SAMPLE BASIC SCIENCE APPLICATION

ABSTRACT:

TITLE: Smooth Muscle Cells' effect on the angiogenic invasion of Human-Umbilical Vein Endothelial Cells in fibrin- based Tissue Engineered Blood Vessels Analogs subjected to 10% 3-D Cyclic Strain

INTRODUCTION:

Tissues greater than 200 μm from a vascular supply are susceptible to both ischemia and subsequent necrosis. Engineered tissues, including tissue engineered blood vessels (TEBVs), have the same requirements. As such, the development of grafts with thicker media layers will likely require the development of a microvasculature within these vessel's walls to nourish the smooth muscle cells (SMCs) of the tunica media which add both structural integrity and elasticity.(1) Recent developments in tissue culture techniques have demonstrated that dynamic mechanical stimulation can augment cellular proliferation, alignment, and matrix protein synthesis. To achieve these same benefits TEBV's have been cultured with dynamic mechanical stimulation. Cyclic strain has been shown to particularly augment the mechanical strength of TEBV's.(2) Preliminary results within the lab of Dr. Howard Greisler have shown that, when compared to static controls, endothelial cells (ECs) seeded alone within fibrin-based TEBVs and cultured in a dynamic environment of 10% cyclic strain demonstrate shorter, more robust, and wider multi-cellular angiogenic sprouts. Additionally, work in the same lab has shown that EC's co-cultured with SMCs (both homogenous distribution and co-aggregated) in a fibrin matrix have both longer and more robust angiogenic sprouting.

HYPOTHESIS:

Within a dynamic culture environment, the homogenous addition of SMCs to the extracellular matrix will augment the development and angiogenic invasion of EC sprouts into a fibrin hydrogel.

METHODS:

TEBVs will be formed from fibrin, over silicone sleeves. Prior to formation of the TEBV and pelleting of the ECs, both cell types' identities will be confirmed using immunohistochemistry and then labeled seperatly with two distinct membrane bound immunoflourescent surface dyes. A tubular hydrogel will be formed from a suspension of human-umbilical vein EC (HUVEC) pellets (2k cells/pellet, 20 pellets/mL), canine carotid SMCs (100k /mL), fibrinogen (2.5mg/mL), thrombin (0.32 U/mL), heparin (5U/mL), vascular endothelial growth factor (VEGF) (1 ng/mL), fibroblast growth factor-1 (FGF-1) (10ng/mL), and aprotinin (100KIU/mL). The hydrogel TEBV analog will then be mounted within a bioreactor chamber. The constructs will then be covered with assay media comprised of all of the same reagents and concentrations as the TEBV except the cells, fibrinogen and thrombin. This media will be replaced every 3 days. A Windkessel effect pump system will deliver fluid pulsations through the bioreactor distending the TEBV another 10% of the silicones sleeves' original volume. Angiogenic invasion will be documented at Day 7 by measuring average sprout length and sprout number by both light and confocal fluorescence microscopy. The samples that have large multi-cellular sprouts will be sent for TEM sectioning and imaging to confirm lumen formation

ANTICIPATED RESULTS:

ECs and SMCs co-cultured in a fibrin-based TEBV subjected to 10% 3-D cyclic strain will likely demonstrate a robust network of angiogenic sprouts that are longer than sprouts formed by endothelial cells alone and wider than sprouts formed in co-culture without mechanical stimulation.

Research Plan:

Background and rationale

Tissue Engineered Blood Vessels: The current standard for small diameter (<6mm) functional arterial grafts in vascular medicine is the autologous graft. The use of these grafts is limited in many patients since a suitable replacement is not available due to vascular disease, amputation, or previous harvests. Experiments have shown that with lower flow velocities in the smaller grafts, the incidence of thrombogenesis, aneurysm formation, and atherosclerotic plaque formation is increased. There is currently a need for an alternative option for small diameter functional arterial grafts, and this absence has spurred interest into TEBVs.(3) Originally described by Weinberg and Bell, TEBVs are comprised of a complex network of cells and tubular support scaffolding.(4) Within these constructs, scaffolding materials typically include synthetic alternatives as well as biologic hydrogels such as fibrin and collagen. Fibrin hydrogels have been shown to stimulate ECs and pericytes (i.e. vascular SMCs and fibroblasts) to up-regulate cellular proliferation, synthesis of cytokines and both elastin and collagen genesis.(5)

Dynamic Stimulation: Dynamic mechanical stimulation, within a bioreactor cell culture, has been shown to promote tissue organization in engineered constructs.(6) Cyclic 2-D uniaxial strain has been shown to augment ECs migration, cytokine production, proliferation, matrix remodeling and inhibit EC apoptosis.(7) Dynamic culture techniques have shown that subsequent mechanical stimulation after hydrogel formation increases the constructs mechanical structural characteristics (i.e. burst pressure, suture retention strength, etc).(2) The dynamically conditioned TEBVs are able to develop these characteristics secondary to proliferation of the cellular components of tunica media (i.e. SMCs). As these structural layers thicken to handle their mechanical load (>200 μm) a microvascular support network akin to native vessels vasa vasorum must be considered to allow nutrients and gas exchange into the deeper layers of tissue. (1)

Angiogenesis: Angiogenesis is the formation of new capillary blood vessels from a pre-existing vasculature. This process consists of cellular differentiation, proliferation, and matrix invasion and subsequent tubulogenesis. Angiogenesis is influenced by cytokines present within the extra cellular matrix, cytokines secreted in a paracrine fashion from local supporting cells such as pericytes and mechanical stimulation.(8, 9) The ultimate cellular response to stimulation is determined secondary to summation of all these complex signaling pathways.

Co-Culture with SMCs and ECs: In vitro, ECs and SMCs secrete cytokines, which may locally influence their culture environment in a paracrine fashion. This stimulation has been shown to augment angiogenic invasion cell proliferation; for example, SMCs have been shown to locally secrete growth factors (e.g. VEGF, PDGF, etc.).(6) Co-cultures using myoblasts and ECs have been shown to stimulate lumen formation as well as vessel network formation.(8)

An understanding of any one of these pathways would be useful in the development of a microvascular network, and understanding how these processes occur in concert could potentially provide a more accurate picture of how angiogenesis both occurs *in vivo* and may be manipulated to augment the development of TEBVs with structural characteristics that more closely resemble native tissue. This experiment investigates the effects of a 3-D co-cultured fibrin hydrogel under 3-D cyclic strain. The effects of SMCs on angiogenesis The effects of SMCs on angiogenesis muscular human artery.

Hypothesis and specific aims

It is our hypothesis that within a dynamic culture environment the homogenous addition of SMCs to the extracellular matrix will augment the development and invasion of EC's sprouts into a fibrin hydrogel.

SPECIFIC AIM #1: Develop/ identify a co-culture model for angiogenesis.

Dr Greisler's lab has extensive experience with *in vitro* angiogenesis and in particular within fibrin hydrogels. Fibrin is a relevant scaffold material to use in this study as it has been shown to have greater organization and alignment than other biopolymers (i.e. collagen) which improves

matrix deposition which makes the mechanical properties of TEBVs more closely resemble a human artery.(5)

In this study, HUVECs will be used due to well documented angiogenic behavior and they have been shown to be capable of producing very stable networks of microvessels in engineered tissue.(10)

SMCs are added to more accurately study the local environment of ECs within the tunica media of native and engineered blood vessels. It has been shown that SMCs produce matrix compounds (i.e. elastin and collagen) in fibrin hydrogels which both structurally stabilizes and adds compliance to engineered blood vessels.(5) Additionally, work within Dr Greisler's lab (manuscript in preparation) indicates that pelleted ECs and SMCs co-cultured in static fibrin hydrogel demonstrate greater overall sprout lengths and durability over 7 days.

Although human SMC may represent greater potential future relevance, canine carotid SMCs were chosen due to their accessibility and due to Dr. Greisler's lab's experience and expertise with handling these cells. These data will also be relevant to future canine implant studies.

SPECIFIC AIM #2: Develop/ identify a bioreactor capable of generating 10% cyclic distension.

Recently, Dr. Greisler's lab developed a bioreactor system with a Windkessel effect-based pump used to generate 10% cyclic distension in TEBV analogs**.** This same system shall be employed in this experiment. Ten percent mechanical cyclic strain has been reported as a value sufficient to elicit mechanical cellular stimulation.(11)

SPECIFIC AIM #3: Identify and quantify sprouting

The population of cellular components used in these vascular analogs will be confirmed immunohistochemically prior to use. In hydrogels with a thickness >1 mm immunohistochemical identification of angiogenic sprouts with fluorescently labeled antibodies (>150kD) after fabrication will not be readily achievable. This fact is due to steric limitations of the antibody causing poor penetration and subsequent removal. As such identifying the angiogenic sprouts immunohistochemically will likely result in prohibitively low signal and high background fluorescence even with confocal microscopy. Although sectioning and subsequent staining is possible, the integrity of the very fragile capillary sprouts present within these hydrogels could be in jeopardy during the sectioning process. As such, pre-labeling each cell type with a specific fluorescent surface label could identify sprout behavior within intact hydrogels Dr Greisler has shown that PKH can be successfully used to track endothelial cells for >45 days with minimal signal loss and cell viability loss.(12) Although sprouting may occur with convolutions and in a 3-D fashion, quantifying the pellet's sprouting by means of a 2-D image and radial grid is reasonable if both treatment and control groups are quantified in the same fashion.(13)

Research design and methods

Cell Culture: HUVEC's, will be seeded at $5.0x10^5$ cells/flask and grown in fibronectin coated ($2.5mg/cm^2$) t75 flasks in complete EGM media (comprised of proprietary concentrations of: EGM base medium and bovine brain extract, recombinant human Epidermal Growth Factor (rhEGF), hydrocortisone, Gentamicin, and amphotericin) until 75% confluent. The cells will then be transitioned to an M199-based media for the remaining culture until experimental use. The transition media will be comprised of M199 supplemented with L-Glutamine, Hepes salts, Earle's salts, 20% fetal bovine serum, streptomycin/penicillin (100 µg/mL), and fungisone (250 µg/mL). This solution will be passed through a 0.22µ filter and then further enhanced with rhEGF (10ng/mL). This protocol allows HUVECs to be cultured *in vitro* without obvious phenotypic de-differentiation in passages 2-6 and retains viability in M199 based hydrogels. SMCs previously obtained from the media of canine carotid arteries by Dr. Greisler's lab well established IACUC approved canine protocols will be used in this model. SMC's will be cultured in similar fashion with two exceptions. First SMCs will not require fibronectin coated t75's, and the SMC culture media will be comprised of DMEM supplemented with 10% fetal bovine serum, sodium pyruvate (1mM), Gentamicin (50 mg/mL), and non-essential Amino acids (0.1 mM). Cultures will be incubated at 37°C, 100% humidity, and 5% CO_2 with regular media changes every 3 days. Cells will be transferred from culture flasks using a short digestion (≤10 minute) trypsin-EDTA. For consistent cellular behavior, only passages 2-6 will be used in these experiments.

Cell Identification: Prior to experimental use, EC and SMC populations will be identified by immunohistochemistry. ECs will be labeled with a 1^{O} antibody of either rabbit anti-vWF/Factor VIII or rabbit anti-human cd31 and subsequently labeled with the fluorescent 2^{O} antibody flouroscein conjugated goat anti-rabbit. SMCs will be identified with anti-smooth muscle α-actin 1^{O} antibodies and fluorescent rhodamine labeled goat anti-mouse 2^{O} antibodies. Cultures, >95% pure, will be used for angiogenesis assays.

Cell Labeling: Separate EC and SMC populations will be labeled prior to pelleting with either membrane bound immunoflourescent PHK 26 or 67. These surface dyes, 20mM, and BAEC's will be suspended in Diluent C, and the labeling process halted with the complete growth media. The PHK 26 or 67 stains will later be detected with TRIT-C and FIT-C imaging, respectively

Pellet Formation: An EC cell pellet will be created by placing 37.5 µL of 1% sterile methylcellulose solution into a cell suspension comprised of 2,000 HUVEC's in 112.5 µL growth media. The (1:3) ratio of methylcellulose to cell suspension will result in a solution with a final concentration of 0.25% methylcellulose. This solution will be placed in a 96-well round-bottom culture plate and incubated 24 hours allowing cell aggregation. Pelleted ECs will be transferred from their round bottom culture plate to a centrifugation tube and spun at 250 RPM for two minutes. After the supernatant solution is removed, the pellets will be ready for use in TEBVs.

TEBV Formation: All TEBVs will be formed in a culture hood using strict sterile technique. The labeled EC pellets will be re-suspended in M199 medium supplemented with L-Glutamine, Hepes salts, and Earle's salts. Labeled SMCs will be added to the solution along with fibrinogen, heparin, growth factors, and aprotinin. Thrombin dissolved in an equal volume of the same base medium will then be added to the suspension yielding a final concentration of EC pellets (20 pellets/mL), SMCs (100k /mL), Fibrinogen (2.5mg/mL), Thrombin (0.32 U/mL), Heparin (5U/mL), Vascular Endothelial Growth Factor (VEGF) (1 ng/mL), Fibroblast Growth Factor-1 (FGF-1) (10ng/mL), and Aprotinin (100KIU/mL). As soon as the components are thoroughly mixed they will be added to a TEBV mold. The mold will be comprised of five parts 1) an outer glass cylinder (3cm long x 8 mm I.D.), 2) an inner glass mandrel (3cm long x 3mm O.D.), covering the mandrel 3) a sterile silicon sleeve (2 cm x 3.15 mm I.D.x 3.55 mm – 50 D) that has been etched in 10N HCl for 1 hour followed by buffered washes until neutral pH,7) two sterile silicone stoppers for both open ends of the outer cylinder, and 5) an 18 G needle to equalize the pressure within the mold and air evacuation as both stoppers close the cross-linking hydrogel into the mold. The mold will then be gently rolled for 4 minutes to ensure equal distribution of EC pellets and individual SMC's. After 8 minutes the hydrogel is careful removed from the mold and placed within assay media comprised of all same reagents and concentrations as the hydrogel except fibrinogen or thrombin for another 8 minutes to allow final equilibration. Manipulated by their silicone sleeves alone these TEBV analogs will then be attached to the inlet and outlet mounting pins within the bioreactor chamber and sutured into place. The constructs will then be covered with assay media, which will be replaced every 3 days.

Dynamic culture: The bioreactor, sealed with exception of 0.22µ filter vent port will be placed within a 37°C incubator with 100% humidity, and 5% CO_2. The external portion of each TEBVs inlet and outlet mounting pin will be attached to sterile silicone tubing and perfused with phosphate buffered saline until all air has been removed from the perfusion tubing. At this point, Dr Greisler's lab novel Windkessel effect pump system will be engaged delivering fluid pulsations through the perfusion tubing sufficient enough to distend the thin silicone sleeves by 10% of the silicones sleeves' original volume.

Quantification: Angiogenesis will be documented at Day 7 by measuring average sprout length, sprout number by fluoroscopy at 4x microscope and a color digital camera. Images will then be transferred to Adobe® Photoshop® v 5.0 software where a 36 spoke radial grid (one every 10°) will be overlaid over each image. The number of sprouts that cross a grid line and the distance of furthest crossing will be documented. Additionally the number of sprouts >30 µm in diameter will also be calculated. The sprout ultra-structure will be analyzed confocal microscopy with subsequent 3-D reconstructions of angiogenic sprouts using the NIH's Image J software. The samples that appear to have large multi-cellular sprouts will be sent for TEM sectioning and imaging to confirm lumen formation.

Statistics: The average length of sprout, number of sprouts, and number of sprouts with diameter >30 μm will be tabulated. The values for static and dynamic culture conditions will be compared using student t-test with equal variance. P values < 0.05 will be considered significant.

Anticipated results and interpretation

Within a dynamic culture environment the homogenous addition of SMC's to the extracellular matrix will augment the development and invasion of EC sprouts into a fibrin hydrogel.

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