Aetiology of Late Age-Related Macular Disease

7

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Core Messages

- Choroidal neovascularization (CNV) grows in response to induced growth factors, including vascular endothelial growth factor (VEGF)
- Reasons for growth factor expression are not well elucidated at present
- Examination of identified causes of growth factor release with known physiologic information of the aging eye has led to several theories, some of which are more likely than others
- Oxidative damage can explain many aspects of late age-related macula disease
- A sequence of specific steps involved in formation of late age-related maculopathy can be constructed by integrating present knowledge
- The development of treatment and prevention strategies depends on knowledge of disease pathogenesis; understanding the pathogenesis is a basic step in creating a cure

7.1 Introduction

Late age-related macular disease is the largest cause of visual loss among older adults in industrialized countries. This disease entity comprises two main components involved in age-related macular degeneration. Patients may develop choroidal neovascularization, which is a growth of vessels, proliferation of a number of cell types including the retinal pigment epithelial cells, along with recruitment of inflammatory cells such as neutrophils and macrophages. The concept of choroidal neovascularization by its very name highlights the vascular aspects of the process, guided by the chief method of diagnosis, angiography, and the accompanying signs such as leakage and bleeding. However, the temporal and spatial sequence of cytokine expression, endothelial and inflammatory infiltration, endothelial cell proliferation, maturation, matrix remodelling, and apoptosis is quite similar to a wound healing response. The non-neovascular change that leads to significant loss of visual acuity is the development of geographic atrophy. Regions of retinal pigment epithelial cell death occur with atrophy of the overlying retina and underlying retinal pigment epithelium. The shared epidemiologic risk factors, the common occurrence of one of these disorders in one eye with the other being present in the fellow eye, and the common occurrence of both forms of AMD in one eye suggests they share some common aetiobiologic phenomena. While control of some aspects of the neovascular forms of AMD appears to be an attainable goal, the increasing prevalence and lack of any known treatment makes geographic atrophy an increasingly important public health problem.

[1] The author has no financial interest in this chapter.

7.2 Epidemiologic Factors

The most significant risk factor for AMD is age, but additional important risk factors have been identified. A positive family history [89, 111, 200], cigarette smoking [89, 226, 203], and hypertension [1, 133, 226] are risk factors that have been fairly consistently found as risk factors for the development of exudative AMD. Additional risk factors found with varying degrees of consistency among studies [114] include increased C-reactive protein [188], increased white blood cell count [113], increased intake of vegetable fat, mono- and polyunsaturated fatty acids, increased intake of linoleic acid [33, 186], increased intake of fat [187], increased intake of baked goods [187], female gender [112, 201, 203], hyperopia [1, 12], and blue iris colour [89, 226]. Black race [35, 68], increased intake of docosahexaenoic acid (curiously the most polyunsaturated fatty acid) [33], higher intake of fish [186, 187, 202], nuts [187], and dark green leafy vegetables [185], and higher levels of serum carotenoids [226] have been associated with a lower risk. The Eye Disease Case Control Study only had a handful of women using oestrogen replacement, but these patients seemed to have a lower risk for neovascularization compared to women not using oestrogen [226].

7.3 Genetic Factors

There is a higher risk for the development of late age-related maculopathy in people with a positive family history [89, 111, 200]. This raises the possibility of finding a gene or genes that may be linked to macular degeneration. Genetic investigation into age-related macular degeneration is hindered because the disease occurs in older individuals who are unlikely to have parents or grandparents alive for comparative testing. Mutation of the Stargardt disease gene (ABCR) was found by Allikmets and associates [6] to be associated with AMD (in particular the non-neovascular subtype), but this same association was not found by other researchers [40, 237]. The APOE epsilon4 allele has been found to be associated with a decreased risk, and the epsilon2 allele was associated with a slight increase in risk for AMD [110, 204]. This association was not found by others, however [156, 183]. Macular degeneration is a complex disease, in that there are a number of possible genetic, epigenetic, dietary, and environmental factors all interacting to confer a risk for the development of disease in any given individual. Because there are probably a large number of polymorphisms of many different genes that potentially could be related to the development of AMD (in the context of various other genetic, epigenetic and environmental factors), it is likely that there is no single gene defect responsible for more than a minority of cases of AMD. It is also possible that with different genotypes there are different pathophysiologic mechanisms that produce a generic choroidal neovascular response.

7.4 Structurally Induced Changes Associated with Aging

Some cells in the body are capable of ongoing replication, while others like the RPE have very limited ability to divide before reaching replicative senescence [60]. Under most conditions individual RPE cells persist for the life of the individual. Located between the choroid and the retina, the RPE acts in the absorption of light passing

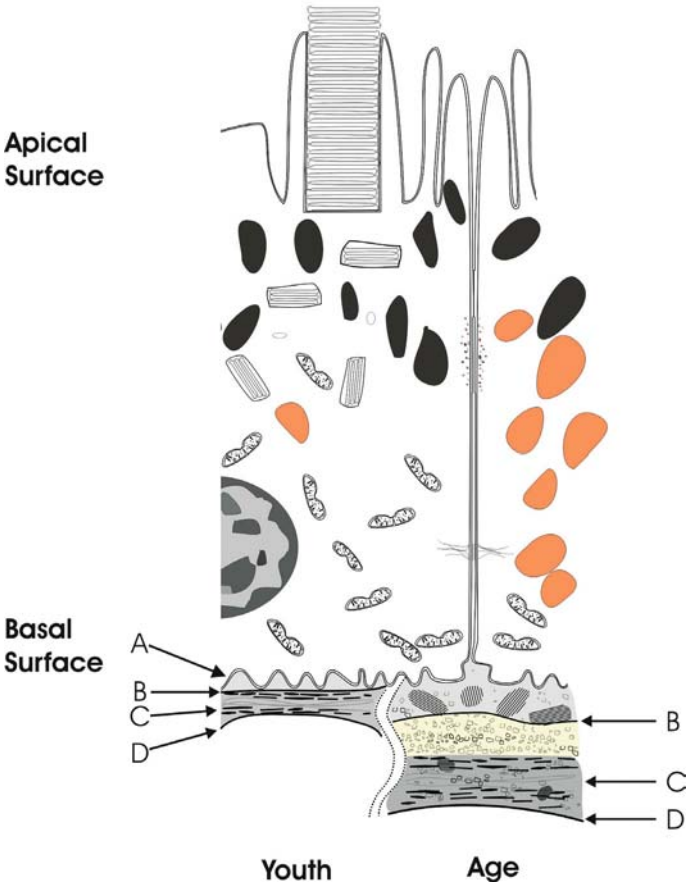

Fig. 7.1. Aging of the RPE and Bruch's membrane. *A* Plasma membrane, *B* basement membrane, *C* trilaminar core of Bruch's membrane, *D* basement membrane of choriocapillaris. Often what is referred to as Bruch's membrane is a five layered structure comprising *G*, *H*, and *I*. Bruch's membrane and associated structures undergo a number of changes with aging (*right*). In between the plasma membrane and the basement membrane an accumulation of material, including wide-spaced collagen, occurs. This material is called basal laminar deposit. External to the basement membrane a material called basal linear deposit accumulates. This material has a high lipid content with membranous debris. Mounds of this material are visible as soft drusen. With age there is also an increased amount of lipofuscin in the RPE cell as well as thickening, calcification, and potential fracture (not shown) of Bruch's membrane

through the retina, regeneration of visual pigments, formation of the outer blood-ocular barrier, upkeep of the subretinal space including fluid and electrolyte balance, phagocytosis of spent outer segment discs [251], maintenance of the choriocapillaris, and scar tissue formation. It is estimated that during a 70-year lifetime each RPE cell will phagocytize 3 billion outer segment discs [134]. Most of the discs appear to be degraded quickly in lysosomes of young healthy individuals. However, with time incompletely degraded membrane material builds up in the form of lipofuscin within secondary lysosomes or residual bodies [25, 165]. Lipofuscin is a diverse group of molecular species [49], yellow to brown in colour and autofluorescent, that accumulates in all postmitotic cells, especially in the RPE (Fig. 7.1) [50, 243]. The presence of lipofuscin may act as a cellular aging indicator [24, 54, 241], and its quantity in tissues may be estimated by the amounts of autofluorescence present [41, 234]. The topographical distribution of autofluorescence as an indicator of lipofuscin content shows that the macular region has much more lipofuscin than the periphery [78]. Light irradiation of RPE cultures accelerates the formation of lipofuscin-like fluorophores, with a colour and fluorescence similar to the lipofuscin found in older cells. The formation of this pigment is nearly eliminated in oxygen-free conditions [126]. The for-

mation of lipofuscin increases with vitamin E deficiency and is reduced by vitamin A deficiency [248].

The structure of one component of lipofuscin, *N*-retinylidene-*N*-retinylethanolamine (A_2E), has been characterized and appears to be formed from vitamin A and ethanolamine in a ratio of 2:1. Precursors to A2E are formed in the outer segments prior to phagocytosis [55, 130]. There appears to be numerous other components of lipofuscin and many of these appear to be derived from free radical induced oxidation of macromolecules, particularly proteins and lipids, with subsequent molecular rearrangement and cross-linking to themselves or other macromolecules [248]. The RPE is unusual in the amount of retinoids and polyunsaturated fatty acids that each cell must process through life. The indigestible portions of what is phagocytized daily contribute to the formation of lipofuscin. In older individuals up to 25 % of the volume of RPE cells may be occupied by lipofuscin. Room for normal cellular machinery is consequently limited. However, lipofuscin is not an inert filler material. Components of lipofuscin inhibit lysosomal protein degradation [48], are photoreactive [59, 240], producing a variety of reactive oxygen species (ROS) and other radicals [59], have detergent properties, and lipofuscin may induce apoptosis of the RPE [220]. Blue light damage to RPE cells is proportional to the amount of light given and the amount of lipofuscin within the RPE cells [191, 210]. There is an age-related loss in RPE cells, particularly in the fovea and mid-periphery [155]. With time the RPE cells decline in function and number, forcing ever hindered, lipofuscin engorged, cells to provide metabolic maintenance for the retina.

Deposition of material under the basal surface of the RPE contributes to Bruch's membrane thickening with age. Basal laminar deposit accumulates between the RPE cell plasma membrane and its basement membrane [67, 69]. Basal laminar deposit is a complex composite that contains granular electron-dense material, coated membrane bodies, and wide-spaced or long-spaced fibrous collagen [93, 229, 230]. Although the characteristic material in basal laminar deposit is collagen, transgenic mice with APO*E3 formed basal laminar deposits when fed a diet high in fat and cholesterol [100]. Basal linear deposit accumulates external to the basement membrane of the RPE and comprises vesicles and membranous debris. Accumulation of basal linear deposit is the most frequent histopathologic correlate of soft drusen [66, 181]. Contributing to the age-related thickening of Bruch's membrane is an increase in collagen, particularly in the outer collagenous layer [82, 229]. In a histologic study of 95 specimens of normal human maculae aged 6–100 years, Bruch's membrane thickness increased by 135 %, from 2.0 to 4.7 µm over the 10 decades examined [167]. In a study by Spraul et al., Bruch's membrane in eyes with exudative AMD showed a greater degree of mineralization and more fragmentation than did age-matched controls [212].

Analysis of Bruch's membrane specimens has shown an exponential increase in the amount of lipid present with age of the donor [81] (Fig. 7.2). There is also a decrease in the hydraulic conductivity [145, 213], occurring somewhat earlier in age than the inflection point for the rise in extractable lipid from Bruch's membrane. Using eximer laser ablation, Starita and co-workers found the region accountable for the decreased hydraulic conductivity appeared to be located in the inner portion of Bruch's membrane, the same location of maximal lipid accumulation [214]. The amounts of lipid, as well as the predominant decrease in hydraulic conductivity,

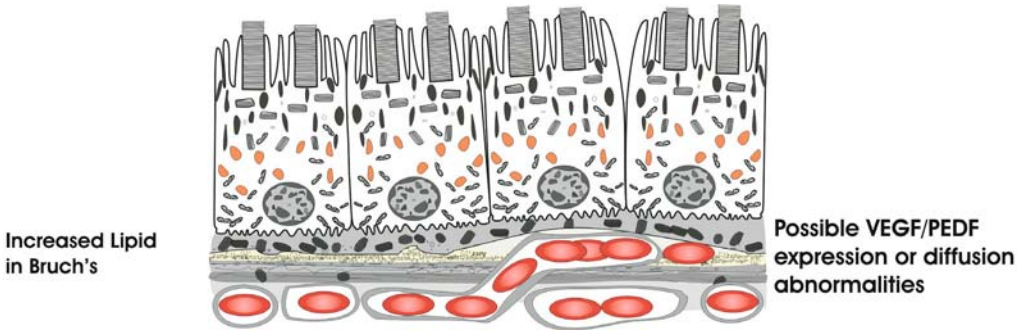

Fig. 7.2. Accumulation of lipids in Bruch's membrane as a potential cause of CNV in AMD. Although the diffusion of growth factors secondary to lipid accumulation in Bruch's membrane has been proposed as a potential cause of CNV, the exact mechanism by which this is supposed to occur has not been defined

occurs more in Bruch's membrane specimens from the posterior pole as compared to the periphery [81]. Although Bruch's membrane has been found to contain neutral fats [192], the predominant class of lipids identified by one group of workers was phospholipids [81]. Pauleikhoff and associates found that a high content of neutral fat was associated with a lack of fluorescein staining and fibronectin [157]. On the other hand, a high proportion of phospholipid was associated with strong fluorescein binding and the presence of fibronectin. Pauleikhoff and associates thought the composition of the lipids found was consistent with a cellular and not a blood origin [157]. Pauleikhoff and associates [159] also found an age-related decrease in adhesion molecules, laminin and fibronectin that appeared to be inversely correlated with the lipid content of Bruch's membrane. The decrease in hydraulic conductivity may lead to the formation of serous RPE detachments, as the RPE cells pump fluid out toward the choroid, against a Bruch's membrane made more hydrophobic by the accumulation of lipid. Curcio and associates [157] found that the predominant lipid deposited in Bruch's membrane was esterfied cholesterol, similar to deposition in other membranes throughout the body that occurs with age. They believed the high proportion of cholesterol esters indicated a blood rather than a cellular origin for the lipid. Ultrastructural examination revealed the cholesterol accumulated within 80-nm particles densely packed within a thin layer external to the basement membrane of the RPE [116]. The particle size appeared to be larger than the pore size of the basement membrane, suggesting that the particles probably either did not pass as such from the RPE toward the choriocapillaris, or that they were inhibited from passing from the choriocapillaris to the RPE.

Histologic evaluation of choriocapillaris in aging eyes by Ramrattan and associates [167] has shown that there appears to be an age-related decrease in the lumenal diameter and vascular density. However, in a study by Spraul and associates, eyes with AMD showed fewer large choroidal vessels in the submacular choroid, but a higher density of the submacular choriocapillaris than controls without AMD [212]. The RPE seems to play a role in maintaining the vitality of the choriocapillaris [116]; perhaps with senescence of the RPE there is a corresponding degradation of the choriocapillaris.

Summary for the Clinician

- RPE cells process photoreceptor outer segments, and retain waste material
- Build-up of waste has the potential to cause harm
- Bruch's membrane thickens with age and accumulates lipid, especially cholesterol
- Vascular alterations with age are not consistent in histologic reports
- Predictable alterations occur with aging that may set the stage for pathologic consequences

7.5 Pigment Epithelium-Derived Factor (PEDF)

The RPE constitutively expresses VEGF, and also produces another factor, pigment epithelium-derived factor (PEDF) [39], that has neutrophic, neuroprotective [90], and antiangiogenic effects [14]. Hypoxia is a well known mechanism that results in increased VEGF expression. Retinal hypoxia can decrease the expression of PEDF by Muller cells [47]. Intraocular injection of PEDF directly or viral vector increasing local production of PEDF results in inhibition of ocular neovascularization [61, 144]. It has been proposed by several authors that the amounts or relative proportion of the expression of these VEGF and PEDF may allow neovascularization to occur [80, 153, 209].

Experimental evidence to date does not support the contention that the ratio of PEDF to VEGF is the permissive event in the generation of ocular neovascularization. While some studies have shown decreased levels of PEDF in ocular tissues during various types of neovascularization [80, 170], most studies have shown a simultaneous increase in VEGF and PEDF during active neovascularization [44, 135, 136, 137, 152]. In addition, in all studies the concentration of PEDF measured appeared to be at least an order of magnitude higher than that required to inhibit neovascularization. There may be possible explanations for these observations. First VEGF may upregulate secretion of PEDF in an autocrine manner [154]. Many of the antiangiogenic effects of PEDF were determined using FGF2 (fibroblast growth factor 2) as a growth factor. In a study using VEGF, the growth factor for endothelial cells, PEDF, seemed to have a synergistic effect on endothelial proliferation [88]. While VEGF is necessary and sufficient for angiogenesis, other factors such as FGF2 are also commonly present. This suggests that if this effect is true the control of angiogenesis is more complicated than the simple ratio of two different cytokines.

Summary for the Clinician

- PEDF is a neutrophic, neuroprotective, and antiangiogenic substance made by RPE
- However, PEDF increases during active neovascularization in many studies
- Unclear interaction effects are present among angiogenic and anti-angiogenic cytokines
- Control of angiogenesis is more complicated than simple ratios of PEDF to VEGF

7.6 Does the Simple Accumulation of Lipid Explain Why CNV Occurs?

A possible cause of CNV may be gleaned from the histopathologic observation of the deposition of basal laminar and basal linear deposit. The most common histopathologic correlate to soft drusen is the accumulation of membranous debris in

basal linear deposit [67, 66, 181]. Soft drusen are an ocular risk factor for the development of CNV in AMD. There are several main ways that the presence of deposited material may play a role in the development of CNV. It is possible that the presence of deposits, particularly lipids, may affect the ability of growth factors produced by the RPE to diffuse through Bruch's membrane. In particular it is possible that the diffusion of factors could either selectively partition into the lipid layer or be blocked from passing through the lipid rich area. The two possibly involved factors would be VEGF, which stimulates the growth of vessels, and PEDF, which inhibits neovascularization. Examination of the histopathology of CNV in AMD and the topography of VEGF found in the eye would seem to argue against either of these two possibilities. CNV generally grows up to and into the inner portion of Bruch's membrane [107]. If a mediator inhibiting neovascularization was selectively concentrated in this area, one would not expect the newly growing vessels to actively grow to, then into, the same layer. Histopathologic examination of CNV in AMD shows that while the new vessels grow under the basal laminar deposit, basal linear deposit is not commonly found in most specimens. This may imply that the basal linear deposit was never present, it was lost in processing, or that the CNV was growing into the layer previously occupied by the basal linear deposit and was replacing or removing the deposit. Since the clinical correlate of mounds of basal linear deposit is soft drusen, and since soft drusen are known ocular risk factors for CNV, the later interpretation seems more likely. Neovascularization may penetrate through the RPE or may start as vessels growing outward from the inner retina toward the subretinal space. In either of these two situations the newly growing vessels seem to seek to proliferate in the outer retina as a separate plane to the aforementioned vessels that grow in the region occupied by the basal linear deposit.

Summary for the Clinician

- **Diffusion of VEGF and PEDF may be altered by lipid accumulation in Bruch's membrane**
- **CNV grows to and into the inner portion of Bruch's membrane**
- **CNV can break through into the subretinal space**
- **Diffusional hindrance of inhibitors or promoters of angiogenesis by lipid in Bruch's membrane does not explain the growth characteristics of CNV**

7.7 Ischaemia and Angiogenesis

Age-related decrease in delivery or diffusion of oxygen or metabolites to the macular region may occur, and has been theorized as the key event in the initiation of compensatory mechanisms that ultimately leads to the formation of new vessels in AMD. Neovascularization is an important cause of blindness in a number of ocular diseases such as diabetic retinopathy, and neovascular glaucoma, vein occlusions, and in each case neovascularization have been linked to ischaemia. By logical extension, CNV has been theorized to be caused by ischaemia.

Blood vessels grow in adult tissue by expansion of the vascular tree through angiogenesis, a process where new vessels sprout from pre-existing vessels. The actual ischaemic event is signalled by an increase in adenosine [75, 195, 221], which may bind to one of at least four receptors. This binding leads to increased vascular endothelial growth factor (VEGF) in an action mediated by hypoxia-inducible factor-1 (HIF-1), a transcription factor that binds to one or

more areas in the hypoxia response element [138, 140, 179, 235, 249]. There are several hypoxia-inducible genes including those for erythropoietin, VEGF, inducible nitric oxide synthase, glycolytic enzymes, and glucose transport proteins. The most important of these for vessel growth is VEGF [3, 5, 12, 18, 20, 43, 46, 120, 131, 132, 162, 171, 190, 194, 252]. There are many different isoforms of VEGF caused by differential RNA splicing. Although VEGF is sufficient for new vessel growth, a variety of other growth factors are commonly found in association [56].

At the initiation of angiogenesis, gaps begin to form between endothelial cells of the capillary wall, and the endothelial cells themselves first develop areas of fenestrations [46, 171]. These changes start within minutes after exposing vessels to VEGF. The capillary becomes more permeable, allowing plasma proteins, particularly fibrinogen, to extravasate [36]. Clotting of the fibrinogen leads to the creation of fibrin, which forms a provisional matrix to support the newly growing vessel. The endothelial cell forms a bud, with the advancing edge expressing integrins. With the aid of matrix metalloproteinases the endothelial cells degrade the extracellular matrix. The advancing cells move away from the pre-existing vessel toward the angiogenic stimulus. The endothelial cells in the vascular sprout proliferate, and a lumen forms. Anastomotic connections between neighbouring sprouts form a capillary loop. At this stage the cells form a thin-walled pericyte-poor capillary that eventually starts to produce new basement membrane. Production of vessels starts with the secretion of VEGF, but a large number of different cytokines play a role in the development of a blood vessel. Withdrawal of VEGF, or blocking VEGF of the receptor, causes suppression of vascular growth and regression [103, 115] at this stage.

Hypoxia in retinal cell cultures induces VEGF [4]. Animal models of neovascularization show increased VEGF levels from induced hypoxia and these increased levels were spatially and quantitatively correlated with the resultant neovascularization [4, 43, 139]. Inhibition of VEGF caused suppression of ocular neovascularization in an animal model [5]. Many tested patients with ischaemic retinal diseases leading to neovascularization had increased levels of VEGF in their vitreous and these levels declined after successful laser photocoagulation [3]. Autopsy specimens confirmed the presence of VEGF in diabetic eyes [132]. Choroidal neovascular membranes that were surgically removed showed immunohistochemical evidence of VEGF [131]. Experimental choroidal neovascularization induced by laser photocoagulation also shows VEGF expression [124]. Injection of an adenoviral vector encoding VEGF into the subretinal space has caused experimental CNV in rats [13, 211]. One study showed the indocyanine green angiographic grading of CNV activity was correlated with the amount of immunohistochemical staining for VEGF in excised specimens [20]. Injection of an anti-VEGF aptamer and of an anti-VEGF antibody fragment caused angiographic regression of choroidal neovascularization [15, 53]. The mean visual acuity still declined in a randomized trial looking at the effects of the anti-VEGF aptamer, suggesting that antiangiogenic treatment may not be a sufficient treatment for choroidal neovascularization.

Summary for the Clinician

- **Angiogenesis is induced by hypoxia (and other stimuli)**
- **Coordinated cascade of events ultimately causes VEGF secretion**
- **VEGF is spatially and temporally correlated with induced angiogenesis**

- **Blocking VEGF causes regression of neovascularization**
- **Steps involved in ischaemia induced angiogenesis are well defined**

7.8 Ischaemia and CNV

Because of the weight of the basic and clinical science linking ischaemia to VEGF production, and in turn VEGF production to neovascularization, it may be very logical to presume the same factors may play a role in the development of CNV. Indeed there are many clues suggesting decreased blood flow occurs in the aging choroid, especially in patients with AMD (Fig. 7.3). Laser Doppler studies have shown that patients with AMD, defined as having ten or more large drusen, had decreased blood flow, but no change in velocity when compared with age-matched controls without ten or more large drusen [70]. In contradistinction, Mori and co-workers, using a Langham ocular blood flow computerized tonometer, found no decrease in ocular blood flow in patients with non-exudative AMD, but did find a statistically significant decrease in pulse amplitude and pulsatile ocular blood flow in patients with exudative AMD as compared with age-matched controls [143]. Tonographic methods of ocular blood flow measurement are based on assumptions about the relationship between intraocular volume and resultant intraocular pressure, from which ocular blood flow is estimated [117]. Comparisons between individuals also include assumptions that factors that may alter the pressure/volume relationship such as scleral rigidity and axial length do not vary among individuals. Yang and associates found the interindividual variation of peak ocular blood flow determined by the ocular blood flow tonograph was so large that valid comparisons between individuals may not be possible [247].

Patients with age-related macular degeneration have been found to be more likely to have choroidal watershed filling defects during fluorescein [29] and indocyanine green angiography [158, 172] than controls, although the controls were not matched on important factors such as hypertension [172]. Besides the alterations in

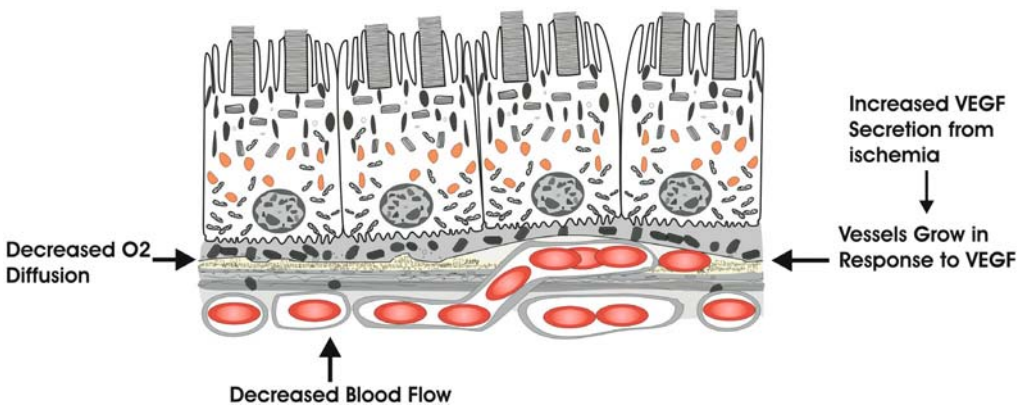

Fig. 7.3. Ischaemia as a potential cause of CNV in AMD. Decreased diffusion of O_2 due to increased thickness and altered composition of Bruch's membrane has been proposed to lead to increased VEGF production. This would then lead to angiogenesis and neovascularization arising from the choriocapillaris

blood flow, it has been theorized that age-related changes in Bruch's membrane may also limit the diffusion of oxygen and therefore create an ischaemic environment. The RPE cells lying on top of drusen were thought of as being particularly ischaemic [158], which would lead to VEGF secretion and formation of CNV [158].

There are some aspects of the physiology of the outer retina and RPE in which the histologic appearance and growth pattern of CNV do not appear to support the ischaemic theory. In a prospective study of choroidal filling defects, patients were more likely to develop geographic atrophy, not CNV [163]. A large histopathologic study found that the luminal cross-sectional area and choriocapillaris density is higher in AMD patients than in non-AMD controls [212]. The blood flow through the choroid is the highest, and the oxygen extraction from haemoglobin is one of the lowest of any tissue in the body. Less than 1 volume percent of the oxygen in the blood is extracted from the choroidal blood flow. Consequently the resultant pO_2, at the level of the choriocapillaris, is maintained at a level higher than any other perfused tissue. The oxygen diffusion through the RPE and retina has been measured in several species [2, 26, 77, 128, 129, 245, 246], and follows a consistent pattern. The pO_2 levels of the RPE are very high because of its close approximation to the choriocapillaris. The pO_2 decreases linearly with distance from the choriocapillaris to the inner portion of the photoreceptors. Under normal physiologic conditions the pO_2 at the inner portion of the photoreceptors approaches 0 mmHg in the dark and is somewhat higher in light. One possible reason for this design may be to lower the oxygen tension in the outer retina to decrease the amount of oxidative damage there, because of the inherent high susceptibility to oxidative damage conferred by the extraordinarily high proportions of both polyunsaturated fatty acids and retinoids in the outer segment membranes. In measurements of the constitutive secretion of VEGF in the eye, the RPE makes a prominent amount of VEGF [108]. On the other hand, the photoreceptors make little VEGF. Under normal circumstances, then, the RPE is exposed to an exceptionally high pO_2, but secretes VEGF. The inner portions of the photoreceptors are exposed to a very low pO_2, but do not produce much VEGF. This paradox cannot be explained by simple ischaemia.

It is possible that lipid deposition in Bruch's membrane may limit the diffusion of oxygen. It has been theorized by some that this induces RPE ischaemia with the subsequent production of VEGF. However, organisms are designed with the strategy, refined through evolution, of O_2 diffusing through lipid membranes. Indeed analysis has shown lipid membranes are not a rate-limiting step in oxygen diffusion [216–219], because the diffusion through lipid membranes approaches that of water [218]. Although the lipids in Bruch's membrane are not necessarily in the form of lipid membranes, there is not much available evidence to support the assertion that the presence of lipids in Bruch's membrane leads to RPE ischaemia. Thickening of Bruch's membrane may cause a decrease in the pO_2 at the level of the RPE because of an increase in distance from the choriocapillaris to the RPE, but the RPE would still have a much higher pO_2 than the photoreceptors. Even so, excessive VEGF production at the level of the photoreceptors as studied in transgenic mice showed that there was a growth of vessels extending from the middle retinal layers to the outer retina, but no development of CNV [232].

In one study RPE cells exposed to 5 % O_2 produced 1.3 times more VEGF than when exposed to normal atmospheric oxygen

levels [12]. In another study human RPE cells exposed to 3 % O_2 increased the secretion of VEGF by a factor of approximately 3 as compared to atmospheric conditions, and the increase was statistically significant [4]. (However, normal tissue levels of oxygen are far below that found in room air.) Bovine RPE cells cultured in the same conditions did not produce a statistically significant increase in VEGF [4]. Studies on O_2 delivery by the choroid have shown that as perfusion decreases, the oxygen extraction from the choriocapillaris increases [128]. Under normal conditions little of the O_2 in the choriocapillaris blood is extracted, so there is a significant reserve. Because of this process the change in oxygen flux at the level of the RPE shows much less change under conditions of decreased perfusion than what ordinarily is expected. Although experimental study has shown that RPE may increase VEGF production to a certain degree when exposed to levels of oxygen lower than room air, the O_2 levels used in experiments may not be physiologically relevant for understanding how CNV develops secondary to AMD.

The growth patterns of CNV suggest there is more involved than just ischaemia driven neovascularization. Excised choroidal neovascular "membranes" show significant participation by cells other than the vascular endothelium including a variety of inflammatory cells such as lymphocytes, macrophages, and foreign body giant cells [160, 161]. The histopathologic picture of CNV in AMD looks similar to granulation tissue or a wound-repair response [212]. In one study the amount of VEGF in CNV was found to be proportional to the number of macrophages in the specimen [123], a finding that is difficult to explain by any ischaemia theory and suggests inflammation is important in CNV secondary to AMD. In animal models of CNV depletion of the monocyte cell lines inhibits experimental choroidal neovascularization [52, 95, 176]. During the development of experimental CNV using a laser model, CD18 and ICAM-1 are expressed; targeted disruption of either of these inhibits the development of CNV [177]. Animal models of CNV have been developed that mimic many aspects of CNV in AMD. These mice monocyte chemoattractant protein-1 or its cognate C-C chemokine receptor-2 developed drusen, lipofuscin accumulation, geographic atrophy, and choroidal neovascularization [7]. Depletion of neutrophils further inhibits the development of CNV [227]. All of these factors strongly suggest integral involvement of inflammatory cells in the development of CNV. Finally ischaemia-based theories do not adequately explain the typical later stages of CNV in AMD – the formation of scarring and regression of vessels. With time the neovascularization appears to "burn out", leaving a cicatricial mass almost completely devoid of vessels. If ischaemia is the only cause for the vessels to grow, then once the CNV does grow the capillaries of the CNV recapitulate the anatomy of choriocapillaris and overlying neurosensory retina. One would not expect these vessels to make an abrupt regression, which would be expected to increase the amount of ischaemia present. However, this growth pattern is analogous to that seen in a wound healing response (Fig. 7.4).

There is a strong link between ischaemia and VEGF mediated angiogenesis. Patients with AMD may have decreased blood flow as compared with those who do not have AMD, but the decrease in blood flow has yet to be firmly linked with a significant amount of ischaemia. In addition, the growth patterns of CNV, the regression of active neovascularization later in the disease process, many of the histopathologic findings, and many findings in animal models are not explainable by ischaemia.

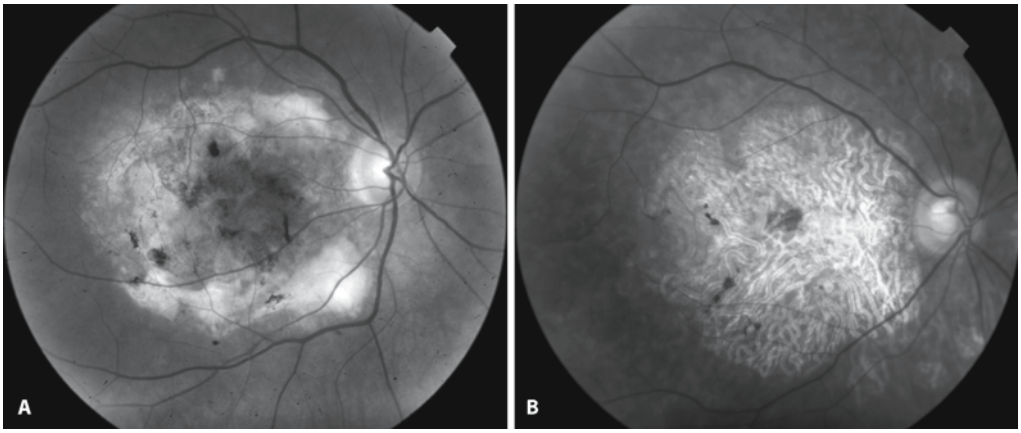

Fig. 7.4 A, B. Endstage CNV. The ischaemia theories proposing the recruitment of additional vessels as the aetiology of CNV in AMD have a very difficult time explaining pictures such as these. In **A** there is a hyperplastic scar, but not much in the way of visible vessels. In **B** spontaneous resolution of the CNV has led to a complete absence of not only the CNV but also the RPE. This process probably came about secondary to massive apoptosis. Although hyperplastic scarring, remodelling, and apoptosis are common events in a wound healing response, they are not expected as an angiogenic response to simple ischaemia. This suggests that there are other factors in addition to, or other than, simple ischaemia

Readily identifiable ischaemia, such as choroidal vascular occlusion seen in toxaemia of pregnancy or malignant hypertension, is not associated with CNV. Ischaemia of the retina is not associated with CNV either. The implication is that ischaemia is not sufficient to explain CNV in AMD and there must be other factors involved.

Summary for the Clinician

- **It seems logical to presume CNV is related to ischaemia from possible choroidal vascular changes with age, thickening of Bruch's membrane; however:**
- **RPE cells are normally exposed to very high pO_2, but constitutively make VEGF**
- **Photoreceptors are exposed to very low pO_2, but do not make much VEGF**
- **Inflammatory cell interaction is very important in the development and progression of CNV**
- **CNV growth patterns and eventual regression suggest a wound healing response**
- **Ischaemic conditions of retina and choroid are not associated with increased risk of CNV**
- **Ischaemia may play a part in the initiation and evolution of CNV, but does not explain many characteristics of CNV in AMD**

7.9 Oxidative Stress

Although light induced free radical oxidation in the photoreceptors has been known for almost 30 years, the full realization of the effects of oxidative damage is still being elucidated. There is tremendous interest in oxidative damage as an integral component in the aetiology of several seemingly diverse diseases ranging from atherosclerosis to Alzheimer's disease to cancer. In the fol-

lowing section successive steps in progression of oxidation will be illustrated along with response on the cellular level and then on larger degrees of scale.

We are a carbon-based life form that burns carbon-based molecules to stay alive. In the process free radicals are produced by intention in a process designed to stay within mitochondria. A free radical is any atom or compound that has an unpaired electron. For quantum mechanical reasons atoms like to have paired electrons. A free radical is not necessarily an ion, which is an atom or compound with an excess charge, be it positive or negative. Ordinarily four electrons (and four associated protons) are required to reduce O_2 to form two molecules of water. In most interactions with organic molecules, oxygen preferentially accepts electrons one at a time for quantum mechanical reasons. Each of these electron additions results in a potentially reactive molecule. The stepwise series of reductions producing metabolites of oxygen occurs as electrons are donated to oxygen in the electron transport chain in the mitochondria.

The addition of one electron to oxygen results in the formation of the superoxide anion, which is represented as O_2^-. The walls of the mitochondria are curiously leaky to oxygen radicals produced during metabolism. Large amounts of superoxide leak from the walls of mitochondria, such that about 1 % of oxygen used in respiration actually leaks from the mitochondria in the form of superoxide. In older subjects the proportion is greater [27, 71]. This potentially exposes the cellular constituents to internally generated oxidative attack. Further reduction of the superoxide (with the addition of two hydrogen ions) produces hydrogen peroxide. Continued reduction leads to the formation of the hydroxyl radical, which is particularly reactive. The final reduction yields water.

When photosensitizers absorb light they are elevated to a higher energy state called a triplet state. This excess energy can be transferred to oxygen, creating singlet oxygen, which is another reactive species. Photosensitizers can be exogenous chemicals or endogenous compounds such as porphyrins or lipofuscin. There are a number of protective enzymes that help in detoxifying reactive oxygen species (ROS as mentioned earlier). In addition to enzymes, various antioxidants may intercept ROS and chemically reduce them into less reactive molecules. The reason the mitochondrial wall is leaky to ROS is not known, especially considering the toxic nature of the ROS. It is possible that the superoxide leaked may act as a chemical messenger. It is also possible that the ROS leaks for some other purpose. It has been shown that there is an inverse correlation between the amount of superoxide leak and the expected life span of an organism across a large number of species. This has raised speculation that lifespan for a given species is intrinsically controlled, in part, by the amount of ROS leakage through mitochondria [74, 118, 119].

There are a number of sources of ROS in any organism besides the oxidative machinery in the mitochondria. The NADPH oxidase system, particularly the p47 phox subunit, produces singlet oxygen and hydrogen peroxide as part of the respiratory burst in macrophages and neutrophils. A similar enzyme has been found in vascular endothelial cells. Superoxide can be produced by xanthine oxidase, nitric oxide synthase [231], as a by-product in the production of prostaglandins, from exposure to light, ionizing radiation, pollution, cigarette smoke, and even ischaemia [22]. ROS are generated as a second messenger for some cytokines and hormones [150, 225]. ROS looks to find a source of electrons and can find them in cells in the form of nucleic acids, proteins, carbohydrates, and lipids,

and this reaction often leads to molecular damage.

ROS attack on proteins directly alters the chemical composition of the protein, may secondarily affect protein configuration, and can also lead to cross-link formation. Breakdown of these altered proteins is more difficult and can inhibit normal proteosome function. Inappropriate oxidation of lipids represents a special case for several reasons. The vulnerability of a fatty acid to oxidative damage is related to the number of double bonds that are present. One double bond increases the susceptibility by a factor of 100 [58]. Each successive double bond increases the possibility in proportion to the total number of double bonds. The predominant polyunsaturated fatty acid (PUFA) found in the cell membrane of the photoreceptor outer segments, docosahexaneoic acid, is the most unsaturated fatty acid in the body in that it has six double bonds. Peroxidized lipids can participate in reactions with other lipids to generate additional lipid peroxides in a process known as propagation reactions. Thus one peroxidized lipid molecule may lead to a progeny of other peroxidized lipids. Each of these peroxidized PUFAs is reactive in their own right in a way analogous to ROS. Oxygen can attack any of the double bonds in a PUFA and thereby create a reactive molecule capable of a large number of permutations of interactions and breakdown products. The end result of lipid peroxidation is the creation of a diverse family of daughter molecules, many of which retain the ability to react with other molecules. In the process, though, they cross-link to the molecules they react with to produce abnormal conjugates.

These interactions may produce a number of untoward effects. For example, bonding to a protein may affect the functional ability of the protein by binding to its active centre, altering its tertiary or quaternary structure, or by changing the hydrophobicity. The ability of a peroxidized lipid to attack a protein molecule is proportional to the number of double bonds in the fatty acid [169]. Lipid peroxides lead to an increase in cell membrane rigidity, and contribute to aging of the membrane [34]. Lipid peroxides may damage cellular organelles and membranes [9, 34, 87]. Oxidatively damaged lipid may bind to more than one protein, creating large, interlinked, molecules. Lipid derived molecules irreversibly altered by oxidative effects are known as advanced lipoxation endproducts or ALEs. Analogous endproducts derived from carbohydrates are known as advanced glycation endproducts or AGEs. Many AGEs may resemble, and be quite similar to, those formed from lipids, the ALEs [57, 109, 168]. Because of the unusual structure caused by oxidative damage and cross-linking, and because these molecules have the potential to damage proteosomes, the cell may have a difficult time breaking these molecules down. The indigestible material, particularly lipid peroxides and their metabolites [105, 106], is compartmentalized as lipofuscin granules [16, 102, 238], and the accumulation of lipofuscin is increased in proportion with greater RPE O_2 exposure [239]. The production of lipofusin in the RPE is compounded because of the high concentration of retinoids, molecules with double bonds that are used to capture energy from light, in the outer segments.

To help protect against inappropriate oxidation, there are basically three levels of protection: molecular, cellular, and over a larger scale, a tissue level. On the molecular level the cell has antioxidant vitamins and enzymes. These include vitamins C and E, superoxide dismutase, catalase, glutathione transferase [196], glutathione reductase, and glutathione peroxidase. The antioxidants may limit inappropriate oxidation in

the first place, or may terminate propagation reactions. Vitamin E, a lipophilic free-radical scavenger, may do both. In homogeneous solutions, β-carotene is a potent free-radical scavenger, in vitro; the in vivo effects of β-carotene are less well defined.

On a cellular level two main responses may occur. The cell may try to adapt to the oxidative stress by increased activation of such transcription factors as nuclear factor κB (NF-κB) [182] and activator protein 1 which help control gene expression of antioxidant enzymes. Oxidative stress itself alters the activity of matrix metalloproteinases and collagenases, possibly playing a role in tissue remodelling induced by oxidative stress [197]. Exposure to ROS also may induce apoptosis, which can be blocked with antioxidants [96]. Interestingly, the process of apoptosis is actually mediated by ROS; the mitochondria undergo a permeability transition and leak ROS into the cell, and the resultant oxidative damage causes cellular suicide [97].

Over larger levels of scale, increasingly sophisticated responses may occur. ROS and peroxidized lipids increase the production of VEGF [121, 142], which is involved in supporting vascular endothelial cells as well as promoting the formation of new vessels. RPE cells show a dose related increase in VEGF mRNA levels when exposed to superoxide, and this response could be blocked with antioxidants [121]. Exposure of cultured RPE cells to repeated doses of near ultraviolet light reduces RPE proliferation, similar to that seen in RPE senescence. These same cells showed increased lipofuscin content, an "age" pigment, and the cells also expressed less PEDF [126]. The scavenger receptor system [86, 199] is responsible for recognizing and binding to oxidatively damaged molecules, including AGEs and ALEs. It is involved in a diverse number of processes particularly in the recognition of old erythrocytes [178]. When erythrocytes age, lipid peroxide products accumulate within the cell membrane, and because of the associated cross-linking, the cell membrane becomes more stiff. Scavenger receptor recognizes these abnormalities and works to remove old erythrocytes from the circulation. A similar process of attempted removal of abnormally oxidized material may lead to atherosclerosis. Oxidation of LDL produces a variety of peroxidized molecules, which are recognized by the scavenger receptor system. Macrophages and smooth muscle cells bind oxidized LDL (oxLDL) as an initiating event in atheroma formation. Oxidatively damaged LDL may form under a number of different conditions such as hypertension and exposure to cigarette smoke, transition metal ions, and pollutants, and its oxidation may be inhibited by antioxidants [250]. Under ordinary circumstances when a cell binds LDL through an LDL receptor, the receptor is downregulated through a negative feedback loop triggered by rising levels of intracellular cholesterol. When macrophages are exposed to oxLDL, they phagocytose the oxLDL through alternate receptors that are part of the scavenger receptor system, including CD-36 [28, 62, 98, 148, 242].

Instead of downregulation of CD-36, phagocytosis of oxLDL causes an upregulation of CD-36 expression through a positive feedback loop [73]. Important effects that occur on binding to CD-36 are the secretion of VEGF, vascular cell adhesion molecule-1 (VCAM-1), and release of monocyte chemoattractant protein-1 (MCP-1) [193]. Other receptors have been characterized, including a receptor for AGE, known as RAGE. Similar to CD-36, RAGE binds its ligand, AGE, which causes an upregulation of more RAGE [223], the expression of a number of pro-inflammatory cytokines, evidence of increased oxidative stress, NF-κB activation [244] and expression of

VEGF. These actions could be inhibited by administering a soluble receptor for RAGE or with antioxidants [19, 189]. This may have importance in ocular diseases [79, 147, 189]. Excised CNV specimens have been found not only to express AGE, but also RAGE [72, 94].

While it may seem counterintuitive that ROS, lipid peroxides, and advanced end-products can stimulate VEGF production, the response does seem to fit into a larger strategy where the body takes aggressive steps to contain, neutralize, and rid itself of oxidatively damaged material. Not only do these molecules increase the secretion of VEGF, but they can cause vascular endothelial cells to form capillary tubes much more efficiently, through a mechanism that apparently does not involve the upregulation or release of angiogenic growth factors [125].

Summary for the Clinician

- **Many mechanisms exist to combat oxidative stress and the damage caused by oxidative injury**
- **Bruch's membrane has no intrinsic means of protection against oxidative damage to contained lipids**
- **Oxidative stress can lead to VEGF secretion**
- **Oxidative stress can lead to senescence and apoptosis**
- **Oxidative stress and damage has the potential to induce many findings seen in late AMD**

7.10 Oxidative Damage and CNV

Oxidized lipids are formed in the photoreceptor outer segments as a normal part of daily life. Scavenger receptors [45, 175], in particular CD-36, are present on the RPE cell and participate in phagocytosis of spent outer segment segments. It is possible that ordinary everyday exposure of the CD-36 receptor to oxidized lipids in the photoreceptor outer segments helps maintain the constitutive secretion of VEGF by RPE cells. Excessive secretion of VEGF by RPE cells, however, may be one factor responsible for the initiation of CNV. This raises the possibility that excessive exposure to oxidative damage may lead the RPE cells to secrete excessive VEGF. Animal models of increased excretion of VEGF by RPE cells can produce CNV [13, 211].

Ueda and associates have previously shown that a 10-µg injection of linoleic hydroperoxide, a lipid peroxide derivative, into a corneal pocket leads to corneal neovascularization from the limbus [228]. In addition, Armstrong and co-workers found injection of 50–600 µg of linoleic hydroperoxide into the vitreous cavity caused retinal neovascularization that persisted for 4 weeks [11]. Following the injection of linoleic hydroperoxide, there was a cascade of cytokines secreted including VEGF. This brings us back to Bruch's membrane, which has an exponential increase in lipids with age, the lipid seems to preferentially accumulate in the same region where the neovascularization grows to, and Bruch's membrane has no known intrinsic mechanism offering protection against oxidative damage for the lipids accumulating there. Perhaps oxidative damage to lipids in Bruch's membrane is important in the aetiology of CNV in AMD.

The eye has a dioptric mechanism to focus light, which can stimulate photo-oxidative reactions, it has a high oxygen flux through Bruch's membrane, and there are a plethora of potentially susceptible lipids in the retina, and perhaps in Bruch's membrane, to enter into oxidative reactions. To evaluate this aspect we looked at Bruch's membranes from autopsy eyes and measured the total amount of peroxidatively

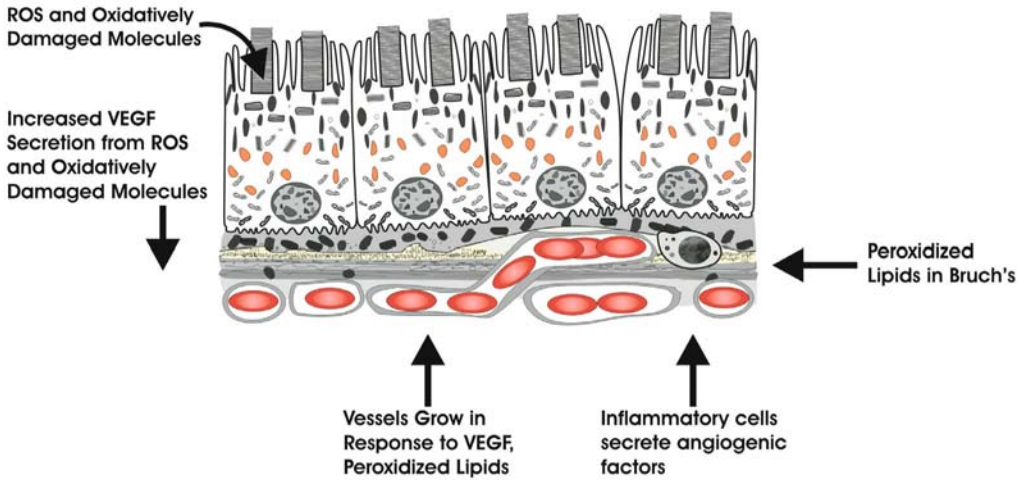

Fig. 7.5. Oxidative damage as a potential cause of CNV. Exposure of RPE cells to ROS (reactive oxygen intermediates) can lead to increased VEGF secretion. It is possible that CD36 mediated binding of outer segment discs does as well. Lipid peroxides, which can stimulate the formation of new vessels, increase in amount in Bruch's membrane with age

damaged molecules with the fluorometric thiobarbituric acid assay and characterized the lipids further with high pressure liquid chromatography [208]. We found the total amount of peroxidized lipids increased exponentially in Bruch's membrane with age. We also found PUFAs occurred in Bruch's membrane, and that peroxidation products of linoleic acid were the most common peroxidized PUFAs present, similar to that seen in atherosclerotic lesions. Peroxidized docosahexaenoic acid was also found, indicating a cellular origin of at least some of the lipids present. In a study being prepared for publication, we found that although lipid peroxides are found in Bruch's membrane specimens from the macular region, the Bruch's membrane specimens from the periphery of the same eyes contain very low levels. In a separate study, subretinal injection of linoleic hydroperoxide caused CNV in rabbits [222] (Fig. 7.5).

Lipid peroxides appear to increase with age in Bruch's membrane, but they do so in other tissues, principally in atherosclerotic lesions in arterial walls. Although on histopathological examination, cholesterol, both free and esterfied, appears to be the predominant lipid present, the overwhelmingly large proportion of peroxidized lipid present is derived from PUFAs such as linoleic acid [164, 215]. In atherosclerotic vessels, the body mounts an aggressive cell-mediated approach to contain the oxidized material [23, 224], principally using vascular endothelial cells and macrophages. The oxidized materials stimulate production of VEGF by these cells [91, 92, 104, 166], in an effort that is thought to maintain the vitality of the vascular endothelial cells [122]. VEGF may inhibit the apoptosis of a number of cell types [101]. VEGF production there leads to neovascularization of the plaque [99, 151], starting early in the formation of the plaque. It is thought the body's ability and tendency to aggressively remove oxidized lipids arises from an evolutionary derived process based on the strategy to remove old or oxidatively damaged cells, using oxidatively damaged lipids

in the plasma membrane as an identification system by the scavenger receptor system [23]. Unfortunately an atherosclerotic plaque represents a mother load of the same damaged lipids – up to 30% of linoleic acid (the principal PUFA of cell membranes) contained in atherosclerotic plaques is in a peroxidized state [215]. The presence of these lipids elicits a series of events, often self-reinforcing, where the body tries to contain or remove the offending material. Perhaps some of the same sequence of events occurs in the eye as well. This is not to say that the stages leading to development of CNV in AMD are identical to that seen in atherosclerosis. However, the body has a number of defined strategies and methods of dealing with degenerating cells and tissue, and many of the same strategies and methods that are used in atherosclerosis of vessel walls are also used in the eye. Perhaps these oxidatively damaged molecules help elicit the invasion of neovascularization in Bruch's membrane as they do in atherosclerotic lesions. Injection of these same lipids has led to ocular neovascularization in the rabbit [11, 208, 228].

There are other oxidative mechanisms that may be operative at the level of the outer retina, RPE cell, or Bruch's membrane other than those involving inappropriate oxidation of lipids. However, if lifelong increase in oxidatively damaged molecules, particularly lipid peroxides, is a principal risk factor for the development of CNV, strategies to prevent CNV need to counter this build-up. One strategy may include a lifelong diet rich in carotenoids [226], which are selectively accumulated in the macula. These molecules function both to absorb blue wavelengths, and also as antioxidants. The Age-Related Eye Disease Study [10] found that supplementation with beta-carotene, vitamins C and E, copper, and zinc in patients at risk was associated with a reduction in neovascularization and visual acuity loss as compared with controls. Antioxidants may indeed act as "antioxidants", by scavenging free radicals and reducing inappropriately oxidized macromolecules. These "antioxidants" also function to alter gene expression [42, 63, 127, 233], alter cell signalling proteins such as protein kinase C [65], alter the valence of metal ions in the active centre of enzymes [42], induce apoptosis in certain cell lines [146], cause maturation of other cell lines, reduce or induce expression of a variety of antioxidant enzymes [64], and bind to structural proteins [17], so there may be other mechanisms to consider.

There are two established ocular findings that are risk factors for the development of CNV: focal hyperpigmentation and soft drusen. Recently a study of fundus autofluorescence derived from lipofuscin has shown that fellow eyes of patients with CNV have higher mean levels of autofluorescence than do patients who do not have CNV [206]. The focal areas of hyperpigmentation in these patients were found to have high levels of autofluorescence and had absorption characteristics, suggesting the pigment seen was derived, at least in part, from lipofuscin. The histopathologic correlate to focal hyperpigmentation is detached pigment cells in the subretinal space. These areas of hyperpigmentation were autofluorescent, suggesting lipofuscin accounted for at least some of the observed pigment. The finding of hyperautofluorescent, hyperpigmented spots in the fellow eye was particularly associated with retinal angiomatous proliferation in the fellow eye. Recently in a study imaging patients with retinal angiomatous proliferation with optical coherence tomography and autofluorescence photography, the location of the angiomatous proliferation was seen to be topographically associated with pigmented hyperautofluorescent structures in the outer nuclear layer [207]. It was thought these

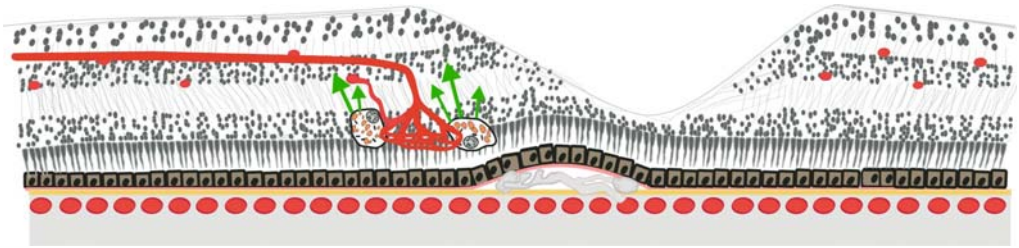

Fig. 7.6. Patients with retinal vascular anastomosis to the neovascular process, which has a number of names including deep retinal vascular anomalous complexes (RVAC) and retinal angiomatous proliferation (RAP). These patients have focal hyperpigmentation that is hyperautofluorescent. Optical coherence tomography reveals particulate densities in the outer retina. It is proposed that these densities represent oxidatively stressed cells (icons in the outer retina) containing lipofuscin that are producing VEGF (*green arrows*), something both oxidatively stressed macrophages and RPE cells do. The secretion of VEGF in the outer retina causes recruitment of new vessels from the retinal circulation. It has been proposed that these patients often do not have concurrent occult CNV; however, careful inspection of late phase fluorescein and indocyanine green angiograms would suggest otherwise

structures were macrophages or detached RPE cells laden with lipofuscin. Since either of these cell types can secrete VEGF when subjected to oxidative stress, it was theorized that these cells may be secreting VEGF in the outer retina. This would be expected to cause recruitment of the retinal vessels as they grow down the VEGF gradient, leading to formation of a RAP lesion. In rabbits, injection of lipid peroxide in the subretinal space caused migration of RPE cells into the subretinal space and outer retina and these RPE cells had phagocytized droplets of lipid peroxide [222]. Optical coherence tomography suggests that either RPE cells detach or macrophages migrate into the subretinal space (Fig. 7.6).

The origin of drusen is a perplexing and contested issue. Analysis of the lipids in Bruch's membrane suggested to some that they were of cellular origin while other investigators thought the lipids must have had a vascular origin. Through a very detailed analysis, Hageman and co-workers [8] determined that cellular remnants from degenerate RPE cells contribute to inflammatory stimulus, and these remnants may act as a nidus for drusen formation. In a proteomic analysis of drusen dissected from Bruch's membrane, oxidative protein modifications, including protein cross-links, were found. In particular carboxyethyl pyrole adducts, which are formed from oxidation products of docosahexaenoic acid, were found more frequently in AMD eyes than in age-matched controls [37]. Also crystallins, which are nonsecreted heat shock proteins that are synthesized by the retina and RPE [21, 37], were more likely to be found in drusen of eyes with age-related macular degeneration. Many of the altered proteins could have been derived from either the blood or RPE cells. The accumulation of material in the first place may be related to altered Bruch's membrane physiology from accumulated cross-links with oxidatively damaged lipid and protein, and subsequent inflammatory sequelae [8, 21, 37]. One would expect that there is a bidirectional flux of lipid through Bruch's membrane over time and that there may be a selective partitioning of molecules within the altered Bruch's membrane, contributing to the formation of drusen, particularly those containing lipid, such as soft drusen. The principal

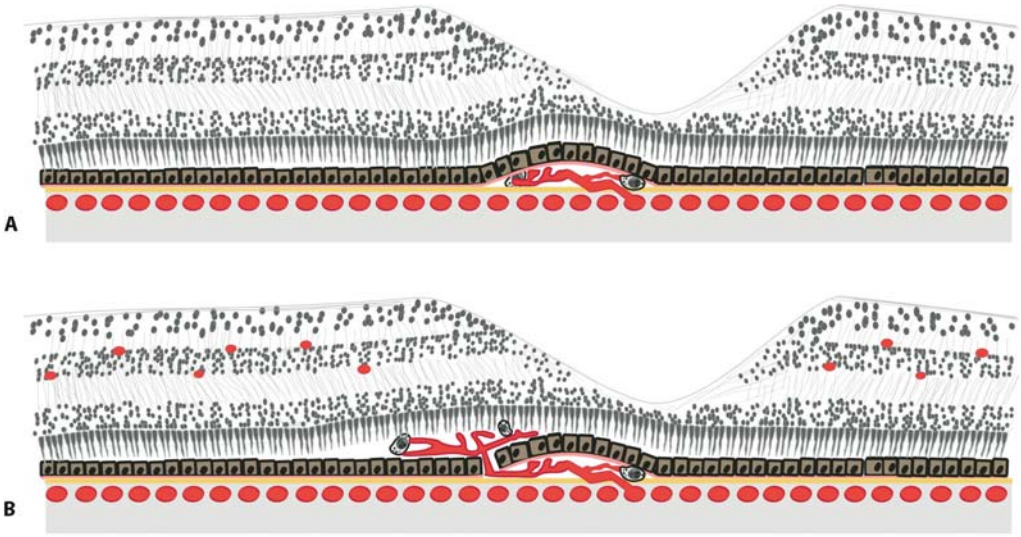

Fig. 7.7 A, B. Invasion of CNV. Once the invading tissue reaches the inner portion of Bruch's membrane (**B**) and potentially the outer retina space, there may be some influence on VEGF production by the low O_2 tension there

material leading to the formation of soft drusen, basal linear deposit, represents a cache of lipids and other materials that could be the target of oxidative attack. There are no intrinsic cellular mechanisms to counter this attack; however, it is certainly possible that vitamins and antioxidants may offer some protection.

While there is compelling evidence, some of which is circumstantial, linking CNV in AMD to oxidative stress and damage, other mechanisms are certainly operative. Once the invasion of tissue begins, especially if it reaches the subretinal space, it will experience the physiologically normal, but low oxygen tension and may itself produce growth factors to perpetuate endothelial proliferation. In addition, hypoxia promotes migration and tube formation by the bone marrow derived endothelial progenitor cells (Fig. 7.7).

Summary for the Clinician

- **Experimental evidence shows oxidative damage to lipids in Bruch's membrane**
- **Mechanisms of dealing with oxidative damage, as evidenced in other diseases such as atherosclerosis, may also be operative in the eye**
- **Clinically observable alterations, such as increased lipofuscin, occur in AMD**
- **Patterns of lipofuscin deposition are associated with specific types of CNV**
- **Antioxidants reduce incidence of some aspects of late AMD**
- **Initiation, continuation and pattern of neovascular growth appear to be explainable by pathophysiology within the local milieu**

7.11 Oxidative Damage and Geographic Atrophy

More than 100 different types of oxidative lesions to DNA have been described, including single- and double-strand breaks and the development of a variety of cross-link lesions [76]. The maintenance of genome integrity is extremely important not only in avoiding the production of mutations in progeny of the organism, but in the potential progeny of the cell. Many types of DNA damage can be fixed through the coordinated action of a number of different proteins, but other types cannot be repaired with guaranteed fidelity. The cell responds to genomic damage through repair processes employing a large number of proteins. In addition, the cell may turn off growth and replication until the repair process is complete [205].

Some cells may be permanently induced into a senescent state or may die through apoptosis. Cellular senescence occurs in most cell lines as a consequence of increasingly limited proliferative potential and eventual growth arrest with shortening of the telomeres [205]. Induced cellular senescence causes a premature decline in replicative potential from cell cycle arrest without death [30]. Senescent cells are not responsive to growth factors and have altered gene expression, protein synthesis, and cellular morphologies as compared with non-senescent cells [30, 31]. Induction of senescence occurs with the production of tumour suppressor proteins, in particular p53 and pRb (mutation of the Rb gene can lead to retinoblastoma), which among other things arrests the affected cells at checkpoints in their cell cycle. Continued oxidative injury can cause senescent cells to undergo apoptosis, or cellular suicide. Induced cellular senescence and apoptosis are seen as adaptive responses to the onslaught of genomic damage, where the organism trades cell death to prevent the possibility of replication of mutated cells (cancer). This has been called the Samurai law of biology, where it is better to be dead than wrong [198]. Production or activation of p53 encourages senescence, while inactivation of p53 can lead to rescue from senescence, with an increase in the tendency for carcinogenesis [32]. Oxidative stress increases the activation of p53 and pRb and also increases the rate of telomere shortening [180]. Oxidative damage not only affects nuclear DNA, but also mitochondrial DNA, where mutation affects the efficiency of energy production and increases the propensity for additional ROS production [27, 71, 141].

Extension of these concepts to age-related macular degeneration may explain a number of factors. The accumulation of oxidative damage has been suggested as a cause of CNV, but the same oxidative damage may induce senescence [85] and an aging phenotype, with possible apoptosis [253] in RPE cells as well. Oxidative damage can lead to an increased accumulation of lipofuscin within RPE cells, a finding linked with the development of geographic atrophy. This series of events may explain geographic atrophy, where there is a localized well demarcated area of "atrophy" of the retina and choriocapillaris sandwiching a region of absent RPE cells. This hypothesis may explain the seemingly illogical response in which adjacent RPE cells do not replicate and fill in areas vacated by apparently dying fellow RPE cells. It is possible that in the area of atrophy affected RPE cells have been lost through apoptosis and cannot be replaced by adjacent RPE cells because they themselves are in senescence. Of interest is that the autofluorescence of the RPE cells immediately adjacent to geographic atrophy are increased, indicating a

larger lipofuscin load, and in follow-up these areas of increased autofluorescence are more likely to undergo "atrophy" [83]. Geographic atrophy is frequently seen in fellow eyes with CNV, also implying a common aetiologic link.

Summary for the Clinician

- **Oxidative damage can induce senescence and apoptosis**
- **Apoptotic cell loss leads to zones of cell loss**
- **Bordering RPE cells, which presumably have induced senescence, do not migrate or replicate to fill the defect**
- **Operative principles developed through evolution concerning oxidative damage and potential for DNA damage may influence the development of late AMD**

7.12 Summary

This review critically examined a number of different theories in light of known physiologic concepts. No one specific theory by itself explains all aspects of the development of CNV in AMD. Integration of a number of aspects from differing theories, particularly oxidative damage, as delineated appears to explain many aspects, however. There are still a number of questions that face all of these diseases in terms of prevention and treatment.

Although aging, in part, may be the result of an accumulation of genetic defects that do not necessarily inhibit reproduction, there is increasing evidence that much of the aging phenotype is also the result of oxidative stress and the induced cellular adaptation responses. A principal risk factor for degenerative aspects of aging appears to be life itself. Aging is a problem that has challenged biologists and philosophers for centuries. Aging has deterministic and stochastic aspects, something the most ancient of philosophers knew. Clearly there are numerous factors involved and many of these are coded into our genetic structure. Certainly genes are powerful navigators of our fate, but the course can be modified by our interventions. AMD affects the quality of life, particularly in aged people who may have other infirmities. With increasing life spans and an increasing number of aged people, the incidence of macular degeneration is expected to rise. Development of a comprehensive hypothesis for the aetiology of late AMD is an iterative process over time, but is central to developing treatments for this debilitating disorder.

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