THE DEVELOPMENT AND CHARACTERIZATION OF A NOVEL 3D ANGIOGENESIS TISSUE MODEL FOR TESTING AN ANTI-ANGIOGENESIS DRUG

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CHAPTER 1

INTRODUCTION

Diabetic Retinopathy (DR) is a microvascular complication associated with diabetes mellitus. DR is an eye disease that occurs as a result of chronic hyperglycemia, which causes vascular damage, increased vascular permeability, vascular leakage, and edema [1]. Diabetes mellitus or just diabetes is a chronic condition caused by relative or absolute deficiency of insulin in the body. DR was responsible for blindness in 5% of the world population in 2002 with approximately five million people becoming blind [2]. During this time period it was also the third leading cause of blindness in working age adults in the United States. In the American Indian population in Oklahoma, diabetes has attained an epidemic status. As a result, DR had a higher prevalence of visual impairment in Oklahoma Native Americans than other ethnic groups [3].

When the blood glucose level is elevated in the body, it can lead to a production of growth factors. Among the many growth factors present, vascular endothelial growth factor (VEGF) has been recognized as the primary mediator of vascular alteration in DR [4, 5]. Several clinical studies have shown how increase in VEGF concentrations within the eye are linked to DR [6-11] VEGF expression is induced by hypoxia, hyperglycemia, oxidative stress, high glucose, tissue ischemia, all occurring in diabetes. Therefore in order to reduce the morbidity and mortality associated with DR, it is important to develop

therapeutic strategies for controlling the disease. A potential new therapy for DR is the development of anti-angiogenesis drugs that would inhibit the angiogenic function of VEGF, thereby reducing VEGF levels within the eye.

Angiogenesis is a physiological process involving the growth of new capillary blood vessels from pre-existing blood vessels. This involves basement membrane degradation, cell migration and alignment, lumen formation, loop formation, blood flow initiation, and a new basement membrane formation [12]. It is a fundamental step that occurs during wound healing, organ formation and embryonic development, and pathologically in disorders such as rheumatoid arthritis [13], diabetic retinopathy and cancer (tumor growth and metastasis) [14, 15]. *In vitro* tissue models are used for studying angiogenesis because they create an environment suitable for cell growth that closely recapitulates what transpires *in vivo.* An *in vitro* angiogenesis model could be two dimensional (2D) or three dimensional (3D). For the studies carried out in this research, a 3D *in vitro* tissue model was utilized because it provided the third dimension that captures the physiological complexity unavailable in 2D models. To mimic the *in vivo* environment, *in vitro* models were used to control biological conditions, as well as analyze isolated processes that contribute to angiogenesis [15, 16]. These models have also effectively quantified blood vessel formation in 3D. A 3D *in vitro* angiogenesis model involves using cells of endothelial type grown or embedded within an extracellular matrix component (ECM) and adding angiogenic agent(s) that would induce cell sprouting, proliferation, migration and differentiation. An example of a 3D angiogenesis model can be described by Nehls and Drenckhahn [17]. In this model, endothelial cells were seeded on gelatin-coated microcarriers and implanted within a fibrin gel or matrigel

[18]. The cells were observed to form multicellular capillary-like structures in response to basic fibroblast growth factor (bFGF) and VEGF added to the system. Another model involves embedding aorta rings in fibrin or collagen gels in the absence of serum and protein supplements [19].

Selecting a suitable ECM and angiogenic agent is crucial for the development of a 3D tissue model because ECM provides vascular support essential for maintaining the organization of vascular endothelial cells into blood vessels [20] and angiogenic agents are responsible for stimulating blood vessel growth in the body [16]. Literature has shown VEGF as the key regulator in both physiological and pathological angiogenesis. This cytokine was selected because of its potent factor to stimulate endothelial cell proliferation, differentiation and migration in blood vessel formation [21]. Collagen was also selected because it provides a matrix environment suitable for blood vessel formation [22, 23] and constitutes the major protein in the ECM [24]. *In vitro* angiogenesis models utilize endothelial cells grown on a 3D extracellular matrix environment. These cells are the most suitable because they line the interior surface of the blood vessel and their cellular functions are involved in many angiogenic events [15]. Human umbilical vein endothelial cells (HUVECs) were the type used because they are readily obtainable and simple to isolate and culture [25].

This research was divided into two studies. The first study was to develop a 3D angiogenesis tissue model to study the effect of a novel anti-angiogenesis drug (CLT-003, a thalidomide analogue). Thalidomide is a potent inhibitor of new blood vessels (angiogenesis) [26]. Thalidomide analogues were created to decrease the harmful side effects associated with thalidomide. For the first tissue model, HUVECs were grown on

type 1 collagen matrix in a 96 well solid plate format using a growth factor cocktail as the stimulant. The effect of the drug was studied on HUVEC viability, proliferation, number of sprouts and migration. Limitations of the first tissue model to fully measure the effect of CLT-003 on angiogenesis led to the development and characterization of a novel 3D angiogenesis vascular tissue model. The second study employed a Transwell® membrane well to create a model that better represents physiological conditions. The Transwell® membrane plate format was used because of its ability to grant independent and easy access to both sides of the monolayer, in the upper and lower compartment of the membrane, and the simplification in taking measurements. The Transwell® assay is often used in *in-vitro* angiogenesis models to study endothelial cell migration in response to growth factors, [16] but literature has shown that there have been challenges during the set up and the quantification stage [15]. Some of these challenges involving the quantification of the stages of angiogenesis have been overcome in the new model used in this research. The 3D tissue model was characterized by varying collagen thickness and VEGF concentration added to the system. These two variables were investigated on HUVEC viability, proliferation, migration and number of sprouts. The new model gave an improved *in vitro* assessment of the angiogenic process because it mimicked *in vivo* events such as viability, proliferation, migration and sprouting. The most suitable collagen thickness and VEGF concentration that gave the best results in stimulating HUVEC viability, proliferation, migration and number of sprouts would be used to study the effect of the anti-angiogenesis drug within the model.

The next chapter presents a literature review of the diabetic retinopathy disease, its association with angiogenesis, VEGF and the different types of tissue models used for

studying angiogenesis. The information provided gives a better understanding of the development of the 3D tissue model that was used in this research.

CHAPTER 2

LITERATURE REVIEW

2.1 Diabetic Retinopathy

Diabetic retinopathy (DR) is characterized by reduced retinal capillary blood flow, retinal and vitreous hemorrhage, reduced visual acuity and eventually blindness. It is a disease caused by damage to the small blood vessels in the retina, the light sensitive layer of tissue lining the back of the eye. The retina converts light energy falling on it into electrical impulses that can be analyzed by the brain.

The earliest stage of DR shows mild non-proliferative abnormalities characterized by increased vascular permeability. It is also known as background DR and consists of microaneurysms only. Then it progresses to moderate and severe non-proliferative diabetic retinopathy (NPDR) characterized by blurred vision, floaters, loss of central or peripheral vision, and flashes of lights. Floaters are black peppery spots in the vision. The last stage is known as proliferative diabetic retinopathy (PDR). This stage is characterized by neovascularization (as seen in figure 2.1 below), blurred vision, floaters, loss of central or peripheral vision, and flashes of light. The onset is typically more sudden and blurring of vision is more severe than NPDR [27]. Neovascularization is the growth of new blood vessels. PDR is more common in patients with type 1 diabetes than

type 2 diabetes. The newly formed blood vessels are very fragile, and as a result, flame-shaped intraretinal hemorrhages are commonly found during opthalmoscopic examination. One of the mechanism by which diabetic retinopathy causes blindness is by retinal and vitreous hemorrhage through pooling of blood in the back of the eye that obscures vision [28]. This may occur suddenly and resolve spontaneously, but if not treated timely, it may lead to permanent loss of eyesight.

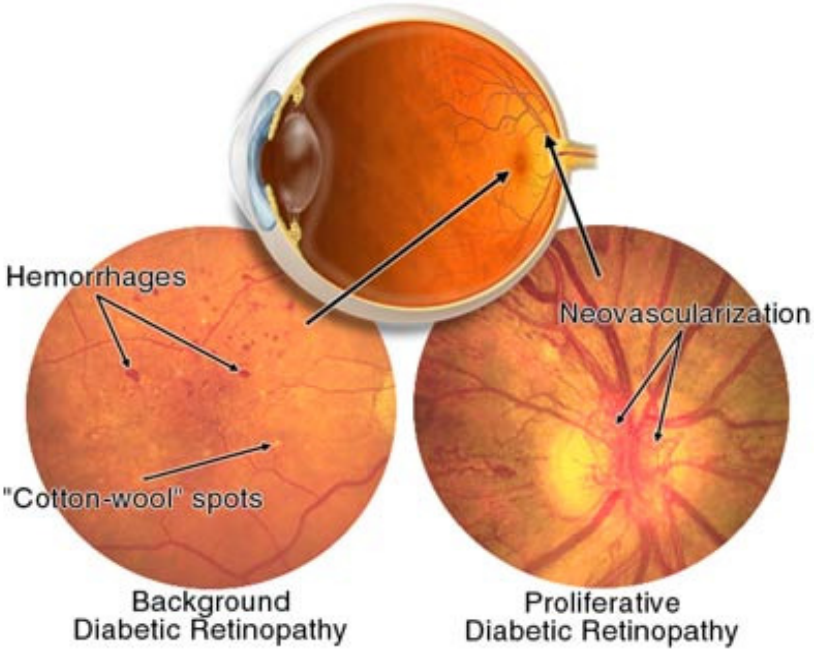


Figure 2.1 A diagram showing the progression of diabetic retinopathy [29]

An example of normal vision and vision with progressive diabetic retinopathy is shown in figure 2.2 below. As the disease progresses a condition known as Diabetic Macular Edema (DME) occurs. DME can be defined as the collection of intraretinal fluid in the macular area of the retina, which manifest as slow degradation in and blurring of vision and distorted images [30].

Figure 2.2 A scene viewed by a person with normal vision and diabetic retinopathy[31]

According to the World Health Organization (WHO), DR is one of the leading causes of blindness in the world. In 2002, DR was shown as the fifth leading cause of blindness worldwide and accounts for 4.8% of the world population. In the United States, it is the third leading cause and accounts for 17%, with age-related macular degeneration (AMD) and glaucoma in first and second place, respectively [2]. The development and progression of retinopathy in an individual is dependent upon the duration of diabetes. Almost all patients with type 1 diabetes and over 60% of patients with type 2 diabetes will develop some degree of retinopathy after the first 20 years of having the disease [32]. Forty-percent of adults with diabetes age 40 years and above have retinopathy and this condition is vision-threatening in 20 % of these patients [33]. Vision-threatening DR can be classified in patients having one or more of these: severe NPDR, PDR, and DME. People with diabetes that are 50 years and above have two times the risk of vision impairment as people in the same age group without diabetes [34].

The present treatments available for DR: laser photocoagulation and vitrectomy do not restore lost vision. They are only effective at slowing the progression of retinopathy and reducing the risk of severe vision loss. It is therefore important to identify and treat patients early in the disease [27]. However, the techniques involved in these treatments are invasive and are very likely to have side effects. Though new techniques are currently developed, a non-invasive and effective drug treatment that would prevent progression of diabetic retinopathy has not yet been discovered.

Vascular endothelial growth factor (VEGF) plays a fundamental role in the etiology of DR. It serves as the primary mediator of intraocular neovascularization, [4] that is the abnormal or excessive formation of blood vessels in the eye. These blood vessel formations are induced by hypoxia brought about by the blockage of retinal capillaries at the early stages of DR. Hypoxia occurs when there is an inadequate supply of oxygen to tissue and this increases the tissue production of angiogenic factors, including VEGF, which promotes vascular permeability [35]. It is therefore necessary to understand blood vessel formation (angiogenesis) in the body, and the role VEGF plays in the angiogenesis process.

2.2 Angiogenesis Process

Angiogenesis is the formation of new blood vessels, but it differs from neovascularization in that angiogenesis is mainly characterized by the protrusion and outgrowth of capillary buds and sprouts from pre-existing blood vessels. In the human body, a balance exists between angiogenic factors, which stimulate angiogenesis and

anti-angiogenic factors (inhibitors) which inhibit angiogenesis. The balance becomes upset when either the concentration of angiogenic or anti-angiogenic factors outweighs one more than the other. The result is pathological angiogenesis, where there is an abnormal proliferation of blood vessel formation. This can occur in many diseases in addition to DR such as cancer, rheumatoid arthritis, psoriasis and atherosclerosis.

The angiogenesis process involves two consecutive stages namely neovessel growth and neovessel stabilization. A sequence of events occurs during the first stage. These events include dissolution of the basement membrane of the "parent" vessel and its surrounding matrix, migration of endothelial cells within the created space toward the angiogenic stimuli, proliferation of endothelial cells, lumen formation within the endothelial sprout, and formation of loops by anastommoses of sprouts. The second stage entails the cessation of endothelial cell proliferation, reconstruction of a basement membrane around the neovessel, and recruitment and coverage of the immature vessels with pericytes. Both stages are equally significant, because without vessel stabilization, the immature capillary will undergo apoptosis at a very fast rate and deteriorate [36]. Figure 2.3 gives a pictorial explanation on the progression of angiogenesis process, from angiogenic factor production to vascular stabilization.

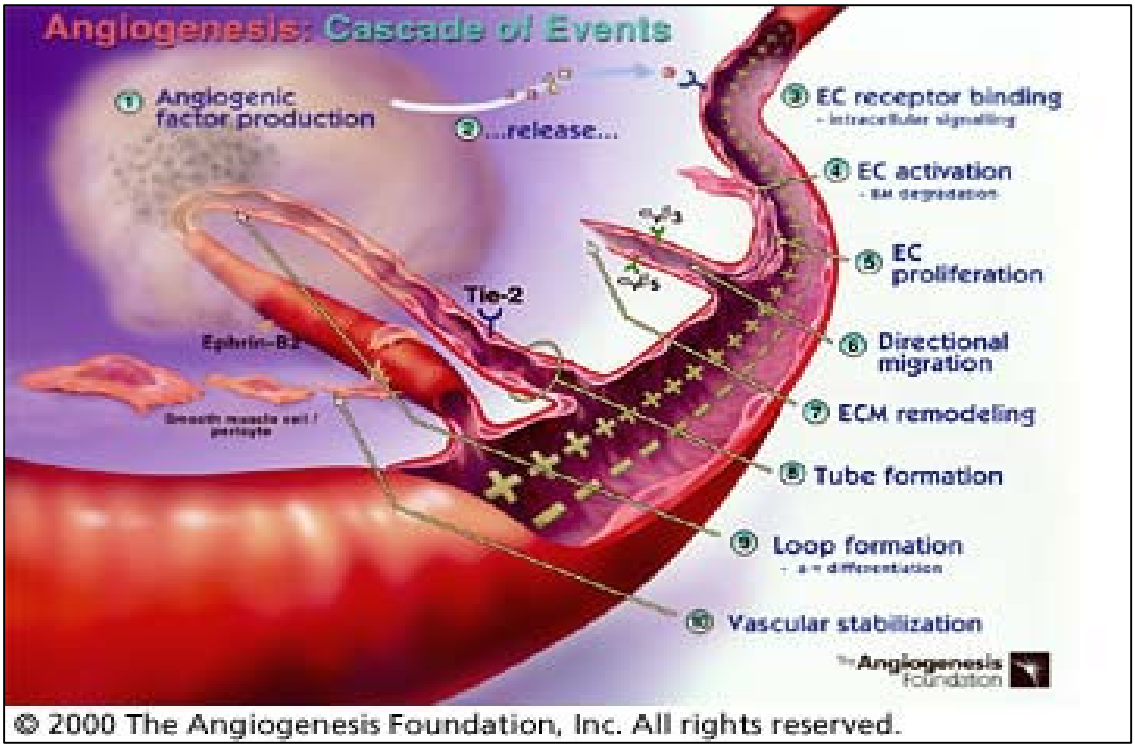


Figure 2.3 Progression of Angiogenesis [37]

2.3 Vascular Endothelial Growth Factor

VEGF, also known as vascular permeability factor (VPF), is an angiogenic, endothelial cell mitogen that stimulates angiogenesis and vasculogenesis i.e. blood vessel formation form a de novo production of endothelial cells [38]. This growth factor is a heparin-binding, homodimeric glycoprotein with a molecular mass of 45-kDa [39] and comes from the sub-family of platelet derived growth factor. The VEGF family consists of seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (PIGF). VEGF-A (typically referred to as VEGF) is the most important member because of the key role it plays in regulating angiogenesis and

vasculogenesis. VEGF-C and VEGF-D are mediators of lymphangiogenesis, while VEGF-E and PIGF also induce angiogenesis [40]. VEGF is produced by many cell types namely retinal pigment epithelial cells, pancreatic duct cells, Schwann cells, retinal capillary pericytes, endothelial cells, glial cells, Müller cells, and ganglion cells [41-45]. The expression of VEGF in these cell types is induced by hypoxia through different mechanisms [44].

2.3.1 VEGF Isoforms and Receptors

VEGF isoforms are a result of alternative exon splicing of a single gene [46]. Exon 1-5 spans the receptor binding domain, while exons 6 and 7 span the heparin-binding domain. There are four major isoforms of VEGF having 121, 165, 189, and 206 amino acids: $VEGF_{121}$, $VEGF_{165,}$ $VEGF_{189,}$ and $VEGF_{206}$, respectively [46-48]. Other isoforms are in existence and represent less frequent splice variants; $VEGF_{145,}$ [49] $VEGF_{183,}$ [50] $VEGF_{162}$ [51] and $VEGF_{165b}$ [52]. These isoforms are characterized based on their heparin-binding affinity to the extracellular matrix. Their ability to bind heparin has an inverse relationship with diffusivity. $VEGF_{121}$ is acidic with no heparin-binding affinity, but is a highly soluble protein. $VEGF_{165}$ is basic and binds to heparin, while $VEGF_{189}$ and $VEGF_{206}$ are more basic than $VEGF_{165}$ and bind very tightly to heparin [53]. $VEGF_{165}$ is a secreted protein, though it has a significant portion bound to the cell surface and extracellular matrix. In contrast $VEGF_{189}$ and $VEGF_{206}$ remain sequestered in the extracellular matrix [54]. However these isoforms may be released in a soluble form by heparin. VEGF interaction with heparin was studied in order to address the biological

significance of its longer isoforms, it was demonstrated that a loss of heparin binding, whether due to alternative splicing or proteolysis, will result in a reduction of the vascular endothelial cell mitogenic activity of VEGF [55].

In order for the angiogenesis process to begin, VEGF will need to bind to its receptors called VEGF receptors (VEGFRs). The binding of VEGF to its receptor induces endothelial cell proliferation, migration, survival and permeability of endothelial cells. These receptors are located on the surface of endothelial cells [56] and also on bone marrow derived cells [57]. When a member of the VEGF family binds to its receptor, dimerization occurs, and they become activated through a process called autophosphorylation. Autophosphorylation is the phosphorylation of a kinase protein catalyzed by its own enzymatic activity. VEGFRs are structured into seven immunoglobulin-like folds in the extracellular domain, a single transmembrane region, and a split tyrosine kinase domain, which is interrupted by a 70-amino-acid kinase insert domain [35]. VEGF binds to two receptors namely VEGFR-1/Flt-1 (fms-like tyrosine kinase) and VEGFR-2/KDR/Flk-1 (kinase insert domain-containing receptor/fetal liver kinase). These receptors along with VEGFR-3/Flt-4 are part of the receptor tyrosine kinase (RTK) family. The expression VEGFRs differs in specific endothelial cell layers. VEGFR-2 is can be found on almost all endothelial cells, while VEGFR-1 and VEGFR-3 can be found on endothelial cells in distinct vascular layers [58]. VEGF-B binds to VEGFR-1, VEGF-C and VEGF-D binds to VEGFR-2 and VEGFR-3, VEGF-E binds to VEGFR-2, and PIGF binds to VEGFR-1.

2.3.2 VEGF Inhibitors

VEGF inhibitors are anti-angiogenic agents that hinder VEGF binding to its receptors that promote angiogenesis. Many VEGF inhibitors have been approved by the United States Food and Drug Administration (FDA) to aid in the treatment of diseases associated with angiogenesis. Some of these inhibitors include Bevacizumab (Avastin), which has been shown to increase the survival rate of patients with cancer, [59] Ranibizumab and Macugen (Pegaptanib), which were also approved by FDA for the treatment of age-related macular degeneration [60, 61].

Thalidomide has also been identified as a strong inhibitor of VEGF and thus angiogenesis. It is a powerful teratogen, immunomodulatory and anti-angiogenic drug that inhibits blood vessel formation in adults and animals [62-64]. Komorowski et al [65] studied the effect of different concentration of thalidomide on VEGF secretion, endothelial cell migration, adhesion and capillary tube formation in vitro. It was observed that 1 μM to 10 μM thalidomide concentrations inhibited VEGF secretion, and thalidomide concentrations of 0.01 μM and 10 μM reduced the number of capillary tubes formed, but did not affect cell proliferation. The inhibitory factor of thalidomide has also been studied in vivo by D'Amato et al [26] using the rabbit cornea micropocket model, where angiogenesis was induced by basic fibroblast growth factor. Due to the side effects associated with using thalidomide, derivatives of the drug called thalidomide analogues were designed to decrease harmful side effects, while enhancing the anti-angiogenic activity of thalidomide. This derivative has been shown from literature to be more powerful than thalidomide. For example, Moreira et al reported that thalidomide

analogue (cc-1069) inhibited endothelial cell proliferation more effectively than thalidomide [66].

2.4 Various Model Systems

A model is a representation of a system that allows for investigation of the properties of the system as well as the prediction of future outcomes. Researchers use experimental models to better understand how biological systems function and the complications associated with them. These models provide an environment for studying human diseases and treatments. The biological model systems can be divided into *in vivo* and *in vitro* models. *In vivo* models include the human and animal models, while *in vitro* models include two dimensional and three dimensional cell and tissue culture models. Examples of these models are shown in figure 2.7 below.

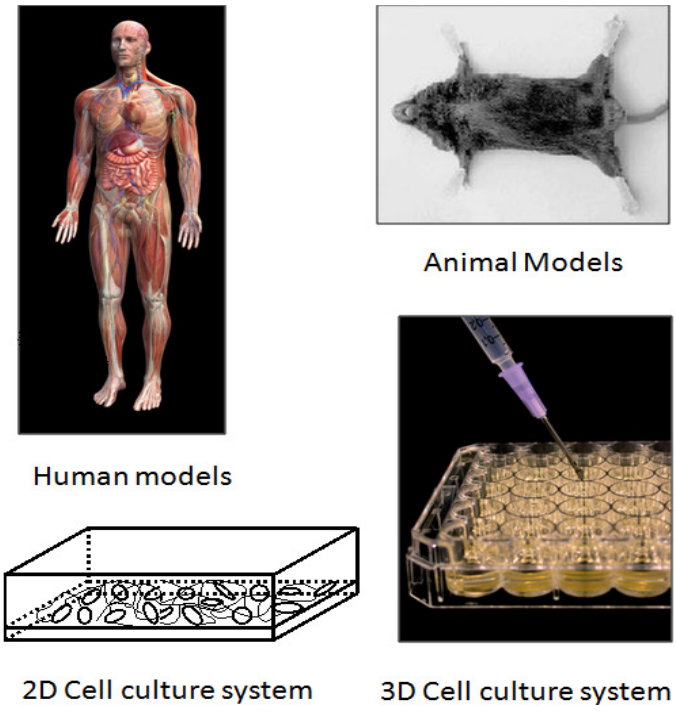


Figure 2.4 The Different Types of Model Systems [67-69]

2.4.1 *In Vivo* Versus *in Vitro* Model Systems

Human models are the ideal models to use in research studies because they are the exact environment where pathological conditions occur. However, the ethical issues involve constitute the major disadvantage associated with using these models. In an unfortunate history of medical research, experiments were performed illegally on human subjects without their knowledge, consent, or informed consent [70] and this led to many untimely deaths. As a result, researchers cannot use human models to study anything that is harmful to human health. In addition, human models are difficult to control due to the complex nature of the human biological system. There is also the issue of variability, which makes it difficult to analyze the effect of one variable at a time.

Animal models have been used as substitutes for human models because of their similarities to human cells, tissue and organ functions. They can be used to develop and test drugs that may be too hazardous to do so in humans. Another advantage of using animal models over human models is the ease of manipulating and controlling variables in order to study their effect on the system. This gives scientists a better understanding of the physiological behavior of the system. The disadvantage of using animal models is that some of the factors, such as receptors and signaling factors present in animals, may behave differently than those present in humans. As a result, an animal model may give a poor prediction of disease and/or treatment mechanisms in humans.

Two dimensional cell culture models provide more control of experimental variables than animal and human models, are less expensive, and easier to use with room for improved reproducibility. They consist of cells grown in cell culture medium in a monolayer in a variety of formats, for example T-25 cm^2 and T-75 cm^2 tissue culture

flasks, Petri- dishes or well plates. A major limitation of the 2D models is that they do not provide the third dimension that correctly mimics in vivo surroundings. In 2D environment, there are no mechanical and biochemical signals, cell to cell communication and tissue-specific architecture [71]. Nonetheless, because of their simplicity, they are still the most popular models used for in vitro studies.

Three dimensional models are more accessible than animal based models and overcome the limitations of 2D cell culture systems. These models capture more of the relevant complexity associated with the 3D microenvironment, similar to that found in vivo where cells proliferate, migrate and differentiate within an extracellular matrix. Three dimensional models have also been shown to replicate in vivo-like responses [72, 73] thus giving a better description of *in vivo* environment than their 2D counterpart.

2.4.2 Two Dimensional Versus Three Dimensional Cell Culture Models

Three dimensional models are becoming popular and very important because they reduce the need for animal experimentation and provide a potential bridge for the gap between 2D cell culture models and human studies. In 2D model systems, cells grown on planar surfaces do not model the 3D living tissues and organs within the human body. Cell functions such as proliferation and differentiation, which involves cell to cell interactions and cell to matrix interactions are lost under 2D conditions [71]. For this reason, the results obtained from using 2D models are not completely reliable when they do not correctly mimic the *in vivo* environment. For example transforming growth factor-β (TGF-β) inhibited endothelial proliferation in 2D systems, but appeared to be ineffective in a 3D culture system [74]. A 3D culture system can provide a higher surface

area, three orders of magnitude higher than 2D cultures. This higher surface area allows cells to attach and arrange themselves. In addition, cells are not limited by contact inhibition in 3D as in 2D; they can attain a higher density closer to that found in tissues, provided that nutrients can be efficiently transported [75]. Research has also shown that cells exhibit different behavior when grown in 3D cell culture. For example, it has been shown that a higher cell density in 3D culture can generate more monoclonal antibody at high titer [76]. Another example shows fibroblast cells with a difference in morphology and migration when suspended in collagen gels; in 2D cell culture, the substrate induced an artificial polarity between the lower and upper surfaces of these naturally nonpolar cells [77, 78]. A 3D ECM has been recognized for epithelial cells because it provides a 3D environment that promotes epithelial polarity and differentiation [79].

2.4.3 *In Vitro* Models of Angiogenesis

The aim of every *in vitro* model is to recapitulate *in vivo* conditions. *In vitro* models are useful tools in i) studying the various steps occurring in angiogenesis, such as cell migration, cell proliferation, and tube formation, ii) modeling pathological conditions, as well as understanding the molecular mechanisms of angiogenesis, iii) testing the effectiveness of angiogenic and anti-angiogenic agents, and iv) the identification and screening of potential drugs [15, 80, 81].

There are two types of in vitro angiogenesis models namely 2D and 3D models. These models are classified according to the way the cells reorganize. In the 2D models, cells develop tubular structures on the surface of the substrate, but in 3D models, they invade the surrounding matrix consisting of a biogel to form 3D structures. The 3D

angiogenesis models simulate *in vivo* environment better than the 2D models because they describe more of the steps involved in the angiogenesis process. Furthermore, the effect of a gradient of diffusion of nutrients, oxygen, and stimulating factors on the events occurring during angiogenesis can be studied in 3D models. Two dimensional models lack this third dimension required for carrying out these studies.

In vitro angiogenesis was initially demonstrated by Folkman in 1980 where bovine capillary endothelial cells cultured in gelatin formed capillary like tubular structures [82]. This has set the pathway for the development of other *in vitro* angiogenesis models, which have been designed to simulate *in vivo* angiogenesis, but due to the complex nature of the *in vivo* process, *in vitro* models have not been successful in fully representing the stages of angiogenesis as a whole [83]. As described by Rakesh Jain and colleagues, an ideal *in vitro* angiogenesis model should have the following characteristics. 1) the release rate, the spatial and temporal concentration distribution of angiogenic factors or inhibitors should be known 2) it should quantitatively measure the structure of the new vasculature 3) it should also quantitatively measure the function of the new vasculature (i.e. endothelial cell migration rate, proliferation rate, canalization rate, blood flow rate, and vascular permeability 4) the in vitro responses should be confirmed in vivo. 5) newly formed and pre-existing host vessels should be clearly differentiated and 6) it should be cost effective, reproducible, quick, convenient and reliable [84]. Several *in vitro* models have been developed that closely resemble angiogenesis within an in vivo environment. They include endothelial cells grown either on the surface or embedded within an extracellular matrix to form 3D tube-like structures in response to angiogenic factors [22, 85-88].

In the human body, cells secrete molecules that interact to form a complex network, which constitute the extracellular matrix (ECM). A suitable ECM must be selected to provide the right structural environment for cells to assemble into tissues. The ECM is responsible for the developmental process of the tissue through interaction with the receptors on the cells. It also provides a scaffold for cell support, a surface for cells to adhere and migrate into the ECM, and serves as a reservoir where growth factors and chemicals required for cell metabolic function can be stored [89-92]. Collagen is the most abundant protein present in the ECM, making up 25 % of total protein body content [93]. It is mainly found in the skin and bone. A number of angiogenesis models use collagen as the ECM because it represents the major environment where angiogenic events occur [94, 95].

Endothelial cells line all the blood vessels in the human body, the entire circulatory system and are the main constituents of capillaries. For this reason, many *in vitro* angiogenesis models utilize endothelial cells grown in cell culture. Another reason is because their cell functions are associated with many angiogenic events, including matrix degradation, proliferation, migration and morphogenesis [16]. *In vitro* angiogenesis models that make use of endothelial cells are advantageous over *in vivo* models in the following ways: 1) they are easier and faster to set up and execute, 2) there is better control and manipulation of biological functions, 3) angiogenic and anti-angiogenic factors are more easily evaluated and 4) overall cost is lower [15]. Furthermore, endothelial cell functions can be directly assessed, without the involvement of other cells or any metabolic processing [81]. The most widely used endothelial cells are human umbilical vein endothelial cells (HUVECs) because of their availability and

simplicity in culture. Consequently, they have been used in many assays and have been shown to have similar behavior with endothelial cells isolated from vascular beds [25].

Vernon and Sage [96] described a quantitative model termed, "the radial invasion of matrix by aggregated cells" (RIMAC), which was used to study endothelial cell migration and sprout formation within 3D collagen matrices. In this model bovine aortic endothelial cells were embedded within collagen gels supported by rings of nylon mesh. The collagen plus support were called assay disks. The disk was immersed in nutrient media, which resulted in radial movement of endothelial cells into the collagen within two to five days. The RIMAC model was characterized by testing the response of the endothelial cells to collagen density and the angiogenic protein bFGF, VEGF and transforming growth factor (TGF-β1). Their results show that lower concentrations of collagen gels (0.3-0.6 mg/ml) support migration and sprout formation of endothelial cells than higher concentrations (2 mg/ml). Their results also show that both bFGF and VEGF increase endothelial cell invasion in collagen in a concentration-dependent manner, while TGF-β1 inhibits invasion for a wide range of concentrations (0.1-30 ng.ml).

Other 3D in vitro models using collagen as ECM was described by Davis et al [23, 88] to assess capillary morphogenesis formed by endothelial cell invasion into 3D collagen matrices. In the first model, endothelial cells were seeded on the surface of collagen gel in a 6 mm culture well insert with a porous membrane. The cells were fixed and stained after 72 hrs of culture and it was observed that endothelial cells invaded into the collagen below the surface of the monolayer and formed capillary sprouts and lumenal structures. The second model was a modification of the first model where the collagen gel described above was placed on top of a different collagen gel with and

without endothelial cells. The top gel was then seeded with endothelial cells to investigate whether invasion was induced by the endothelial cell already undergoing morphogenesis in the lower gel. It was observed that after 72 hrs of culture, endothelial cells on the top gel invaded and underwent morhpogenic changes while migrating into the collagen matrix. It was concluded that endothelial cells during morphogenesis may release autocrine factors that control EC invasion. The third model was developed on a microscope slide so that the morphogenic process could be visualized by means of phase-contrast microscopy. Invasion of EC into the collagen gel was observed in the same manner as the two earlier models described, in addition to tube formation and intracellular vacuoles.

There are also 3D in vitro models that use fibrin as the ECM. An example of one is that developed by Nehls and Drenckhan [17] where endothelial cells from bovine pulmonary arteries were seeded on gelatin-coated cytodex-3 microcarriers embedded in fibrinogen and induced to polymerize by adding thrombin. Endothelial cells were observed to migrate into the fibrin gel forming 3D capillary-like structures. This model was used to test the effect of angiogenic factors VEGF and bFGF on capillary sprouting and it was observed that VEGF and bFGF at 100 ng/ml and 30 ng/ml, respectively, stimulated endothelial cells proliferation followed by increase in capillary-like formations. This model was later modified using matrigel in place of fibrinogen as the ECM because of its variability and sensitivity to small changes occurring during the polymerization of fibrinogen, and also because of the variation that could be associated with the thrombin batch [18]. Matrigel is a scaffold made up of ECM and basement membrane proteins derived from the mouse Engelbreth-Holm-Swarm sarcoma and

enhanced with collagen IV, laminin and nidogen. The matrigel used in this model was a reduced growth factor matrigel, which differs from standard matrigel in that it contains reduced levels of cytokines and angiogenic factors and has overcome the problems associated with increased stimulation in standard matrigel. Endothelial cells were discovered to attach and rapidly form tubules within a short time period (4-12 hrs) when seeded on matrigel [97]. However, there was a lack of lumen formation, and non-endothelial cell, including human fibroblasts and glioblastoma cells, had the same tubules structure when grown on matrigel [98].

2.4.4 *In Vivo* Models of Angiogenesis

In vivo angiogenesis models have been developed to confirm results obtained in vitro because they represent the exact environment where angiogenesis occur. There are several *in vivo* angiogenesis models, but the most current and frequently used ones in today's practice are the chick chorioallantoic membrane (CAM) model, the corneal angiogenesis model, and the subcutaneous implants [99].

The CAM model can be described as the most widely used *in vivo* model for studying angiogenesis [99, 100]. There are two ways to access the CAM of a chick. The first way is by placing the test substance, or the tissue or organ graft, directly on the CAM of a developed 7-9 day chick embryo through a window created by cutting an opening on the eggshell. The second way is by transferring the egg content into a Petri dish prior to applying the test substance [101]. In this case, there is no eggshell involved and is considered as an *in vitro* method, while the former is considered an *in ovo* method. *In ovo* means, "occurring in the egg or embryo". The test substance is prepared either in

slow-release polymer pellets, absorbed by gelatin sponges, or is air-dried on plastic discs [102]. A pictorial diagram of a chorioallantoic membrane is shown in figure 2.4 below.

Figure 2.5 Chick embryo chorioallantoic membrane [103]

The CAM model has been used to study tumor angiogenesis and to test angiogenic and anti-angiogenic factors [104]. Tumor angiogenesis was studied by grafting tumor samples to the surface of the CAM. Grafting was carried out on day 8 of incubation at 37ºC at constant humidity. This evoked a tumor-induced vasoproliferative response after 48 to 72 hours made up of newly formed vessels that assembled towards the graft. Evaluation of new blood vessels was done by means of a stereomicroscope *in vivo* [105-107]. For testing pro- and anti-angiogenic factors, inert synthetic polymers are soaked with these molecules and placed onto the surface of the CAM. Elvax 40 and hydron, first described by Folkman and Langer, are examples of polymers commonly used [108]. Polymers in combination with an angiogenic factor elicit a vasoproliferative response 72 to 96 hours after placement onto the CAM. An increased vessel density was observed around the implant with vessels converging towards the center [109]. On the other hand, when polymers were combined with an anti-angiogenic factor, the vessels were observed to decrease in density and eventually vanished [110].

The corneal angiogenesis model is considered to be one of the best in vivo models of angiogenesis. Since the cornea is normally avascular, the production of an angiogenic reaction is the most convincing illustration of true vascularization [111]. The test tumors or tissues are introduced in the cornea of rabbits, mice, or rats through a pocket made in the cornea at a safe distance from the limbus, as can be seen in figure 2.5. The test substances that would induce angiogenesis in the corneal pocket are introduced in slow-release materials such as the ethylene vinyl copolymer (ELVAX), Hydron, or a sponge material. Due to the avascular nature of the cornea, introducing angiogenic stimulators creates an environment for easy quantification of blood vessels, thus any vessels formed are new vessels [112]. A slit lamp stereomicroscope is used to evaluate the vasoproliferative response. Vessel budding from the limbal plexus is scored as a positive response, occurring after 3 to 4 days. Capillaries were observed to reach the implanted pellet in 7 to 10 days. Implants that do not produce neovessel growth within 10 days are scored as a negative response [113]. The number and growth rate of newly formed blood vessels are used to estimate the intensity of angiogenic activity. An angiogenic score is calculated using the formula of vessel density times the distance from the limbus [114]. Other methods have been reported to improve quantification, such as video data acquisition and computerized image analysis [115-117].

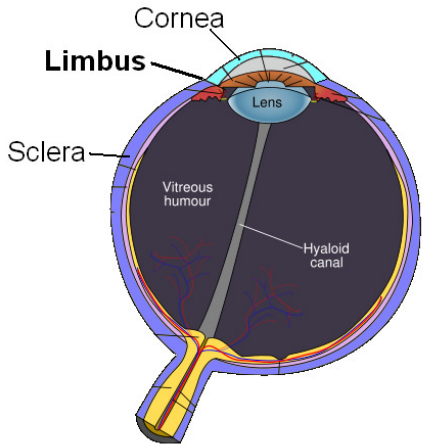


Figure 2.6 Location of the cornea and limbus on the eye [118].

The subcutaneous implant model, which involves trapping the angiogenic factor within a polymer matrix (a sponge-like structure or matrigel plug), has been used to study *in vivo* angiogenesis [119, 120]. One of the characteristics of matrigel is that it is in liquid form at 4ºC and a solid gel at 37ºC. This feature is used in the matrigel plug model, where the liquid containing the test substance is injected subcutaneously, and the matrigel becomes solid under the skin of the test animal [87]. When angiogenic factors are suspended in the gel, this induces an angiogenic response through a slow release of the growth factors to the neighboring tissue. The response can be quantified by measuring the amount of endothelial cell vessel growth or the quantity of hemoglobin in the plug. Since the matrigel is avascular, any vessel formed within the plug can be considered a new vessel [121].

2.4.5 *In Vitro* Versus *in Vivo* Models of Angiogenesis

In vivo models mimic the normal and pathologic states of angiogenesis. *In vivo* models also provide more information than *in vitro* models as a result of the complex cellular and molecular activities of angiogenic reactions, as long as the biology of the assay and the experimental design are pertinent [104]. However, there are several disadvantages associated with modeling angiogenesis *in vivo*. They suffer from batch to batch variability, making it difficult to maintain homogeneity between experiments. They do not have the advantage of controlling one or more variables in order to study the effect of a single variable on the system. This makes quantification of the response from a single variable very difficult in this model. *In vivo* models involve numerous cell types, which does not allow identification of the direct effects on endothelial cell functions only [16]. In addition, they are time consuming, expensive, and require technical expertise in animal handling. The problems associated with in vivo models, limit the number of tests than can be performed at a time. Thus, several *in vitro* models have been developed for carrying out studies that have been difficult to assess *in vivo*.

In vitro angiogenesis models are essential because they create an environment where researchers can have control of many variables associated with the angiogenesis process. They also permit the analysis of isolated processes involved in angiogenesis, unlike their *in vivo* counterparts that model angiogenesis as a whole. *In vitro* angiogenesis models provide an alternative to using animals for testing. They are also less expensive than *in vivo* models, especially when it comes to performing experiments on a large scale [16]. They are easily quantifiable, but interpretation of results obtained should be done with caution. *In vitro* models provide initial information and are subject to validation by

using *in vivo* models, since they represent the exact angiogenesis environment. Also more than one *in vivo* model should be used to confirm in vitro results, due to variations that exist in species or animals used for testing, microenvironments, organ sites, and method of applying the test substances [122].

2.5 Design of a Novel Three Dimensional Vascular Tissue Model

The lack of suitable 3D angiogenesis tissue models that closely mimic physiological conditions useful for studying anti-angiogenic drugs led to the development of a novel 3D tissue model. The novel design consists of endothelial cells cultured on bovine type 1 collagen in a Transwell® permeable support. These supports/inserts are positioned in a 24-well tissue culture plate. They have been used to develop 3D models that would determine migratory or invasive response of endothelial cells to an angiogenesis stimulating or inhibiting factor [123]. Endothelial cells respond to angiogenic factors along a gradient through a process called chemotaxis. Chemotaxis as seen in figure 2.7 below is defined as cell movement towards an extracellular gradient of a bioactive molecules, such as a chemokine or ligand [124].

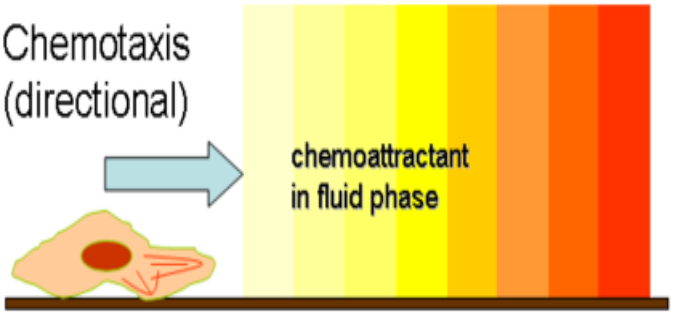
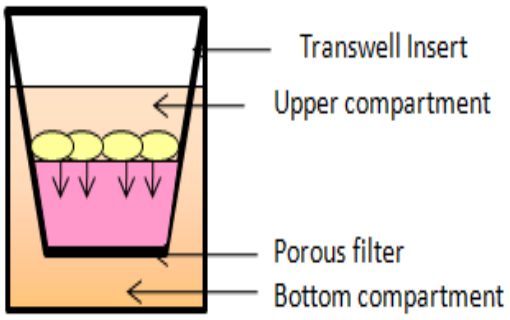


Figure 2.7 Chemotaxis direction of a cell [125]

The Transwell® insert divides the system into two compartments. A porous filter located at the bottom, as can be seen in figure 2.8 below, separates the upper compartment from the lower compartment. The membrane pores are of defined diameter with 3.0 μm selected for the use of endothelial cells. This pore size allows cell migration and diffusion of chemoattractants through the filters to the extracellular matrix, but prevents endothelial cells from migrating through the membrane. The use of a porous filter in a Transwell® assay was first identified by Stephen Boyden, who studied the chemotactic activity of ploymophonuclear leucocytes [126]. The cells were placed in the upper compartment, while the chemoattractant was placed in the lower compartment to induce migration of leukocytes. This Transwell® assay is known as the Boyden chamber or modified Boyden chamber assay and is the most popular assay used for studying cell migration. The main advantage of using this Transwell® is because of the sensitivity to small changes in concentration gradients [127].

The novel 3D system mimics an environment close to physiological conditions. As shown in figure 2.9, blood vessels within the eye are located at the back of the eye. When external agents (drugs) for treating diabetic retinopathy are added from the front they diffuse through the extracellular matrix towards the blood vessels to hinder proliferation and differentiation of endothelial cells. The novel 3D *in vitro* design provides ports at the sides for easy delivery of the anti-angiogenic drug to the bottom compartment. The drug then diffuses through the matrix towards the endothelial cells. Thus, this simulates the *in vivo* environment within the eye and can be used for studying an anti-angiogenesis drug.



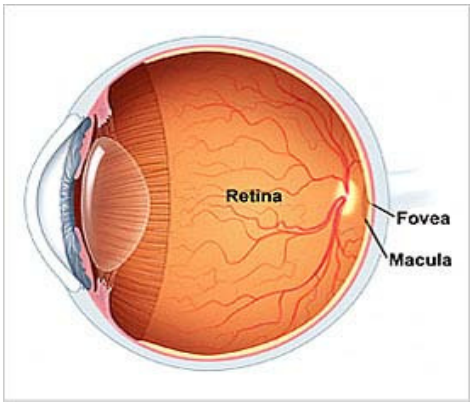


Figure 2.8 Transwell Permeable Support **Figure 2.9 Inside the Human Eye [128]**

PROJECT OBJECTIVES

1. Develop a 3D vascular tissue model to study angiogenesis
2. Study the effect of an anti-angiogenesis drug on endothelial cells in the model
3. Develop a novel 3D angiogenesis tissue model
4. Determine the effect of vascular endothelial growth factor concentration added to the 3D vascular tissue model on angiogenesis
5. Determine the effect of collagen thickness in the 3D vascular tissue model on angiogenesis

CHAPTER 3

METHODOLOGY

3.1 Materials

Human umbilical vein endothelial cells (HUVECs) were purchased from PromCell (Heidelberg, Germany); bovine type 1 collagen was purchased from Advanced BioMatrix (Tucson, Az); fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT); 1X and 10X medium 199, penicillin-streptomycin-glutamine (PSG), trypsin-EDTA and dulbecco's phosphate buffered solution (DPBS) were purchased from Invitrogen Corporation (Carlsbad, CA); human fibronectin was purchased from Biomedical Technologies Inc. (Stoughton,MA); Cell Titer Blue cell viability assay was purchased from Promega Corporation (Madison, WI); vascular endothelial growth factor was purchased from R&D Systems (Minneapolis, MN); 24-well transwell® permeable supports were purchased Corning Incorporated Life Sciences (Lowell, MA); human VEGF ELISA development kit was purchased from Preprotech (Rocky Hill, NJ); Tween 20 and albumin bovine serum (BSA) were purchased from Sigma-Aldrich (St. Louis, MO); ELISA microplates were purchased from Greiner Bio-One (Monroe, NC).

3.2 Cell Culture

HUVECs were cultured in 1X Medium 199 supplemented with 1% PSG and 20% FBS. The cells were grown in a T-75 flask in a humidified 5% carbon dioxide (CO_2), 95% air incubator at 37ºC (standard conditions). These conditions were used for all experiments. The cell culture media was changed every other day until HUVECs were 95 -100% confluent. The time period for HUVECs to become confluent was approximately seven days.

3.3 Development of a Three Dimensional Vascular Tissue Model to Test the Effect of an Anti-Angiogenesis Drug

The 3D vascular tissue model consisted of HUVECs grown on a 3D collagen matrix within a 96 well solid plate. The collagen gel was prepared by mixing 8 parts bovine type 1 collagen (3 mg/ml), 1 part 10x Medium 199 and 5 parts 0.1 M sodium hydroxide in a centrifuge tube. To a single well, 50 µl of the prepared collagen solution was added and incubated for an hour for the solution to gel. To the top of each collagen gel, 100 µl of complete media containing 20% FBS and 1% PSG was added and incubated at standard conditions from 4-24 hrs for the system to attain equilibrium. The HUVECs were seeded on the collagen gel at a density of 100,000 cells/cm² and incubated at standard conditions. The complete media was changed the next day and then every other day until the cells were 95 % to 100 % confluent. The cells became confluent on collagen gels in about five to seven days. Experiments were carried out in triplicate samples. Figure 3.1 shows a diagram describing the development of the 3D tissue model.

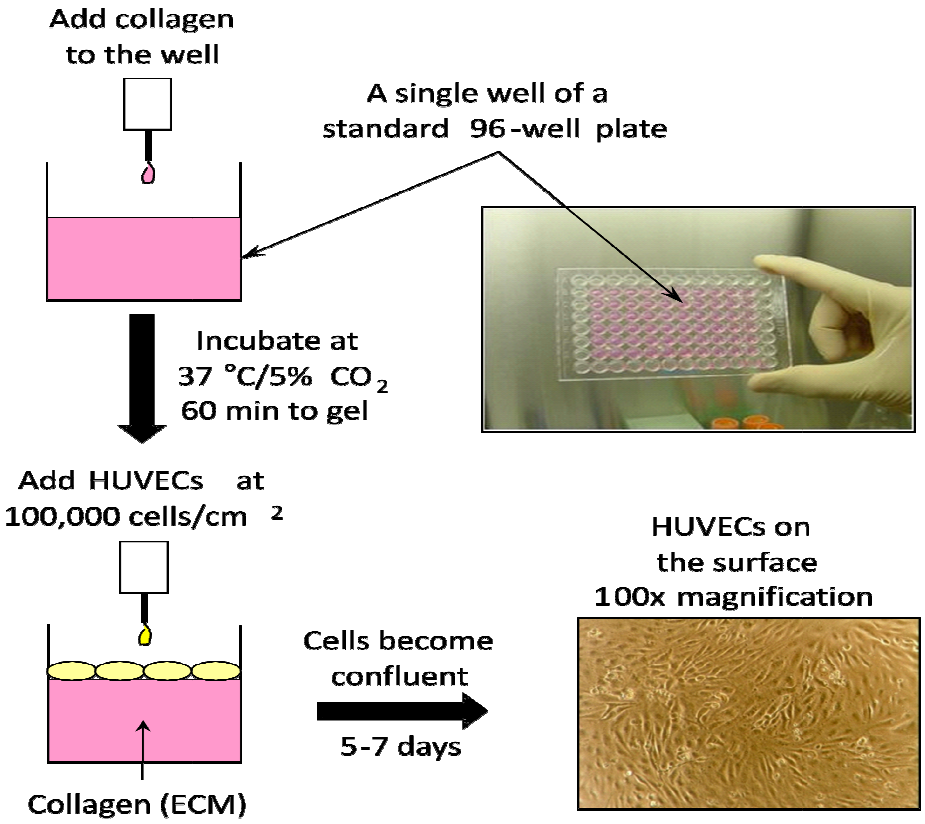


Figure 3.1 3D vascular tissue model development in a 96 well solid plate

The tissue model was used to test the effect of the anti-angiogenesis drug called CLT-003, a thalidomide analogue. To examine the effect of the drug on angiogenesis within the tissue model, cell viability (ability of cells to survive), proliferation (rapid increase in cell number), migration (distance travelled by cells into the collagen) and number of sprouts were analyzed. Various methods were used to evaluate the cellular functions associated with angiogenesis, as described in sections 3.3.1 and 3.3.2 below.

3.3.1 Cell Titer-Blue Viability Assay

This assay was used to measure viability and proliferation of HUVECs at different time points. The Cell Titer Blue viability assay estimates the number of viable cells present in each well by using an indicator dye resazurin, present in a buffered solution, to measure the metabolic capacity of cells. The viable cells reduce resazurin into resorufin, which is highly fluorescent, but the non-viable cells do not reduce the dye and thus give off no fluorescent signal. The reagent is added directly to each well. It is initially dark blue in color and has minimum fluorescence until it changes to a pink color. This indicates that resazurin has been reduced to resorufin, which is highly fluorescence, giving it a pink color. The signal could be measured by either fluorescence or absorbance. Fluorescence is the preferred method used as a result of its higher sensitivity and the fewer calculations involved. Figure 3.2 shows an overview of the cell titer blue viability assay from the first steps of adding the reagent to the final step of measuring the fluorescence signal of each sample.

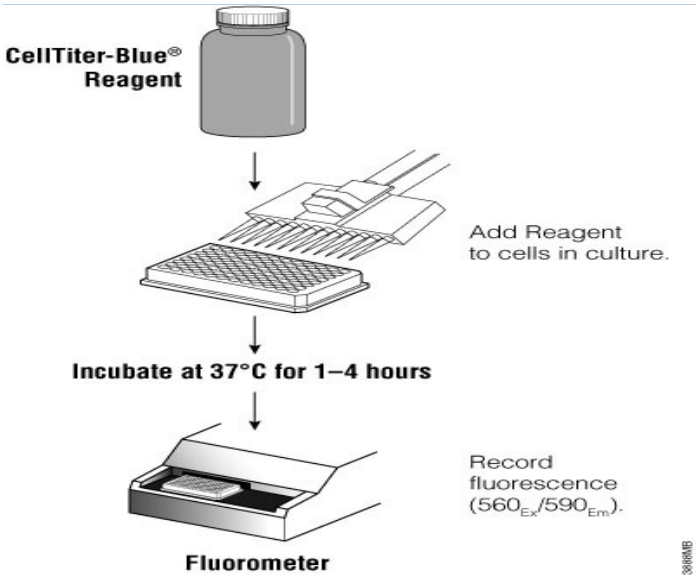


Figure 3.2 Cell Titer-Blue Cell Viability Assay [129]

In the 3D tissue model, 20 µl of reagent was added (at specified time points) directly to the cells in the culture media and incubated for four hours, after which the fluorescence signal was measured using a fluorometer. The fluorescence was measured at excitation 560 nm and emission 590 nm. The fluorescence signal measured was proportional to the number of viable cells. The number of cells were calculated by using a quadratic relationship that exists with measured fluorescence, determined by measuring standards. Standards were used to relate the number of cells per well to the measured fluorescence signal. They were prepared by seeding HUVECs at different seeding densities on collagen and incubating them for four hours. The reagent was added to these samples, and the fluorescence was measured using the spectrophotometer. A graph of the fluorescence signal for the number of cells per well was plotted and the number of cells per well was calculated from the quadratic equation shown in figure 3.3 below.

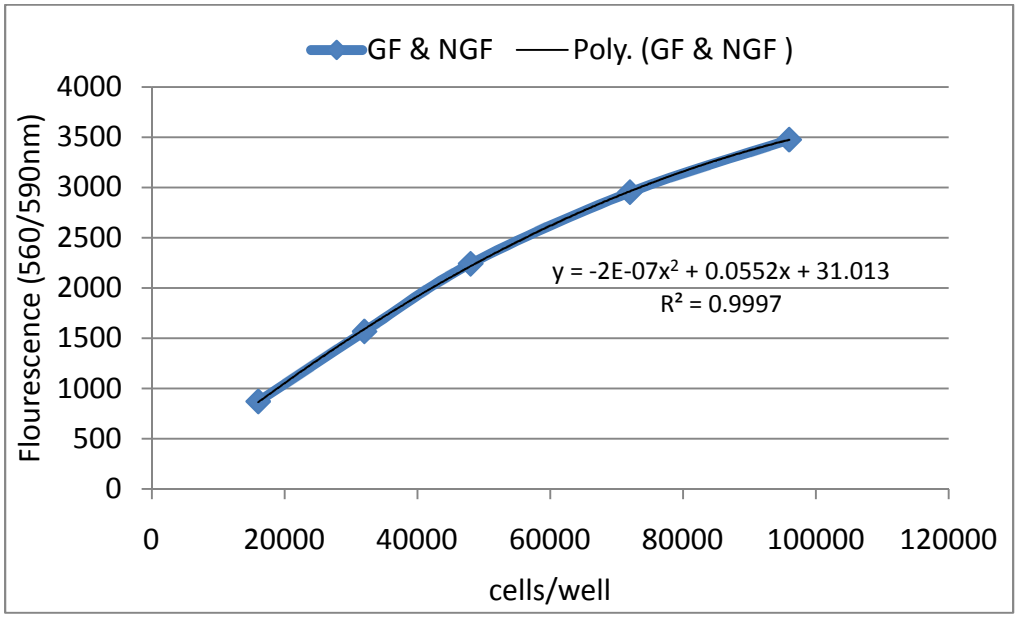


Figure 3.3 Fluorescence measured for a known number of cells after the Cell Titer Blue assay.

3.3.2 Microscopic Evaluation of Migration and Number of Sprouts

A sprout is a branch or a shoot from a growing cell. Number of sprouts were measured by visually counting by using a phase contrast inverted microscope and a cell counter. Sprouts were counted for each well at 100x magnification. Since samples were done in triplicates, the average and the standard deviations were calculated for number of sprouts counted in each well. Below is a picture showing an example of sprouts at 100x, 250x and 400x magnifications, respectively.

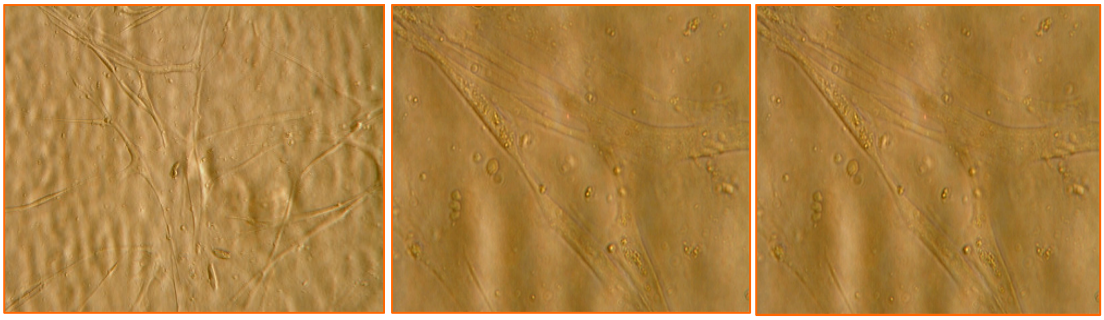


Figure 3.4 Sprouts in the 3D vascular tissue model at 100x, 250x and 400x magnification

Cell migration was measured by calculating the depth of migration from the surface of the collagen into the matrix as shown in figure 3.5. A glass cover slip with a known thickness was used as a standard to calculate the actual cell migration distance. The thickness of the glass cover slip was measured to be 1 mm using a micrometer screw gauge. This thickness corresponds to 3.45 revolutions, using the fine-focus knob of the microscope. By using the standard measurements, the depth of cell migration was determined and the average and standard deviation calculated from triplicate samples.

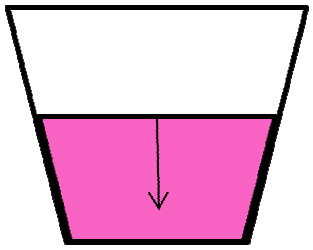


Figure 3.5 Measuring the depth of migration in a single well

3.3.3 Effect of Thalidomide Analogue on Endothelial Cell Viability, Proliferation, Number of Sprouts, and Migration in Growth Factor Model.

Thalidomide analogue, CLT-003 was initially tested in a growth factor model. This model consists of HUVECs grown on collagen gels in a 96 well solid plate, as described in section 3.3 above with the exception of the culture media used was called a Growth Factor Media. The growth factor media contained M199 with 20% FBS, 1% PSG and a growth factor cocktail with the following: 40 ng/ml VEGF, 40 ng/ml bFGF, 50 ng/ml phorbol ester, and 50 µg/ml ascorbic acid. The model was called the growth factor model because of the presence of this growth factor cocktail. In this model, the effect of two concentrations of CLT-003, 32 µM and 64 µM, was tested on cell viability, proliferation, migration and number of sprouts. The wells that had the drug concentrations added to the cells were called "with CLT-003". The control group for this experiment consisted of wells with no drug added to the cells. The control samples were called "without CLT-003". When endothelial cells had attained 100% confluence, within 24 hours the media was changed to growth factor media containing CLT-003. For the control group, the growth factor media was replaced with fresh growth factor media. This day was called day 0. The drug was initially added for a final concentration of 32µM

in the media. Thereafter the growth factor media was changed to fresh growth factor media without drug on all samples on Days 1, 3, and 7 after adding the drug. On day 9, CLT-003 was added again to the test group at twice the initial concentration level, final concentration of 64 μM in the media, and the growth factor media was changed on day 10 and 16. The control group was replaced with fresh growth factor media for the same time points. Separate samples were prepared for the viability and proliferation tests and for the migration and number of sprouts tests. Viability and proliferation tests were carried out on Days 1, 3, 7, 10 and 16. Number of sprouts and migration measurements were carried out on Days 2, 3, 6, 9, 10, 13, and 16. Analyses were performed before the fresh growth factor media and drug media were added to the samples. At the required time point, the viability and proliferation, and migration and number of sprouts were analyzed as described in sections 3.3.1 and 3.3.2 using the cell titer blue viability assay and microscopic evaluation, respectively.

3.3.4 Effect of Thalidomide Analogue on Endothelial Cell Viability, Proliferation, Number of sprouts and Migration in Normal Model.

The second model was called the Normal Model due to the absence of a growth factor cocktail. It was made up of cells cultured in M199 with 20% FBS and 1% PSG. The effect of the two drug concentrations, 32 μM and 64 μM was also tested on cell viability, proliferation, migration and number of sprouts within this model. However, the two drug concentrations were tested separately and only applied to the samples once. As before, the control group for this experiment was samples with media containing no drug. When cells were 95% to 100% confluent, within 24 hours the media were removed from

the samples and fresh media containing either 32 µM or 64 µM of CLT-003 for the test samples or no drug for the control samples were added. This day was called day 0. The media was changed on all samples to fresh media on days 1, 4, 7, 10, and 13. Separate samples were prepared for cell viability and proliferation samples and for number of sprouts samples. These tests were carried out as described in section 3.3.1 and 3.3.2 on days 1, 3, 7, 10 and 14 days after adding CLT-003.

3.4 Characterization of Novel 3D Angiogenesis Tissue Model

A novel 3D angiogenesis model that better mimics physiological conditions than the previous models was developed. It consists of HUVECs grown on collagen gels in Transwell® permeable supports/inserts. These inserts are placed in each well of a 24 well plate. Each insert has a microporous membrane located at the bottom of the insert. The membrane is 6.5 mm in diameter, has a pore size of 3.0 µm, and an insert membrane growth area of 0.33 cm^2. The Transwell® supports are made from polycarbonate and have a nominal membrane thickness of 10 µm and 2 x 10^6 pores/cm^2 nominal pore density. The Transwell® inserts divide the model into two compartments, referred to as the upper and the lower compartments. This format provides access to the top and bottom surface of the collagen matrix for easy delivery of factors, which makes it more advantageous than previous models where factors could only be delivered to the top surface of the matrix. Figure 3.6 shows the Transwell® membrane wells and the process for making the 3D vascular tissue model. Collagen solution (8 parts bovine type 1 collagen (3 mg/ml), 1 part 10x Medium 199 and 5 parts 0.1 M sodium hydroxide) is added to the inserts at 50 µl volume and incubated for one hour at standard conditions to

gel. Complete media, containing 10% FBS and 1% PSG was added to the top of the collagen in each insert and to the bottom chamber below the membrane. The volumes added were 100 µl to the top and 650 µl to the bottom compartment. The addition of these volumes resulted in equal liquid levels for inside and outside the well, in order to establish equilibrium. Then the inserts were incubated for 4-24 hrs prior to cell seeding. Afterwards, HUVECs were seeded on collagen gels at a seeding density of 100,000 cell/cm². The complete media was changed in both compartments every two days until cells were about 95% to 100% confluent. The cells became confluent in about three to five days. The model was characterized by employing changes in collagen thickness and VEGF concentration added to the wells.

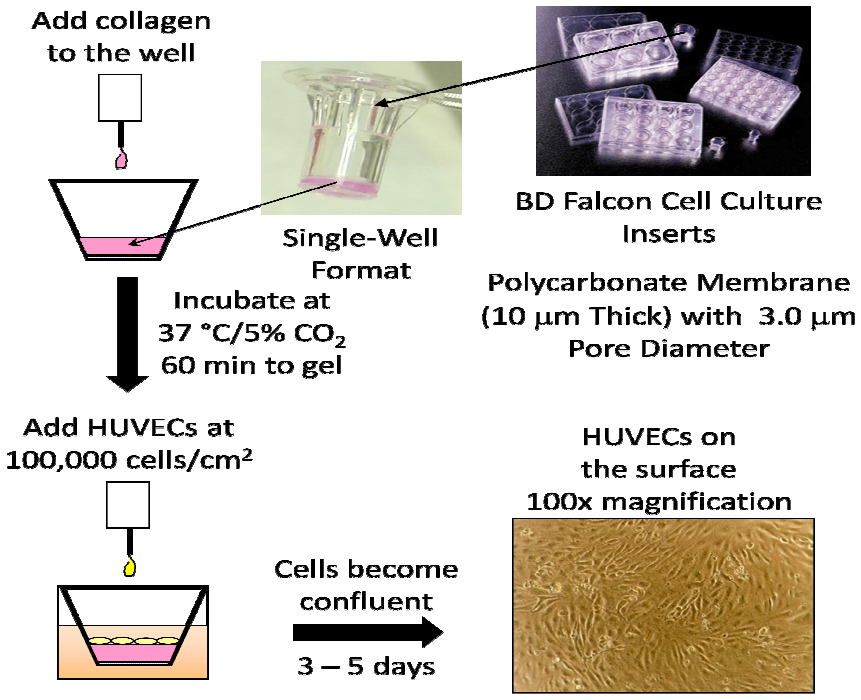


Figure 3.6 Development of a novel 3D angiogenesis tissue model on a Transwell® permeable support

3.4.1 Effect of Vascular Endothelial Growth Factor and Collagen Thickness on Endothelial Cell Viability, Proliferation, Number of Sprouts and Migration

Changes in VEGF concentration and collagen thickness were the two design variables chosen to characterize the new 3D tissue model. VEGF concentration was varied at 5 ng/ml, 50 ng/ml and 100 ng/ml, and collagen thickness was varied at 0.73 mm, 2.01 mm and 4.28 mm. The effect of VEGF concentration added to the model and changes in collagen thickness on endothelial cell viability, proliferation, number of sprouts and migration was studied.

To test the two design variables, for a single experiment, collagen thickness was held constant and VEGF concentration was varied. A control group containing no addition of VEGF was used to normalize the samples, in order to compare them to other experiments with a different collagen thickness. In more detail, the 3D vascular tissue model was constructed by setting the collagen thickness. Collagen thickness was set by controlling the volume of collagen solution added to each well. For the first experiment, 50 μl of collagen solution was added to each well, which resulted in a collagen gel thickness of 0.73 μm. To the top compartment, 100 μl of complete media was added and to the bottom compartment 650 μl of complete media containing either 0, 5, 50, or 100 ng/ml of VEGF was added to their respective wells. VEGF was added to the bottom chamber in order to simulate physiological conditions. In vivo, VEGF is released by cells into the surrounding ECM, which leads to concentration gradients that can stimulate cell proliferation and directional cell sprouting and migration. Delivery of VEGF to the bottom chamber can lead to concentration gradients within the collagen matrix that would stimulate cell proliferation, migration, and sprouting from the HUVES on the top surface.

The plates were incubated and the media was changed with fresh complete media in the top compartment and fresh VEGF growth factor media in the bottom compartment of the plates in their respective wells on Days 3, 6, 9, 12, and 15 post seeding. Prior to media changes on these days, viability and proliferation, number of sprouts, and migration analyses were performed. Viability and proliferation analysis was performed on one set of tissue model samples and migration and number of sprouts analyses were performed on another set of tissue model samples, all following the same experimental conditions. The experiment was repeated for the next two collagen thicknesses of 100 µl and 150 µl collagen solution added to each well, which resulted in 2.01 mm and 4.28 mm collagen gel thicknesses, respectively.

For this model, viability and proliferation was also measured using the cell titer-blue viability assay. Due to the porous membrane at the bottom of the insert, the protocol had to be modified. If the reagent was added to the top of the wells, it would leak through the pores and be diluted in the solution in the bottom compartment. In order to prevent the reagent from leaving the top compartment, parafilm was gently wrapped round the lower portion of the insert and a rubber ring was attached onto this lower portion as shown in figure 3.7 below. The parafilm and the rubber stop were able to hold the reagent for the required analysis time.

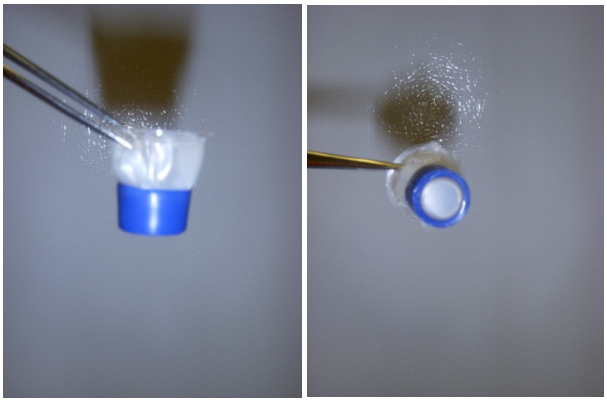


Figure 3.7 Front and bottom view of the membrane insert

At each time point, the culture medium in the top and bottom compartments was aspirated for the wells to be analyzed. Next, the parafilm and rubber ring were attached to the insert as described above. Then 100 µl of fresh complete media was added to the top of the collagen followed by 20 µl of cell titer blue reagent. The plates were placed inside the incubator for four hours. At the end of the incubation period, the reagent in the complete media on top of the gel was mixed gently using a micro pipette and 100 µl of sample was transferred from each well into assigned wells in a 96 well plate. The fluorescence signal for each well was measured using the spectrophotometer. Cell migration and number of sprouts were analyzed as described in sections 3.3.2.

3.5 Statistical Analysis

Data is expressed as mean ± standard deviation for n = 3 samples. Data was analyzed by use of a student's t-test to determine the significance of any difference between the groups tested and the control. A value of $p < 0.05$ was considered significant. Sample measurements were normalized based on the 0 ng/ml VEGF concentration control group.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Development of a Three Dimensional Vascular Tissue Model to Test the Effect of an Anti-Angiogenesis Drug

4.1.1 Effect of thalidomide analogue on viability, proliferation, number of sprouts and migration in the Growth Factor Model

The aim of this study was to develop a three dimensional vascular tissue model, which can be used to study the effect of a novel anti-angiogenesis drug called CLT-003, a thalidomide analogue, on angiogenesis. The 3D *in vitro* model was made up of HUVECs grown on a bovine type 1 collagen matrix in a 96 well solid plate using a growth factor cocktail as the stimulant. HUVECs viability, proliferation, number of sprouts and migration was measured within the vascular tissue model in order to determine the effect of CLT-003 on angiogenesis. Collagen was the extracellular matrix (ECM) selected because it is the most abundant protein in the ECM. Out of the 25 different collagen molecules present, type 1 is the most widely distributed in the body; it accounts for 90% of the collagen in the human body. In addition, it provides the chemical and physical cues required to stimulate morphogenesis and many cell functions [130]. Angiogenic growth factors are essential for promoting the angiogenic events that occur during the angiogenesis process. For this model, the growth factor cocktail based on Davis and

Camarillo's [131] work was chosen to stimulate proliferation and differentiation of endothelial cells. The growth factor cocktail consists of 40 ng/ml VEGF, 40 ng/ml bFGF, 50 ng/ml phorbol ester, and 50 µg/ml ascorbic acid.

Due to the use of a growth factor cocktail, this model is referred to as the Growth Factor model. In this model, the effect of CLT-003 at two concentrations, 32 µM and 64 µM, on endothelial cell viability, proliferation, number of sprouts and migration was tested. When endothelial cells were 100% confluent, the drug was added at 32 µM and the time considered as Day 0. Cell viability and proliferation was measured using the Cell Titer Blue viability assay on Days 1, 3, and 7. It was observed that at the 32 µM concentration of CLT-003, there was no significant change in the angiogenesis mechanisms, thus the drug dosage was doubled and 64 µM was added on Day 9. Viability and proliferation was measured on Days 10 and 16. During the experimental period, the media containing the growth factor cocktail was changed on the same day when viability and proliferation measurements were carried out, on Days 1, 3, 7, 10, and 16. Figure 4.1 shows the effect of the drug on cell viability and proliferation for the various time points. Samples without the addition of CLT-003 served as the control group.

When CLT-003 was added at 32 µM on Day 0, there was a slight decrease in the number of cells per well from Days 1 to 3 of approximately 125,000 cells per well on Day 1 to 115,000 cells per well on Day 3. The control samples on these two days were observed to increase from approximately 154,000 cells per well on Day 1 to 164,000 cells per well on Day 3. This was an indication of the early effects of the drug in reducing cell

viability. The decrease in the number of viable cells per well was significant for the CLT-003 group compared to the control group, but the decrease in the number of viable cells per well for the CLT-003 from Day 1 to Day 3 was not significant.

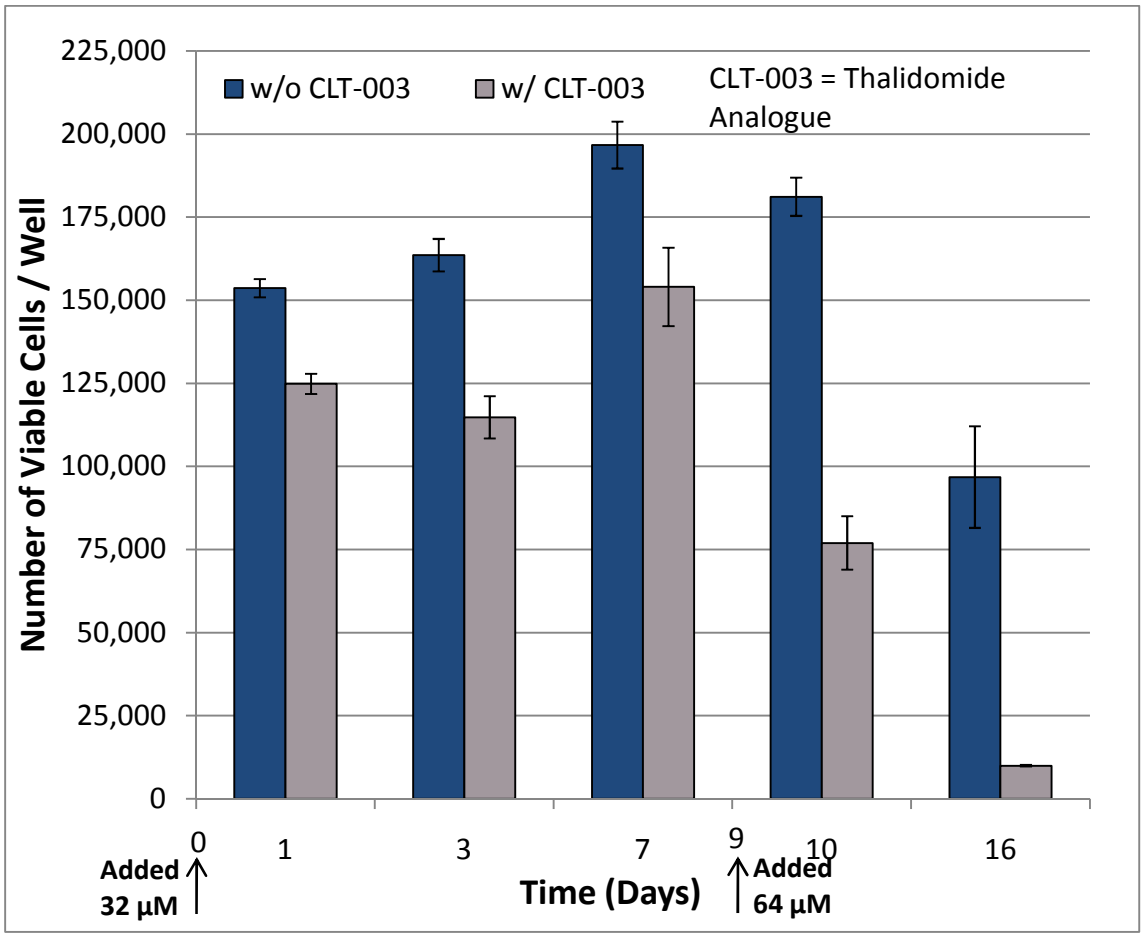


Figure 4.1 Effect of CLT-003 on cell viability and proliferation in the Growth Factor Model. Data shown are mean values ± sd; n=3. The samples with CLT-003 showed a significant decrease in the average number of viable cells per well compared to the samples without CLT-003 ($p<0.05$).

From Day 3 to 7, it can be seen that the number of viable cells increased significantly from approximately 115,000 cells per well to 154,000 cells per well. However, the increase was not as great as compared to the control, which also had an increase of approximately 164,000 cells per well to 197,000 cells per well. This increase does not reflect the normal mechanism of the thalidomide analogues in hindering viability and proliferation of endothelial cells *in vitro* [66]. Therefore, it was concluded that the 32 μM CLT-003 concentration reduced endothelial cell viability and proliferation initially, but the cells started to recover and cell viability and proliferation increased. When the drug concentration added to the tissue model was increased to 64 μM, cell viability and proliferation was significantly reduced, compared to the samples with only 32 μM of the drug and the control. There was a significant decrease from approximately 154,000 cells per well on Day 7 to 10,000 cells per well on Day 16. These results signify that a higher thalidomide concentration produces a greater effect in reducing endothelial cell viability and proliferation. The observed decrease on Day 16 may not be completely attributed to the increase in drug concentration, since the viability of the control samples decreased as well. A significant decrease in number of viable cells per well was also observed for the control samples on Day 10 and Day 16 from approximately 180,000 cells to 97,000 cells per well respectively. This may show that the cells are only viable in this type of culture system and conditions for 16 days. Therefore, the effects of the drug should be tested when the cells are fully viable, within the 16 day limit. It was concluded that CLT-003 at 64 μM had a greater effect on viability and proliferation of endothelial than CLT-003 at 32 μM.

The effect of CLT-003 on endothelial cell sprouting and migration was also studied within the collagen matrix in the model. Figures 4.2 and 4.3 show the average number of sprouts and depth of cell migration with time for samples with and without the drug added. Samples without CLT-003 served as the control. Both the number of sprouts and cell migration were measured microscopically. Cell migration was measured as the distance the cells migrated from the surface of the collagen down into the collagen matrix. The drug was added to the samples at 32 μM on Day 0 and 64 μM was added on Day 9. Cell sprouting and migration measurements were carried out on Days 2, 3, 6, 9, 10, 13, and 16. As shown in figure 4.2, the number of sprouts was observed to increase with time for the control samples. There was an increase of approximately 80, 120, 180, 300, 360, 410, and 600 sprouts per well on Days 2, 3, 6, 9, 10, 13, and 16 respectively. After the addition of 32 μM of CLT-003, no sprouts were observed on the Days 2 and 3 compared to the control sample. However, sprouts were observed on Days 6 and 9 with 29 and 53 sprouts, respectively. Adding CLT-003 at 64 μM on Day 9 removed existing sprouts and prevented cell sprouting for five days. Possible sprout recovery was observed on Day 16 with approximately two sprouts (not visible in the figure). Figure 4.3 shows a similar effect for the depth of cell migration. The control group samples showed enhanced migration of 0.37 mm, 0.35 mm, 0.61 mm, 0.62 mm, 0.60 mm, 0.68 mm, and 0.69 mm on Days 2, 3, 6, 9, 10, 13 and 16, respectively. No migration was observed after adding 32 μM CLT-003, until Days 6 and 9 with 0.35 mm and 0.40 mm migration depth, respectively. There was also significant cell migration of 0.29 mm on Day 16 after adding 64 μM CLT-003 on Day 9.

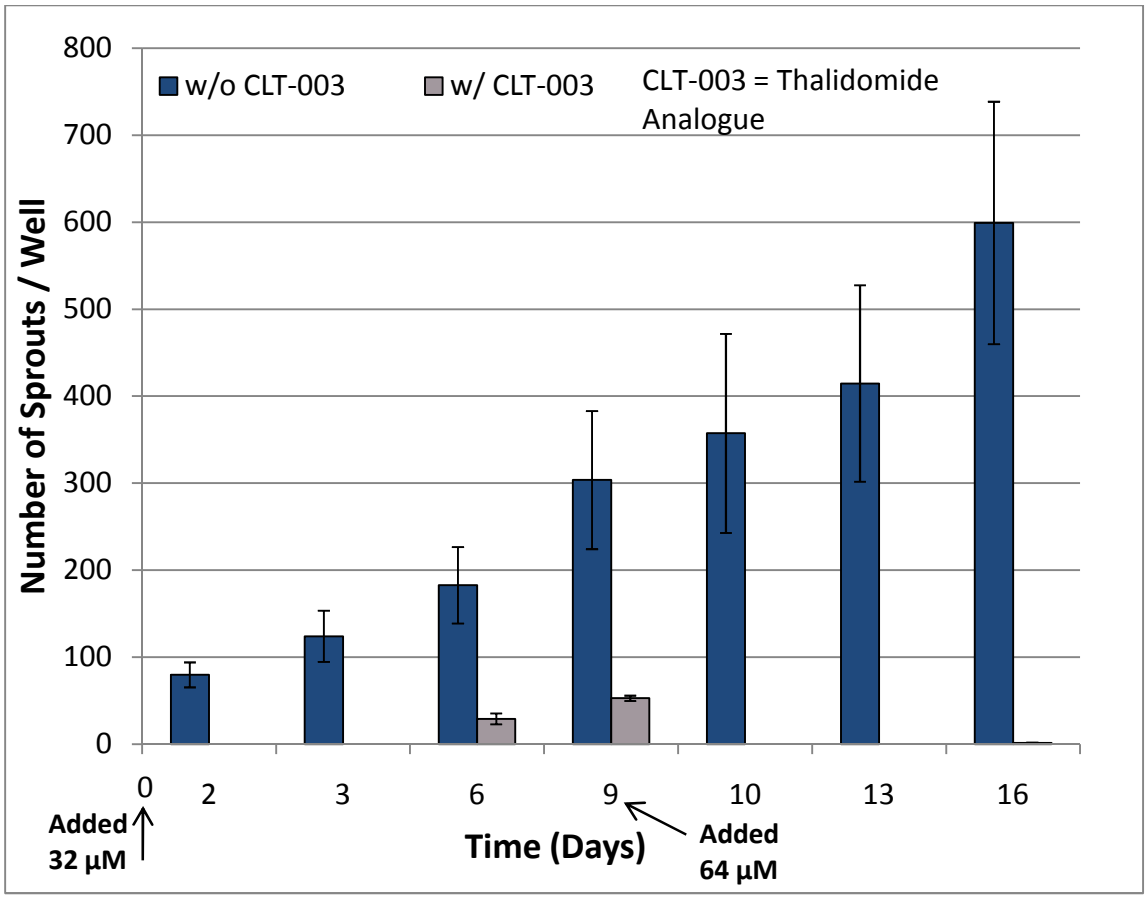


Figure 4.2 Effect of CLT-003 on the number of sprouts in the Growth Factor Model. Data shown are mean values ± sd; n=3. The samples with CLT-003 showed a significant decrease in the number of sprouts per well compared to the samples without CLT-003 ($p<0.05$).

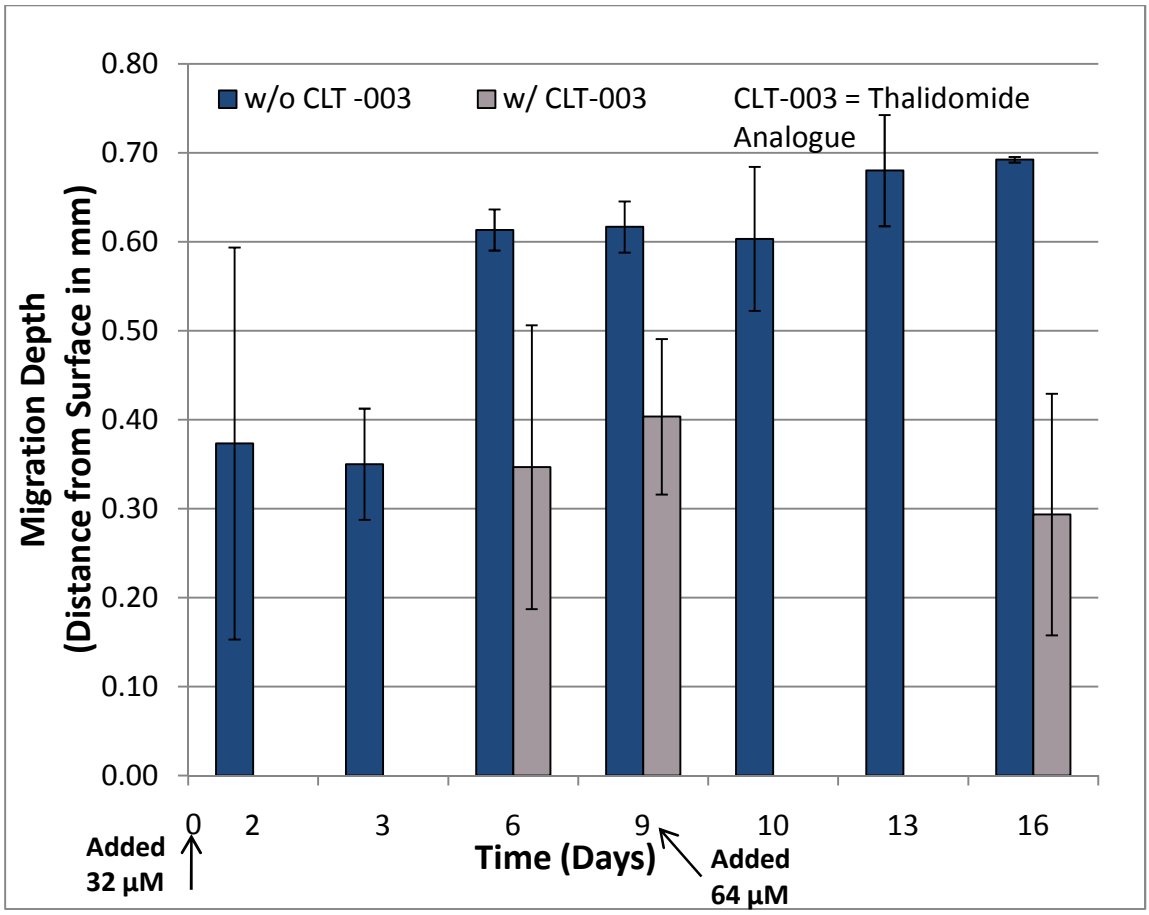


Figure 4.3 Effect of CLT-003 on cell migration in the Growth Factor Model. Data shown are mean values ± sd; n=3. The samples with CLT-003 showed a significant decrease in cell migration compared to the samples without CLT-003 ($p<0.05$).

The recovery observed for both cell sprouting and migration is believed to be the result of adding the growth factor media, which counteracted the normal mechanisms of the drug. It can also be observed that both cell sprouting and migration recovery occurred six days after adding the drug at the lower concentration and seven days after adding the drug at the higher drug concentration. This recovery shows that for this model, CLT-003 may no longer have an effect on cell sprouting and migration occurring after six or seven days. It

is possible that cell recovery in sprouts and migration may be described as a phenomenon known as 'secondary sprouting' where endothelial cells were observed to survive after tube collapse and migration from secondary sprouts occurs [132]. Comparing these results to those for viability and proliferation, it was observed that CLT-003 had a more significant effect on the number of sprouts and cell migration than viability and proliferation, compared to the respective control samples.

Based on the results, it was concluded that the growth factor model was not suitable for studying the effect of CLT-003 on angiogenesis within the tissue model because the growth factor cocktail induced endothelial cell recovery after adding either 32 μM or 64 μM of CLT-003. Since it is believed that the growth factor cocktail competes with the drug, another model was developed by taking the growth factor cocktail out of the system. This model was called the Normal Model.

4.1.2 Effect of thalidomide analogue on viability, proliferation and number of sprouts in the Normal Model

For the Normal Model, CLT-003 concentrations of 32 μM and 64 μM were added to separate samples on Day 0, in order to test the effect of the drug at various concentrations on angiogenesis within the model. Samples were analyzed on Days 1, 3, 7, 10 and 14 after adding the drug. The complete media was changed on Days 1, 4, 7, 10 and 13 after adding the drug. For sample analysis and media changes occurring on the same days, samples were taken for analysis prior to media changes. Samples without CLT-003 added served as the control. Figures 4.4 and 4.5 shows the effect of CLT-003

on endothelial cell viability and the number of sprouts for the two drug concentrations added to the Normal Model, respectively.

As shown in Figure 4.4, compared to the control, a significant decrease in viability was observed when 32 μM and 64 μM CLT-003 were added to the cells. The addition of the higher thalidomide concentration to the samples showed a more significant decrease in cell viability with time compared to the control than for the addition of the lower drug concentration. When comparing cell viability in the Normal Model compared to the Growth Factor Model, no cell recovery was observed, and the drug demonstrated its expected mechanism in reducing cell viability and proliferation. Comparing Day 1 of the two models after adding 32 μM CLT-003, the Normal Model had a more significant decrease in cell viability than the Growth Factor Model with a decrease of approximately 32% compared to 19%, respectively. The viability of the control samples in Figure 4.4 were observed to increase significantly from Day 1 to Day 10 with approximately 76,000 cells per well and 115,000 cells per well, respectively. However, a decrease was observed from Day 10 to Day 14 with approximately 115,000 cells per well and 77,000 cells per well, respectively. A similar effect was also seen in the control samples for the Growth Factor Model. This shows that cell viability and proliferation may be limited to approximately ten days for the solid well plate format either with or without growth factors.

To further demonstrate the effectiveness of the drug on angiogenesis in the model, the number of sprouts was also investigated, and the results are shown in Figure 4.5. After the drug was added, no sprouts were observed on the Day 1 and 3. Possible sprout recovery was observed from Days 7 to 14 for both CLT-003 concentrations. However,

due to the large standard deviation for these samples, the number of sprouts may be insignificant. Any possible recovery may be due to the regular change in complete media, which dilutes the drug within the system.

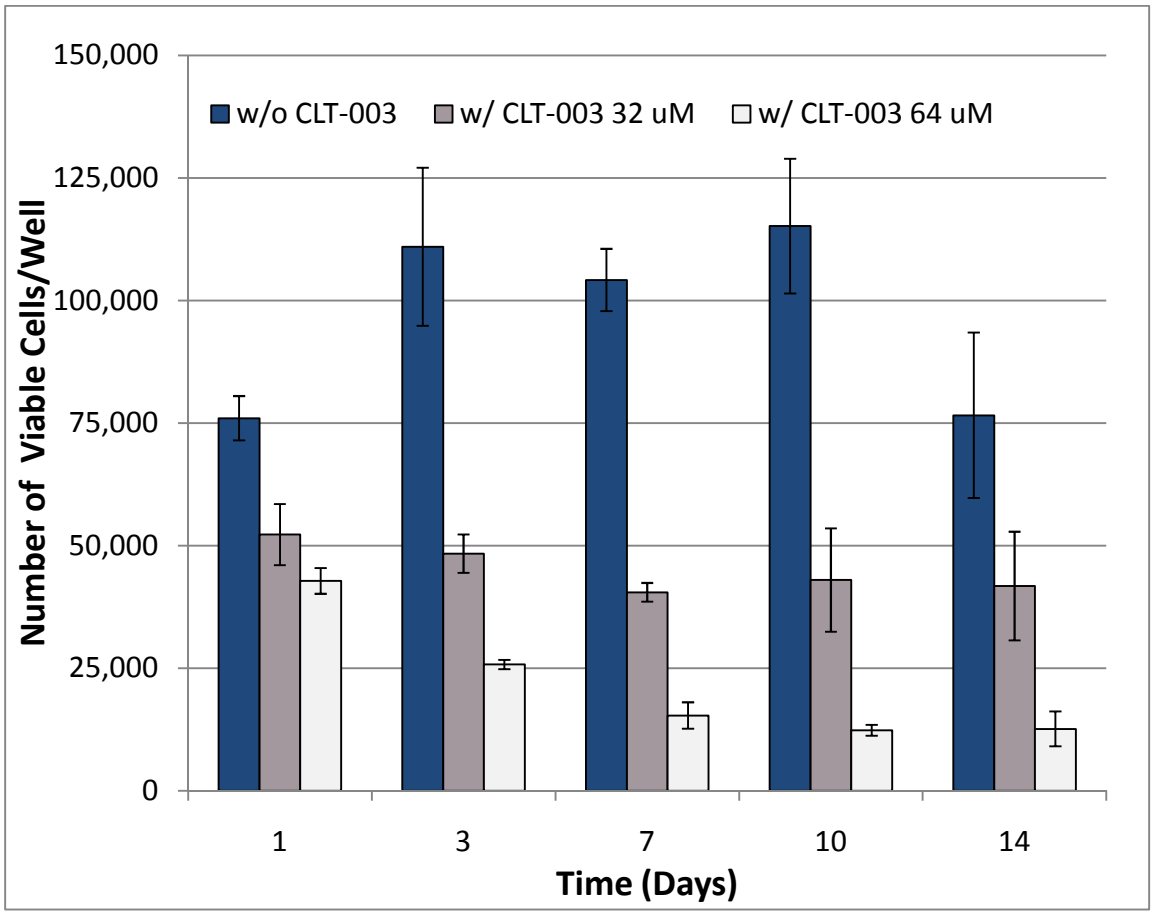


Figure 4.4 Effect of thalidomide analogue on viability and proliferation in the Normal Model. Data shown are mean values ± sd; n=3. The samples with CLT-003 showed a significant decrease in cell viability and proliferation compared to the samples without CLT-003 ($p<0.05$).

Complete media also naturally contains low levels of angiogenic factors, which stimulate cell growth and sprouting. The control samples in Figure 4.5 were observed to also increase with time from approximately 15 sprouts on the Day 1 to 53 sprouts on Day 14.

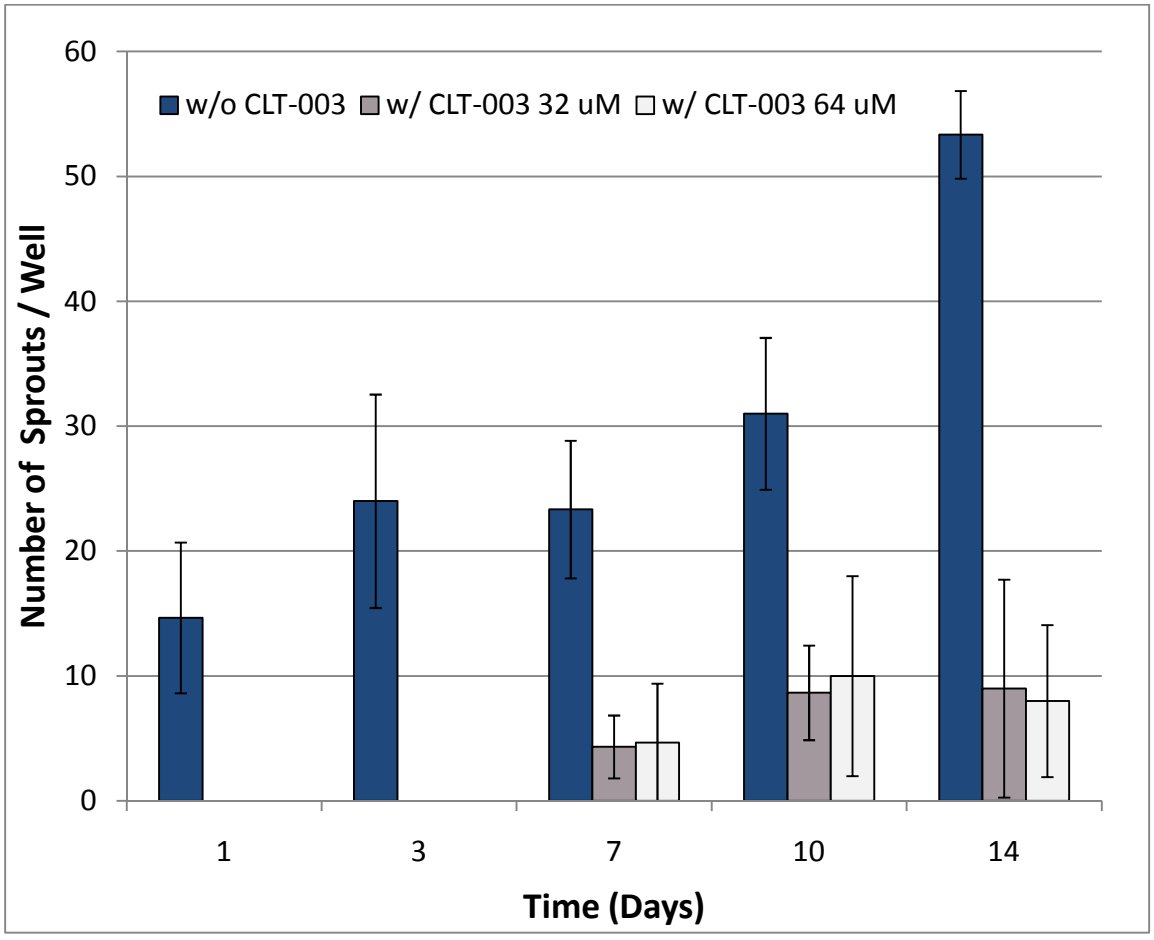


Figure 4.5 Effect of CLT-003 on the number of sprouts in the Normal Model. Data shown are mean values ± sd; n=3. The samples with CLT-003 showed a significant decrease in the number of sprouts compared to the samples without CLT-003 ($p<0.05$).

Without the presence of a growth factor cocktail, CLT-003 at both concentrations significantly reduced viability and proliferation of endothelial cells with time. The Normal Model also showed minimal sprout recovery compared to the Growth Factor Model. Thus from the overall study with CLT-003, it can be concluded that the Normal Model was more suitable for studying the effect of the drug on angiogenesis than the Growth Factor Model. Even though it was a better model, there was still a need to improve the viability of endothelial cells in the control samples for the model. It was also necessary to find an alternative method to deliver the drug to the tissue model that better mimics how the drug is administered to the eye. For drug delivery to the eye, the drug is delivered to the front of the eye and diffuses through the matrix to reach the blood vessels located at the back of the eye. In the solid well plate tissue models, the drug was administered directly to the cells, which corresponds to the drug being delivered directly to the blood vessels, and thus did not give an accurate description of what happens *in vivo*. A better representation of *in vivo* conditions would be delivery of the drug to the collagen matrix side, so the drug can diffuse through the collagen matrix and reach the endothelial cells on the other side. For this reason, a new model that would improve the viability of endothelial cells and allow for the delivery of factors to the matrix site was developed. It is proposed that this new system would be a better physiological model because it gives a better imitation of drug delivery to the eye and angiogenesis occurring within the eye than the other models. The two variables tested in the new model were the concentration of VEGF added to the cells and the collagen thickness. The improved model could then be used to study the effectiveness of CLT-003 and other therapeutic strategies on angiogenesis.

4.2 Characterization of a Novel 3D Angiogenesis Tissue Model.

When designing this model, changes in VEGF concentration and collagen thickness were tested as the design variables because they have been shown from literature and previous work to be important variables affecting the angiogenesis of endothelial cells within a 3D model. HUVECs were chosen for this study because of their use in previous experiments and other studies in literature. Membrane plates were used to develop the 3D model. Each well in the plate contains a permeable membrane insert/support that divides the well into two compartments. A microporous membrane located at the bottom of the insert separates the two compartments and allows passage of factors from the upper compartment to the lower compartment in the well. The membrane plates provide access to a basolateral compartment to deliver a VEGF and access to an apical compartment to deliver complete media in the absence of VEGF. Delivery of VEGF this way would provide easy delivery of nutrients to the cells and it would simulate the diffusion of VEGF through the matrix to reach its receptors located on the endothelial cells on the top surface, similar to as it occurs *in vivo*. The ability to deliver VEGF to the endothelial cells in this manner makes the membrane plate format more advantageous than the solid plate format.

VEGF is an endothelial cell-specific mitogen known to induce proliferation and differentiation of endothelial cells that occurs in angiogenesis. Therefore one of the aims of this study was to investigate the effect of VEGF on HUVECs viability, proliferation, number of sprouts and migration in the novel 3D angiogenesis tissue model. VEGF concentration was varied at 5 ng/ml, 50 ng/ml and 100 ng/ml in order to measure the effect of different levels of stimuli on endothelial cell angiogenesis within the 3D tissue

model. Viability, proliferation, number of sprouts and migration of HUVECs were measured on Days 3, 6, 9, 12, and 15 after the addition of VEGF. VEGF was not added to Non-Growth Factor (NGF) samples and served as the control group. The control group was cultured in media containing 10% FBS and 1% PSG. FBS is an essential requirement for cells, it enables cells to proliferate and live properly within an *in vitro* environment. However, it is not as effective as VEGF, which also stimulates sprouting and migration of endothelial cells.

Collagen is an essential structural protein in the ECM that supports endothelial cell growth and provides a medium for cell movement and interactions. The the second aim was to study the effect of collagen thickness on HUVECs viability, proliferation, number of sprouts and migration in the novel 3D angiogenesis tissue model. The collagen thickness in the tissue model mimics the distance between an endothelial cell and the source of stimulant, i.e. VEGF. The thickness also represents the diffusional distances a factor must travel to reach the cells, which results in local concentration gradients. Collagen thickness was varied at 0.73 mm, 2.01 mm, and 4.28 mm.

4.2.1 Effect of vascular endothelial growth factor on endothelial cell viability and proliferation

Figure 4.6 describes the percent difference in viability and proliferation of endothelial cells with time in response to increasing VEGF concentrations for the tissue model with a collagen thickness of 0.73 mm compared to the NGF control group. The percentage values above zero represents more viable cells compared to the NGF sample and percentage values below zero represent less viable cells compared to the NGF

sample. It can be observed that when 5 ng/ml VEGF was added to cells, it slightly increased viability and proliferation; however, these values were not significant compared to the control.

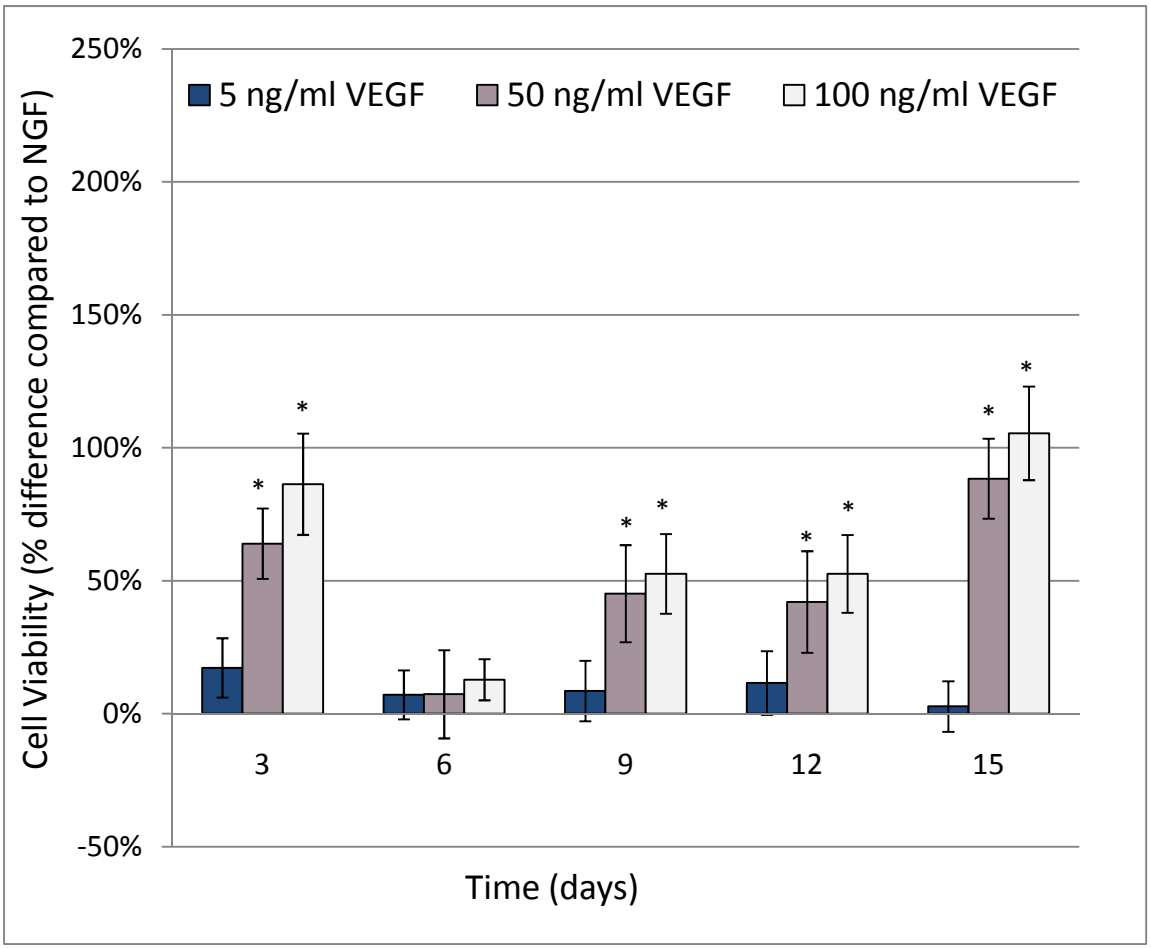


Figure 4.6 Effect of VEGF concentration on viability and proliferation in the tissue model with 0.73 mm collagen thickness. Data shown are mean values ± sd; n=3. *p<0.05 vs. Non-Growth Factor sample.

When 50 ng/ml VEGF was added, a significant increase in viability was observed on Days 3, 9, 12, and 15 with 65%, 45%, 42%, and 88%, respectively. A significant increase was also observed when cells were exposed to 100 ng/ml VEGF concentration on Days 3, 9, 12, and 15 with 86%, 53%, 53%, and 105%, respectively. These numbers indicate that

50 ng/ml and 100 ng/ml VEGF concentrations had a greater effect in stimulating cell viability and proliferation than 5 ng/ml compared to the control. However, it was also observed that 100 ng/ml did not significantly increase viability and proliferation compared to the 50 ng/ml. The results obtained for 0.73 mm collagen thickness suggests that VEGF stimulates viability and proliferation of endothelial cells with an increase in VEGF concentration.

Figure 4.7 describes the percent difference in viability and proliferation of endothelial cells when exposed to various concentrations of VEGF for the tissue model with 2.01 mm collagen thickness compared to the NGF control group. When the collagen thickness is 2.01 mm, 5 ng/ml VEGF slightly increased cell viability and proliferation; however this effect was not significant compared to the control. When 50 ng/ml of VEGF was added to the cells, this gave a significant increase of approximately 73%, 31%, 216%, 170% and 146% on Days 3, 6, 9, 12 and 15, respectively. Adding 100 ng/ml VEGF to the cells also resulted in a significant increase of approximately 76%, 187%, 194% and 156% on Days 3, 9, 12 and 15, respectively. This increase in cell viability and proliferation clearly suggests that 50 ng/ml and 100 ng/ml has the same effect on viability and proliferation in this collagen thickness as shown in the 0.73 mm collagen thickness samples. Also, both concentrations show approximately the same effect on endothelial cells for both collagen thicknesses tested. The reason the 100 ng/ml concentration does not show an increase in cell viability and proliferation compared to the 50 ng/ml concentration may be due to cell receptor saturation. For example, there is a maximum number of receptors on the cell surface to bind with VEGF and the 50 ng/ml VEGF concentration leads to enough VEGF in the system to bind with all of these receptors.

Therefore, the 100 ng/ml concentration leads to excess VEGF in the system that cannot bind with the receptors and does not lead to any further stimulation of the cells.

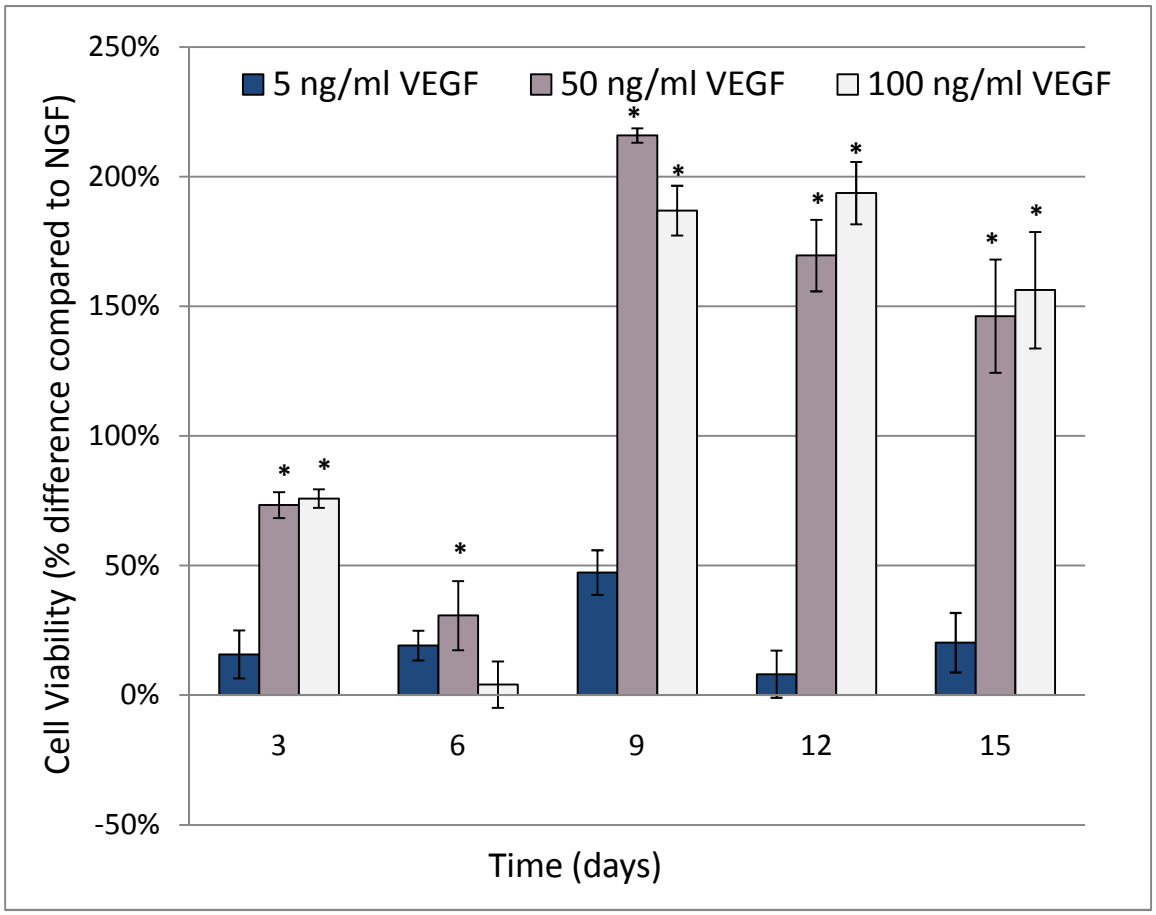


Figure 4.7 Effect of VEGF concentration on viability and proliferation in the tissue model with 2.01 mm collagen thickness. Data shown are mean values ± sd; n=3. *p<0.05 vs. Non-Growth Factor sample.

Figure 4.8 illustrates the percent difference in viability and proliferation of endothelial cells in response to increasing VEGF concentration for the tissue model with a collagen thickness of 4.28 mm compared to the NGF control group. The figure below

shows no significant increase in cell viability and proliferation for this collagen thickness, when compared to the NGF control group. Endothelial cell viability was observed to be less than 25% in this collagen thickness for each VEGF concentration, which is significantly lower when compared to the 2.01 mm and 0.73 mm collagen thicknesses with cell viability and proliferation of approximately 200% and 100%, respectively. The decrease in viability and proliferation could possibly be due to insufficient nutrients and/or oxygen supplied to the cells, leading to a relative decrease in viability and proliferation within the 4.28 mm collagen thickness. Thus, the results suggest that increasing VEGF concentration has a negligible effect on viability and proliferation in 4.28 mm collagen thickness.

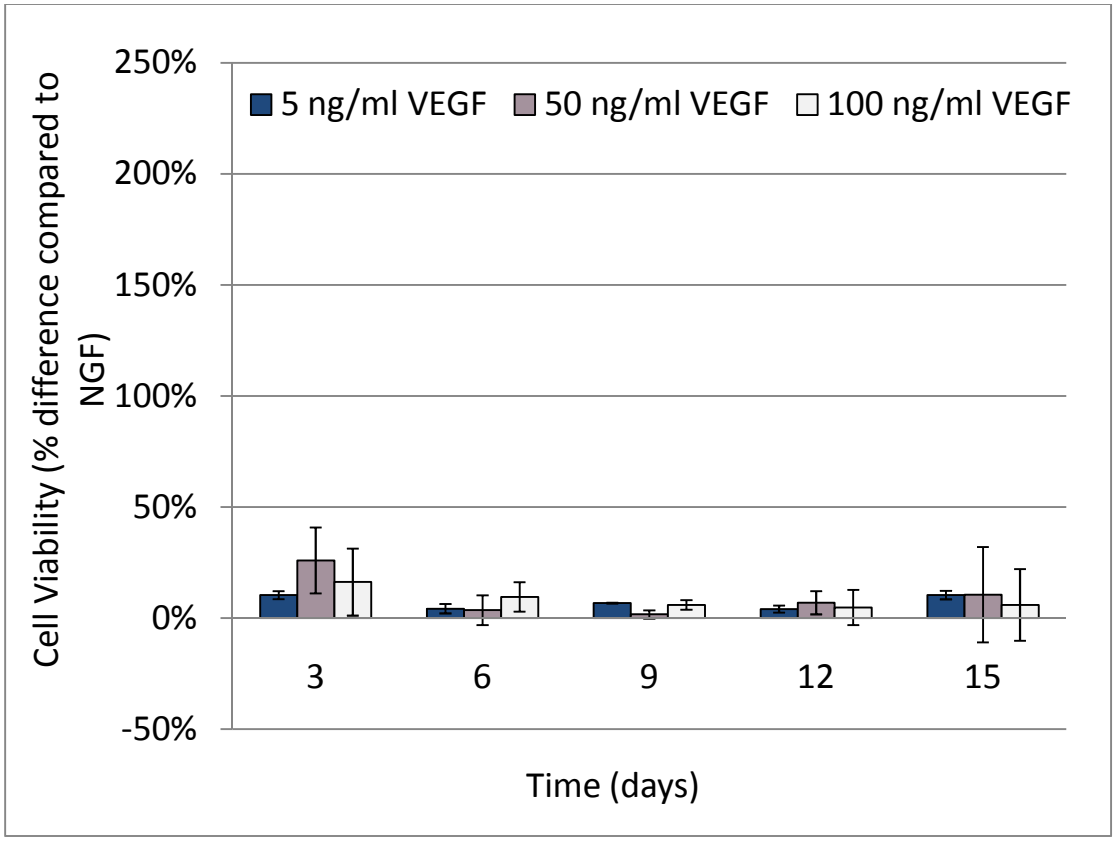


Figure 4.8 Effect of VEGF concentration on viability and proliferation in the tissue model with 4.28 mm collagen thickness. Data shown are mean values ± sd; n=3.

4.2.2 Effect of vascular endothelial growth factor on endothelial cell number of sprouts

The effect of VEGF concentration on the number of sprouts was also examined as shown in Figure 4.9 below. This graph illustrates the total number of sprouts counted per well in response to increasing VEGF concentration in the tissue model with 0.73 mm collagen thickness compared to the NGF control group (0 ng/ml VEGF). When 5 ng/ml of VEGF concentration was added to the cells, a slight increase in sprouts from endothelial cells was observed, but was not significant compared to the control. For the samples with 50 ng/ml VEGF added, there was a significant increase in sprout formation on Days 3 and 6 with approximately 17 and 23 sprouts, respectively. For the samples with 100 ng/ml VEGF added, there was a significant increase in sprout formation on Days 3, 6, and 12 with approximately 19, 11 and 14 sprouts, respectively. These results suggest that the number of sprouts from the cells increased when 50 ng/ml and 100 ng/ml VEGF concentrations were added to the cells, compared to the addition of the 5 ng/ml VEGF concentration. Each VEGF concentration was observed to increase cell sprouting on Day 9, however the samples show large standard deviations, which can be attributed to the high variability in analyzing the triplicate samples. A rapid increase in the number of sprouts was observed from Day 12 to 15 for the control group. Compared to this group, 5 ng/ml VEGF concentration had a - 6% decrease, 50 ng/ml VEGF concentration had an 8% increase, and 100 ng/ml VEGF concentration had a 21% increase in the number of sprouts.

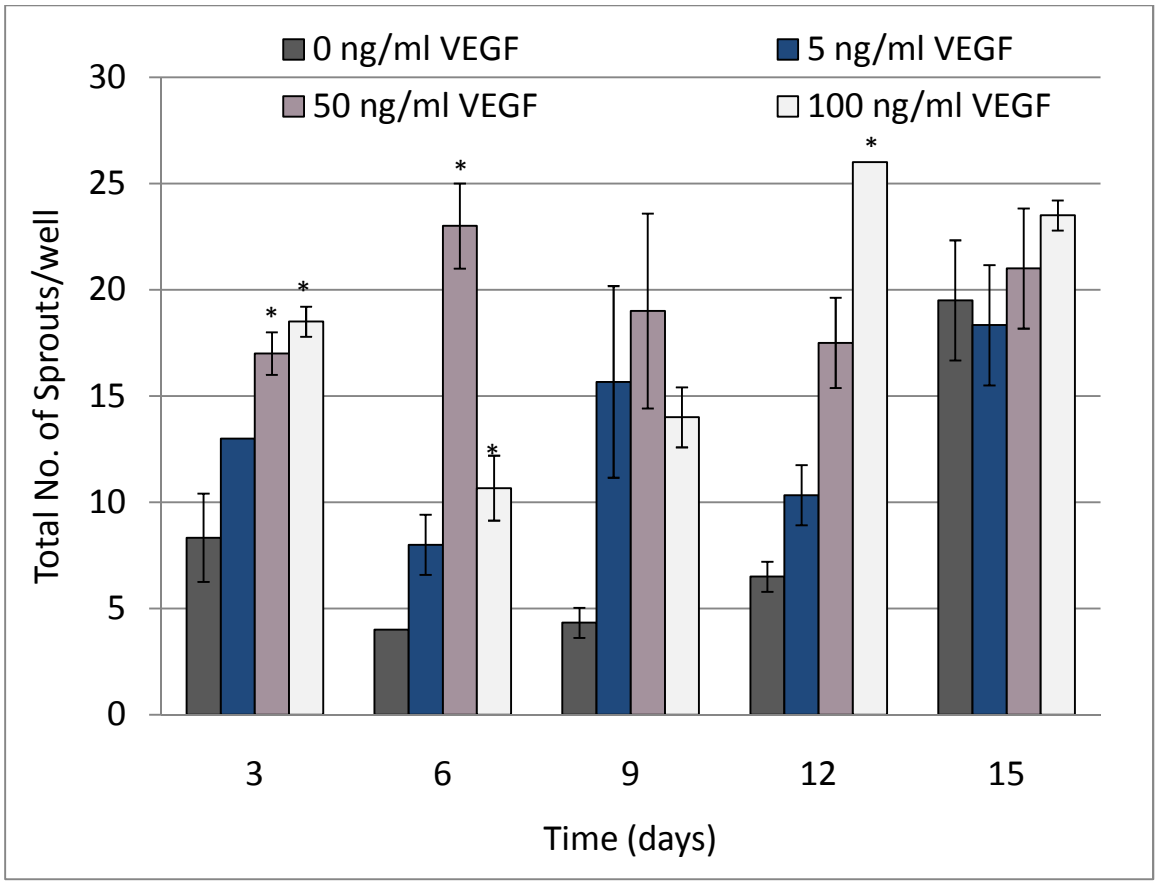


Figure 4.9 Effect of VEGF concentration on the number of sprouts in the tissue model with 0.73 mm collagen thickness. Data shown are mean values ± sd; n=3. *p<0.05 vs. 0 ng/ml VEGF sample.

On Day 15, viability was observed to be as high as 3% for 5 ng/ml VEGF, 88% for 50 ng/ml VEGF, and 100 % for 100 ng/ml VEGF, compared to the control. This suggests that viability and proliferation is independent of the number of sprouts from the cell within the tissue model. The effect of viability and proliferation on the total number of sprouts was also studied within the 2.01 mm and 4.28 mm collagen thicknesses (Data not shown). However, compared to the NGF control, there was no significant number of

sprouts observed for the tissue models with these collagen thicknesses. For this result, VEGF increases number of sprouts in only one collagen thickness, the 0.73 mm.

4.2.3 Effect of vascular endothelial growth factor on endothelial cell depth of migration

Figure 4.10 describes the maximum depth of migration achieved by the endothelial cells in response to increasing VEGF concentrations with respect to the NGF control group. For cell migration analyses in this model, the distance travelled by the cell into the collagen was measured for each triplicate sample and the average was calculated. Though cells are within the same culture conditions, there is a possibility that cell migration in each sample may vary, which would reflect large standard deviation in the data. Compared to the control group, 5 ng/ml of VEGF concentration showed an increase in depth of migration on Days 3, 9 and 15 with 0.59 mm, 0.47 mm, and 0.37 mm, respectively and a decrease on Days 6 and 12 with 0.44 mm and 0.36 mm, respectively. These values suggest that 5 ng/ml VEGF was not potent in enhancing cell migration into the collagen. When 50 ng/ml of VEGF was added, there was an increase in cell migration of approximately 0.66 mm, 0.65 mm, 0.47 mm and 0.46 mm on Days 3, 6, 9, and 15 compared to the control, respectively. However, minimum viability was observed on Days 9 and 12 for the samples exposed to 50 ng/ml VEGF. When 100 ng/ml VEGF was add to the tissue model, there was an increase in cell migration into the collagen with approximately 0.61 mm, 0.62 mm, 0.66 mm, 0.54 mm, and 0.48 mm on Days 3, 6, 9, 12 and 15, respectively. This suggests that 50 ng/ml and 100 ng/ml of VEGF enhanced cell migration into the collagen more than compared to 5 ng/ml of VEGF. The effect of

VEGF concentration on the depth of cell migration within the tissue model was also studied within the 2.01 mm and 4.28 mm collagen thicknesses (Data not shown). However, compared to the NGF control, there was no significant cell migration observed for these thicknesses. In conclusion, VEGF enhanced cell migration in only the 0.73 mm collagen thickness and this was dependent on an increase in VEGF concentration.

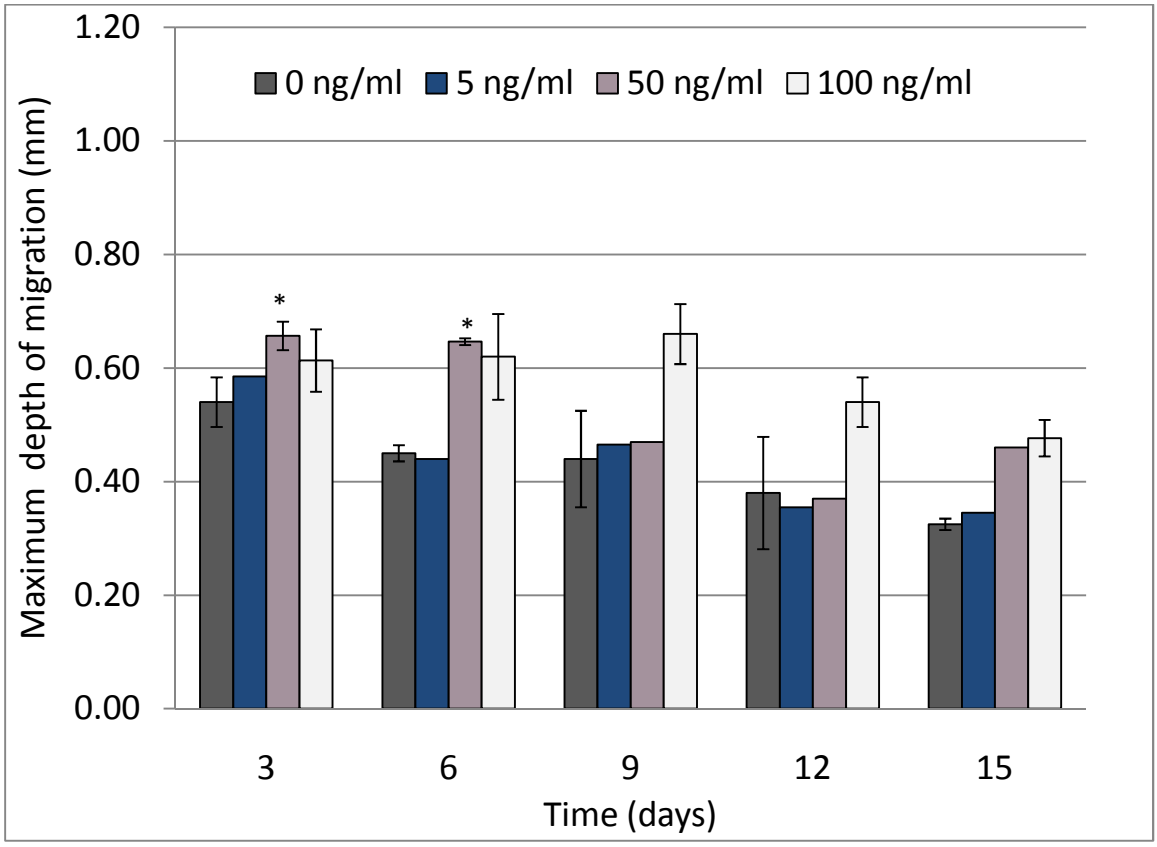


Figure 4.10 Effect of VEGF concentration on depth of migration in the 0.73 mm collagen thickness. Data shown are mean values ± sd; n=3. *p<0.05 vs. 0 ng/ml VEGF sample.

4.2.4 Effect of collagen thickness on endothelial cell viability and proliferation

Figure 4.11, 4.12 and 4.13 illustrates the percent difference in cell viability and proliferation associated with increasing collagen thickness for 5 ng/ml, 50 ng/ml and 100 ng/ml VEGF concentrations compared to the NGF control group, respectively. It can be observed that cell viability and proliferation increased slightly in each collagen thickness for 5 ng/ml. However, taking the standard deviation into consideration, this increase was not significant compared to the control. This shows that the addition of the low concentration of VEGF to the tissue model did not stimulate an increase in cell viability and proliferation in any of the collagen thicknesses tested, compared to the NGF control sample.

According to Figure 4.12 for samples exposed to 50 ng/ml, there was an increase in cell viability and proliferation for the 2.01 mm collagen thickness samples on Days 6, 9, 12, and 15 with 31%, 216%, 170%, and 146% compared to the 0.73 mm collagen thickness samples, respectively. On the other hand, the 4.28 mm collagen thickness samples showed a significant decrease in cell viability and proliferation compared to the 0.73 mm collagen thickness samples on Days 3, 9, 12 and 15 with 26%, 2%, 7% and 11%, respectively. These numbers reassert the multi-functional role collagen plays in supporting endothelial cell viability and proliferation. Since collagen can serve as a storage site for growth factors, increasing the collagen thickness from 0.73 mm to 2.01 mm may provide more storage for VEGF and induce greater cell viability and proliferation. Therefore, increasing collagen thickness to 4.28 mm should show even greater cell viability and proliferation, but this was not the case. The decrease in cell viability and proliferation for the 4.28 mm thickness suggests that there may be other

factors (such as oxygen) responsible for endothelial cell growth as well. For example oxygen is an essential factor known to affect the growth mechanisms of endothelial cells.

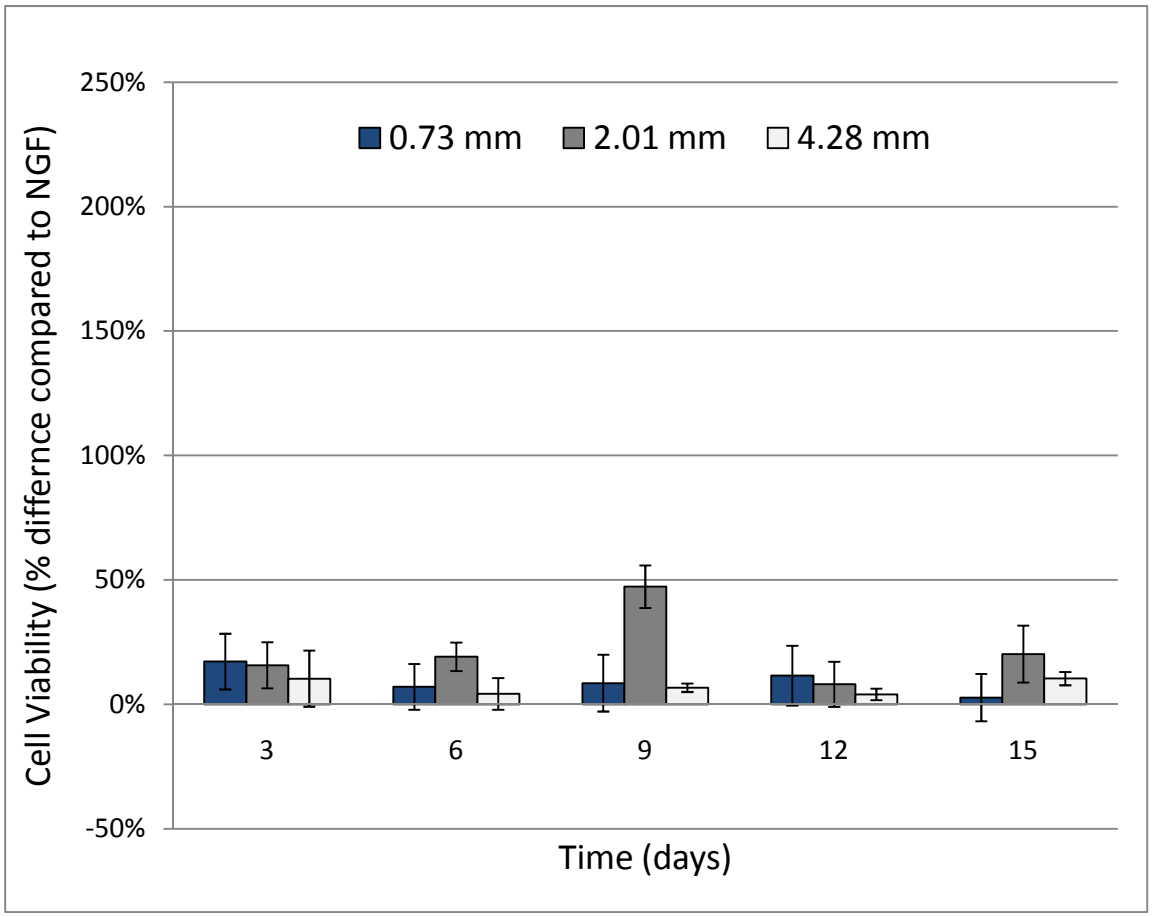


Figure 4.11 Effect of collagen thickness on viability and proliferation for samples exposed to 5 ng/ml VEGF concentration. Data shown are mean values ± sd; n=3

It is possible that as the collagen thickness increases, oxygen diffusion into the collagen is slower relative to its consumption by the cells. Literature has shown that diffusion of oxygen and nutrient delivery is inadequate in tissues greater than 2 mm. [133] This is probably why there is minimum cell viability in the 4.28 mm collagen thickness. Thus,

endothelial cell viability and proliferation is greatest for the 2.01 mm than the 0.73 mm and 4.28 mm collagen thicknesses.

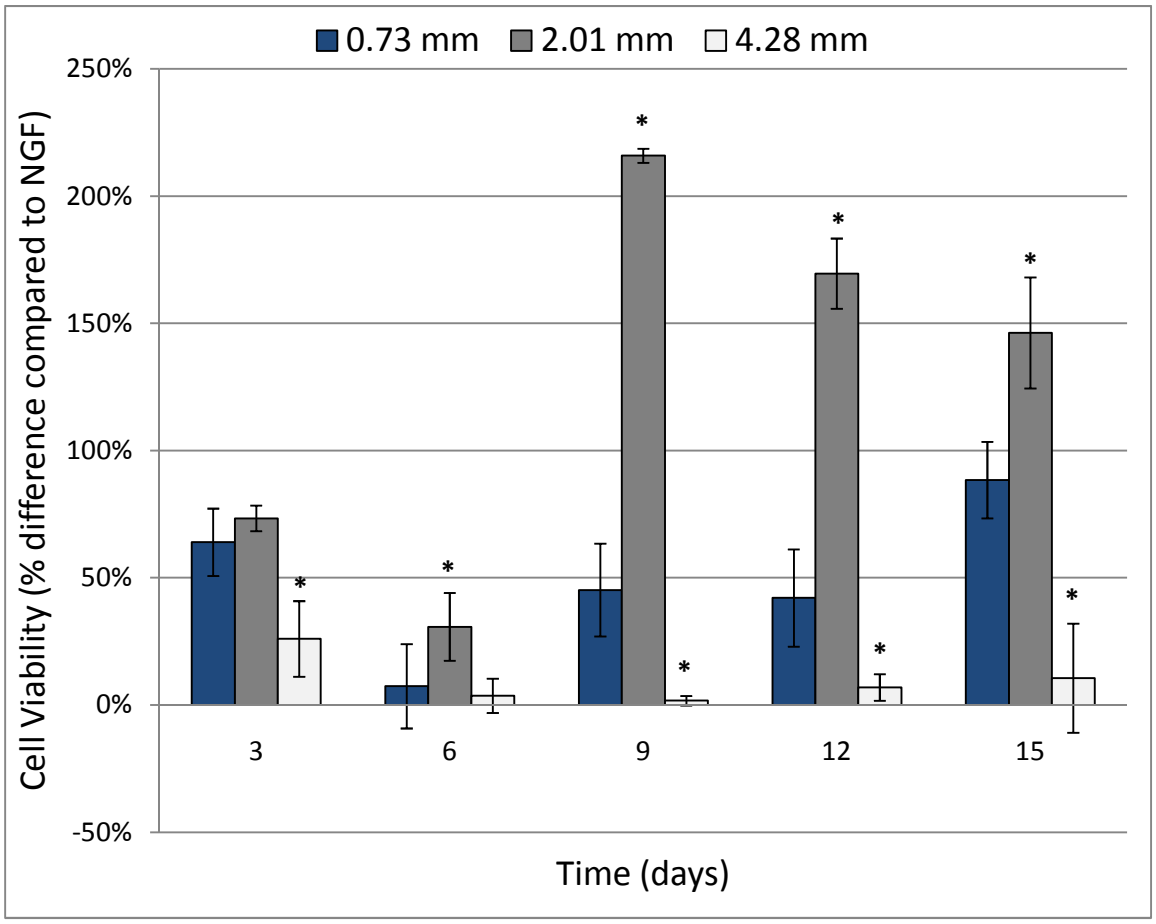


Figure 4.12 Effect of collagen thickness on viability and proliferation for samples exposed to 50 ng/ml VEGF concentration. Data shown are mean values ± sd; n=3, *p<0.05 vs. 0.73 mm collagen thickness sample.

For the samples with 100 ng/ml VEGF, it was observed that the samples with the 2.01 mm collagen thickness had a significant increase in cell viability and proliferation, but the samples with the 4.28 mm collagen thickness was not significant, when compared to the samples with the 0.73 mm collagen thickness. The samples with the 2.01 mm

collagen thickness had on average fourteen times higher viability than the samples with the 4.28 mm collagen thickness, and twice as high as the samples with the 0.73 mm collagen thickness. These results show similar effects on cell viability and proliferation as shown previously.

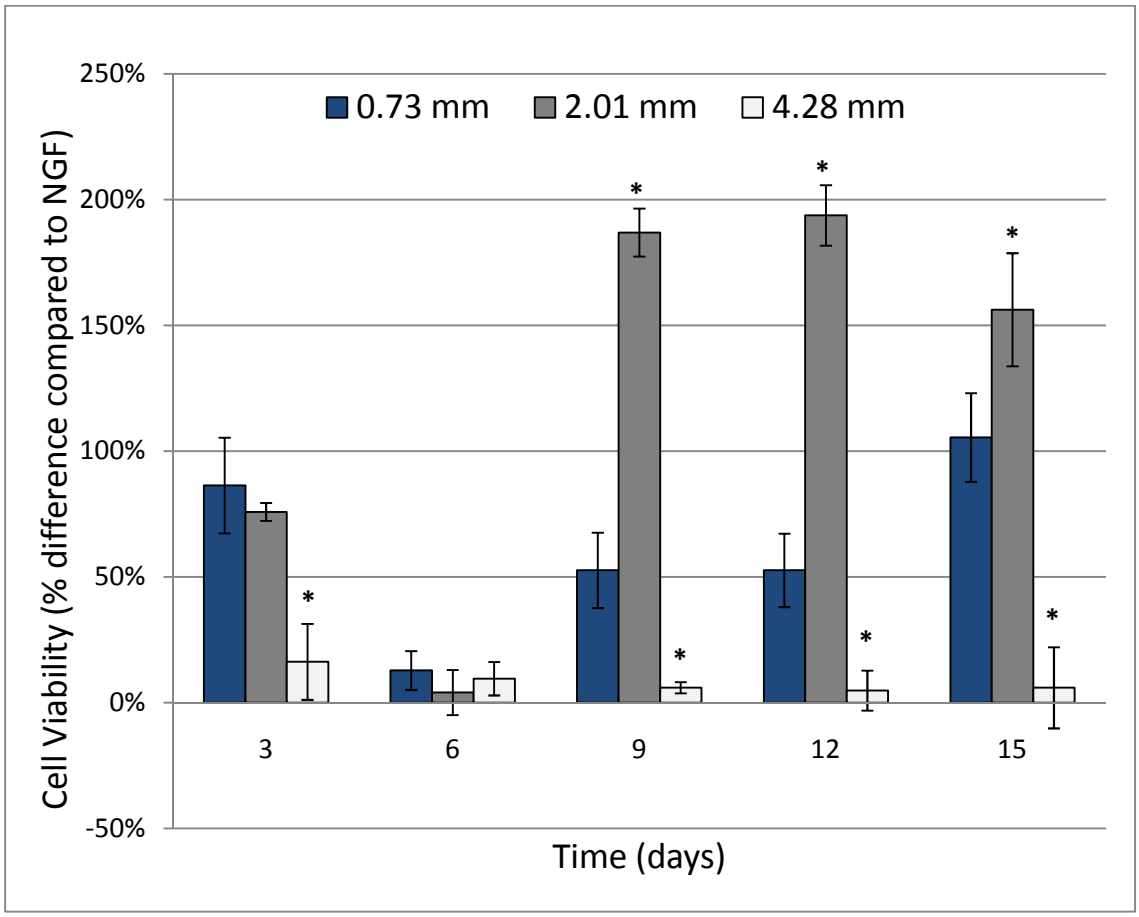


Figure 4.13 Effect of collagen thickness on viability and proliferation for the samples exposed to 100 ng/ml VEGF concentration. Data shown are mean values ± sd; n=3, *p<0.05 vs. 0.73 mm collagen thickness sample.

Another significant observation from the data was the decrease in cell viability and proliferation from Day 3 to 6 for each collagen thickness. For 50 ng/ml VEGF added to the samples, a decrease from 64% to 7%, 73% to 31%, 26% to 4% was observed for

the 0.73 mm, 2.01 mm and 4.28 mm collagen thicknesses, respectively. For 100 ng/ml VEGF added to the samples, a decrease from 86% to 13%, 76% to 4%, 16% to 10% was observed for the 0.73 mm, 2.01 mm and 4.28 mm collagen thicknesses, respectively. The decrease in cell viability and proliferation was due to the increase in cell growth in the control group on Day 6. However, changing the media on Day 6 after carrying out analyses increased VEGF concentration in 5 ng/ml, 50 ngml, and 100 ng/ml samples and as a result cells were observed to increase at a rapid rate on Days 9, 12 and 15.

4.2.5 Effect of collagen thickness on endothelial cell number of sprouts and migration

The effect of collagen thickness on number of sprouts was investigated (data not shown). For 5 ng/ml VEGF added to samples, it was observed that sprouts were stimulated in one sample, the 0.73 mm collagen thickness, as was seen in figure 4.9, but this increase was not significant compared to the control. Also for this group, the effect of collagen thickness on cell migration was observed to be negligible compared to the NGF control group (data not shown). For the samples with 50 ng/ml of VEGF, a significant increase in cell sprouting and migration was observed in the 0.73 mm collagen thickness samples compared to the control, as seen in Figure 4.9 and 4.10, respectively. There was no cell migration or sprouting observed in the 2.01 mm and 4.28 mm collagen thicknesses compared to the control (data not shown). The effect of collagen thickness on sprouting and migration for the addition of 100 ng/ml VEGF to samples was also studied (data not shown). The results showed the same effect observed for samples with 50 ng/ml VEGF; only the 0.73 mm collagen thickness showed significant sprouting and migration.

For both 50 ng/ml VEGF and 100 ng/ml VEGF added to samples, increasing collagen thickness from 0.73 mm to 2.01 mm stimulated higher viability and proliferation, but not sprouting and migration of endothelial cells. Thus, the 2.01 mm collagen thickness had the greatest effect on viability and proliferation, the 0.73 mm collagen thickness had the greatest effect on cell sprouting and migration, and the 4.28 mm collagen thickness had no significant effect in either of the processes.

For the novel 3D angiogenesis system, it was concluded that 50 ng/ml and 100 ng/ml VEGF concentrations were more potent in stimulating viability, proliferation, sprouting and migration compared to the 5 ng/ml VEGF concentration. The higher VEGF concentrations of 50 ng/ml and 100 ng/ml exerted approximately the same effect on cell viability, proliferation, sprouting and migration. Taking cost into consideration, it was concluded that the 50 ng/ml VEGF concentration is the most suitable for this model. It was also concluded that 0.73 mm collagen thickness was the most suitable extracellular matrix environment for angiogenesis in this novel system because it showed significant increases in cell viability, proliferation, sprouting and migration compared to the 2.01 mm collagen thickness, which only showed increases in cell viability and proliferation. These two variables, 0.73 mm collagen thickness and 50 ng/ml VEGF concentration, sets the platform for testing the effect of thalidomide analogue, CLT-003 on angiogenesis within the tissue model.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The main goal of this research was to develop and characterize a novel 3D vascular tissue model that can be used to study the effect of an anti-angiogenesis drug (CLT-003) on human umbilical vein endothelial cells. The motivation for this research was to develop an angiogenesis tissue model that mimicked human physiological conditions to study an anti-angiogenic drug for treating diabetic retinopathy. The newly developed model has great potential for commercialization. It can be used to study the different mechanisms associated with angiogenesis and new therapeutic strategies for controlling the disease.

In vitro tissue models can serve as alternative testing models to animal or human models, especially in cases where either or both fail to provide the information needed. With animal models there is the possibility of uncertainty in results due to the differences in tissue response in some organs. Human models on the other hand cannot be used in studying anything that is considered hazardous to the human health. The use of *in vitro* models has overcome the limitations of both animal and human models in research. There are two types of *in vitro* models; two dimensional (2D) and three dimensional (3D). The 3D *in vitro* models are more advantageous than 2D tissue models because they provide

the third dimension essential for investigating the proliferation and migration steps of angiogenesis. Without the third dimension, 2D *in vitro* models provide limited insight into the complex interactions occurring within *in vivo* angiogenesis. The 3D *in vitro* models are also closer to the *in vivo* environment than 2D models because they take into consideration more steps of angiogenesis process including cell migration and the formation of intricate capillary-like structures. Other factors such as the effect of a gradient of diffusion of nutrients, oxygen, and stimulating factors on the angiogenesis process can also be studied in 3D models.

This project was divided into two parts. The first part was based on developing a 3D tissue model adapted from literature to test the effect of an anti-angiogenesis drug (CLT-003) on endothelial cell viability, proliferation, sprouting and migration. The second part was based on creating a novel 3D angiogenesis tissue model that was characterized by studying the effect of changes in collagen thickness and VEGF concentration on endothelial cell viability, proliferation, sprouting and migration. The main conclusions from this project include:

1) For the 3D tissue model developed using a solid plate format, the normal model demonstrated a more significant effect of CLT-003 on endothelial cell viability, proliferation and sprouting than the growth factor model. Due to the absence of a growth factor cocktail, the normal model was able to demonstrate the normal mechanism of CLT-003 in reducing angiogenesis.
2) A novel 3D vascular tissue model that mimics angiogenesis was successfully developed, and the effect of changes on the design variables of collagen thickness and VEGF concentration on angiogenesis was determined.

3) Increasing VEGF concentration was shown to demonstrate a mitogenic effect in stimulating endothelial cell growth. There was no significant effect observed when 5 ng/ml of VEGF concentration was added to the system. An increase in VEGF concentration from 5 ng/ml to 50 ng/ml showed a significant increase in viability, proliferation, sprouting and migration of endothelial cells. However, an increase in VEGF concentration from 50 ng/ml to 100 ng/ml did not have a significant effect, showing a limit to this mitogenic effect.
4) Varying collagen thickness had an effect on endothelial cell viability, sprouting and migration. Viability and proliferation increased in samples with increasing collagen thickness, from 0.73 mm to 2.01 mm. There was no significant increase in endothelial cell viability and proliferation in samples with 4.28 mm collagen thickness. Endothelial cell sprouting and migration were induced only in the tissue model with 0.73 mm collagen thickness.
5) For the novel system, the optimal conditions that could be used to test the effect of anti-angiogenesis drugs on angiogenesis in the tissue model are 0.73 mm collagen thickness and 50 ng/ml VEGF concentration. The 0.73 mm collagen thickness had significant effects on all the angiogenic events analyzed in this model. For the VEGF concentration, since the two concentrations have approximately the same effect on angiogenesis within the tissue model, it is more economical to select the 50 ng/ml than the 100 ng/ml.

A 3D model to mimic angiogenesis that occurs *in vivo* was initially used to study the effect of a novel anti-angiogenesis drug (CLT-003). The model design included

human umbilical vein endothelial cells (HUVECs) grown on bovine type 1 collagen matrix in a solid plate system. Two drug concentration were used, 32 μM and 64 μM, to study the potent effect of the anti-angiogenesis drug. The first model was called the Growth Factor Model because it used a growth factor cocktail to stimulate angiogenesis. It was observed that CLT-003 had a greater effect on viability, proliferation, number of sprouts and migration of endothelial at the higher CLT-003 concentration than the lower concentration. However, after the drug was added to the cells, the growth factor cocktail stimulated cell recovery, leading to a system that did not illustrate the normal mechanism of the drug. The drawbacks associated with this model led to the development of the second model, called the Normal Model. This model had the same design as the Growth Factor Model but there was no stimulant used to induce viability, proliferation and differentiation of endothelial cells. CLT-003 was shown to significantly affect endothelial cell viability, proliferation and sprouting, which made it more suitable for testing the drug than the Growth Factor Model. However, problems associated with this design included, a significant decrease in endothelial cell viability of the control samples for the duration of the experiment and there was no provision for drug delivery to mimic *in vivo* conditions. Therefore, a more physiological model was developed, and the effect of certain design parameters on angiogenesis tested.

The novel 3D vascular tissue model consisted of HUVECs cultured on bovine type 1 collagen in Transwell® membrane supports. Since this was a new model and was significantly different from previous models, the model had to be characterized before it could be used to test the anti-angiogenesis drug. The effect of two design variables, collagen thickness and VEGF concentration, were characterized. Collagen constitutes the

major protein in the extracellular matrix. Three collagen thicknesses (0.73 mm, 2.01 mm and 4.28 mm) were chosen in order to represent different thicknesses of the extracellular matrix. VEGF plays a key role in pathological and physiological angiogenesis. Three VEGF concentrations (5 ng/ml, 50 ng/ml, 100 ng/ml) were chosen in order to study its mitogenic effect on endothelial cell viability, proliferation, sprouting and migration.

For studying the effect of collagen thickness in the tissue model, viability and proliferation of endothelial cells increased twice as much for the samples with 2.01 mm collagen thickness compared to the samples with 0.73 mm collagen thickness. This demonstrates that extracellular matrix plays an important role in regulating endothelial cell behavior. It employs a direct control over the activity of growth factors through binding and release of certain growth factors. Through this, the extracellular matrix serves as a sequestration and storage site for growth factors, concentrating their activity in the vicinity of cells and protecting them from degradation [134]. The 2.01 mm collagen thickness accommodated more VEGF in the collagen and induced greater stimulation of viability and proliferation than the 0.73 mm collagen thickness. Cell viability and proliferation decreased when increasing the collagen thickness further to 4.28 mm compared to the 0.73 mm collagen thickness. The decrease in cell viability and proliferation for the 4.28 mm collagen thickness can be due to other factors, i.e. the supply of oxygen, that also contribute to endothelial cell viability and proliferation [133]. Endothelial cell migration and the number of sprouts increased for samples with 0.73 mm collagen thickness compared to 2.01 mm and 4.28 mm collagen thicknesses. With reference to this 3D tissue model, the 0.73 mm thickness was chosen as the most suitable

collagen thickness, because it showed a significant effect on all four angiogenic steps: viability, proliferation sprouting, and migration.

For the effect of VEGF concentration on viability, proliferation, sprouting, and migration, a slight increase was observed when 5 ng/ml VEGF was added to the samples, compared to the NGF control sample. When the VEGF concentration was increased to 50 ng/ml, this showed a further increase in endothelial cell viability, proliferation, sprouting, and migration, compared to the NGF control sample. The 100 ng/ml VEGF concentration also showed a significant increase in the viability, proliferation, sprouting, and migration of endothelial cells, compared to the NGF control sample. The function of VEGF as an endothelial cell specific mitogen was to stimulate survival, proliferation and differentiation of endothelial cells. With reference to this 3D model, this effect was displayed in a dose-dependent manner. However, it was found that the 100 ng/ml VEGF concentration did not show a significant increase in viability and proliferation compared to the 50 ng/ml VEGF concentration. There may be a point between these two concentrations, which the receptors on endothelial cells become saturated with VEGF and there is no further mitogenic effect. With reference to this 3D vascular tissue model and taking cost into consideration, the 50 ng/ml VEGF concentration was chosen as the most suitable for endothelial cell stimulation within this model.

The current 3D tissue model was successful in demonstrating the basic angiogenic steps: viability, proliferation, sprouting, and migration. However, this model needs to be improved upon and further characterized so that it can be used as a testing system for the development of new drugs that affect angiogenesis, such as those for the treatment of

diabetic retinopathy. Consequently, future work will be directed towards addressing the following:

1) During angiogenesis, blood vessels supply both oxygen and nutrients that increase stimulation of endothelial cell survival. Oxygen is therefore important in angiogenesis [135-137] and may be a factor that affects endothelial cell growth when varying collagen thickness. Therefore, the effect of saturated oxygen concentration within the tissue model on angiogenesis needs to be characterized.
2) The ECM has a high affinity for many soluble factors, including VEGF. Review of literature shows that angiogenic molecules may be entrapped in the ECM, but they are directly recognized by integrins [138, 139]. Integrins are receptors that mediate between a cell and the surrounding tissue. The interactions of VEGF binding to the ECM through integrins needs to be studied in order to completely characterize the effect of VEGF transport and interaction within the model. This could also reveal why the cell viability and proliferation is greater in samples with 2.01 mm collagen thickness compared to samples with 0.73 mm collagen thickness.
3) Use the new model to test the anti-angiogenic properties of CLT-003 on the mechanisms associated with angiogenesis. This includes studying the effect of the route of delivery of the drug on angiogenesis in the tissue model. The already characterized response to CLT-003 can be used to validate the new model, so that it can be used for the design and testing of new therapeutics for treating diabetic retinopathy.

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VITA

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Candidate for the Degree of

Master of Science

Thesis: THE DEVELOPMENT AND CHARACTERIZATION OF A NOVEL 3D ANGIOGENESIS TISSUE MODEL FOR TESTING AN ANTI-ANGIOGENESIS DRUG

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Title of Study: THE DEVELOPMENT AND CHARACTERIZATION OF A NOVEL 3D ANGIOGENESIS TISSUE MODEL FOR TESTING AN ANTI-ANGIOGENESIS DRUG

Pages in Study: 85 Candidate for the Degree of Master of Science

Major Field: Chemical Engineering

Scope and Method of Study:
Diabetic Retinopathy (DR) is a microvascular complication associated with diabetes mellitus and is the leading cause of blindness in American adults. DR occurs as a result of chronic hyperglycemia, which causes vascular damage, increased vascular permeability, vascular leakage, and edema. When the blood glucose level is elevated in the body, it can lead to a production of growth factors. Among the many growth factors present, vascular endothelial growth factor (VEGF) has been recognized as the primary mediator of vascular alteration in DR. Currently there is no non-invasive and effective drug treatment to prevent or arrest the progression of DR. Studies have shown that agents that block VEGF activity can prevent diabetes-induced retinal damage, thus the development of new drugs to block retinal vascular leakage via targeting VEGF or its receptor has become a potential new therapy for DR. Recently a novel anti-angiogenesis drug, a thalidomide analog, has been developed for the treatment of diabetic retinopathy. The objective of this research was to develop a three dimensional (3D) *in vitro* model to study the effect of an anti-angiogenesis drug on endothelial cell functions associated with angiogenesis. Initially, a model consisting of endothelial cells grown on bovine type 1 collagen gel formed within a 96 solid well plate was tested. Due to drawbacks associated with the model, a novel 3D model was created using Transwell® permeable supports. The new model was characterized by varying collagen thickness at 0.73 mm, 2.01 mm and 4.28 mm and VEGF concentration at 5 ng/ml, 50 ng/ml, and 100 ng/ml.

Findings and Conclusions:
The higher VEGF concentrations of 50 ng/ml and 100 ng/ml showed greater stimulation of cell viability, proliferation, number of sprouts and migration than the 5 ng/ml concentration. However there was no significant increase in the endothelial cell functions for the 100 ng/ml of VEGF compared to the 50 ng/ml of VEGF. The 2.01 mm collagen thickness showed the greatest increase in cell viability and proliferation compared to the 0.73 mm and 4.28 mm thicknesses. The 0.73 mm collagen thickness showed the greatest increase in sprouting and migration compared to the 2.01 mm and 4.28 mm thicknesses. It can be concluded that the 0.73 mm collagen thickness and the 50 ng/ml VEGF concentration result in the optimal conditions suitable for testing the anti-angiogenesis drug in the new model. The 0.73 mm thickness was chosen as the most suitable collagen thickness, because it showed a significant effect on all four angiogenic steps: viability, proliferation sprouting, and migration.

ADVISER'S APPROVAL: Dr. Heather Gappa-Fahlenkamp