Histopathology of Human Age-Related Macular Degeneration and the Development of a Novel Animal Model

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ENGLISH ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly worldwide. Due to the inadequacy of current pharmacotherapies, novel molecular targets must be sought as potential therapeutic candidates. Furthermore, there is a need for more efficient and cost-effective animal models of this pathology in order to accelerate *in vivo* investigations.

Our laboratory is in possession of human choroidal neovascular membranes which we examined for expression of cyclooxygenase (COX)-2. This expression was characterized in retinal pigment epithelial, vascular endothelial, and fibroblast cells and correlated with patient age. We also looked at the feasibility of creating a rabbit laser-injury model to adequately mimic human neovascular AMD.

Our results suggest that anti-COX-2 therapies may be beneficial to some patients with neovascular AMD. Moreover, there is strong potential for the development of clinically relevant choroidal neovascularizaiton in rabbits using the laser-injury technique. This approach may yield a novel, cost-effective AMD model.

FRENCH ABSTRACT

La dégénérescence maculaire liée à l âge (DMLA) est la principale cause de cécité chez les personnes âgées. Bien que plusieurs traitements soient sur la marche, aucun d'entre eux ne permet de soigner la DMLA. C'est dans cette optique que la recherche de nouvelles cibles thérapeutiques continue. De plus l'élaboration de modèles animaux efficaces et abordables de cette pathologie est capital afin d'accélérer les recherches in vivo.

Notre laboratoire possède des échantillons de tissus de patients atteints de la DMLA, que nous avons utilise pour caractérise l'expression de la cyclooxygenase (COX-2). Le niveau d'expression de COX-2 a été déterminé dans l'épithélium rétinal pigmente, l'endothélium vasculaire, les fibroblastes, et mis en corrélation avec l'âge des patients. Nous avons également étudie la possibilité de créer un nouveau modèle animal de la DMLA chez le lapin base sur la destruction de la rétine par un laser.

Nos résultats suggèrent qu'une thérapie anti-COX-2 pourrait être bénéfique pour les patients atteints de DMLA. De plus, il semblerait que la destruction de la rétine par un laser chez le lapin possède un fort potentiel pour induire une signifiante neovascularisation de la choroid. Cette approche pourrait aboutir à la création d'un nouveau et abordable modèle de la DMLA.

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LIST OF ABBREVIATIONS

ABC	avidin-biotin complex
AEC	3-amino-9-ethylcarbazole
BSA	bovine serum albumin
CNV	choroidal neovascularization
CNVM	choroidal neovascular membrane
COX	cyclooxygenase
EC	vascular endothelial cell
FA	fluorescein angiography
FB	fibroblast
ICG	indocyanine green angiography
LDL	low-density lipoprotein
MMP	matrix metalloproteinase
PDGF	platelet-derived growth factor
PDT	photodynamic therapy
PIGF	placental growth factor
PKC	protein kinase C
RPE	retinal pigment epithelium
TBS	tris-buffered saline
TIMP	tissue inhibitor of matrix metalloproteinase
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

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Chapter 1

INTRODUCTION

1.1 Ocular Anatomy

The human eye perpetuates vision through the interaction of light with several distinct ocular structures that work in concert. Light waves from an external source enter the eye through the cornea, the clear, concave-convex tissue at the most anterior border of the eye. The cornea refracts incoming light and transmits it through the fluid-filled anterior chamber, which is delimited posteriorly by the iris, the colored part of the eye. Light is transmitted through the fluid, known as aqueous humor, and subsequently through the pupil, the opening in the center of the iris. Light then passes through the crystalline lens, a clear structure composed of four layers and having a natural curvature that can be adjusted involuntarily to refract the incident light to varying degrees. Light then continues through the vitreous humor, a clear gel that constitutes approximately 80% of the eye's total volume, and ultimately and ideally focuses on the retina immediately posterior to the vitreous. At this point, the incident light rays have been reversed and inverted. The retina is composed of 7 distinct cell types, namely cone, rod, ganglion, amacrine, horizontal, bipolar and glial cells. These various cell types interact with one another to accomplish the common task of converting incident light into electrical signals that are sent to higher brain structures for processing, allowing for what we refer to as vision. Posterior to the retina lies a cellular layer known as the retinal pigment epithelium, which is responsible for many tasks including sustenance of the overlying

neurosensory retina. Posterior to the retinal pigment epithelium is the choroid, a major intraocular vascular network. The retinal pigment epithelium and the choroid are separated by a thin membrane known as Bruch's membrane, which has a significant role in some ocular pathologies, including neovascular age-related macular degeneration. The sclera is the tough, fibrous layer immediately posterior to the choroid and is also the outermost layer of the eye, continuous anteriorly with the cornea.

1.2 Overview of Age-related Macular Degeneration

Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in the elderly in industrialized nations [1, 2]. The hallmark of the more severe form of this disease, known as neovascular AMD, is the aberrant growth of new blood vessels within the eye. These vessels arise from the choroid, the vascular network that lies deep to the neurosensory retina and serves to nourish the retina and absorb scattered light. This aberrant growth, termed choroidal neovascularization (CNV), contributes to the severe visual deficits experienced by individuals afflicted with advanced AMD. As the vessels grow into the subretinal space and retina, blood and fluid can leak out, subsequently causing irreparable damage and vision loss. Aggressive research initiatives are underway with hopes of discovering novel therapeutic strategies that will more adequately manage and prevent progression to advanced disease. Neovascular AMD currently affects nearly 1.75 million individuals in the United States alone [3]. Inevitably, if research efforts fall short, prevalence of this pathology will increase dramatically as the number of people increases in the over 65 age bracket in the coming decades.

AMD is a multifactorial pathology with numerous unknowns yet to be elucidated. A major breakthrough in AMD research was the recent discovery of an increased prevalence of the disease in individuals with a specific polymorphism in the gene encoding complement factor H [4-6], a negative regulator of the complement system. This novel finding substantiated previous studies that suggested a role for inflammation in the prevalence and progression of AMD [7-11]. A follow-up study further demonstrated numerous polymorphisms in the same gene that were associated with a higher risk of

AMD development [12]. Despite continued advances in describing and characterizing disease etiology, current therapeutic strategies are limited to focusing on the control of CNV rather than its prevention. Accordingly, the development of animal models has focused on creating CNV through a variety of means in an attempt to mimic the human pathology. The laser-injury model in primates, first described by Ryan in 1982 [13], has since been adapted for other species, including rat [14-17], mouse [18-21] and pig [22, 23], and is heralded as an effective technique for mimicking the CNV seen in humans with neovascular AMD. The laser-injury technique makes use of surgical ophthalmic lasers - generally argon [15-17], krypton [19, 21, 24] or diode [20, 25, 26] - to create lesions in the retinas of experimental animals. Ideally these lesions create breaks in Bruch's membrane, the thin membrane that separates the pigmented layer of the retina from the vascular choroid. The insult can stimulate a cascade of events that may ultimately result in neovascularization. Moreover, the break in Bruch's membrane provides a conduit for vessel growth into the subretinal space, thereby somewhat imitating the human condition. Other models have made use of transgenic animals [19, 27-29] or subretinal injection of compounds to stimulate neovascularizaiton [30-34], but to date no model is universally recognized as an adequate re-creation of the human disease.

Two diagnostic tests are commonly employed when evaluating the presence and subtype of CNV in patients. Fluorescein angiography (FA) involves administration of fluorescein dye intravenously and subsequent photographing of the ocular circulation using a camera equipped with special filters. If there is any abnormal circulation, swelling, or leakage, diagnosis can generally be made from observations of fluorescein patterns. Indocyanine

green angiography (ICG) is similar to FA in many ways but has the added advantage of allowing the ophthalmologist to identify leaking vessels deeper within the eye [35].

There are limited treatment options that exist for patients with CNV. One option that has proven somewhat effective for selected patients is photodynamic therapy (PDT), whereby a photosensitive dye is injected intravenously, associates with lipoproteins, and localizes in neovasculature due to the increased number of low-density lipoprotein (LDL) receptors in neovascular tissues [36]. The drug is then activated using a laser with a wavelength that corresponds to the peak absorption of the drug. Release of toxic oxygen species ultimately results in occlusion of neovasculature, preventing further damage from blood and fluid leakage. As with many therapies, there exists great variation in the success of PDT. Consequently, treatment focus has shifted to combination therapies employing antiangiogenic or anti-inflammatory drug therapy along with PDT [37-43] in order to achieve greater treatment outcomes. However, published literature is laden with conflicting results. Current studies are aimed at determining the efficacy of various drug combinations with concomitant PDT in achieving desired therapeutic results.

Chapter 2

LITERATURE REVIEW

2.1 Current Pharmacotherapies

There are several drugs that are currently being investigated for disease activity in CNV. The majority of current approaches focus on anti-vascular endothelial growth factor (VEGF) compounds. Previous studies have demonstrated increased expression of VEGF in animal models of CNV [44-46] and in the eyes of AMD patients [47, 48]. Furthermore, VEGF expression has been positively correlated with vessel formation [49] and is absolutely required for the development of CNV in animal models [50]. Thus, it is no surprise that anti-VEGF strategies top the list of AMD treatment hopefuls. Details of three heavily researched anti-VEGF compounds are herein discussed.

Pegaptanib

In 2004, an unprecedented hope for effective disease management was instilled in AMD patients with the FDA approval of a new drug called pegaptanib (Macugen®), a pegylated (polyethylene glycolated) anti-VEGF aptamer that specifically binds one isoform of VEGF known as $VEGF_{165}$ [51]. This conjugation to polyethylene glycol confers advantages in biodistribution of the aptamer to both healthy and inflamed tissues [52]. $VEGF_{165}$ is known to be the primary VEGF isoform affecting CNV growth and vascular permeability, and thus is logically an ideal candidate target. Recent results from multi-center clinical trials suggest that pegaptanib was clinically beneficial in patients with AMD. However, benefit was associated with prolonged therapy in a study comparing

patients treated for 2 years versus 1 year, demonstrating visual benefits associated with continued treatment in the former group [53, 54].

In addition to the requirement for prolonged therapy, the drawbacks of this apparent panacea are well documented. There have been some reports demonstrating that intraocular pegaptanib injections have been associated with retinal pigment epithelial (RPE) tears [55, 56] and even progression of CNV [57]. In a multi-center safety study of pegaptanib over 2 years, serious injection related ocular complications, although rare, were reported [53, 54]. As with any new drug, care must be taken to separate effects observed as a result of the agent being tested versus those of other treatment modalities being given at the same time so as not to misrepresent drug efficacy.

Bevacizumab

A second anti-VEGF compound is the monoclonal antibody bevacizumab (Avastin®), approved by the FDA in February 2004 for the treatment of metastatic colorectal cancer [58] and now being intensely studied for its efficacy in treating CNV. Bevacizumab binds all active forms of VEGF-A, and thus holds the potential of proving more effective than pegaptanib in treating CNV. To date, there are no long-term studies that have been performed to evaluate either toxicity or efficacy of intravitreally administered bevacizumab; however, several short-term studies have been performed. Evidence from many of these studies has demonstrated that intravitreal bevacizumab is well-tolerated, efficient in reducing anatomical signs of CNV, and generally associated with stabilization or improvement in patient vision [59-63]. Another study investigated the safety and efficacy

of systemic bevacizumab, despite the potential risks associated with systemic anti-VEGF therapy, and concluded that therapeutic benefits were obtained [64]. Due to the possible complications associated with systemic treatment, initiation of large-scale clinical trials involving such therapy are questionable.

There has been some suggestion that bevacizumab therapy may benefit patients who do not respond well to pegaptanib therapy [65] although further investigation is required before any conclusions can be drawn. It is worth noting that RPE tears have been observed in patients receiving intravitreal bevacizumab [66] but, like pegaptanib, occurrence is very rare.

Ranibizumab

Ranibizumab (Lucentis™) is a humanized monoclonal antibody fragment that binds all active isoforms of VEGF-A [67, 68]. The drug binds to VEGF and prevents its association with its receptors VEGFR-1 and VEGFR-2 in much the same way as bevacizumab (both drugs are derived from the same murine anti-VEGF antibody) [69]. Ranibizumab, although having been specially designed for intraocular administration, is significantly more expensive than its close relative bevacizumab [70]. However, ranibizumab has lived up to expectations as several studies have verified its safety and unprecedented efficacy [68, 71, 72], and even superior efficacy to PDT in one comparison study [73]. The positive pooled results obtained from phase III clinical trials led to the June 2006 FDA approval for the use of ranibizumab to treat neovascular AMD [43, 67]. As with other anti-VEGF therapies,

some complications have been seen with ranibizumab therapy [71], but the rate of serious complications reported has been minimal.

2.2 Emerging Pharmacotherapies

The search for a universal treatment regimen for CNV is ongoing and will likely involve the union of two or more therapies. Further research is needed to identify other treatment avenues that may be useful in combination with established therapies in order to obtain the best possible treatment outcome. The following drugs are some of the most promising emerging therapies.

Triamcinolone acetonide

Triamcinolone is a corticosteroid [74] and thus exerts an anti-inflammatory effect by preventing the formation of prostaglandins and leukotrienes through the release of lipocortin, which reduces arachidonic acid release by inhibition of phospholipase A2 [75]. Experimental studies have demonstrated the ability of triamcinolone to decrease VEGF expression in cultured human RPE cells under oxidative stress [76] and thus may prove effective in the fight against CNV. Intravitreal triamcinolone has also proven to be a potent inhibitor of CNV in animal models [77, 78]. These findings suggest a beneficial role for triamcinolone in the fight against CNV.

Although some studies are still investigating triamcinolone as a monotherapy [79, 80], the focus has largely shifted to combination therapies with PDT. Justification for this combination lies in the assumption that triamcinolone, being a corticosteroid, will decrease the inflammatory and exudative reaction that follows PDT, thereby acting synergistically with PDT to improve visual outcome [39, 69, 81, 82]. This effect has been seen *in vitro* where triamcinolone effectively suppressed a PDT-induced pro-angiogenic

response, in part by preventing an increase in VEGF expression [83]. A number of studies have investigated the efficacy of combining intravitreal triamcinolone with PDT to determine if any additional benefits may be conferred to the patient over PDT alone. There is still no general consensus about the efficacy of this combination, but some studies have demonstrated that the combination therapy is more effective in terms of number of necessary re-treatments, and furthermore report increases in visual acuity over PDT monotherapy [38, 40, 41, 74]. Despite the apparent synergistic effect of this combination, there have been many reports of severe cataract and glaucoma development during the clinical investigations [38-40, 74, 81]. One study found that the combination of PDT and triamcinolone was ineffective in preventing a considerable decrease in visual acuity [39], while another illustrated a case of acute retinal necrosis following the administration of triamcinolone as an adjunct to PDT [84]. Clearly, risk versus reward needs to be considered if this form of combination therapy is to be employed in its current state.

Anecortave acetate

Anecortave acetate is an angiostatic cortisene that exerts its action by inhibiting metalloproteinases, consequently blocking the migration of proliferating endothelial cells [69, 85]. This drug is devoid of conventional steroidal pharmacological properties, which reduces the risk of developing increased intraocular pressure and progression of cataracts [69]. Thus, it has a clear advantage over triamcinolone if it proves comparatively effective in treating CNV. In a safety trial involving 128 patients, anecortave acetate was shown to be clinically safe following administration and re-administration at 6-month intervals [86]. Another study demonstrated the clinical benefit of anecortave acetate versus a placebo in

AMD patients [85]. A phase III randomized, double-blinded study compared safety and efficacy of anecortave acetate with PDT and found no significant difference in the number of responders that lost fewer than 3 lines of vision at month twelve [87]. Taken together, these results suggest that anecortave acetate holds potential as a therapeutic agent to treat CNV. Future studies will undoubtedly investigate the efficacy of anecortave acetate both as a monotherapy and in combination with other established and emerging treatments.

Squalamine

Squalamine is a broad-spectrum steroidal antibiotic isolated from tissues of the dogfish shark and first characterized and described by Moore *et al* in 1993 [88]. An independent investigation yielded novel antiangiogenic properties of squalamine in multiple animal models of cancer, attributed in part to the blocking of mitogen-induced proliferation and migration of endothelial cells while having no observable effect on unstimulated endothelial cells [89]. The unique action of squalamine led researchers to hypothesize a potential widespread value of the antibiotic in diseases characterized by neovascularization [89]. Investigations of the efficacy of squalamine in ocular pathologies began with a study by Higgins *et al* in which inhibiting retinal neovascularization in a mouse model of oxygen-induced retinopathy using squalamine was investigated [90]. The authors concluded that subcutaneously-administered squalamine significantly improved retinopathy and may be a novel agent for effective treatment of ocular neovascularization. Subsequently, Genaidy and colleagues demonstrated that intravitreal administration of squalamine did not inhibit iris neovascularization in a primate model,

yet systemic squalamine did [91]. A more recent study showed that systemically administered squalamine partially reduced neovascularization in a rat model of CNV [92]. Future clinical trial data will yield more information about the usefulness of squalamine in treating human CNV while also providing information about the most efficacious route of delivery.

Rapamycin

Rapamycin is an immunosuppressive agent produced by *Streptomyces hygroscopicus* that was first isolated more than 30 years ago and initially revered for its antifungal properties [93-95]. Several years later, a study investigating corneal allograft rejection demonstrated that systemic rapamycin effectively inhibited corneal neovascularization in a rat model [96]. This finding opened the door for additional studies on the antineovascular properties of rapamycin. In 2002, Guba *et al* reported that rapamycin was effective in inhibiting tumor growth and angiogenesis in *in vivo* mouse models, and demonstrated an association between decreased production of VEGF and an inhibited response of vascular endothelial cells to stimulation by VEGF [97]. Recent reports suggest that intraocular rapamycin delivery can also effectively inhibit corneal neovascularization [98, 99]. Dejneka and colleagues demonstrated in 2004 that systemic rapamycin inhibited both retinal and choroidal neovascularization in mouse models [100]. Interestingly, this group observed increased Flt-1 (VEGFR-1) protein levels after rapamycin treatment concomitant with inhibition of ocular neovascularization. This finding not only supports the growing body of evidence that VEGFR-1 may have an antiangiogenic role [101-104] but also demonstrates

how rapamycin could effectively act as an adjunct to current anti-VEGF therapies to prevent or treat neovascular disease.

2.3 Alternate Molecular Targets

Continued research into alternate molecular targets is necessary until a sound treatment regimen can be established for AMD patients. A vast array of targets are currently under investigation. Here we present a brief overview of some of the most promising targets.

Vascular endothelial growth factor receptors (VEGFR)

The importance of VEGF in the development of CNV has been well established. Several studies have unequivocally demonstrated this fact, and consequently very specific drugs have been developed that target and inhibit VEGF binding to its receptors. *In vivo*, VEGF binds to and activates VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1), both being tyrosine kinase receptors [105]. These receptors represent possible novel therapeutic targets in the fight against CNV.

To date, the role of VEGFR-1 in CNV development has not been completely elucidated. In 2003, Rakic *et al* reported that systemic administration of an anti-VEGFR-1 antibody was efficient in inhibiting experimental CNV in a mouse model [106]. In agreement with these findings, a recent publication demonstrated that intravitreous or periocular injections of siRNA directed against VEGFR-1 resulted in a significant reduction in CNV area [107]. Conversely, Nozaki *et al* demonstrated that intravitreous injection of anti-VEGFR-1 antibody did not reduce CNV in a mouse model [101]. Furthermore, this group demonstrated that by using a VEGFR-1-specific ligand, placental growth factor-1 (PIGF-1), a dose-dependent suppression of CNV could be seen. The further demonstration that

VEGF signaling through VEGFR-1 could have an antiangiogenic function [101] is a novel concept that certainly merits further investigation as well as careful consideration when designing future experiments. Another recent report reviews past research and suggests that VEGFR-1 has a dual role: a negative role in embryonic angiogenesis and a positive role in adulthood [108]. There is also evidence that the VEGR-1 gene encodes mRNA for both a full-length receptor and a soluble form of the protein which carries only the extracellular domain [109, 110]. The suggestion that the soluble VEGFR-1 could act as a natural VEGF inhibitor certainly warrants further investigation [108]. Despite continuing research, a definitive role for VEGFR-1 in neovascularization remains to be explicated.

Unlike VEGFR-1, the role of VEGFR-2 appears much more clear-cut. It is generally accepted to be the major positive signal transducer for both physiological and pathological angiogenesis [108], and consequently has become the target of numerous antiangiogenic strategies. A few studies have investigated the role of a non-specific kinase inhibitor (PKC412) in blocking VEGFR-2-mediated signaling. Animal models have revealed inhibition of CNV [23] and complete blockage of retinal neovascularization [111]. PKC412 is known to inhibit VEGF and platelet-derived growth factor (PDGF) receptor tyrosine kinases as well as several isoforms of protein kinase C (PKC) [111, 112], and thus may exercise its antiangiogenic effect via mechanisms beyond VEGFR-2 inhibition. Other non-selective VEGFR-2 inhibitors have also been found to effectively suppress the development of CNV [25]. To determine whether selective VEGFR-2 inhibition would similarly inhibit CNV formation, Takeda *et al* investigated the therapeutic capability of such an inhibitor, SU5416, both *in vitro* and *in vivo*, and

concluded that this selective inhibition may be beneficial for treating CNV [113]. Evidently, VEGFR targeting may be a logical adjunct to the current anti-VEGF therapies.

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of extracellular matrix degrading proteinases [114] that have been shown to be associated with angiogenesis [115-117]. MMP-2 and -9 are within the subgroup of MMPs known as gelatinases and have traditionally received much of the MMP-associated attention in cancer research in part because of their roles in angiogenesis [118-120]. The importance of MMPs in pathology, however, is not exclusive to their roles in tumor angiogenesis. Zeng and colleagues demonstrated expression of MMP-2 and -9 in choroidal neovascular membranes using immunohistochemistry and a corresponding lack of expression of these gelatinases in normal retinas [121]. The authors concluded that MMP-2 and -9 may be involved in degradation of Bruch's membrane, thereby facilitating vessel perforation into the membrane. Another study investigated the effects of known CNV-related factors (tumor necrosis factor-alpha, VEGF, and fibronectin) on the secretion of gelatinases from cultured RPE cells [122]. In this study, the authors demonstrated expression of MMP-2 but not MMP-9 in the absence of these exogenous factors. Up-regulation of MMP-2 expression and initiation of MMP-9 expression in the presence of these exogenous CNV-related factors was observed and highlights a key role of gelatinases in the pathogenesis of CNV. *In vivo*, natural MMP inhibitors known as tissue inhibitors of matrix metalloproteinases (TIMPs) exert their effects by forming complexes with MMPs, thereby inhibiting their bioactivity and ultimately decreasing angiogenesis [123-125]. Some

studies have investigated the potential for synthetic MMP inhibitors to inhibit CNV induction and progression [26, 126]. The results from one such study suggests that administration of a selective MMP inhibitor may prevent the formation of CNV as demonstrated in a laser-injury model, however, no statistically significant benefits were observed for previously established CNV [26]. The authors suggested that MMP inhibition may be beneficial in combination with PDT in preventing the recurrence of CNV from temporarily closed new vessels. In this manner, MMP inhibitors may prove to be potent adjuncts to currently established treatment modalities.

Cyclooxygenases

Cyclooxygenase (COX) is involved in the conversion of arachidonic acid to prostaglandins and is therefore an attractive target in many pathologies involving an inflammatory component, including numerous ocular diseases [17, 127-130]. Cyclooxygenase exists as three isoforms: COX-1, COX-2, and COX-3. To date, COX-3 has not received a great deal of attention. COX-1 is expressed in various tissues and is integral to normal, non-pathologic processes. COX-2, however, is expressed almost exclusively in neoplastic or injured tissues [131], with its expression being induced by various inflammatory stimuli such as mitogens and cytokines [132]. Furthermore, COX-2 has been shown to modulate the expression of the VEGF ligand and its receptors [133, 134].

In a study of experimentally-induced CNV in mice, it was shown that non-selective inhibition of COX-1 and -2 could suppress CNV formation [135]. Building on these results, another study effectively demonstrated that selective COX-2 inhibition suppressed

experimental CNV in a mouse model, indicating that selective inhibition of COX-2 elicits an antiangiogenic response *in vivo* [136]. This conclusion is consistent with findings in other forms of angiogenesis, both neoplastic [127, 128, 137-139] and non-neoplastic [17, 129, 130, 140-142]. Despite these promising observations, some studies have demonstrated a lack of efficacy of both COX-1 and COX-2 selective targeting in inhibiting laser-induced CNV in an animal model [17]. Thus, further investigation into the efficacy of COX inhibition is needed to determine therapeutic potential.

Chapter 3

MATERIALS AND METHODS

3.1.1 Human Specimens

Formalin-fixed, paraffin-embedded sections human choroidal neovasuclar membranes (CNVM) from 16 cases of AMD were collected from the archives of the Henry C. Witelson Ocular Pathology Laboratory and Registry, McGill University, Montreal, Canada. This study involved specimens from six females (mean age = 71.3 years) and ten males (mean age = 67.7 years) who underwent surgical resection of CNVM between 1995 and 2004. Surgical intervention was deemed necessary for these patients by their respective ophthalmologists. Specimens were kindly provided to The Henry C. Witelson Ocular Pathology Laboratory for use in CNV-related histopathological investigations.

3.1.2 Immunohistochemistry

Formalin-fixed, paraffin-embedded sections of the collected specimens were immunostained with an anti-COX-2 monoclonal antibody (dilution 1:400; Zymed Laboratories, San Francisco, CA, USA; clone COX 229) according to the avidin-biotin complex (ABC) technique. Sections were deparaffinized in xylene and re-hydrated through graded ethanol washes. A ten-minute incubation in boiling citrate buffer (pH 6.0) was used for antigen retrieval. To block endogenous peroxidase, incubation with 3% hydrogen peroxidase in methanol for 5 minutes was performed. Non-specific binding was blocked with a 30-minute wash with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS, pH 7.6). The anti-COX-2 mouse mAb was applied to sections of each specimen and incubated overnight at 4°C. Anti-vimentin mouse mAb (dilution 1:400; Ventana Cat# 790-2917) was applied to serial sections of each specimen to confirm tissue antigenicity. Next, the slides were incubated with rabbit anti-mouse secondary mAb E0354 (dilution 1:500; DAKO, Ontario, Canada) for 30 minutes at 37°C. Sections were then incubated with horseradish peroxidase-conjugated ABC complex (DAKO, Ontario, Canada) for 30 minutes at 37°C. Immunostaining was visualized using 3-amino-9-ethylcarbazole (AEC) chromogen (DAKO, Ontario, Canada). Finally, the slides were counterstained with Giu-II haematoxylin and cover-slipped. Sections of colon cancer and tonsil were used as positive controls for COX-2 and vimentin staining, respectively.

3.1.3 Histopathology

Sections were analyzed for COX-2 expression in retinal pigment epithelial (RPE) cells, vascular endothelial cells (EC), and fibroblasts (FB). Samples were independently graded as either positive or negative for COX-2 expression in each of these cell types by two different pathologists. Vimentin-stained sections were also analyzed for general expression to confirm tissue antigenicity and were similarly graded as negative or positive.

3.2.1 Animals

Eight female Dutch-belted rabbits (Covance Research Products, Denver, PA) aged approximately 11 months and weighing 2.0 - 2.2kg each were used in this study. These animals were individually housed at the Montreal General Hospital Animal Care Facility for the duration of the experiment. Prior to the initiation of the study, all investigators partaking in the project successfully completed the required animal training workshop and exam established by the Animal Care Committee at McGill University. All animal research was conducted in accordance with the guidelines set forth by the Canadian Council on Animal Care, the McGill University Animal Care Committee, and the Association for Research in Vision and Ophthalmology.

The eight rabbits were subdivided at random into two equal groups that were named Group A and Group B. All animals were subjected to the same experimental and housing conditions throughout the experiment, regardless of group, with exception of the time of euthanasia. All animals in Group A were euthanized 14 days post-surgery while those in Group B were euthanized 28 days post-surgery. General animal health was assessed daily by the animal care staff and ocular safety was assessed fundoscopically by a trained ophthalmologist immediately post-surgery and weekly thereafter.

3.2.2 Surgical Procedure

Animals were pre-anesthetized using a combination of Butorphanol (10mg/ml; 1mg/kg) and Acepromazine (1mg/ml; 25mg/kg) administered subcutaneously 20 minutes prior to induction of anesthesia with Thiopental sodium 2.5% (0.5 ml bolus and 0.25 ml to effect) via catheter. The following details of surgical procedure will be described for a single rabbit but were the same for all rabbits unless otherwise noted. After anesthesia induction, the rabbit was placed on the surgical table in a prone position with its head resting on the built-in head rest on the laser/ophthalmoscope apparatus (Laser Elite 532nm, Coherent AMT, Kitchener, Ontario) (Figure 1). One investigator held the rabbit's head so that it was stationary in the apparatus throughout the surgical procedure. Laser photocoagulation was performed in the right eye of each rabbit by a trained ophthalmologist while the left eye of each rabbit was left as a control. Laser burns were created using the following parameters: 532nm wavelength, 100um spot size, and 0.1 sec pulse duration. Four different burn patterns were created around the optic nerve head in each eye with each pattern corresponding to a specific laser power setting (Table 1). The same burn patterns were used in each rabbit and were created in the same location relative to the optic nerve head. The right eye of the first rabbit initially received seven peripheral laser burns in order to establish a reasonable range for laser power settings. The ideal setting would result in a burn that creates a readily apparent blister (indicative of a break in Bruch's membrane) followed by no or little bleeding. Laser settings that were too low failed to produce anything resembling a blister and those that were too high resulted in extensive hemorrhage. Once the ideal range had been determined, four power settings within that range were chosen for experimentation. A pediatric surgical contact

lens was placed against the cornea and the laser was administered through this lens. After the laser burns had been applied, the rabbit was immediately placed in front of a fundus camera (Kowa RC-XV) (Figure 1). Fundus images were obtained using a digital camera and attachment to the fundus camera apparatus in order to confirm and digitally document the exact location and morphology of laser burn patterns.

Pattern	Power (mW)	Spot Size (μm)	Duration (sec)
A – 5 on dice	75	100	0.1
B – 6 on dice	100	100	0.1
C – pentagonal	150	100	0.1
D – circle	250	100	0.1

Table 1. Parameters used for laser injury in all rabbits involved in this study.

3.2.3. Fundoscopy

Fundoscopic examination was performed by a trained ophthalmologist to ensure ocular health of the experimental animals. Each animal was sedated prior to examination using the cocktail previously described for pre-anesthesia (Section 3.2.2 Surgical Procedures). The burn patterns were assessed immediately following laser injury with concomitant assessment of potential inflammatory complications secondary to the surgical procedure. Fundus pictures were taken immediately post-surgery and at each weekly examination. To obtain digital photographs, a Sony DSCW50 digital camera was attached to the fundus camera. Photographs were catalogued by rabbit number and date for future reference.

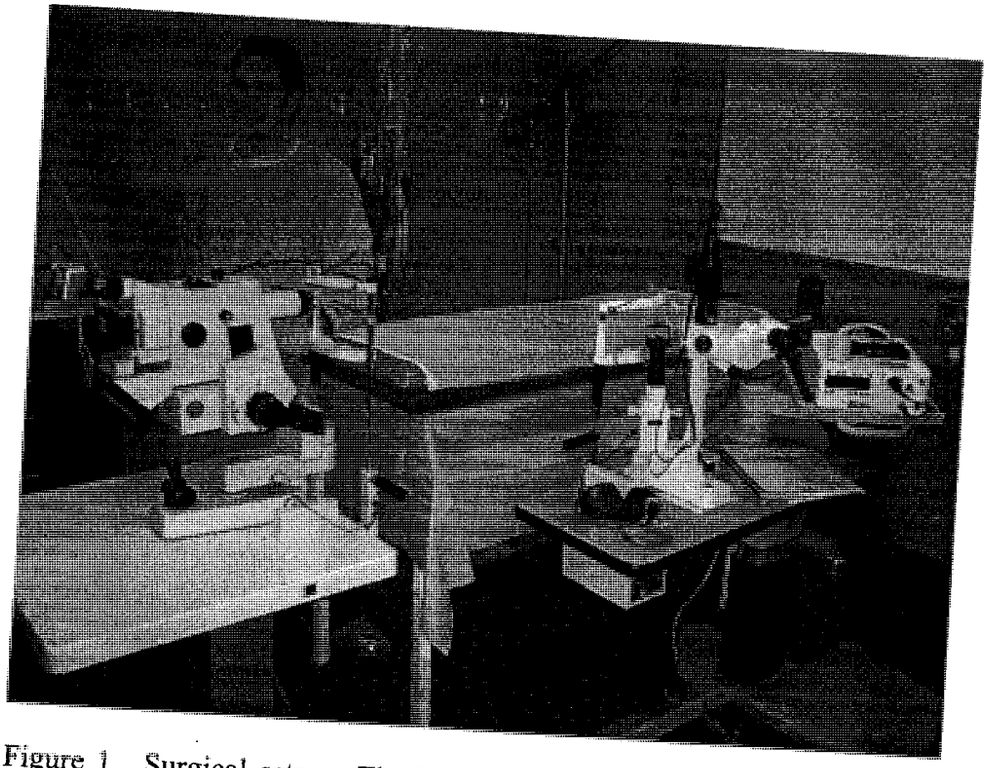

Figure 1. Surgical setup. The laser system (right) was purchased from Coherent AMT, Kitchener, Ontario, for this study. The fundus camera (left) was obtained for research use from the Department of Ophthalmology, Montreal General Hospital, Montreal, Quebec.

3.2.4. Euthanasia and Tissue Processing

After 14 days and 28 days for Group A and Group B respectively, all animals in the group were sacrificed via exsanguination followed by anesthetic overdose. Once the animals were confirmed to be dead by the animal care personnel, both eyes from each animal were enucleated and immediately placed individually in sterile surgical specimen collection containers embedded in ice. During the enucleation procedure, the lateral rectus muscle was left in tact in order to properly orient the eye at the time of processing. Eyes were then placed in formalin and allowed to fix for 24 hours before processing. All tissue processing was carried out by a trained ocular pathologist to ensure specimen integrity. The portion of the globe anterior to the ciliary body was dissected away from the rest of the globe and discarded. The remaining tissue was cut into four quadrants by making two perpendicular cuts through the optic nerve head. These quadrants corresponded to the areas in which the known laser patterns were created. Each tissue piece was then placed in a plastic cassette and taken for paraffin embedding. Separate paraffin blocks were made for each tissue piece and all blocks were subsequently sectioned in 4um thick sections. A library of labeled slides was created for subsequent analysis.

3.2.5. Histopathology

Sections from all eyes were hematoxylin and eosin (H&E) stained and examined by a trained ocular pathologist for morphological changes attributable to the laser injury. These changes included disruption of retinal pigment epithelium and pigment dispersion, breaks in Bruch's membrane, vascular endothelial and fibroblast proliferation, and choroidal neovascularization. Each section was examined and observations were recorded with respect to specimen and group (A or B).

Chapter 3

RESULTS

4.1 Human Specimens

Eleven of 16 (69%) human CNVM specimens showed positive staining for COX-2 in RPE cells, with six (38%) staining positive for COX-2 in vascular endothelial cells and six (38%) showing expression in fibroblasts (Figure 2). The five sections that were negative for COX-2 expression in RPE cells were also negative in the other two cell types (Figure 3). The five negative sections were derived from the three oldest female patients and second and third oldest male patients (Table 2). There was a statistically significant difference ($p=0.0097$) in mean age between the COX-2 positive (65.6 years) and the COX-2 negative (76.8 years) patients.

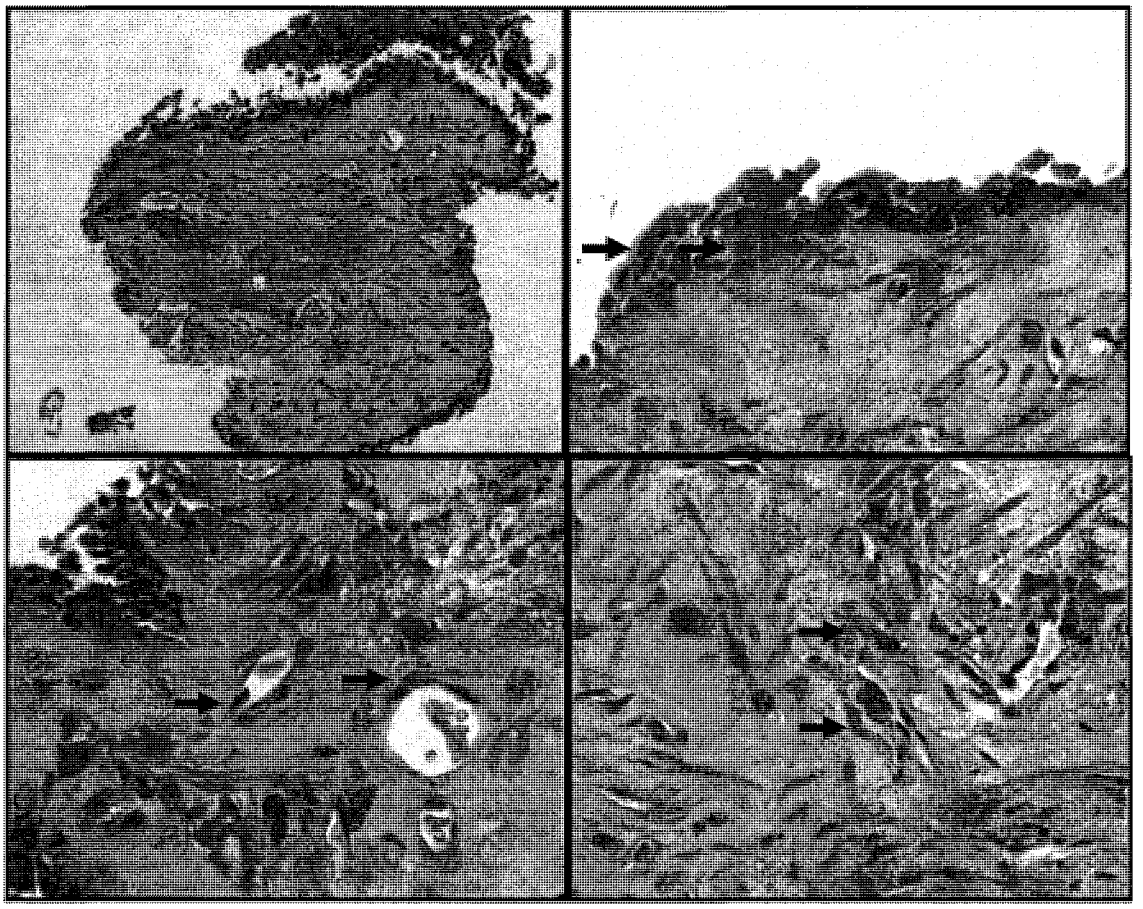

Figure 2. (Top left) Human choroidal neovascular membrane staining positive for COX-2 in all three cells types assessed (100x). (Top right) Higher magnification (640x) shows the positive staining in RPE cells, (Bottom left) vascular endothelial cells, and (Bottom right) fibroblasts. Arrows are indicating only a fraction of positive cells in each figure.

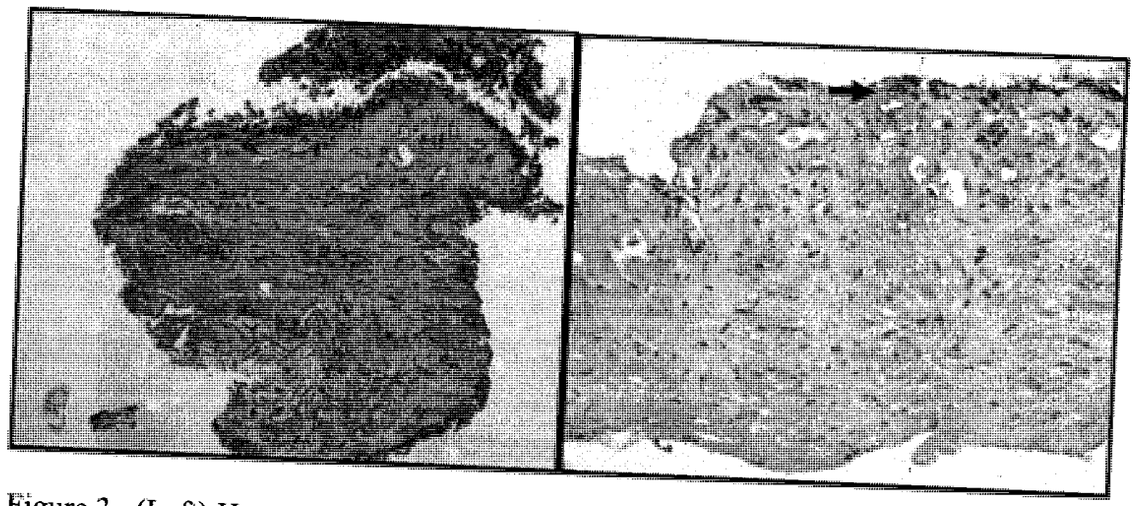

Figure 3. (Left) Human membrane (100x) staining strongly positive for COX-2 expression in RPE cells, fibroblasts, and vascular endothelial cells. (Right) Membrane (200x) showing no expression of COX-2 in any cell type. The pigment observed in RPE cells is intrinsic.

Patient	Sex	Age	RPE	EC	FB
1	F	58	+	+	+
2	F	61	+	+	+
3	F	74	+	-	+
4	F	76	-	-	-
5	F	77	-	-	-
6	F	82	-	-	-
7	M	57	+	+	+
8	M	59	+	-	-
9	M	60	+	-	-
10	M	63	+	+	-
11	M	68	+	+	+
12	M	68	+	-	-
13	M	70	+	-	-
14	M	73	-	-	-
15	M	76	-	-	-
16	M	83	+	+	+

Table 2. Patient samples and observed COX-2 staining. RPE = retinal pigment epithelial cells; EC = vascular endothelial cells; FB = fibroblasts. The patient data shaded in grey highlights those patients with no detectable COX-2 expression in any cell type assessed.

4.2 Laser Injury

The surgical procedure resulted in definitive blister formation secondary to the majority of 250mW laser burns but surgical blisters were not observed for the 75mW, 100mW, or 150mW laser settings despite the obvious white burns created (Figures 4 and 5). In both Group A and Group B, all of the 150mW and 250mW lesions were readily detected during histopathological analysis of specimen sections, while none of the 75mW or 100mW lesions were found. The architectural changes in the RPE and retina were similar for both groups at the 150mW setting (Figure 6) but markedly different at the 250mW setting (Figure 7). With the 150mW setting, there was noticeable disruption of retinal architecture and mild disruption of RPE causing moderate pigment dispersion. The extent of RPE disruption, however, was insufficient to be accompanied by breaks in Bruch's membrane. In both groups, the retina was clearly detached from the underlying RPE and no signs of neovascularization were evident. With the 250mW setting, there was obvious disruption of RPE and Bruch's membrane - as this membrane is continuous with the basement membrane of the RPE which was clearly destroyed with this laser power - in both groups, accompanied by the expected pigment dispersion. Moreover, lesions in both groups demonstrated anastamosis between the damaged retina and RPE layers, an expected step in the development of CNV when using the laser-injury technique. The remarkable difference between Group A and Group B was that the Group B lesions showed characteristic signs of early stages of CNV development while Group A lesions did not (Figures 7 and 8).

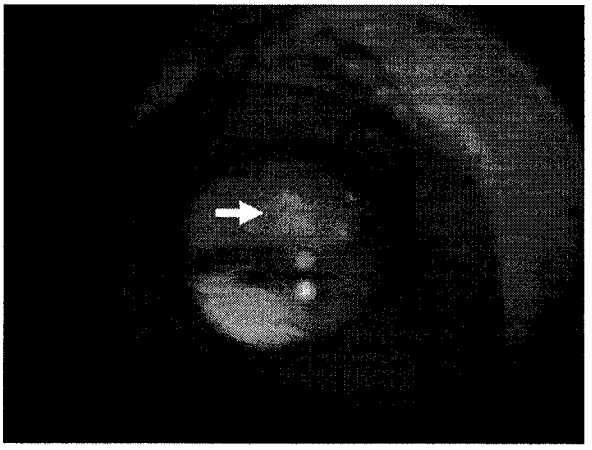

Figure 4. Fundoscopic image of a pentagonal laser burn pattern.

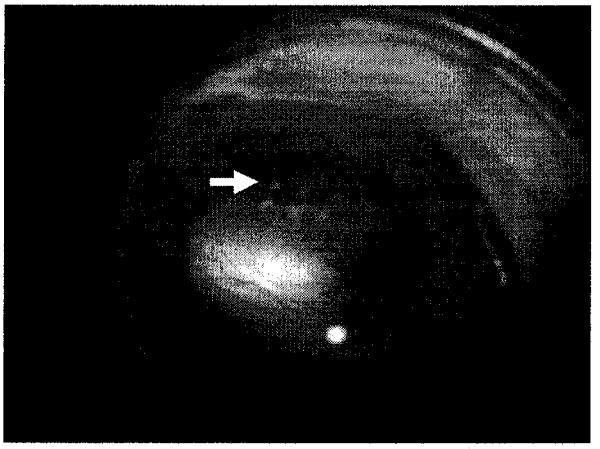

Figure 5. Fundoscopic image of a 6-on-dice burn pattern.

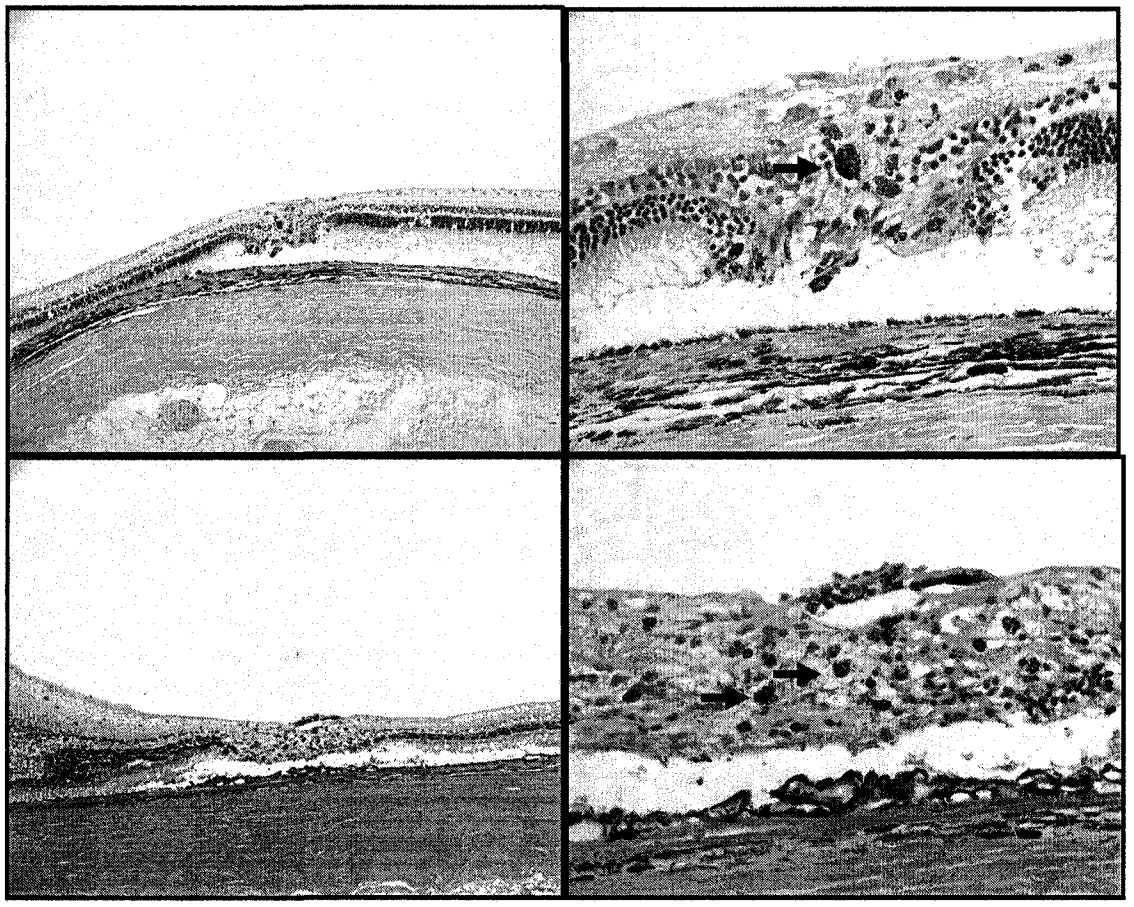

Figure 6. Lesions resulting from 150mW power setting. (Top left) Lesion from a Group A rabbit at 100x magnification and (Top right) 400x magnification. (Bottom left) Similar lesion in a Group B rabbit at 100x and (Bottom right) 400x magnification. Note the pigment dispersion in the high magnification images (arrows).

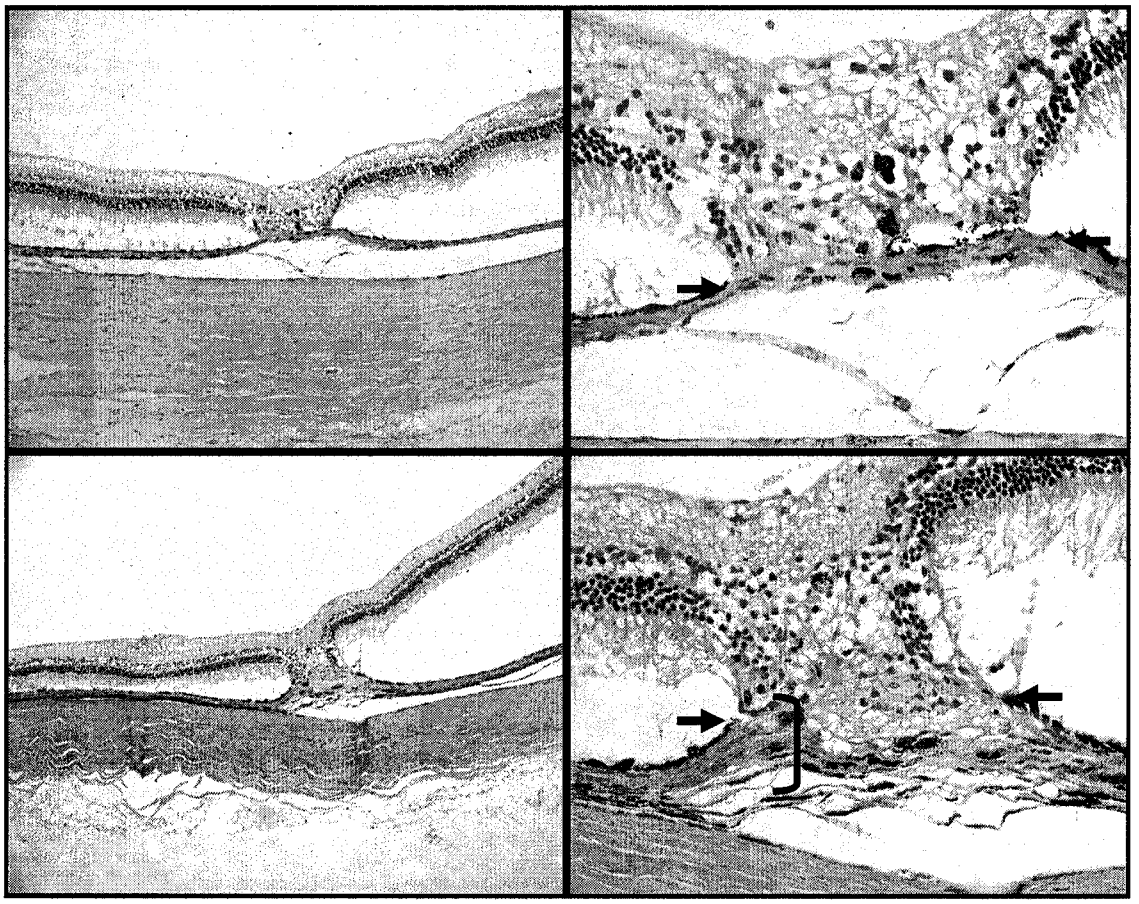

Figure 7. Lesions resulting from 250mW power setting. (Top left) Lesion from a Group A rabbit at 100x magnification and (Top right) 400x magnification. Note the clear break in RPE delineated by the arrows. (Bottom left) Similar lesion in a Group B rabbit at 100x and (Bottom right) 400x magnification. Arrows demarcate the boundaries of the disrupted RPE while the bracket highlights the area of early CNV development.

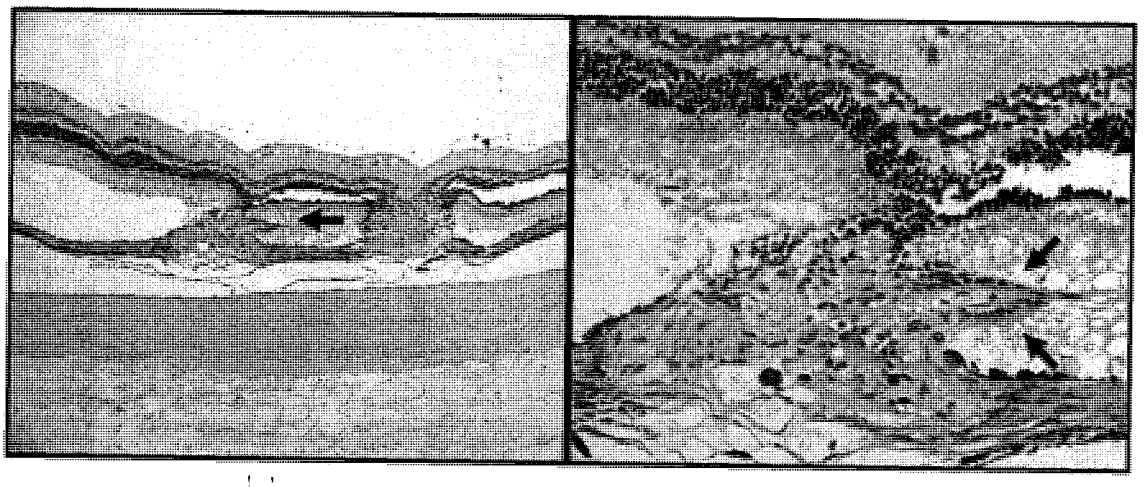

Figure 8. Lesions from 250mW power setting highlighting the mushroom-like extension of fibroblasts and endothelial cells denoted by the arrows in the (Left) 100x and (Right) 400x magnification photos.

Chapter 5

DISCUSSION

5.1 Human Membranes

Figure 2 highlights the COX-2 positive staining in the three cell types assessed. COX-2 expression has previously been characterized in cultured, untreated human RPE cells and was found to be weakly expressed at the mRNA level while undetectable at the protein level by means of immunocytochemistry [143]. The same study further demonstrated that the combination of IL-1β with various cytokines was sufficient to induce COX-2 protein expression, confirming that COX-2 is inducible in RPE cells. Another study demonstrated that such COX-2 induction in RPE could be achieved with several different growth factors [144]. It is therefore conceivable that COX-2 may play a role in the pathogenesis of CNV as it appears to be induced in RPE cells in 69% of the cases studied in this paper. COX-2 has also been shown to be inducible in human fibroblasts[145] and endothelial cells[146]. It is known that VEGF is one of the principal factors involved in the induction of endothelial cell COX-2 expression[147] and thus it is plausible that, since VEGF expression is necessary for CNV development[148], COX-2 expression may be induced in endothelial cells early in disease pathogenesis. Consequently, anti-COX-2 efforts may prove efficacious in delaying CNV onset and progression.

Table 2 highlights the statistically significant difference in mean age of COX-2 positive versus COX-2 negative patients. Previous studies have demonstrated that basal COX-2 expression increases with age in human monocytes[149] and several rat tissues including

vascular smooth muscle cells[150], aortic endothelial cells[151], cardiac myocytes[152], and kidney cells[153]. It appears that although COX-2 is generally recognized as an inducible enzyme in times of tissue injury or neoplasia[131], it has basal expression in some tissues that may serve some physiological significance. An age-dependent increase in COX-2 expression could similarly occur in ocular tissues and may facilitate the onset of CNV development in older individuals. Moreover, the significant correlation between COX-2 and VEGF expression and their association with angiogenesis [154-157] suggests a possible role for COX-2 not only in CNV onset but also throughout the process of CNV development. All of these things may be true but they fail to explain why there is an absence of COX-2 expression in some of the oldest patients. In this study, the specimens lacking COX-2 expression were derived from the three oldest female and the second and third oldest male patients, suggesting that COX-2 expression may be reduced in older AMD patients compared to their younger counterparts.

It is quite possible that the COX-2 negative patients were not truly negative, but rather had levels of COX-2 expression too low to be detected by immunocytochemistry. It is likely that these patients did have higher COX-2 levels at one point in time, but that these levels decreased with disease progression. A similar observation was seen in patients with Alzheimer's disease (AD) where neuronal COX-2 expression was increased in patients with moderate grade AD but decreased in patients with end stage AD when compared to non-demented controls[158]. This study also showed that these patterns of COX-2 expression were evident in subjects with other demetia and thus may not be specific to AD. Thus, it is possible that some of the older patients in our study had been

living with the disease longer than the rest of our patient population, or simply that their disease state was more advanced. This hypothesis would seem to agree with the results previously reported in AD[158]. Another possibility is that there may be a difference in the two groups in terms of genetic predisposition to a dysregulation of inflammatory responses. The recent reports of genetic polymorphisms that confer an increased risk for developing AMD relate to regulation of the complement system[4-6]. Similar genetic predispositions may affect COX-2 expression, although evidence of this possibility has yet to surface. Further investigation will need to be done to elucidate the mechanisms underlying the observed difference in COX-2 expression and its correlation with patient age.

5.2 Rabbit Model

Figures 4 and 5 show some of the characteristic burn patterns that were created around the optic nerve head. These patterns allowed for easy post-mortem identification of the laser setting employed and were created specifically to avoid any potential confusion about this issue. Interestingly, none of the 75mW or 100mW lesions were identifiable in any specimen upon histopathologic examination. The most probable explanation of this apparent phenomenon comes from the understanding of how the laser technique works. The laser heat is absorbed by the retinal pigment epithelium and is dispersed to the overlying neurosensory retina. The more powerful the incident laser light, the more residual damage there is to the retina. It is likely that in our model, the 75mW and 100mW power settings were inefficient at causing significant retinal damage. As observations of such damage are the clear indicators of burn regions to the investigator, their absence makes such regions almost completely unidentifiable when using low-power microscopy. The 150mW and 250mW laser settings, however, were efficient at causing retinal damage in all rabbits and thus resulted in easily identifiable lesion sites characterized by cellular debris and fragmented nuclei, a characteristic morphology resulting from the laser injury technique[159]. The 150mW lesions in both groups resulted in similar types of retinal and RPE damage (Figure 6). The pigment dispersion that was observed was a result of the damage done to the RPE cells by the laser injury, causing the intrinsic pigment to be dispersed to the surrounding tissue. There was no observable obliteration of the RPE layer in any of the 150mW lesions, indicating that this power setting was inadequate to cause a break in Bruch's membrane (which is continuous with

the basement layer of the RPE). Moreover, there were no distinct histopathological differences between Group A and Group B at this laser setting.

Figures 7 and 8 show typical lesions for the 250mW setting in Group A and Group B rabbits. In both cases there are observable anastamoses between the retina and RPE layers. With sufficient damage to the RPE layer, these anastomoses are made possible. It is for this reason that no anastomoses are seen in the lesions created by the 150mW setting. Pigment dispersion in the lesion sites is indicative of at least some RPE cell damage (which was seen with the 150mW setting), but the obvious discontinuity of the RPE at the lesion site is confirmation that both the RPE and Bruch's membrane have been destroyed. These histopathological findings confirmed our *in vivo* fundoscopic observations – the formation of a subretinal blister general accompanies the successful rupture of Bruch's membrane when using the laser-injury technique. Such blisters were only observed following 250mW laser burns and thus we could have predicted that only burns made by the 250mW laser setting would yield obvious breaks in Bruch's membrane upon histopathological analysis. This correlation between blister formation and rupture of Bruch's membrane has been previously described[19, 159]

Unlike the case with the 150mW burns, the 250mW burns resulted in notable histopathological differences when comparing Groups A and B. Group B lesions resulted in more extensive endothelial cell and fibroblast migration than Group A lesions, a precursor step to CNV development[159]. This migration was often seen in a 'mushroom' pattern, which is what may be expected of CNV. Moreover, many of the Group B lesions

had obvious vessel growth in the direction of the lesion (Figure 7), once again setting them apart from the Group A lesions. When considering the fact that the only experimental difference between Group A and Group B rabbits was the time of sacrifice post surgery, it is evident that the observed differences between these two groups are entirely time-dependent. This time dependence is a well-characterized phenomenon in other CNV laser-injury models[13, 160] and is something that we anticipated prior to beginning this study, leading us to establsih the different time points for sacrifice.

5.3 Conclusions

The expression of COX-2 in 69% of the human choroidal neovascular membranes studied supports the theory that inflammation is an important component in the development and progression of neovascular AMD. The absence of detectable COX-2 expression in some of the oldest patients may have some disease state-specific or genetic underpinnings, but further research is necessary before any concrete conclusions can be drawn. Specific targeting of COX-2 may represent a novel therapeutic approach to managing the inflammatory component of this disease. Moreover, anti-COX-2 strategies may prove to be beneficial in preventing disease progression in high-risk patients.

A rabbit laser-injury model of neovascular age-related macular degeneration is promising based on the results obtained in our study. The observation of histopathological changes characteristic of choroidal neovascular membrane formation puts the rabbit model on par with other established models of early stages of AMD pathogenesis. It is likely that a repeated experiment with increased temporal endpoints will yield a model that is clinically relevant and easier to manage than established rodent models, while being associated with only a fraction of the costs of a primal model. The successful establishment of such a model would unquestionably benefit and accelerate *in vivo* AMD studies.

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APPENDIX

Health Canada
Santé Canada

Healthy Environments and Consumer Safety Branch
Direction générale, Santé environnementale et sécurité des consommateurs

Address Locator 3502B
Ottawa ON K1A 1B9

Your file Votre référence

Our file Notre référence

2006-02-13

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Dr. Miguel N. Burnier
Faculty of Research/Medicine
McGill University
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Montréal QC H3A 2B4

Dear Dr. Burnier:

In response to your recent request for an exemption to use a controlled substance, the following exemption is being granted to you pursuant to section 56 of the *Controlled Drugs and Substances Act* (CDSA) on the basis that such exemption is necessary for a scientific purpose.

Subject to the terms and conditions herein, you are hereby exempted from the application of subsection 4(1) of the CDSA with respect to the controlled substance listed below. This effectively allows you to possess the substance, subject to the terms and conditions herein.

Subject to the terms and conditions herein, you are hereby exempted from the application of subsection 5(1) of the CDSA as it applies to the administration to an animal of any controlled substance that you are allowed to possess as a result of the exemption above, if such administration is done in accordance with the research project submitted.

The exemption herein is only applicable if you comply with the following conditions:

(1) You may only possess the controlled substance listed below, up to the quantity indicated:

Substance Name	Maximum Quantity
Ketamine hydrochloride 100 mg/ml	3 x 10 ml

Canada

.../2