INTERACTIONS OF ANGIOGENIC MICROVESSELS
WITH THE EXTRACELLULAR MATRIX

by

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ABSTRACT

Angiogenesis, the formation of new blood vessels from existing ones, is critical to development, repair, tumorigenesis, and artificial construct design. It is regulated by soluble factors, cell-matrix interactions, and mechanical loading. Sprouting endothelial cells degrade their basement membrane and surrounding extracellular matrix (ECM) by matrix metalloprotease (MMP) activity, migrate along ECM components, and deposit a new basement membrane to form a patent capillary network. It is recognized that survival and integration of tissue engineered grafts depend on their vascularity. A better understanding of the angiogenic process and its interactions with the ECM is critical from a basic science perspective and in the fabrication of vascularized grafts. The angiogenic process by virtue of the associated invasion and growth of new vasculature can influence the local mechanical properties. The objective of this dissertation research was to study the effects of angiogenesis on mechanical properties of 3D in vitro vascularized constructs and relate the alterations to changes in gene expression and proteolytic activity at similar culture times, and to characterize the role of different mechanical boundary conditions on the morphology of microvascular networks. The dynamic mechanical properties of cell-free collagen constructs were evaluated at different collagen concentrations, strain magnitudes, and strain rates. The mechanical properties were shown to only alter minimally over 7 days in culture. Thus, any change in the mechanical properties of vascularized constructs was attributed to angiogenesis.

An increase in MMP gene expression and proteolysis paralleled the decrease in normalized dynamic stiffness of vascularized collagen constructs on the 6th day of culture. This was followed by a rise in dynamic stiffness, accompanied by a reduction in proteolysis in spite of elevated expression of MMP mRNA. The alignment of angiogenic sprouts from microvessels in a 3D in vitro model under different boundary conditions was also examined. Significant microvessel orientation was observed along the direction of anchorage and stretch, recapitulating in vivo behavior of vasculature in soft tissues such as muscles, ligaments and tendons. Free floating constructs lacked any preferred orientation. The results of this work provide fundamental insights into the process of angiogenesis, the interaction of angiogenic microvessels with the ECM, and the influence of mechanical conditioning on angiogenic microvessels, and will be beneficial in engineering of functional constructs with defined orientation of vasculature.

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CHAPTER 1

INTRODUCTION

The Vascular System and Angiogenesis

The blood circulatory system is central to the transport and distribution of nutrients and oxygen to the tissues and the removal of by-products of metabolism in multicellular organisms. Equally critical is the role of the circulatory system in maintaining temperature homeostasis, supporting humoral communication, and adjustments in the levels of oxygen and nutrients to tissues in different physiological demand states [1]. In the early stages of embryonic development, diffusion is sufficient for exchange of nutrient and wastes. With progressive development, the vasoformative cells or angioblasts form the hematopoietic system and the primordial vasculature. This process of blood vessel formation from precursors is called vasculogenesis. Progressively, with complex structures developing in the embryo, these preformed vascular elements give rise to new blood vessels. This process of growth of vessels from existing ones is called angiogenesis [2-5]. A less discussed process in this regard is the process of arteriogenesis, whereby changes in circulatory dynamics influence the morphology of vasculature [6]. It is currently accepted that circulating endothelial cell (EC) precursors can localize in the areas of angiogenesis or high endothelial cell turnover like regions of altered flow, shear, etc., and recapitulate vasculogenesis [7]. However,

angiogenesis is thought to be the primary mechanism of blood vessel growth in the adult tissues.

Angiogenesis chiefly occurs at the level of microvessels. In the human body, about 64% of the total circulatory blood volume is distributed in the veins, venules and venous sinuses and about 7% is in the arterioles and capillaries [8]. Though misleading, this low 7% of the arteriolar volume covers about 2500 cm² in cross-sectional area, but the average length of these units is only about 0.3 to 1 mm, constituting more than 80% of the total vascular surface area [8, 9]. Physiological endothelial cell turnover is low, with a typical replication of less than 1% per day [10] except in cases of wound healing, exercise, and uterine vessels during ovulation or pregnancy [2, 4]. Pathological angiogenesis can be categorized as lack of adequate vascularization as in postinfarction scarring of myocardium or nonhealing wounds in peripheral vasculopathies, aggressive (re)vascularization as in chronic inflammation or tumor growth, and vascularization (non metaplasia) at nonvascular sites like diabetic retinopathy [11, 12]. The tissue healing response is characterized by a large increase in local vasculature, especially in soft tissues [13, 14]. It is thus apparent that angiogenesis is a pivotal process in maintenance of the vascular network and occupies a critical place both in the physiological as well as pathological growth of blood vessels.

Motivation

Angiogenic endothelial cells degrade the basement membrane during sprouting and network formation and also produce extracellular matrix elements, leading finally to a mature, patent, and perfusion capable capillary network [3, 15]. Though there has been substantial investigation into the process of angiogenesis, its molecular mechanisms, and

regulation (see reviews - [2, 3]), little is known about the interaction of angiogenic endothelial cells with the matrix in a mechanistic sense. Matrilysis and remodeling associated with angiogenesis can influence the mechanical properties of the host tissue. Substantial evidence supports the direct or indirect regulatory influence of mechanical loading on angiogenesis [15-20]. In soft tissue healing where there is constant exposure to mechanical stimuli, it is imperative to understand the interdependence of angiogenesis and mechanical stimuli from a basic science as well as clinical interventional perspective.

The current challenge is believed to be in the utilization of information on the interactions between the soluble factors, the ECM molecules, various signaling pathways, and mechanical influences of the environment in vivo, towards the engineering of vascularized grafts [15]. The local microvasculature at the implant site influences functional efficiency of several engineered grafts like drug delivery systems, biosensors and tissue replacement grafts (biomaterial or otherwise) as reviewed extensively by Sieminski et al. [3]. The reduced viability of engineered grafts like artificial skin and polymer delivery systems have been attributed, at least partially, to an inadequate microvasculature integrated into these grafts, and the focus is consequently shifting to prevascularized grafts [4, 5]. It has been suggested that graft survival is initially dependant on diffusion, followed by inosculation of host vessels and graft vessels, and finally by neovascularization within the graft substance [6]. This coupled with the demonstrated influence of external mechanical forces on cellular (endothelial or other) orientation and phenotypic responses could guide design of better vascularized grafts that structurally and functionally mimic the host tissue. Given its importance in development, repair, tumorigenesis, and design of artificial constructs, significant resources have been

directed at understanding three-dimensional morphology, especially the vascularity of soft tissues like muscles [21], ligaments [14, 22, 23] and tendons [24, 25], where vasculature is oriented in the direction of strain and along the direction of extracellular matrix. Cellular orientation is the cornerstone of engineering three-dimensional functional tissue equivalents, mimicking the host tissue, and is usually achieved by mechanical loading or contact guidance of growing constructs [26-30]. The overall objective of this dissertation research is to elucidate the relationships between angiogenic microvessels and the extracellular matrix (ECM) using an in vitro model of angiogenesis that uses preformed vascular elements [31].

Research Goals

The central role of angiogenesis in health and disease states has prompted several studies into the molecular mechanisms of angiogenesis and identification of potential regulatory target pathways [32-34]. More recently, the contribution of specific ECM mediated regulation of EC tube formation was recognized by altered growth of vasculature on different substrates [35-37]. The concept of engineered vascularized constructs, either intended as a vascular graft, as a part of the vascular supply of a different functional graft, or of angiogenesis evaluation models [38], has formed the paradigm for engineering artificial constructs. Though it is clear that angiogenesis influences tissue mechanics and can also be influenced by signaling from the ECM based on the current understanding of tensional homeostasis [15], the relationships between the two have not been explored in detail. The first objective of this dissertation research was to design a viscoelastic test system to measure mechanical properties of engineered hydrogel constructs. Secondly, the research sought to understand the interplay between

angiogenesis, its regulators and ECM mechanical properties using gene expression, construct stiffness, vascular density and proteolytic activity as the measured parameters. Finally, the research examined the response of preformed vascular elements to mechanical conditioning in vitro as they spontaneously grow into patent capillary networks.

Summary of Chapters

This research explores the relationship between angiogenesis and the extracellular matrix by relating changes in material properties of vascularized constructs with progressive angiogenesis, to the changes in the gene expression profile and proteolytic activity in these constructs over the same time periods, and by characterizing the changes in vascular morphology under external mechanical loading. Chapter 2 provides a working knowledge of the angiogenic process, its molecular regulators, control by ECM molecules, and the influence of external mechanical forces on vascular morphology. Relevant research on endothelial cell mechanotransduction, cell-matrix interactions, genes regulating angiogenesis and prevascularized constructs of defined structural and functional characteristics are also presented.

Collagen type I is a commonly used tissue engineering substrate in an attempt to approximate a physiological ECM with low immunogenicity. The significant water content of these hydrogel substrates makes them viscoelastic in nature. Cell growth modifies the structure of these substrates by compaction [39]. Chapter 3 describes the characterization of the viscoelastic mechanical properties of cell free ‘native’ collagen constructs to establish baseline mechanical properties of such constructs over the duration of culture as well as the effect of substrate concentration on construct mechanics. Thus

any changes in the mechanics of the gels over time with cultured vessels can be unambiguously attributed to the changes induced by the angiogenic process itself. The mechanical testing methods and test system described in this chapter are used for tracking alterations in construct mechanics by angiogenic microvessels as described in Chapter 4.

Matrilysis, new matrix deposition, and production of cell-matrix interaction molecules can alter the tissue mechanical properties. We have for the first time characterized the alterations in tissue mechanical properties during angiogenesis in vitro. Chapter 4 relates the changes in construct mechanics over a 10-day period of angiogenesis in vitro to the changes in expression of genes regulating angiogenesis as well as the total proteolytic activity observed in these constructs. This research demonstrates the dynamic and temporal influence of growing vessels on mechanics of engineered scaffolds, in the absence of any external loading or mechanical forces and has implications to the understanding of mechanics of healing soft tissue which experience a multifold local increase in vascularity.

Quantification of angiogenesis assumes significance in the identification of pro- or antiangiogenic drugs, study of tumor morphology, and characterization of vascularity in engineered matrices for implants, and other investigations into the regulation and control of the angiogenic process. Most current methods in angiogenesis imaging vary considerably in the parameters estimated and focus on measuring only small representative areas of vasculature. Chapter 5 describes the use of a recently developed image processing method within a commercial package [40, 41], to quantify vessel morphology over a large volume of engineered constructs subjected to different mechanical boundary conditions. The primary focus of this section of the dissertation

was to identify the changes in orientation, length, diameter, etc. of angiogenic vessels in response to anchorage, static and cyclic stretch as compared to a free floating construct as the baseline. Finally, the salient findings of the dissertation are discussed in Chapter 6, along with some of the possible limitations of this work.

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CHAPTER 2

BACKGROUND AND LITERATURE REVIEW

Foreword

The process of angiogenesis has been the subject of intense research over the last few decades. More recently, the focus has shifted from understanding the process and its regulation to an application oriented approach with a view to generation of appropriate anti- or proangiogenic treatment regimens for various diseases. The importance of angiogenesis in the field of tissue engineering is underscored by the fact that until very recently only three tissue engineered products were available for clinical use, with several more in development for urology and wound healing. The success of most of these products could be attributed at least in part to their avascular nature or their sustainability on diffusive transport until vascularization is established [5]. However, the major bottleneck in tissue engineering complex three-dimensional structures is establishment of an efficient vascular supply. Since the recognition of this bottleneck, it has continued to be a topic of active interest over the last decade [6]. A prevascularized graft, engineered to match the local vascular topology and tissue architecture including its material properties, could be a tailored solution to this problem. This dissertation research adds both to the basic knowledge in angiogenesis in an in vitro model and its mechanistic relationship with the ECM, as well as provides insights into generation of constructs with oriented vasculature of defined morphology.

Structure of Microvasculature

As described earlier, angiogenesis mainly occurs in capillaries and other microvessels. A typical microvessel may be of arterial, venous or capillary origin and shows different anatomical organization based on this structural and functional distinction. The basic structure of a microvessel is an endothelial cell tube, and it is called a nascent vessel during angiogenesis [3]. In capillaries, this EC tube is invested by a basement membrane consisting of laminin and collagen IV [4, 7, 8] among other ECM molecules, and has a sparse layer of pericytes within this basement membrane. The mural cell coverage on arterioles and venules is higher than on capillaries, with arterioles having the highest smooth muscle cell coverage, which secrete their own basement membrane (Figure 2.1) [3, 9]. With vessel maturation, the cell-cell contacts between endothelial cells as well as their association with the pericytes become stronger and transform the leaky nascent vessels into well formed conduits. Capillaries, with the thinnest walls, are ideally suited for nutrient exchange, and any outgrowth of endothelial cells from this structure must be accompanied by a destabilization of this structure and an invasion of the surrounding matrix.

Quiescent Endothelium and the Angiogenic Switch

The low turnover rate (over a thousand days) of vascular endothelium in most tissues has promulgated the idea of a quiescent endothelium [9-11]. The mechanisms of maintenance of quiescence are not clear but related studies offer an insight into the possible regulatory paths, including loss or change in laminar flow and the consequent steady shear [12, 13], or integrin mediated adhesions to the ECM that mediate the internal levels in cAMP (second messenger system) signaling in response to external forces as

Figure 2.1. Anatomical structure of vasculature. (A). Nascent endothelial cell tube (B). Capillaries, (C). Venules or arterioles. Reprinted by permission from Macmillan Publishers Ltd: [Nature Medicine], Jain. R [3], copyright (2003).

compared to simple receptor occupancy as in a homeostatic endothelium [14]. Quiescence could also be thought of in terms of a dynamic equilibrium between pro and antiangiogenic factors which are tightly regulated in physiological states. This leads to the idea of an ‘angiogenic switch’, whereby quiescent endothelial cells switch to invasive and migratory phenotypes in physiological and pathological angiogenesis.

Normal capillary endothelial cells are retained within their surrounding basement membrane composed of Collagen IV, VIII, and laminin and a contact with collagen I, the

chief constituent of ECM may be a trigger to the angiogenic phenotype [15]. Soluble growth factors, hypoxia or other factors signaling a requirement of increased oxygenation could be considered primary causes of this switch. VEGF-A has been shown have a non-redundant role in angiogenic switching apart from its other regulatory activities [16]. This phenotype is marked by an increased lytic activity by MMPs [17] or plasminogen activator systems [18], but their relative contributions are not clear [19]. A significant role for MMP-2 & 9 has been demonstrated [20, 21] with a concomitant reduction in tissue inhibitors of metalloproteases (TIMP) mRNA [22]. This leads to the important question of the initiation, progression and termination of the angiogenic response.

Angiogenesis Overview

Angiogenesis can be initiated by increase in the production of angiogenic factors or by the reduction of angiogenesis inhibitors, but hypoxia is considered the most common cause [23]. Chief among the angiogenic factors secreted by inflammatory cells, pericytes or tumor cells are the soluble cytokines VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor), PDGF (platelet derived growth factor), TGF- β (transforming growth factor), TNF- α (tumor necrosis factor), and interleukins [1]. They act either by directly binding to their specific receptors on endothelial cells to induce a response or by acting on other cells that amplify or regulate this response. As described later, the specific interactions between the ECM and integrins form another wing of the regulatory mechanism. In typical feedback fashion, the byproducts of most molecular modulators of angiogenesis can induce an inhibitory effect on angiogenesis. For example, protease activity byproducts like angiostatin from plasmin, tumstatin, arrestin, and camstatin from collagens, and PEX from MMP-2 inhibit proliferation and

migration during angiogenesis [1, 3]. A soluble form of VEGF receptor VEGFR-1 acts as a VEGF antagonist [16]. Angiopoietin-1 is a ligand of the Tie-2 receptor, an EC specific receptor tyrosine kinase, that activates its signaling while Angiopoietin-2 is a competitive ligand that cannot activate signaling [11]. Angiopoietin-2 may thus be involved in destabilization of vessels during angiogenesis [24] and may be considered one of the earliest steps in the angiogenic switch. The angiogenic process is broadly divided into an initiation phase, during which the capillary plexus formation takes place, and a resolution phase that includes inhibition of EC proliferation and vessel maturation by formation of tight cell-cell junctions and secretion of a basement membrane [25].

Physiological angiogenesis is thought to begin with local vasodilatation under the influence of nitrous oxide (NO), regulated by vascular endothelial growth factor (VEGF), hypoxia or other proangiogenic stimuli. The transport of plasma proteins into the extravascular tissue is greatly increased, aided by formation of vesiculo-vacuolar organelles and loosening of interendothelial cell junctions involving a redistribution of PECAM-1 (platelet endothelial cell adhesion molecule) and vascular endothelial (VE)-Cadherin [26]. VE-Cadherins are specific to the endothelial cells, and their disruption interferes with vessel maturation and induces apoptosis [27]. The mature vessel with its well formed basement membrane must be first destabilized to allow for outgrowth of endothelial cells into the surrounding matrix. This involves a general relaxation of the microvessel structure and migration of pericytes and smooth muscle cells away from the EC tube (Figure 2.2) [24, 26]. A consequence of this increased permeability is the leakage of provisional ECM components like fibrinogen, fibronectin, and vitronectin into the ECM space to form temporary migration scaffolds for migrating endothelial cell

Figure 2.2. Overview of angiogenesis. Influence of angiogenesis on mechanical properties of the extracellular matrix.

sprouts [28].

Cell invasion into the ECM is preceded by matrilysis (Figure 2.3) and effected by three major categories of matrix cleavers- MMPs, lysosomal cysteine proteases, and serine proteases like the plasmin system. The plasminogen system – consisting of Plasminogen- the zymogen form, Plasmin- the activated form of the serine protease, and plasminogen activators (t/uPA) have a significant role in angiogenesis [18]. Plasmin can cleave several ECM proteins like laminin, fibronectin, thrombospondin, etc. and can

Figure 2.3. Matrilysis and sprout formation. Panel [A-C] focal matrilysis and vessel destabilization, sprout formation, and neovasculature maturation. Reprinted by permission from Wiley-Blackwell Publishers Ltd, UK: [J Cell Mol. Med.], Rundhaug [1], copyright (2005).

activate MMPs. Inhibition of plasminogen activator has been shown to reduce angiogenesis in vivo in genetically altered mice [29]. Collagen cleavage is one of the major roles of MMPs in angiogenesis [28, 30] and seems essential to endothelial cell invasiveness [17]. In vitro angiogenic sprouting can be blocked by blocking MMPs [15]. ECM constitution dictates the choice of proteases involved in this process, e.g., fibrin barriers are broken down by MT1-MMP or fibrinolytic systems while collagen I matrices invoke a MMP-2 synthetic response [26]. Basement membrane degradation involves uPA and MMPs [10].

Angiogenic endothelial cells migrate and proliferate to form sprouts that invade this local area of matrilysis (Figure 2.3) [31]. Endothelial cell migration and proliferation is stimulated by a variety of growth factors including VEGF, bFGF, angiopoietin-1 & 2, and epidermal growth factor (EGF). Most mitogens are nonspecific but VEGF has high specificity to endothelial cells [26]. TIMP-2 has been shown to have an anti-MMP independent, but protein tyrosine phosphatase dependant (PTP) inhibitory role on endothelial cell proliferation [32]. The control of the extent of vascularization is not

exactly understood but chemotactic gradients, tissue hypoxia and acidosis, along with a host of other regulatory factors, are involved [33]. The temporal precedence of migration over proliferation, with mitosis confined to growing sprout ends, has been suggested [31, 33, 34]. Recently, Gerhardt et al. have provided evidence that the tip of the growing sprout, called the tip cell, is nonproliferative and has a gene expression profile different from the remaining stalk cells enclosing a lumen [33]. Ingber et al. hypothesized that local basement membrane and ECM degradation changes the local matrix compliance and triggers a change in the tensional homeostasis of the cell with its neighbors and the ECM, thus causing a change in the cellular cytoskeleton and biochemistry that ultimately drives the angiogenic growth process [34]. The demonstration of several lamellipodia probing the matrix from the ‘apical’ cell lends further credibility to this hypothesis [33]. Capillary lumen formation requires polarization of ECs and is established by cell-cell contacts and cell-matrix contacts [10]. Endothelial cell tube formation is a dynamic process involving progression as well as regression of growing sprouts [7, 35].

The final step in angiogenesis is the maturation of new endothelial cell tubes by acquisition of a mural cell cover and secretion of a basement membrane (Figure 2.2) [4, 8, 26, 36]. Both ECs and fibroblasts produce matrix molecules and direct their assembly [3]. Leaky vasculature forms the temporary matrix, followed by secretion of specific ECM molecules like fibronectin, laminin, and collagen III and finally the secretion of collagen I by fibroblasts during the wound remodeling stages [3]. Pericytes are directly in association with endothelial cells and may even form direct connections via holes in the basement membrane, allowing for direct transfer of mechanical forces in addition to their perceived role in regulating vessel diameter and maturation [37]. Ang-1 facilitates

this maturation by promoting interactions between endothelial and peri-endothelial cells [24]. Part of vessel maturation is optimal branching and pruning of the newly developed vasculature to meet the local demands. PDGF-B is also involved in formation of a mural cell cover [3]. Typically, immature vessels lacking pericyte cover or basement membranes are ideal targets for apoptosis [38] and may play a significant role in the regulation of extent of vasculature remodeling by counteracting the effect of EC proliferation [12]. Apoptosis may also be anchorage dependant with inputs from alterations in the tensional equilibrium and contributions from the integrin $\alpha_v\beta_3$ [35]. Lack of cell-cell contact, destabilization of microtubular network preventing ECM remodeling, and blocking of the integrin $\alpha_v\beta_3$ induces higher apoptotic rates in vitro [35].

Growth Factors in Angiogenesis

VEGF, in different isoforms, and its receptor, VEGF-R2/KDR, are central to the process of angiogenesis. VEGF is a strong mitogen for vascular endothelial cells and plays a critical role in cell proliferation, differentiation, sprouting and migration. VEGF is mitogenic to other cells involved in angiogenesis like mononuclear cells and vascular smooth muscle cells (VSMC) and increases synthesis of MMP-1 and MMP-9 in vascular smooth muscle cells, facilitating their invasion into the ECM [39] or the vascular basement membrane. ECM bound bFGF is necessary for proliferation and migration [40]. PDGF secreted by the endothelial cells recruits mural cells and thus leads to vessel maturation with basement membrane deposition [41, 42]. Angiogenesis depends on VEGF [21] for initiation and PDGF for maintenance of blood vessels and prevent dissociation of pericytes from endothelial cells during angiogenesis [20, 43-45]. Platelet derived microparticles, consisting of VEGF, bFGF, and PDGF, can induce angiogenesis

[45]. Growth factors may also be involved in the phenotypic changes seen in response to external loading. VEGF, TGF- β , and PDGF have been shown to increase transiently after cyclic stretch [46, 47]. TGF- β also upregulates VEGF mRNA levels [46]. Electrical stimulation of muscle cells have demonstrated an increase in the VEGF production [48]. VEGF and other growth factor production by a tissue may thus serve as an indicator for requirement of increased vasculature and set up a directed chemotaxis to promote angiogenesis with ECs not being a major source [44, 49]. Blocking bFGF or PDGF reduces angiogenesis but does not completely block it [50]. bFGF and PDGF have additional roles in supporting the proliferation of ECs and maintaining the vascular endothelium, respectively [43].

VEGF binding sites are localized on endothelial cells [51] in adult rat tissue, even in the absence of active angiogenesis suggesting a role for VEGF in maintaining the vascular endothelium [50]. VEGFR-2 is the primary receptor mediating most angiogenic functions like increasing permeability, proliferation and migration [44]. The capillary-like structure forming ability of endothelial cell spheroids may be expressed via its transduction by the VEGFR-2/KDR complex [52-54]. VEGFR-2 is upregulated in endothelial cells seeded in a three-dimensional collagen matrix, which increases cell sensitivity to VEGF [55, 56]. Secretory form of VEGFR-1 is deposited in the ECM to become a matrix associated protein that specifically binds to $\alpha_5\beta_1$ integrin on ECs to promote cell spreading and three-dimensional vascular morphogenesis [57]. This spreading is however slower than that on fibronectin [57]. VEGF also has been shown to regulate assembly of focal adhesion complexes (FAK) in ECs [58].

Matrilysis and its Regulation in Angiogenesis

Two major proteinase systems are implicated in matrix degradation; the zinc dependant family of endopeptidases called Matrix Metalloproteases (MMPs), and the Plasminogens [59]. The pivotal role of MMPs is underlined by the inhibition of angiogenesis and matrilysis in response to MMP inhibition both in vitro and in vivo [15, 60]. Matrilysis can release matrix sequestered growth factors [25]. The collagenases (MMP-1, 3, 13), gelatinases (MMP-2, 9) and membrane bound MMPs (MT-MMP-1 or MMP-14) are thought to play a role in angiogenesis [1, 2, 61]. MMP production is largely dependent on ECM environment [10, 15, 26]. The dogmatic notion of matrilysis is that the interstitial fibrillar collagens I, II and III are degraded by the fibrillar collagenases MMP-1, 2, 13 [62], and the degraded fragments are broken down by gelatinases MMP-2 and 9. However, there is evidence to show that TIMP free MMP-2 can degrade soluble fibrillar collagens [62, 63].

MMP-2 is secreted as an inactive zymogen, and is subsequently activated by a mechanism involving TIMP-2, MMP-14, and an activated MMP-2 (Figure 2.4A). The involvement of membrane bound MMP-14 effectively localizes this process of activation to the cell surface where invasive activity is required [60]. Nakahara et al. demonstrated that MMP-14 can activate both bound and soluble MMP2 in cultures, but only MMP-14 localization to cell protrusions called invadopodia was necessary for cell invasiveness [64]. MMP-2 and 9 have a fibronectin type domain that binds to gelatins, collagen and laminin [62]. MMP-2, via its PEX domain binds to integrin $\alpha_v\beta_3$ which also localizes its activity by an MMP-14 independent mechanism [2]. MMP-2 regulation may depend on

the MMP-14/TIMP-2 ratio and an excess of MMP2 in solution may displace TIMP-2 from the cell surface [65].

The amount of MMPs in the environment is tightly regulated. Small amounts of MMP-2 are required for initial morphogenesis, but a sustained excess of this activity prevents tubulogenesis and causes reabsorption of vasculature by loss of matrix supporting scaffold [66, 67]. Under physiological conditions, matrilysis by MMPs is regulated at the level of transcription, activation of zymogens and inhibition by TIMPs or endogenous protein fragments. Tissue inhibitors of metalloproteases (TIMPs) form noncovalent 1:1 stoichiometric bonds with MMPs to inhibit their activity [61]. Expression of TIMP-1 is inducible, while TIMP-2 is constitutively expressed [61]. Other endogenous inhibitors of MMPs include tissue pathway inhibitor-2 and α_2 -macroglobulin [25]. Another rate limiting step, especially in vivo, is the cleavage of collagen I at specific sites [25, 30]. More recently, ECM tension has been shown to play a role in susceptibility to matrilysis, possibly linked to the accessibility of specific degradation sites [68].

Focal Matrilysis: Paradox of Simultaneous

Matrilysis and Cell Attachment

Fibroblasts form oriented tracts, starting from the random fibrils in freshly reconstituted collagen gels [69] by cellular traction [70, 71]. Realignment of collagen fibers [72] and straightening of kinks in the collagen structure first at the fibrillar [73], then at the molecular level, followed by molecular gliding and ultimate fibril structure disruption under loading has been demonstrated [74]. Enzyme cleavage sites on collagen have been attributed to susceptible areas in its helical structure. There are a limited

number of cleavage sites on the native collagen molecule for eukaryotic collagenases [75]. Enzymatic degradation of collagen depends on availability of specific motifs, which may conceivably be altered in a collagen fiber under loading [76].

The paradox of how cell generated forces can cause matrix remodeling while at the same time providing sufficient matrilysis for cellular invasion has not been directly addressed until recently [60], though possible mechanisms based on integrin and membrane bound MMP localization and activation were suggested [77, 78]. Wolf et al. recently demonstrated the co-localization of MT1-MMP and β_1 integrin on the forward leading edge of cell processes followed by a trailing proteolytic zone responsible for the actual matrix remodeling [60]. It is suggested that the anterior advancing lamella, via the actin and integrin systems, transfers the tractional forces and pulls the fibers towards the cell body and the transient MT1-MMP clumping does not cleave fibrillar collagen (steric hindrance) and functions mostly as an adhesive anchor. However, the cell body shows similar clumping of integrin and MT1-MMP and is actively involved in matrilysis [60]. A similar non-RGD dependant MMP-2 binding was demonstrated for integrin $\alpha_v\beta_3$, that can localize MMP-2 to the cell surface to support local invasion [78], especially on denatured or interstitial collagenase cleaved collagen, which has the cryptic binding sites for integrin $\alpha_v\beta_3$ exposed (Figure 2.4B). A local regulatory mechanism is established with MMP breakdown fragment PEX, also competitively binding to the same site [79].

Figure 2.4. MMP activation and regulation. (A). Surface localization of MMP-14 and activation of MMP-2. (B). MMP-14 independent localization of MMP-2. Reprinted by permission from ASCI: [Journal of Clinical Investigation], Stetler-Stevenson [2], copyright (1999).

ECM and Integrins in Angiogenesis

The extracellular matrix in question during angiogenesis consists of both the basement membrane lining the vessels (pericellular matrix) and the surrounding collagen I fibrillar stromal matrix. The pivotal role of local matrixlysis has been described above and it is clear that endothelial cell invasiveness is central to angiogenesis. This has formed the basis of antiangiogenic therapies by administration of MMP inhibitors [80]. The feedback regulation of angiogenesis by the associated degradation products has also been discussed above. Accompanying this matrixlysis is the deposition of various matrix

elements that switch from a temporary fibrin based matrix to a more permanent collagen I and III based stromal matrix and a collagen IV and laminin based basement membrane matrix [4, 8, 28]. Collagen I is a major secreted product during angiogenesis in vitro [81]. Collagen VIII may serve as a molecular bridge between different matrix molecules. It has been associated with rapidly proliferating cells [82]. Collagen VIII is less adhesive to cells and may thus play a significant role in promoting cell migration [83]. Additionally, other matricellular proteins mostly secreted by the endothelial cells, can modulate the cellular adhesion and deadhesion responses and regulate tube formation [28]. Decorin, a proteoglycan that is not synthesized by quiescent endothelial cells, is causally involved in the formation of capillary like structures, and is localized around the endothelial cords and cavities in the angiogenic phenotype [84, 85]. Decorin inhibits cell binding to collagen and fibronectin and promotes migration, which in turn may be responsible for cord like alignment [86]. Integrin mediated adhesion of ECs to fibrillar collagens may be a prerequisite to induction of Decorin translation [87]. Decorin expression seems to be under regulation of interleukins rather than growth factors like VEGF or FGF [87, 88]. Tenascin C, a matrix protein promoting endothelial cell migration [89], cord lengthening, branching and complex network formation, has a cytoprotective role [90], and shows regulation by VEGF [91] and MMPs [92].

The type of ECM can influence in vitro angiogenesis, though the mechanisms are yet unclear. Montanez et al. found differences in the lumen morphogenesis between Matrigel and collagen I based angiogenesis models [93]. Madri et al. demonstrated differences in endothelial cell phenotypic expression between collagen I and basement membrane based matrices [8]. Endothelial cells grown on collagen I matrices showed

more confluency, higher proliferation, lower production of basement membrane constituents, and late formation of fewer tube like structures (TLS) as compared to basement membrane based matrices. Additionally, cells cultured on the basement membrane surface of washed amnionic membrane showed significant TLS formation, while those cultured on the stromal side showed higher invasiveness into the stroma, but not through the basement membrane [8].

The family of transmembrane proteins called integrins is present at the junction of the ECM and the cell cytoskeleton. These integrins also form a part of the mechanosensory arm of the endothelial cells [28, 34]. Specific integrin subtypes bind to corresponding ligand epitopes on the ECM and alter downstream intracellular signaling. For example, cleavage of collagen IV of the basement membrane eliminates binding sites recognized by $\alpha_1\beta_1$ (ligand- helical collagen, AGGA) and reveals cryptic sites recognized by $\alpha_v\beta_3$ integrins (ligand- denatured collagen, RGD), which in turn show a dramatic increase in expression in angiogenic endothelial cells compared to their near absence on quiescent ones [28, 77, 94, 95]. Integrins also bind MMP2 in a non-RGD dependent manner and may serve to localize proteolysis in tandem with an invasive migration as reviewed by Eliceiri et al. [77]. Integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$, upregulated in response to VEGF, are receptors for both collagen and laminin, suggesting a modulation of cell surface adhesivity for migration. A similar ‘angiogenic’ integrin is $\alpha_5\beta_1$, and antibody based blocking any of these integrin types has been shown to reduce tumor growth and angiogenesis (review - [80]). This has been suggested as a potential antiangiogenic therapy [80].

ECs are linked to each other by tight junctions and adherence-type junctions, mediated by transmembrane calcium dependent VE- Cadherins [27]. VE- Cadherins may not be required for the initial assembly of EC tubes, but are essential once flow induced shear develops on the endothelial cell tubes [27]. This adhesion molecule is however a required part of the VEGFR-2/ VEGF-A complex that prevents apoptosis [27]. N- Cadherin may mediate the adhesion between mural cells and ECs [27]. A strong correlation exists between cryptic site exposure and activation of proMMP2 [96]. Thus, matrix remodeling during angiogenesis can exert a regulatory role by exposure of such cryptic sites, especially considered with evidence of shifting integrin ligand profiles from a predominantly collagen binding type (β_1) to one that is required for angiogenesis, survival and growth ($\alpha_v\beta_3$) [28, 94].

Mechanical Interactions Between Cells and the ECM

The in vivo environment is mechanically dynamic, exposing the endothelial cells to a variety of physiological forces like luminal fluid shear, radial distension and the accompanying circumferential stretch, and abluminal stretch from the surrounding ECM, especially in soft tissues. There is ample evidence supporting the interaction of cells with the surrounding matrix in a reciprocal manner whereby the cell traction generated within a substrate is regulated by the substrate material properties and the cell in turn responds to changes in the matrix properties [97, 98]. A decrease in stiffness of load deprived tendons and an upregulation of proteases has been shown to be ameliorated by low magnitude and low frequency cyclic strain [99-101]. Compensatory overloading of muscles in vivo has been shown to influence the local vascularity, and has been shown to be caused by a significant contribution of abluminal stretch rather than increased fluid

shear [102]. Several studies in vitro on fibroblasts and endothelial cells have demonstrated changes in cell migration, proliferation [103], orientation [104, 105], expression of adhesion molecules [106], and signalling or production of cytokines [103, 107], MMPs [104, 108], t/uPA (transient rise and then fall) [109], or protease inhibitors [109] in response to different magnitudes, frequencies, or duration of loading. Mechanical strain may be responsible for maintaining a functional phenotype of cells exposed to constant forces like VSMC (Vascular smooth muscle cells) and alterations in any perceived forces may lead to a phenotypic shift [110]. VSMC increase matrix production in response to mechanical loading [110]. Shear stress has been shown to cause smooth muscle cells to transdifferentiate and express EC markers like vWF and even capillary like structure formation [105].

In vitro, the contractile forces generated by endothelial cells and fibroblasts to remodel collagen I were found to be similar to that generated by dermal fibroblasts and within an order of magnitude of smooth muscle cells [111, 112]. Subconfluent and migratory cells were more potent at traction than confluent or quiescent cells, and the effect was diminished by MMP inhibition [111]. Substrate rigidity, a function of its (protein) concentration, was shown to regulate the capillary like structure (CLS) forming ability of endothelial cells, arguably by their ability to sustain multicellular retraction [98]. Lower matrix concentrations (less rigid) [95, 98, 113] and free floating constructs [97] were shown to support more florid CLS growth. A hitherto unknown level of substrate adhesivity or stiffness supporting retraction was shown to be essential in tubulogenesis using varying densities of fibronectin on the seeding substrate [98]. Network formation and morphology of endothelial cells were better with increasing

substrate thickness and thus its mechanical properties [114]. Long paths of condensed matrix fibers were shown between cell aggregates, formed in the direction of overlapping tractional force fields, according to the ‘two-center’ effect as originally described by Weiss. P in 1934 (Figure 2.5) [4, 81, 114]. This was substrate dependent and collagen I showed much less alignment than basement membrane [114]. Sieminski et al. have recently demonstrated that the relative magnitude of cell traction with respect to the substrate, rather than the absolute substrate stiffness, modulates capillary morphogenesis [97]. Simple changes in substrate concentrations could alter more than the matrix stiffness, like the availability of adhesion sites, or direct biochemical effects of possible cross-linkers added to increase stiffness. Altering mechanical boundary conditions to alter the substrate mechanical properties has been suggested as an effective alternative to isolate the effects of substrate stiffness [97]. Additionally, the alterations in mechanical properties of the extracellular matrix and the construct as a whole, in response to

Figure 2.5. The two-center effect. (A). Overlapping tractional force fields augment to align matrix (B). Cell alignment and migration along guidance path. Originally proposed by Weiss. P, 1934, leading to matrical tracks. Reprinted by permission from Springer Science: [In vitro Cell Dev. Biol.], Vernon RB [4], copyright (1999).

angiogenesis from prevascularized elements, have never been examined. An in vitro three-dimensional angiogenesis model from microvessels would thus provide an ideal system for the examination of changes in construct mechanics with angiogenesis, as well as the influence of external mechanical boundary conditions on the process of angiogenesis.

In Vivo and In Vitro Models of Angiogenesis

In vivo models of angiogenesis have been categorized by Jain et al. [115] as 1.) microcirculatory preparations in animals; 2.) vascularization into compatible matrices; and 3.) excision of vascularized tissue. Most relevant for in vivo observation of angiogenesis is the microcirculatory model, which has been further subdivided into 1.) chronic transparent chambers (e.g., rabbit ear chamber [116], dorsal skin-fold chambers [117], cranial windows [118, 119] in rats and mice); 2.) exteriorized tissue preparations (e.g., chorioallantoic membrane [30], rat or rabbit mesentery, hamster cheek pouch); and 3.) in situ preparations (e.g., corneal pockets [120] or iris implants) [9, 115, 121]. Some advantages of using in vivo preparations include close approximation to human pathophysiology, the ability to study flow, permeability, and effectiveness of therapeutics and toxicity in addition to the routine analysis of vessel morphology like length, diameters and branching density. The greatest challenge to using such nonhuman models, however, is from the difficulty in separating the different limbs of the responsible control, regulatory, or signaling system from the complex background environment in vivo possibly confounded by inflammatory responses [9, 121].

In vitro models of angiogenesis find several applications, e.g., drug activity and toxicity testing, studying EC differentiation during tubulogenesis, and identification of

molecular and regulatory mechanisms involved in angiogenesis [122]. One of the most commonly used models of angiogenesis in vitro is the culture of endothelial cell monolayers on various substrates or membranes as typical two-dimensional cultures. Endothelial cells, in the absence of any flow or other cell types, spread to confluence or near confluence depending on the substrate used, to form tube or cord like structures which given time interconnect to form a tubular network and hence referred to as the “tubulogenesis” assays [9]. Some of the earliest work in angiogenesis was accomplished on simple two-dimensional cultures consisting of a tissue culture grade substrate typically with a thin layer of ECM substrate component [8, 98]. An obvious drawback to this approach is the lack of a physiological context environment.

With the growing understanding that cell functions and secretory activities (normal phenotypic expression) may differ between two- and three-dimensional substrates [15], many models have adopted a three-dimensional variation of this tubulogenesis process. In brief, endothelial cells seeded on the surface of three-dimensional matrices like collagen I, Matrigel or fibrin gels invade the matrices on reaching near confluence to form endothelial cell cords as in the two-dimensional system (review - [9]). Endothelial cells from several sources like bovine aorta, human umbilical vein, or microvascular networks have been shown to produce cord like invasions into the matrices including lumen formation as discussed earlier (Page 19). Variations to this method include the use of cell clusters or cell spheroids coated on microbeads [123] as well as co-culture of different cell types [124]. Mural cells or pericytes play a significant role in angiogenesis as discussed above. A pure endothelial cell model without any feedback from the physiologically associated pericytes is seen as a significant drawback

to these systems [9]. An alternative approach, typically used in quantification of angiogenesis (automated) is the aortic or venous ring culture model, wherein thin rings or these large vessel explants are seeded in a three-dimensional matrix [125]. The presence of pericytes and other mural cells alone, however, may not be a significant improvement, especially since angiogenesis is typically thought to occur in the microvasculature.

It is thus clear that there is no ideal in vitro angiogenesis system that mimics physiology perfectly [122]. That said, the spontaneous angiogenesis observed from microvessel fragments in a three-dimensional collagen gel, in the absence of blood flow, as shown by Hoying et al. can be considered to be a step closer to recapitulating the physiologic process given the presence of mural cells and intact preformed microvessels as parent fragments [7]. This system of in vitro angiogenesis from preformed microvessels is best characterized as an ‘organ culture’ model. The ‘parent’ microvessels, obtained by partial digestion of epididymal fat pads as described by Hoying et al., include the full spectrum of microvascular elements, i.e., arterioles, venules, and capillaries, with their associated perivascular cells and basement membranes [7]. Growth of new vessel ‘sprouts’ is typically observed on the second day of culture, with dissociation of pericytes and destabilization of the parent vessels to allow sprouting and vessel elongation, as described earlier (Figure 2.2). The sprouts continue to elongate as patent tubes that branch and anastomose with other vessels, forming a complex vascular network filling up the gel (substrate) space by the tenth day of culture. Though the degree of maturation in these newly formed vessels is variable, implantation of these cultures (7-day-old constructs) in vivo has demonstrated their rapid inosculation with the

host vasculature [126] and currently provides an ideal model for the study of three-dimensional angiogenesis.

Mechanical Testing of Engineered Constructs

Most three-dimensional tissue culture substrates can be classified as natural or artificial hydrogels, characterized by their large water content. Collagen type I, the chief constituent of ECM in vivo, offers an ideal substrate to investigate cell-ECM interactions [15]. The characterization and control of construct mechanical properties is now thought of as an integrated and desirable outcome for engineered grafts, in addition to functional and morphological mimicking of native tissue. Construct strengthening or recapitulation of native tissue morphology has been reported for artificial tendons [127] and vascular grafts [108]. A thorough understanding of construct material properties in the presence and absence of cells is paramount to identify parameters for such interventional strategies. Once construct mechanical properties are known, the mechanical behavior can be represented by appropriate models of solids, liquids, or viscoelastic materials [128]. Such a model can also be used to study the mechanical function of cell cytoskeleton and matrix proteins or adhesion sites. This could also be the method of choice to characterize and optimize prospective graft tissues.

Several methods of mechanical testing or conditioning have been reported in literature based on the physical nature of the substrate, and the current discussion is confined to the methods relevant to testing hydrogels. Typically parameters like yield stress, ultimate stress and material modulus have been used as indicators of mechanical properties. Typically, the strain-rate dependence of viscoelastic materials makes it necessary to investigate parameters like storage and loss moduli, relaxation functions,

hysteresis, and normalized stress relaxation curves to completely characterize the construct material properties. Rheology has been the method of choice for estimation of mechanical properties of low concentration gels (substrates) [129, 130]. Other methods like laser trap microrheometry, manipulations of magnetic microparticles, stretching of substrates on elastic membranes, and direct tensile and shear tests have been used to characterize viscoelasticity of such constructs [131-133]. Some of the drawbacks of these methods include limited strain rates and magnitudes, limitations in concomitant force measurements, limitations of sample shape and aspect ratio, strain heterogeneity, and strain anisotropy. Response of the constructs to cyclically varying strain at different frequencies has been used widely to measure the complex moduli and thus the associated model constants according to the linear viscoelasticity theory [134-137]. However, the restricted applicability of these systems in tissue engineered constructs which typically are nonlinear with large deformations, necessitates the application of similar strain rate based analysis at different equilibrium strain magnitudes, where the linear viscoelasticity theory holds for the frequency analysis within the individual strain levels [134, 138].

Cell Orientation Under Stretch

A dynamic mechanosensory system in cells that responds to endogenous or externally applied forces has been well documented in various tissue culture models ranging from cell monolayers on deformable substrates to cells seeded in three-dimensional matrices as discussed earlier. The cell orientation response in itself has been studied in much detail, with an effort to identify the mechanisms leading to organized three-dimensional structures in vivo. Whether contact guidance or ECM modulation by

traction takes precedence in the orientation response is still unclear, with current evidence supporting a role for both (Figure 2.6).

Reconstituted collagen hydrogels with a predominant fluid fraction usually show a random distribution of collagen fibers [69]. In these matrices, cells have been shown to sense their environment and orient by internal traction along the direction of maximum resistance to deformation [139, 140], and migrate towards higher density of collagen [141], associated with higher stiffness [142]. Contraction and realignment of the extracellular matrix and cells can be explained both by the contact guidance theory [143] as well as by the tensegrity and tensional homeostasis theory [144]. Contact guidance on

Figure 2.6. Influence of the mechanical environment on angiogenesis. Changes in matrix and vessel orientation, vessel morphology, and possible interdependence.

prepatterned surfaces is widely used to achieve cellular orientation in vitro. Endothelial cells in general prefer to align along the direction of microgrooves with a ridge height and width preference [145, 146]. A similar patterning effect is induced by (pre) alignment of matrix fibers and graded orientation of cells can be achieved by altering amount of collagen fibrillar orientation [147]. Matrix (collagen) fiber structure and forces influence cell shape [148] via the cellular actin and microtubular framework as proposed in the tensegrity models. Since cell shape is determined by the internal traction exerted by the cytoskeleton and resisted by the attachment points to the ECM [149-151], such matrix alignment also occurs secondary to cell contractility or locomotion, where growing cell processes by way of their lamellopodia or adhesion complexes, attach to the matrix fibers exerting traction and orienting them to form ‘matrix migration pathways’ [70, 142, 152, 153]. Such matrix alignment has been shown to extend a substantial distance from the cell processes and may form similar ‘tracks’ of aligned matrix fibers even when cells are separated without interconnections between them [71, 154]. This process can be envisaged to occur until cells sense an optimum matrix reaction force below which cells continue exerting traction in an attempt to maintain ‘tensional homeostasis’ [143, 144, 155]. A minimum force, corresponding to about 20% of the endogenous forces generated by cells themselves, has been shown to be required to elicit a cellular mechanical response [156].

Orientation of cells under a strain field is thought to be an avoidance response where cells attempt to shield themselves from external forces that disturb their tensional equilibrium. The stress shielding response as discussed by Eastwood et al [157] suggests that orientation of cells along the direction of maximum principal strain allows the cells

the minimize their exposure to the three-dimensional strain field, especially when a long thin bipolar morphology is adopted, as in fibroblasts. A similar argument of minimizing area exposed to strain has also been put forth in explaining cell orientation perpendicular to stretch direction. The cause of the apparent discrepancy between the cellular responses to strain is yet unclear. The prevalent hypothesis seems to be that the alignment response of cells is an attempt to minimize the stress and injury experienced by the cells due to strain [158, 159]. This, however, cannot explain the orientation of cells along the direction of stretch, since this effectively puts the cells with their long axis along the stretch direction. A survey of few studies showing an orientation perpendicular to strain reveals that most were performed using silicone or other extensible films overlaid with cells directly or with cell seeded matrices though some had favorable size aspect ratios [158, 160-164]. As suggested by Eastwood et al., it must be perceived while considering similar studies of orientation on free matrices or substrates adhered to extendable films that this three-dimensional strain will be dependent on the shape and aspect ratio of the constructs and their boundary conditions and not necessarily on the direction of loading alone. Considering the tensegrity theory and the experiments that lend it credence in conjunction, it is clear that any increased forces on the cells would automatically act in line with the cell tractional forces and cause a shift in the internal force balance between the actin fibers in tension and microtubules in compression [150]. The alignment of matrix fibrils under strain and the fact that angiogenic endothelial cells have fragmented basal lamina and project pseudopods [165] into the surrounding ECM to form adhesion complexes indicate that an initial alignment response could be the result of contact guidance followed by development of a dynamic tensional homeostasis and further

reinforced by formation of ‘matrix guidance pathways’. The retraction of cut ends and concomitant loss of orientation [157] could be seen supporting this hypothesis, where loss of external forces causes a larger internal tension unsupported by the microtubular compression, leading to loss of orientation and rounding up of cells followed by cytoskeleton remodeling.

Alignment of cells in response to multiple simultaneous cues can point to possible mechanisms inducing cell orientation and the precedence of the stimuli in eliciting the same. On grooved surfaces, stretch perpendicular to the grooves has been shown to increase the orientation perpendicular to stretch direction (along the grooves) as compared to stretch along the groove direction [166]. Mudera looked at contact guidance and mechanical loading [167], and determined that cells not in direct contact with a contact guidance fibronectin strand perpendicular to stretch direction could be realigned (up to 80%) along the direction of stretch. They also demonstrated an increase in MMP activity in the dual cue zone possibly arising from the cells oriented along the contact guidance strand that were unable to reorient along the stretch direction, arguably in an attempt to remodel the surrounding matrix. It is thus conceivable that three-dimensional tissue architecture is the result of such precisely timed complex interactions of different stimuli and unique cell responses. This considered with other similar observations of alignment of endothelial cells, fibroblasts, neuritis [168], and tenocytes [169] on three-dimensional ECM derived matrices under static or cyclic stretch indicates that the gradient of cellular tensional homeostasis under resting or external loading conditions, may play a significant role in three-dimensional tissue architecture development in conjunction with contact guidance from the underlying extracellular matrix.

Quantitative Characterization of Angiogenesis

Recognition of the importance of angiogenesis in health and disease has led to investigations on the regulation and control of this process, necessitating a quantitative approach based on image analysis. In addition to tissue engineering, a quantitative assessment of angiogenesis and vascular morphology benefits studies of tumor angiogenesis [170-172], changes in vascularity [173, 174] and developmental biology [175]. Different models of angiogenesis are prevalent for their unique applications as discussed earlier, e.g., the Chorioallantoic membrane of chick embryo, aortic ring of vena caval explants in three-dimensional substrates [176, 177], tube like structures from invaginating confluent endothelial cell monolayers and endothelial cell coated spheroids [154, 158, 178]. Angiogenesis in a three-dimensional substrate from preformed vascular elements however offers a closer approximation to in vivo physiological conditions, while retaining many features of the aforementioned systems [179]. Many studies on vascular [158, 176, 178] or cellular morphology are based on two-dimensional image analysis techniques (of an arguably three-dimensional phenomenon in case of angiogenesis). The angiogenic process is quantitatively compared using parameters like vessel density, vessel orientation, number of branches, and vessel lengths. Automated image analysis of vasculature is gaining importance both in vivo [174, 180] and in vitro [176, 181, 182] with recent development of numerous automated thresholding and feature analysis algorithms for oriented tissue [183-185]. Most methods however are relatively recent and benchmark parameters and applications to angiogenesis quantification are relatively limited. A 'Blockwise' processing method to speed up analysis of large image mosaic stacks was recently reported and incorporated into a commercially available

software package, Amira™ [186]. The defining feature of this algorithm was its ability to process images in ‘blocks’ of subimages optimized for memory utilization on standard computers, and preserve global and local skeleton properties. Another advantage of this process was the processing of image blocks with and without overlapping, such that the inner blocks needed to be processed only once, and thus was computationally beneficial.

Pathological Angiogenesis and Tissue Engineering

Angiogenic microvessels are an integral part of the physiological granulation tissue during wound healing. This process also has clinical relevance in many disease states [10]. Peripheral vascular diseases alone affect 15% of population over the age of 55 [122]. Some of the common vascular disorders are [187]: 1.) Chronic wound healing (impaired) or ulcers due to insufficient vasculature or venous stasis leading to pressure ulcers [188]; 2.) Peripheral and cardiac ischemia, characterized by poor perfusion of the periphery or of the cardiac tissue; 3.) Rheumatoid arthritis, the hallmark of which is an invading vascular pannus into the cartilage matrix [189, 190]; 4.) Retinal vasculopathies like diabetic retinopathy and macular degeneration where fluid extravasation, edema, and fragile capillaries cause vitreal hemorrhage or retinal detachment, leading to blindness [191]; 5.) Tumor vascularity.

Several pro and antiangiogenic strategies are being attempted to regulate the local vascularity. Treatment of peripheral ischemia is being addressed using angiogenic growth factors like VEGF [3, 192]. Fibroblasts embedded in a three-dimensional collagen matrix implanted on epicardial surface of infarcted hearts have been shown to promote vascularization to a distance of about 300 μm or about 28% of ventricular wall thickness in SCID mice [193]. Engineered vessels formed by seeding grafts with

endothelial cells remain immature after implantation [3]. The alternative could be the use of preformed vascular networks, elaborated in vitro. It has been suggested that cells do not survive beyond a 100 μm radius based solely on diffusion [194]. This assumes special significance in attempts to recapitulate the three-dimensional functional morphology of complex organs and their vasculature [191].

Contributions of Dissertation Research

Compelling evidence in the literature reviewed above points to a close interaction between angiogenic endothelial cells, the surrounding extracellular matrix, and external mechanical loading. Though molecular regulators of angiogenesis continue to be well described, an adequate description of the mechanical properties of angiogenic tissue and its temporal relationship to other molecular factors is not available. A significant part of this dissertation research addresses these questions. The role of external mechanical forces on endothelial cell morphology to guide three-dimensional tissue organization has received much attention in recent years, especially in the areas of vascular and musculoskeletal tissue engineering. A large volume of literature exists on the effects of mechanical loading of different cells in two- or three-dimensional matrices mostly relating to changes in their phenotypic expression and orientation in response to fluid shear, tensile or radial stretch. Though construct vascularity has repeatedly been shown to be pivotal to graft survival, there have been no studies on the role of external mechanical loading on preformed vascular elements. Engineering of three-dimensional structures like muscle and tendons have been reported and an organized vascular network would be an ideal complement to these engineered systems. The relative magnitudes of stress involved in these systems and the relative contribution from contact guidance is

unclear. The final section of this dissertation research is aimed at understanding this problem from a basic science perspective.

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CHAPTER 3

DESIGN AND APPLICATION OF A TEST SYSTEM FOR VISCOELASTIC CHARACTERIZATION OF COLLAGEN GELS¹

Abstract

Characterization and control of the mechanical properties of the extracellular matrix is critical to the interpretation of the results of in vitro studies of cultured tissues and cells and for the design of functional engineered constructs. In this work a viscoelastic tensile test system and custom culture chambers were developed and characterized. The system allowed quantification of strain as well as the stresses developed during cyclic viscoelastic material testing. Finite element analysis of the culture chambers indicated that the tensile strains near the actuated ends of the gel were greater than the strains experienced by material in the center of the culture chambers. However, the strain was uniformly distributed over the central substance of the gel, validating the assumption that a homogeneous strain state existed in the central region of the chamber. Viscoelastic testing was performed on collagen gels that were created with three different collagen concentrations. Results demonstrated that there was a significant increase in the dynamic stiffness of the gels with increasing equilibrium strain, collagen

¹ Reprinted by permission from Mary Ann Liebert Inc. publishers, copyright (2004): [Tissue Engineering, Vol. 10, No. 1-2], Krishnan, L., Weiss, J. A., Wessman, M. D., Hoying, J. B., "Design and application of a test system for viscoelastic characterization of collagen gels," pp: 241-52.

concentration and frequency of applied strain. With increasing strain rate, the loss modulus dropped initially and then increased at higher rates. Mechanical testing of gels at different time intervals up to 7 days after polymerization demonstrated that the material properties remained stable when appropriate environmental conditions were maintained. The ability to characterize the viscoelastic properties of gels after different periods of culture will allow the quantification of alterations in gel material properties due to changes in cell cytoskeletal organization, cell-matrix interactions and cellular activity on the matrix. Further, the test device provides a means to apply controlled mechanical loading to growing gel cultures. Finally, the results of this study will provide guidance to the design of further experiments on this substrate.

Introduction

In vitro constructs composed of tissue components and/or cells and extracellular matrix (ECM) provide simplified systems for the study of cell-matrix interplay in health, embryonic development and in pathological states such as inflammation and tumorigenesis [1]. Cell-matrix interactions play a pivotal role in the mechanical properties of tissue constructs - the very presence of the cells in the ECM constructs can increase the stiffness of the matrix, and cells can in turn modify the matrix and alter its material properties [2-4]. This is particularly significant when studying the process of wound healing due to a massive infiltration of new microvasculature at the injured site followed by an alteration of the extracellular matrix by metalloproteinases and other proteases secreted by endothelial and inflammatory cells [5]. Cells can also alter ECM properties directly. As an example, endothelial cells grown in ECM constructs can contract the construct [6] and produce a provisional matrix [7]. Conversely, stretching of

the ECM via an attached substrate induces cell alignment as well as other varied responses such as synthesis of Prostaglandin E [8, 9] associated with matrix reorientation [3]. For all of these cases, the material and functional properties of the ECM and the overall construct are directly altered.

Collagen is the main constituent of many ECMs and cell-populated collagen gels have been used extensively for in vitro systems [10] and for model studies of healing [11]. Collagen gels are viscoelastic as demonstrated by creep, stress relaxation, hysteresis and strain rate dependence. The viscous nature of these constructs arises from both inherent viscoelasticity of the solid phase and fluid movement resulting in viscous drag between the solid and fluid phases during loading [3]. For practical applications of functional tissue engineered constructs, it is essential to characterize and control the mechanical properties of these constructs. Numerous studies have assessed the response of cells to mechanical stimulation and many novel devices have been designed to apply static as well as dynamic forces to growing cell cultures. There have only been few instances of extensive characterization of the ECM constructs which are used for study of cell mechanics and interactions based on the use of various indirect techniques like micropipettes, markers [11], viscometry [10] etc.

Mechanical stretching devices have been used for the in vitro study of mechanotransduction, focusing either on the cellular or the ECM responses [12]. These devices have taken a variety of approaches: deformable elastic membranes where deformation is induced by vacuum, hydrostatic pressurization, platen or prong displacement; uniaxial [8, 9, 13], biaxial [14] or radial stretching systems; fluid shear test systems [15], piezoelectric crystals [16]; the use of commercial materials test machines

[6], and similar devices [17, 18]; rheology [10], markers and finite element analysis [1, 11] etc. A detailed review of the various systems is provided by Brown [12]. Almost all of these systems are designed to provide mechanical stimulation to growing cells or cultures and typically do not have the ability to simultaneously quantify the applied strains and resulting forces simultaneously [16]. Other limitations include strain heterogeneity, strain anisotropy, reactive stresses induced by fluid shear at the interfaces, fluid pressurization or acceleration [19]. Very little work has been performed to characterize the frequency-dependent viscoelastic behavior of cell-ECM constructs or even the isolated ECM constructs. Many of the devices that are currently used for mechanical conditioning are inadequate for use in viscoelastic materials characterization.

Our laboratories are investigating the effects of angiogenesis on the ECM. An *in vitro* model of angiogenesis in collagen gels has been developed to isolate the effects of angiogenesis on ECM material properties. In support of this work, the objectives of this study were to design and characterize a new materials test system and culture chambers for the viscoelastic characterization of collagen gels, to analyze the strain distribution in the loaded culture chambers using finite element analysis, and to examine the effects of collagen concentration, strain rate and equilibrium strain level on the viscoelastic response of collagen gels. Based upon the viscoelastic response of native biological soft tissues, we hypothesized that there would be a moderate increase in dynamic stiffness as a function of strain level and frequency and that the phase angle would be relatively unaffected by changes in strain level and strain rate.

Materials and Methods

For the intended application, the test system needed to be able to quantify the applied strain and the resulting forces under a range of cyclic strain rates. Further, the test chambers needed to provide a region of homogeneous strain during uniaxial extension for characterization of the viscoelastic material properties.

Chamber Design

Custom culture chambers (65.0 x 18.0 x 12.7 mm) were CNC machined from sheets of Acrylic and Lexan (Regional Supplies, Salt Lake City, UT) – materials that do not adhere to collagen (Figure 3.1). A glass microscope slide (75 x 25 mm) formed the bottom of the chambers to enable microscopic evaluation of growing gels. Chambers were attached to the microscope slide with biocompatible silicone glue (Med 1041, Nusil, Carpinteria, CA). The gels were polymerized around a fixed horizontal anchor post (6.0 x 8.5 x 3.0 mm) and a moveable actuating post (5.0 x 17.0 x 3.0 mm), yielding a gage length of 20 mm (4:1 aspect ratio). Four holes (1.6 mm dia.) in both the actuating and anchor posts provided a mechanical link between the polymerized collagen gel and the test system. Elongation of the gels required free space behind the actuating post. A large reservoir was incorporated into the design for this purpose. During polymerization, the reservoir was occluded with a removable block. The actuating post was held upright with two 316 stainless steel pins (Small Parts Inc, Miami Lakes, FL) that passed through apertures in the chamber sides. These pins also ensured that a consistent specimen length was maintained between each test. The actuating post was attached to a 0.25 N load cell (accuracy ± 0.00012 N, Transducer Techniques, Temecula, CA). Specimen thickness was measured midway between the anchor bar and the flag using digital calipers. An average

of measurements taken at the edge of the chamber and in the center of the gel was used to account for meniscal effects at the edges of the gel.

Finite Element (FE) Modeling

A half-symmetry FE model of the gel and anchor posts was created using commercial mesh generation software (XYZ Scientific Applications, Livermore, CA) to simulate the equilibrium tensile test configuration in terms of application of forces and boundary conditions (Figure 3.1B). The anchor posts were modeled as rigid and the gel was represented as a hyperelastic neo-Hookean material [20] with the following strain energy function W : [21]

$$W = G(\tilde{I}_1 - 3) + \frac{K}{2}[\ln(J)]^2 \quad (3.1)$$

Here, G is the shear modulus, $\tilde{I}_1$ is the first deviatoric invariant of the right Cauchy deformation tensor, [22] K is the bulk modulus of the material and $J = V/V_0$ is the local volume ratio. The shear modulus G was chosen based on the experimental values for tangent equilibrium modulus E for the gels polymerized with 3.0 mg/ml collagen at 6% equilibrium strain (7314 Pa) and K using the relationship from linear elasticity, $G = 3EK / (9K - E)$. As the volumetric (bulk) material behavior of the collagen gels was unknown, the bulk modulus K was varied over three orders of magnitude so that the ratio of $G:K$ was 10:1, 100:1 and 1000:1. The holes in the anchor and actuating posts were included in the model and the gel was modeled as passing through the holes (Figure 3.1C). Two types of boundary conditions were considered between the gel and the anchor posts. First, we examined the case of perfect bonding between the posts and the

Figure 3.1. Test chamber and FE model. (A). Custom Acrylite chambers with polymerized collagen I gel and culture media. Actuating post (1) is held in position by stainless steel pins. The second post (2) is attached to the base of the chamber. The reservoir (3) contains only culture media and provides space for stretching of tensile test region, between labels (1) and (2). The posts contained four small holes near their base to allow polymerization of the collagen gel through the posts. Dashed line indicates region of gel represented in finite element analyses. (B). Finite element mesh used to analyze the transfer of strain from the actuating posts to the center of the specimen. (C). Cross-section at the level of the posts as indicated by solid arrows in panel B. In panels B and C, the FE mesh used for the gel material is the light color and the FE mesh used for the post is the dark color.

gel. Second, the case of no adhesion between the front of the anchor block and the interior of the holes and the gel material was investigated; rather, sliding contact surfaces were used to simulate the transfer of load between the gel and actuating/anchor posts on those surfaces during actuation. The nonlinear, implicitly integrated FE code NIKE3D (LLNL, Livermore, CA) was used for all FE analyses [23]. The motion of the movable anchor block was discretized in quasi-time and an incremental-iterative nonlinear solution procedure was used (see, e.g., [24]).

Preparation of Collagen Gels

Sterile rat-tail collagen type I (BD Labware, Bedford, MA) was mixed with concentrated DMEM (Gibco, Grand Island, NY) to yield a final concentration of either 1.5, 3 or 4.5 mg/ml of collagen and 1X DMEM. Streptomycin (100 µg/ml, Invitrogen, Gibco BRL), Penicillin (100 units/ml, Invitrogen, Gibco BRL) and Fungizone (0.25 µg/ml, Invitrogen- Gibco BRL) were added to yield the final DMEM solution. The collagen solution was prepared in a sterile environment at 4°C, the pH was adjusted to 7.4 with 1M NaOH and 1.5 ml of this solution was pipetted into custom culture chambers (see below). Care was taken to ensure that air bubbles were removed. The solution was polymerized at 37°C and 100% humidity in a sterile environment for 30 minutes. The gels were then covered with 2.5 ml of the 1X DMEM solution. The gels were incubated at 37°C in a 5% CO₂ environment. Gels were either tested on the same day of preparation or at specific time intervals after the polymerization and storage in an incubator.

Twenty-two separate collagen gels and chambers were used to evaluate the effect of culture period, while six chambers each were used for the three different collagen

concentrations. Chambers used for evaluation of the effect of culture period were tested either on Day 1 (N=11) or Day 7 (N=11) of culture. The chambers with different concentrations of collagen were tested on the day of polymerization.

Mechanical Test Device

A mechanical test device was designed and constructed using a piezoelectric stage (Piezojena, Hopedale, MA) as a mechanical actuator to generate sinusoidal motion profiles at specified frequencies (Figure 3.2). The piezo stage enabled controlled microscopic displacements at a variety of frequencies while minimizing the effects of heat and electric fields [16]. Culture chambers were mounted on the piezo stage with the long axis of the chambers aligned with the movement axis of the stage. The piezo actuator was attached to two translation stages (Newport, Irvine, CA) to allow positioning

Figure 3.2. Material test system. (A). Left panel - the tensile test apparatus used to perform viscoelastic testing of the collagen gels. A vertical positioning stage (a) facilitated alignment of the load cell (b) with the actuating post embedded in the polymerized gel. The chamber (c) was attached to the piezoelectric actuator (d). Side-to-side alignment and application of equilibrium tensile strains larger than the movement range of the piezoelectric actuator were applied with an x-y translation stage (e) equipped with micrometer heads. Displacement and strain were measured using an LVDT (f) White arrows indicate movement directions of translation stages and actuator. (B). Right panel – close-up of the interface between the load cell and actuating post (black arrow).

during setup. The bottom stage also allowed application of lateral (y-axis) movements larger than the movement range of the piezo stage. Displacement was measured using an LVDT (Schaevitz, Hampton, VA). The load cell was mounted on a separate platform attached to a z-axis positioning stage, eliminating motion-induced artifacts. The piezo stage was driven by amplified signals (12V40 amplifier, Piezosystems, Hopedale, MA) from a programmable digital function generator (Tektronix, Wilsonville, OR). Force and displacement data were collected using an A/D card and the Labview software (National Instruments, Austin, TX).

Testing Protocol

Before testing, each gel was placed in a acrylic test enclosure and allowed to equilibrate to ambient temperature in the enclosure (25-27°C). The CO₂ concentration in the enclosure was maintained at 5% during testing. Each chamber was mounted on the piezo stage and the actuating post was secured to the load cell interface. The load cell stage was then raised approximately 150 μm so that the actuating post did not touch the chamber base. The gel was allowed to equilibrate for 5 minutes and then the load cell was re-zeroed. The gel was preconditioned by first stretching it to 6% strain and then allowing it to stress-relax for 20 minutes. The zero-load length was then re-established and the gel was stretched to 2% of its initial length at approximately 1%/sec and allowed to stress-relax until the change in force was within the noise band of the load cell signal (less than 0.0001 N/sec). This typically required 20 minutes, with larger strain levels requiring a longer relaxation time. This was followed by cyclic sinusoidal stretching at frequencies of 0.05, 0.1, 1.0 and 5.0 Hz with an amplitude of $\pm 0.5\%$ strain. The entire procedure was then repeated at equilibrium strain levels of 4 and 6%. The strain

amplitude of $\pm 0.5\%$ was chosen based on preliminary testing that demonstrated a linear response of the gels to this amplitude – in other words, when a sinusoidal strain-time input was applied, the resulting stress-time signal was well-described by a sine wave in terms of time variation and the positive and negative portions of the signal amplitude were nearly identical about the equilibrium stress level. Small sinusoidal strain oscillations about an equilibrium strain allow application of linear viscoelastic theory for determination of dynamic stiffness and phase angle, enabling the assessment of the effects of strain rate on the modulus and energy dissipation characteristics of the material.

Data Reduction and Statistical Analysis

Equilibrium stresses were calculated from the forces measured during the relaxation tests at 2, 4 and 6% and the initial cross-sectional area for all three collagen concentrations. The resulting set of equilibrium stress-strain points for each collagen concentration were fit to a third-order polynomial. The slope of this curve was determined at each strain level to obtain the equilibrium tangent modulus. The equilibrium tangent modulus represents the modulus due to the elastic part of the material response, after all viscoelastic effects have subsided.

Cyclic stress-time and strain-time data were fit to a four-parameter sine function (Sigmaplot, SPSS Inc., Chicago, Illinois):

$$f = Y_o + A \sin \left(\frac{2\pi}{b} t + \phi \right) \quad (3.2)$$

Here, Y_o is the equilibrium stress or strain level, A is the amplitude, $2\pi/b$ is the frequency of oscillation (radians), and ϕ is the phase angle (radians). When fitting the

stress-time data, the value of b obtained from the fit of the strain-time data was used. The dynamic stiffness M (Pa) and phase angle ϕ were calculated as [25]:

$$M = \frac{A_{\sigma}}{A_{\varepsilon}}; \quad \phi = \phi_{\sigma} - \phi_{\varepsilon}. \quad (3.3)$$

Here, $(A_{\varepsilon}, \phi_{\varepsilon})$ and $(A_{\sigma}, \phi_{\sigma})$ are the amplitudes and phases of the strain-time and stress-time data, respectively. The dynamic stiffness M is the ratio of the amplitude of the stress response to that of the strain input, and thus represents a measurement of the viscoelastic tangent modulus of the material for a particular strain rate and amplitude. The phase angle ϕ describes the lag in time between the stress and strain sine waves and is a direct measurement of energy dissipation by the material. For a purely elastic material, $\phi = 0$, indicating that the stress-time and strain-time signals are completely in phase, while for a fluid, $\phi = \pi/2$, indicating that the stress response leads the strain by 90 degrees. Viscoelastic materials will exhibit a phase angle between these two values.

Three one-factor ANOVAs were used to assess the effect of collagen concentration on equilibrium stress at each strain level. A two-factor repeated measures ANOVA was used to assess the effect of strain level (repeated measure) and collagen concentration on the equilibrium stiffness of the gels. Two-factor repeated measures ANOVAs were used to examine the effect of equilibrium strain level and excitation frequency on dynamic stiffness and phase at each time point. To evaluate the effect of culture period, a two-factor repeated measures ANOVA was performed at each strain level and effect of interactions between the factors (day and frequency) was also evaluated. A two-way ANOVA was used to evaluate the effect of concentration,

frequency and interactions between these factors. The effects of equilibrium versus dynamic calculation of modulus (E versus M , repeated measure) and collagen concentration on the modulus of the material were assessed at each strain level using a two-factor repeated measures ANOVA. Significance was set at $p \leq 0.05$ for all comparisons. When significance was found, Tukey tests were performed between the different levels of each factor.

Results

Integrity of all gels was maintained during the mechanical testing. There were no measurable variations in pH or molarity over the culture periods and evaporation of medium was negligible. In the course of the pilot tests as well as over 50 tests with these gels, there was no damage to the gels during the mechanical testing. By running a sharp #11 surgical blade around the edges of the gel just prior to testing we ensured that the gels were free of all the recesses of the chamber. During loading, gels stretched at the anchor points, but the gels did not separate from the anchors due to polymerization around the posts. Preliminary tests examining the effect of repeated testing of the same gels at similar strain levels and different frequencies did not demonstrate any effect of the testing itself.

Finite Element Simulations

Regardless of the chosen bulk modulus or boundary conditions, the finite element simulations demonstrated that a highly uniform uniaxial strain field was always present in the center 80% of the tensile test region of the sample (Figures 3.3A and 3.3B). The bulk modulus had a minimal effect on the strain at the center of the sample and thus results are

Figure 3.3. Results of finite element simulations. (A). Axial strain distribution for the case of perfect bonding between the posts and collagen gel. (B). Axial strain distribution for the case when the gel is allowed to separate from the interior face of the posts and is not bonded in the holes through the posts. (C). Graphs of applied axial strain versus strain in the center of the sample for the two boundary conditions considered.

not shown. The boundary condition between the posts and the gel also had minimal influence on the relationship between applied actuator strain and the strain experienced by the sample in the center, although it did alter the distribution of axial strain near the posts. In both cases there was a strong linear relationship ($R^2 = 0.999$).

Equilibrium Stress-Strain Behavior

The shape of the equilibrium stress-strain curves from tests of the gels with different concentrations of collagen was nearly linear for the lowest collagen concentration and became mildly upward concave for the highest collagen concentration (Figure 3.4). The average equilibrium tangent modulus E calculated from the static equilibrium stress-strain data for a collagen concentration of 3.0 mg/ml at 6% strain was

Figure 3.4. Equilibrium stress-strain behavior of collagen gels with different concentrations of collagen, Day 0 tests (mean $\pm$ sem).

1538.5±130.5 Pa. The average peak stresses were between two and three times the equilibrium stresses and hence the tangent modulus itself will be about two to three times higher than the tangent equilibrium modulus. The trends were otherwise parallel to those observed for the tangent equilibrium modulus as shown in Figure 3.4. It would however be necessary to account for the rate dependant viscous elements prior to making any estimate of the tangent modulus from the tangent equilibrium modulus and vice versa. There was a significant effect of collagen concentration on the equilibrium stress at all three strain levels ($p < 0.001$ in all three cases). Tukey tests between levels revealed that there were significant differences between the 3.0 and 4.5 mg/ml collagen concentrations at all three strain levels ($p < 0.001$ in all cases) but not between the 1.5 and 4.5 mg/ml collagen concentrations.

Effect of Collagen Concentration on Viscoelastic Properties

There was a significant increase in dynamic stiffness with increasing concentrations ($p < 0.001$ for 2,4,6%) and with increasing frequency ($p = 0.015$, 2% and $p = 0.045$, 4 and 6%) with interaction between frequency and concentration at 4 and 6% ($p = 0.712$, 2% and $p = 0.028$, 4 and 6%) (Figure 3.5). The stiffening effect was more marked with increasing concentrations of collagen than with increasing strain levels. There was also a significant effect of concentration on the phase shift at each strain level ($p = 0.003$, 4 and 6%) except at 2% strain ($p = 0.681$) and with increasing frequencies ($p = 0.015$, 2% and $p = 0.045$, 4 and 6%), with a significant interaction between concentration and frequency at 4 and 6% ($p = 0.028$ for 4 and 6%) (Figure 3.6). At lower strain levels and frequencies, the phase difference was higher for gels with lower collagen concentration, and the difference becomes less pronounced with increasing strain levels

Figure 3.5. Dynamic stiffness of gels with different concentrations of collagen, Day 0 tests at (A). 0.05, (B). 0.1, (C). 1.0 and (D). 5.0 Hz (mean $\pm$ sem).

Figure 3.6. Phase shift of gels with different concentrations of collagen, Day 0 tests at (A). 0.05, (B). 0.1, (C). 1.0, and (D). 5.0 Hz (mean $\pm$ sem).

and frequency. There was a large decrease in the phase shifts for all concentrations at 0.1 Hz and then an increase again at higher frequencies to values close to those at 0.05 Hz. At this trough at 0.1 Hz there was also a reversal of the concentration effect, with the 4.5% gels showing larger phase shifts than the 1.5% gels.

Effect of Equilibrium Strain Level and Strain Rate on Viscoelastic Properties

When plotted against excitation frequency, the dynamic stiffness showed a mild positive slope on a log–log scale. There was a significant increase in dynamic stiffness

with equilibrium strain level for both Day 1 and Day 7 groups ($p < 0.001$), indicating that the rate-dependent modulus of the material is influenced by its position on the equilibrium stress-strain curve. There was a significant effect of strain rate on phase shift ($p = 0.002$ for Day 1, $p = 0.005$ for Day 7), but there was no effect of strain level on the phase delay.

Dynamic Stiffness Versus Equilibrium Tangent Modulus

The modulus values calculated from the equilibrium stress response of the gels were considerably lower than the dynamic stiffness values determined from the cyclic viscoelastic test data. For instance, at a collagen concentration of 3 mg/ml, 6% prestrain and strain rate of 5 Hz, the dynamic stiffness was 21.84 ± 0.21 KPa, while the equilibrium modulus was 1.53 ± 0.01 KPa. Thus, over 90% of the material response at 5 Hz was due to the viscoelastic part of the material behavior (Figure 3.7).

Effect of Test Day on Viscoelastic Properties

There was no significant effect of postpolymerization test day on the dynamic stiffness in repeated dynamic testing about equilibrium strain levels of 2, 4 and 6% ($p = 0.346$, 0.155 and 0.249 , respectively) or on the phase shift ($p = 0.531$, 0.939 and 0.855 , respectively) (Figure 3.8).

Figure 3.7. Effect of collagen concentration on stiffness. (A). equilibrium tangent modulus and (B). dynamic stiffness, Day 0 tests at 5 Hz excitation (mean $\pm$ sem).

Figure 3.8. Effect of culture period. Top row: Log-log plots of dynamic stiffness as a function of frequency for Day 1 (A) and Day 7 (right) gels. There was an increase of the stiffness with increasing equilibrium strain levels, but there was no effect of test day. Bottom row: Semi log plots of phase angle as a function of frequency for Day 1 (C) and Day 7 (D) gels. The phase angles showed a drop at 0.1 Hz and then again increased at 1 and 5 Hz. All data are for a collagen concentration of 3.0 mg/ml.

Discussion

The objectives of this study were to design and evaluate custom culture chambers and a dynamic mechanical test system for measurement of the viscoelastic material properties of collagen gel constructs, and to determine the effects of collagen concentration and period of culture time on the viscoelastic properties of the gels. There was a significant effect of frequency and equilibrium strain level on the dynamic stiffness and phase angle of the gels, and the dynamic stiffness increased nonlinearly with increasing concentrations of collagen. The results of this study demonstrate that, under appropriate conditions, the material properties of native collagen gels can be maintained in culture with only minimal changes up to at least 7 days.

The uniaxial tensile test system offers distinct advantages for evaluation of the material properties of ECM constructs as well as for tissue or cell mechanics. The major advantage of the present system is the ability to apply arbitrary strain waveforms, both static and dynamic, at different rates as well as the quantification of the associated stresses. The close approximation that we achieved to a routine tensile test specimen with this chamber and the actuating mechanism also ensures that we minimized errors arising due to variables introduced by shear [13] involved in gripping the substrates and strain heterogeneity. Collagen gels are also known to be stiffer at the point of anchorage due to sticking of the collagen to the substrates [17]. To avoid difficulties associated with adherence of the collagen to the culture chamber sides and bottoms, materials that do not adhere to collagen were used in the chamber design – actuation was achieved via polymerization of the gels around the anchor bars as opposed to relying on adhesion.

The FE simulations showed that the amount of strain applied to the gel is related to the boundary conditions at the interface between the posts and the collagen gel. When perfect bonding was assumed, the strain at the center of the sample was nearly identical to that applied by the actuating post. When this condition was relaxed to allow for transfer via only contact between the gel and posts, the strain at the center of the specimen was still very close to the applied axial strain, but the distribution of strain near the posts showed higher gradients. This result is to be expected due to stress concentrations near the posts. Regardless of the amount of strain transferred, the strain field in the central $\frac{3}{4}$ of the gage length was homogenous and linearly related to the applied actuator strain, and this is the most important characteristic required for a tensile test configuration. Observations of the amount of separation between the gels and the posts during the experiments suggest that the true boundary conditions between the posts and the gel lie somewhere between perfect bonding and transfer solely via contact. In the modeling, we assumed isotropic hyperelastic material behavior for the gel to model the equilibrium stress-strain behavior of the material. Although the gels are clearly viscoelastic and consist of both a fluid and solid phase, the assumed material behavior provides a reasonable approximation to the equilibrium material behavior for the purposes of determining the homogeneity of the strain field under conditions of small sinusoidal perturbations about an equilibrium strain configuration.

The equilibrium stress-strain behavior of the gels demonstrated a linear relationship at the lower collagen concentrations (1.5 and 3.0 mg/ml), with the relationship showing more upward concavity at the highest collagen concentration of 4.5 mg/ml. This change in the shape of the curve is likely due to an increased propensity for

the collagen fibrils to crosslink at the higher collagen concentration, resulting in more recruitment of random fibers along the stretching direction during extension of the gel [26-28]. A similar trend was observed for the changes in dynamic stiffness and equilibrium tangent modulus with increasing collagen concentration. The dramatic difference between the equilibrium tangent modulus and the dynamic modulus demonstrates the contribution from the rate dependent viscous part of the constructs.

The collagen gels exhibited a moderate strain rate-dependent dynamic stiffness and were more viscous at low and higher strain rates, with the change in frequency-dependence occurring at about 0.1 Hz. The frequency dependent trends in both dynamic modulus and phase shift in our study are consistent with those reported by Wakatsuki for cell-populated matrices [18]. Our reported values for dynamic stiffness are higher than values previously reported for the complex moduli of these gels. The differences are likely due to the much higher collagen concentrations used in the present work. Additional issues that could play a role include the differences in test temperature and the specific methods used to compute the modulus. When comparing the present results to other data in the literature (e.g., [11, 18]), it is important to note that the present data were collected at 25-27°C. At a temperature of 37°C, the temperature most often associated with incubators, the stresses can be predicted to be lower based solely on the rheological behavior of collagen solutions [27].

The properties of collagen are influenced by temperature, pH and osmolarity of the nutrient solution, fiber diameter, associated cells and extracellular constituents. It has been demonstrated that fiber diameter can influence collagen mechanical properties [29]. Fiber diameter, however, has been shown to be unaffected by changes in pH changes

over a range of temperatures, and it has also been suggested that the viscoelastic properties of collagen depend more on the fiber lengths rather than their diameters [29]. This, in combination with the results of this study, demonstrates that the rise in stiffness of the gels with strain level and strain rate is not merely due to variations in environmental conditions causing an increase in cross linking. Another factor that needs to be considered is the theory of aging of collagen fibers with time and the increase in stability of collagen fibers even after completion of polymerization mainly due to hydrophobic interactions. The only available rheological data was acquired after a period of only three hours of aging [30].

Tests of the collagen gels were carried out after a particular period of culture, rather than serially. This was intentional, as it is anticipated that serial testing of the vascularized gels will affect the growth of the microvessel fragments via mechanical conditioning. The dramatic effects of mechanical loading on cell and tissue metabolism have been demonstrated repeatedly (e.g., [3-5, 17, 31-33]). Although the present system can be readily adapted for repeated testing inside an incubator, our intended application is for the measurement of changes in ECM properties due to growth. Attempts to remove the gels from the incubator, perform mechanical testing, and then return the gels to culture resulted in contamination of the gels. The strategy of testing the gels once after a fixed period of culture avoids both of these difficulties.

Dynamic linear viscoelastic analysis is a useful tool for investigation of strain- and rate-dependent material properties [34]. By subjecting a material to small sinusoidal perturbations about an equilibrium strain at different frequencies, the linear viscoelastic material properties of the gel can be quantified. Changes in dynamic stiffness with

frequency are representative of the effect of strain rate on material modulus and changes in damping (phase shift) are representative of changes in hysteresis or energy dissipation. In the present data analysis, it is assumed that the strains in the gel are homogenous, that the material is isotropic and that the assumptions of linear viscoelastic theory apply for perturbations about a particular equilibrium strain level. The latter implies that when a sinusoidal strain-time input was applied, the resulting stress-time signal was well described by a sine wave in terms of time variation and it was symmetric about the equilibrium stress level. It should be noted that the system is in no way restricted to linear viscoelastic materials characterization, and that other dynamic strain profiles and magnitudes can be applied readily with the piezoelectric actuator. Such an approach could be used to characterize the nonlinear viscoelastic response of the material using more advanced viscoelastic theories to describe the resulting data (e.g., [35-38]).

This study developed a tensile test machine that allows viscoelastic characterization of ECM constructs and expands on the capabilities of other systems reported in the literature by allowing the simultaneous quantification of both applied dynamic strains and resulting stresses. The system was used to evaluate the viscoelastic material properties of collagen type I constructs as a representative extracellular matrix system. The data generated from this work can be used to formulate a model of the viscoelastic nature of these collagen gels, which can subsequently be used to describe its constitutive behavior. This model can then be extended to encompass the variations induced by culturing of cells in such constructs. Our system acquires special importance when we consider the studies establishing the relationship of mechanical stimuli to cell growth and differentiation [17, 39-41]. This work will also benefit other areas of tissue

mechanics where culture of cells in a physiologically significant and mechanically dynamic environment is desired [8, 9, 32, 33, 39]. We are presently working to evaluate the effects of microvasculature growth on gel material properties. When combined with the results of the present study, these data will enable the formulation of a model to predict the constitutive behavior of such ECM constructs and in formulation of functional engineered constructs.

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CHAPTER 4

INTERACTION OF ANGIOGENIC MICROVESSELS WITH
THE EXTRACELLULAR MATRIX¹

Abstract

The extracellular matrix (ECM) plays a critical role in angiogenesis by providing biochemical and positional cues as well as by mechanically influencing microvessel cell behavior. Considerable information is known concerning the biochemical cues relevant to angiogenesis, but less is known about the mechanical dynamics during active angiogenesis. The objective of this study was to characterize changes in the material properties of a simple angiogenic tissue before and during angiogenesis. During sprouting, there was an overall decrease in tissue stiffness followed by an increase during neovessel elongation. The fall in matrix stiffness coincided with peak MMP mRNA expression and elevated proteolytic activity. An elevated expression of genes for ECM components and cell-ECM interaction molecules and a subsequent drop in proteolytic activity (although enzyme levels remained elevated) coincided with the subsequent stiffening. The results of this study show that the mechanical properties of a scaffold tissue may be actively modified during angiogenesis by the growing microvasculature.

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Introduction

Angiogenesis, the growth of new vasculature from existing blood vessels, is central to normal physiological and healing processes. Angiogenesis occurs by either intussusception, where the parent vessel splits into daughter vessels, or more commonly by endothelial cell sprouting from parent vessels. During angiogenesis, normally quiescent capillary endothelial cells (EC) [1, 2] are stimulated to switch to an angiogenic phenotype [3]. Angiogenic endothelial cells take on invasive and proliferative phenotypes, degrade the basement membrane and invade the extracellular matrix (ECM) [4]. These cells form new vessel sprouts that guide the growing neovessel through the ECM. Eventually, the neovessels mature with the development of pericyte coverage and form perfusion-capable capillary networks [5].

The ECM influences the process of angiogenesis through two mechanisms. The first is by acting as a biochemical regulator of endothelial cell phenotype. Considerable work has demonstrated the importance of outside-in signaling that occurs from the ECM via the integrins in endothelial cells. For example, the ligation of the $\alpha_v\beta$ integrins is essential to prevent apoptosis in angiogenic endothelial cells [1, 6]. Also, matrix molecules, such as those comprising the basement membrane, decorin and tenascin C, signal endothelial cells to establish and maintain capillary-like structures during and following angiogenesis. ECM molecules such as hyaluronan can inhibit angiogenesis and reduce endothelial cell migration and adhesion [7]. Finally, the ECM may indirectly regulate the phenotype of endothelial cells by acting as a reservoir for bound growth factors and proteases thereby affecting the bioavailability of these important biochemical signals. The second mechanism by which ECM can regulate angiogenesis is by serving

as a structural framework to support sprout and neovessel structure and function. Matrix flexibility or stiffness is a significant modulator of metalloproteinase activity in endothelial cells [8, 9]. Metalloproteinase activity is an essential aspect of angiogenesis [10]. In addition, matrix molecules, such as fibronectin, may be responsible for inducing tissue alignment and providing architectural clues for the neovessels [11, 12]. Finally, the ECM provides a foundation from which intracellular stresses, critical to endothelial cell function, are generated.

The invasive and proliferative activities of angiogenic ECs may have the propensity to influence the material properties of the ECM. Endothelial cells produce MMPs 2, 9, 13 and 14 that degrade the basement membrane and remodel the surrounding ECM to facilitate capillary ingrowth [13]. Given how the ECM plays a central role in angiogenesis, the endothelial cell-dependent effects on ECM may in turn influence angiogenesis. However, little is known about this dynamic interaction between the extracellular matrix and angiogenic vessels and its impact on wound healing. A better understanding of this interaction will also lead to discerning the underlying mechanisms affecting the mechanical properties of healing or healed tissues, as well as facilitate the design of functionalized tissue engineered constructs. The objective of this study was to investigate alterations in the mechanical properties of three-dimensional vascularized collagen constructs due to angiogenesis and to interpret these changes in the context of measurements of microvessel proliferation, proteolysis and gene expression.

Materials and Methods

In Vitro Model of Angiogenesis

A three-dimensional model of angiogenesis was adapted for use in the present study [14]. Rat microvessel fragments were isolated from epididymal fat pads of retired breeder Sprague-Dawley rats (>500g). The pads were minced and subjected to limited digestion with 2 mg/ml of *Clostridium* collagenase (Worthington Biochemicals, Lakewood, NJ) and 2 mg/ml bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) in Dulbecco's cation free phosphate buffered saline (DCF-PBS, pH 7.4) at 37°C. The digestion solution was centrifuged to obtain a pellet. The pellet was washed twice and resuspended in DCF-PBS containing 0.1% BSA and then sequentially filtered through 500 µm and then 30 µm sterile nylon membrane filters in a Collector tissue sieve (Thermo EC, Milford, MA). The first filtration step eliminates undigested particulate debris and the second step retains larger vessel fragments of interest, while allowing single cells and smaller fragments to pass through. The 30 µm membrane was flushed with 0.1% BSA DCF-PBS and the fragments were collected in a sterile petri dish. Forty microliters of this suspension was pipetted on a glass slide and the fragments were counted systematically throughout the entire drop. The final yield of fragments in the suspension volume was determined from an average of at least two such counts. The suspension was then centrifuged to obtain a pellet of rat fat microvessel fragments. Fragments consist of arterioles, venules and capillaries with abluminally-associated smooth muscle cells and pericytes [14].

Sterile rat tail collagen type I (Discovery Labware, BD Biosciences, Bedford, MA) was mixed with filter sterilized Dulbecco's modified Eagle medium (DMEM,

GIBCO-Invitrogen, Carlsbad, CA) to yield final concentrations of 3 mg/ml of collagen in 1X DMEM. Microvessels were suspended in the collagen solution at 15,000 fragments/ml. Of this suspension, 1.1-1.5 ml was pipetted into custom culture chambers and polymerized at 37°C and 95% humidity for 30 min (Figure 4.1) [15]. The custom chambers have fixed anchors near one end and a mobile post near the other end with holes (1 mm dia) to allow the collagen solution to pass through. There is a reservoir of media at one end, which is blocked during polymerization. Gels are overlaid with 1X DMEM containing 10% (v/v) fetal bovine serum (Intergen, Purchase, NY) and incubated until time for mechanical testing or RNA extraction. Approximately 30% of the media was changed on the third day of culture and every other day thereafter. Cultures for mechanical testing and RNA extraction were used on Days 1, 6 and 10 during which the gels contracted from the sides of the chamber (Figure 4.1).

Figure 4.1. Custom culture chambers with vascularized constructs. (A). Top: Day 6 of culture. Contraction of gels away from the vessel wall was evident at Day 6. (B). Bottom: Day 10 of culture. By Day 10 the constructs have contracted away from the chamber walls but integrity with the anchor points was maintained.

Viscoelastic Testing of Vascularized Gels

Dynamic viscoelastic testing was carried out using a piezoelectric actuated test system (Figure 4.2) and our established protocol [15]. Twenty-four vascularized gels and 15 native gels (no vessels) were tested. Eight vascularized gels each were tested at Day 1, Day 6 and Day 10 of culture, while 6, 4 and 5 native gels polymerized from the same collagen were tested at the same culture periods.

To quantify changes in geometry, construct cross sectional areas were measured at the middle third and an average of multiple measurements was used. The cross-sectional area of each vascularized construct was normalized by that of the corresponding

Figure 4.2. Mechanical test device. a – x-y positioning stage. b – z-positioning stage. c – LVDT (Linear Variable Differential Transformer). d – load cell. e – piezoelectric stage.

native gel (V/N) to obtain a normalized cross-sectional area for graphical display. A one-way ANOVA was used to test for any effect of culture time on the normalized cross-sectional area.

Gels were stretched between a fixed anchor post on the custom culture chamber and a mobile anchor interfaced to a load cell. The testing protocol consisted of preconditioning at 6% strain, followed by sinusoidal testing at 3 strain levels (2, 4, 6%) and 4 strain rates (0.05, 0.1, 1, 5 Hz). Viscoelastic properties of the constructs were calculated using the principles of linear viscoelasticity [16]. Dynamic stiffness (M) and phase shift (ϕ) of the constructs were calculated as in Equation 4.1:

$$M = \frac{A_\sigma}{A_\epsilon}; \quad \phi = \phi_\sigma - \phi_\epsilon. \quad (4.1)$$

Here, $(A_\epsilon, \phi_\epsilon)$ and (A_σ, ϕ_σ) are the amplitudes and phases of the strain-time and stress-time data, respectively. The dynamic stiffness M represents the tangent modulus of the material for a particular strain rate and amplitude. The phase shift ϕ describes the lag in time between the stress and strain sine waves and is a direct measurement of energy dissipation by the material. For a purely elastic material, $\phi = 0$, indicating that the stress-time and strain-time signals are completely in phase, while for a fluid, $\phi = \pi/2$, indicating that the stress response leads the strain by 90 degrees. Viscoelastic materials exhibit a phase angle between these two extremes.

The dynamic stiffness of the vascularized constructs was normalized to that of the native constructs at each culture time to compute a ratio of vascularized to native gel (V/N). The phase shift difference of native gels was subtracted from that of the

vascularized constructs at each culture time to compute a phase difference between vascularized and native gels (V-N). Multiple 2-way ANOVAs were used to assess the effect of culture time and strain level for the individual test subsets and the normalized data. Kruskal-Wallis analysis was used when assumptions of normality or equal variance were violated.

Effects of MMP-9 on Material Properties of Native Collagen Gels

The effect of MMP-9 on the material properties of native collagen gels was determined by viscoelastic testing of gels before and after the addition of MMP-9 to the media overlaying the gels. Mechanical testing of sixteen native collagen gels was carried out 24 hours after polymerization. Media in the chambers was then replaced with culture media containing 0.1% Thiomersal, Penicillin-Streptomycin (100 U/ml) and Amphotericin B (0.25 ug/ml). MMP-9 activity on the substrate was verified by zymography. Activated human recombinant MMP-9 (67 kDa form, PF140, EMD Biosciences) was added to the media in eight of the chambers (4 ug per chamber) and the remaining eight gels were used as controls. Mechanical testing was repeated after 24 hours. Multiple 2- way RM-ANOVAs (repeated measures ANOVA) were used to examine the effect of test time and MMP-9 treatment on the mechanical properties of the gels.

Microvessel Fragment Density

Three gels each from Days 1 and 6 of culture, and four gels from Day 10 of culture were analyzed for vessel density. Vascularized gels were fixed in 1% formalin

and processed into paraffin. Five sections, 10 μm thick, were taken every 8th section, from the central third of the gel and mounted in paraffin. The sections were deparaffinized and stained with rodent endothelium specific fluorescein conjugated GS-1 lectin (Vector Labs, Burlingame, CA). The total numbers of GS-1 positive structures were counted at 4X magnification with a Nikon Diaphot inverted microscope. Four fields were evaluated per section and the density expressed as the number of vessels per unit area (mm^2). The mean vessel density per unit area was evaluated from five sections per sample at each time point. One-way ANOVA was used to analyze the effect of culture time.

Proteolytic Activity

Media and culture lysates were evaluated for gelatinolytic activity. MMP extraction was based on established protocols [17, 18]. Collagen gels were weighed and homogenized (OMNI International, Marietta, GA) for 15 seconds in low-calcium Triton X buffer (10 mM CaCl_2 in 0.25% Triton X- 100, 1 ml / 200 mg tissue wet weight, Sigma-Aldrich, St. Louis, MO). The homogenate was centrifuged for 30 minutes at 4°C and the supernatant containing unbound MMPs was collected. The pellet was resuspended in high calcium buffer (50 mM Tri-HCl, 100 mM CaCl_2 , 0.15 M NaCl, pH 7.4), heated at 60°C for 6 minutes, and centrifuged for 30 minutes at 4°C to dissociate bound collagenases. Six vascularized and native gels were analyzed for proteolytic activity using the EnzCheck collagenase assay kit (Molecular Probes, Carlsbad, CA). Culture media was collected during periodic media changes, stored at -70°C and pooled media was used for analysis. Fluorescence due to substrate lysis was quantified using a SynergyHT multiwell plate reader (Bio-Tek, Winooski, VR). *Clostridium* collagenase

was used to generate a standard curve. Each reaction volume of 200 μ l was made up of 10 μ l of DQ-gelatin (1 mg/ml), 90 μ l of buffer (50 mM Tris-HCl, 5 mM CaCl₂, pH 7.4), and 100 μ l of clostridial collagenase, pooled media, or supernatants from tissue lysates or heat extractions. The reaction mix was incubated overnight at room temperature and the fluorescence read after 12 hours and expressed as average absolute Units of activity in each subset (media and tissue lysates) per time point. The total genomic DNA in the tissue lysates was determined fluorimetrically using the Quant-iT DNA assay kit (Molecular Probes, Eugene, OR). The total proteolytic activity of each sample (media and lysates) was normalized to the amount of DNA in the sample and the activity expressed as U/ μ g of DNA. One-way ANOVAs were used to analyze the effect of culture time.

Quantitative Polymerase Chain Reaction (PCR)

Six vascularized gels each were cultured for 1, 6 and 10 days. Additionally, pooled microvessel fragments equivalent to the number used for six gels were harvested to serve as Day 0 controls. Samples were stored in RNALater (Ambion, Austin, TX). RNA was extracted using a phenol based protocol [19]. Further processing was carried out in RNEasy MINI elute columns (Qiagen, Valencia, CA). RNA was eluted from the column in 52 μ l of nuclease free water. Two microliters of the solution was used to quantify RNA using Ribogreen (Molecular Probes- Invitrogen, Carlsbad, CA). The remaining RNA solution was treated with DNase I (Sigma-Aldrich, St. Louis, MO) and then processed through an RNeasy MinElute cleanup kit (Qiagen, Valencia, CA). RNA was reverse transcribed to cDNA using the EndoFree RT kit (Ambion, Austin, TX).

Primers (Biosearch Technologies, Novato, CA) were designed for 18 genes of interest and 5 housekeeping genes [Glyceraldehyde phosphate dehydrogenase (GAPDH), Hypoxanthine-Guanine phosphoribosyl transferase (HPRT), Ubiquitin-C (UBC), Dynactin-2 and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (YWH)], covering the broad categories of MMPs [Matrix metalloproteinases (MMPs 2, 9, 13), Membrane bound MMP (MT1-MMP)] and their inhibitors [(Tissue inhibitors of MMPs) TIMPs 1 and 2]; growth factors [Vascular endothelial growth factor (VEGF) and its receptor, basic fibroblast growth factor (bFGF), bone morphogenic protein (BMP-1), platelet derived growth factor (PDGF)]; matrix proteins (collagens 1, 3 and 8); and cell-matrix interaction proteins [decorin (DCN), tenascin C (TNC), fibronectin (FN), hyaluronic acid synthase (HAS)] using nucleotide sequences identified from the NCBI database and Primer3 [20]. A Blast search was performed on the primer pairs to confirm target specificity. PCR reactions were optimized individually with rat skeletal muscle cDNA as the template

Quantitative PCR was carried out on a thermal cycler (RotorGene 3000, Corbett Research, Australia). Each 10 µl reaction consisted of UDG Supermix (Invitrogen, Carlsbad, CA), 1.5-6 mM primers (forward/reverse), SYBRGreen dye (Molecular Probes, Carlsbad, CA), and 5 µg cDNA except in case of controls. Reactions were carried out in triplicate and grouped so that all samples for a specific target gene were analyzed in a single run. Reaction efficiency for each target was calculated from a standard curve of pooled cDNA. Expression efficiencies were calculated from a standard curve for each target. Relative expression levels were calculated using the method of Vandesompele et al. [21]. Briefly, the best housekeeping genes that did not alter significantly over the

experimental conditions were determined using the GeNorm software and a normalization factor was calculated for each sample. The relative expression levels of these targets were the ratios of their raw expression levels to this normalization factor, allowing direct comparison of expression levels between both different treatment effects and different target genes. One-way ANOVAs were used to compare the normalized data for significant differences when the data were normally distributed. The Kruskal-Wallis test was used when data were not normally distributed.

MMP Zymography

Six additional vascularized gels (2 each at Days 1, 6 and 10 of culture) were analyzed via MMP zymography to assess the presence of MMP-2 and MMP-9 protein. For each construct, the culture media was removed and saved at -20°C in 50 µl aliquots. Each gel was homogenized using a tissue homogenizer (Tissue Tearor, BioSpec Products, Inc., Bartlesville, OK) in 200 µl of zymography loading buffer (80 mmol/L Tris-HCl (pH 6.8), 4% sodium dodecyl sulphate (SDS), 10% glycerol, and 0.01% bromophenol blue) and centrifuged at 12,000 rpm for 10mins in 1.5 ml microfuge tubes. The supernatants were removed and stored at -20°C. Equal amounts of protein (MicroBCA, Pierce, Rockford, IL) were separated by electrophoresis in a 0.75mm SDS 7% PAGE gel containing 0.2% porcine gelatin. An additional lane was loaded with 2 µl of purified native MMP-2 and MMP-9 enzymes diluted 1:200 (Sigma, St. Louis, MO) for each run to serve as migration standards. Following electrophoresis, SDS was removed by soaking in 75 ml of renaturing solution (2.5% TritonX-100) for 45 minutes. The gels were then preincubated in developing buffer (50 mmol/L Tris-HCl (pH 7.5) and 10 mmol/L CaCl₂) at room temperature for 30 minutes followed by incubation overnight at

37°C in fresh developing buffer to develop the zymogram. Zymograms were imaged with a digital camera using back illumination. Pixel intensities for select bands were determined from the images using the image analysis package Metamorph® (Molecular Devices, Sunnyvale, CA).

Results

In Vitro Model of Angiogenesis

Angiogenesis began predictably on the 3rd day of culture with endothelial cells breaching the abluminal walls to form sprouts both at the ends and mid regions of parent vessels. Newly formed translucent endothelial cell cords were evident by the 6th day, progressing or regressing dynamically to ultimately form vascular networks by the 10th day (Figure 4.3). The resulting microvessel networks, characterized previously [14, 22], have a collagen IV basement membrane, stain for vWF and integrate with host vessels after in vivo implantation [22].

Constructs contracted away from the chamber walls as early as the 6th day,

Figure 4.3. Phase contrast micrographs of rat microvessel fragments, illustrating representative microvessel sprouting and growth over time in cultures. (A). freshly isolated microvessel fragment showing lumen (arrow) and blood cells. (B). early sprout formation (*) at Day 1. (C). translucent invading neovasculature at Day 7 (*), elongating into the collagen gel from parent vessel (arrow). (D). same field as panel C, showing well formed vascular networks and anastomoses at Day 10.

resulting in decreases in cross-sectional area (Figure 4.4). There was a significant effect of culture time on the normalized cross-sectional area ($p < 0.001$). In particular, the Day 10 normalized cross-sectional area was significantly less than that at Days 1 and 6 ($p < 0.001$ in both cases).

Microvessel Fragment Density

Vessel density increased gradually from the 1st day to the 6th and 10th days of culture (Figure 4.5). The vessel density at 10th day was significantly higher than the density at 1st ($p < 0.001$) and 6th days of culture ($p = 0.018$).

Figure 4.4. Effect of culture time on normalized cross sectional area (ratio of vascularized to native constructs). There was a significant decrease in cross sectional area at Day 10 of culture (Bars: Standard Error).

Figure 4.5. Effect of culture time on microvessel density. Day 10 cultures had significantly higher microvessel density than Day 1 and Day 6 cultures (Bars: Standard Error).

Mechanical Properties of Collagen Gels

We sought to quantify changes in the mechanical properties of the vascularized constructs that occurred during angiogenesis. To accomplish this, we performed viscoelastic testing of vascularized and native gels at three different times during culture. In general, there was a strain level dependent increase in dynamic stiffness for both vascularized as well as native constructs at all test times ($p < 0.001$). There was also a strain rate dependent stiffening of the constructs (Figure 4.6A). The V/N ratio for dynamic stiffness fell significantly ($p < 0.001$) from Day 1 to Day 6 and increased significantly ($p < 0.001$) at Day 10 for all strain levels and strain rates. Overall there was no significant effect of strain level ($p = 0.042$, $\alpha^c = 0.025$) and the strain rates ($p = 0.076$)

on the difference in phase shifts over the duration of culture ($p = 0.040$, $\alpha^c = 0.025$) (Figure 4.6B). A ‘Bonferroni correction’ for the value of α was used for this comparison to get a final $\alpha = 0.05$. The median of all the phase shifts was 0.255 radians with 0.314 radians as the 75th percentile.

Effects of MMP-9 on Material Properties

of Native Collagen Gels

Since it was hypothesized that changes in the mechanical properties of the vascularized constructs may be due at least in part to MMP activity on the collagen matrix, we examined the effects of MMP-9 treatment on the mechanical properties of native gels. The posttreatment values for each gel were normalized to its pretreatment values to get a paired ‘Post/Pre’ ratio. The MMP-9 treatment caused a highly significant reduction (by about 50% - Figure 4.7) in the dynamic stiffness of the native collagen gels ($p = 0.001$, $p = 0.003$, $p = 0.003$ with $\alpha = 0.05$ for strain levels of 2, 4, and 6% respectively). Control groups did not show a significant difference in dynamic stiffness between test times ($p = 0.316$, $p = 0.686$, $p = 0.219$ for strain levels of 2, 4, and 6% respectively) and the normalized ratio was close to 1.0 (Figure 4.7). There were no significant changes in the phase shift differences for the MMP-9 or control groups.

Quantitative PCR

Gene expression studies were performed to assess the time-profile of expression of genes for MMPs, ECM components, cell-ECM interaction genes and growth factors during the culture period. In general, maximum expression of MMPs occurred at Day 6 of culture, remaining relatively unchanged at Day 10 (Figure 4.8A). There was a

Figure 4.6. Results from viscoelastic mechanical testing of vascularized constructs. (A). Normalized dynamic stiffness as a function of culture time, strain level and frequency. The normalized stiffness is the ratio of that of the vascularized gels to that of the native constructs at the same time point. (B). Phase shift difference as a function of culture time, strain level and frequency. The difference in phase shift is the phase shift of the vascularized constructs minus the phase shift of the native gels at the same time point (Bars: Standard Error).

Figure 4.7. Effects of MMP-9 on material properties of native collagen gels. Post treatment data normalized pairwise to pretreatment data. (Bars: Standard Error).

significant increase in MMP-2 expression level between Day 1 and Day 6 ($p < 0.001$), Day 1 and Day 10 ($p = 0.002$) and Day 6 and Day 10 ($p = 0.009$). MMP-9 showed a significant increase at day 6 as compared to Day 1 ($p = 0.038$). MMP-13 mRNA was elevated at Day 6 ($p = 0.003$) and Day 10 ($p = 0.002$) in comparison to the levels at Day 1. There were no significant effects of culture time on expression levels for MT1-MMP (MMP-14, results not shown), TIMP-1 or TIMP-2.

Three of the four growth factors did not show a significant change in mRNA levels throughout the culture period (Figure 4.8B). Only PDGF showed a significant peak in mRNA on Day 1 of culture ($p = 0.004$ and 0.006 with respect to Days 6 & 10, respectively), returning to baseline levels by Day 6.

There were no significant changes in mRNA levels for collagens I, III or VIII (Figure 4.8C), but there were significant changes in the expression of cell-matrix interaction genes (Figure 4.8D). There was a significant upregulation of message for decorin, tenascin C and fibronectin with culture time. Decorin levels increased through the culture period ($p < 0.001$ for all comparisons), while fibronectin ($p < 0.001$, $p = 0.002$) and tenascin C ($p < 0.001$ for both cases) mRNA levels at day 6 and day 10 were significantly higher than their day 1 levels. Hyaluronic acid synthase, an anti-angiogenic molecule, was significantly downregulated from its day 1 levels at both day 6 and day 10 ($p = 0.006$, $p < 0.001$).

Proteolytic Activity and MMP Zymography

We assessed MMP enzyme levels and proteolytic activity in the cultures to determine whether there were temporal differences between gene expression profiles and active enzyme levels. The total normalized proteolytic activity showed a significant increase on the 6th day compared to both the 1st ($p = 0.011$) and 10th ($p = 0.045$) days of culture (Figure 4.9A). Figure 4.9 (B and C) shows the results for one of the two sets of constructs analyzed for specific MMP-2 and MMP-9 activity. Consistent with the gene expression changes, there was an increase in the amount of MMP-2 and MMP-9 protein able to be activated from day 1 to day 6. This was true for both diffusible enzyme (in the media) and nondiffusible enzyme (in the construct). In addition, there was a greater proportion of MMP-2 enzyme to proenzyme in the day 6 and day 10 constructs as compared to day 1 constructs (Figure 4.9D). Interestingly, MMP enzyme levels remained elevated in the day 10 samples, despite the drop in measured overall protease

Figure 4.8. Gene expression as a function of culture time. (A). MMPs and their inhibitors. (B). collagens. (C). cell-matrix interaction genes. (D). growth factors (Bars: Standard Deviation). See text for details.

Figure 4.9. Quantification of proteolysis. (A). Proteolytic activity combined for the media and tissue lysates, normalized to DNA content. * = significant difference, (Bars: Standard Error). (B & C). MMP zymography results for the construct lysates (cell/ tissue lysates) and media, respectively. Lane labeled “M” represents MMP-9 and MMP-2 standard purified enzymes. Results were obtained at Days 1, 6 and 10 (d1, d6, d10). Both MMP-2 and MMP-9 enzymatic activity were sharply increased at Days 6 and 10. The two bands for MMP2 represent a proenzyme form and an enzyme form. (D). Densitometric measurements of select band intensities from the two replicate construct experiments.

activity. This suggests that the decrease in protease activity is likely due to the activity of protease inhibitors and not reduced MMP protein levels or conversion from the pro-enzyme to the active enzyme forms.

Discussion

The changes in microvessel density of the vascularized constructs over culture time provide a global picture of the progression of growth and proliferation. There was a continued increase in the microvessel density with increasing culture time (Figure 4.5), while phase contrast micrographs showed that this increase during the early culture periods was due to initial sprouting, followed later by the elongation and anastomosis of new sprouts. By Day 10 of culture, there was a dramatic and significant decrease in construct cross-sectional area to approximately 20% of that of the non-vascularized control gels (Figure 4.4). This result is consistent with the results for many in vitro culture models involving endothelial cells (e.g., [9], and the cause is an increase in cell-matrix traction forces [23, 24]. This reduction in cross-sectional area was readily visible to the naked eye. Despite substantial contraction by the later culture times, the vascularized constructs remained anchored to the attachment posts. When the results for microvessel density and cross-sectional area are considered together, it is clear that the overall increase in microvessel fragment density was due to a combination of microvessel growth and proliferation and compaction of the microvessel constructs.

Cellular remodeling exerts a strong influence on ECM mechanical properties due to matrilysis, cell adhesion and the resulting compression and reorientation of the ECM. These effects are evident in our results for the dynamics stiffness of the constructs. The dynamic stiffness of the native constructs in this study were comparable to those of our

earlier study [15]. The Day 6 cultures showed a mild reduction in cross-sectional area (Figure 4.4) but a significant reduction in dynamics stiffness in comparison to Day 1 values (Figure 4.6). This reduction in stiffness parallels an increase in microvessel density (Figure 4.5) and the peaks in proteolytic activity (Figure 4.9) and mRNA expression levels for MMPs 2, 9 and 13 (Figure 4.8). Taken together, these results strongly suggest that proteolysis was responsible for the reduction in dynamic stiffness at Day 6 of culture. To confirm that MMP enzymatic degradation could decrease the dynamic stiffness of the collagen constructs, we treated native collagen gels with activated MMP-9 enzyme. MMP-9 treatment resulted in a significant reduction in the dynamic stiffness of the native collagen gels, with a reduction in magnitude that was similar to that observed in the in vitro studies (Figure 4.7). These results demonstrate that global MMP activity can reduce the dynamic stiffness of the collagen gels.

Enzyme cleavage sites on collagen have usually been attributed to weaker or susceptible areas in its helical molecular structure. There are a limited number of cleavage sites available on the native collagen molecule for digestion by eukaryotic collagenases, and this is generally attributed to their specificity of local motifs in a relatively narrow region of the substrate [25]. Enzymatic degradation of collagen is thus dependent on the availability of these specific motifs, which may be altered under loading. This has been unequivocally demonstrated by Ruberti et al. [26] in a type I/V heterotypic collagen structure of the bovine corneal stroma, where fibrils under low tensile load were preferentially cleaved by a collagenase (Clostridiopeptidase A). The contraction of vascularized constructs, especially by Day 10, in turn could lead to a reduced susceptibility to enzymatic digestion, especially by the rat MMPs (as opposed to

bacterial enzymes with multiple cleavage sites) and contribute to the increased stiffness observed at Day 10.

The mechanical measurements for the vascularized constructs at Day 10 demonstrate an increase in normalized dynamic stiffness, even in relation to the Day 1 measurements. This was accompanied by a substantial reduction in cross-sectional area (Figure 4.4) and continued increase in microvascular density (Figure 4.5). The apparent paradox between high MMP mRNA levels, reduction in construct area, and reduction in observed proteolytic activity can be resolved by considering the effects of syneresis and cell-ECM interactions. As the microvessel fragments contract the gel, they force liquid from the gel. Since dynamic stiffness is normalized by cross-sectional area, a reduction in the fluid phase results in an effective increase in solid phase fraction. It is likely that better cell-ECM contacts, engineered by the molecules DCN, FN, TNC and HAS, also contribute to the stiffening of the constructs at Day 10. In addition, fibronectin deposition promotes clustering of focal adhesion complexes, resulting in stronger adhesion of endothelial cells to the matrix, thus increasing the contribution of the active cellular phase to the overall dynamic stiffness of the vascularized constructs. It is also possible that anisotropy induced by gel contraction affected the mechanical properties in these constructs. Studies on matrices with high aspect ratios and similarly restrained cell seeded constructs [27], demonstrate a higher level of orientation and associated strength [28, 29] between anchorage posts. The sharp drop in cross-sectional area (Figure 4.4) and increased vascular density by Day 10 of culture in the present study might indicate a similar behavior, progressively inducing vascular and collagen orientation along the

direction of anchorage. This could have introduced anisotropy to the vascularized constructs and thus increased their stiffness along the direction of anchorage.

The results for gene expression of MMPs, their inhibitors and protease activity illustrate the progression of the microvessel fragments from a relatively quiescent phenotype immediately after isolation to an invasive phenotype. Normal capillary endothelial cells are quiescent and retained within their investing basement membrane. The switch of ECs to the angiogenic phenotype is marked by increased lytic activity by MMPs [1, 30] and plasminogen activator systems. One of the major roles of MMPs in angiogenesis is collagen cleavage [1, 10], which is a prerequisite for EC invasiveness and angiogenesis [30]. MMP-9 may play a significant role in switching to the angiogenic phenotype [2]. The significant increases in mRNA levels of gelatinases (MMPs 2, 9) and the major rodent endothelial interstitial collagenase (MMP-13) [30] at Day 6 of culture support an invasive angiogenic switch. Peak MMP mRNA levels have been found at the 6th day of EC co-cultures with fibroblasts, with a down-regulation by the 12th day [31]. Our results suggest a switch from a general invasive phase to a more selective invasion at later culture times. This is supported by our data for total proteolytic activity, which peaks at Day 6, and falls at Day 10. MMP-2 is known to bind native Collagen I [32], making this protease an excellent effector of focal matrix degradation/remodeling.

MMP-2 and MT1-MMP are normally endogenously expressed by microvessel endothelial cells in culture, and increase with formation of ‘sprout’ like outgrowths, especially from confluent monolayers [5, 31]. The constant level of MT1-MMP over 10 days of culture in the present study suggests either that MT1-MMP is not the bottleneck in the activation of MMP-2 or that invasion is more widespread than just local

proteolysis. However, considering the MMP activity over the entire culture period, it is seen that at day 10, only MMP-2 continues to increase significantly, while MMP-9 & 13 levels do not differ significantly from day 6 levels. In addition, there were no significant changes in the mRNA levels of TIMPs over the culture period. Our results for TIMP-2 expression are consistent with those of another study examining in vitro angiogenesis from isolated ECs in a collagen matrix [5].

Increases in gene expression for molecules responsible for cell-ECM adhesion and interaction are characteristic of the proliferation phase of angiogenesis. There were significant increases in mRNA levels for fibronectin (FN), decorin (DCN) and tenascin C (TNC) at Days 6 and 10 of culture, with a concomitant reduction in hyaluronic acid synthase (HAS), an enzyme catalyzing the synthesis of the glycosaminoglycan hyaluronan. Decorin, localized around endothelial cords and cavities, is causally involved in capillary formation and reduction of apoptosis [33, 34]. It inhibits cell binding to collagen and fibronectin and promotes migration, which in turn may be responsible for cord like alignment [35, 36]. Similar roles have been suggested for tenascin C [37, 38]. Hyaluronan has an angiogenesis inhibiting role [39], which explains the downregulation of HAS over culture time in our study. Matricellular proteins like TNC and DCN modulate EC-ECM interaction and regulate deadhesion and intermediate adhesion steps, favoring motility and reducing anoikis [40]. It is interesting to note that the plateau of TNC mRNA level noted at Day 10 follows the plateauing effect of TNC on tube lengthening and EC migration [41].

This study examined expression of fibroblast growth factor (bFGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and its

receptor (VEGF-R) and bone morphogenic protein (BMP1) all of which have been linked to angiogenesis. Overall, the results for growth factor gene expression indicate a highly proliferative phenotype. The initiation of angiogenesis depends on VEGF, while PDGF is necessary for maintenance and to prevent dissociation of pericytes from endothelial cells during angiogenesis [2, 42, 43]. Biological functions of VEGF are transduced by VEGF-R2, a tyrosine kinase receptor expressed predominantly on endothelial cells [44]. VEGF receptors are predominantly distributed on adult rat ECs and expressed even on nonangiogenic vessels with detectable VEGF mRNA levels [2, 45]. VEGF is highly inducible by hypoxia and serum [3]. PDGF showed a significant increase on the 1st day of culture while the others did not demonstrate a significant increase. This result is consistent with studies of the culture of isolated ECs [46]. VEGF production by a tissue or group of cells may thus serve as an indicator for requirement of increased vasculature and set up chemotaxis to promote angiogenesis, with ECs not being a major source of VEGF [42]. The difference in growth factor profiles may be attributed to the difference in angiogenesis from formed vascular elements and EC cultures in their requirement of growth factors.

In summary, the results of this study demonstrate that angiogenesis in vitro can have dramatic effects on the material properties of a substrate material through the mechanisms of matrilysis, syneresis and consolidation/compaction. Questions that remain to be addressed include the relative contribution of local versus global MMP enzymatic degradation and the influence of mechanical boundary conditions and mechanical loading on the dependent variables investigated in this study. It is thus

evident that a finely regulated relationship exists between angiogenesis, the ECM and its mechanical properties.

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CHAPTER 5

EFFECT OF MECHANICAL BOUNDARY CONDITIONS ON ORIENTATION OF ANGIOGENIC MICROVESSELS¹

Abstract

Mechanical forces are important regulators of cell and tissue phenotype. We hypothesized that mechanical loading and boundary conditions would influence neovessel activity during angiogenesis. Using an in vitro model of angiogenesis sprouting and a mechanical loading system, we evaluated the effects of boundary conditions and applied loading. The model consisted of rat microvessel fragments cultured in a three-dimensional collagen gel, previously shown to recapitulate angiogenic sprouting observed in vivo. We examined changes in neovascular growth in response to four different mechanical conditions. Neovessel density, diameter, length and orientation were measured from volumetric confocal images of cultures exposed to no external load (free-floating shape control), intrinsic loads (fixed ends, no stretch), static external load (static stretch) or cyclic external load (cyclic stretch). Neovessels sprouted and grew by the third day of culture and continued to do so during the next three days of loading. The numbers of neovessels and branch points were significantly increased in the static stretch

¹ Reprinted by permission from Oxford Journals, copyright (2008): [*Cardiovascular Research*, Vol. 78, No. 2], Krishnan, L., Underwood, C. J., Maas, S., Ellis, B.J., Kode, T. C., Hoying, J. B., and Weiss, J. A., "Effect of mechanical boundary conditions on orientation of angiogenic microvessels," pp: 324-32, May 1, 2008 ePub

group when compared to the free-floating shape control, no stretch or cyclic stretch groups. In all mechanically loaded cultures, neovessel diameter and length distributions were heterogeneous, while they were homogeneous in shape control cultures. Neovessels were significantly more oriented along the direction of mechanical loading than those in the shape controls. Interestingly, collagen fibrils were organized parallel and adjacent to growing neovessels. Externally applied boundary conditions regulate neovessel sprouting and elongation during angiogenesis, affecting both neovessel growth characteristics and network morphometry. Furthermore, neovessels align parallel to the direction of stress/strain or internally generated traction, and this may be due to collagen fibril alignment induced by the growing neovessels themselves.

Introduction

Physiological organ morphology is achieved by migration and orientation of cells under the influence of chemotaxis, haptotaxis, and mechanotaxis. Both externally applied and internally generated mechanical forces influence cell migratory, proliferative, and secretory activities and matrix orientation [1-3]. The current understanding of the morphogenesis of natural three-dimensional tissue structure is limited. Given its importance in development, repair, tumorigenesis, and design of artificial constructs, significant resources have been directed at understanding the three-dimensional morphology of the microvasculature, especially the vascularity of soft connective tissues [4, 5], where vasculature is often oriented along the direction of strain experienced in vivo and/or structural features of the extracellular matrix. Cellular orientation in engineered tissue is often achieved by mechanical loading or contact guidance [6-9].

Mechanical forces affect the behavior of nearly all cell types (e.g., [7, 10-12]). The mechanical environment of endothelial cells influences migration, proliferation and tube formation [11, 13], protease and other secretory activities [14], cellular and cytoskeletal organization [15], expression of genes and surface molecules [16], and cell signaling [12]. A large body of literature has focused on the responses of endothelial cells as monolayers, spheroids or cords, cultured in natural or engineered matrices and surfaces, to biochemical and biomechanical stimuli [11-18]. These studies have shown that cells align both along [19, 20] and transverse [15, 17, 18, 21] to the direction of principal stretch in response to mechanical loading. The causes of this discrepancy are elusive and have been attributed to cell seeding density, matrix density, genetic framework, cell age, two-dimensional vs. three-dimensional substrates, cell subpopulation and the nature of loading. The angiogenic response of intact preformed microvessels to mechanical perturbations is relatively unexplored.

The objective of the present study was to determine the morphological changes in sprouting microvessels in a three-dimensional in vitro model of angiogenesis [22] due to alterations in construct boundary conditions and external loading, in previously unaligned collagen matrices. We quantified the orientation of microvessel segments in free-floating cultures, in cultures under traction against fixed anchors, and in cultures under externally applied strain. This is the first study to examine the orientation of multicellular microvessel structures rather than individual cells or cell clusters. The results of this study demonstrate that neovessel orientation and during angiogenesis depends more on culture boundary conditions than externally applied strains.

Materials and Methods

In Vitro Culture Model

The three-dimensional in vitro angiogenesis model has been characterized in our prior publications [22-25]. Unlike angiogenesis models that use isolated endothelial cells, this model uses isolated microvessel fragments. “Parent” vessels contain associated perivascular cells and retain their basement membrane after initial harvest and seeding in three-dimensional collagen gels. Sprouting begins predictably at Day 2-3. Pericytes disassociate from parent vessels and endothelial cells, consistent with observations that perivascular cell withdrawal relaxes the parent endothelial cell tube and permits sprouting and vessel elongation during angiogenesis. Sprouts elongate as patent tubes, branching and anastomosing with other vessels, and forming a new vascular network that ultimately fills the construct. Neovessel constructs form a functional vascular tree when implanted [23], rapidly inosculating with the recipient host circulation after implantation and carrying blood.

Epididymal fat pads from male retired breeder Sprague-Dawley rats (>500g) were aseptically harvested conforming to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996), and washed with Leibowitz (L-15) media containing 2% fetal bovine serum. Fat pads were minced into ~1 mm³ pieces and subjected to limited digestion with 2 mg/ml Clostridium collagenase (Worthington Biochemicals, Lakewood, NJ) and 2 mg/ml bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) in Dulbecco’s cation free phosphate buffered saline (DCF-PBS, pH 7.4) at 37°C for 4 minutes. The solution was immediately diluted with L-15 media to halt digestion and centrifuged. The pellet was washed twice and resuspended in L-15 media

with 2% serum and sequentially filtered through a 350 μm and then a 30 μm sterile nylon filter. The larger filter eliminates undigested particulate debris while the fine filter retains larger vessel fragments, allowing single cells and smaller fragments to pass through. The 30 μm filter was transferred to a sterile Petri dish and washed with serum containing L-15 media to suspend the fragments. The solution was analyzed for number of microvessels and centrifuged to obtain a pellet of microvessel fragments. Reagents were obtained from GIBCO (Grand Island, NY) unless mentioned otherwise.

Rat tail collagen type I (BD Biosciences, Bedford, MA) was mixed with concentrated Dulbecco's modified Eagle medium (DMEM, GIBCO-Invitrogen, Carlsbad, CA) to yield a final concentration of 3 mg/ml in 1X DMEM. Microvessels were suspended in collagen solution at 15,000 fragments/ml, and 1.2-1.5 ml of this suspension was pipetted into custom culture chambers and polymerized at 37°C and 95% humidity for 30 minutes (Figure 5.1). Labtek II chambers (Nunc-Nalge, Rochester, NY) were modified to accommodate a 5×20 mm specimen between fixed and mobile anchor posts, based on our earlier design [26]. A removable Teflon mold was used to cast the collagen constructs and was then removed after polymerization. The fixed post was attached to the chamber base near one end (Loctite[®] 3301, Henkel Corp., Rocky Hill, CT). Stainless steel pins were inserted through holes in the sides of the chamber for temporary anchorage of the mobile post. The acrylic posts had holes (1 mm diameter) to allow the collagen solution to pass through. Gels were overlaid with serum free culture media composed of 1:1 mixture of 1X DMEM (low glucose) and F12 nutrient mixture with additional components (Insulin 5 $\mu\text{g/ml}$, Transferrin 100 $\mu\text{g/ml}$, Progesterone 20 nM, Selenium 30 nM, and Putrescine 100 μM) [27], rhVEGF (10 ng/ml, PeproTech, Rocky

Hill, NJ), and Gentamycin (50 $\mu\text{g/ml}$). Three chambers and one chamber without the acrylic anchors were prepared simultaneously and allowed to grow undisturbed for three days. The interval between preparation of microvessel seeded collagen constructs and the beginning of mechanical intervention was based on the time required for initial sprout formation as we previously reported [22, 24]. On the third day, culture dimensions were measured with digital calipers, photographed, and given a 70% media change.

Mechanical Conditioning and Experimental Groups

The mechanical conditioning device used two motorized linear actuators for simultaneous testing of two constructs within the incubator (Aerotech, Pittsburgh, PA, range = ± 25 mm, resolution = ± 5 nm) (Figure 5.1A). Chambers were mounted on raised platforms to minimize any influence of heat, magnetic or electric fields and vibration. Two conditioning regimens and two boundary conditions were evaluated (Figure 5.1E). For each experiment, one construct was subjected to 6% “Static Stretch” (SS group) for three days and the second to 6% “Cyclic Stretch” (CS group) at 1 Hz for 12 hours every 24 hours for 3 days. This cyclic routine was chosen since vasculature in tissues that are subjected to cyclic stretch such as muscle and tendon experience a recovery period during the sleep cycle. The free floating constructs without acrylic anchors, referred to as “Shape Control” (SC), were placed on the conditioning platform along with the SS construct. Similarly, the anchored but unstretched construct, referred to herein as “No Stretch” (NS), was placed on the cyclic conditioning platform along with CS construct. To maintain comparability across different groups, four constructs, one for each group, were cast using the same starting material. Some samples became contaminated due to

Figure 5.1. Mechanical conditioning setup. (A). Mechanical conditioning system with raised platform mounted on motorized actuator. A stationary vertical stage is interfaced with the mobile anchor post by a beam. (B). Modified Labtek culture chamber with fixed anchor, mobile anchor supported by pins and removable Teflon mold. The Teflon mold was removed after collagen polymerization to yield a free floating tensile test specimen. An unanchored shape control (C). and a typical anchored construct (D). (E). Schematics show the four different test configurations in side view – (1). Shape Control, (2). No Stretch, (3). Static Stretch and (4). Cyclic Stretch.

the open culture system and hence the final number of samples in each group over 9 experiments were SC – 9, NS – 8, SS - 6, and CS – 6.

Confocal Microscopy

On the 6th day of culture constructs were cut free from the anchor posts with a scalpel, gels were measured and then fixed in 4% formaldehyde. Note that fixation caused a reduction in construct volume (approximately 20% or 7.5% along each dimension, nearly uniform). Microvessels within the gel were permeabilized with 0.1% Triton-X-100 in PBS. Endothelial cells were stained with 2 μ g/ml Isolectin IB4-Alexa 488 conjugate (Invitrogen, Carlsbad, CA). Confocal microscopy of six fields adjacent to

the centers of the top and bottom surfaces of the constructs was performed on an Olympus FV1000 with a 10X objective. Individual z-axis images were acquired to a depth of 300 μm at 2.5 μm intervals and 512x512 resolution.

Volumetric Image Reconstruction and Skeletonization

Image stacks from the six adjacent fields (1.27x1.27 mm each) forming a 2x3 grid were stitched together to generate a single mosaic (3.8x2.5x300 μm) (Figure 5.2). The Amira™ software (Mercury Computer Systems, Carlsbad, CA) was used for image analysis and skeletonization. Intensity artifacts in images with slice depth [28] were

Figure 5.2. Overview of image processing. (A). Six adjacent confocal image stacks were stitched together to obtain a mosaic of the center of the construct. (B). Final skeletonized output with largest continuous vessel highlighted.

reduced using an algorithm that fit an exponential curve to average intensities. Mosaic stacks were further deconvolved using a point spread function generated from the numerical aperture ($NA = 0.4$, 10X – air), wavelength of light (520 nm) and the refractive index of the gel [29]. An automated thresholding algorithm based on a Gaussian distribution fit to the image histogram was used to binarize each mosaic stack (ImageJ, NIH). A size filter of $600 \mu\text{m}^3$ was applied to remove debris and small cell clusters. Images were then skeletonized [30, 31]. A Chamfer distance map was generated as a part of this process, and these data were used to determine average diameter of each segment [30].

Data Analysis

Coordinates of points from the skeletonization, forming line equivalents of the vasculature, were processed by a custom C++ program (available at <http://mrl.sci.utah.edu>) to calculate morphometric parameters, including branch points, end points, individual segments, and vessels (all connected segments). Total vascular volume was determined from lengths and diameters. Additional parameters were orientation with respect to stretch axis, segment and total network length, vessel diameters, number of branches, junctions, segments, segment lengths, and vascular volume fractions. A minimum vessel size of $150 \mu\text{m}$ length was used to avoid smaller segments and debris. This cut off was based on microvessel dimensions at the time of seeding, which were about $150 \mu\text{m}$ long. A segment length cutoff of $25 \mu\text{m}$ was used to eliminate branches that were not much larger than the average vessel diameters. Segment orientation was examined along the direction of stretch (X-axis, θ) and through the culture depth (Z- axis, δ). Additionally, each segment was projected onto the XY-plane

and the angle of the projected segment with respect to the X-axis was determined (Projected-X, ϕ). Segments were sorted into 10° bins from 0-90°. Results were expressed as percentage of total fragments per bin and as percentage of length-weighted segments per bin. The angle distribution of segments was compared between groups and within groups using 2-way ANOVAs and Tukey tests (or Kruskal-Wallis ANOVA and Dunn's post hoc tests for nonparametric distributions) with significance at $\alpha=0.05$.

Confocal Reflectance Microscopy

Confocal reflection microscopy [32] was performed to visualize collagen fibril orientation in the presence and absence of anchorage and external loading, and modulation by growing microvessels. Samples were illuminated with a 633 nm laser and reflected light was collected between 632-633 nm. Several image stacks (1 micron step size x 50) were collected for each condition. To see whether fibril orientation was induced by the boundary / loading conditions, gels lacking microvessels were prepared and subjected to the same mechanical regimens. After 6 days these gels were fixed while still attached to the anchors.

Results

In Vitro Culture Model

All constructs showed well formed sprouts by Day 3 and well established vascular networks by Day 6 (Figure 5.3A). Total volume reduction between the 3rd and 6th day of culture was 60% for free-floating SC constructs and 40% for the other three groups ($p=0.001$, ANOVA). The SC constructs reduced to about 32% of their Day 3 length, while the length of constructs in the other groups was maintained by the anchoring posts.

The average height reduction in anchored construct groups was 17.6% and not significantly different from the SC constructs. Similarly, there was 27.6% reduction in width of the anchored constructs, which was not significantly different from SC constructs. SC constructs showed a near uniform percent contraction along the two directions.

Construct Vascularity and Complexity

Microvessel network morphometry at Day 6 was determined from the 3D image reconstructions (see Figure 5.2). Qualitatively, the SC constructs showed random microvessel orientation, while the NS, SS and CS constructs showed various degrees of orientation along the construct long axis. The number of vessels per construct was highest in the SS group and lowest in the SC group (Figure 5.3B). All treatment groups had significantly more vessels per construct than the SC group (Kruskal-Wallis ANOVA, $p < 0.05$). Among the three anchored groups, the SS constructs had the highest vessel count, followed by the CS and NS groups. However, these differences were not significant. The vascular volume fraction (construct volume occupied by vessels) of the SS group was 1.62 times higher than the SC group ($p = 0.015$, Tukey post hoc) while the NS and CS groups, were 1.39 and 1.51 times higher, respectively. SS constructs had more branches and vessel ends than the SC group ($p = 0.015$ and $p = 0.003$ respectively, Kruskal-Wallis ANOVA) (Figure 5.3B). There were no differences in branches and endings between any of the anchored (stretched or unstretched) groups. Similarly, average branch points and end points per vessel were not significantly different between treatment groups (1 way ANOVA/ Ranks test). The SS group thus showed greater

Figure 5.3. Construct vascularity and complexity. (A) 3D images of representative vascularized constructs: SC, Shape Control; NS, No Stretch; SS, Static Stretch; CS, Cyclic Stretch. (B) Construct vascularity and vascular morphometry measurements, expressed as total number of vessels per construct, volume fraction of total, number of vascular branch points and end points per construct and per vessel. Values are normalized to values of shape control.

network complexity than SC constructs but was not significantly different in terms of vascular volume fraction, branching and terminations from the other anchored groups.

Vascular Orientation

Preferred orientation of microvessels was observed in the NS, SS and CS groups (Figure 5.4). In the projected X angle distribution, the SC constructs displayed a random orientation and had a near uniform distribution of segment lengths across all angle bins. Vessel orientation in all anchored constructs was significantly different than the SC (Kruskal-Wallis ANOVA, $p < 0.001$) (Figure 5.4B). In anchored constructs, neovessels aligned with the stretch direction, with approximately 40% of the segments falling in the 0-10 degree bin. About 70% of segments were oriented along the X-axis (0-30°) and the remaining 30% were distributed from 40-90°.

Orientation relative to the X-axis was similar to the projected X angle distribution, with the SC group being significantly different than the other groups (Kruskal-Wallis ANOVA, $p = 0.003$) and showing nearly equitable distribution of segment lengths across all orientations (Figure 5.4C). Overall a higher percentage of segment lengths of NS, SS, and CS groups were oriented along the stretch direction and lower percentages were oriented orthogonal to stretch than shape controls. Interestingly, irrespective of external boundary conditions, most segments (about 80%) were oriented along the horizontal or XY plane (Figure 5.4D, $p = 0.03$, Kruskal-Wallis ANOVA). In this comparison, the significant difference lay between the SC and CS groups (Dunn's Test, $p < 0.05$), but the general distribution remained the same for all groups. In all cases, the degree of orientation orthogonal to the vertical direction was significantly greater than that expected for a random distribution (Post-hoc Tukey, $p < 0.05$) and could explain

Figure 5.4. Construct orientation quantification. (A). Schematic of construct orientation convention. The long axis of the construct was aligned with direction of stretch or anchorage. (B). Projection of the segment along the Z-axis on the XY-plane was used to calculate projected angle (ϕ , *Projected-X*).

Figure 5.4. Continued. (C). Orientation along X-axis (θ , horizontal, direction of stretch) and (D). Z-axis (δ , vertical, transverse to stretch) were directly determined based on segment orientation in these planes.

similarities between results for segment orientation along the X-axis and projected X-axis. Results without length weighting were similar (data not shown).

Segment Length Distribution

There were no significant differences in segment length distribution between groups (Kruskal-Wallis ANOVA, $p=0.524$) (Figure 5.5A). About 80% of segments were shorter than 100 μm and about 50% were in the 25–50 μm range. Median segment lengths ranged from 53–62 μm (Figure 5.5A-Inset). Interestingly, when median segment lengths were analyzed as a function of segment orientation, differences were observed between groups (Figure 5.5B, Kruskal-Wallis ANOVA, $p<0.001$). Anchored groups had longer segments along the direction of stretch or anchorage than the SC group, but were shorter than the shape controls orthogonal to this axis, with a transition point around the 40-50 degree bin. Vessels in the SS group were significantly shorter than those in the NS and CS groups across all angle bins (multiple comparisons, $p<0.001$). The SC group did not show significant differences across angle bins, while all other groups did show significant differences ($p<0.001$).

Segment Diameter Distribution

There were no significant differences in segment diameter distribution between groups (Figure 5.5C, Kruskal-Wallis ANOVA, $p=0.838$). About 70% of segments were less than 10 μm in diameter and nearly 80% were smaller than 15 μm . However, when the distribution of median diameters was examined as a function of segment orientation, there were significant differences between groups (Figure 5.5C, 2-way ANOVA, $p<0.001$). The NS, SS, and CS groups had smaller diameters along the stretch direction,

Figure 5.5. Segment length and diameter distributions. (A). Segment length distribution. Inset – Median segment lengths over treatment groups. (B). Segment length distribution across incremental angle bins. Orientation along the Projected-X direction is shown.

Figure 5.5. Continued. (C) Segment diameter distribution within constructs. Inset – Median segment diameters over treatment groups. (D) Segment diameter distribution across incremental angle bins. Orientation along the Projected-X direction is shown.

and increasingly larger diameters away from the stretch direction, with the SS (Tukey, $p=0.002$) and CS (Tukey, $p<0.001$) groups being significantly thinner than the NS group. Thus, the microvessels were thinner along the direction of stretch and were significantly thinner under the influence of static stretch and cyclic stretch.

Diameter Distribution Changes with Segment Length

There was a correlation between median segment diameter and segment length for all groups (data not shown). Longer segments had smaller diameters and vice versa. However, there was a significant effect of mechanical boundary conditions on the distribution of segment diameters with respect to segment lengths (2-way ANOVA, $p<0.001$). The SS and CS constructs had the thinner segments, compared to SC and NS groups (Tukey, $p<0.001$). Neither the SS and CS nor SC and NS groups differed significantly from each other irrespective of segment lengths.

Collagen Fiber Distribution

Since boundary conditions caused neovessel orientation, we examined the structure of the collagen matrix using confocal reflection microscopy (Figure 5.6). Constructs without microvessels were imaged as controls (Figure 5.6, upper panel). In constructs without vessels, fibrils were well defined and randomly oriented in the SC and NS groups. Constructs in the SS and NS groups appeared to have some fibrils with more orientation along the direction of strain. After 6 days in vitro, fibrils in constructs containing microvessels were less well defined, likely due to matrix remodeling. Although collagen fibrils were less defined, they appeared to be aligned along the long

Figure 5.6. Reorganization of collagen matrix by microvessels. Representative images of the collagen matrix of gels without microvessels and gels with growing microvessels, subjected to identical stretching regimes after 6 days in culture. The collagen matrix in gels containing microvessels is more condensed making the individual collagen fibrils more difficult to distinguish (Sections chosen to avoid microvessels).

axis in NS, SS and CS constructs. Nonanchored SC constructs did not show this orientation.

Discussion

The major finding of this work is that boundary conditions and mechanical loading can affect the morphometry of neovessel networks that are formed from angiogenic microvessels in a 3D culture model. Anchoring produced the largest effect in terms of neovessel orientation, while static and cyclic mechanical stretch did not cause a significant change in orientation over anchoring alone. To our knowledge this is the first time the effect of construct anchoring has been demonstrated for angiogenic vessels from preformed microvessels.

Studies of fibroblast cells in 3D culture also observed alignment of collagen fibrils and fibroblasts over time [6, 33]. Similarly, long paths of condensed matrix fibers or ‘matrix migration pathways’ were shown between endothelial cell aggregates or other cell clusters, formed in the direction of overlapping tractional force fields [34, 35], [1, 36, 37]. Reconstituted collagen gels usually have random orientation. Isolated cells seeded in collagen gels orient along the direction of maximum resistance to deformation by internal traction [38]. These internal contractile forces generated in vitro by endothelial cells and fibroblasts are similar to that of dermal fibroblasts and within an order of magnitude of smooth muscle cells [39, 40]. The potency of these tractional forces and the formation of capillary like structures were dependent on monolayer confluence, migratory or quiescent phenotypes, MMP inhibition, substrate properties like concentration, thickness and rigidity, and anchorage [35, 39, 41-44]. Cells align with fibrils via contact guidance and generate traction force along the fiber direction, creating

a positive feedback loop, ultimately resulting in cell and fibril alignment between anchor points. Such ECM alignment may extend to about 20 μm from cell processes [2]. These observations are supported by several other studies where cellular traction against anchors was sufficient to cause cellular [20] and matrix alignment [1, 45] and cell associated fiber bundling [46]. In contrast to those studies that examined alignment of single bipolar cells, the present study examined the orientation of growing microvascular segments, which are multicellular structures.

Cell orientation in an active strain field is generally attributed to an avoidance or shielding response, i.e., the cells tend to orient perpendicular to the direction of stretch [47, 48]. Cyclic changes in cell length with axial strain, anisotropic strain fields, anchorage, and relative magnitude of cell traction with respect to the substrate rather than the absolute substrate stiffness have been shown to alter cellular orientation and modulate capillary morphogenesis [44, 47]. In the present study, static and cyclic stretch increased the degree of alignment along the stretch direction; however the increase over anchoring was minimal and not significant. Thus, these externally applied mechanical strains did not appear to have a significant influence on microvessel orientation. Internal traction forces generated by the microvessels may have been sufficient to induce alignment in the NS group.

The results of this study on multicellular angiogenic microvessels differs from the results of other studies on isolated endothelial cells and fibroblasts [10, 17, 18, 21, 49, 50]. Endothelial cells grown on 2D elastic substrates orient their primary axis perpendicular to the direction of cyclic strain. In one study, the multicellular cord-like structures formed by invaginations of endothelial cells from surface monolayers into

deeper layers of the matrix oriented perpendicular to the direction of cyclic strain [21], hypothesized to be an attempt to minimize the stress and injury experienced by the cells due to strain at least in 2D settings under cyclic strain. The question thus arises: why do microvessels in collagen gels align along the direction of traction/stretch as opposed to perpendicular to this direction? Possible explanations are that alignment of underlying collagen matrix prevents reorientation by cyclic strain, the viscoelastic nature of collagen gels fail to maintain stress on the cells during application of the cyclic strain, or that the response of multicellular angiogenic constructs to mechanical strain and fixed tractions is fundamentally different from that of isolated cells on 2D membranes. Although cell reorientation in response to strain has been observed in several studies, the interaction of cells with substrate topology via contact guidance can override this effect [48]. However, matrix alignment does not seem to be the cause of or at least precede cellular alignment under the influence of cyclic stretch [20]. Lower density collagen gels (1mg/ml) have been shown to align under the influence of external strain [51]. The results of the present study showed that SC and NS gels had random fibril orientation, while some alignment parallel to stretch direction was observed under static or cyclic strain (Figure 5.6, upper panel). However, alignment is seen in the no stretch group when growing microvessels are present (Figure 5.6, lower panel). Therefore, if anchoring alone can induce alignment of multicellular neovessels and the collagen matrix, then addition of cyclic stretch may not be able to reorient cells away from the aligned matrix. The viscoelastic nature of the collagen constructs [26] may also play a role. There may be a fall in the actual tissue equilibrium tension due to stress relaxation, but the gels may still retain a strain-induced collagen alignment [51]. Alignment of endothelial cells, fibroblasts, neurites [52], and

tenocytes [7] on 3D matrices under static or cyclic stretch indicate that cellular tensional homeostasis may play a significant role in 3D tissue architecture development, in conjunction with contact guidance from matrix structure.

We examined vessel volume fraction, segment length and segment diameter in the microvessel cultures. Although there were no significant differences in volume fraction of vessels across the groups, the average volume fraction across all construct groups (Mean = 0.75% of total volume) was similar to those found in vivo [53]. Diameter values were in good agreement with previous literature [54, 55]. Segment length distributions were broad, but similar to one another with median lengths in 53-62 μm range. These values are slightly lower than the reported 93 μm in fibrin ingrowth models [55] and 87–119 μm in rat mesenteric capillaries [56], possibly due to differences in vascular complexity and anastomosis. These observations suggest that this in vitro model reasonably replicates the morphometric characteristics of angiogenic microvessels in vivo.

It should be noted that diameter measurements from the image data can be influenced by the thresholding process and image resolution. The final voxel resolution of image mosaic stacks ($2.5 \mu\text{m}^3$) was a function of the 10x objective, the image resolution and Z direction step size. Although this combination allowed for imaging of a large field of view, the diameter of the vessels was dependent on a small number of voxels. The thresholding process could affect measured diameters; thus an automated thresholding algorithm was used to minimize such errors. Nevertheless, the diameters reported in this study are in good agreement with the literature [54, 55].

Application of engineered grafts in medicine has demonstrated that graft survival is often dependent on the ability to recruit host vasculature. Vascular supply networks to and within an implant form a significant bottleneck in graft integration. A prevascularized graft, engineered to match local vascular topology, tissue architecture and material properties could provide a solution to this problem. Angiogenesis in a 3D substrate from preformed vascular elements offers a closer approximation to in vivo physiological conditions. Our results demonstrate that changing the boundary conditions of a vascularized 3D gel construct can induce substantial organization of angiogenic neovessels. Alignment along the direction of anchorage and strain and the associated perivascular condensed matrix fibrils recapitulate the 3D architecture of soft tissues such as muscles and ligaments [4, 5]. Further research is needed to elucidate the changes in gene and protein expression that may be responsible for the observed phenomena, as well as to quantify changes in collagen fibril morphometry over time.

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CHAPTER 6

DISCUSSION

Summary

Measurement of mechanical properties of cell free constructs allows the use of the results to quantify the forces of cell traction indirectly by quantification of cell induced matrix deformation [1]. Direct quantification of cell free matrices and comparison with cell seeded constructs however can be considered optimal for delineation of contributions from the cells, matrix, and matrix remodeling [2]. Accordingly, the first aim of this research was to build and characterize a mechanical test setup to test viscoelastic mechanical properties of a collagen I based hydrogel, a commonly used tissue culture substrate, to determine the effects of collagen concentration and duration of culture, on construct mechanics. Dynamic linear viscoelastic analysis is an ideal tool to characterize the strain and rate dependant material properties [3]. The mechanical behavior of many materials can be approximated as linear under small perturbations about a reference configuration, as required for application of linear viscoelastic models, but may be nonlinear at higher stresses [4]. The analysis method presented here did not assume linearity over large strains, but at each equilibrium strain level tested, the sinusoidal perturbations were chosen to be within the linear range. Further, the conformity of the construct dimensions to standard high aspect ratio tensile test specimens, with anchorage at either ends without clamps, ensured a homogenous strain distribution area in the

construct middle without complications from clamp shear [5] or stiffening of constructs at the point of anchorage [6].

The collagen constructs exhibited a moderate rate dependent stiffening as reported previously [2]. At higher collagen concentrations, the equilibrium stress strain and dynamic stiffness curves shifted to the left and showed an upward concavity, deviating from the near linear relationship in the lower concentration groups. This may be attributed to increased availability of neighboring fibrils for physical association and entanglement (crosslinks), resulting in greater recruitment of random fibers [7-9]. The dramatic difference between the equilibrium and dynamic stiffness demonstrates the large contribution from the rate dependant viscous part (aqueous and intrinsic viscoelasticity) of the constructs. By validating the maintenance of material properties over typical culture durations, any changes in the mechanical properties of vascularized constructs during culture could be attributed directly to the alterations caused by growing microvessel fragments. Though not reported in this study, these results could be directly used to formulate constitutive models of viscoelasticity, which can subsequently be used to describe and predict the constitutive behaviour of such constructs.

Comparison of material properties of angiogenic microvessels after 1, 6, and 10 days of angiogenesis revealed a fall in the normalized dynamic stiffness for all frequencies and strain level on the 6th day of culture followed by a stiffening to its original day 1 levels. The reduction in stiffness on the 6th day is accompanied by a peak in MMP mRNA and active protein, an increase in signalling for fibronectin, and only a mild reduction in cross sectional area, suggesting that fall in stiffness was primarily proteolysis mediated. The possible role of global proteolytic activity in reduction of

matrix dynamic stiffness was supported by the effects of treating cell free constructs with exogenous MMP-2. The increase in stiffness on the 10th day of angiogenesis was paralleled by a concomitant reduction in MMP activity, a large decrease in cross sectional area, a stable level of expression of MMPs 9, 13, and 14 with no changes in TIMP levels from the 6th day, and an increase in mRNA for fibronectin, decorin and tenascin C. The deposition of a temporary matrix could indeed result in better cell matrix contacts, improve 3D organization of the angiogenic tubules, and compliment the compaction and syneresis accompanying the florid sprouting and network formation. The dynamic stiffness is normalized to the cross sectional area, and could be influenced by the greater gel compaction by the 10th day in conjunction with a fibronectin mediated clustering of focal adhesion complexes and a consequent increase in contributions of an active cellular phase [2].

In cellular constructs, cellular traction against fixed anchors can cause alignment of matrix fibers between anchor points [10]. Progressive traction can also reorient matrix fibers in the anchorage direction and introduce anisotropy as a part of the compaction process, ultimately strengthening the constructs in the direction of restraint [6, 11, 12]. Progressive contraction of the constructs supports the notion that a similar mechanism exists within the growing angiogenic constructs. A nonconforming alignment of cells in a strain field has been shown to increase the production of MMPs, arguably in an attempt to realign the cells [13]. It is conceivable that the peak in proteolysis seen on the 6th day reflects this phenomenon. The presence of enzyme specific degradation motifs for digestion by eukaryotic collagenases could also offer a protective role, reducing

degradation in strained or loaded fibers [14, 15], thus opening up only the unaligned fibers to the increased degradative activity by the aforementioned mechanism.

The orientation of fibroblasts, endothelial cells, and EC tube like structures has already been demonstrated as extensively reviewed in Chapters 2 and 5. This dissertation research is the first to demonstrate the effect of mechanical boundary conditions on preformed vasculature. These results suggest that anchorage alone is sufficient stimulus in a high aspect ratio construct to induce vessel orientation. External loading, either static or cyclic, was not found to add significantly to the observed orientation response though other morphological changes like vascularity, branch length, and diameters were shown to be influenced by mechanical loading. The microvascular alignment along with perivascular matrix condensation recapitulates the 3D architecture of soft tissues such as muscles, bone and ligament [16, 17]. There are conflicting reports suggesting both a stiffening [18] as well as a softening of the cell [19] in response to stretch. The role of mechanical forces as the predominant factor in causing cell alignment is also debated [10, 20]. Cell orientation in a strain field is attributed to an avoidance response, whereby orientation along the strain direction reduces the perceived 3D strain [6]. The significant alignment produced by traction against fixed anchors alone points to matrix realignment to form guidance paths [21-24] and re-establishment of a dynamic tensional homeostasis in response to altered tensional forces [10, 25].

Contact guidance along established guidance channels like collagen fibers can be sufficient to override any reorienting influence of external mechanical forces [20]. A stiffening of the cell cytoskeleton has been demonstrated in response to stretch [18]. Considering this in the light of the recent evidence by Sieminski et al. that relative

magnitude of the cell traction against the cell substrate is involved in mechanoregulation, it may be that the cells respond only to a narrow range of relative stiffness change, with superimposed mechanical stiffening not playing a significant role [26, 27]. The viscoelastic nature of our constructs may be responsible for a rapid decrease in the actual stresses seen by these microvessels, some of the effects of which can be expected to be counteracted by the actively contracting cells. However, a similar response was seen in response to continuous cyclic stretch for 12 hours/day over 3 consecutive days. There may be a fall in the actual tissue equilibrium tension due to stress relaxation, but the gels may still retain a strain-induced collagen alignment [28]. This research demonstrates that external mechanical loading does not improve alignment over anchored constructs (internal traction), and the preliminary evidence of matrix compaction along aligned vessels supports the notion of contact guidance being a significant regulatory influence even in the presence of external strain fields, even to the extent of preventing any stretch induced realignment [20]. Viewed in the light of current knowledge in soft tissue wound healing [29-32] and efforts at improving graft morphologically and functionally [33], this research has direct applicability in tissue engineering functional vascularized constructs either as a vascular supply bridge or as a part of other cell co-culture systems.

Limitations

Estimation of Material Properties

Stress relaxation properties indicate dimensional stability and structural rearrangement within the material and are a measure of the relative viscoelasticity [34]. The material test device used for mechanical testing of constructs depended on manual actuation to the required large strains (2, 4, and 6%). This nonuniform ramp-rate results

in variations in peak stresses, which unfortunately compromises the ability to generate accurate normalized stress relaxation curves [4, 34]. Although the use of this device was limited to terminal mechanical testing, an appropriate temperature maintenance chamber could permit testing at physiological temperatures and prolong cell viability.

Angiogenesis Model

The microvasculature used in the angiogenesis model was obtained from rats classified as ‘retired breeders’ for cost justification and only a limitation on weight range was imposed. An effect of age in gene expression and angiogenic ability has since been demonstrated (2003) between young (6 to 8 weeks) and older rats (26 to 40 weeks) [35, 36]. To the best of our knowledge, there is however no evidence on differences existing between rats of older age groups with a less dramatic age difference. The use of preformed microvascular elements [37] could be considered an advantage and a limitation. In many earlier studies on molecular mechanisms of angiogenesis, cell lines or marker evaluated endothelial cells have been used to assure uniformity in cell responses to intervention. The gene expression, proteolysis, matrix compaction, and alignment responses in the studies in this dissertation research cannot hence be ascribed to a single cell type. Although this may be seen by many as a possible limitation, this research views it in the light of physiological angiogenesis which does not progress with endothelial cells alone and requires the regulatory control of pericytes and mural cells as described earlier. The lack of any of form marker or dye based identification of endothelial cells from mural cells in this research in favor of existing characterizations on this model [37, 38] is however conceded as a limitation in the present research.

Another possible limitation of this study is that it does not examine the anisotropy in relation to the mechanical properties in these constructs, arising out of vascular orientation and/or collagen fibril orientation. However, studies by Nirmalanandhan, Butler et al. [39] on matrices with high aspect ratios and similarly restrained cell seeded constructs [6, 40, 41], demonstrate a higher level of orientation and associated strength (See Shi and Vesely [11, 12, 42]) between anchorage posts, and support our arguments as described in the earlier section. Proteolytic activity was quantified and correlated with mRNA for different MMPs, but a lack of localization of the respective MMPs in the substrate limits the scope of this work in understanding the actual mechanisms of alterations in construct stiffness. Similarly, the lack of quantification of t/ uPA enzymes, though a limitation, must be considered in the view of overwhelming evidence pointing to the predominant role of MMPs in angiogenesis associated matrilysis in vitro.

Quantification of Orientation

The lack of a measure of matrix stiffness, either in the form of an end-point mechanical test or by active measurement of forces during the intervention (stretch) limits the scope of this study to address the key question of contributions from active cellular forces towards perceived construct stiffness, especially in the light of recent evidence presented by Sieminski et al. as described earlier [26]. Along similar lines, a more rigorous quantification of the matrix orientation associated with each mechanical boundary condition would significantly help in clarifying the role of contact guidance vis-à-vis the mechanical boundary conditions. The diameter measurements in the study were a function of the thresholding process and image resolution. To include a wider area of the constructs in the quantification and thus obtain maximum number of

microvessel fragments for analysis, a 10X objective with final voxel resolution of $2.5\ \mu\text{m}^3$ was used, which limits the accuracy of diameter measurements. Nevertheless, the diameters reported in this study are in good agreement with the literature [43, 44].

Future Work

With the quantification of the gene expression profile, proteolytic activity, mechanical properties of cell free and vascularized constructs, and the microvasculature orientation under different boundary conditions, we are in a position to use these as inputs to a computational model to predict angiogenesis. Information on the early phase of sprout growth, rate, and progression is however limited. This is currently being addressed by using the vessel quantification methods established in this dissertation research using green fluorescent protein (GFP) expressing rats. Possible uses of these models are in predicting the behaviour of the vasculature in complex and varying substrates to guide further experimentation, e.g., in engineering the parenchymatous substrate base for artificial liver or pancreas [45]. Identification of the areas of substrate lysis as well as the specific lytic enzymes involved, in addition to the local mechanics, will further contribute to the basic understanding of how angiogenic microvessels modulate the local matrix environment and maintain the fine balance between the amounts of matrix lysis required for invasion while at the same time maintaining sufficient anchorage to allow traction for migration and matrix compaction. Further, the response of microvascular networks to other boundary conditions like, confinement and loading along the short axis, annular confinement, alteration in orientation of substrates under varying strain field and substrate densities is not well understood for preformed vasculature. A better understanding of the microvascular morphology under these

conditions and combinations thereof will significantly improve our current understanding of the factors that influence 3D architecture in vivo and improve our ability to engineer morphologically and functionally efficient grafts.

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