Tear Film VEGF in Dogs with Vascularizing Corneal Disease

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ABSTRACT

This body of work encompasses two studies: the collection of canine tears and tear film vascular endothelial growth factor-A (VEGF) via a novel polyester rod and the measurement of VEGF concentrations in tears from dogs with normal and vascularized corneas.

The first study evaluated use of the polyester rod for tear collection in dogs, determined fluid volume and VEGF recovery characteristics of the rod, as well as potential binding of VEGF to the rod itself. Wicked volumes eluted after centrifugation were determined. Tears were then harvested from ophthalmologically normal dogs using rods and glass capillary tubes. Tears were eluted from the collection media and assayed for tear film VEGF using a commercially available canine VEGF sandwich ELISA kit. Dilutions of VEGF standard were also wicked passively into the rods or drawn into capillary tubes. The dilutions were eluted and assayed along with tear samples. Total holding capacity of the rods approximates 65µL. 100% recovery of a wicked solution volume is possible, regardless of full or partial saturation. VEGF is detectable at biologically active concentrations in the normal canine tear film (mean 8.4 +/- 3.3 ng/mL). At concentrations greater than 0.625 pg/mL, percent recovery of VEGF from both rods and capillary tubes was greater than 90%. Percent volume recovery is adequate for polyester rods as is percent VEGF recovery, which did not differ appreciably from capillary tubes. VEGF is detectable in normal canine tears. Polyester rods are an easy and efficacious method for canine tear collection

The second study evaluated tear film VEGF concentration bathing vascularized and non-vascularized canine corneas. Tear samples were harvested from eyes of dogs with vascularizing corneal disease (including unaffected eyes of dogs with unilateral vascularization) and normal dogs. Vascularization scores were assigned to diseased eyes. VEGF concentration was evaluated using the same commercial kit as above. Mean tear film VEGF concentration of diseased eyes

was 6.53 +/- 0.77 ng/mL (geometric mean, 95% CI) and did not differ significantly from control eyes (6.01 +/- 1.0 ng/mL). Tear film VEGF concentrations in unaffected eyes of dogs with unilateral disease were significantly higher (9.78 +/- 1.52 ng/mL) than control and vascularized eyes. VEGF concentrations of diseased eyes did not differ significantly by disease process, degree of corneal vascularization, or use of topical or oral anti-inflammatory medications. In conclusion, canine tear film VEGF concentrations exceed biologically active concentrations of VEGF, but do not correlate with state of corneal vascularization. VEGF-related control of corneal vascularization may be mediated by VEGF receptor expression and regulation or a balance between proangiogenic and antiangiogenic VEGF splice variants indistinguishable by current testing methods.

DEDICATION

This work is dedicated to my family, especially my mother, my father, my sister and brother-in-law, and their children Matt, Josh, and Natalie. Finally, to my dogs Tulip Pearl, Ina Mae, and Georgie. Their unending support and late night snuggles have made this work possible.

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VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
NP	Neuropilin
PlGF	Placenta growth factor
MMP	Matrix metalloproteinase
EGF	Epithelial growth factor
NGF	Nerve growth factor
TGF	Transforming growth factor
KGF	Keratinocyte growth factor
IGF-1	Insulin-like growth factor-1
FGF	Fibroblast growth factor
PDGF	Platelet-derived growth factor
IL	Interleukin
IFN	Interferon
TNF	Tumor necrosis factor
PKC-β	Protein kinase C-beta
HIF	Hypoxia inducible factor
vHL	von Hippel-Lindau
bFGF	Basic fibroblast growth factor
COX	Cyclooxygenase
CSK	Chronic superficial keratitis
KCS	Keratoconjunctivitis sicca
AMD	Age-related macular degeneration
CNV	Choroidal neovascularization
ROP	Retinopathy of prematurity
PDR	Proliferative diabetic retinopathy
HSV	Herpes Simplex Virus
PIFM	Pre-iridal fibrovascular membrane
SCCED	Spontaneous chronic corneal epithelial defect

SPK	Superficial punctate keratopathy
IMMK	Immune-mediated keratitis

I. CHAPTER 1: INTRODUCTION

A. VEGF

Vascular endothelial growth factor (VEGF) protein is a potent mediator of blood vessel formation in both health and disease. It is an essential mitogen and survival factor for vascular endothelial cells, promoting growth of arteries, veins, and lymphatic vessels.[1,2] Its integral role in vasculogenesis begins with early development of the embryo and continues through adulthood. During development, VEGF expression is tightly regulated, guiding normal hematopoiesis and angiogenesis of the cardiovascular system.[1] In the adult mammal, angiogenesis is a critical component of healthy tissue repair and growth and the female reproductive cycle.[3] However, imbalances in VEGF expression have untoward consequences. *In utero,* small increases in VEGF protein cause severe abnormalities; loss of even one VEGF allele results in cardiovascular defects and embryonic death. Mice null for VEGF receptors die *in utero* as a result of severe blood vessel abnormalities.[1,4] Dysregulated angiogenesis in the adult has been associated with several human diseases, including gingivitis, rheumatoid arthritis, various ocular diseases, and cancer.[2-8] Furthermore, VEGF protein homologs have been identified in the Orf virus, displaying VEGF-like activity that contributes to the pathology of these diseases.[1,9]

The term vascular endothelial growth factor actually refers to a family of gene products that control vasculogenesis, including placenta growth factor (PlGF), VEGF-A, VEGF-B, VEGF-C, and VEGF-D.[1-3] VEGF-A, commonly referred to as simply VEGF, and VEGF-B mediate blood vessel angiogenesis; VEGF-C and VEGF-D regulate lymphatic angiogenesis.[1] VEGF-E also exists, and refers to the VEGF homolog found in the Orf virus genome.[9]

VEGF-A is a 45 kDa heparin-binding homodimeric glycoprotein and is the most common isoform.[1,3,10] In humans, the VEGF-A gene is located on chromosome 6p21.3 and is composed of 8 exons and 7 introns.[1,3] Alternative splicing of the 8 exons allows several isoforms of VEGF-A to exist.[3] The different forms are denoted $VEGF_{xxx}$ and, in humans, 4 are recognized: $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$.[1,4] All isoforms contain exons 1-5, which contain the receptor-binding domain; exons 6 and 7 span the heparin-binding domain. Exons 6-8 are omitted from or added to the sequence, depending on the isoform.[1,2,4,10] The heparin-binding domain mediates the

mitogenic activity of VEGF[11,12] and the extent to which the isoform is membrane-bound influences its bioactive capabilities.[1] Thus, the amino acid sequence determines the bioactive characteristics of the isoform.

$VEGF_{121}$ is highly acidic, does not bind heparin or extracellular matrix well, and is freely diffusible.[1,3,4] $VEGF_{165}$ is heparin binding and exists in both a bound form (to both extracellular matrix and cell membranes) and a secreted form.[1,3,4] Isoforms 189 and 206 have a higher affinity for heparin than isoform 165, are highly basic and are sequestered almost exclusively in the extracellular matrix.[1,3,4] The proteolytic enzymes heparinase and plasminogen release bound VEGF into the local environment. Heparinase displaces extracellular matrix bound isoforms from their binding sites, whereas plasminogen cleaves near the carboxyl terminus.[1] These proteolytic mechanisms can increase the proportion of bioactive VEGF fragments in the microenvironment and, as a result, are important regulators of VEGF bioavailability.[1]

Less frequent splice variants of VEGF-A include $VEGF_{145,}$ $VEGF_{162}$, and $VEGF_{183}$.[1,3,4] $VEGF_{145}$ has two heparin-binding domains, though has a lesser affinity for heparin than isoforms 189 and 206.[1,4] Isoform 145 also has characteristics that allow it to bind extracellular matrix and its expression appears to be limited to reproductive organs.[3,4] Although controversial at this time, an anti-angiogenic isoform subset of $VEGF_{xxx}$ is thought to exist, denoted $VEGF_{xxxb}$.[1,13,14] $VEGF_{121b}$, $VEGF_{145b,}$ $VEGF_{165b}$, $VEGF_{183b}$, and $VEGF_{189b}$ have been recognized in normal lens, sclera, retina, and iris tissue,[15] and ocular vitreous fluid.[15,16] $VEGF_{165b}$ in particular has also been identified in normal colonic tissue,[6] and renal tissue.[17] Like $VEGF_{165}$, $VEGF_{165b}$ appears to be the most common b isoform and has been shown to account for more than 50% of the total VEGF in normal tissue.[6,13,16,17] $VEGF_{165b}$ differs from $VEGF_{165}$ by 6 amino acids at the C-terminus of exon 8.[13] As a result, the C-terminal domain is altered, but VEGF receptor binding and dimerization ability remains.[13] $VEGF_{165b}$ binds to VEGF receptor-2 (VEGFR-2) with the same affinity as $VEGF_{165}$, but does not fully activate the receptor.[13,18] Thus, the activity of $VEGF_{165b}$ is proposed to be inhibitory in nature, hindering endothelial cell proliferation and migration.[6,13,15,17] Despite several studies affirming the existence of the b isoforms, a recent paper Harris et al in 2012 has called their existence into question. This group was consistently unable to detect b isoforms in mouse and human cells and tissues, suggesting that any $VEGF_{xxxb}$

transcripts detected were likely amplification artifacts resulting from injudicious PCR primer design.[14]

Various receptors for VEGF-A exist, the most prominent of which, in angiogenesis, are the ligand-activated tyrosine kinases VEGFR-1 (also referred to as Flt-1), VEGFR-2 (also referred to as KDR or Flk-1), and the inhibitory soluble Flt-1 variant (sFlt-1).[1,3,4] VEGF binding sites are present on vascular endothelial cells, bone marrow-derived cells, and tumorigenic cells. The precise function of each receptor is variable, and may depend upon the cell type on which the receptor is found, as well as the life development stage of the human or animal.[1]

VEGFR-1, or Flt-1, is expressed in trophoblasts, monocytes, and renal mesangial cells in addition to vascular endothelial cells.[4] It binds VEGF-A, VEGF-B, and PlGF,[1,3] and is thought to inhibit angiogenic activity by acting as a decoy receptor.[19,20] However, Flt-1 null mice die *in utero* due to a failure of endothelial cells to organize into vascular channels.[1] In addition, while activation does not induce cell proliferation, it does induce cell migration.[4] Therefore, Flt-1 may have a dual function dependent on the physiologic circumstances.[1] The soluble form of VEGFR-1, denoted sFlt-1, also exists as a result of alternative splicing. Similar to Flt-1, sFlt-1 inhibits VEGF activity by acting as a decoy receptor, binding VEGF such that it is rendered unavailable to VEGFR-2 (Flk-1) receptors.[19,20]

VEGFR-2 (Flk-1) is the primary mediator of the mitogenic, angiogenic, and permeability-enhancing effects of VEGF.[1,3] It is found on endothelial cells, hematopoietic stem cells, megakaryocytes, and renal progenitor cells.[4] VEGFR-2 binds VEGF-C, VEGF-D, and VEGF-A, albeit with a lower affinity than VEGFR-1.[1,3] Activation induces a mitogenic response and cell migration.[4] Evidencing this, Flk-1 null mice die *in utero* due to a failure to develop blood islands and organized blood vessels.[4]

Other receptors such as VEGFR-3 and neuropilins also have the ability to bind VEGF. VEGFR-3 is expressed in lymph vessels and binds VEGF–C and VEGF –D, regulating lymphangiogenesis.[4] Neuropilins are integral receptors for blood vessel development; neuropilin null mouse embryos are unable to form a viable cardiovascular system.[4] Neuropilins (NP) are

found on endothelial cells and are VEGF-isoform specific in their binding; both NP-1 and NP-2 bind VEGF-A_{165}.[4] NP-1 may act as a co-receptor, enhancing the binding of VEGF-A to Flk-1 and increasing VEGF-mediated chemotaxis.[1,3,4] NP-1 does not bind $VEGF_{121}$; if $VEGF_{165}$ can bind to both NP-1 and VEGFR-2, this may partly explain why $VEGF_{165}$ is a better mitogen.[4]

Angiogenesis is essential for normal embryonic growth and, in healthy mature human individuals, VEGF is essential for normal wound healing, placental development, cyclical change in the female reproductive cycle, muscle and adipocyte tissue growth.[1,3,4,13,21] Angiogenesis begins with enzymatic degradation of the basement membrane of existing vasculature. Endothelial cells proliferate and then migrate toward the angiogenic stimulus, at the same time fibrin and fibronectin is extravasated.[3,4] This protein meshwork is thought to form a scaffold for new endothelial cells, which then bud into new vessels.[1,3,4] Pericyte coverage and lumen formation ensues.[3]

In vitro, the VEGF family promotes vasculogenesis of arteries, veins, and lymphatics; endothelial cells are stimulated to invade collagen gels and form capillary-like structures.[1] Both *in vitro* and *in vivo*, VEGF is a survival factor for endothelial cells, preventing apoptosis by inducing apoptosis inhibitors (Bcl-2, XIAP, survivin) in developing new vessels prior to coverage by pericytes.[1,3] This is particularly true in embryonic or newly formed vessels and, as they mature, vessel pericyte coverage takes place and dependence on VEGF for survival is lost.[1] VEGF-A isoforms 121, 145, and 165 all induce proliferation of endothelial cells and initiate *in vivo* angiogenesis.[4] In addition to being a mitogen and survival factor, VEGF-A induces vascular leakage and endothelial cell fenestration in some vascular beds.[1] Furthermore, VEGF induces secretion of interstitial collagenases such as matrix metalloproteinase-1 (MMP-1) such that endothelial cell invasiveness is facilitated.[3]

While endothelial cells are the primary targets of VEGF, non-endothelial cells such as retinal pigmented epithelial cells, pancreatic duct cells, and Schwann cells are also affected by VEGF activity.[1] VEGF stimulates release of alveolar cell surfactant and may have a neuroprotective role as well.[1] Finally, VEGF stimulates hematopoiesis, affects skeletal growth and endochondral bone formation.[1] Administration of anti-VEGF antibodies or sFlt-1 to mice results in glomerular

malfunction, endothelial cell detachment and hypertrophy.[1]

Although VEGF is necessary for normal vascular growth and maintenance, many studies have documented that overexpression of VEGF can lead to pathologic vascular proliferation. In humans, elevated plasma VEGF levels are correlated with tumor angiogenesis and metastasis, as well as immune-mediated disease such as lupus erythematosis and rheumatoid arthritis.[22-24] Circulating VEGF levels have also been used as a prognostic indicator in neoplastic disease.[25,26] In dogs, elevated plasma levels of VEGF have been noted in several neoplastic conditions.[27-33] More recently, dogs diagnosed with systemic inflammatory response syndrome (SIRS) that also had concurrent elevated VEGF levels were less likely to survive.[34]

The angiogenic influence of VEGF is regulated on several different levels, including gene promotion, translation and transcription, receptor availability and the local tissue environment. VEGF mRNA expression is up regulated by a number of cytokines, including epidermal growth factor, transforming growth factor α (TGF-α), transforming growth factor β (TGF-β), keratinocyte growth factor (KGF), insulin-like growth factor-1(IGF-1), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF).[1,4] Suspected autocrine or paracrine release of these factors, coupled with local hypoxia, are thought to regulate VEGF release into the microenvironment.[1] During tissue repair and inflammation, cytokines such as interleukin-1α (IL-1α) and interleukin-6 (IL-6), induce VEGF expression in several cell types, mediating angiogenesis and vascular permeability.[1,4] Hydrogen peroxide (H_2O_2), as released by neutrophils that invade the environment, potentiates VEGF production.[4] Likewise, UV-B radiation stimulates VEGF production as part of the wound repair mechanism.[4] Nitric oxide, itself up regulated by VEGF, stimulates VEGF production and the two molecules together additively affect vessel permeability and vasodilation.[1,4]

Tissue hypoxia is a potent stimulator of VEGF expression. Hypoxia inducible factor (HIF)-1 appears to be the main mediator of hypoxic VEGF induction.[4] When tissue oxygen tension decreases, HIF-1 binds to enhancer elements stimulating increased VEGF production.[1] HIF-1 is inactivated by the von Hippel-Lindau (vHL) tumor suppressor gene; this is evidenced by mutations in or inactivation of the vHL gene leading to increased VEGF expression as mediated

by HIF-1.[1,4] In addition, during hypoxia, VEGF mRNA is stabilized by proteins that bind to the 3' untranslated region[35], and evidence suggests that alternative transcription initiation sites exist in the 5' untranslated region and may be utilized during hypoxic conditions.[36]

Oncogenes affecting VEGF levels include mutations of *ras* and the *Wnt* signaling pathway associated with colonic adenomas.[1,4] p53 tumor suppressor gene normally inhibits VEGF production; mutations of p53 can, therefore, lead to increased production of VEGF.[3,4] Hormonal potentiators of VEGF gene expression include thyroid-stimulating hormone in carcinoma cell lines, adrenocorticotropic hormone, gonadotropins in the ovary, human chorionic gonadotropin (hCG) in leydig cells, androgen and progestins.[1,4]

Together, VEGF receptor expression and bioavailability of VEGF protein ultimately determine the extent of angiogenesis. Transcription of VEGFR-1 is enhanced by hypoxia, as is VEGFR-2 expression. Plasminogen activation can increase the bioavailability of VEGF protein. Heparin and heparinase can mobilize extracellular matrix-bound VEGF into the diffusible form where it can more easily activate receptors. And finally, VEGF competes with PlGF for sFlt-1. When PlGF is up regulated, it potentiates VEGF effects by displacing it from sFlt-1, making it more bioavailable to VEGFR-2.[1,4]

Inhibitors of VEGF expression include interleukin-10 (IL-10), interleukin-13 (IL-13), and intact tumor suppressor genes as previously mentioned (p53, vHL, *ras*, Wnt-signaling pathway). Neovascularization is further suppressed by interferon-α (IFN-α), interferon-γ (IFN-γ), interleukin-4 (IL-4), interleukin-12 (IL-12) and leukemia inhibitory factor (LIF), all of which block secretion of angiogenic cytokines and chemokines.[1,3,4,21] Vessel formation due to VEGF may be decreased or inhibited by corticosteroids, calcineurin inhibitors such as cyclosporine A and tacrolimus, and cyclooxygenase (COX) inhibitors.[37-43] And finally, as mentioned above, $VEGF_{165b}$ splice variants and the soluble sFlt-1 receptor may help maintain an anti-angiogenic state.[6,13,19,20]

B. Cornea

Vision begins with light entering the eye. The retina is stimulated, light energy is converted into

chemical energy via retinal photoreceptors, the central nervous system pathways relay this message to the brain, which summates and interprets this stimulus as a specific image. The cornea is instrumental in this visual process; if light is not efficiently and effectively conducted through the cornea, vision impairment results. In order to achieve its role in vision, the cornea must both allow light to pass and accurately refract the light such that it is appropriately focused on the retina. The cornea achieves these goals by maintaining the appropriate level of transparency, curvature, and thickness. Changes to any one of these three factors can affect structure and function, and therefore vision.

The cornea has four major components common to most species: epithelium, stroma, Descemet's membrane, and endothelium.[44-48] Humans and non-human primates have a fifth layer called Bowman's layer, which is a thin acellular zone located posterior to the epithelial basement membrane.[44-47] Temporal embryologic sequencing is crucial to correct development of the cornea. At approximately day 25 during embryologic development in the dog, the anterior edges of the optic cup migrate under the surface ectoderm as the lens vesicle detaches.[49] Surface ectoderm migrates into the defect left behind by the lens vesicle and becomes the epithelium.[46,49] Neural crest mesenchyme then fills the future anterior chamber and gives rise to the corneal stroma and endothelium.[45,46] The adjacent lens vesicle is necessary for endothelium induction, and collagen fibrils and fibronectin are secreted by developing stromal keratocytes.[46,49] The cornea is relatively transparent by day 65 of gestation in the dog and the eyelids open approximately 2 weeks after birth.[46,49]

The mammalian cornea is generally elliptical to circular in shape and has a greater horizontal diameter than the vertical diameter.[45] In the dog, the cornea is thickest peripherally, measuring approximately 0.5 to 0.65 mm.[45,50-52] Centrally, the cornea thins to 0.45-0.55 mm thick.[50,52] The cornea is innervated by the long ciliary nerve originating from the ophthalmic branch of the trigeminal nerve (cranial nerve V).[45,52-54] The anterior stroma and epithelium are richly innervated, with a nerve plexus located both anterior and posterior to the epithelial basement membrane.[46,47,53,55] The superficial layers also have pain receptors and the stromal layers have pressure receptors.[52]

The corneal epithelium in the dog is composed of three cell morphologies.[44-46,48,52] The basal cell layer is composed of a single layer of columnar cells.[44,45,48,52] The adjacent cell surfaces have small interdigitating infoldings with desmosomal attachments and are attached to its secreted basement membrane via hemidesmosomes, laminin, and anchoring collagen fibrils.[48,52] Within the epithelium, the basal cells are the only cells to undergo mitosis.[45,52] Basal cell numbers are replenished from progenitor cells at the limbus and migrate centripetally at approximately 120 μm/week.[46] The basal cell layer turns over every 7 to 10 days.[46,52]

Anterior to the basal cell layer lie 2-3 layers of polyhedral cells referred to as wing cells.[45,46,48,52] Similar to the basal cell layer, adjacent cells are attached by hemidesmosomes.[48] Two to three layers of non-keratinized squamous cells form the topmost layer of the epithelium with interdigitating processes in the canine species.[48] In other domestic species, more layers may be present.[52] Squamous cells are connected to each other via zonula occludens tight junctions, preventing movement of fluid and ions from the tear film into the corneal stroma and protecting the deeper layers of the cornea from pathogens.[46,52] Squamous cells contain surface microvilli, to which the mucous/glycocalyx layer of the tear film is attached.[52,56] Other cells in the epithelial layer include leukocytes of hematopoietic origin[52], thought to be inactive dendritic cells.[57] These leukocytes are located throughout the epithelium, although greater numbers are found closest to the limbus.[57]

Subjacent to the epithelium is Bowman's layer, which is most prominent in human and non-human primate corneas.[46,47,52] It is a thin acellular zone in the anterior stroma produced by the epithelium and is thought to confer stiffness and strength to the cornea.[46] In most domestic species, however, Bowman's layer is a small area of randomly oriented collagen fibrils.[47,48] Of the domestic species, ruminants have the closest approximation to a true Bowman's layer in the cornea.[47,52]

The corneal stroma comprises approximately 90% of the corneal thickness and lies posterior to the epithelium.[52] It is composed of multiple layers of fibrous lamella, each of which are made up of parallel collagen fibrils that run the diameter of the cornea.[44-46,48,52] The lamellae are oriented at angles to each other, with the more anterior layers lying at acute angles to each other and

marked interweaving compared to the posterior layers.[46,52] The posterior two-thirds of the corneal lamellae lay at increasingly orthogonal angles to each other.[46,48,52] Type I collagen is the predominant form found in the stroma, although type III, V, VI, and XII are also found; type III and XII are thought to be present during development.[52] Fibrils are made of collagen, and glycosaminoglycans and other glycoproteins interconnect the fibrils.[46,52] The stroma is relatively acellular, but fibrocytes (keratocytes) are found between lamellae and few leukocytes are also present.[46,52] The keratocytes have a dendritic shape and communicate with each other via gap junctions.[46]

Descemet's membrane lines the interior of the stroma and is secreted by a single monolayer of cuboidal cells, the endothelium.[46,52] Despite macula occludens tight junctions and lateral gap junctions, the endothelium is a high-permeability barrier – in contrast to the epithelium – that allows certain ions and water to pass through.[46] Na^+/K^+ ATPase, and possibly carbonic anhydrase, pump sodium and calcium ions into the aqueous humor and corneal stromal water passively follows.[45,46,52,58] The corneal stroma maintains a state of relative dehydration as a result of the endothelial energy-consuming pump action. Endothelial cells do not proliferate over time; instead, endothelial cell density has been shown to decrease with age in multiple species.[50,51,59,60] As endothelial cells die, surrounding adjacent cells hypertrophy and merge to fill in any gaps left behind. This results in increasing polymegthism and polymorphism from their original homogenous hexagonal shape.[51,52,60]

The aqueous humor, limbal vessels, and tear film supply the majority of nutrition for the avascular cornea.[46,58] The corneal epithelium acquires oxygen for aerobic glycolysis from the tear film and glucose from both the limbal vessels and tear film.[46,58] The endothelium and deep stroma obtain oxygen from the aqueous humor.[46,58] The keratocytes are relatively inactive and thus have few metabolic needs. In contrast, the endothelium has a high metabolic need as a result of its pump activity. Endothelial cells contain a large amount of mitochondria and endoplasmic reticulum, and primarily utilize anaerobic glycolysis, the citric acid cycle, and the pentose phosphate pathway for energy needs.[46,58]

As would be expected, the mechanism of corneal healing depends on the layer that has sustained

damage. Following epithelial loss, the first phase of epithelial wound closure consists of nearby cells sliding into the defect, whether the defect is superficial or deep stromal.[45,46] The epithelium then proliferates, both vertically through basal cell mitosis and horizontally via centripetal migration of progenitor basal epithelial cells.[45,46,52] In contrast, the corneal endothelium in most species does not undergo mitosis; rather, nearby cell hypertrophy compensates for adjacent cell loss.[45,46,52]

Epithelial and endothelial repair is completed relatively quickly, in days to weeks. Stromal repair, in contrast, occurs over months to years. When the stroma is initially wounded, edema of the collagen matrix follows and surrounding keratinocytes become metabolically activated.[45,46,58] Inflammatory cells migrate into the area within a few hours in response to keratinocyte death from the original wound.[45,58] Activated keratinocytes undergo fibroblastic change, producing collagen, glycosaminoglycans, and fibronectin, which stimulate cell adhesion, migration, and protein synthesis.[45,46,52] The first phase of stromal healing results in the production of a disorganized stromal matrix (scar); this occurs in approximately the first 6 months of stromal healing.[46] The second phase of healing is thought to occur over years, and is called the remodeling phase. Hyaluronic acid fills interlamellar spaces and gaps, gradually decreasing as proteoglycans are reformed and normal collagen fibril spacing is returned.[52] During this time, the cornea continues to remodel, resulting in improved corneal transparency and increased wound strength.[46] Various growth factors and cytokines are produced by the epithelium and other tissues and conveyed by the aqueous tear film, including epithelial growth factor (EGF), fibroblast growth factor (FGF), interleukin-1 (IL-1), nerve growth factor (NGF), transforming growth factor beta (TGF-β), insulin, and retinol, all of which facilitate healing, particularly in the early stages.[45,46] TGF-β is thought to be one of the most important of these factors, stimulating fibrotic repair and helping to create corneal stromal scarring.[46] Over time, matrix metalloproteinases (MMPs) foster remodeling of collagen lamellae such that the ultimate long-term outcome is optimization of corneal clarity.[61] Corneal vascularization occurs at various stages of corneal insult and its subsequent repair, made possible by the various factors discussed later in this section.

As previously stated, optimal vision begins with the clear cornea. In fact, under normal

conditions, 99% of light entering the cornea is transmitted.[52] Factors contributing to corneal clarity include a non-keratinized epithelial layer, regularly arranged small collagen fibrils, hypocellularity of the corneal stroma, keratocytes with few intracytoplasmic organelles and water soluble cytoplasmic crystallins, and a relative state of dehydration.[45] In addition, the healthy cornea contains no blood vessels or lymphatic vessels, no pigmentation, and relatively few white blood cells.[45,46,52,61]

Although several characteristics allow the cornea to be clear, avascularity of this specialized tissue is maintained via a balance of anti-angiogenic and pro-angiogenic factors. Anti-angiogenic factors include angiostatin, endostatin, pigment epithelium derived factor (PEDF), Fas ligand (Fas-L), and thrombospondin-1 (TSP-1).[61-65] Angiostatin is a 38 kDa fragment of plasminogen that inhibits proliferation of endothelial cells and tumor growth by up-regulating apoptosis in cells undergoing mitosis; however, it does not affect resting endothelial cells.[66] In humans, plasminogen is produced locally by the corneal epithelium[67] and angiostatin is present in tears.[63] Angiostatin is one of the few anti-angiogenic molecules studied in multiple species. A recent study by Pearce et al in 2007 evaluated the normal eyes of cats, dogs, cattle, horses, pigs, and rats for expression of angiostatin in ocular tissues. Angiostatin was present in the corneal epithelium of all species but cattle.[65] Endostatin inhibits fibroblast growth factor and VEGF-induced corneal neovascularization.[63] PEDF is present in corneal stroma and inhibits basic fibroblast growth factor (bFGF)-induced vascularization.[61,63,64] PEDF has been detected in the tear film in healthy human patients.[63] Fas-L is highly expressed in human cornea, inducing apoptosis of invading inflammatory and endothelial cells that are Fas-L positive.[61,68] Fas-L thus serves as a barrier of sorts, providing the corneal protection from angiogenesis.[61] TSP-1 is normally present in the human cornea and reduces secretion of VEGF from macrophages.[61,64]

Genes thought to play a role in corneal clarity include Kruppel-like transcription factor (Klf-4), the paired box gene family group 6 (*PAX6*), and Destrin.[61] Klf-4 is responsible for maintaining epithelial integrity and normal corneal hydration.[61] Corneas in Klf-4 null mice lose transparency and develop corneal edema and fragile epithelium.[61] Mutations in the *PAX* gene family result in ocular malformations, anterior segment dysgenesis, and spontaneous corneal neovascularization in humans.[69] Autosomal recessive mutations in Destrin leads to corneal epithelial hyperplasia,

neovascularization, and lymphatic vascularization in mice.[61]

Although VEGF-A is primarily responsible for mediating angiogenesis, complex dynamics between VEGF and its receptors, and potentially its splice variants, also have an anti-angiogenic function. Work done by Ambati et al in 2006 and 2007 resulted in the theory that sFlt-1, the soluble form of VEGFR-1 (Flt-1) receptor, functions as a decoy receptor, thereby maintaining corneal avascularity.[19,20] They proposed that sFlt-1 binds available VEGF, rendering it less available for binding the pro-angiogenic receptor VEGFR-2.[19,20] The Florida manatee *(Trichechus manatus latirostris*), has a naturally vascularized cornea, arising during the prenatal period and persisting into adulthood.[70] The Antillean manatee (*Trichechus manatus manatus*) also has a vascularized cornea.[19] Interestingly, sFlt-1 is not expressed in the corneal tissue of the Florida or Antillean manatee.[19] Furthermore, the dugong (*Dugong dugon*) and the Asian (*Elephas maximus*) and African (*Loxodanta africana*) elephants – the phylogenetically closest related species to the manatee – do not have vascularized corneas, and express sFlt-1 in their corneal tissue.[19]

In addition to sFlt-1, VEGFR-1 and certain splice variants of VEGF are thought to have anti-angiogenic activity. While controversial, the splice variant of $VEGF_{165}$ known as $VEGF_{165b}$ is thought to bind to VEGFR-2 with the same affinity as $VEGF_{165}$. However, it does not fully activate the receptor and is thus proposed to be inhibitory in nature.[6,13]

While the pre-eminent factor controlling angiogenesis appears to be VEGF, other chemokines, interleukins, MMPs and molecules also play a role. These factors have been documented in both humans and animal models. Blood supply to the cornea originates from the ophthalmic artery (primarily internal ophthalmic in humans, and external ophthalmic in canines)[71], proceeds to the ciliary arteries, and terminates as superficial vessels near the limbus.[52,63,71] Corneal vascularization arises from these peri-limbal vessels.[63] VEGF is required for angiogenesis and is most often induced by local hypoxia or inflammation. Corneal tissue, tear film, inflammatory cells, and other ocular tissues are all potential sources of VEGF. Vascular endothelial cells proliferate when stimulated by VEGF and invade the tissue, forming early vessels. The tissue is made more permeable to budding vessels as MMPs degrade extracellular matrix and vascular

basement membranes.[61,63,64] MMPs are proteolytic enzymes located in the extracellular matrix and can behave as pro-angiogenic molecules under the correct conditions.[63] They are expressed by corneal fibroblasts and, although they are present at the onset of tissue damage, they continue to be expressed several months after the injury.[61,63,64]

During corneal insult, inflammatory mediators (IL-1, TNF-α) and cells infiltrate the corneal tissue, stimulating further angiogenesis. Local nitric oxide (NO) causes vasodilation and interacts with VEGF, as does the prostaglandin-cyclooxygenase system.[63] Macrophages enhance inflammation by recruiting more inflammatory cells and producing pro-angiogenic factors themselves, such as multiple VEGF isoforms and macrophage migratory inhibitory factor.[61,63] This promotes endothelial cell interactions and increases the angiogenic effects of leukocytes. Basic fibroblast growth factor (bFGF) stimulates proliferation and migration of endothelial cells, as well as extracellular matrix degradation.[61,63,64]

In canines, corneal vascularization arises due to a variety of conditions. These include immune-mediated or inflammatory processes, neoplasia, chronic irritation, chemical burns, ulceration and perforation, as well as intraocular disease (e.g. glaucoma, uveitis).[45,72,73] Vascularization may precede or occur secondary to corneal lipid or calcific corneal degeneration.[45,74] Immune-mediated corneal disorders include chronic superficial keratitis (CSK, otherwise known as pannus or Uberreiter syndrome), superficial punctate keratitis, and the more general clinical diagnosis of immune-mediated keratitis, often used in cases where no specific underlying clinical syndrome can be diagnosed.[45,72]

CSK is associated with the German shepherd, Belgian shepherd, Greyhound and other breeds. CSK is characterized by profound progressive, potentially blinding corneal vascularization and inflammatory cell infiltration, originating most commonly at the ventral-lateral limbus.[75-77] $CD4^+$ lymphocytes and plasma cells are the predominant cell types present.[78] Ulceration of the epithelium is not usually a feature of the disease. UV light damage to corneal proteins with subsequent immune attack has been proposed as the underlying cause.[76,79,80] High altitude and an early age of onset have been correlated with increased disease severity and diminished response to treatment.[45,72,75,77,80]

Superficial punctate keratitis is an immune-mediated disease that presents with superficial punctate corneal opacities or defects, often bilateral and symmetrical.[45,72] It can be ulcerative or non-ulcerative, often is recurrent and can progress to involve the entire cornea. Vascularization is not a major component of the disease, but can be present.[72] It is most commonly seen in longhaired dachshunds and, as is the case with CSK, UV light is thought to play a role in the pathogenesis of this disease.[45,72]

Immune-mediated keratitis, while better defined in the horse, occurs in the canine as well. The term is generally used as a clinical diagnosis when a classic syndrome such as CSK cannot be diagnosed and other underlying causes cannot be identified. It is characterized by mild to severe vascularization of the cornea, beginning at the limbus and extending axially. The vascular response often includes the entire cornea. Inflammatory infiltrates can sometimes accompany the vessels. Such keratitis has been seen at the Virginia-Maryland Regional College of Veterinary Medicine in conjunction with pigmentary keratitis, post-operative phacoemulsification, possible allergic conjunctivitis, and in various breeds. Keratitis can also be seen as an extension of scleritis (necrotizing or nodular granulomatous) or episcleritis, both of which are also defined as immune-mediated diseases.[45,73]

Vascularization may accompany neoplastic cell infiltration of the cornea. Squamous cell carcinoma, lymphoma, and hemangioma/hemangiosarcoma have all been reported in the dog and corneal tumor infiltration may be associated with vascularization.[45] Chronic frictional irritation from hairs abrading the corneal surface (i.e. entropion, trichiasis, ectopic cilia), chronic tear film deficiencies, whether qualitative or quantitative as in keratoconjunctivitis sicca (KCS), and inadequate distribution of tear film across the cornea due to absence of lid protection (i.e. exposure keratitis, neuroparalytic keratitis) can also lead to corneal vascularization. This neovascularization likely occurs through both hypoxic and inflammatory mechanisms as the corneal tissue responds to repeated abrasion and damage.

Ulceration of the cornea often leads to vascular infiltration, again likely through inflammatory mechanisms. The severity of vascularization associated with ulceration depends on a number of

factors, including depth and chronicity of ulceration, as well as whether complicating factors, such as infection or keratomalacia, occur. Corneal lipid or calcific degeneration secondary to pathologic corneal change is often preceded or accompanied by vascularization.[45,74] Degeneration occurs after the cornea has sustained damage or inflammation and may result from various chronic corneal conditions.[45,74] Finally, intraocular diseases such as anterior uveitis and glaucoma can lead to corneal vascularization, arising from deep limbal vessels in this instance. Corneal vascularization may also accompany anterior lens luxation. Vascularization in this case is most likely a result of uveitis and keratitis secondary to the displacement of the lens itself.

Current therapy to reduce corneal vascularization in dogs includes topical corticosteroids, topical immunomodulators such as tacrolimus and cyclosporine A.[45,81] Corticosteroids are used to treat corneal inflammation and cause regression of corneal vascularization. Corticosteroids exert their effects on the cyclooxygenase and lipooxygenase pathways, both of which occur in the cornea and conjunctiva. Metabolites of these pathways (prostaglandins, thromboxanes, arachidonic acid, and leukotrienes) are inhibited or attenuated by the use of corticosteroids.[81,82] Corticosteroids impair acute inflammation by decreasing vasodilation, reducing capillary permeability, inhibiting leukocyte migration, and decreasing fibroblast formation.[81] Topical ophthalmic corticosteroids come formulated in acetate, alcohol, and sodium phosphate formulations. Acetate and alcohol formulations tend to have better corneal penetration than do sodium phosphate formulations.[81] Common topical corticosteroids used in veterinary medicine include prednisolone acetate or phosphate and dexamethasone alcohol or phosphate products. Corticosteroids are clinically used to diminish corneal vascularization resulting from many of the disease entities listed above, including immune-mediated keratitis, scleritis, episcleritis, chronic irritation, and neoplastic infiltrates.

The topical immunomodulators tacrolimus and cyclosporine A inhibit signal transduction pathways that lead to T-cell activation.[81,83] Tacrolimus and the related drugs pimecrolimus and rapamycin are macrolide antibiotics derived from *Streptomyces spp*, whereas cyclosporine A is isolated from the fungus *Tolypocladium inflatum*.[83] Both types of drugs inhibit the calcium-activated protein phosphatase calcineurin, albeit through slightly different binding mechanisms. Calcineurin is necessary for appropriate transcription of lymphokine mRNA expression; thus

these immunomodulators more specifically affect inflammation mediated by T-cells.[81,83]

Despite the use of these topical drugs, corneal vascularization in canine patients may persist or worsen in the face of treatment. In an attempt to maintain or improve vision in canine patients, it is important to expand our understanding of the molecular drivers of corneal vascularization in these wide-ranging disease conditions in order to propose alternative therapeutic modalities that may target the mediators of corneal vascularization. Evaluation of levels of VEGF in pre-corneal tear film and its role in canine corneal vascularization is therefore the purpose of this study.

C. Ocular tear film

The precorneal tear film in the dog is approximately 7-10 μm thick and has several different functions, all of which are integral to maintaining corneal transparency and health.[84] It provides a clear refractive surface, removes debris and exfoliated epithelial cells, prevents bacteria and pathogens from adhering to the corneal surface, and nourishes the cornea.[84] It is composed of 4 distinct layers: a glycocalyx layer, a mucous layer, an aqueous layer, and a lipid layer.[58,85] Its secretion is regulated by both parasympathetic and sympathetic nerves that innervate the lacrimal gland, cornea, and conjunctiva.[84,85] Secretion is balanced by evaporation of tears from the corneal surface and drainage of tears through the nasolacrimal duct system.

The glycocalyx layer closest to the corneal surface is composed of a network of polysaccharides emanating from the epithelial microvilli.[56,58,85] Proteins attached to the carbohydrate side chains, referred to as mucins, are categorized as being either membrane spanning or secreted.[84-86] Membrane-spanning mucins create the glycocalyx itself by having an intracellular tail and a membrane-spanning domain. They are produced by and adhered to the stratified squamous cells of the cornea and conjunctiva. Membrane-spanning mucins are stored in small secretory vesicles in the cytoplasm; secreted mucins are either gel-forming molecules secreted from goblet cells or soluble mucins secreted by the lacrimal gland.[85] Together, the epithelial microvilli, glycocalyx and mucins hydrate the cornea, stabilize the tear film, and prevent pathogen adherence.[56,84,85]

The mucous layer is comprised of gel-forming mucin, membrane-spanning mucin, proteins,

electrolytes and water.[58,85,86] Mucin is secreted by the goblet cells of the conjunctiva that are most densely located in the nasal lower lid and fornix in the canine.[84,87] Electrolytes and water are secreted by stratified squamous cell and goblet cells of the conjunctiva.[85] The mucous layer is distributed through blinking and drains down the nasolacrimal duct. It provides a smooth refractive surface, holds the aqueous layer to the epithelium, decreases shear forces, and contains urea, salts, glucose, immunoglobulins, enzymes, leukocytes and cellular debris.[58,84] Goblet cell secretion is controlled by both the number of goblet cells present as well as sensory nerve stimulation in the cornea and conjunctiva. Sensory stimulation induces a neural reflex, leading to stimulation of parasympathetic and sympathetic efferent axons, which then results in mucin secretion.[85]

Tubuloacinar glands of the lacrimal gland, accessory lacrimal glands, and the nictitating membrane secrete the aqueous portion of the tear film. Individually, they are responsible for 61.7%, 3.1%, and 35.2% of the aqueous secretions, respectively.[88] The aqueous layer supplies glucose, oxygen, electrolytes, and water to the superficial cornea. Aqueous tear removes metabolites such as carbon dioxide and lactic acid as well as debris and bacteria.[84] The release of aqueous tear in humans is also triggered by sensory stimulation, again followed by a neural reflex resulting in parasympathetic and sympathetic nerve stimulation of the lacrimal gland.[85]

The lipid layer is the fourth and outermost layer of the pre-corneal tear film. It is produced by the meibomian glands located along the lid margins. The meibomian glands are modified sebaceous glands with acini that lead to a central ductule. The openings of the ductules are visible on the lid margin. There are approximately 20-40 glands along the lid margin in a dog. Lipids are stored in intracellular vesicles, and cells burst to release their contents in a holocrine fashion. Secretions are composed of lipid and protein and are referred to as meibum. Meibum should be liquid at lid temperature and it is generally released via blinking action of the lids. Although blood vessels and nerves surround acini, the precise neural and hormonal control of lipid release is not known. The lipid layer reduces evaporation of the tear film, aids in corneal/conjunctival lubrication, enhances stability of the tear film, prevents tear overflow, and helps form a water-tight seal during closure of the eyelids.[84,85]

The tear film contains a variety of cytokines, proteins, and inflammatory cells with the critical function of supporting corneal homeostasis. The aqueous layer is 98.2% water and 1.8% solids, including electrolytes, glucose, urea, polymers, and proteins.[84] In humans, several attempts have been made to quantify and characterize the tear film protein profile. Membrane array characterization has identified at least 80 chemokines, cytokines, and growth factors.[89] Constituents include IL-2, IL-4, IL-5, IL-10, IFNγ, TNFα, epidermal growth factor, lactoferrin, plasminogen, amylase, lysozyme, and VEGF.[89-92] VEGF is a normal component of the tear film in humans, with reported basal concentrations ranging from approximately 2.4 to 3.0 ng/mL,[93] 5-6 ng/mL,[94] and more recently as high as 19 ng/mL.[95] Elevated levels have been noted after surgical procedures such as keratectomy and in association with keratitis and conjunctivitis.[95-97]

The tear film in dogs is typically measured clinically via the Schirmer tear test (STT). This comprises use of a small piece of wicking paper placed in the conjunctival sac for one minute. When done without topical anesthesia, the paper irritates the cornea and conjunctiva, and by doing so, stimulates and measures reflex tearing. When the paper is placed in the conjunctiva of an individual that has been topically anesthetized, the paper is thought to wick only basal levels of tearing. These are known as the STT I and II.[98,99]

In animals, fewer studies have evaluated tear film proteins, and, as a result, knowledge of the protein profile is likely incomplete at this point in time. In dogs, IgA, IgG, IgM, albumin, lysozyme, lactoferrin, lipocalin, epidermal growth factor, transforming growth factor, cells, transferrin, ceruplasmin, glycoproteins, matrix metalloproteinases, and antibodies have all been identified in canine tears.[100-109] Canine tear film has not yet been evaluated for the presence of VEGF. Therefore, the purpose of the study conducted and reported here is to identify/quantify VEGF in canine pre-corneal tear film and to determine if the level of VEGF increases with corneal neovascularization.

II. CHAPTER 2: LITERATURE REVIEW

A. VEGF in human ocular disease

Within the context of human ophthalmology, VEGF protein is expressed in several ocular tissues, including retinal pigmented epithelium, Müller cells, vascular endothelium and uvea, ganglion cells, and corneal endothelium, epithelium, and, albeit weakly, keratocytes.[2,110,111] VEGFR-1 receptors are expressed in monocytes, vascular endothelial cells, and corneal endothelial cells; VEGFR-2 receptors are found on vascular endothelial cells, hematopoietic stem cells, and retinal cells.[4,111] Under neoplastic, hypoxic, and inflammatory conditions, angiogenic balance is tipped in favor of ocular vascularization, often with blinding and painful sequellae. Age-related macular degeneration, retinopathy of prematurity, diabetic retinopathy, and various corneal diseases are theorized to have a critical vascular proliferative component largely mediated by VEGF.[2,111-113]

Age-related macular degeneration (AMD) is the leading cause of blindness worldwide. The neovascular form of AMD only comprises approximately 10% of the cases of AMD, but accounts for approximately 90% of cases afflicted with blindness.[2,114] It is associated with neovascularization originating from the choroidal vasculature (choroidal neovascularization, CNV) which extends into the subretinal space. Functional vision impairment occurs particularly when CNV occurs beneath the macula.

There is a genetic predisposition associated with AMD; however, since the disease tends to develop later in life, environmental factors are thought to play a role as well.[2] The widely accepted theory that VEGF mediates pathologic choroidal vascularization is supported by three main facts: VEGF is increased secondary to stimuli such as oxidative stress and inflammation that have been linked to AMD and CNV; pigment epithelium derived factor plays a role in AMD/CNV and is known to regulate VEGF expression and receptor binding; and finally, success of anti-VEGF therapy as a treatment for AMD/CNV suggests that VEGF is a primary mediator of the condition.[2]

In contrast to AMD, pathologic vascularization in retinopathy of prematurity (ROP) and

proliferative diabetic retinopathy (PDR) originates from the retinal vessels and occurs due to retinal hypoxia and ischemia, respectively.[1,2,114] In the neonate, VEGF and placental growth factor mediate normal developmental retinal vascularization that is generally complete at birth. Premature individuals, however, have an incompletely vascularized retina at birth. Normal retinal vascular growth and development is hindered by supportive therapeutic oxygenation shortly after birth. The increased percentage oxygen inhaled with the subsequent hyperoxic retinal microenvironment signals down regulation of VEGF and thus decreased normal developmental retinal vascularization.[1,2,4,114] After oxygen therapy is discontinued, however, the retinal microenvironment changes to one of hypoxia, stimulating increased VEGF expression and the formation of hyperpermeable retinal vessels that grow into the vitreous.[1,2,4,114] Vision disturbance stems from a number of consequences, including vessel hemorrhage into the vitreous and, in some cases, vitreal traction band formation resulting in retinal detachment.[1,2,114]

Diabetic retinopathy is the most frequent complication of diabetes mellitus and is a leading cause of blindness in developed countries.[2] Up to 75% of patients have signs of diabetic retinopathy within 15 years of diagnosis.[2] Clinical progression is related to ischemia. Oxidative damage and inflammation affects retinal vessels; leukocytes adhere to vessel walls, platelets aggregate, and retinal blood flow is altered.[1,2,4,114] Retinal capillaries become occluded, which leads to local hypoxia and increased VEGF production. VEGF stimulates existing vessels to become leaky, unstable, and non-perfused, resulting in microaneurysms and hemorrhage.[1,2,4,114] Proliferative neovascularization can also occur across the internal retinal surface and pathologic capillaries may extend into the vitreous. Similar to ROP, intraretinal and intravitreal hemorrhage may occur and tractional retinal detachment may follow.[1,2,4,114]

In addition to traditional therapies such as laser photocoagulation or anti-oxidants, which slow or stop posterior segment vascularization, anti-VEGF therapies are being developed to address the underlying pathogenesis of these chorioretinal vasculopathies. Injectable anti-VEGF therapies have shown great promise in reversal of or slowing the progression of ocular vascularization in humans and in animal models.[1,2,4,115-118] VEGF can be targeted in multiple ways: inhibition of factors thought to up regulate VEGF; agents that reduce VEGF production; agents that bind VEGF, such as monoclonal antibodies or aptamers; and finally, agents that inhibit VEGF

receptors.[1,2,4] Particularly in diabetic retinopathy, the enzyme protein kinase C-beta (PKC-β) up regulates VEGF production.[2] The drug LY333531, a PKC-β-specific inhibitor, has shown promise in large clinical trials in reducing vision loss and the need for laser treatment in PDR.[2] Agents that reduce VEGF production include squalamine and rapamycin. Squalamine is an anti-neoplastic and anti-angiogenic amionsterol; rapamycin is a macrolide fungicide. Both have been found to decrease CNV in a laser-induced injury model in the rat.[1,2]

Probably most well known anti-VEGF therapies are the treatments comprising the group of monoclonal antibodies that bind VEGF. Bevacizumab (Avastin®), an antibody that has been approved for use in the systemic treatment of some cancers, has also been injected intravitreally for the successful treatment of AMD and PDR.[2,116,119,120] Ranibizumab (Lucentis®) is also an FDA-approved monoclonal antibody used to treat AMD via intraocular injection. Macugen® (pegaptanib sodium) is an aptamer that preferentially binds to the heparin-binding domain of the VEGF protein and has been successfully used for AMD.[115] It is the first drug approved for intravitreal use and has been marketed to selectively inhibit the 165 isoform of VEGF.[2,115,121] Another form of VEGF binding therapy is a molecule referred to as VEGF Trap (aflibercept). VEGF Trap is a combination molecule, bringing together the immunoglobulin binding domains of VEGFR-1 and VEGFR-2 fused to the Fc-fragment of human IgG.[121] It has a high affinity for all forms of VEGF and PlGF, both in tissues and in circulating fluids.[121] Finally, since VEGFR-2 mediates a majority of angiogenic processes, agents that inhibit its function have also been developed. SU5416 has been shown in animal models of CNV to effectively inhibit the VEGFR-2 kinase component.[1,2]

Abundant evidence exists supporting the role of VEGF in posterior segment disease and the pathogenesis of several conditions has been well studied, as outlined above. Elevated VEGF levels in the posterior segment, however, can also lead to vascular proliferative disease in the anterior segment of the eye. The structures likely to be vascularized include the iris and the iridocorneal angle. Vascular proliferation in this region of the eye may then lead to decreased aqueous humor outflow and potential neovascular glaucoma; this form of glaucoma often results in a devastating visual outcome.[122-125] Studies performed in nonhuman primates suggest that iris neovascularization can be mediated by elevated VEGF concentrations in the vitreous resulting

from widespread posterior segment ischemia.[122,125] It is generally regarded that neovascular glaucoma usually occurs secondary to ischemic posterior segment diseases such as central retinal vein occlusion and proliferative diabetic retinopathy.[123,124]

Corneal neovascularization can also lead to vision impairment due to disorganization of corneal collagen fibers resulting in changed refractive status and transparency. Pathologic vascularization of the cornea originates from the conjunctival and episcleral limbal vessels.

In humans, several inflammatory and vascularizing corneal conditions have been associated with increased VEGF levels. In 2000, work done by Philipp et al demonstrated increased VEGF concentration within vascularized corneas as compared with normal control corneas.[111] In addition, expression of VEGF protein was increased in inflamed corneas, particularly within corneal epithelial cells and vascular endothelial cells of newly formed vessels in the stroma.[111] Further work done by Kvanta et al in 2000 and Amano et al in 1998 also documented increased VEGF expression in a rat model of corneal inflammation.[110,126] Increased VEGF corneal levels have been associated with infectious keratitis such as Herpes Simplex Virus (HSV), Herpes Zoster keratitis, and fungal keratitis; atopic keratoconjunctivitis; vascularized post-traumatic or post-ulceration scars; rejected allografts; chemical burns; and corneal surgery such as photorefractive keratectomy.[111,113,127-130]

The most studied corneal vascularizing disease associated with increased VEGF protein expression is Herpes Simplex Virus (HSV). A recent review by Giménez et al postulated that surface ocular infection likely results from direct inoculation of the eye by HSV-1, but HSV-1 can spread to the eye from other areas of the body in immunocompromised individuals.[127] Initial infection results in replication of the virus in epithelial cells for 5-6 days, followed by the virus establishing latency in the trigeminal ganglion. The review further states that recrudescence of latent HSV-1 occurs intermittently, with repeated epithelial inflammatory episodes potentially progressing to stromal keratitis. Stromal keratitis is most likely to impair vision by corneal vascularization and inflammatory cell infiltration, and is typically more difficult to control than surface corneal disease. Infection leads to increased expression of chemokines and VEGF protein, although their source remains unknown. Potential sources include infected epithelial

cells, adjacent uninfected epithelial cells, or infiltrating inflammatory cells.[113,127] Much of the HSV-1 keratitis pathology is T-cell induced; T-cells may be reacting to damaged stromal tissue auto-antigen formation in the cornea and are also responsible for releasing IL-17, a chemokine which contributes to VEGF production in the local environment.[127,131] Vascularization may be due to the increase in VEGF protein itself and/or a decrease in binding activity or decreased production of sFlt-1, a decoy receptor that binds VEGF and thus prevents vascularization.[112,127]

Sources of corneal VEGF studied both *in vitro* and *in vivo* include inflammatory cells invading the cornea (both polymorphonuclear cells and macrophages), corneal epithelial cells, corneal stroma, and vascular endothelial cells.[110,111,113,126,128,131-133] The corneal tear film, owing to its close proximity to the cornea, conjunctiva, and limbus, is also a potential source of both VEGF and soluble receptors, as well as vascularization potentiating chemokines. VEGF has been evaluated in tears following pterygium removal and photorefractive keratectomy in man. VEGF concentrations of tears surrounding eyes with the sclera left exposed following pterygium removal had increased levels of VEGF compared to levels prior to surgery as well as compared to those whose surgical site was grafted with amniotic membrane.[96] Individuals treated with photorefractive keratectomy also had significantly increased tear levels of VEGF following the procedure.[95,134] Most recently, patients with vascularized corneas due to limbal stem cell deficiency were documented as having elevated tear film VEGF levels as compared to patients with normal corneas.[94]

In human ophthalmology, anti-VEGF therapies have been developed as topically applied or subconjunctival injectable forms to combat corneal neovascularization. Bevacizumab and ranibizumab have been used with positive short-term results. Overall efficacy of anti-VEGF therapy for corneal vascularization is likely influenced by degree of scarring, chronicity, and extent of corneal vascularization.[135-139]

B. VEGF in canine ocular disease

The role of VEGF in canine ocular neovascularization is just beginning to be elucidated. In contrast to many chorioretinal vascularizing human ocular diseases, proliferative vascularization of the canine eye is not commonly seen in the posterior segment. Only one report has

documented the existence of a rare syndrome of intravitreal neovascular proliferation with presumed secondary glaucoma.[140] More typically, canine inherited or breed-related vitreoretinopathies lead to membrane formation within the vitreous and adjacent retina, but vessel formation is not a prominent feature.[141-143]

Similar to anterior segment vascularization described in humans, however, proliferative angiogenesis in the domestic dog frequently occurs on the iris face or the cornea. Surface iris vascularization is commonly referred to as pre-iridal fibrovascular membrane (PIFM) formation. As with the phenomenon in humans, PIFM formation in dogs often leads to complications that may cause ocular pain and/or blindness and sometimes necessitates eye removal.

Pre-iridal fibrovascular membrane formation was first described in domestic animals by Peiffer et al in 1990.[144] More than 1400 enucleated globes from dogs, cats, horses, and cattle were histologically examined for this feature. When present, pre-iridal membranes appeared to arise from endothelial buds in the anterior iris stroma, a conclusion confirmed by a later study examining the characteristics of PIFM formation immunohistochemically.[145] Peiffer et al further described the membranes as extending over the face of the iris and, in some cases, the iridocorneal angle; membranes were characterized as cellular, vascular, or fibrous.[144] The study further speculated on membrane pathogenesis and significance by evaluating the percentage of enucleated globes affected with PIFM. Approximately 14% of globes affected with chronic glaucoma, 21% of globes with retinal detachment, and 29% of globes with intraocular neoplasia had developed PIFM.[144]

From a clinical perspective, extension of the membrane across the iridocorneal angle was a particularly interesting finding, suggesting that secondary glaucoma may be a direct consequence of PIFM formation. Furthermore, several intraocular diseases were now linked to fibrovascular membrane formation. It was not clear, however, whether the PIFM was a cause of the pathology or a result of it. A later study by Moore et al in 2003 evaluated canine eyes enucleated or eviscerated following complications after phacoemulsification surgery.[146] Reasons cited for enucleation or evisceration were glaucoma and uveitis, affecting 86% and 82% of eyes, respectively. Histologically, 86% of the globes had PIFM development.[146] Prior to surgery, no

globes had clinically detectable glaucoma or PIFM formation.

Another study by Scott et al in 2013 looked specifically at eyes enucleated due to intractable secondary glaucoma development post-phacoemulsification surgery across a variety of breeds.[147] Evidence of PIFM formation was noted in 75% of Boston terrier globes and 70% of Labrador retriever and Bichon frise globes. The lowest percentage of PIFM formation seen was in the shih tzu breed at 28%.[147] Along with lens fiber regrowth and epithelial membranes, endophthalmitis, and health of the corneal incision, PIFM development was assessed as contributing to the failure of cataract surgery and secondary glaucoma formation.[144,146]

PIFM formation has also been linked to secondary glaucoma development in dogs with primary lens luxation.[148] In this study, only 4 of 13 enucleated eyes had anterior lens luxation at the time of initial assessment, whereas 9 of 13 had posterior subluxation or full luxation. 9 of 13 eyes were glaucomatous at the time of evaluation, and 3 of the remaining 4 dogs developed high intraocular pressure within 4 months. At the time of enucleation, 77% of eyes had PIFM formation.[148]

Work by Zarfoss et al attempted to elucidate factors leading to fibrovascular membrane development by examining the histologic structure and staining qualities of PIFMs.[149] 36 diseased globes and 4 normal globes were evaluated for comparison.[149] Diseases represented included lens-induced uveitis, retinal detachment, intraocular neoplasia, corneal perforation, severe hyphema, or vitreal gliovascular membranes. All PIFMs were characterized by endothelial cells, spindle cells, lymphoplasmacytic inflammatory cells, and extracellular matrix. PIFM vessels and spindles cells stained positive for VEGF and COX-2, leading to the conclusion that VEGF and COX-2 may play a role in fibrovascular membrane development. Strength of VEGF staining was subjectively increased in diseased eyes compared to normal eyes. In normal eyes, corneal epithelium, corneal endothelium, intraocular vessels, iridal musculature, nonpigmented ciliary body epithelium, retinal pigment epithelium, and most layers of the retina (except the outer nuclear layer) consistently stained positive for VEGF protein. In diseased eyes, additional structures positively staining for VEGF included corneal keratocytes and the outer nuclear layer of the retina. The anti-VEGF antibody used in this study detected multiple VEGF

isoforms, including isoforms 121, 165, and 189.[149]

Recent work performed by Sandberg, et al demonstrated increased expression of VEGF in the aqueous humor of dogs with a variety of intraocular diseases (glaucoma, retinal detachment, lens luxation, and intraocular neoplasia).[150] A significant association between aqueous humor VEGF concentration and PIFM formation was documented in diseased eyes. Intraocular VEGF concentrations did not correlate with plasma levels of VEGF, suggesting that VEGF production is regulated locally in the ocular microenvironment. Aqueous humor concentrations in normal eyes averaged 10.6 pg/mL and ranged from 2.9 to 39.0 pg/mL. Diseased eyes without PIFM had a mean aqueous humor VEGF concentration of 417 pg/mL (57.7-3020 pg/mL 95% CI), whereas mean aqueous humor VEGF concentration in diseased eyes with fibrovascular PIFM was 5720 pg/mL (791-41400 pg/mL 95% CI).

Subsequent work identified VEGF receptor expression in canine ocular tissues.[151] VEGFR-1 was constitutively expressed in corneal epithelium, corneal endothelium, limbal vascular endothelium, iris stroma, iris constrictor and dilator musculature, iris and ciliary body vascular endothelium, ciliary body nonpigmented epithelium, lens epithelium, retinal pigment epithelium, choroidal vascular endothelium and smooth muscle, multiple layers of the retina, scleral vascular endothelium, and optic nerve astrocytes. Staining for VEGFR-1 was similar in diseased eyes. VEGFR2 expression, on the other hand, was limited in ocular tissues. In normal eyes, VEGFR2 was not expressed in ciliary body endothelium or choroid and was not up regulated with disease in the retina, choroid, sclera, ciliary body, and iris stoma. VEGFR2 expression was slightly increased in diseased eyes in corneal vascular endothelium, limbus, and in any PIFM present. These results suggest that VEGF receptors likely play a role in both homeostatic and pathologic angiogenesis in canine eyes. These studies, together with knowledge of human ocular vascularizing disease, suggests that anti-VEGF therapies may have a role in controlling ocular neovascularization in veterinary patients.

Despite these advances in knowledge, the role of VEGF in canine corneal vascularization remains undefined. VEGF protein has been detected in normal corneal epithelium, corneal endothelium and is thought to be up regulated in diseased corneal epithelium and endothelium.[149]

In disease, corneal keratocytes also begin to express VEGF protein.[149] Furthermore, VEGF receptors, particularly VEGFR2, are increased, albeit slightly, in diseased eyes in corneal vascular endothelium and limbus.[151] Given that VEGF protein appears to be increased in intraocular vascularization,[150] it is likely that VEGF may also be up regulated in corneal vascularization. However, in what exact corneal tissues or fluids VEGF is upregulated is unknown. What is also is unknown is the relative proportion of VEGF splice variants present in normal and vascularized corneas, VEGF receptor presence, and whether the tear film, which plays an essential role in corneal health and homeostasis, also changes its composition in corneal disease with regard to VEGF concentrations.

Canine corneal vascularization is commonly noted in conjunction with several intraocular and surface ocular disease conditions, such as chronic superficial keratitis, keratoconjunctivitis sicca, corneal ulceration, glaucoma, intraocular tumor, uveitis, and other immune-mediated conditions. Current therapy for vascularizing corneal disease includes treatment of underlying conditions, topical and/or oral corticosteroids, topical calcineurin inhibitors, and topical and/or systemic COX inhibitors (non-steroidal anti-inflammatory drugs). Despite aggressive treatment, a percentage of these patients will nonetheless experience progressive corneal vascularization, ultimately leading to visual compromise. Therefore, investigation into underlying mechanisms and novel treatment strategies is warranted. Establishing the role of VEGF as a mediator of canine vascularizing corneal disease would provide specific rationale for treatment of these conditions with VEGF inhibitors. The study reported herein aims to evaluate VEGF concentration in tears of dogs with and without corneal vascularization. Our group is currently in the process of developing a recombinant canine sFlt-1 receptor molecule intended for application in clinical disease of dogs, including vascularizing corneal disease.[152]

C. Tear film collection and analysis

Assessment of tear components has been historically difficult due to the small volume of tear that can be dependably collected. In addition, tear contents can be diluted or contaminated by unintended reflex tear stimulation and irritation of the conjunctiva during collection.[90,153,154] In veterinary species, there is the added complication of unpredictable movements of the patient during collection and growing intolerance to the procedure itself. In humans, tear collection via

capillary tubes is generally considered the gold standard; based on work done by our group, however, capillary tubes are slow to draw fluid and it is difficult to acquire adequate volumes using this method. Cellulose sponges have been used to collect tears in humans, ruminants, and dogs with success.[109,155,156] However, such sponges may cause irritation of the conjunctiva leading to tear contamination. Likewise, Schirmer tear test strips can be utilized to collect tear samples, but cause confounding changes in protein concentrations due to reflex tearing and irritation in humans.[153,154,157] In dogs, use of cotton swabs has also been reported, which may also cause irritation.[101] Polyester rods, on the other hand, are quick wicking, easy to use, and have excellent protein recovery characteristics in humans.[158,159] Since the use of polyester rods in dogs has not been reported, we designed and performed a pilot study to evaluate the feasibility of using polyester rods in dogs. In the study, we evaluated recovery of tear fluid volume and tear film VEGF concentrations from the polyester rods. We also evaluated whether there was substantial binding of VEGF to the rod material itself.

III. CHAPTER 3: COLLECTION AND RECOVERY OF TEAR FILM VEGF PROTEIN IN DOGS USING A NOVEL POLYESTER POROUS ROD: A PILOT STUDY

A. Abstract

The purpose of this pilot study was to evaluate use of the polyester rod for tear collection in dogs, determine fluid volume and VEGF recovery characteristics, as well as potential binding of VEGF to the rod itself. Twenty polyester rods were weighed before and after wicking saline to the rod's full capacity, as well as smaller, known volumes. The volume eluted after centrifugation was determined via manual pipetting. Tears were then harvested from ophthalmologically normal dogs using rods, followed by harvesting with glass capillary tubes. Tears were eluted from the collection media via centrifugation and assayed for tear film VEGF using a commercially available canine VEGF sandwich ELISA kit. Dilutions of the supplied VEGF standard were created and the individual dilutions were wicked passively into the rods or drawn under negative pressure into capillary tubes. The dilutions were eluted and assayed along with tear samples from normal dogs. Total holding capacity of the rods approximates 65μL. 100% recovery of a wicked solution volume is possible, regardless of full or partial saturation. VEGF is detectable at biologically active concentrations in the normal canine tear film (mean 8.4 +/- 3.3 ng/mL). At concentrations greater than 0.625 pg/mL, percent recovery of VEGF from both rods and capillary tubes was greater than 90%. Percent volume recovery is adequate for polyester rods as is percent VEGF recovery, which did not differ appreciably from capillary tubes. VEGF is detectable in normal canine tears. Polyester rods are an easy and efficacious method for canine tear collection.

B. Introduction

The precorneal tear film in the dog is approximately 7-10 μm thick and has several different functions, all of which are integral to maintaining corneal transparency and health.[84] It provides a clear refractive surface, removes debris and exfoliated epithelial cells, prevents bacteria and pathogens from adhering to the corneal surface, and nourishes the cornea.[84] In addition, the tear film contains a variety of cytokines, proteins, and inflammatory cells all with the critical function of supporting corneal homeostasis. In disease, composition of the tear film can alter markedly, affecting overall corneal stability, health, and vision.[102,160,161]

The canine tear film aqueous layer is 98.2% water and 1.8% solids, including electrolytes, glucose, urea, polymers, and proteins.[84] In humans, several attempts have been made to quantify and characterize the tear film protein profile, as well as standardize the collection and processing of tears.[160] Membrane array characterization has identified at least 80 chemokines, cytokines, and growth factors.[89] Constituents include IL-2, IL-4, IL-5, IL-10, IFNγ, TNFα, epidermal growth factor, lactoferrin, plasminogen, amylase, lysozyme, and VEGF.[89-92] VEGF, a potent mediator of vascularization in both health and disease, is a normal component of tear film in humans, with reported basal concentrations ranging from approximately 2.4 to 6 ng/mL,[93,94] and as high as 19 ng/mL.[95]

In animals, fewer studies have evaluated tear film proteins, and, as a result, knowledge of the protein profile is likely incomplete at this time. In dogs, IgA, IgG, IgM, albumin, lysozyme, lactoferrin, lipocalin, epidermal growth factor, transforming growth factor, cells, transferrin, ceruplasmin, glycoproteins, matrix metalloproteinases, and antibodies have all been identified in canine tears.[100-109] The canine tear film has not yet been evaluated for the presence of VEGF.

Assessment of tear components has historically been difficult due to the small volume of tear that can be dependably collected. In addition, tear contents can be diluted or contaminated by unintended reflex tear stimulation and irritation of the conjunctiva during collection.[90,153,154] In veterinary species, there is the added complication of unpredictable movements of the patient during collection and growing intolerance to the procedure itself. In humans, tear collection via capillary tubes is generally considered the gold standard; based on work done by our group, however, capillary tubes are slow to draw fluid and it is difficult to acquire adequate volumes using this method. Cellulose sponges have been used to collect tears in humans, ruminants, and dogs with success.[109,155,156] However, such sponges may cause irritation of the conjunctiva leading to tear contamination. Likewise, Schirmer tear test strips can be utilized to collect tear samples, but cause confounding changes in protein concentrations due to reflex tearing and irritation in humans.[153,154,157] In dogs, use of cotton swabs has also been reported, which may also cause irritation.[101] Polyester rods, on the other hand, are quick wicking, easy to use, and have excellent protein recovery characteristics in humans.[158,159]

Since the use of polyester rods in dogs has not been reported, we designed and performed a pilot study to characterize and evaluate the feasibility of using polyester rods in dogs for tear protein recovery. In this study, we evaluated recovery of tear fluid volume and tear film VEGF protein concentrations from the polyester rods, both from standard VEGF dilutions and tears from normal dogs. We also evaluated whether there was substantial binding of VEGF to the rod material itself.

C. Materials and Methods

Bonded polyester fiber rods (Transorb® Wicks R-15643) were acquired from Filtrona Porous Technologies in Richmond, VA. Twenty rods were utilized to evaluate mean dry weight of the rods, mean wet weight when saturated to capacity, and total fluid holding capacity of the rods.

Twenty polyester rods were weighed prior to saturation with sterile saline and the results tabulated. The same 20 rods were then held with forceps at one end while the opposite end was submerged in sterile saline. The rods were deemed saturated to capacity once the fluid visibly expanded the entire rod. The fluid was then eluted via centrifugation. To prepare the saturated rod for elution, the tip of a 0.7 mL tube was manually punctured with a 20-ga needle to create a drainage hole. Each 0.7 mL tube was placed in an intact 1.7 mL micro-centrifuge tube, and the saturated rod placed within the 0.7 mL tube. Rod contents were eluted through the drainage hole of the smaller tube and into the larger tube via centrifugation for five minutes at 3500 rpm at 20°C. The post-elution rod was then re-weighed. The volume eluted was also manually pipetted to determine the volume recovered.

To evaluate percent recovery of volumes measuring less than the full capacity of the rods, various known volumes were wicked into the rods and subsequently eluted via the above-described centrifugation protocol. Volumes of 5 µL, 10 µL, 20 µL, 30 µL, 40 µL, 50 µL, and 60 µL were wicked into rods in triplicate, eluted, and the recovered volume measured via manual pipetting.

Fifteen healthy dogs aged 24-36 months were then evaluated and deemed ophthalmologically

normal via slit-lamp biomicroscopy (Kowa Co. Ltd, Japan), indirect ophthalmoscopy (Heine, Germany), Schirmer tear testing (Intervet, Roseland, NJ), and measurement of intraocular pressure (Tonovet, Tiolat oy, Helsinki, Finland). Tears were harvested from both eyes of each dog with polyester rods followed by tear harvesting with glass capillary tubes (Fig. 1). Rods were held to the lid margin for approximately 30 seconds. Tears were eluted from the collection media via centrifugation at 3500 rpm for 5 minutes at 20°C and immediately stored at -80ºC until assayed for the presence of VEGF protein. A commercially available canine VEGF sandwich enzyme-linked immunosorbant assay (ELISA) designed for serum or cell culture supernatant samples was utilized (Quantikine, CAVE00, R&D Systems, Minneapolis, MN). Tear samples were processed according to the manufacturer's instructions for cell culture supernatant samples.

Since the minimum volume needed for consistent detection of VEGF is unknown, right and left eye samples were pooled for each collection method to increase the tear volume available for use in the assay. If the total tear volume for both eyes did not equal or exceed 5 µL, the samples were not used in the VEGF assay. Samples were diluted to a final volume of 200 µL, as specified for the assay, using the kit diluent appropriate for cell culture samples. Final concentration of sample VEGF was calculated on the basis of the original sample volume and the dilution factor used for each assayed sample. All assays were performed in duplicate.

In addition to tears, 2-fold dilutions of the supplied VEGF positive control were created according to the manufacturer's instructions and ranged from a 1:2 dilution of concentrate (1250 pg/mL) to a 1:128 dilution (19.53 pg/mL). Each individual standard dilution was wicked passively into 2 separate polyester rods until completely saturated; in addition, each dilution was drawn under negative pressure into a capillary tube until filled. The standard dilutions were subsequently eluted by centrifugation at 3500 rpm for 5 minutes at 20°C. Twenty-five microliters of these standardized eluates were then assayed for VEGF in duplicate along with the tear samples from normal dogs. The tear sample volumes used in the assay varied due to the wide range of tear volumes harvested from each dog.

D. Results

Mean dry weight of the rods was 40.5 +/- 0.86 mg; mean wet weight after saturating the rod with

sterile saline was 108.0 +/- 1.28 mg (mean +/- standard deviation). Total fluid holding capacity of the rods approximated 65 µL. Mean volume holding capacity as measured via manual pipetting was 62.8 +/- 3.21 µL; mean volume holding capacity based on weighing rods before and after wicking fluid was 67.3 +/- 1.55 µL (mean +/- standard deviation). The difference between these two mean total volume capacities is likely due to pipetting error when performed manually. Post-centrifugation weight of the same rods used for fluid wicking yielded an average 40.6 +/- 0.84 (mean +/- standard deviation), which is nearly identical to the average dry weight of rods prior to wicking. The average percent recovery of all wicked fluid volumes was equal to or greater than 93% (Table 1).

Tear film collection via polyester rods was attempted in 15 dogs, but samples were acquired from only 13 dogs. Tear volume was collected via polyester rods from 22 eyes of the 13 dogs, averaging 11.2 +/- 10.3 µL (average +/- SD) collected per eye (range 2-30 µL). Right and left eye samples were pooled. Pooled samples with a total volume less than 5 µL were not assayed. Also, rod samples from one dog were mishandled during processing and thus were not assayed. Thus, tear volumes from only 16 of the 22 eyes (i.e., 8 dogs) collected via rods were utilized in the VEGF assay. The average tear volume via rods used in the VEGF assay was 26.2 +/- 20.9 µL (average +/- SD), range 8-58 µL.

Tear collection from 8 of 15 dogs did not yield any sample via capillary tubes. Tear volume collection via capillary tubes was performed successfully on 12 eyes from the 7 remaining dogs, averaging 4.8 +/- 3.56 µL (average +/- SD) per eye (range 2-10 µL). Again, right and left eye samples were pooled. Pooled samples with a total volume less than 5 µL were not assayed. Thus, tear volumes from only 6 of the 12 eyes (i.e., 3 dogs) collected via capillary tubes were utilized in the assay. The average tear volume via capillary tubes used in the assay was 15.0 +/- 7.0 µL (average +/- SD), range 7-20 µL.

Average VEGF concentration detected in the tear film of healthy dogs was 8.4 +/- 3.3 ng/mL (mean +/- SD), range 5.1-16.2 ng/mL, across collection methods. Average VEGF concentration detected in the tear film as collected via polyester rods was 8.99 +/- 3.56 ng/mL (mean +/- SD), whereas that of capillary tubes was 6.9 +/- 2.04 ng/mL (mean +/- SD). No statistical analysis of

these values was performed due to small sample numbers (Fig. 2).

VEGF concentration of the recovered dilution eluates was similar to the actual dilution VEGF concentration, both from capillary tubes and rods (Fig. 3). Results of the VEGF assay revealed that, at moderate concentrations (≥1:4 dilution, 625 pg/mL) of the standard dilutions, percent recovery of VEGF protein from both rods and capillary tubes was greater than 90% (Table 2). Neither recovery via capillary tubes, nor via the rods, detected VEGF concentrations less than the 1:32 standard dilution (78.125 pg/mL). Overall, average percent recovery of the standard VEGF protein dilutions from rods was 89.6% +/- 9.12% (range 66.8-103.7%). Average percent recovery of VEGF from capillary tubes was comparable to that of rods at 81.7% +/- 21.4% (range 54.0-97.0%).

E. Discussion

This study describes an alternative methodology of tear collection in animals and serves as a pilot study for the quantification of VEGF protein in the tear film. Tear collection in animals is difficult due to unpredictable movement and behavior. It is also challenging to acquire sufficient volumes within a restricted time frame, such as might be encountered in the clinical setting. The method of tear collection described herein is easy and quick, facilitating the acquisition of workable tear volumes in a short amount of time.

In humans, tear collection via glass capillary tubes is considered the gold standard. With capillary tubes, it is possible to avoid stimulation of reflex tearing which might alter tear film composition; likewise, protein binding to the glass tube is theorized to be minimal, a desirable trait when attempting to accurately determine tear film components. However, the time it takes to collect a reasonable volume of tears renders capillary tube use somewhat impractical, especially in a veterinary setting. Long collection times may result in accidental contact with the cornea or tear film break-up, both of which can lead to reflex tearing.[160] In animal patients in particular, the impracticality of long collection times is amplified due to low patient tolerance and compliance. Other methods, such as Schirmer tear test strips, are clinically more feasible since patients tend to tolerate the strip well and tear recovery is fast;[160] however, the tear film components may be altered as a result of corneal and conjunctival epithelial cell damage and capillary breakage from

the strip itself.[92,153,162,163] Tissue irritation such as this has likely contributed to the varying reports of total tear protein concentration throughout the literature.[160] Thus, development of novel methodologies for tear collection in animals is warranted.

Polyester rods have been utilized in few studies examining various tear film components.[159,164,165] A study by Jones et al introduces the use of polyester rods as a novel method of tear collection in humans. In this study, Jones et al used polyester porous rods to wick the tear meniscus at the lateral inferior lid and compared the results to wicking with glass capillary tubes.[159] Tear collection rate in microliters/minute was significantly faster than that of capillary tubes, by greater than three times. Typical tear volumes collected with the capillary tubes averaged 3.3 μL whereas collection with the rods averaged 5.5 μL. In this study, collection rate was not determined. However, tear volumes collected from dogs in this study via polyester rods far exceeded the volumes reported in the Jones study, ranging from 8 to 58 μL. The rods were easy to use and atraumatic to the cornea, and were held to the tear meniscus a relatively short period of time (less than 1 minute). The average tear volume recovered with polyester rods was nearly twice that of capillary tubes, though sample numbers were too small to carry out statistical analysis. Partial saturation of the rods, as is likely to occur when attempting to wick tears, yielded near-complete recovery of all wicked volumes. This suggests that use of the polyester rod in a veterinary clinical setting may yield relatively ample amounts of tear film fluid for subsequent analysis, and that recovery of even very small amounts of tear volume is possible.

The Jones et al study also evaluated protein recovery of the rods and capillary tubes, specifically for lactoferrin and epidermal growth factor. Both tear collection methodologies were similar in their protein recovery efficacy; however, the polyester rods showed a trend of enhanced protein recovery, though this difference was insignificant.[159] Similarly, a study performed by Esmaeelpour et al chose to use cellulose acetate rods for tear collection over polyester rods due to suspected contaminant release from the rods that may have lead to an increase in measured bovine serum albumin concentrations.[158] However, in a review by Zhou et al in 2012, enhanced protein recovery was not listed as a potential disadvantage, of either polyester or cellulose acetate rods.[160] In the study reported herein, comparable protein recovery efficacy between the rods and capillary tubes was also demonstrated. Interestingly, there was an enhanced protein

recovery pattern noted with the polyester rods in this study as well, when comparing the concentration of recovered VEGF standard dilutions from the two collection media. Percent recovery of VEGF was higher for rods on average, and was more pronounced at lower VEGF concentrations. In addition, the range of percent recovery of standard VEGF protein dilutions wicked by rods was 66.8% to 103.7%. Thus, the enhanced recovery characteristic of rods noted in the previously mentioned studies may hold true for this pilot study as well. Enhanced recovery may be due to charge characteristics of the rod or affinities for certain proteins.

VEGF, a potent mediator of vascularization in both health and disease, is a normal component of tear film in humans, with reported basal concentrations ranging from approximately 2.4 to 6 ng/mL,[93,94] and as high as 19 ng/mL.[95] This study detected average concentrations of approximately 8.4 +/- 3.3 ng/mL as collected via polyester rods and capillary tubes in tear volumes greater than 5 μL. This suggests that canine tear film VEGF concentration is likely similar to that of humans and that VEGF is likely detectable in even smaller tear volumes than used in this study. What's most interesting, however, is that the VEGF tear film concentrations reported in the human literature, as well as this study of the canine tear film, are beyond the biologically available concentration needed to stimulate angiogenesis.[166] In the case of corneal tissue, this high level of basal tear film VEGF is intriguing since the cornea is, in health, completely avascular. It is unclear what role tear film VEGF may play in corneal health and/or disease. However, elevated tear film VEGF concentrations have been documented in various human vascularizing corneal diseases.[94-96]

In conclusion, this pilot study lays the foundation for further work utilizing polyester rods in canine tear film collection. Percent tear film volume recovery was >93% for polyester rods at all volumes wicked. Percent VEGF recovery was also suitably high at greater than 90%, and did not differ appreciably from capillary tubes. Binding between VEGF protein and the rod itself appears to be minimal. Furthermore, VEGF was readily detectable in normal canine tears. Polyester rods proved to be an easy, reliable, and efficacious method for collecting canine tears for VEGF assays. More thorough study is needed, however, to analyze the difference in detection of canine tear film VEGF concentrations between capillary tubes and polyester rods. Furthermore, investigators should be aware of a possible enhancement effect of protein recovery.

IV. CHAPTER 4: TEAR FILM VEGF IN DOGS WITH VASCULARIZING CORNEAL DISEASE

A. Abstract

This study measured vascular endothelial growth factor-A (VEGF) concentrations in tears from dogs with normal and vascularized corneas. Tear samples were harvested from both eyes of dogs with vascularizing corneal disease (including non-vascularized eyes of dogs with unilateral vascularization). Tears from eyes of ophthalmologically normal dogs served as controls. Vascularization scores were assigned to diseased eyes based upon slit-lamp biomicroscopy and photodocumentation. VEGF concentration was evaluated using a commercially available enzyme-linked immunosorbant assay (ELISA). Tears were collected from 63 eyes of control dogs, 45 non-vascularized eyes of dogs with unilateral disease, and 101 eyes with vascularizing corneal disease. Disease categories included keratitis, corneal stromal ulceration/perforation, superficial ulceration/SCCED, endothelial degeneration, corneal lipid dystrophy/degeneration, corneal facet, uveitis, glaucoma, and anterior lens luxation. Mean tear film VEGF concentration of diseased eyes was 6.53 +/- 0.77 ng/mL (geometric mean, 95% CI) and did not differ significantly from control eyes (6.01 +/- 1.0 ng/mL). Tear film VEGF concentrations in non-vascularized eyes of dogs with unilateral disease were significantly higher (9.78 +/- 1.52 ng/mL) than control and vascularized eyes. VEGF concentrations of diseased eyes did not differ significantly by disease process, degree of corneal vascularization, or use of topical or oral anti-inflammatory medications. Canine tear film VEGF concentrations detected by ELISA exceed known biologically active concentrations of VEGF, but do not correlate with state of corneal vascularization. VEGF-related control of corneal vascularization may be mediated by VEGF receptor expression and regulation or a balance between proangiogenic and antiangiogenic VEGF splice variants indistinguishable by current testing methods.

B. Introduction

Vascular endothelial growth factor (VEGF-A, hereafter referred to as VEGF), a 45 kDa heparin-binding dimeric glycoprotein, is a potent mediator of blood vessel formation in both health and disease. It is an essential mitogen and survival factor for vascular endothelial cells, promoting growth of arteries, veins, and lymphatic vessels.[1,2] Various receptors for VEGF exist, the most

prominent of which, in angiogenesis, are the ligand-activated tyrosine kinases VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and the inhibitory soluble Flt-1 variant. VEGF is secreted in response to low oxygen tension, epidermal growth factor, tumor necrosis factor-α, placental derived growth factor, inflammatory cytokines IL-1α and IL-6, several hormones (TSH, gonadotropins, sex steroids), and, finally, oncogenes.[1,2,167,168] Vessel formation due to VEGF may be decreased or inhibited by corticosteroids, calcineurin inhibitors such as cyclosporine A and tacrolimus, and COX-inhibitors.[37-43]

Although VEGF is necessary for normal vascular growth and maintenance, it has been well documented that overexpression of VEGF can lead to pathologic vascular proliferation. A balance thus exists between pro-angiogenic and angiostatic factors. While VEGF has been detected in healthy individuals, elevated levels have been noted in numerous disease conditions. In humans, elevated plasma VEGF levels are correlated with tumor angiogenesis, metastasis, and immune-mediated disease such as lupus erythematosis and rheumatoid arthritis.[22-24] Circulating VEGF levels have also been used as a prognostic indicator in neoplastic disease.[25,26] Treatment of these conditions with therapy targeting VEGF has demonstrated tumor growth arrest and even regression in some cases.[119,120] In dogs, elevated plasma levels of VEGF have been noted in several neoplastic conditions.[27-33] More recently, dogs diagnosed with systemic inflammatory response syndrome (SIRS) and concurrent elevated VEGF levels were less likely to survive.[34]

Within the context of ophthalmology, VEGF is expressed in several ocular tissues, including retinal pigmented epithelium, Müller cells, vascular endothelium and uvea, ganglion cells, and corneal endothelium and epithelium.[2,110,111] Under neoplastic, hypoxic, and inflammatory conditions, angiogenic balance is tipped in favor of ocular vascularization, often with blinding and painful sequellae. Age-related macular degeneration, retinopathy of prematurity, diabetic retinopathy, and certain corneal diseases all have a critical vascular proliferative component largely mediated by VEGF.[2,111-113] Topical and injectable anti-VEGF therapies have shown great promise in slowing the progression of both intraocular and corneal vascularization in humans and in animal models.[115-118] The role of VEGF in canine ocular neovascularization, however, is just beginning to be elucidated.

Canine corneal vascularization is commonly noted in conjunction with several intraocular and surface ocular disease conditions, such as chronic superficial keratitis, keratoconjunctivitis sicca, corneal ulceration, glaucoma, intraocular tumor, uveitis, and other immune-mediated conditions. Current therapy includes treatment of underlying conditions, topical and oral corticosteroids, topical calcineurin inhibitors, and topical or systemic COX inhibitors (non-steroidal anti-inflammatory drugs). A percentage of these patients will nonetheless experience progressive corneal vascularization despite treatment, ultimately leading to visual compromise. Therefore, investigation into underlying mechanisms and novel treatment strategies is warranted.

The value of anti-VEGF therapy has been demonstrated in many human vascularizing ocular diseases including age-related macular degeneration, diabetic retinopathy, and vascularizing corneal disease. Anti-VEGF therapy may also have clinical utility for canine diseases. Recent studies of canine ocular disease have shown increased VEGF aqueous humor levels in intraocular tumor formation, uveitis, secondary glaucoma, and other intraocular inflammatory diseases. VEGF levels associated with corneal disease, however, have not been evaluated. To examine the potential role of tear film VEGF in the pathophysiology of corneal vascularization and to assess the clinical viability of anti-VEGF therapy in canine corneal vascularization, we proposed evaluating VEGF protein levels in tear film recovered from eyes with both normal corneas and vascularized corneas. We hypothesized that VEGF is a component of the normal tear film and that tear collection and evaluation is a reliable method for detecting and quantifying VEGF on the ocular surface. Furthermore, we hypothesized that tear film VEGF is increased in the presence of corneal vascularization. Finally, we hypothesized that tear film VEGF concentration is correlated with severity of corneal vascularization.

C. Materials and Methods

a. Animals

With client consent, 79 client-owned dogs with corneal vascularization were enrolled in the study. In addition, 32 healthy, ophthalmologically normal dogs served as controls. Control tear samples were harvested from client-owned and hospital staff/student-owned pets. For both diseased and control dogs, tears were harvested from both eyes whenever possible. The Virginia Tech Institutional Animal Care and Use Committee approved all animal procedures employed in

this study. Dogs with disease conditions known or suspected to be associated with elevated systemic VEGF concentrations, including neoplasia and diabetes mellitus, were excluded from the study.

b. Experimental protocol

All study subjects received a complete ophthalmic examination by a Diplomate of the American College of Veterinary Ophthalmologists or ophthalmology resident (KRB). The exam consisted of slitlamp biomicroscopy (Kowa, Co. Ltd, Japan), indirect ophthalmoscopy (Heine, Germany), Schirmer tear testing (Intervet, Roseland, NJ), measurement of intraocular pressures (Tonovet, Tiolat oy, Helsinki, Finland), and corneal fluorescein staining (Ful-Glo, Akorn, Inc., Lake Forest, IL). Age, gender, and breed of the sample subject, clinical diagnosis, and current medical treatments were recorded. Duration of medical treatment and duration of disease were not recorded. To compare the disease severity between patients, the length, extent, and depth of corneal vascularization was documented both in the paper record and with photographs using a digital camera (Digital Camera FinePix S3Pro, Fuji Photo Film Co., Ltd., Minato-Ku, Japan).

Vascularized eyes were given a vascularization score of 1 to 4 in each of the following groups: vessel length, diameter, density, and corneal depth. Vessel length was characterized with respect to extension toward the axial cornea: 0) none, 1) perilimbal, 2) ¼ axial, 3) ½ axial, and 4) axial. Vessel diameter was described as being 0) none, 1) ghost, 2) thin, 3) medium, and 4) large. Vessel density was characterized with respect to both number of vessels and branching of vessels. Those with <10 vessels/quadrant of cornea were considered to have sparse vascularization (1); those with 10-20 vessels/quadrant or <10 vessels present with extensive branching were considered mildly affected (2); those with moderate vascular density were characterized by 20-40 vessels/quadrant or 10-20 vessels with extensive branching (3); and severe vascular density was characterized by >40 vessels/corneal quadrant and/or 20-40 vessels with extensive branching. Vascularization scores (sum of the three above listed criteria) of 6-8 were considered mild, those of 9-11 were considered moderate, and those with scores of 12-15 were considered severely affected.

Tears were harvested using bonded polyester fiber rods (Transorb® Wicks R-15643, Filtrona

Porous Technologies, Richmond, VA). Rods are 2 x 32.4 mm and were held at the medial or lateral canthus of each eye for 60 seconds to allow tear wicking while minimizing ocular contact. Once harvested, tear-filled rods were placed immediately into 1.7 mL micro-centrifuge tubes and refrigerated at 4°C until the tear sample could be recovered from the rod. At the time of tear sample elution, the tip of a 0.7 mL tube was manually punctured with a 20-ga needle to create a drainage hole. Each 0.7 mL tube was placed in an intact 1.7 mL micro-centrifuge tube, and the rod placed within the 0.7 mL tube. Rod contents were eluted through the drainage hole of the smaller tube and into the larger tube via centrifugation for five minutes at 3500 rpm at 20°C. All samples were eluted from rods within 24 hours of sample harvesting. Eluted tears were immediately stored at -80°C until subjected to VEGF ELISA assay.

Tears were assayed for VEGF using a commercial kit designed for detection of canine VEGF-A (Quantikine, CAVE00, R&D Systems, Minneapolis, MN). Samples were processed according to the protocol for processing cell culture samples. Tear sample volumes used for the assay ranged between 1 and 5 μL, based upon the available sample volume for each case. Samples were diluted to a final volume of 200 μL, as specified for the assay, using the kit diluent appropriate for cell culture samples. Final concentration of sample VEGF was calculated on the basis of the original sample volume and the dilution factor used for each assayed sample. All assays were performed in duplicate.

c. Statistical analysis

Normal probability plots showed that VEGF concentrations were skewed while paired differences (in VEGF concentrations) between left and right eyes were normally distributed. As a result, a log (base e) transformation was applied to VEGF concentrations before group comparisons.

Statistical analysis consisted of descriptive statistics for both control and diseased groups. Groups were further described by breed, sex, disease process, and severity of corneal vascularization. A mixed model analysis of variance of the logarithm of VEGF concentration was performed between diseased and control groups, and within the diseased group by disease process, vascularization score, age, and therapy utilized. The linear model specified groups to be

compared as a fixed effect, individual dog as a random effect (adjusts for pairs/clusters of eyes within dog), and Kenward-Roger as the denominator degrees of freedom. Where appropriate, Tukey's procedure was selected to adjust for multiple comparisons. For each of the ANOVA models, residual plots were inspected to verify that the errors followed a normal distribution with a constant variance. A p-value of ≤ 0.05 was considered significant. All data analyses were in conjunction with the Laboratory for Study Design and Statistical Analysis within the VMRCVM using a commercial statistical program (SAS version 9.3, Cary NC, USA).

A second assessment was performed to assess differences between two eyes of the same individual via a paired t-test. In dogs with unilateral vascularizing disease, tear film VEGF concentration of the unaffected eye was compared to the diseased eye of the same dog. In control dogs, tear film VEGF concentration was compared between right and left eyes.

Finally, paired t-tests were also carried out to compare the tear film VEGF concentration of both the left and right eye of dogs in the mild vascularization group, the moderate vascularization group, and the severe vascularization group.

D. Results

a. Descriptive data

All cases and pertinent information used in the study are displayed in Tables 3-5. Sixty-three control samples were collected from 32 dogs of 12 different breeds. The most common breeds sampled were mixed breed dogs (16/32, 50%), Labrador retrievers (5/32, 15.6%), and Staffordshire terriers (2/32, 6.3%); all breeds represented are displayed in Table 6. Mean age of control dogs was 5.68 +/- 3.55 years (mean +/- SD), ranging from less than 1 year to 18 years old. Dogs were split into three age groups: fifteen dogs aged less than 1 year to 4 years old; 13 dogs were aged 5 to 9 years old; and 4 were aged greater than 10 years old. Fifteen dogs were spayed females, 14 were castrated males, 2 were intact males; one intact female was sampled. Schirmer tear test data was available for 56 samples. The average tear test value of control samples was 22.7 +/- 4.18 mm/min (mean +/- SD), with a range of 11 to 31 mm/min.

One hundred-one diseased samples were collected from 79 dogs with vascularized corneas

(hereafter referred to as diseased samples). The 79 dogs represented 30 breeds that are displayed in Table 7. The most common breeds sampled were mixed breed dogs (16/79, 20%), boxers (7/79, 8.8%), and pugs (5/79, 6.3%). Mean age of diseased dogs was 8.57 +/- 3.49 years (mean +/- SD), ranging 9 months to 16 years. Again, three age groups were formed: fourteen dogs were less than 1 to 4 years old; 31 dogs were 5 to 9 years old; and 34 were greater than 10 years old. Two dogs were intact females, 37 dogs were spayed females, 35 were castrated males, and 5 were intact males. Schirmer tear test data was available for 94 samples. The average tear test value of diseased samples was 20.41 +/- 5.84 mm/min (mean +/- SD), with a range of 6 to 32 mm/min.

Of the 101 diseased samples, 34 were categorized as mildly vascularized, 53 classified as moderately vascularized, and 14 were severely vascularized. A picture representative of each vascularization score is shown in Fig. 4. Diseases sampled included: stromal ulceration and perforation (9), spontaneous chronic corneal epithelial defect (SCCED) or superficial corneal ulcer (19), non-ulcerative keratitis (43), endothelial degeneration (9), corneal lipid degeneration/dystrophy (5), corneal facet (7), uveitis (11), glaucoma (24), and anterior lens luxation (8). The non-ulcerative keratitis group was a category comprised of cases with a diagnosis of keratoconjunctivitis sicca (KCS), superficial punctate keratopathy (SPK), immune-mediated keratitis (IMMK), chronic superficial keratitis (CSK, pannus), pigmentary keratitis, and qualitative tear film deficiencies.

Forty-five samples were collected from the unaffected eye of 45 dogs with unilateral vascularizing disease; hereafter, these samples are referred to as the unaffected group. The most common breeds represented in this sample set were mixed breed dogs (8/44, 18%), boxers (6/44, 13.6%), and Australian shepherds (4/44, 9%). Mean age of dogs with unilateral vascularizing disease was 8.51 +/- 3.56 years (mean +/- SD), ranging from 9 months to 16 years old. Three age groups were formed: Six dogs were less than 1 year to 4 years old; 17 dogs were 5 to 9 years old; and 21 were10 years old or greater. 24 were spayed females, 16 were castrated males, 4 were intact males, and none were intact females. Schirmer tear test data was available for 41 samples. The average tear test value of unaffected samples was 20.54 +/- 4.58 mm/min (mean +/- SD), with a range of 6 to 28 mm/min.

b. Tear film VEGF concentration

Sample amount used in the ELISA assay had a significant effect on the calculated tear film VEGF concentration. Of all tested samples, 0.5 µL of sample was used for 5 samples, 1 µL was used for 23 samples, 2 µL was used for 69 samples, 3 µL was used for 15 samples, 5 µL was used for 95 eyes, and 6 µL was used for 2 eyes. The geometric mean VEGF tear film concentration for each amount is listed in Table 8. An overall upward trend of VEGF concentration existed as the amount of sample decreased. Mean VEGF concentrations from 0.5 µL samples were significantly elevated compared to all other volumes, $p \leq 0.0329$; 1 µL samples were significantly elevated compared to 5 µL samples, $p = 0.0015$ (Fig. 5). Each sample type (control, unaffected, or diseased samples), however, had a relatively even distribution of sample types by volume. (Fig. 6) The mean sample amount used in the ELISA assay for the control, unaffected, and diseased samples was 3.51 +/- 0.42 µL, 2.82 +/- 0.47 µL, and 3.44 +/- 0.34 µL, respectively (mean +/- 95% CI). There was no significant difference between the mean sample volume used for the control and diseased groups, $p = 0.97$. However, the difference between sample volume used for unaffected eyes and control eyes, as well as that between unaffected eyes and diseased eyes, approached significance at $p = 0.08$ and $p = 0.06$, respectively.

Tear film VEGF concentration of diseased samples was 6.53 +/- 0.77 ng/mL (geometric mean +/- 95% CI) and was not significantly different from that of control eyes (6.01 +/- 1.0 ng/mL, $p = 0.7$). In dogs with unilateral vascularizing corneal disease, the VEGF concentration of unaffected samples was 9.78 +/- 1.52 ng/mL. This was significantly higher than both control and diseased samples ($p < 0.0001$), (Fig. 7). Comparison of the left and right eyes of control dogs found no significant difference ($p = 0.1028$) between mean tear film VEGF concentrations. In individuals with two diseased eyes, the VEGF concentrations of these bilaterally affected eyes were not significantly different from control eyes (Table 9).

The degree of corneal vascularization, as determined by the assigned corneal vascularization score, did not significantly correlate with tear film VEGF concentrations. As shown in Fig. 8, the tear film VEGF concentration of mildly vascularized eyes was 7.09 +/- 1.38 ng/mL (geometric mean +/- 95% CI), moderately vascularized eyes had a VEGF concentration of 6.17 +/- 0.96

ng/mL, and severely vascularized eyes had a VEGF concentration of 6.16 +/- 1.78 ng/mL. The geometric mean tear film VEGF concentration between vascularization score groups was not significantly different ($p > 0.6$), nor was the VEGF concentration of the vascularization score groups significantly different from that of control eyes (6.01 +/- 1.0 ng/mL) ($p > 0.59$).

In general, extent or character of vascularization did not correlate with tear film VEGF concentration. The geometric mean +/- 95% confidence interval tear film concentration of eyes with perilimbal vascularization (6.51 +/- 2.15 ng/mL) was not significantly different from eyes with vessels extending ¼ axially (6.32 +/- 1.18 ng/mL), ½ axially (5.69 +/- 1.38 ng/mL), or fully axially (7.14 +/- 1.41 ng/mL), with $p \geq 5359$ for all interactions. Additionally, none of these groups differed significantly from control samples ($p \geq 0.6907$) (Fig. 9).

Similarly, vessel density was not strongly correlated with tear film VEGF concentration. Sparsely vascularized eyes had significantly elevated tear film VEGF compared to severely vascularized eyes ($p = 0.049$); other interaction were, however, insignificant (p-value ranged from 0.0745 to 0.9988). The geometric mean +/- 95% CI tear film concentration of sparsely vascularized eyes was 7.54 +/- 1.52 ng/mL; that of mildly vascularized eyes was 7.18 +/- 1.4 ng/mL; that of moderately vascularized eyes was 5.76 +/- 1.34 ng/mL; and that of severely vascularized eyes was 4.93 +/- 1.22 ng/mL for those with a severe density of vessels (Fig. 10).

Vessel depth likewise did not largely correlate with tear film VEGF concentration. Samples from eyes with deep stromal vessels had significantly higher tear film VEGF concentrations compared to control eyes ($p = 0.0016$) and eyes with vessels located in the anterior ($p = 0.002$) or mid-stroma ($p = 0.0002$). All other interactions were not significantly different; p-values ranged from 0.26 to 0.98. The geometric mean +/- 95% CI VEGF tear film concentration of superficially vascularized eyes was 7.90 +/- 3.93 ng/mL; that of eyes with anterior stromal vessels was 6.42 +/- 0.90 ng/mL; that of eyes with mid-stromal vessels was 5.32 +/- 1.24 ng/mL; and that of eyes with deep stromal vessels was 15.38 +/-7.10 (Fig. 11).

Finally, vessel size did not markedly impact tear film VEGF concentrations. Vascularized eyes with large vessels had significantly elevated tear film VEGF concentrations compared to control

samples ($p = 0.0483$) and samples with thin vessels ($p = 0.0266$). All other interactions were insignificant, with p-values ranging from 0.19 to 0.97. The geometric mean +/- 95% CI tear film VEGF concentration of samples from eyes with ghost vessels was 8.44 +/- 3.52 ng/mL; that of eyes with thin vessels was 6.06 +/- 0.86 ng/mL; that of eyes with medium vessels was 6.63 +/- 1.70 ng/mL; and that of eyes with large vessels was 11.22 +/- 4.67 ng/mL (Fig. 12).

Schirmer tear test (STT) values were available for 56 control samples, 94 diseased samples, and 41 unaffected eyes from dogs with unilaterally vascularized corneas. The mean STT value for the control group was 22.76 +/-1.6 mm/min, 20.60 +/- 1.1 mm/min for the diseased group, and 19.97 +/-1.5 mm/min for the unaffected group. The STT value for the unaffected group was significantly lower than the control group, $p = 0.038$. No other difference in STT values between groups was significant (Fig. 13). Within the diseased group of samples, VEGF tear film concentration was inversely proportional to STT value with a high level of significance, $p < 0.0001$ (Fig. 14).

No significant difference in tear film VEGF concentration was noted between disease groups (Fig.15). P-values between groups ranged between 0.31 and 1.0. The geometric mean tear film VEGF concentrations for each group are displayed in Table 10. No disease group differed significantly from the control group.

Patients were documented as being on various medications, both topical and oral. Information on duration of treatment with a particular medication was not available.

Topical anti-glaucoma medications recorded included a β-blocker (timolol maleate 0.5% ophthalmic solution), carbonic anhydrase inhibitors (dorzolamide HCl 2% ophthalmic suspension, brinzolamide 1% ophthalmic suspension), combination β-blocker/carbonic anhydrase inhibitor (timolol maleate 0.5%/dorzolamide HCl 2% ophthalmic suspension), a prostaglandin analogue (latanoprost 0.005% ophthalmic solution), epinephrine 1% ophthalmic solution, and pilocarpine 2% ophthalmic solution.

Topical antibiotics recorded included aminoglycosides (gentamicin 0.3%, tobramycin 0.3%

ophthalmic solutions), fluoroquinolones (ofloxacin 0.3%, ciprofloxacin 0.3% ophthalmic solutions), combination antibiotics (neomycin-polymyxin B-bacitracin or neomycin-polymyxin B-gramacidin, polymyxin B-oxytetracycline), and combination steroid-antibiotic preparations (neomycin-polymyxin-dexamethasone).

Topical miconazole 1% antifungal was used. Topical steroidal preparations recorded included prednisolone acetate and dexamethasone. No patient was documented as being treated with topical non-steroidal anti-inflammatories. Topical immunomodulators (calcineurin inhibitors) included cyclosporine (Optimmune® 0.2% ophthalmic ointment; 1% and 2% cyclosporine compounded preparations, The Prescription Center, Fayetteville, NC) and tacrolimus 0.02% or 0.03% (compounded preparation, The Prescription Center, Fayetteville, NC). Other topical medications used included canine serum, Remend™ (Virbac Animal Health), non-medicated eye lubricant, and atropine 1% ophthalmic solution or ointment.

Oral steroidal medication recorded included prednisone. Oral non-steroidal anti-inflammatories drugs (NSAIDs) recorded included carprofen tablets and meloxicam liquid suspension. Oral immunomodulating medications included cyclosporine (Atopica, Novartis Animal Health) and doxycycline. Oral antibiotics included doxycycline and amoxicillin. Other oral medications recorded included soloxine, aminophylline, lignin, and melatonin.

Of the medications recorded, those theorized to have a potential effect on VEGF tear film concentration included oral and topical corticosteroids, oral and topical calcineurin inhibitors (cyclosporine, tacrolimus), and oral NSAIDs. Of the 101 diseased samples from vascularized eyes, 16 were treated with topical steroids; 14 were treated with topical calcineurin inhibitors; 4 were treated with oral steroids, 8 were treated with oral NSAIDs, and 2 with oral calcineurin inhibitors (cyclosporine); 65 samples were not treated with any medication.

No significant difference in tear film VEGF concentration was noted between medication groups. P-values ranged from 0.53 to 1.0. When controls were added to the statistical analysis, no significant difference was found between samples that had received medication and the control group. The geometric mean of tear film VEGF concentration of diseased samples with no topical treatment was 5.97 +/- 0.97 ng/mL; that of the topical steroid group was 7.41 +/- 2.31

ng/ml; that of the topical calcineurin inhibitor group was 8.40 +/- 3.14 ng/ml; that of the oral steroid group was 7.19 +/- 4.36; that of the oral NSAID group was 6.51 +/- 2.72; and that of the oral calcineurin inhibitor group was 7.85 +/- 10.17 (Fig.16).

Of diseased samples, 18 were from dogs less than 1 year to 4 years old; 38 were from dogs 5-9 years old; and 45 were from dogs 10 years or more. Tear film VEGF concentrations were not significantly different between age groups. As shown in Fig. 17, VEGF levels were 6.30 +/- 1.94 ng/mL in dogs aged 4 or fewer years; 7.47 +/- 1.56 ng/mL in dogs 5-9 years old; and 5,86 +/- 1.14 ng/mL in dogs 10 years old or greater.

Tear film VEGF concentrations did not differ significantly between sexes; p-values ranged from 0.50 to 1.0 (Fig.18). Of diseased samples, 3 were from intact females, 46 were from spayed females, 5 were from intact males, and 47 were from castrated males. The geometric mean of tear film VEGF concentration of the intact female samples was 12.80 +/- 10.96 ng/mL, while that of female spayed samples was 6.42 +/- 1.21 ng/mL. The geometric mean of tear film VEGF concentration of intact male samples was 6.17 +/- 3.47 ng/mL, and that of castrated male samples was 6.38 +/- 1.23 ng/mL.

c. Vascularization score

The mean vascularization score of the diseased samples in each medication group was calculated and statistically evaluated. Sixty-five diseased samples received no medication; 16 received topical steroids; 14 received topical calcineurin inhibitors; 4 received oral steroids; 8 received oral NSAIDs; and 2 received oral calcineurin inhibitors. No statistical significance in vascularization score was found between any medication groups. Mean vascularization score of samples receiving no topical medication was 9.52 +/- 0.48 that of samples receiving topical steroids was 9.52 +/- 0.90; that of samples receiving topical calcineurin inhibitors was 9.57 +/- 1.07. Those receiving oral steroids had a mean vascularization score of 9.51 +/- 1.69; those receiving oral NSAIDs had a mean score of 9.21 +/- 1.20; and those receiving oral calcineurin inhibitors had a mean score of 7.0 +/- 3.15. P-values ranged from 0.61 to 1.0 (Fig. 19).

Among disease groups, vascularization scores did not differ significantly. All disease

vascularization scores are displayed in Table 11, and all interactions are displayed in Fig. 20. P-values between groups ranged from 0.68 and 1.0.

E. Discussion

Corneal vascularization is a common sequela to several ocular surface and intraocular diseases. This study attempted to examine the role of VEGF in canine vascularizing corneal disease by evaluating the tear film as a possible source of VEGF protein. As originally hypothesized, VEGF protein was detected as part of the normal canine tear film, a seemingly incongruous finding given that an avascular, clear cornea is essential for maintaining ocular health and vision. Nonetheless, it is a similar finding to several studies documenting VEGF as part of the normal healthy tear film in humans.[89,93-95,97] Current literature in human medicine reports mean tear film VEGF concentrations in normal eyes as ranging between approximately 2.4 ng/mL and approximately 5-6 ng/mL.[93,94] A third investigation evaluating the tear film concentration of VEGF bathing normal corneas prior to photorefractive keratectomy reports median concentrations as high as 19 ng/mL.[95] The study presented here identified geometric mean tear film concentrations of 6.01 +/- 1.0 ng/mL in control eyes. These concentrations are approximately 2-3 times the levels found in human basal tears as reported by Carreno et al in 2010, are comparable to the levels found by Zakaria et al in 2012, and approximately 3-fold less than the median levels reported by Vesaluoma et al in 1997. This suggests that the ophthalmologically normal domestic canine has similar levels of tear film VEGF as compared to normal humans.

Interestingly, the study presented herein found similar levels of tear film VEGF in both control and vascularized canine eyes, with no significant difference between the two sample populations. This finding was unexpected and seemingly contradicts the human literature. In humans, studies investigating tear film VEGF concentrations suggest that VEGF release is elevated after photorefractive surgery and tear film VEGF concentration is elevated after pterygium removal.[95,96,134] In addition, Zakaria et al documented tear film VEGF as being significantly elevated in vascularized eyes when compared to normal controls.[94] When this latter assertion is examined more closely, however, it seems this may be true only when certain types of tear samples are analyzed. Samples in the Zakaria study were collected in multiple ways and in

different contexts to investigate the origin of tears and tear film pro-angiogenic cytokines. Basal tears (i.e., those tears comprising the lacrimal lake and tear film absent any provocation for tearing) were carefully collected from the lateral canthus with the patient sitting upright, without stimulation of the ocular surface. In addition, reflex tears were collected from the upper lateral conjunctival fornix while the patient was supine and stimulated with a noxious bright light. This allowed collection of newly released tears emanating from the lacrimal gland ductual openings, specifically. Following this, reflex tears were again collected using a similar stimulus, this time while the patient was sitting; tears collected in this manner originated from the inferior medial canthus. Finally, tears were collected via corneal bath from topically anesthetized corneal surfaces, specifically. These latter samples were meant to represent the corneal epithelial cell secretions alone. When samples are separated out by collection method and context of collection, results of the Zakaria et al study are actually similar to the findings in the study presented herein. Neither basal tears nor reflex tears demonstrated significantly elevated VEGF levels in vascularized eyes compared to normal control eyes. Corneal bath tears, however, had a significantly elevated level of VEGF concentration compared to controls. In the canine study reported here, tears were collected only from the existing lacrimal lake, taking care to avoid stimulation of reflex tearing and conjunctival irritation which might impact tear film VEGF concentration. Tears collected in this study are therefore most akin to the basal tears collected by Zakaria et al, or to some degree, albeit unintentionally, the reflex tear samples. When reviewed in this manner, it becomes apparent that the results of this canine study most likely are similar to and support results from the Zakaria study in humans. Furthermore, the study reported herein documented an overall trend of decreased tear film VEGF concentration with increasing Schirmer tear test values among diseased samples. This inverse relationship between STT and tear film VEGF concentration was highly significant. This may imply that tear film VEGF is elicited primarily by tissues other than lacrimal tissues (e.g. corneal or conjunctival epithelium, inflammatory cells) and increased tearing thus dilutes tear film VEGF.

The conclusion that tear film VEGF concentration was similar in both normal, healthy corneas and vascularized corneas may be supported biologically by several other theories as well. VEGF tear film concentrations reported in the human literature, as well as in this study of the canine tear film, are within the biologically available concentration needed to stimulate angiogenesis.[166]

In the case of corneal tissue, this high level of basal tear film VEGF is interesting since the cornea is, in health, completely avascular. Therefore, it stands to reason that corneal vascularization may be mediated via VEGF receptor availability and regulation, as well as the concentration of splice variants, rather than absolute VEGF protein concentration in the pre-corneal tear film. Soluble Flt-1 is considered an important inhibitor of VEGF activity by binding extracellular, non-membrane bound VEGF protein. Via this mechanism, sFlt-1 is thought to contribute to inhibition of corneal vascularization and maintenance of corneal clarity.[19,20,70] In states of corneal vascularization, sFlt-1 may be down regulated such that existing levels of extracellular VEGF are then more bioavailable to pro-angiogenic cellular receptors such as VEGFR-2.

Alternatively, perhaps sFlt-1 is not down regulated in vascularized corneal conditions, but rather the local extracellular environment is inundated by an increased release of non-membrane bound, and thus bioavailable, VEGF protein. Studies evaluating VEGF tear film concentration in humans following photorefractive keratectomy showed an increase not in the absolute tear film concentration, but in the release of VEGF into the tear film. Following surgery, patients' tear fluid flow was significantly higher than pre-operative values. As a result, tear film release of VEGF was calculated in pg/min by multiplying the tear film VEGF concentration by the tear fluid flow into the capillary tube. Within 2 days of surgery, release of tear film VEGF increased approximately 5 times, although the absolute concentration of tear film VEGF remained the same.[95,134] This would potentially overwhelm constitutively expressed tear film sFlt-1, leaving residual VEGF protein available for binding to pro-angiogenic receptors.

Conversely, VEGFR-2, which is considered a primary mediator of angiogenic activity, may be up regulated in corneal tissue and within the endothelial cells of the vessels themselves in vascularizing corneal disease. Recent work performed by Binder et al found that VEGFR-2 was not widely expressed in normal ocular tissue, aside from retinal and scleral vascular endothelium.[151] However, VEGFR-2 was expressed in corneal vascular endothelial cells in the majority of eyes with corneal vascularization. In addition, VEGFR-2 expression was increased in the limbal vascular endothelium in eyes affected with primary glaucoma, uveitis glaucoma, and neoplastic glaucoma when compared to controls.[151] Since corneal vascularization originates from

existing limbal vasculature, up-regulation of VEGFR-2 in this area may facilitate corneal neovascularization.

An additional potential explanation for the findings presented here, including that of physiologically significant VEGF concentrations in the tear film of unaffected normal eyes, is alternative splicing of VEGF. An alternative isoform of VEGF ($VEGF_{165}$), termed $VEGF_{165b}$ differs from $VEGF_{165}$ by 6 amino acids at the C-terminus and is thought to have anti-angiogenic activity.[6,15-18,169] In the case of human colorectal cancer, greater than 90% of VEGF in unaffected colonic tissue in patients with colorectal cancer is $VEGF_{165b}$, as detected by PCR and ELISA.[6] In tissues affected by colorectal cancer, however, $VEGF_{165b}$ is down regulated and $VEGF_{165}$ is up regulated, leading to increased vascularization and potential tumor metastasis.[6] Studies investigating the role of anti-angiogenic isoforms in ophthalmic disease have also shown a shift from the $VEGF_{165b}$ isoform to the angiogenic $VEGF_{165}$ isoform. This has been demonstrated in both central retinal vein occlusion and diabetic retinopathy,[15,16] as well as studies of retinal neovascularization in mice.[170] Thus, the balance of angiogenic and anti-angiogenic isoforms is purported to determine whether angiogenesis takes place. It is possible then that in health, the $VEGF_{165b}$ isoform predominates in the tear film. With corneal vascularization, however, the $VEGF_{165}$ isoform may indeed be up regulated while the b isoform is down regulated. To the authors' knowledge, the ELISA kits used in this study do not distinguish between the $VEGF_{xxx}$ and $VEGF_{xxxb}$ splice variants. Recently, the idea of anti-angiogenic VEGF isoforms has become somewhat controversial. Despite several studies affirming the existence of the b isoforms, a recent (2012) paper by Harris et al has called their existence into question. This group was consistently unable to detect b isoforms in mouse and human cells and tissues, suggesting that any $VEGF_{xxxb}$ transcripts detected were likely amplification artifacts resulting from injudicious PCR primer design.[14]

In addition to documenting similar levels of tear film VEGF in both control and vascularized canine eyes, no definitive correlations could be made between tear film VEGF concentration and extent of disease, type of disease, medications in use, or other parameters using the current model which was not adjusted for STT value. No specific breeds were strongly over-represented in the sample population. Only corneal vessel depth was associated with an elevated tear film

VEGF concentration; however, even this positive relationship between deep corneal vessels and increased tear film VEGF levels is difficult to correlate, as availability of tear film VEGF protein to the deep corneal layers has yet to be determined. Interestingly, Zakaria et al also found that human tear film VEGF concentration was not correlated with extent of vascularization, as determined by image analysis.[94] In the study reported herein, differences in corneal vascularization were assessed via a vascularization score for each eye based on multiple components that typify corneal vascularizing disease severity. While a more accurate way to quantify corneal vascularization might have been to employ similar image analysis to that of Zakaria et al and determine percent of cornea affected, such an approach has significant limitations in dogs. Accurately ascertaining surface area involvement from a photograph ideally requires that the photograph be obtained perpendicular to the area being evaluated. Due to patient movement and the significant difficulty of obtaining consistent photographic angles of all portions of the cornea on awake clinical patients, an image analysis approach was discounted. Additionally, vessel density, particularly with vessels affecting multiple layers of the cornea cannot be reliably ascertained from a two-dimensional photograph; thus, slitlamp biomicroscopy, while introducing a degree of subjectivity, was determined to be the most practical method of corneal vascularization assessment. Nevertheless, this method of evaluating extent of canine corneal vascularization resulted in similar findings to Zakaria et al, who found no link between the percentage of cornea affected by vascularization, nor any relationship between the size of the vessels and tear film VEGF.

Despite the lack of relationship between tear film VEGF and other parameters, this study detected significantly higher tear film VEGF concentration in unaffected eyes of dogs with unilateral vascularizing corneal disease, compared to control eyes and eyes with vascularized corneas. In these unaffected eyes, tear film VEGF concentration averaged approximately 1.5 times the levels found in control or diseased tear film samples. Zakaria et al reported a similar finding in basal tears and reflex tears. In these samples, the tear film of unaffected eyes had an elevated VEGF concentration when as compared to control eyes.[94] Although this finding was not significant, VEGF concentrations were also increased 1.5-fold. This trend of increased levels in the contralateral normal eye of a unilaterally vascularized individual also existed for the pro-angiogenic cytokines IL-6 and IL-8, which were increased 1.3- and 1.6-fold as compared to

controls, respectively. In this canine study, evaluation of STT findings across diseased, control, and unaffected samples, including the significantly lower STTs in unaffected eyes, as well as the inverse relationship of tear film VEGF concentration to STT results, suggest that the elevated VEGF concentrations found in the tear film of unaffected eyes may be, at least in part, due to reduced dilutional effects.

However, if the unaffected eyes truly have elevated levels of tear film VEGF, what biological purpose these elevated tear film levels might serve is unclear, as are the underlying mechanisms of this finding. Our lack of understanding of the sources of tear film VEGF, of control of VEGF secretion and activity, as well as VEGF receptor expression in corneal disease, all contribute to the apparent paradox of this finding. One explanation could be that while only one eye is affected by disease, perhaps tear film VEGF concentrations or release of VEGF into the tear film is stimulated equally in both eyes; that is, the eyes respond in a unified manner to a given stimulus. The diseased eye may then have a concurrent change in VEGF receptor expression, sFlt-1 and/or VEGFR-2, which favors angiogenesis. A combination of these receptor responses may further be complemented by changes in the vasoactive or vasoinhibitory cytokine profile of the tear film. The increased VEGF protein would then only be consumed in the affected eye, thus resulting in an elevated VEGF protein content in the unaffected eye tear film and a constant VEGF concentration in the tear film of the vascularized eye. Arguing against this line of thinking is the theory that VEGF is locally produced and released in the ocular environment in an autocrine or paracrine fashion.[1] Alternatively, if lower amounts of tears have a higher VEGF concentration, perhaps disease contributed to increased tear production in the diseased eye. This would result in dilution of VEGF in the diseased eye, resulting in a lower tear film concentration. Given the data presented in this study, this dilution effect may accurately explain the reason for increased VEGF concentrations in the unaffected eye and lower concentrations in the control and unaffected eyes.

In this study, appropriate sample numbers were obtained for conclusions to be drawn between the three main sample types (control, patient unaffected, and patient diseased). However, adequate case numbers of specific disease entities and cases treated with certain medications were lacking. This lack of sample size and diversity impacted the study's ability to draw clear

associations between different underlying disease processes and effects of medications on tear film VEGF levels. In addition, as with many previous tear film studies, it was difficult to acquire large tear volumes from all patients. For example, it was possible to harvest greater than 30 microliters of tear film from some animals, while from others the maximum volume obtained was less than 5 microliters. As a result, different volumes of tear sample were used in the ELISA assays. Statistical evaluation of the effect of tear film sample amount on VEGF tear film concentration indicated a significantly higher concentration when very small tear volumes (0.5 and 1 μL tear samples) were used as compared to larger volumes (5 and 6 μL tear samples). Theoretically, a set of samples could have artificially inflated or depressed detected VEGF concentration levels depending on the sample volume used for the assay. In addition, since the assay is manufactured to use with 200 μL samples, using extremely small samples and diluting to the volume necessary for the assay could introduce error into the calculation of VEGF concentration. To extrapolate the VEGF concentration of an optimal 200 μL tear sample, all tested tear film volumes were multiplied by the appropriate factor to reach 200. Any error in assessing the true tear film VEGF concentration (including volumetric error or assay error) would be amplified by that same factor. Mitigating this systematic error, however, is the relatively even distribution of sample volumes used within each sample type group. Furthermore, the mean volume of sample utilized for the assay was not significantly different between sample type groups. Statistical analysis overall was not adjusted for sample amount due to collection methodology (timed collection versus volume as an endpoint for sample collection), and has also not been adjusted for STT values.

In conclusion, the findings of this study, while they do not suggest outright that topical VEGF inhibitor therapy would alleviate corneal vascularization, do not contradict the use of this mode of therapy either. In fact, if the postulations above regarding potential epithelial cell secretion of VEGF, as well as increased receptor availability and consumption of VEGF, are true, then topical therapy targeting VEGF may indeed still be integral to corneal clarity and disease therapy. Subsequent investigations should attempt to elucidate the source of the VEGF in tear film, the cytokine profiles of the tear film, tear film concentrations of sFlt-1, and corneal epithelial and stromal concentrations of VEGF protein and receptors in vascularizing corneal disease. Further investigations into changes in the pro-angiogenic cytokine and analyte profiles

of the unaffected contralateral eye in those with unilateral disease might also provide further insight into bilateral ocular response to disease, even if the clinical disease is unilateral in nature.

V. CHAPTER 5: CONCLUSIONS AND FURTHER RESEARCH

In conclusion, the pilot study reported herein lays the foundation for further work utilizing polyester rods in canine tear film collection. Percent tear film volume recovery was >93% for polyester rods at all volumes wicked. Percent VEGF recovery was also suitably high and did not differ appreciably from capillary tubes. Recovery of VEGF from the rods was greater than 90% and binding between VEGF and the rod itself appears to be minimal. Polyester rods proved to be an easy, reliable, and efficacious method for collecting canine tears for VEGF assays.

Follow up work to the pilot study found that VEGF tear film concentration were high in both normal dogs and dogs with vascularizing corneal disease. Tear film VEGF concentration did not correlate with disease status, extent of vascularization, or medication status. Corneal vascularization does not appear to be controlled simply by the amount of VEGF protein in the sampled tear film; rather, corneal vascularization may be controlled by corneal epithelial cell VEGF secretion, tissue concentration of VEGF protein and/or VEGF receptors in tissue or by tear film expression. Specific disease processes do not appear to affect tear film VEGF concentrations.

The results of this study do not suggest that topical VEGF inhibitor therapy would not be effective; in fact, as postulated above, it may still very well be effective by rendering VEGF protein less available to primed receptors. However, if the appropriate receptors are located deep within corneal tissue or within the vascular endothelium itself, then topically applied anti-VEGF therapies may need enhanced penetration characteristics to allow them to reach target areas within corneal tissues. Future studies in this area may focus on sources of tear film VEGF, contributions of the epithelial secretion portion of the canine tear film, the cytokine profiles of the tear film, tear film concentrations of sFlt-1, and corneal epithelial and stromal concentrations of VEGF protein and receptors in vascularizing disease. Evaluating this may thus give further insight into VEGF-mediated corneal vascularizing disease and anti-VEGF therapies.

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APPENDIX A: TABLES

Table 1. Percent recovery of multiple volumes from bonded polyester fiber rods. Various known volumes measuring less than full rod capacity were wicked and eluted in triplicate; average volume eluted and percent recovery of volume is displayed below. Total volume holding capacity of the rods is approximately 65 µL.

Volume Wicked µL	Mean Volume Eluted µL +/- SD	Mean % Recovery +/- SD
5	4.67 +/- 0.58	93.3%
10	10.0 +/- 0.0	100%
20	19.67 +/- 0.58	98.3%
30	28.33 +/- 0.58	94.4%
40	39.0 +/- 0.0	97.5%
50	48.0 +/- 1.0	96.0%
60	59.33 +/- 1.15	98.9%

Table 2. Recovery of VEGF standard dilution via polyester rod or capillary tube. Individual VEGF standard dilutions were wicked into 2 polyester rods and the eluates were assayed in duplicate. Each dilution was also drawn into a capillary tube and the eluates were assayed in duplicate. Actual concentration recovery and percent recovery of the VEGF standard dilution concentration is displayed below.

Dilution	Dilution Concentration pg/mL	Polyester Rod	Capillary Tube	Polyester Rod Mean % Recovery +/- SD	Capillary Tube Mean % Recovery
1:2	1250	1228.71	1213.04	98.3 +/- 7.58	97.04
1:4	625	566.68	568.0	90.7 +/- 2.50	90.88
1:8	312.5	249.31	178.86	79.8 +/- 2.45	57.24
1:16	156.25	120.38	84.44	77.0 +/- 14.45	54.04
1:32	78.125	None Detected	None Detected	None Detected	None Detected
1:64	39.0625	None Detected	None Detected	None Detected	None Detected
1:128	19.53125	None Detected	None Detected	None Detected	None Detected