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Experimental Treatments for Neovascular Age-Related Macular Degeneration

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1. Introduction

Age related macular degeneration (AMD) is the leading cause of severe visual loss in adults older than 60 years (1, 2). It is estimated that approximately 30% of adults older than 75 years have some sign of AMD and around 10% develop advanced stages of the disease. More than 1.6 million people in the United States currently have one or both eyes affected by an advanced stage of AMD and it is estimated that there are another 7 million individuals "at risk" (1). Due to rapid aging of the population in many developed countries, this number is expected to double by the year of 2020 (1, 3). Although neovascular AMD only accounts for about 10–20% of the overall AMD incidence, this subtype is responsible for 90% of cases of severe vision loss (20/200 or worse) (4, 5).

Neovascular AMD is characterized by the presence of choroidal neovascularization (CNV) and is associated with retinal pigment epithelium detachment (PED), retinal pigment epithelium (RPE) tears, fibrovascular disciform scarring, and vitreous hemorrhage(4).

Choroidal neovascularization is an intricate process controlled by myriad angiogenic agents such as growth factors, cytokines, and extracellular matrix (ECM) components. Several growth factors have been implicated in pathologic vessel formation in ocular diseases, such as age-related macular degeneration, including fibroblast growth factor (FGF), platelet-derived growth factor (PEDF), tumor necrosis factor (TNF-α) and vascular endothelial growth factor (VEGF)(6). Additionally, it is hypothesized that an inflammatory process is behind the pathogenesis of AMD. It was found that extracellular depositions of diffuse basal laminar and linear deposits (BLD) between the cytoplasmic and basement membrane of the RPE are significantly associated with CNV formation (4, 5, 7). Histological studies of these BLDs proved the presence of complement complexes C3, C5b-9, MMP- 2, MMP-9, and vitronectin (8). Further support of this hypothesis came from genetic studies where

mutations/polymorphisms were found in genes coding for the alternative complement pathway regulator (Factor H and Factor H related proteins) and complement pathway proteins (complement component C2, factor B, and toll-like receptor 4).

Several focal treatments have been proposed and extensively studied to prevent the severe visual loss in neovascular AMD patients including laser photocoagulation (9), photodynamic therapy (PDT) (10) and the combination of PDT with intraocular injections of triamcinolone acetonide. Despite anatomical success, there is a low chance for visual improvement when these treatments are used. In recent years, research has provided new insights into the pathogenesis of macular disease. Today less destructive treatments directly targeting CNV and its pathogenic cascade have become available (8, 11). Antibodies against VEGF uniquely offer a significant chance of increase in visual acuity to patients affected by neovascular AMD.

Currently, inhibition of VEGF-A is the first choice of therapy for neovascular AMD, which not only stabilizes, but also improves visual acuity. The most effective preparations, bevacizumab (Avastin, Genentech Inc, South San Francisco, California) or ranibizumab (Lucentis, Genentech Inc), are recombinant monoclonal antibodies (Fab) that neutralize all biologically active forms of VEGF (12). Two Phase III clinical trials (MARINA and ANCHOR) studied ranibizumab for the treatment of CNV associated with neovascular AMD (13-15). In both of these studies, ranibizumab was administered every 4 weeks (fixed schedule) for up to two years without monthly imaging. Both trials demonstrated prevention of substantial vision loss (lost < 15 letters) in more than 90% of subjects. Additionally, approximately 30% to 40% of the subjects experienced substantial visual acuity gains (gain > 15 letters). Though these dramatic results have revolutionized the treatment of neovascular AMD, the monthly treatment schedule used in the clinical trials has a number of drawbacks including the high number of injections and the lack of efficiency in some patients who do not respond to anti-VEGF therapy (12).

Therefore it is important to continue the study of the CNV physiopathology in order to find new molecules involved in the angiogenesis. In this way it will be possible to develop new drugs to reduce the treatment frequency and to treat patients that don't respond to anti-VEGF therapy.

2. Animal models of choroidal neovascularization

The development of animal models of CNV has paralleled and contributed to the understanding of the biology of this condition. In addition, these models have also been developed in order to test new treatments.

a. Laser induced models of CNV

The first CNV model was developed in primates (16), and coworkers later developed a rat model of CNV in 1989 (17). Those authors created argon laser photocoagulation spots (647 nm, 100 mm, 50e100 mW, 0.1 s) through a dilated pupil with a coverslip over the cornea. The created spots break the Bruch's membrane, with a central bubble formation with or without intraretinal 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 coworkers also developed a rat model of CNV in 1989 (18). Also, a diode laser may be used to create the CNV (532 nm, 100 mm 50e100 mW, 0.1 s) and this model has been used to assess aging as it relates to CNV formation.

b. Surgically induced models of CNV

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 (19-21).

c. Transgenic and knockout mouse models of CNV

Although there are several transgenic mouse models AMD (22), 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 Bruch's membrane in the development of CNV (22). The advantages of these models are the ability to study various biologic components of CNV by comparing 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.

3. Retina cytotoxicity assays for new drugs

a. "In vitro" assays

Toxicity is a complex event *in vivo*, where there may be direct cellular damage, physiological effects, inflammatory effects and other systemic effects. Currently, it is difficult to monitor systemic and physiological effects *in vitro*, so most assays determine effects at cellular level, or cytotoxicity (23).

New drugs have to go through extensive cytotoxicity testing before they are released for the use (24, 25). Today there is a continuous search for methods to determine the toxicity by using *in vitro* tests, trying to reduce the number of experiments involving animals (26). Important live-cell functions, including apoptosis, cell adhesion, cell migration and cell proliferation, can be monitored with various *in vitro* tests by using colorimetric and fluorescence assays (27, 28). The most frequently used cell lines are: human retinal pigment epithelial cells (ARPE-19), rat neurosensory retinal cells (R28), rat retinal ganglion cells (RGC-5)(29, 30), the immortalized Muller cell line (MIO-M1) (31) and human umbilical vein endothelial cells (HUVEC) and rabbit aorta endothelial cells (6, 32, 33).

Many of these processes lead to changes in intracellular and membrane components that can be followed with appropriately responsiveness by indicators that could be detected by microscopy, flow cytometry or with a microplate reader. Because cytotoxicity could not be easily defined in terms of a single physiological or morphological parameter, it is often desirable to combine several different measures, such as enzymatic activity, membrane permeability or oxidation–reduction potential. The most common assay to determine the cytotoxicity is the viability assay. The viability is principally used to measure the proportion of viable (life and function) cells after a drug exposure. Most tests verify the cell membrane

integrity by dye exclusion, as Naphtalene Black and Trypan Blue as well as by dye uptake as fluorescein diacetate and propidium iodide (PI) (34, 35). In the first one viable cells are impermeable to the dye, and the analysis is performed by light microscopy. In the second test viable cells uptake diacetyl fluorescein and hydrolyze (esterase) in fluorescein that fluoresce in green, and the nucleus of the non-viable cells are stained by the PI that fluoresce in red, the analysis could be performed both by fluorescence microscopy and flow cytometry (36).

Cell viability also can also be measured by MTT reduction (37) using a microtitration assay in 96 multiwell plates. The reduction of tetrazolium salt (yellow) is reduced in metabolically active cells to form insoluble purple formazan crystals. Other assays include acidotropic stain using acridine orange that concentrates in acidic organelles in a pH-dependent manner. Under fluorescence microscope it is possible to see the metachromatic green or red fluorescence of acridine orange to assess cell viability (38).

Besides viability the apoptosis research is a powerful tool for drug toxicity screening. Apoptosis is the programmed cell death and is characterized morphologically by compaction of the nuclear chromatin, cell-permeability and production of apoptotic bodies. The characteristic observed in apoptotic cells is the fragmentation of the chromatin, degradation of the nuclear envelope and nuclear blebbing, resulting in the formation of micronuclei. A different assay frequently used is the APO-BrdU TUNEL (Terminal Deoxynucleotide Transferase dUTP Nick End Labeling) where DNA strands of apoptotic cells are labeled with BrdUTP, once incorporated into the DNA, BrdU can be detected by an anti‑BrdU antibody conjugated with a enzyme or a fluorescent probe using immunohistochemistry or immunofluorescence (39).

Annexin V is a protein that binds phosphatidylserine located at the cell surface and used to detect apoptotic cells. In apoptotic cells phosphatidylserine is exposed to the outer of the plasma membrane being detected by the annexin V conjugated with a fluorophor. Fluorescent cells could be observed in fluorescence microscope or flow cytometer (33, 40).

b. “In vivo” assays

Retinal toxicity can be evaluated by intravitreal injections of drugs in rats, mice, rabbits and non-human primates. The safety and efficacy of intravitreal drugs can be analyzed in choroidal neovascularization (CNV) in the laser-induced rat model (6, 41). The investigation of toxicity in animal models using the standard tools of light microscopy (LM) and histopathological analysis makes critical benchmarks for the study of development of the angioproliferative disease. In this way is possible to observe the functional and morphological alteration results of drug toxicity *in vivo*.

Microscopic studies using light, electron or confocal microscopy are common methods used for retinal biocompatibility studies. For microscopy analysis, it is essential to know the normal retina morphology of the animal species analyzed. Histological studies, using light or electron microscopies could be descriptive or analytical.

Clinical evaluation is also an important method to evaluate the retinal toxicity of new drugs. The occurrence of a transient or permanent toxic reaction can be documented by the retinal appearance, function or histological findings in experimental eyes (42). Ocular examinations include slitlamp for anterior segment and detailed dilated fundus examinations (42, 43).

Electrophysiological testing is an effective and objective method to assess the status of the visual pathways. The electroretinogram (ERG) is obtained by recording, through a contact lens electrode on the cornea, the electrical potential generated by the retina in response to a brief stimulus (flash or flicker) of light. ERG is one of the most important examinations for retinal biocompatibility in experimental models, since it is a functional and objective test. In animals, behavioral assessment of visual function is a difficult parameter to be evaluated. Currently, the basis of retinal evaluation for pharmacological and toxicological effects of intravitreally-administered drugs in animals consists of ERG associated with histopathology by light and electron microscopy (44, 45). Toxicity testing can be obtained in rodent as well as non-rodent species for extrapolation to humans for determining risk and safety (46).

4. Therapeutic Monoclonal Antibodies

Monoclonal antibodies (mAbs) can be used therapeutically due to the binding to molecular targets with high specificity. In ophthalmology, therapeutic mAbs have been introduced recently to treat inflammatory and angiogenic diseases. The rationale for mAb application in ophthalmology also is based on a recent understanding of the molecular biology of various ocular diseases (12).

a. Monoclonal Antibody anti-tumor necrosis alpha

Recent evidences have shown that the cytokine TNF-α participates actively in the pathogenesis of inflammatory, edematous, neovascular and neurodegenerative ocular, and extra ocular diseases. In addition, the central pathogenic role of TNF in medicine is supported by the clinical efficacy of TNF-α antagonists such as infliximab in randomized controlled trials for various diseases including rheumatoid arthritis (RA) and Crohn's disease (47). Furthermore, although TNF-α is barely detectable in the serum of healthy humans at levels of 10 fg/ml, in patients with systemic inflammatory or neoplastic diseases, the levels increase markedly to 50 pg/ml (48).

Consecutive studies have described the role of infliximab in the treatment of ocular inflammation. Single or multiple infusions of infliximab at concentrations of 3–10 mg/kg within a 2- to 36-month period have been efficacious in preventing ocular attacks, decreasing relapses, diminishing concomitant corticosteroid use, and controlling disease activity in patients with idiopathic uveitis or uveitis associated with juvenile arthritis, ankylosing spondylitis, Behcet's disease, sarcoidosis, or Crohn's disease (12).

Regarding ocular neovascularization, one patient with Behcet's disease with uveitis and retinal neovascularization treated with systemic infliximab had regression of new vessels after 8 months. A series of patients receiving 5 mg/kg of infliximab infusions for inflammatory arthritis had remarkable regression of CNV due to AMD (49, 50). The preventive and therapeutic effects of infliximab and etanercept have been studied in a rat model of laser-induced CNV as reported previously by other reports and by our research group (6, 51). In the study by Olson et al., both anti-TNF agents given prophylactically decreased the size and leakage of CNV lesions in these animal models, although in one study only etanercept induced reduction of CNV (52). We performed intravitreal injection of escalating doses of infliximab from 10 to 320 μg in rats after laser-induced CNV. At lower doses, infliximab promoted significant reduction of neovascular complex. However, at

higher doses, it induced no effect compared to the control group. These results suggested that either the pro-angiogenic effect of anti-TNF mAb might occur only at higher doses or that in a lower dose some antiangiogenic indirect effect may be seen. Clinical studies have shown a marked elevation in vitreous levels of TNF-α in patients with PDR (53, 54). Experimental studies in a rat RD (retinal detachment) model showed that anti-TNF agents might reduce leukocyte adhesion, blood–retina barrier breakdown, and endothelial injury. The association between TNF-α and pathologic intraocular neovascularization may be explained by direct transmembrane-TNF stimulation of blood vessel growth, or TNF-α-induced expression of isoform VEGF-C, which may protect retinal endothelial cells from apoptosis (55).

b. Monoclonal Antibody anti-platelet derived growth factor

Vascular endothelial cells release PDGF-B, which in turn induce recruitment, proliferation, and survival of pericytes, glial cells, and RPE cells (56). Newly established pericytes along with retinal cells provide survival signals for endothelial cells, and more importantly, pericytes may promote the scarring process following CNV (57). Mural cell recruitment to the growing endothelial tube is regulated by PDGF-B signaling; interference with this pathway causes disruption of endothelial cell–mural cell interactions and loss of mural cells. Therefore, antagonists of PDGFs with or without VEGF antagonists may reduce scarring and neovascularization. Moreover, inhibition of both VEGF-A and PDGF-B signaling may be more effective than blocking VEGF-A alone in causing vessel regression in multiple models of neovascular growth (58-60). A clinical trial phase 1 is evaluating the safety of a monoclonal antibody anti PDGF injected intravitreously for the treatment of neovascular AMD (E10030- Ophthotech Corporation, clinical trial NCT00569140) (61).

c. Monoclonal Antibody anti-integrin α5β1

Components of the ECM play an important role in angiogenesis and CNV formation by helping to facilitate endothelial cell migration. Integrins are heterodimeric transmembrane proteins, composed of alpha and beta subunits, which interact with the ECM. Both αvβ3 and α5β1 integrins have been shown to play a role in angiogenesis and their expression is upregulated in activated vascular endothelial cells (62). Inhibition of α5β1 integrin may be of particular interest for the treatment of neovascular AMD because of its expression in RPE, macrophages, and fibroblasts in addition to endothelial cells. Wang et al. demonstrated that an integrin α5β1 inhibitor (ATN-161) was able to inhibit CNV leakage and neovascularization in a laser induced CNV model (63).

d. Monoclonal Antibody anti-basic fibroblast growth factor (b-FGF)

FGFs are a family of heparin-binding growth factors involved in wound healing and embryonic development. The basic-FGF form, also referred to as b-FGF, may be a more potent angiogenic factor than VEGF or PDGF (64). In the eye, FGF is localized within the lacrimal gland, retina, lens, photoreceptors, aqueous humor, vitreous, and corneal epithelium. In both retina and RPE cells, FGF induces changes in cellular proliferation and *in vivo* angiogenesis. Most uveal melanoma cell lines express FGF subtypes including b-FGF to various extents, and increased FGF expression along with other growth factors was reported in an animal model of retinal detachment (65, 66).

An anti-FGF mAb (no registered brand name to date, BioWa, Princeton, NJ, USA) was developed recently for future application on the treatment of various cancers. Although no study has reported if that anti- FGF agent is useful in ocular pharmacology, some potential indications for the application of anti-FGF mAb based on FGF function can be proposed as adjuvant chemotherapy for ocular melanoma, in conjunction with other mAbs such as anti-TNF to treat PVR associated with rhegmatogenous retinal detachment, and to reduce the chance of PCO after cataract surgery (12, 67). More investigation should unravel the usefulness of anti-FGF mAbs in PCO or PVR, because so far the absence of a cause–effect relationship has not been settled. In addition, other mediators may play a more important role than FGF in these entities.

5. Angiostatic compounds

a. Heparin mimetics

Choroidal neovascularization is a complex process controlled by numerous angiogenic agents such as growth factors, cytokines and ECM components, including glycosaminoglycans (GAGs) (68, 69). GAGs can interact with a diverse range of proteins leading to various biological activities, including angiogenesis (69). Among the sulfated GAGs, heparin and heparan sulfate (HS) have been involved in the modulation of the neovascularization that takes place in different physiological and pathological conditions (70-73). This modulation occurs through the interaction of GAGs with angiogenic growth factors, such as VEGF, FGF, TGF-β, IFN-γ and TNF-α. This property of GAGs to bind and modulate angiogenic growth factors provides a strong reason for studying and designing new synthetic GAG analogs, or discovering GAG-like natural compounds, endowed with angiostatic properties. Sulfated oligosaccharides, which are structural mimics of HS or heparin, are potential drug candidates because these compounds may interfere with the role HS plays in the process of angiogenesis. Heparin is known for its anticoagulant activity, but it also has a strong anti-inflammatory effect also (74, 75).

Recently, we have shown that a heparinoid isolated from marine shrimp presenting negligible anticoagulant and hemorrhagic activities was able to reduce over 60% the neovascularization areas in the laser induced CNV after intravitreal injection. Also this compound is capable of reducing acute inflammatory processes in an animal model (76).

Studies using intravitreal injection of PI88 (phosphomannopentaose sulfate) showed that this compound is capable to reduce the neovascularization area in laser induced experimental CNV in 50% (77). Intravitreal injection of heparin also can reduce the size of the CNV, but the hemorrhagic complications are imminent (33, 78).

The pharmacological and biochemical properties of the heparinoids point to these compounds as compelling drug candidates for treating neovascular AMD.

b. Blockage of complement cascade

Immunological factors are involved not only in the pathogenesis of AMD, but also in its treatment of this disease. Genetic polymorphisms in different complement proteins can increase the risk for developing AMD (e.g., lack of factor H in patients with Y402H

mutations) (79). There are three pathways of complement activation and all of them activate a final common pathway (C3). Lipofucsin and basal lipid deposits between Bruch's membrane and the retinal pigment epithelium (RPE) cell layer may act as a stimulus for the local activation of the complement system. This may lead to a further growth of the deposits due to the strong chemotactic activity of complement activation products with an influx of inflammatory cells (80). Furthermore these activated RPE cells release angiogenic stimuli leading to choroidal neovascularization (81).

Several agents that modulate different parts of the complement system are in clinical trials. In general, these agents work either by replacing a defective complement component as factor H, that is the central soluble activation inhibitor of the alternative complement pathway, or by blocking the complement pathway C3, the POT-4 (79, 82).

c. Kinase inhibitors

Another approach to inhibit angiogenic growth factors as VEGF is through inhibition of the downstream signaling pathways targeting the tyrosine kinases. Several inhibitors were tested and a case in point is the intravitreally administered Vatalanib, a VEGF receptor inhibitor that binds to the intracellular kinase domain (83). Other kinases inhibitors currently in development include pazopanib, sorafenib, motesanib, TG100801, as well as AG013736 (84-86).

Sorafenib is an orally active multikinase inhibitor that inhibits the serine/threonine kinases activity of the VEGF receptor. The CNV area in sorafenib-treated rats was significantly reduced in a dose-dependent manner (85). Sorafenib is in phase III trials for renal-cell carcinoma patients.

6. Small interference RNA (siRNA)

RNA interference is a technology that allows the silencing of genes in animals using therapeutic double-stranded RNA molecules. siRNA molecules induce gene silencing by binding to complementary target RNA molecules in association with the nucleolytic cytoplasmic protein complex known as the RNA-induced silencing complex (87). Nowadays, siRNA is being designed to reduce the production of angiogenic molecules providing potent therapies for ocular neovascularization in patients with AMD. siRNA can be injected into the vitreous cavity or at the subretinal space to treat choroidal neovascularization. This delivery produces local silencing of a gene with small chance for a systemic effect on the same gene (88, 89).

The targeted genes for CNV treatment are mostly VEGF and VEGF receptors (90-92). The silencing of hypoxia inducible factor-1alpha (HIF-1alpha), that regulates the VEGF expression in hypoxic conditions of ocular angiogenesis is also under investigation to treat CNV (93). siRNA targeting the TGF-β, involved in fibrotic scars, seems to be another great potential to treat AMD (94). Furthermore genes associated to photoreceptors degeneration (apoptosis mediators) *c-Jun*, and *Bax* are being tested for futures therapies (95).

A phase I study to investigate the safety, tolerability, pharmacokinetics of a single intravitreous injection of Sirna-027 (siRNA-mediated VEGF silencing) in 26 patients with choroidal neovascularization was completed, and stabilization or improvement in visual acuity and foveal thickness was observed (90).

7. Gene therapy

Gene-based therapy is defined as the introduction, using a vector, of nucleic acids into cells with the objective of changing gene expression to prevent or reverse a pathological process (96). Pro- and antiangiogenic factors regulate the pathogenesis of the ocular neovascularization. Gene transfer to increase expression of endogenous antiangiogenic proteins has the potential to provide long-term stability in patients with AMD (97). There are two routes of administration of viral vectors: intravitreous injection and subretinal injection. The main vectors used for gene transfer are adenovirus, adeno-associated virus (AAV) and lentivirus (96).

Genes encoding antiangiogenic proteins are genetically inserted in viral vectors. The viral vectors infect animal cells and the overexpression of the antiangiogenic protein can be detected. Pigment epithelium-derived factor (PEDF) is a serine proteinase inhibitor from cultured retinal pigmented epithelial cells, which posses a combination of neurotrophic, antitumoral and antiangiogenic activities. Intravitreous or subretinal injection of adenoviral vector expressing human PEDF suppressed the development of retinal neovascularization (98). In the rat CNV model, the gene transfer of PEDF using ultrasound-mediated microbubbles was able to inhibit effectively the CNV (99).

The secreted extracellular domain of VEGF receptor-1, sFlt-1, a soluble form of the Flt-1 VEGF receptor has been used effectively in recombinant adenovirus (Ad)- and recombinant adeno-associated virus (AAV)-mediated antiangiogenic gene therapy to inhibit angiogenesis in CNV animal models (100, 101). The expression of sFlt-1 was associated with the long-term regression of neovascular vessels in mice and monkey (102).

Endostatin is C-terminal fragment derived from collagen XVIII that inhibits tumor angiogenesis (103). Systemic injection of adenoviral vectors containing a sequence coding for murine endostatin, and the mice injected had the serum levels of endostatin raised up to 10-fold and had nearly complete prevention of CNV (104). Subconjunctival injection of recombinant adeno-associated viral vector expressing human angiostatin reduced alkali burn-induced corneal angiogenesis (105).

Intravitreal adenovirus-mediated gene transfer of 15-Lipoxygenase-1, an oxidizing enzyme producing reactive lipid hydroperoxides, efficiently inhibited VEGF induced neovascularization and pathological changes in rabbit eyes (106).

8. Conclusions

The treatment of AMD up to 2000 was limited to vessel destructive procedures that did not improve the visual acuity. The development and testing of therapeutic agents that prevent or delay the progression of AMD is urgently needed, from the standpoint of patient care and quality of life, as well as cost savings. The development of new therapies targeting the angiogenic components of CNV could have a significant impact on the health and quality of life of AMD patients. Moreover combination therapy will possibly replace monotherapy as the treatment of choice in order to reduce the frequency of treatment and prevent the late-stage complications of neovascular AMD.

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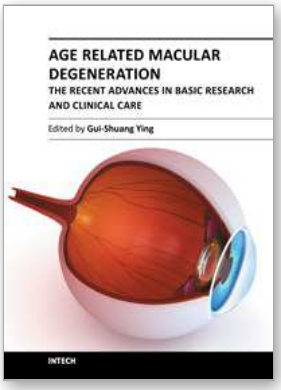

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Age-related Macular Degeneration (AMD) is the leading cause of vision loss and blindness in the developed countries. In the past decade, great progress has been made in understanding the pathobiology and genetics of this blinding disease, as well as in finding new therapies for its treatment. These include the discovery of several genes that are associated with the risk of AMD, new anti-VEGF treatments for wet AMD and new imaging techniques to diagnose and monitor the AMD. All chapters in this book were contributed by outstanding research scientists and clinicians in the area of AMD. I hope this timely book will provide the basic scientists and clinicians with an opportunity to learn about the recent advances in the field of AMD.

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