

EFFECTS OF ANGIOGENESIS ON THE MATERIAL PROPERTIES OF THE EXTRACELLULAR MATRIX: CORRELATION WITH GENE EXPRESSION

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INTRODUCTION

Angiogenesis, the formation and growth of new blood vessel sprouts from existing vessels, is the process by which the additional vascular elements are formed from an initial vascular plexus. Quiescent endothelial cells are stimulated to exhibit invasive, migratory and proliferative phenotypes. Sprouting endothelial cells degrade their basement membrane and form contacts with and migrate along extracellular matrix (ECM) components. Later, endothelial cells deposit a new basement membrane resulting in a patent, perfusion-capable capillary.

Little is known regarding the effects of angiogenesis on the material properties of the ECM. Characterization of the interaction of capillaries with the ECM can provide new insight into wound healing and neoplasia. The objective of this study was to quantify changes in ECM material properties due to angiogenesis using a 3D in vitro angiogenesis model. Studies of gene expression were performed to investigate the possible causes of changes in material properties of the vascularized gels with culture period.

METHODS

In vitro Angiogenesis Model

An established 3D angiogenesis model formed the basis for the in vitro studies [1]. Briefly, microvessel fragments (30 μm mean length) were isolated from epididymal fat pads of SD rats. Isolated vessel elements contain associated perivascular cells and spontaneously grow as patent tubes through the elaboration of numerous vessel "sprouts" after seeding in collagen gels. These vessels continue to grow into a new vascular network that ultimately fills the gel space. Angiogenesis begins, predictably, at Day 4 of culture and forms a uniform vascular network by Day 14. Vessels were seeded in Type I collagen gels at a density of 15,000 frags/ml. Gels were polymerized in custom culture chambers (Figure 1) and incubated at 37°C, 95% humidity. A total of 16 vascularized collagen gels and 11 native gels (no vessels) were tested. Eight vascularized gels were tested at Day 1 and Day 6 of culture, while 6 and 5 native gels were polymerized from the same collagen and tested at the same times.

Mechanical Testing of Vascularized Collagen Gels

Custom culture chambers were CNC machined from acrylic (Figure 1). A glass microscope slide formed the bottom of the chambers. Gels were polymerized around a fixed anchor post and a moveable actuating post, yielding a 20 mm gage length. Holes in the actuating and anchor posts provided a mechanical link between the gel and the test system. The actuating post was attached to a 0.25 N load cell (accuracy ± 0.00012 N).

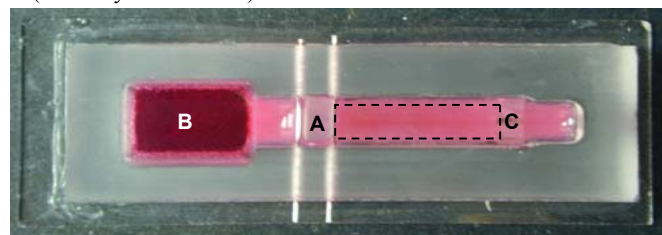


Figure 1. Test chamber with polymerized collagen. Actuating post (A) is held in position by pins. Reservoir (B) contains only culture media, allowing elongation of test region (dashed line). Anchor (C) attaches to chamber base.

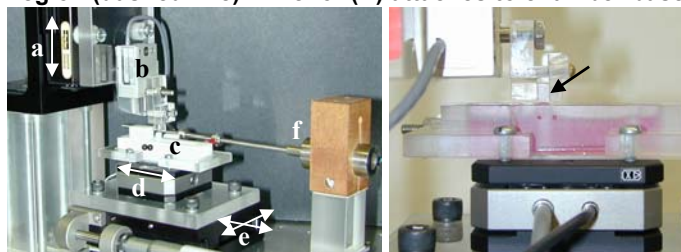


Figure 2. Left - apparatus for viscoelastic testing of collagen gels. Vertical stage (a) aligned the load cell (b) with actuating post. Chamber (c) was attached to piezo actuator (d). An x-y stage (e) allowed alignment/application of equilibrium strains. Displacement was measured using an LVDT (f). Right - load cell interface (black arrow).

A custom material testing device was designed and constructed to perform dynamic viscoelastic tensile testing (Figure 2). Actuation was achieved via a piezoelectric stage driven by a function generator. Load and displacement were monitored continuously with a load cell and an LVDT. Gels were preconditioned via a stress relaxation test at 6% of the initial length. Each gel was then stretched to 2% strain and allowed to stress relax for 8 min. This was followed by sinusoidal strain oscillations (1% amplitude, 0.05, 0.1, 1.0, 5.0 Hz) about the equilibrium strain level. The procedure was repeated at equilibrium strains of 4 and 6%. Linear viscoelasticity theory was used to calculate dynamic stiffness (M , Pa) and phase shift (ϕ , radians) as a function of strain and frequency [2]:

$$M = \frac{A_{\sigma}}{A_{\epsilon}}; \quad \phi = \phi_{\sigma} - \phi_{\epsilon} \quad (1)$$

($A_{\sigma}\phi_{\sigma}$) and ($A_{\epsilon}\phi_{\epsilon}$) are the amplitude and phase of the strain-time and stress-time data, respectively. Multiple one and two-way ANOVAs were used to assess the effects of frequency and culture period on dynamic stiffness and phase at each strain level.

Quantitative Polymerase Chain Reaction (qPCR)

Twelve additional vascularized gels were polymerized in a 48-well culture plate. Six gels each were harvested at Day 1 and Day 6 of culture and RNA was extracted with the RNeasy Lysis kit and protocol (Tel-Test Inc, Friendswood, TX). RNA was transcribed into cDNA using Superscript Reverse Transcriptase (Gibco, Gaithersburg, MD). Primers were designed for 5 targets that have been reported to regulate ECM degradation during angiogenesis (MMP1, MMP2, MMP9, TIMP1, and TIMP2) and one marker of angiogenesis initiation (VEGF). qPCR was performed on a Corbett Research RotorGene system, with Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Carlsbad, CA) and SYBR green (Molecular Probes, Inc, Eugene, OR). Reactions for each target were optimized individually and final acquisitions were performed in triplicate with no-template and no-RT controls. GAPDH was used as a housekeeping gene. Relative expression ratios between Day 1 and Day 6 were determined via the method of Pfaffl [3] and expression levels were compared statistically using pairwise fixed reallocation randomization tests [3].

RESULTS

Small sprouts at the end of the seeded fragments were already evident at Day 1 of culture (Figure 3, left panel), visible as smooth elongations from the rougher, parent vessels. Extensive microvessel sprouts with bifurcations were evident at Day 6 (Figure 3, right panel), with many gels showing contraction from the chamber wall.

There was a significant decrease in dynamic stiffness between Day 1 and Day 6 ($p < 0.001$). In contrast, there was no change in the

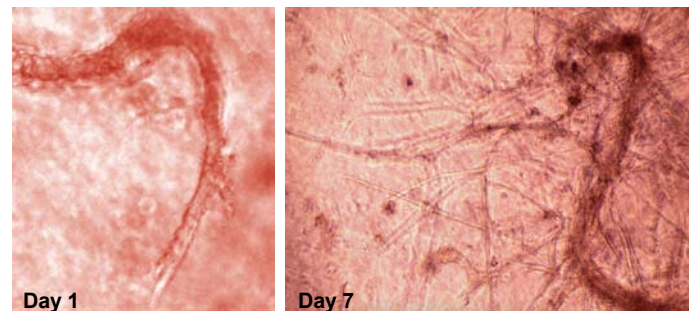


Figure 3. Phase contrast micrographs of microvessel fragments in collagen gels. Left panel shows initial sprouting at Day 1, while right panel shows formation and branching of a microvascular network at Day 6. 200x mag.

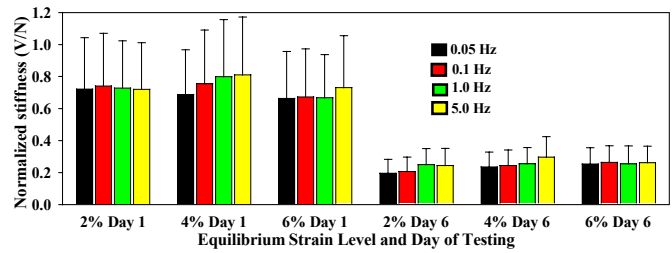


Figure 4. Normalized dynamic stiffness of vascularized gels.

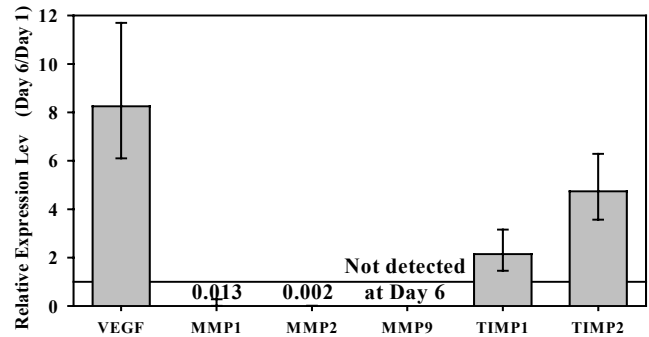


Figure 5. Relative expression levels of qPCR targets.

dynamic stiffness of the native gels between test days. This effect was clearly seen when dynamic stiffness of vascularized gels was normalized by the corresponding native gels (Figure 5, mean $\pm$ stdev).

VEGF showed a robust upregulation between Day 1 and Day 6 ($p=0.01$). Contrary to expectations, all MMP targets were significantly downregulated ($p < 0.001$ -MMP1; $p=0.03$ -MMP2) between Days 1 and 6, while the TIMPs were significantly upregulated ($p < 0.001$ -TIMP1; $p=0.03$ -TIMP2). No template was detectable at Day 6 for MMP9.

DISCUSSION

This study demonstrated that angiogenesis alters the material properties of the ECM. Although the results demonstrated a downregulation of MMPs and upregulation of TIMPs between Day 1 and Day 6, it is still possible that MMP action on the ECM is responsible for the decrease in dynamic stiffness. The Day 1 qPCR results may already represent elevated mRNA expression levels in comparison to freshly harvested vessels. The delay from mRNA transcription to protein translation and activation needs consideration. To address these possibilities, we plan to examine gene expression at additional time points and investigate MMP protein activity.

These results may have important implications for wound healing in musculoskeletal soft tissues, where there is likely a tradeoff between the amount of angiogenesis that is needed for proper wound healing and the degree of ECM degradation. The latter has the potential to compromise the healing and result in inferior material properties.

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