP2 and TIMP2 were all expressed at a higher level on 173Pa scaffolds than on 50Pa or 1345Pa scaffolds. This correlates with cell growth rate, and it is expected that more proliferatory cells would show increased remodeling of their environment.

In confluent cells, our data show that endothelial cells on softer scaffolds produce significantly more of several common, key ECM molecules, including the α1 and α5 chains of collagen IV, fibronectin, biglycan and perlecan, than cells on stiffer scaffolds. However, the ratios between these molecules is similar between types of scaffolds, suggesting that while the cells on softer scaffolds are producing *more* matrix than those on stiffer scaffolds, the ECM itself is similar. One ECM gene which did not follow this pattern was elastin, which was expressed at higher levels on the stiff substrate versus softer ones, in both confluent and subconfluent cells.

Unlike ECM protein genes, matrix remodeling genes (specifically TIMP1 and TIMP2) were expressed at higher levels on both 50Pa and 1345Pa scaffolds than on 173Pa scaffolds. The remodeling data taken together with the ECM protein data suggest that it is possible confluent endothelial cells on softer scaffolds may be trying to stiffen their substrate by producing more structural ECM proteins, while cells on stiffer matrices may be trying to achieve the opposite affect by producing more elastin – a molecule which would increase the compliance of their substrate.

Substrate dependent ECM production is found across cell types. Extracellular matrix formation in various cell types is dependent on the material on which they sit. For example, fibroblasts\(^5\) and embryonic stem cells\(^6\) produce different ECM when cultured in 2D vs. 3D. Chondrocyte ECM production changes with alterations in 3D structure\(^5\).
and with substrate stiffness. Fibroblasts and endothelial cell differentially remodel their ECM in 2D when cultured on substrates of varying stiffness. Finally, endothelial cells in vivo differentially express genes for ECM molecules depending on the stiffness of the artery from which they came.

The type of extracellular matrix changes seen in our system – an increase in the overall amount of ECM but few changes in its composition – suggests that the endothelial cells are not being changed on a fundamental level. The type of extracellular matrix produced by endothelial cells corresponds with their functional state. For example, an extracellular matrix rich in laminin will promote quiescence compared to one rich in collagen I, which promotes angiogenesis. Similarly, tumor endothelial cells, which are highly dysfunctional, produce excess collagen I and III. The fact that the extracellular matrix produced by our endothelial cells is compositionally similar suggests that their base functionality has not changed.

In addition to extracellular matrix, integrins – the interface between the cell and the matrix – were examined as well. Two types of integrins were examined in this thesis: $\alpha_3\beta_3$ and $\alpha_5\beta_1$. The main target of both of these integrins is fibronectin, one of the ECM proteins most affected by stiffness, although both bind to other molecules as well. $\alpha_\nu\beta_3$ also binds to vitronectin. It also binds to VEGFR2, and increases the action of VEGF-A. $\alpha_5\beta_1$ binds to Tie2 and potentiates the action of angiopoietin, which is involved in the maturation of blood vessels. $\alpha_\nu\beta_3$ is generally found in immature focal complexes such as those found on migrating cells, while $\alpha_5\beta_1$ tends to be found in the more mature focal adhesions (which contain proteins such as vinculin, etc. in addition to integrins). Both are involved in angiogenesis, but at different stages. $\alpha_\nu\beta_3$ is found on proliferating cells.
actively forming vessels and is involved in the prevention of apoptosis, while $\alpha_5\beta_1$ is associated with the maturation of new blood vessels (a more quiescent phenotype) and is also found in quiescent EC. This is not an either/or situation, however, and angiogenic cells can express both types of integrins at the same time.

The $\alpha_5\beta_1$ integrin, was expressed less in cells grown on stiff substrates compared to softer substrates. This was seen both at the gene expression level by RT-PCR data as well as by flow cytometry. The $\alpha_v\beta_3$ integrin, also a fibronectin receptor, was also affected, though in a different way. Unlike the $\alpha_5\beta_1$ integrin, gene expression of $\alpha_v\beta_3$ was not significantly different between scaffolds. Also, with integrin $\alpha_5\beta_1$, all cells expressed some amount of the integrin but the level of surface expression (as measured by the geometric mean of fluorescence recorded by flow cytometry) varied between groups. Integrin $\alpha v \beta 3$ appeared to be regulated in more of an on/off fashion. All cells which expressed the protein exhibited similar levels of surface presentation, but the proportion of cells expressing this integrin varied with stiffness.

The integrin expression results seen in this study differ from the results seen for other cell types in the literature. Most studies examining the effect of substrate modulus on integrin and/or focal adhesion expression reported that cells on stiffer substrates tend to produce more of these molecules. It is possible that in our system, the effect of remodeling, in the form of ECM production, outweighs the effect of substrate modulus itself in affecting integrin expression.

Changes in extracellular matrix gene expression correlate well with changes in smooth muscle cell inhibition. Gene expression of many extracellular matrix proteins, including collagen IV $\alpha_1$, collagen IV $\alpha_5$, fibronectin, and perlecan, the major core
protein for HSPGs in endothelial cells, was linearly correlated to smooth muscle cell inhibition. Although these correlations only involved three points, the fits were excellent, with $r^2 \geq 0.95$. These strong correlations suggest that scaffold modulus may regulate extracellular matrix production and smooth muscle cell inhibition through the same pathways.

Integrin expression and smooth muscle cell inhibition correlate in interesting ways. Integrin $\alpha_\delta \beta_3$ appears to correlate with smooth muscle cell inhibition, but integrin $\alpha_5 \beta_1$ expression levels are especially strongly related to smooth muscle cell inhibition. There is a linear relationship between both $\alpha_5$ and $\beta_1$ integrin gene expression and smooth muscle cell inhibition ($r^2 > 0.99$ for both). Additionally, when the cell surface expression levels of $\alpha_5$ integrin are plotted versus smooth muscle cell inhibition or HSPG secretion, an interesting pattern appears. At the low integrin expression levels of high modulus scaffolds, inhibition is flat. However, once integrin expression has reached its maximum and plateaus at low moduli (<= 173Pa), both inhibition and HSPG secretion begin to increase. (Figure 46) It is should be noted that this region of increasing HSPG secretion and smooth muscle inhibition correlates with the mechanically sensitive region of the HSPG/smooth muscle inhibition versus modulus curves as well.
Figure 45: Smooth Muscle Cell Inhibition Correlates with Integrin $\alpha_5\beta_1$ Expression. 
Top: Integrin $\alpha_5$ (Left) and $\beta_1$ (Right) subunit gene expression strongly correlates with cell number normalized smooth muscle cell inhibition. Bottom: Below a critical integrin $\alpha_5$ surface expression, smooth muscle cell inhibition is independent of modulus.

Based on the data presented in this chapter, it is possible to propose the following model for modulus control of endothelial cell function. In vivo endothelial cells are part of both 2D and 3D structures – they line the interior of blood vessels in a 2D sheet, but the vessels themselves course through tissues as 3D structures. The endothelial cells in these 3D structures are subject to several types of mechanical forces and parameters –
contraction and compression of the surrounding tissue, shear flow on the inside of the vessel, and the modulus of the surrounding tissue and vessel wall. Normally, endothelial cells are able to exist in their preferred, low energy state by producing a specific set of extracellular matrix molecules and integrins to balance these forces. This balanced state would also correspond to secretion of a specific level of inhibitory and stimulatory molecules.

In our system this balance has been disturbed by changing the modulus of the scaffold on which the cells sit. (In vivo the balance may be disturbed by the stiffening of tumor stroma or the creation of areas of turbulent flow around atherosclerotic plaques, for example.) In the proposed model (Figure 47), in response to the disturbance, cells attempt to restore the balance of forces and their preferred basal state by altering their expression of extracellular matrix and integrins. Changes in integrin number and composition alter intracellular signaling and have downstream effects on cell function including changes in secretion of both inhibitory and stimulatory factors. These changes in stimulatory and inhibitory function are different and do not change in lockstep. Growth stimulatory function (FGF2, PDGF-BB secretion) is largely conserved, while growth inhibitory function (heparan sulfate secretion, smooth muscle inhibition) undergoes larger changes. The magnitude of their change was related to the specific changes in integrin expression caused by the change in modulus.

The two types of integrins examined in this thesis, $\alpha_v\beta_3$ and $\alpha_5\beta_1$, are associated with different cellular phenotypes. $\alpha_v\beta_3$ is only associated with a stimulated phenotype: cells which are actively proliferating and migrating. $\alpha_5\beta_1$ on the other hand, can be associated with a more stable phenotype: quiescent cells with stable focal adhesions.67-69
It is these stable, non-proliferating endothelial cells which produce growth inhibitors such as heparan sulfates.

The actual changes in inhibitor and stimulator secretion were not linearly correlated to integrin expression. Integrin mediated intracellular signaling is complex and downstream functions can be affected by many factors, including signals from integrins not studied in this work and autocrine/paracrine signaling. Rather, the magnitude of integrin expression alteration corresponds to changes in secretion. In our system, $\alpha_5\beta_1$ expression alteration was much larger than change in $\alpha_4\beta_3$ with change in modulus. In turn, the inhibitory secretion associated with a stable, non-proliferating phenotype (like that of $\alpha_5\beta_1$ expressing cells) was affected more than secretion of growth factors, which can be associated with a more activated, proliferative phenotype (such as that of $\alpha_\delta\beta_3$ expressing cells).
Figure 47: Proposed Model of Control of Cell Function By Substrate Modulus: Top: Cells exist in balance with the forces of their environment, and express a specific set of integrins and ECM, and have a given secretory function. Middle: Change in substrate modulus upsets the balance. Bottom: In response, cells alter ECM and integrin expression, which has differential downstream effects on growth inhibition and stimulation based on the new complement of integrins.
CONCLUSIONS

Utilizing carbodiimide crosslinked and autoclaved gelatin scaffolds, we isolated the effect of modulus on endothelial cells cultured in 3D. Substrate rigidity affected proliferation, growth factor secretion, extracellular matrix production, integrin expression and inhibition of smooth muscle cell growth. Correlation was observed between integrin expression and functional smooth muscle cell inhibition. The data support the model that when the natural balance between the cell and mechanical forces of its environment are altered, the cell responds by producing an altered ECM and set of integrins. Change in integrin expression leads to differential downstream effects on stimulatory and inhibitory secretion. The magnitude of these effects are related to the magnitude of change in integrin expression. In the next chapter, we begin to explore whether this same type of response to substrate mechanics is present in the inflammatory response system of endothelial cells.
CHAPTER 4: EFFECT OF SCAFFOLD MODULUS ON RESPONSE TO INFLAMMATORY STIMULI

INTRODUCTION

In Chapter 3 it was found that scaffold modulus affected both the growth stimulatory and growth inhibitory functions of endothelial cells, mediated by changes in integrin expression. This raises the question of whether scaffold modulus can modulate other vascular functions in the same way, or if the effect is limited to growth regulation.

A good target to be regulated in the same manner is the inflammatory system. Like the growth regulatory system, the endothelial cell inflammatory response consists of two complementary systems: immune stimulators (cytokines) and response units (surface receptors such as ICAM-1 and VCAM-1). Additionally, integrin expression is known to be related to the activation of the NF-kB pathway, which is involved in the production of cytokines as well as the expression of surface adhesion molecules such as ICAM and VCAM.70

In order to answer the question of if and how scaffold modulus affects this system, we examined several aspects of the endothelial cell inflammatory system. Cytokine secretion (including MCP-1, IL-6 and IL-8) was examined, as was cell surface expression of adhesion molecules ICAM and VCAM along with their soluble counterparts. Finally, changes in endothelial cell inflammatory function were assessed by examining alterations in the ability of endothelial cells to induce CD4+ T cell proliferation. Changes in expression of these factors were then compared with changes in integrin expression in an attempt to discern if inflammation may be regulated in a similar manner to growth.
METHODS

Cell Culture and Conditioned Media Preparation

Cells were engrafted onto scaffolds and cultured to confluence as outlined in chapter 2. Conditioned media from endothelial cells activated with TNF-α (a pro-inflammatory cytokine) was prepared for use in assays of secreted cytokines. Confluent, 1x2cm scaffolds were incubated in 10mL full growth media (Endothelial Cell Growth Medium + 7% FBS + 1% PS) supplemented with 10ng/mL TNF-α for 24 hours. Conditioned media was then collected, centrifuged to remove any particulates, and the supernatant frozen at -80°C until use. Cells were recovered from scaffolds and counted using a Coulter Counter.

Cytokine Secretion

To measure how an endothelial cell response to an immune stimulus is modulated by substrate stiffness, cytokine secretion in response to 10ng/mL TNF-a was quantified. Conditioned media containing full growth supplements plus TNF-α was prepared as described above. Concentrations of several different factors, including IL-6, IL-8, MCP-1, sICAM and sVCAM, were all measured using ELISAs from R&D according to kit directions.

Surface Receptor Flow Cytometry

The presentation of ICAM-1 and VCAM-1 - cell surface adhesion receptors for leukocytes – in response to an immune stimulus was measured by flow cytometry. Confluent scaffolds were incubated in complete growth media supplemented with
10ng/mL TNF-α for 24 hours. Scaffolds were then digested with collagenase and cells recovered by centrifuging and counted. These cells were washed 2x with ice cold PBS + 2% heat inactivated FBS + 0.09% sodium azide (Cell Staining Buffer, BD). 3x10^5 cells from each sample were resuspended in 100μl of staining buffer and the appropriate volume of FITC conjugated antibody (either mouse anti-human ICAM-1 or mouse anti-human VCAM-1) added. After briefly vortexing to mix, cells were incubated on ice for 45 minutes in the dark with shaking. Cells were then centrifuged and washed 2x with ice cold staining buffer to remove excess antibody. Finally, cells were resuspended in 500μl of 1% paraformaldehyde in PBS. 10^4 cells were analyzed by flow cytometry using a FACScalibur instrument and CellQuest software (Becton Dickinson, San Diego, CA). All samples were compared to similarly prepared cells stained with the appropriate FITC conjugated isotype controls.

**T Cell Proliferation Assay**

In order to determine if scaffold modulus affected functional response to inflammatory stimulus, endothelial cell induction of T cell proliferation was measured. Endothelial cells were cultured on scaffolds under normal culture conditions for 16 days and then exposed to 1000U/mL IFN-γ for 48 hours. Cell growth was subsequently arrested using 50μg/mL mitomycin C. CD4+ T cells were isolated from fresh human blood using a negative selection kit (Miltenyi). Isolated T cells were labeled with 10μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) for 10 minutes at 37°C in phenol red free RPMI1640, and washed three times. Labeled CD4+ T cells were cocultured with scaffolds at 5x10^5 T cells per 1.25x10^5 EC (in triplicate for each scaffold
type), in RPMI medium without phenol red, and maintained for six days. At the end of
the co-culture period, scaffolds were washed extensively, and T cells pelleted by
centrifugation. T cells were resuspended in PBS and analyzed by flow cytometry. With
this method, proliferation (i.e. division) of T cells results in less intense staining per cell
and a shift to the left (i.e. decrease) in mean fluorescence intensity.

Statistics

Statistical analyses were performed with Prism (GraphPad) or Excel (Microsoft)
software. Data are expressed as mean +/- SEM unless noted. Comparisons between
groups were made by ANOVA followed by Tukey’s multiple comparison test. A value of
p < 0.05 was considered statistically significant.
RESULTS

ICAM-1 and VCAM-1 Expression Are Dependent on Scaffold Modulus

ICAM-1 and VCAM-1 expression was assessed in two ways. First, soluble ICAM-1 and VCAM-1 (sICAM-1, sVCAM-1) were measured using a colorometric ELISA kit. Second, cell surface expression was measured using flow cytometry. sICAM-1 had a binary distribution. At lower moduli (<= 508Pa), sICAM-1 expression was relatively high, and was significantly lower at high moduli (>= 1062Pa) (p = 0.002 by ANOVA between these two groups). (Figure 48) Maximum expression was 43.9 +/- 6.1 ng/10^6 cells at a modulus of 376Pa. Minimum expression was 19.3 +/- 12.3 ng/10^6 cells at a modulus of 1345Pa. The percentage of cells positive for ICAM as measured by flow cytometry was not significantly dependent on modulus (p = 0.09 by ANOVA), although the linear correlation between the two was very high (r^2 = 0.943). However, if scaffolds are sorted into the same groups as the sICAM samples (<=508Pa, >=1062Pa), the difference between these two groups is significant (p = 0.003). (Figure 48)

sVCAM-1 was also dependent on modulus (p = 0.02 by ANOVA). (Figure 49) Expression was low at very low moduli (minimum of 33.6 +/- 1.8 ng/10^6 cells at 49.9Pa), but reaches a maximum of 55.3 +/- 2.3 ng/10^6 cells at 173Pa and then decreases linearly to 27.7 +/- 4.7 ng/10^6 cells at 1345Pa. VCAM as measured by flow cytometry follows a different pattern, but is also dependent on modulus (p = 0.004 by ANOVA). (Figure 49) Percent positive expression was highest on scaffolds of 49.9Pa, with 18.75 +/- 0.2 percent positive cells. Positive expression then falls monotonically with modulus to a low of 1.6 +/- 0.4 percent positive cells at 1345Pa. A logarithmic curve can be fit to the data with an
$r^2 = 0.94$. The reason for the differences between soluble and surface VCAM-1 expression patterns is not clear.

**Figure 48: ICAM-1 Expression is Dependent on Scaffold Modulus.** Top: Soluble ICAM-1 levels fall into two groups: high at low moduli, and low at high moduli. The difference between the two groups is significant ($p = 0.002$ by ANOVA). Bottom: Cell surface ICAM-1 correlates strongly with modulus, but dependence on modulus is only significant ($p = 0.003$) when divided into two groups as with sICAM-1.
Figure 49: VCAM-1 Expression is Dependent on Scaffold Modulus. Top: sVCAM secretion is dependent on scaffold modulus ($p = 0.01$ by ANOVA). sVCAM expression is linearly correlated to modulus at $\geq 173 \text{Pa}$ ($r^2 = 0.98$). Bottom: Cell surface expression (%) positive cells) is dependent on modulus ($p = 0.004$), and can be fit with a logarithmic curve ($r^2 = 0.943$).
Cytokine Secretion By Endothelial Cells is Independent of Scaffold Modulus

Three cytokines (MCP1, IL6, and IL8) were assayed for in the conditioned media of TNF-a stimulated endothelial cells using colorometric ELISA kit. In general, the results indicate that the level of these cytokines is very low, and that their secretion is independent of scaffold modulus.

MCP-1 secretion was independent of modulus (p = 0.58 by ANOVA). (Figure 50) Secretion ranged from 3.05 +/- 0.58 ng/10^6 cells at 49.9Pa up to 3.76 +/- 0.08 ng/10^6 cells at 508Pa, but this difference was not statistically significant. (It should be additionally noted that MCP-1 secretion on all scaffolds was much lower than on two-dimensional controls [13.4 +/- 0.8 ng/10^6 cells, data not shown]. Compared to the difference between secretion on scaffolds and in 2D, the changes induced by modulus are extremely small and almost certainly functionally significant in any case.)

Similarly, IL8 secretion was also independent of modulus (p = 0.08 by ANOVA). (Figure 51) Secretion ranged from 104.0 +/- 6.1 ng/10^6 cells at 508Pa up to 142.4 +/- 12.9 ng/10^6 cells at 1345Pa. Like with MCP1, secretion on all scaffolds was much lower than on two-dimensional controls [746.5 +/- 97.4 ng/10^6 cells, data not shown]. The changes induced by modulus are extremely small when compared to the difference between 2D culture and culture on scaffolds, and are not likely to be functional.

Finally, IL6 secretion was found to be dependent on scaffold modulus (p = 0.02 by ANOVA), and the data was fit fairly well with a parabolic curve (r^2 = 0.696) with a minimum between 508Pa and 1062Pa. (Figure 52) However, the absolute difference between scaffolds was small, ranging from 9.5 +/- 1.9 ng/10^6 cells at 508Pa up to 16.2 +/- 3.6 ng/10^6 cells at 49.9Pa, a difference which is not statistically significant by itself.
(Again, this difference is very small in absolute terms, and is dwarfed by the difference in secretion between 2D culture [221.2 +/- 66.4 ng/10⁶ cells, data not shown] and culture on scaffolds – a difference of a order of magnitude.)

In general, it appears that cytokine secretion is not strongly affected by scaffold modulus.

\[\text{Figure 50: MCP1 Secretion is Independent of Scaffold Modulus.} \text{ There does not appear to be a correlation between MCP1 secretion and modulus, and the absolute difference between highest and lowest values is small.}\]
**Figure 51: IL8 Secretion is Independent of Modulus.** There does not appear to be a correlation between IL8 secretion and modulus, and the absolute difference between highest and lowest values is small.

**Figure 52: IL6 Secretion May Be Dependent on Modulus.** 1-way ANOVA suggests that IL6 secretion is dependent on modulus ($p = 0.02$), and the data can be fit fairly well with a parabolic curve ($r^2 = 0.696$). However, the difference between the lowest and highest levels is not significantly different, and the absolute differences are small.
Endothelial Cell Induced T Cell Proliferation Is Significantly Affected By Scaffold Modulus

CD4$^{+}$ T cell proliferation in response to exposure to endothelial cells from scaffolds of various stiffness was measured using a CFSE analysis method, where decreased proliferation is correlated with an increase in T cell mean fluorescence intensity (MFI).

MFI was maximal (and T cell proliferation minimal) at 49.9Pa, with a value of 65.9 +/- 4.8. MFI was minimal (and T cell proliferation maximal) at a scaffold modulus of 1345Pa, with a value of 153.7 +/- 4.7. MFI values increase monotonically (and proliferation decreases monotonically) with increasing modulus. The data is well fit with a logarithmic curve, with $r^2 = 0.98$. (Figure 53) Overall, the data indicate that proliferation is decreased when T cells are exposed to endothelial cells grown on stiff scaffolds when compared to exposure to endothelial cells from softer scaffolds.

CD4$^{+}$ T cell proliferation can be stimulated by a number of endothelial cell surface markers, including ICAM-1 and VCAM-1. Our data show that MFI is linearly correlated with cell surface expression of both ICAM-1 ($r^2 = 0.81$) and VCAM-1 ($r^2 = 0.95$). (Figure 54)
Figure 53: Endothelial Cell Induced T Cell Proliferation Is Increased When Endothelial Cells Are Cultured on Softer Scaffolds. Top: Mean fluorescence intensity of T cells, which decreases with increasing T cell number and proliferation, is minimal at 49.9 Pa and increases with increasing scaffold stiffness.
Figure 54: T Cell MFI is Linearly Related to Endothelial Cell Surface Expression of ICAM-1 and VCAM-1. Top: T cell MFI decreased linearly (i.e. cell number increased) with an increase in endothelial cell ICAM-1 expression. Bottom: T cell MFI decreased linearly (i.e. cell number increased) with an increase in endothelial cell VCAM-1 expression.
<table>
<thead>
<tr>
<th>Factor</th>
<th>Function</th>
<th>Shape of Fit</th>
<th>Minimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP-1</td>
<td>Recruits monocytes to sites of injury and infection</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>IL-8</td>
<td>Neutrophil chemotaxis and activation</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>IL-6</td>
<td>Monocyte chemotaxis and activation</td>
<td></td>
<td>828Pa</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Adhesion to, and migration into, vessel wall by leukocytes</td>
<td></td>
<td>1345Pa</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Involved in adhesion of leukocytes to the vessel wall</td>
<td></td>
<td>1345Pa</td>
</tr>
<tr>
<td>CD4(^+) T Cell Proliferation</td>
<td>In vivo, activated endothelial cells induce proliferation of these cells which are involved in many aspects of the immune response. This function is greatly decreased in 3D culture compared to 2D culture.</td>
<td></td>
<td>1345Pa</td>
</tr>
</tbody>
</table>

Table 5: Summary of Endothelial Cell Immune Response Data
DISCUSSION

Prior to completing the work of this chapter, there was reason to believe that scaffold modulus would affect cytokine secretion. Cytokine secretion by endothelial cells is regulated by the NF-kB pathway, which is upregulated by \( \alpha_5\beta_1 \) integrin expression.\(^7\) Additionally, previous work (unpublished) from our lab has suggested that integrins \( \alpha_5\beta_1 \) and \( \alpha_4\beta_3 \) are important in the production of cytokines by endothelial cells. When these integrins are neutralized, or when endothelial cells are cultured on Gelfoam, where levels of these integrins are naturally lower than in 2D culture (unpublished), production of cytokines is decreased. As our data (figures 39-40) suggest that integrin expression levels are dependent on substrate modulus we examined how substrate modulus affects the secretion of cytokines by endothelial cells in response to inflammatory stimuli.

There was essentially no dependence of cytokine (MCP1, IL8, IL6) secretion on modulus. Although IL-6 was statistically dependent on modulus, the absolute range of values was very small, and unlikely to be functionally significant. This does not necessarily contradict the finding discussed above of the integrin dependence of cytokine secretion. Integrin expression on 3D gelatin scaffolds is in general lower than in 2D culture, as is cytokine secretion.\(^7\) It is possible that any affect of integrin expression has already been saturated just by the transition from 2D to 3D culture, and a further reduction in integrin levels would have no effect.

The expression of soluble and surface expressed ICAM-1 and VCAM-1, which are also regulated by the NF-kB pathway, by TNF-\( \alpha \) stimulated endothelial cells was also examined. ICAM-1 (Intercellular Cell Adhesion Molecule 1) is a cell adhesion molecule involved in the adhesion of leukocytes to, and migration into, the blood vessel wall.
following an inflammatory stimulus. ICAM-1 is constitutively expressed on endothelial cells, but is strongly and quickly upregulated by stimulation with cytokines such as TNF-α. ICAM-1 binds to a number of ligands present on immune cells, including Macrophage Adhesion Ligand-1 (Mac-1) and Leukocyte Function Associated Antigen-1 (LFA-1). These are both integrins containing a β2 chain found only on leukocytes.1

Like ICAM-1, VCAM-1 (Vascular Cell Adhesion Molecule 1) is involved in the adhesion of leukocytes to the vessel wall. Although similar in function to ICAM-1, VCAM-1 is not constitutively expressed on endothelial cells, and is transcriptionally upregulated by inflammatory cytokines including TNF-α. VCAM-1 also has different targets on leukocytes than ICAM-1. A major target of endothelial cell VCAM-1 is integrin α4β1, also known as Very Late Antigen 4 (VLA-4), expressed on leukocytes.71

ICAM-1 and VCAM-1 are also markers of atherosclerotic disease, as both are expressed on atherosclerotic plaques. Additionally, blood levels of soluble versions of ICAM-1 and VCAM-1 are predictive for atherosclerosis in healthy and high risk patients, respectively.71 Since the scaffolds used in this work spanned in modulus from that of a healthy vessel up to the low end of measured values for atherosclerotic plaques (see Chapter 3), we were interested to see how our scaffolds would affect expression of these molecules.

Soluble and surface ICAM-1 expression fell into two groups - high expression levels at low moduli, and low expression levels at high moduli (≥ 1062 Pa). Surface expression correlates with integrin α5 surface expression (geometric mean of fluorescence, r² = 0.8), as well as α6β3 surface expression (percent positive cells, r² = 0.77). Like ICAM-1, VCAM-1 surface expression was also dependent on substrate
modulus, and correlated linearly with $\alpha_\beta$ percent positive cells ($r^2 = 0.79$). When VCAM-1 surface expression was compared to $\alpha_5$ surface expression, however a pattern similar to that of smooth muscle cell inhibition versus $\alpha_5$ surface expression was seen: a low level plateau at low $\alpha_5$ expression, followed by a sharp increase in VCAM when $\alpha_5$ plateaus. This may indicate that similar pathways are involved in integrin mediated regulation of both smooth muscle cell inhibition and inflammatory response, although it may also point to integrin mediated upregulation of the NF-kB pathway.

The functional effect of the change in endothelial cell inflammatory state in response to changes in scaffold modulus was examining by measuring CD4$^+$ T cell proliferation. CD4$^+$ T cells serve a wide variety of roles in the immune system, functioning as helper T cells (assisting in the functions of other immune cells), memory T cells (which “remember” past infections), and regulatory T cells (help keep T cell mediated immunity in check). Immunologically activated endothelial cells express cell surface markers (principally MHCII – an antigen presenting molecule, although ICAM and VCAM have both also been implicated) which induce proliferation of these cells. Past work has shown that embedding endothelial cells in gelatin scaffolds greatly reduces this endothelial cell mediated induction of CD4$^+$ T cell proliferation.

In this work, we showed that endothelial cell induced proliferation of CD4$^+$ T cells is greatly affected by scaffold modulus. Endothelial cells cultured on stiff scaffolds induced significantly less T cell proliferation than endothelial cells cultured on softer scaffolds. Although VCAM and ICAM do not directly induce CD4$^+$ T cell proliferation, the decrease in MFI of T cells was linearly related to both VCAM-1 ($r^2 = 0.92$) and ICAM-1 ($r^2 = 0.72$) expression. The ICAM-1 and VCAM-1 data combined with the T
cell proliferation data suggests that the overall inflammatory state of the endothelial cell is reduced when cultured on stiffer scaffolds compared to softer ones.

Although more data is needed, it seems that the model developed in Chapter 3 for growth regulation may also applicable to the endothelial cell inflammatory response. Changes in the balance of forces on the cell lead to altered ECM and integrin expression, leading to changes in the downstream functionality of the cell, with different responses being altered in different ways. In the case of inflammation, stimulatory cytokines change only slightly in response these alterations in modulus, and therefore integrins, while larger changes in cell adhesion molecules and induction of T cell proliferation are seen.
CONCLUSIONS

The data in this chapter indicates that scaffold modulus is able to regulate some aspects of the endothelial cell inflammatory response, namely the expression of cell adhesion molecules ICAM-1 and VCAM-1, and the induction of CD4+ T cell proliferation. On the other hand, cytokine expression was found to be unaffected by scaffold modulus. Changes in adhesion molecule expression correlated with endothelial cell integrin expression, suggesting that integrin mediated signaling is involved in inflammatory regulation.

The data presented in this chapter offers an intriguing hint that modulus mediates control of inflammatory response in a manner similar to the regulation of growth, but there are still many questions left to answer. Future studies will focus on the pathways involved in the regulation of ICAM-1, VCAM-1 and T cell proliferation by substrate modulus. There are several possibilities. The NF-kB pathway is a natural target, as it is intimately involved in the inflammatory response, and has been shown to be altered in response to integrin expression. Alternatively, we will examine whether modulus mediated regulation of smooth muscle cell inhibition and inflammatory response are regulated through some common cellular pathway. If the NF-kB pathway is responsible for the changes in ICAM-1 and VCAM-1 expression, it would also be interesting to determine why cytokine expression was not affected, though adhesion molecule expression was.
CHAPTER 5: CONCLUSIONS AND FUTURE WORK

SUMMARY

In this thesis, we examined the hypothesis that substrate physical properties can affect the functionality of endothelial cell in three dimensional culture, and that these changes are mediated by changes in the interface between the cells and their substrate. Chapter 2 attempted to use gelatin scaffolds to examine the effect of a range of physical properties, including pore size, density and modulus. Cell functions such as growth, secretion of TGF-β1, prostacyclin, glycosaminoglycans and heparan sulfate proteoglycans were all affected to some degree by changes of substrate properties. However, the alteration of multiple scaffold properties confounded the analysis. Based on the observation that modulus seemed to have had the most effect, in Chapter 3 we utilized scaffolds varying in only modulus to examine the effect of changes in substrate mechanical properties on cell biology. Various aspects of endothelial cell biology relating to the regulation of smooth muscle cell growth (including secretion of growth factors and heparan sulfate proteoglycans) were altered by scaffold modulus, and that these changes in regulatory factors translated into a functional difference. Additionally, it was discovered that endothelial cell extracellular matrix gene expression and integrin expression were altered by substrate modulus, and that these changes correlated with the changes in smooth muscle cell growth regulation. Based on these data, we offered a model whereby changes in scaffold modulus upset the normal balance of forces on the cell. The cell attempts to return to its preferred state by altering extracellular matrix and integrin expression. The changes in integrin expression then have differential effects on
the stimulation and inhibition of cellular growth. Stimulatory function was conserved, changing only slightly, as was avb3 integrin associated with a proliferatory phenotype. The change in inhibitory function was much larger, and is of similar magnitude to that of the change in α5β1 integrin, which is related to a more quiescent, less proliferative cellular phenotype. In Chapter 4, we attempted to determine if this same paradigm could be applied to cellular response to inflammatory stimuli. Initial data indicates that some aspects of endothelial cell inflammatory response, namely cell surface expression of ICAM-1 and VCAM-1, are altered in a manner which mirrors changes in integrin expression and possibly can be explained by a similar model as growth regulation. Additionally, induction of CD4+ T cell proliferation was found to be greater by endothelial cells cultured on soft scaffolds compared to those grown on stiffer ones. These data correlated well with ICAM and VCAM cell surface expression.

**FUTURE WORK**

Based on these data, future work will follow two lines of inquiry. The first line of inquiry will be to further examine how endothelial cell inflammatory response is modulated by substrate modulus. Initial work has shown that cell surface adhesion molecules and T cell proliferation are affected, while cytokines are not, and that some of these changes seem to mirror those seen in the smooth muscle cell inhibition system. Work in this area will involve seeking other functional changes, and determining if these follow the same pattern as well.

Second, although it appears that alterations in integrin expression may be related to changes in cellular function, the mechanism by which this occurs is not known. There
are many different pathways which have been shown to be activated by changes in mechanical forces, through integrins and intermediaries such as PKC, RhoA, Ras/Rac, etc., including the MAP kinase cascades and NF-kB pathway. Activation of several of these pathways could explain the data of this thesis. For example, activation of the ERK 1/2 and p38 MAP kinase pathways by mechanical stimuli has been implicated in the increased inhibition of smooth muscle cell growth through the TGF-β pathway, which also increases extracellular matrix deposition. Increased activation of the NF-kB pathway could explain the increases in ICAM-1 and VCAM-1 expression. Although changes in substrate modulus have not been specifically implicated in these systems, it seems likely that changes in substrate stiffness could be thought of as analogous to shear stress or cyclic strain. Elucidation of which pathways are involved will be an important next step to understanding how changes in substrate mechanics are translated into functional differences.
CHAPTER 6: REFERENCES


