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Animal Models of Choroidal and Retinal Neovascularization

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Abstract

There have been numerous types of animal models of choroidal neovascularization (CNV) and retinal neovascularization (RNV). Understanding the pathobiology of CNV and RNV is important when evaluating and utilizing these models. Both CNV and RNV are dynamic processes. A break or defect in Bruch's membrane is necessary for CNV to develop. This may be induced with a laser, mechanically via surgery, or in the setting of transgenic mice. Some of the transgenic mouse models spontaneously develop RNV and/or retinal angiomatous proliferation (RAP)-like lesions. The pathogenesis of RNV is well-known and is generally related to ischemic retinopathy. Models of oxygen induced retinopathy (OIR) closely resemble retinopathy of prematurity (ROP). The streptozotocin (STZ) rat model develops features similar to diabetic retinopathy. This review summarizes general categories and specific examples of animal models of CNV and RNV. There are no perfect models of CNV or RNV and individual investigators are encouraged to choose the model that best suits their needs.

Keywords

choroidal neovascularization; retinal neovascularization; animal models

1. Introduction

The development of animal models of choroidal neovascularization (CNV) and retinal neovascularization (RNV) has paralleled and contributed to the understanding of the biology of these conditions. These models have also been developed in order to test new treatments. Although the models recapitulate many of the clinical manifestations of CNV and RNV in humans, the time to develop, course of progression, size and appearance of the lesion vary among the models. In this review, various categories of animal models of CNV and RNV are presented along with specific examples in each category, including their strengths and weaknesses. This review is meant to be comprehensive, but not encyclopedic. It should be used as a guide for investigators to further elucidate the biology of CNV and RNV with emphasis on evaluation of novel treatments for these conditions.

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2. Animal Models of Choroidal Neovascularization

2.1 Overview of Choroidal Neovascularization

Choroidal neovascularization (CNV) represents a non-specific response to a specific stimulus (Grossniklaus and Green 2004). CNV is encountered in numerous chorioretinal diseases (Green and Wilson 1986; Green 2006) and is a dynamic process with initiation, maintenance and involution stages (Grossniklaus et al 2002). The involutional stage is characterized by a decrease in cytokine production with associated scarring and fibrosis. The CNV may arise de novo or reactivate in areas of inactive CNV. Our laboratory has estimated that CNV in patients with age-related macular degeneration grows at a rate of approximately 0.2 disc areas (DAs) per month (unpublished data). There are varying degrees of inflammation in the CNV depending on the underlying disease and dynamic stage of CNV evolution (Grossniklaus and Green 2004).

Understanding the pathobiology of CNV is important with regard to developing suitable animal models of the process. The evolution of CNV begins with a break or defect in Bruch's membrane. This may be secondary to a traumatic break, a degenerative process, tissue traction and/or inflammation (Penfold, Killingsworth and Sarks 1984, Spraul and Grossniklaus 1997, Grossniklaus et al 2000, Kilarski et al 2009). When this occurs, choriocapillary endothelial cells, pericytes, fibrocytes and inflammatory cells are introduced into the sub-retinal pigment epithelium (sub-RPE) and/or sub-retinal spaces. There are inflammatory, angiogenic and extracellular matrix components of the CNV. These components exist together in a microcosm where there are competing factors that induce or suppress the processes. The combination of endothelial cell growth and coverage by pericytes surrounded by the extracellular matrix (ECM) as mediated by vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF) seems essential for CNV formation (Greenberg et al 2008).

The systems involved with the formation of CNV may be divided into three general categories: inflammation, angiogenesis and proteolysis. These systems are interrelated. Several inflammatory subsystems have been implicated in CNV, including the complement system, cytokines, and chemokines (Hageman et al 2001, Mullins et al 2000). Pro-angiogenic factors that stimulate the proliferation of endothelial cells in CNV include VEGF, basic fibroblast growth factor, (bFGF) and platelet derived growth factor (PDGF) (Amin et al 1994, Kvant et al 1996, Lopez et al 1996). The proteolysis component includes a provisional fibrin matrix laid down in the area that will become the physical location of the CNV (Schlingemann 2004). The fibrin acts as a scaffold upon which the CNV will grow. The fibrin arises from conversion of circulating fibrinogen by tissue factor (TF). The TF cell receptor is a co-activator of serine proteases promoting both thrombin and fibrin. TF is also involved in inflammation, cell adhesion and VEGF secretion (Carmeliet and Collen 1998). Although thrombospondin has anti-angiogenic properties, it also enhances release of the pro-angiogenic molecule, matrix metalloproteinase 2 (MMP-2), from the extracellular matrix (Bein and Simons 2000). Cells critical for the pathogenesis of CNV include macrophages and RPE. Macrophages appear to play a key role in this process, as they express the angiogenic cytokines VEGF and TNF α (Penfold et al 2001, Grossniklaus et al 2002). The RPE is a critical regulator of the process, as it expresses VEGF, MCP1 and IL8, all of which regulate monocyte and endothelial cell recruitment (Elner et al 1996, Kvant et al 1996, Lopez et al 1996, Grossniklaus et al 2002). The dynamic stages of CNV (initiation, maintenance and involution) depend on cell signals being turned on or off during the process (Grossniklaus et al 2002). These opposing signals include pro- and anti-inflammatory, angiogenic and proteolytic. Oxidative damage-induced inflammation may initiate age-related macular degeneration (Hollyfield et al 2008) and there appear to be immune-related mechanisms involved with angiogenesis in CNV (Ferguson and Apte 2008). The most widely studied inflammatory cascades in age-related macular degeneration are the complement pathways (Schol et al 2008, Baird et al 2008, Park et al

2009) and there are competing proteins that potentiate or inhibit these pathways. VEGF is the primary initiator of angiogenesis in CNV (Kvanta et al 1996, Lopez et al 1996, Grossniklaus et al 2002). Angiogenic remodeling factors include angiopoietin 1 and 2, ephrin 1, the tyrosine kinases Tie 1, basic fibroblastic growth factor (bFGF), platelet derived growth factor (PDGF), KDR/Flt-1, and others (Folkman et al 1996, Holash et al 1999, Gal and Yancopoulos 1999, Wada et al 1999). Endogenous angiogenic inhibitors include PEDF, angiostatin and endostatin (Cao 2001). Pigment epithelium-derived factor (PEDF) is a naturally occurring anti-angiogenic protein (Mori et al 2001). Interestingly, PEDF also exhibits anti-inflammatory properties by modulating macrophage activity (Zamiri et al 2006). The MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs) are involved with pro- and anti-angiogenesis as well as pro- and anti- tissue degradation (Sven et al 1998, Bergers et al 2000). These dynamic processes of inflammation, angiogenesis, and proteolysis relate to the initiation, maintenance, and involution stages of CNV formation (Grossniklaus et al 2002) and should be kept in mind when assessing animal models of CNV. There are many conditions that lead to CNV in humans, including age-related macular degeneration, ocular histoplasmosis syndrome, myopia and idiopathic CNV. The clinical features of these conditions should also be considered when creating animal models of CNV.

In addition to recapitulating the pathobiology of human CNV, it is desirable that animal models of CNV be efficient and reproducible, stable and sustainable over time, exhibit similar pathologic findings including growth patterns to human CNV (Grossniklaus and Green 2004), be inexpensive to produce and be able to be followed *in vivo* with imaging techniques including fluorescein angiography (FA) and ocular coherence tomography (OCT). There are generally three categories of animal models of CNV: laser induced models, surgically induced models and genetically engineered animals. These models may be modified by introduction of cytokines, age, diet and other factors. The following will summarize pertinent features of each of these types of models for CNV with specific examples including their strengths and weaknesses.

2.2 Laser and Light Induced Models of CNV

2.2.1 Rat and Mouse—After the first models of CNV were developed in primates (Ryan 1979), Dobi and associated created a rat model of CNV in 1989 (Dobi et al 1989). Those authors created argon laser photocoagulation spots (647nm, 100µm 50 to 100 mW, 0.1s) through a dilated pupil with a coverslip over the cornea. The spots created breaks in Bruchs membrane, with a central bubble formation with or without intra-retinal or choroidal hemorrhage. There was fluorescein angiographic evidence of CNV in 24% of the created lesions. Examination of enucleated eyes by light and electron microscopy showed pathologic evidence of CNV in 60% of the lesions. Frank and co-workers also developed a rat model of CNV in 1989 (Frank, Das and Weber, 1989). That model used a krypton laser (blue green or green wavelength, 500µm, 50mW, 0.02s) to create breaks in Bruchs membrane and CNV. Campochiaro's group and other groups essentially used the same technique to create experimental CNV in mice, with minor modifications to the laser spot parameters. For instance, using a krypton laser in C57BL6 mice with modified settings including a smaller spot size, higher intensity, and shorter duration (blue green or green wavelength, 50µm 50 to 350 to 400mW 0.05s) resulted in a higher frequency (100%) of histologic evidence of CNV (Tobe et al 1998, Seo et al 1999). The rat laser induced CNV model has been used to study the role of tissue inhibitor metalloproteinase 3 (Takahashi et al 2000) and tissue factor (Bora et al 2003) in CNV formation. It has also been used in pharmacologic intervention experiments (Yanagi et al 2002). VEGF has been shown to be the major stimulator of CNV in the mouse model (Figure 1) (Kwak et al 2000). Immunohistochemical staining in this model, especially in the context of transgenic mice, has been used to study various components of CNV including basic fibroblast growth factor 2 (FGF2) (Tobe et al 1998) and matrix metalloproteinases (Berglin et al 2003). Image analysis

software may be used to analyze the histologic area of CNV in these models, especially in the context of testing potential pharmacologic therapies of CNV (Seo et al 1999). The model has evolved to where the current standard of measurement is determining the area of CNV by confocal scanning electron microscopy image analysis of flat-mounts of the CNV/RPE and choroid after intravenous infusion with fluorescently labeled dextran. A diode laser may be used to create the CNV (532 nm, 100µm 50 to 100 mW, 0.1s) and this model has been used to assess aging as it relates to CNV formation (Espinosa-Heidmann et al 2002), macrophage contribution to CNV formation (Sakurai et al 2003, Espinosa-Heidmann et al 2003), and gene therapy (Bainbridge et al 2003), siRNA targeting (Reich et al 2003), novel delivery platforms, such as AAV mediated transfections (Mori et al 2002) and nanoparticles (Singh et al 2009) in the treatment of CNV. Advantages include that the model is relatively simple to create, inexpensive, reproducible, efficient, may be used in conjunction with immune manipulation (Bora et al 2005) or transgenic mice to elucidate the pathobiology of CNV (Sengupta et al 2003, Rakic et al 2003), and experiments may be done in short time frames, such as several weeks. Disadvantages include the artificial nature of the model, the small size of the rat/mouse eye compared to the human eye, mice and rats do not have a macula, and laser photocoagulation has been used to treat CNV as well as create it. Nevertheless, the rat/mouse laser model of CNV is the currently the standard animal model of CNV for most treatment evaluation experiments. Recently, Albert and co-workers described CNV formation in a cyclic light induced rat model (Albert et al 2010). In that model, Albino rats were exposed to 12 hours of 3000-lux cyclic light for 1, 3 or 6 months. Results showed microscopic sub-RPE neovascularization at 1 month, extension of the neovascularization into the outer retina at 3 months, and multiple areas of neovascularization with associated connects with retina vessels and tissue bands at 6 months. The retinas of the light-exposed animals immunostained for 4-hydroxy-2-nonenal (HNE), a measure of ω-6 polyunsaturated fatty acid peroxidation, indicating oxidative stress. The cyclic light also induced other retinal atrophy and drusen-like deposits. An advantage to this model is the potential to study the biologic progression of CNV in non-genetically altered animal model without laser or mechanical perforation of Bruch's membrane.

2.2.2 Primate, rabbit and pig—The primate model of experimental CNV, created by Ryan and colleagues, was termed “subretinal neovascularization” at the time and was the first animal model of CNV (Ryan 1979, Ryan 1982, Miller, Miller and Ryan 1986). Original work in this area included transscleral and transvitreal injection of collagenase and hyaluronidase into the subretinal space (Ryan 1979). This resulted in mechanical or enzymatic damage to Bruch's membrane. These invasive techniques resulted in low incidences of CNV and led to the development of laser disruption of Bruch's membrane as a model of CNV. Ryan was able to induce CNV in *Macaca speciosa* monkeys using argon laser (488nm and 514nm, 50 to 200µm, 200-950mW, 0.1 to 0.5s) disruption of Bruch's membrane. Approximately 40% of the monkeys developed angiographic evidence of CNV and up to 90% developed histologic/ultrastructural evidence of CNV. These studies provided early experimental evidence of the role of ischemia (later studies showing VEGF in response to ischemia), inflammation including macrophages, and the RPE (Miller, Miller and Ryan 1986) in the role of CNV formation. The primate laser model of CNV played an essential role in the development of photodynamic therapy as a treatment for CNV (Miller et al 1995). It has also been used, in conjunction with a surgically induced rabbit model of CNV to evaluate an integrin inhibitor to treat CNV (Zhan et al 2009). Criswell and co-workers created CNV in new-world squirrel monkeys and compared them with CNV created in old-world cynomolgus *Macaca speciosa* monkeys (Criswell et al 2004). With their optimized laser parameters for the squirrel monkeys (532nm, 0.05s, 75µm, 650mW), they elicited fibrovascular tissue (CNV) in 65% of squirrel monkey laser spot sites compared with 37% of cynomolgus laser spot sites (Figure 2). Many of these CNV lesions expanded beyond the size of the original photocoagulation sites including diffuse

subretinal fibrovascular tissue. Advantages of the primate model include the close approximation of the monkey retina and macula to the human, size of the primate eye for drug delivery studies, and utility of the model for development of human clinical trials (such as for PDT and anti-angiogenics). Disadvantages include the expense of the animals/animal care/husbandry, length of experiments and ethical issues regarding use of primates when rodents (mice and rats) are reliable models. An intermediate laser-induced CNV model between rodents and primates has been developed. This is the rabbit model of laser-induced CNV (El Dirini et al 1991). This model avoids the expense and ethical issues of the primate model, although the rabbit lacks a macula and the rabbit retina is supplied by a medullary ray, unlike the vascular supply of the retina to primates and rodents. The model utilized subretinal endophotocoagulation to create histologically identified CNV. Another intermediate laser-induced CNV model is the pig (Saishin et al 2003, Kiilgaard et al 2005). Saishin and coworkers used a diode laser (75 μ m, 400mW, 0.1s) to create defects in Bruchs membrane in the pig eye and establish histologic evidence of CNV in 100% of lesions. Image analysis of histologic sections was used to evaluate drug delivery for the treatment of the CNV (Saishin et al 2003). Kiilgaard and coworkers studied xenon laser versus diode laser versus mechanical disruption of Bruchs membrane in a pig model of CNV (Kiilgaard et al 2005). They found histopathologic evidence of CNV in 54%, 83% and 100% of the animals, respectively (Kiilgaard 2005). The pig eye is approximately the same size as the human eye and the retina vascularization is similar to the human. Like the rabbit, the pig circumvents the cost and ethical issues of the primate. The utility of the pig is for drug delivery experiments. However, the pig lacks the advantages of high throughput, short duration experiments as available in rat and mouse models of laser induced CNV.

2.3 Surgically Induced Models of CNV

2.3.1 Rat and Mouse—Subretinal and/or choroidal neovascularization has been immunologically and mechanically induced in rat and mouse models, primarily by injection of synthetic peptides, viral vectors containing VEGF, cells and inert synthetic materials. Chan and colleagues reported and illustrated subretinal neovascularization in 10% of mice immunized with interphotoreceptor retinoid-binding protein (IRB) and developed experimental autoimmune uveoretinitis (EAU) (Chan et al, 1990). Sakamoto and co-workers immunized Lewis rats with a hind foot pad injection of an IRBP-derived synthetic peptide and intravenous *Bordetella persusis* (Sakamoto et al 1994). They found histologic evidence of inflammatory destruction of Bruchs membrane and subretinal neovascularization in 38% of the rats. This model may be similar to ocular inflammatory diseases that result in subretinal neovascularization, such as punctate inner choroidopathy, Harada disease, and others, and supports the concept that inflammatory disruption of Bruchs membrane may be associated with CNV. Spilbury and co-workers performed injections of a recombinant adenovirus vector expressing rat VEGF₁₆₄ driven by a CMV promotor into the subretinal space of rats (Spilbury et al 2000). They found fluorescein angiographic and histologic evidence of CNV in 80% of the eyes, although they could not exclude the possibility of needle puncture to Bruchs membrane as helping to cause the CNV. Baffi and co-workers injected 5 to 10 μ l of an adenoviral vector expressing VEGF₁₆₅ into the subretinal space of the rat eye (Baffi et al 2000). Examination by fluorescein angiography, histology and electron microscopy showed CNV in most of the eyes. Those authors used the FITC-labeled dextran retinal flatmount technique to examine some specimens, which is now the standard technique. Wang and colleagues performed similar studies, using a 2 μ l injection of an adenoviral vector expressing human VEGF₁₆₅ (or GFP) with a CMV promoter into the subretinal space in rats (Wang et al 2003). Their extensive study demonstrated VEGF expression in the RPE and angiographic/histologic evidence of CNV in approximately 90% of the injected rats. Additionally, electroretinogram (ERG) a and b waves were reduced by approximately 50% in the rats with CNV compared with controls. It is likely that the subretinal injection itself causes trauma to

Bruchs membrane sufficient for CNV. Our laboratory has induced choroidal neovascularization in C57BL6 mice by subretinal injection of retinal pigment epithelium and polystyrene microbeads (Figure 3) (Schmack et al 2009). In our study, we found that the combination of RPE, which expressed VEGF, and polystyrene beads, resulted in larger CNV lesions than RPE or the polystyrene beads alone. Additionally, subretinal injection of PBS resulted in small CNV lesions, thus supporting the concept that the trauma of subretinal injection alone damages Bruchs membrane and may result in CNV. Subretinal/RPE injection of matrigel, a basement membrane extract, that solidifies after implantation in tissue and stimulates local angiogenesis, is reported to induce CNV in B6 and Ccl2 deficient mice with 31% and 53% incidences, respectively. That report supports the involvement of macrophages and chemotaxis in CNV (Shen et al, 2006).

2.3.2 Rabbit—Some of the most extensive work regarding the creation of CNV after subretinal injections has been performed in rabbits. Early studies showed that subretinal injection of vitreous would induce retinal pigment epithelial proliferations and microscopically identified CNV in rabbit eyes (Zhu et al, 1989). In 1995, Kimura and co-workers injected 50 μ l suspensions of 2.5 μ l bFGF impregnated gelatin microspheres into the subretinal space in rabbits via a transscleral route (Kimura et al 1995). They found angiographic and histologic evidence of CNV in 83% to 100% of the eyes (Kimura et al 1995, Kimura et al 1999). The injection technique created a break in Bruchs membrane in their model. The cellular response in that model was studied by immunohistochemistry, which showed CD31 positive endothelial cells first present at 14 days after inoculation and forming neovascular channels that persisted for up to 8 weeks (Kimura et al 1999). Other studies have shown that a break in Bruchs membrane is needed for traumatically induced CNV in the rabbit (Hsu et al 1989). Tamai and co-workers found that subretinal injections of 12.5 to 25 μ g of linoleic acid hydroperoxide (LHP) into the subretinal space in rabbit eyes induced CNV (Tamia et al 2002). They demonstrated angiographic evidence of CNV in 46% of the eyes, and TNF α , PDGF, VEGF, TGF β , bFGF and IL1 α were upregulated by the LHP. The areas of the CNV in Kimura's and Tamai's models were relatively small. There have been several models created that produce large areas of CNV in the rabbit eye. Ni and coworkers injected a 50 μ l cocktail of endotoxin, growth factor, heparin beads, FGF-2, 100ng LPS with 50 μ g of heparin sepharose beads transvitreally into the subretinal space of the rabbit eye (Figure 4) (Ni et al 2005). Fluorescein angiography, ocular coherence tomography (OCT) and histology demonstrated two types of extensive CNV growth in 100% of the eyes. Primary CNV extending into the subretinal space and associated with injury to Bruchs membrane at the injection site was visible by 2-weeks and stable at 3-months post-injection. Secondary CNV extending into the subRPE space away from the injection site developed in all eyes by 8-months post-injection. The CNV persisted for up to 3 years. The CNV was characterized by initiation, growth and maintenance stages, similar to human CNV (Grossniklaus et al 2002), and was inhibited by subcutaneous dexamethasone. Qui and coworkers performed transvitreal injections of 10 μ l of Matrigel and 750 μ g of VEGF into the subretinal space of the rabbit eye (Qui et al 2006). In addition to fluorescein angiography and histology, they used OCT to demonstrate CNV in 100% of eyes at 1 to 9 weeks post-inoculation. Unlike Ni's model, the CNV in Qui's model was relatively small. Qui and coworkers noted that the Matrigel acted as a scaffold upon which the CNV grew. Julien and coworkers performed transvitreal injections of 5×10^6 IU of a high capacity adenoviral vector construct expressing VEGF-A¹⁶⁵ driven by an EF1 α HTLV promoter into the subretinal space of the rabbit eye (Julien et al 2008). Those investigators found angiographic evidence of CNV in 85% of the injected eyes, which was confirmed by histology, immunohistochemistry and electron microscopy. ICG angiography and electron microscopy showed intra-choroidal changes in the model. The CNV in that model, like Qui's, was relatively small compared with Ni's model. An advantage to the rabbit model is that potential large size and long duration of the experimentally induced CNV. Disadvantages include a lack of ability

for high throughput experiments, lack of a macula and lack of retinal vasculature similar to the human eye.

2.3.3 Pig—The mechanically-induced pig model of CNV has mainly been developed in Denmark (Kiilgaard et al 2005, Lassota et al 2006, Lassota et al 2007, Lassota 2008, Lassota et al 2008). Earlier work by Sourbanc and coworkers showed that suprachoroidal injection of basic fibroblast growth factor caused intrachoroidal angiogenesis, but not CNV, since there was no break in Bruchs membrane (Sourbanc et al 1994). As previously mentioned, Kiilgaard and coworkers showed that in pigs, CNV arose after xenon laser photocoagulation, diode laser photocoagulation and surgical debridement of the RPE followed by mechanical rupture of Bruchs membrane in 54%, 83% and 100% of eyes, respectively, thus confirming that a break in Bruchs membrane is needed for CNV development (Kiilgaard et al 2005). The mechanical model was induced by transvitreal subretinal debridement of the RPE with a retinal scraper after retinal detachment with subretinal injection of isotonic saline. Further investigation of that mechanically induced model by fundus examination, fluorescein angiography, and histology showed correlation of the location of the fibrovascular tissue (CNV) with fundus and angiographic findings (Figure 5) (Lassota et al 2006). Studies showed histologic evidence of CNV in 100% of eyes using a technique in which Bruchs membrane was perforated and the RPE was left intact compared with RPE removal alone and RPE removal/Bruchs perforation (Lassota et al 2007). Although all three techniques resulted in CNV, perforation of Bruchs without prior RPE removal was the easiest to reproduce and involved the fewest variables. Mechanical disruption of Bruchs membrane with a retinal perforator alone was found to reliably produce relatively large areas of CNV in the pig model. The fundus, fluorescein angiographic, and histologic aspects of that model were studied over an extended period of time (Lassota 2008, Lassota et al 2008). The lesion was angiographically visualized in 92% of eyes and the vasculature of the pig retina more closely approximates the human retina than does the rabbit retina (Lassota 2008, Lassota et al 2008). Immunohistochemical studies showed cytokine production and configuration of the CNV to be analogous to human CNV. Additionally, the pig eye, like the rabbit eye, is large enough to perform for drug delivery experiments. Disadvantages are similar to the rabbit model of mechanically induced CNV.

2.3.4 Primate—Ryan originally attempted to create CNV in a primate model by subretinal injection of collagenase and hyaluronidase into the subretinal space (Ryan 1979). Cui and colleagues further explored the feasibility of a primate CNV model using subretinal injections (Cui et al 2000). That group injected an 80µl suspension of VEGF-impregnated gelatin microspheres transvitreally through a retinotomy into the subretinal space of *Macacca mulatta* monkeys. They were able to find fundus and angiographic evidence of CNV in 92% of the injected eyes. The CNV was relatively small, and there has been limited use of this model due to expense and ethical issues. This model is more difficult to produce and does not offer any advantages over the laser CNV model in the primate.

2.4 Transgenic and Knockout Mouse Models of CNV

2.4.1 VEGF₁₆₄RPE65—Although there are several transgenic mouse models of age-related macular degeneration (AMD) (Rakoczy et al 2006), only a relatively few of the models spontaneously develop CNV. It has become apparent that overexpression of VEGF by the retina or RPE is not enough to elicit CNV in these models and there is a central role of compromised Bruchs membrane in the development of CNV (Rakoczy et al 2006). The advantages of these models are the ability to study various biologic components of CNV by comparison with controls and cross breeding experiments. Disadvantages relate to the length of time for the CNV to develop, the relatively small percentages of eyes that develop CNV and the small size of the CNV. These models have supported the concept that the best models of CNV are those that incorporate physical disruption of Bruchs membrane into the model

(Rakoczy et al 2006). Schwesinger and colleagues described a transgenic mouse expressing VEGF₁₆₄ driven by an RPE65 promoter (Schwesinger et al 1994). All of the mice developed histologically identified intrachoroidal vascular anomalies, which were described as intrachoroidal neovascularization, although they did not develop CNV. Some consider the intrachoroidal vascular anomalies in this model to represent developmental (vasculogenic) changes, and not neovascularization.

2.4.2 *Ccr2/Ccl2* deficient—The *Ccr2/Ccl2* deficient mouse model of AMD, developed by Ambati and co-workers, has received considerable attention (Ambati et al 2003, Takeda et al 2009). In that model, transgenic mice deficient in either *Ccl2* or *Ccr2* fail to recruit macrophages to the area of the RPE and Bruchs membrane. This allows for accumulation of C5a and IgG, both of which induce VEGF production. Histologic, immunofluorescent and ultrastructural examination of either of these transgenic mice showed that approximately 25% of the mice developed microscopic areas of CNV (Ambati et al 2003). These findings have helped with the understanding of the pathobiology of CNV, especially with regard to macrophage recruitment. Although the areas of CNV are very small, recent work has shown that CCR3 is specifically expressed in choroidal neovascular endothelial cells in either of these deficient mice (Takeda et al 2009). *In vivo* imaging of CCR3-targeted quantum dots locate the CNV that is not able to be visualized by standard fluorescein angiography (Figure 6) (Takeda et al 2009). This is a good example of how an animal model of CNV may translate to the possibility of imaging and possibly treating human CNV.

2.4.3 *ApoE* overexpression—Our laboratory and others have demonstrated AMD-like changes in Bruchs membrane and the area of the RPE in hypercholesterolemic mice (Dithmar et al 2000). Interestingly, Malek and co-workers have shown that in *ApoE4* over-expressing transgenic mice fed a high-fat cholesterol rich diet and allowed to age 65-127 weeks developed CNV in addition to drusen-like and basal laminar-like depositis (Figure 7) (Malek et al 2005). The CNV developed in 19% of male and 18% of female mice and was demonstrated by fluorescein angiography, histology, immunohistochemistry, and electron microscopic examination. This model is an important model to study mechanisms of spontaneous CNV formation in the setting of AMD-like lesions.

2.4.4 *Cp*^{-/-} *Heph*^{-/-} *Y* knockout—Hahn and co-workers have shown that disruption of ceruloplasmin and hephaestin causing iron overload causes AMD-like changes in transgenic mice. These mice developed RPE changes and photoreceptor degeneration. The mice developed funduscopically evident lesions at the level of the RPE and 100% of the mice developed histologically identified subretinal neovascularization, although it is not apparent if the neovascularization arose from the retina or choroid (Hahn et al 2004). Additionally, the mice do not appear to develop the drusen-like or basal laminar deposit-like lesions as seen in AMD.

2.4.5 Spontaneous *Bst* chromosome 16 mutant—The ocular histopathologic findings in the spontaneous *Bst* chromosome 16 mutant mouse have been extensively studied by Smith and coworkers (Smith et al 2000). This mouse was found to spontaneously develop retinal lesions identified by fundus examination in the Jackson Laboratories in Bar Harbor, Maine. The spontaneous belly spot and tail (*Bst*) mutation arose in C57BLKS inbred strains of mice. The eyes of the mice were studied by fundus examination, fluorescein angiography and histology. Approximately 88% of the mice developed histologically identified subretinal neovascularization. The neovascularization was associated with RPE abnormalities, retinal hamartoma-like lesions, and connections of the neovascularization with the retina and choroid through defects in Bruchs membrane. Although the neovascularization was age-related, there were no drusen-like or basal laminar deposit-like lesions in this mouse, thus the

neovascularization appears to be more similar to that seen in retinal angiomatous proliferation (RAP) than CNV in AMD.

2.4.6 *Vldlr* $-/-$ targeted mutant—This mouse was developed by Dr. Joachim Herz at the University of Texas, Southwestern. These are homozygous strains of mice with targeted mutations for very low density lipoprotein receptor gene (*Vldlr*^{Tm1Her}). The mice develop new blood vessels in the area of the outer plexiform layer of the retina and choroidal anastomoses by 3 months (Heckenlively et al 2003). Like the spontaneous *Bst* mouse, these appear similar to RAP lesions.

2.4.7 *Ccl2/Cx3cr1*-deficient mice—Chan and coworkers have generated a *Ccl2/Cx3cr1* deficient double knockout (DKO) transgenic mouse that developed increased N-retinylidene-N-retinylethanolamine (A2E) and decreased ERp29 levels in the RPE (Chan et al 2008). These mice, when fed a diet low in omega-3 polyunsaturated fatty acids (PUFAs), developed funduscopically and histologically-ultrastructurally visible RPE changes and drusen-like lesions. Approximately 15% of the mice developed histologic evidence of neovascularization (Figure 8). The lesions in this mouse develop in 6 weeks, a short time span in the transgenic mouse world, thus making it a potentially attractive model for spontaneous development of neovascularization in a transgenic mouse, although further work is warranted to study the nature of this neovascularization.

2.4.8 *Tet/VMD2/VEGF* & *Tet/VMD2/VEGF/Ang2*—In a sophisticated set of experiments, Oshima and coworkers studied CNV in double (*Tet/VMD2/VEGF*) and triple (*Tet/VMD2/VEGF/Ang2*) transgenic mice (Oshima et al 2004). Adult mice with increased expression of VEGF in the RPE had normal retinas and choroids and did not develop CNV. However, subretinal injection of an adenoviral vector containing an expression construct for *Ang 2* resulted in histologically identified CNV in 100% of the *Tet/VMD2/VEGF* mice whereas *Tet/VMD2/VEGF/Ang2* triple transgenic mice did not spontaneously develop CNV. The investigators used immunohistochemistry and fluorescein dextran perfused flatmounts to demonstrate the presence and size of CNV. This model provides the important information that overexpression of VEGF is not sufficient for the development of CNV; there must be mechanical and/or other factors at the level of Bruch's membrane to induce CNV. Exogenously administered tetracycline or doxycycline turns off the genetic construct in the transgenic animal. One caveat is that doxycycline downregulates CNV and animal models using *Tet-on/Tet-off* for CNV should be carefully evaluated.

2.4.9 *Rhodopsin* promoter *VEGF* overexpression—Okamoto, Tobe and colleagues generated *rhodopsin* promoter/*VEGF* fusion gene transgenic mice (Okamoto et al, 1997, Tobe, Okamoto et al 1998). Progeny of these mice crossbred with C57BL/6 mice developed angiographically visible intraretinal neovascularization that extended into the subretinal space. This lesion, similar to RAP, demonstrated that overexpression of VEGF in the retina is sufficient to cause intraretinal and subretinal neovascularization. It is possible that neovascularization may originate in the choroid of the animal as well as the retina thus resulting in chorio-retinal anastomosis. Some investigators choose to use both a laser-induced CNV model, such as in a rat or mouse, and a transgenic model, such as this *rhodopsin* promoter/*VEGF* mouse, when evaluating new treatments for CNV.

2.4.10 senescent *SOD1*^{-/-} mice—Imamura and co-workers studied the fundus, fluorescein angiographic, histologic, ultrastructural and immunohistochemical features of the retina, RPE, Bruch's membrane and choroidal areas of Cu, Zn-superoxide dismutase (*SOD1*) deficient mice (Imamura et al, 2006). These investigators found that there was fundus and histologic evidence of CNV in 8.3% and 10% of these senescent *Sod*^{-/-} mice, respectively. A

marker of oxidative damage to DNA, 8-hydroxy-2'-deoxyguanosine (I-OHdG) was detected in the RPE of senescent *Sod*^{-/-} but not in controls. The CNV appeared to connect with retinal vessels in 16 month old compared with 12 month old mice.

2.5 Summary of Animal Models of CNV

Representative animal models of CNV are shown in Table 1. There is no ideal animal model of CNV. The models studied to date indicate that VEGF overexpression is insufficient for the development of CNV; there needs to be a compromise to Bruchs membrane, either mechanically or biologically induced, to induce CNV. Furthermore, there appears to be a central role in Bruchs membrane in the formation of human CNV. All models require compromise to Bruchs membrane, VEGF expression and inflammatory cytokine mediation. The emphasis of which plays a predominant role in the CNV formation depends on the model. For instance, some models are based on laser and/or mechanically induction of a break in Bruchs membrane, which is the dominant feature in those models. Some models are based on over-expression of VEGF, either by subretinal introduction of VEGF or materials impregnated with VEGF, or transgenic animals with over-expression of VEGF driven by retina or RPE specific promoters. Other models are based on inflammatory cytokine production as the dominant feature causing CNV, such as those that utilize subretinal injection of biomaterials and possibly those based on oxidative stress. Investigators should be aware of the relative importance of the various components of CNV formation (i.e. mechanical injury to Bruchs membrane, VEGF production, inflammatory cytokine mediation) when utilizing an animal model of CNV.

Each currently available model has advantages and disadvantages as mentioned above. Considerations when choosing an animal model are related to what the model will be used for. Small, reproducible models, such as the mouse laser-induced CNV model, may be chosen for high throughput drug screening studies. These models are reasonably consistent and result in CNV in approximately 80% of lesions. Laser induction of CNV may be performed in transgenic mice as well, thus providing the ability to study various contributions of inflammatory, angiogenic and proteolytic pathways to CNV formation. The rabbit model that includes delivery of angiogenic factors and insult to Bruchs membrane results in CNV that may persist for months. That type of model may be useful for longitudinal studies, where doses and durations of pharmacologic agents need to be studied *in vivo* using angiography and/or OCT. These larger models, such as the rabbit and pig, are also useful for drug delivery studies. If a model is needed with a macula, the primate may be used, although there are cost and ethical issues pertinent to primate use. The three features needed for successful animal models of CNV are 1) sustained angiogenesis by VEGF production; 2) an inflammatory cytokine component; 3) compromise of Bruch's membrane. A given laboratory's capabilities including budget, animal housing facilities, and experimental goals are all important when deciding which animal model of CNV to utilize.

3. Animal Models of Retinal Neovascularization

3.1 Overview of Retinal Neovascularization

The unique feature of the retina with dual blood supply makes it possible to identify two types of neovascularization: retinal neovascularization, which originates from the retinal vessels, usually extends through the internal limiting membrane (ILM) and grows into the vitreous (under some conditions, the vessels grow in the opposite direction into the subretinal space); and choroidal neovascularization, as described in previous section.

The pathogenesis of retinal neovascularization is better understood than that of CNV (Campochiaro, 2000) (Figure 9). Numerous clinical and experimental observations have

indicated that ischemia is the main cause for retinal neovascularization (Michaelson, 1948; Ashton, 1957; Shimizu et al, 1981). Attenuation of retinal vessels resulting in retinal ischemia is a common feature shared by each of the diseases where retinal neovascularization develops, including diabetic retinopathy, retinopathy of prematurity (ROP), central retinal vein occlusion, and branch retinal vein occlusion, diseases that are referred to as ischemic retinopathy.

One retinal neovascular disease that is developmental in origin is retinopathy of prematurity (Smith, 2002). Retinal vascularization completes by 36 weeks of gestation in the nasal quadrant and slightly later in the temporal quadrant. If an infant is born prematurely before the retinal vascularization is completed, normal vascular maturation process can be disrupted. This neovascularization process occurs in two phases: a vaso-obliterative phase and a vaso-proliferative phase (Ashton et al, 1954). After birth, the premature infant is exposed to the relative hyperoxia of extrauterine environment (55-80 mmHg of PO₂) compared to the *in utero* condition (35 mmHg of PO₂), which is exacerbated by supplemental oxygen therapy. During this exposure to hyperoxia, the expression of hypoxia-driven angiogenic factors is downregulated, leading to an arrest or retardation of existing retinal blood growth (Madan and Penn, 2003). Increased metabolic requirement of the developing retina stimulate new vessel growth in response to hypoxia caused by undervascularized retina. This is followed by the vaso-proliferative phase of compensatory, often uncontrolled neovascularization. These new vessels, due to inappropriate development and patterning, cannot sufficiently oxygenate the retina and can bleed easily, causing the degeneration of retinal ganglion cells and photoreceptors (Ashton, 1966; Hellstrom et al, 2001). The vaso-attenuative effect of hyperoxia in neonatal retina with subsequent neovascularization has been used to generate animal models of ischemic retinopathy that not only recapitulates critical events of ROP, but also those of proliferative diabetic retinopathy.

Diabetic retinopathy is the leading cause of acquired blindness in young adults and one of the most severe ocular complications of diabetes (The Early Treatment Diabetic Retinopathy Study Research Group, 1987). In early stages, retinal capillaries are damaged as a result of the microvasculopathy characteristic of diabetes, leading to retinal hypoxia. This is followed by the vaso-proliferative stage of diabetic retinopathy where abnormal, immature new blood vessels grow along the retina and into the vitreous. These vessels can easily bleed, sometimes complicated by fibrovascular membrane and tractional retinal detachment. The microvascular damage also increases vascular permeability. Retinal edema and thickening in the macular area leads to significant visual loss, which is the most common cause of vision loss in non-proliferative diabetic retinopathy (Saraf, 2001). There are currently no perfect models for diabetic retinopathy; the most commonly used model for diabetic retinopathy is the model induced by intraperitoneal injection of streptozotocin in the rats. The diabetogenic action of the nitrourea derivative streptozotocin (STZ) is used to induce diabetes in the rats, and the animals later develop features similar to diabetic retinopathy (Sosula et al, 1972). However, OIR models are used to study specific pathological events that commonly occur during diabetic retinopathy (Feit-Leichman et al, 2005).

Occlusion of retinal veins by either laser photocoagulation or photodynamic therapy can be performed to induce retinal neovascularization associated with retinal vein occlusion. Other animal models includes transgenic mouse models with overexpression of vascular endothelial growth factor (VEGF), intraocular injection of pro-angiogenic molecules, and inflammation-associated models.

3.2 Oxygen-induced Models of Retinal Neovascularization

Animal models of oxygen-induced retinopathy (OIR) have become major models for pathological angiogenesis resulting from ischemia. This model has been developed first in the

kitten (Ashton et al, 1954), then extended to other animal species, including rat (Penn et al, 1993), mouse (Smith et al, 1994), beagle puppy (McLeod et al, 1996), and zebrafish (Cao et al, 2008). Each of the models in different animal species shares the same basic mechanism by which exposure to hyperoxia during early retinal development leads to the arrest or retardation of normal retinal vascular development. When the animals are returned to the normoxic environment, they are under a relative hypoxic situation where the retina now lacks its normal vasculature that is required to adequately support the neural tissue in normoxic conditions. This ischemic situation results in unregulated, abnormal neovascularization. The neovascular response is very consistent, reproducible and quantifiable, and it has become integral for studying disease mechanisms and potential treatments for ischemic retinopathy. Neovascularization occurs at the junction between the vascularized and nonvascularized retina, followed by normalization of the vascular pattern with regression of the abnormal vessels in mice (Smith et al, 1994). Retinal detachment which can be seen in the late stage of ROP occurs in a beagle puppy OIR model, but not in the mouse or kitten OIR model (McLeod et al, 1998; Madan and Penn, 2003).

This ischemia-mediated pathological neovascularization makes the OIR models useful for studying other ischemic retinopathies such as diabetic retinopathy, the leading cause of blindness in younger adults. The mouse OIR model described by Smith et al has become the most common model for studying pathologic angiogenesis associated with ROP (Smith et al, 1994). The use of this model has been extended to the general study of ischemic retinopathies and related anti-angiogenic treatments.

3.2.1 mouse—The most widely used models of OIR are the mouse model originally described by Smith in 1994 (Smith et al, 1994) and the rat model developed by Penn (Penn et al, 1993). The main advantage of the mouse OIR model is the ease of genetic manipulation; transgenic mice can be used to directly study the roles of different genes on pathological neovascularization. Other advantages are the reproducibility of the phenotype, relative costs, the size of the animals, and a reliable method of quantifying retinal neovascularization (Smith et al, 1994; Aguilar et al, 2008). A major disadvantage of this model is the fact that in mice, the central retinal vessels, rather than the peripheral vessels, are obliterated during exposure to hyperoxia. This is different from what happens in ROP where the peripheral retinal vessels fail to develop. Despite this key difference, the mouse OIR model very closely recapitulates the pathologic events that occur during ischemia-induced neovascularization (Aguilar et al, 2008).

C57BL/6J mouse pups and their mothers are placed in 75% oxygen beginning at postnatal day 7 (P7), and remain in the oxygen chamber for 5 days with constant 75% oxygen levels. Neovascularization is noted five days after return to room air. Some strains of mice, such as BALB/cByJ, react differently to this protocol and do not develop pathological neovascularization at all despite hyperoxia-induced vascular obliteration (Ritter et al, 2006). The original model utilized alternating high/low oxygen exposure; however, similar exposure to constant levels of 75% oxygen for 5 days followed by 5 days in room air demonstrated comparable results (Smith et al, 1994) (Figure 10).

3.2.2 rat—While the mouse model utilizes exposure to the constant hyperoxia for 5 days before returning to the room air, the rat OIR model uses alternating hyperoxia-hypoxia cycles. The main advantage of the rat OIR model is that similar to the ROP, the peripheral vasculature becomes obliterated upon exposure to hyperoxia, whereas the central retinal vessels are obliterated in the mouse model (Madan and Penn, 2003) (Figure 11). Removal from oxygen exposure to room air results in neovascular growth at the boundary between vascular and avascular areas in the mid-peripheral retina.

The protocol of the oxygen exposure, both duration and age at which the rat pups are initially exposed to hyperoxia, is different from the mouse OIR protocol. Penn and coworkers generated the rat OIR model by exposing the animals to the alternating cycle of 80% and 40% oxygen for 12-h for the first 14 days followed by exposure to the room air (Penn et al, 1993). The revised protocol also uses alternating oxygen cycles, but these cycles now utilize 12-hour cycles of 50% and 10% oxygen which show a higher incidence and severity of neovascularization than 80%/40% cycle (Madan and Penn, 2003). The severity of neovascularization can be augmented with increased CO₂ concentrations or with growth retardation (Holmes and Duffner, 1996; Holmes et al, 1997). The rat OIR model has been used extensively to identify factors involved in hypoxia-induced neovascularization as well as to test various ocular angiostatics. (Penn et al, 2001; Penn and Rajaratnam, 2003).

3.2.3 feline model—The kitten model was first introduced by Ashton and coworkers, and was one of the first to be used for the study of oxygen-induced retinal neovascularization (Ashton et al, 1954). The kittens are exposed to 70-80% of oxygen within hours after birth for 96 hours, and then returned to room air. The vascular changes occur in successive stages: closure and obliteration during hyperoxia, vasoproliferation induced by hypoxia, and normalization after the relief of hypoxia with distinct cellular mechanisms and stimuli (Chan-Ling et al, 1992).

3.2.4 canine model—McLeod and colleagues studied the pattern of vaso-obliteration in the canine model of OIR. All vascular components showed constriction after 1 hour of hyperoxia, and capillary closure peaked by 24 hours. The pattern and severity of the reaction of the developing retinal vessels to hyperoxia is similar to that observed in the kitten and the premature babies (McLeod et al, 1996). When the newborn pups were exposed to 95-100% oxygen for 4 days and returned to room air until 22-45 days of age, tractional retinal folds and intravitreal vascularized membranes were present in the eyes (McLeod et al, 1998).

3.2.5 zebrafish—Cao and colleagues generated a model of hypoxia-induced retinal angiogenesis in the adult zebrafish. Nitrogen gas was perfused directly into the water in a sealed aquarium. Adult *Fli-EGFP-Tg* zebrafish were first placed in normoxic water and the O₂ tension was gradually reduced to the final 10% air saturation. The fish were exposed in the hypoxic condition from 3-15 days. At day 12, retinal neovascularization reached a plateau of maximal angiogenic responses (Cao et al, 2008).

3.3 Vascular occlusion models of retinal neovascularization

3.3.1 rat—Retinal vein occlusion by laser photocoagulation or photodynamic therapy has been served as a useful model of preretinal neovascularization (Shen et al, 1996; Ham et al, 1997; Saito et al, 1997). Occlusion of retinal veins in rats causes microvascular changes similar to the findings seen in branch retinal vein occlusion clinically, which includes venous dilation, vascular tortuosity, and extravasation of fluid from retinal vessels. These result in reduced blood flow, retinal ischemia, and subsequent neovascularization within the retina or iris. Depending on the number of retinal veins treated, the model can be a model of either branch retinal vein occlusion or central vein occlusion.

After anesthesia, rose bengal dye is injected through tail vein or sodium fluorescein is injected intraperitoneally and laser spots are applied to two or three major vein as they emerge from the optic nerve up to one disc diameter. The laser parameters should be adjusted to achieve venous occlusion; if the laser photocoagulation is too weak, occlusion will not be achieved, and too strong settings can extensively damage other areas of the retina or cause bleeding from the vein. Retinal edema became evident 10-30 minutes after treatment in the sectors associated

with the occluded veins, and later resulting in retinal neovascularization and tractional retinal detachment.

3.3.2 pig—Laser induced retinal vein occlusion in pigs has also been shown to cause retinal neovascularization. Retinal neovascularization occurred after occlusion of a single retinal vein or multiple retinal veins with argon laser coagulation (Pournaras et al, 1990) or photodynamic therapy (Danis et al, 1993). Retinal hemorrhage and edema were observed hours after the occlusion, and later preretinal and intravitreal new vessels developed on the ischemic areas.

3.4 Transgenic Mouse Models of Retinal Neovascularization

3.4.1 *rho/VEGF* Transgenic Mice—As mentioned previously (see 2.4.9), Okamoto and coworkers observed the development of intraretinal and subretinal neovascularization in the transgenic mice with *VEGF* driven by the *rhodopsin* promoter (*rho/VEGF* mice).

Overexpression of VEGF in the retinal photoreceptors demonstrated new vessels originating from the deep capillary bed of the retina extending into the subretinal space. This model may be related more closely to the disease entity seen in patients with retinal angiomatous proliferation (RAP) (Miller, 1997; Okamoto et al, 1997). Tobe et al showed that VEGF transgene mRNA was first detected in the retina on postnatal day 6 (P6). On P14, sprouts from retinal vessels in the deep capillary bed were found, reaching the subretinal space by P18. Invasion of blood vessels from the choroid was not seen (Tobe et al, 1998).

3.4.2 *rho/rtTA-TRE/VEGF* or *IRBP/rtTA-TRE/VEGF* Transgenic Mice—Ohno-Matsui et al used the reverse tetracycline transactivator (rtTA) inducible promoter system couple to either the *rhodopsin* or interphotoreceptor retinoid-binding protein (IRBP) promoter to control the time of onset of *VEGF* transgene expression in the retina. Without doxycycline, the adult transgenic mice showed little VEGF expression. The addition of doxycycline to the mice resulted in prominent *VEGF* transgene expression with evidence of more extensive neovascularization. It was also associated with total retinal detachment and higher ocular levels of *VEGF* mRNA and protein compared to those of *rho/VEGF* mice (Ohno-Matsui et al, 2002).

3.4.3 *VEGF165 (hVEGF)* overexpressing mice—Lai et al developed four human *VEGF165 (hVEGF)* over-expressing transgenic mouse lines with phenotypes ranging from mild to severe retinal neovascularization. These transgenic mice were generated via microinjection of a DNA construct containing the *hVEGF* gene driven by a truncated mouse *rhodopsin* promoter. One transgenic line with low *hVEGF* showed mild changes such as focal fluorescein leakage, microaneurysms, venous tortuosity; three other lines with high *hVEGF* levels presented with severe phenotypes, as well as extensive neovascularization, hemorrhage, and retinal detachment (Lai et al, 2005) (Figure 12).

Bai et al investigated the role of the Müller cell-derived VEGF in ischemia-induced retinal neovascularization in conditional VEGF knockout mice. The transgenic mice were generated by mating Müller cell-expressing Cre mice with floxed VEGF mice, and the VEGF production in the Müller cell was disrupted by feeding 2 mg/ml doxycycline from embryonic day 15 to postnatal day 1. Disruption of VEGF in Müller cell did not cause any detectable retinal degeneration with significant inhibition of the ischemia-induced retinal neovascularization.

3.4.4 *Nrl^{-/-}Grk1^{-/-}* double knockout mice—A double knockout *Nrl^{-/-}Grk1^{-/-}* mice were generated to study the cone G-protein coupled receptor signaling pathway. G-protein coupled receptor kinase (Grk1) is involved in the inactivation and normal recovery of cone pigments, and the neural retina leucine zipper knockout mouse (*Nrl^{-/-}*) retina is biochemically

“all-cones”, because rod phototransduction components are absent in this retina. The absence of Grk1 expression on the *Nrl*^{-/-} showed age-related and light independent cone dystrophy. Retinal neovascularization was observed in all retinal layers by 1 month of age, which was mediated by inflammatory pathway (Yetemian et al, 2010).

3.5 Intraocular injection of pro-angiogenic molecules

3.5.1 Rabbit—Retinal neovascularization was induced after intravitreal injection of fibroblast. Tissue-cultured skin fibroblasts were injected into the vitreous cavity in rabbits, and strand formation in the mid-vitreous cavity was observed, extending toward the retina. Once it attached to the retina, neovascularization began with growth of vessels along the fibrous strand. New vessels regressed after three months (Tano et al, 1981). Antoszyk and coworkers modified the technique by producing partial posterior vitreous detachment with repeated injection and aspiration of hyaluronidase. Rabbit eyes were injected with repeated injection and aspiration of hyaluronidase before injection of homologous tissue-cultured dermal fibroblasts. Ninety-five percent of the eyes developed definite neovascularization by day 14 (Antoszyk et al, 1991). A drawback of this technique is that the neovascular response is more traumatic and inflammatory, rather than ischemic, and retinal detachment is needed for the development of neovascularization (Miller, 1997).

3.5.2 Mouse—In order to recapitulate the vascular changes in the diabetic retinopathy, a binary recombinant adeno-associated virus construct producing green fluorescent protein (GFP) and VEGF under the control of the human cytomegalovirus promoter was injected into the subretinal space of C57BL6 mice. The neovascular changes were more gradual and less pronounced in these mice than other transgenic mouse models with high VEGF levels. This model demonstrated that the development of different stages of diabetic retinopathy is closely correlated with an increased VEGF level in the retina (Rakoczy et al, 2003).

3.6 Other Models of Retinal Neovascularization

3.6.1 Inflammatory Models of Retinal Neovascularization—As mentioned previously (see 2.3.1), the inflammatory model consists in immunization of rats with interphotoreceptor retinoid binding protein peptide to induce mild inflammation. The inflammation was most prominent on day 14, with inflammatory cells in the anterior and posterior segment of the eye. The inflammation decreased in intensity on day 18, but branches of retinal vessels were seen extending into the choroid. On day 45, there was no appreciable inflammation, but new vessels were present in 38% of the eyes (Sakamoto et al, 1994).

3.6.2 Radiation retinopathy model—Irvine and Wood studied the clinical and histological changes of the experimental radiation retinopathy in monkeys. The first change observed after radiation was the focal loss of capillary endothelial cells and pericytes, and as the areas of capillary loss became confluent, large areas of retinal capillary nonperfusion appeared. Later, intraretinal neovascularization and rubeosis iris with neovascular glaucoma developed (Irvine and Wood, 1987).

3.6.3 Diabetic retinopathy model—In early stages of STZ-diabetic rats, there is capillary basement membrane thickening as well as capillary dilatation (Sosula et al, 1972). Vascular occlusion of retinal capillaries and increased vascular leakage from retinal blood vessels are also present (Bek, 1997; Zhang et al, 2005; Masuzawa et al, 2006).

A spontaneously diabetic strain of Sprague-Dawley (SD) rat was recognized in 1988 and named as the Spontaneously Diabetic Torii (SDT) rat. Male SDT rats develop hyperglycemia and glycosuria at approximately 20 weeks of age. The cumulative incidence of diabetes is 100% by 40 weeks of age in the male rats, whereas 33.3% by 65 weeks of age in the female rats

(Shinohara et al, 2000). Histologic examination showed cataract and large retinal folds mimicking tractional retinal detachment with extensive leakage of fluorescein around the optic disk (Takehashi et al, 2006). The SDT rat develops retinal neovascularization without retinal ischemia, which can be attributed to the change in the vitreo-retinal interface, where the persistent vitreous adhesion can be served as the platform for vascular proliferation (Yamada et al, 2005).

3.7 Summary of Animal Models of Retinal Neovascularization

Representative animal models of retinal neovascularization are shown in Table 2. Animal models of retinal neovascularization are well established compared to those for choroidal neovascularization. Ischemia plays a central role in the development of retinal neovascularization. Disease processes in which retinal neovascularization occurs, including diabetic retinopathy, ROP, central retinal vein occlusion, branch retinal vein occlusion, are collectively referred to as ischemic retinopathies. The methods inducing ischemia in animal models include hyperoxia, laser, inflammation, and radiation. There has been a prevalence of rodent studies versus other species in recent years. These models are well characterized, readily quantifiable, and easily reproducible.

2. Conclusions and Future Directions

The two main types of periretinal neovascularization are choroidal neovascularization (CNV) and retinal neovascularization (RNV). CNV originates in the choroid from the choriocapillaris circulation, penetrates through Bruch's membrane, and grows into the subRPE and/or subretinal space. RNV originates in the retina from the retinal circulation and extends onto the posterior hyaloid face of the vitreous or into the vitreous. CNV is generally well-confined, like due to a limited extent of cytokine diffusion and RPE control of the size and extent of the lesion. RNV grows in a more haphazard, wildfire-like manner, likely due to diffusion of angiogenic cytokines into the vitreous. A third type of periretinal neovascularization may occur in the setting of CNV and/or RNV and when it forms anastomoses between the retinal and choroidal circulation, it may be classified as retinal angiomatous proliferation, or RAP. When evaluating animal models of CNV and RNV, one should consider these features. Many laser, surgical, dietary and transgenic models of CNV and RNV have been developed and are well described. There is no perfect animal model of CNV or RNV. Investigators are encouraged to carefully consider the hypotheses to be tested, strengths and weaknesses of the various models, available funding, facilities and time available prior to choosing an animal model of CNV or RNV. This will facilitate experimentation and provide the best opportunity for meaningful results. Animal models of CNV and RNV provide an intermediate step between *in vivo* experimentation and human clinical trials. These current animal models and those that will be developed will continue to provide usefully scientific information for the foreseeable future. The key will be to match scientific interest and funding availability with the model. For those interested in biologic mechanisms, transgenic and knockdown models will continue to be useful. For those interested in drug development, animal models that quickly develop CNV or RNV, such as laser-induced models will continue to be important, especially since these animal models usually develop neovascularization in days to weeks as opposed to the usual months to years it takes for transgenic and knockdown models. As new drugs are developed and become successful, it will become increasingly more difficult to prove superiority of one drug versus another. However, in order to do this, animal models will continue to be needed to test safety and efficacy. Targeted drug delivery to the back of the eye is a rapidly growing area that will require larger animal models of CNV and RNV, such as in the rabbit or pig, in order to simulate the size of the human eye for introduction of investigational drug delivery devices. New, reproducible animal models will continue to be developed, especially larger animals for drug delivery studies.

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Figure 1. Mouse laser model of CNV

A. Control mouse with laser induced CNV (between arrows). B. A mouse is treated with a drug that inhibits VEGF and PDGF develops smaller CNV (between arrows) after laser induction. (From Kwak et al, 2000) C. A flat mount preparation of laser induced CNV in the mouse after perfusion with fluorescent dextran shows the horizontal extent of the CNV. (From Singh et al, 2009)

Figure 2. Cynomolgus monkey laser model of CNV

Part 1. Fluorescein angiography 21 days after laser induction of CNV shows partial localized (A and B) or mixed localized and diffuse CNV (C and D) Part 2. Histologic examination of enucleate eyes shows CNV between the retina and a reflected layer of RPE (A), extension of the CNV into the retina (B), and numerous vascular channels within the CNV which also contains pigmented macrophages (C and D). (From Criswell et al, 2004)

Figure 3. Mouse surgical model of CNV

A. After subretinal injection of RPE and polystyrene beads, CNV is located between the retina and a reflected layer of RPE. Vascular channels are indicated by arrows. B. There are vascular channels (arrows), mononuclear inflammatory cells, and beads (asterisks) located within the CNV. C. A fluorescent labeled dextran flatmount preparation shows the extent of the CNV. (From Schmack et al, 2009)

Figure 4. Rabbit surgical model of CNV

Part 1. A color fundus photograph (a), fluorescein-dextran angiogram (b) and OCT show that there is a separation of the neurosensory from the underlying Bruchs membrane and choroid. Part 2. CNV is present in the subretinal space 1 week (a) and 3 months (b) after subretinal injection of 100 ng FGF-2/100 ng LPS. There are inflammatory cells present (arrows) in the retina, CNV, and surrounding Heparin-sepharose beads at the injection area in the 1 week old CNV. There are giant cells (open arrow) in the 3 month old CNV. (From Ni et al, 2005)

Figure 5. Pig surgical model of CNV

Part 1. A fundus photograph (upper left) and fluorescein angiogram early (upper right), middle (bottom left) and late (bottom right) phases show a yellow, early hyperfluorescent with late leakage CNV lesion in the pig model with perforation of Bruch's membrane only. Part 2. Histology and immunohistochemical staining for CD34 that shows vascular channels shows that the CNV lesion is smaller and has fewer vascular channels after RPE removal/Bruch's perforation (upper and lower left) compared with Bruch's membrane perforation alone (upper and lower right). (From Lassota et al 2007)

Figure 6.

Mouse CNV in Ccr2/CCl2 transgenic model Electron micrographs of CNV shows endothelial cells (E) and fibrocytes (F) from the choroid breaking through Bruch's membrane and extending into the sub-RPE (R) space. Photoreceptors (PR) are present internal to the RPE. (From Ambati et al, 2003)

Figure 7. Mouse ApoE overexpression transgenic model of CNV

Part 1. Early (a), mid (b) and late-phase (c) fluorescein angiogram demonstrates area of hyperfluorescence and leakage indicative of CNV. Part 2. Histology showing CNV under RPE (a), extending from the sub-RPE space into the retina (b and c), and extensive CNV (d). (From Malek et al, 2005)

Figure 8. Mouse Ccl2Cx3cr1 transgenic mouse model of subretinal neovascularization

There is neovascularization in the deep retina with uninvolved underlying RPE (A). These vascular channels (arrow) are present under the photoreceptors and appear to arise from the area of the RPE (arrow) (B). Courtesy of Chi Chao Chan MD

Figure 9. Schematic diagram of the cascade of events that have been implicated in the development of retinal neovascularization

Abbreviations: HIF-1, hypoxia-inducible factor 1; IGF-1, insulin-like growth factor 1; PDGF-BB, platelet-derived factor B chain homodimer; TGF-βs, members of the transforming growth factor beta super family; VEGF, vascular endothelial growth factor; VEGFR1, VEGF receptor 1. (From Campochiaro, 2000)

Figure 10. Time course of retinal vascular response to hyperoxia in fluorescein-dextran perfused flat mounts

Left: normal P7 retina before hyperoxia exposure. Note area of nonperfusion in periphery where blood vessels have not yet developed. Right: P14 retina after 5 days of hyperoxia exposure, followed by 2 days in room air. Note increased perfusion in the periphery and dilation and tortuosity of radial vessels and persistent absence of central perfusion. (Adapted from Smith et al,1994)

Figure 11. Retinal flat mount of rat OIR model

Peripheral neovascularization is seen in the rat retina. (From Madan and Penn, 2003)

Figure 12.

Characterization of different transgenic mouse lines by color fundus photography, fluorescein fundus angiography, fluorescein micrographs of flat mounted, fluorescein labeled dextran perfused eyes, and light micrographs of hematoxylin and eosin stained paraffin sections. (From Lai et al, 2005)

Table 1**Animal Models of Choroidal Neovascularization**

reference	animal	method	success rate	quantification	mechanism
Dobi et al 1989	rat	krypton laser	24%-60%	FA-path	Bruchs
Frank et al 1989	rat	krypton laser	43%	path	Bruchs
Tohe et al 1998	mouse	krypton laser	57%	path	Bruchs
Seo et al 1999	mouse	krypton laser	100%	path	Bruchs
Albert et al 2010	rat	cyclic light	n/a	path	cytokines
Ryan 1979	monkey	argon laser	40-90%	FA-path	Bruchs
Criswell et al 2004	monkey	diode laser	37-65%	path	Bruchs
El Diringi et al 199	rabbit	subretinal argon laser	100%	path	Bruchs
Saishin et 2003	pig	diode laser	100%	path	Bruchs
Kilgaard et al 2005	pig	xenon or diode laser	54% to 83%	path	Bruchs
Chan et al 1990	mouse	immunization	10%	path	cytokines
Sakamoto et al 1994	rat	immunization	38%	path	cytokines
Spilsbury et al 2000	rat	subretinal injection	86%	path	VEGF
Baffi et al 2000	rat	subretinal injection	most eyes	path	VEGF
Wang et al 2003	rat	subretinal injection	90%	path	VEGF
Schmack et al 2009	mouse	subretinal beads	100%	path	cytokines
Shen et al 2006	mice	subretinal matrigel	31% to 53%	path	cytokines
Zhu et al 1989	rabbit	subretinal vitreous	31%	path	cytokines
Kimura et al 1995	rabbit	subretinal microspheres	83%-100%	FA-path	cytokines
Hsu et al 1989	rabbit	penetrating retinal injury	n/a	path	Bruchs
Tamai et al 2002	rabbit	subretinal linoleic acid	46%	FA	cytokines
Ni et al 2005	rabbit	transvitreal subretinal beads	100%	FA, OCT-path	cytokines
Qui et al 2006	rabbit	subretinal matrigel/VEGF	100%	FA	cytokines
Julien et al 2008	rabbit	transvitreal subretinal VEGF	85%	FA	VEGF
Lassota et al 2006	pig	subretinal Bruchs perforation	100%	path	Bruchs
Cui et al 2000	monkey	subretinal microspheres	92%	FA	VEGF
Ambati et al 2003	mouse	transgenic	25%	path	cytokines

reference	animal	method	success rate	quantification	mechanism
Malek et al 2000	mouse	transgenic	18-19%	FA/path	cytokines
Hahn et al 2004	mouse	knockout	100%	path	cytokines
Smith et al 2000	mouse	spontaneous mutant	88%	path	Bruchs
Heckenlively et al 2005	mouse	targeted mutant	n/a	n/a	Bruchs
Chan et al 2008	mouse	transgenic	15%	path	cytokines
Oshima et al 2004	mouse	transgenic	100%	path	Bruchs
Okamoto et al 1997	mouse	transgenic	n/a	FA	VEGF
Inamura et al 2006	mouse	transgenic	8.3% - 10%	path	cytokines

n/a=not available; FA=fluorescein angiography; path=pathology; OCT=ocular coherence tomography

Table 2**Animal Models of Retinal Neovascularization**

reference	animal	method	success rate	quantification	mechanism
Smith et al, 1994	mouse	hyperoxia (75% oxygen)	100%	path	ischemia
Penn et al, 1993	rat	cyclic oxygen (80%/40%)	66%	path	ischemia-reperfusion
Holmes et al, 1997	rat	cyclic oxygen (80%/10%) + high CO ₂	85%	path	ischemia
Ashton et al, 1954	kitten	hyperoxia (70-80%)	100%	path	ischemia
McLeod et al, 1996	puppy	hyperoxia (95-100%)	N/A	path	ischemia
Cao et al, 2008	zebrafish	hypoxia (10%)	N/A	path	ischemia
Shen et al, 1996	rat	argon laser + rose bengal	39%	FA	ischemia
Ham et al, 1997	rat	argon laser + rose bengal	17%	FA	ischemia
Saito et al, 1997	rat	argon laser	70%	FA + path	ischemia
Pournaras et al, 1990	pig	argon laser	N/A	PO ₂ measurement	ischemia
Danis et al, 1993	pig	argon laser + rose bengal	100%	FA + path	ischemia
Okamoto et al, 1997	mouse	transgenic	N/A	FA	VEGF
Ohno-Matsui et al, 2002	mouse	transgenic	47-100%	path	VEGF
Lai et al, 2005	mouse	transgenic	N/A	FA + path	VEGF
Tano et al, 1981	rabbit	intravitreal injection of fibroblast	72%	fundus + path	inflammation
Antoszyk et al, 1990	rabbit	intravitreal injection of fibroblast + hyaluronidase	95%	FA	inflammation
Rakoczy et al, 2003		subretinal injection of recombinant adeno-associated virus-mediated VEGF	90%	FA + path	VEGF
Sakamoto et al, 1994	rat	immunization	38%	path	cytokines
Irvine & Wood, 1987	monkey	radiation	N/A	fundus + path	ischemia
Shinohara et al, 2000	rat	spontaneous	100%	path	hyperglycemia

N/A=not available; FA=fluorescein angiography; path=pathology