AGE-RELATED MACULOPATHY

A BIOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDY

Mike Kliffen

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AGE-RELATED MACULOPATHY
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Den geneeskunstenaar past bescheidenheid

Alleen in het laboratorium mag en kan hij [de geneeskundige] het zuiver wetenschappelijk systeem volgen, aan het ziekbed past een andere houding. Daar moet hij steeds begrijpen, dat hij maar bij uitzondering blijft staan op den vasten bodem der inductieve wetenschap, maar meestal zich begeeft in de nevelige sferen van empirie, opinies en vermoedens. Dat het gevoel van stelligheid en zekerheid, en de meeningen a priori, in het laboratorium gewettigd, hier meestal misplaatst zijn en vervangen moeten worden door een bescheiden en onbevangen afwachten der gebeurtenissen, en accepteeren van alle feiten, hoe onverklaarbaar ook.

Ned. Tijdsch. Geneeskd. 1894; 38 I: 316

CONTENTS

ABBREVIATIONS

AMD	age-related macular degeneration
AP	alkaline phosphatase
ARM	age-related maculopathy
BLD	basal laminar deposit
Con A	concanavalin A
DBA	dolichos bifloris agglutinin
FGF	fibroblast growth factor
FLSC	fibrous long-spacing collagen
GAGs	glycosaminoglycans
GalNAc	N-acetylated galactosamine
GlcNAc	N-acetylated glucosamine
HNK-1	human natural killercell-1
ICZ	inner collagenous zone
IRBP	interphotoreceptor retinoid binding protein
LSC	long-spacing collagen
M_r	molecular weight
NeuNAc	N-acetylated neuraminic acid
OCZ	outer collagenous zone
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
pI	isoelectric point
PNA	peanut agglutinin
RCA-I	ricinius communis agglutinin
RPE	retinal pigment epithelium
SBA	soybean agglutinin
TGF	transforming growth factor
UEA-I	ulex europaeus agglutinin
VEGF	vascular endothelial growth factor
WGA	wheat germ agglutinin

CHAPTER 1

General Introduction

1.1 Introduction

Age-related maculopathy (ARM) is an age-related degenerative disorder of the central part of the retina, the macula lutea (yellow spot). Essentially, ARM is a clinical diagnosis based on funduscopical changes. It is customary nowadays to call the late stages of ARM, geographic atrophy and neovascular (disciform) macular degeneration, age-related macular degeneration (AMD)(Bird, et al., 1995). These late stages result in severe and irreversible visual impairment and tend to progress to the center of the macula (the fovea), where visual acuity is affected most. Because AMD is usually a bilateral disorder, affected people loose their ability to read, drive, and watch television (Macular-photocoagulation-study-group, 1993). The prevalence of AMD is 1-7% in people over 60, up to 14% over age 85. Obviously, the number of people affected by AMD will enlarge, because of the increasing age of the population (Ferris, 1983; Klein, et al., 1992; Leibowitz, et al., 1980; Vinding, 1989; Vingerling, et al., 1995c). In fact, AMD is the most common cause of blindness in the elderly in the western world and it is estimated that 640.000 people in the USA aged 75 years or older have signs of AMD (Klein, et al., 1992), while in The Netherlands 57.000 persons suffer from AMD in at least one eye (Vingerling, et al., 1995d). Although this disease has been known for more than a century (Haab, 1888), knowledge on the pathogenesis of the affliction is still incomplete. Some risk-factors for ARM have been found in epidemiologic studies. Current smokers have a three times increased risk to develop this disease (Vingerling, et al., 1995b), and cardiovascular diseases and early-menopause are reported to be risk-factors as well (Vingerling, et al., 1995a; Vingerling, et al., 1995e). There is still no adequate therapy for the majority of people disabled by AMD. Therefore, it is necessary to intensify the research on ARM and AMD in order to understand the pathogenesis of this disease. This knowledge may eventually lead to an adequate therapy or to preventive measures for this severely invalidating disease.

1.2 Aim of the Thesis

Age-related maculopathy comprises many histopathological changes, some of which are reflected by abnormalities that can be seen clinically by ophthalmoscopy. There is, however, a lack of data correlating clinical signs with the many different histopathological changes encountered in this disease. The main aim of this thesis is to investigate the constituents and origin of the extracellular deposits in ARM that appear to be correlated with the development of AMD. This is a step in analyzing the pathogenesis of the deposits and to find possible ways to prevent or treat them.

1.3 Preview

The morphologic changes in ARM are reviewed, and illustrated with light-microscopical, electron microscopical, and funduscopical pictures in chapter 2 of this thesis. Histologically, the most prominent aging changes in early stages of ARM are drusen and basal laminar deposit (BLD), both extracellular deposits, that are assumed to be important in the development of ARM (Bird, 1992; Green and Enger, 1993; Sarks, et al., 1994; Sarks, 1976; Sheraidah, et al., 1993; van der Schaft, et al., 1992a). Already in 1976, an association between the amount of BLD and retinal pigment epithelium/photoreceptor degeneration has been described (Sarks, 1976). It is assumed that BLD is produced by the retinal pigment epithelium (RPE) for reasons unknown (Löffler and Lee, 1986; Sarks, et al., 1988; van der Schaft, et al., 1991). The question whether BLD is formed because of degeneration of the RPE or the RPE degeneration is a consequence of a mechanical barrier of BLD, drusen, and/or thickening of Bruch's membrane remains to be elucidated. Other morphological changes in ARM are thickening of Bruch's membrane (Sarks, et al., 1988; van der Schaft, et al., 1992b) and atrophy of the choriocapillaris (Feeney-Burns, et al., 1990; Ramrattan, et al., 1994). Although the histopathologic characteristics of these changes are well documented, the chemical composition has only been partly resolved. The most relevant biochemical data from the literature on BLD and drusen are given in chapter 3. Chapters 4, 5, and 6 focus on our research that has been performed to reveal more of the biochemical composition of the structures related to ARM.

Hypoxia of the macular area has been assumed to be a factor in the pathogenesis of ARM (Dastgheib and Green, 1994; Giovannini, et al., 1994; Melrose, et al., 1987). Aging changes in the choriocapillaris and thickening of the diffusion barrier by drusen and BLD may lead to insufficient oxygen supply in the RPE and photoreceptors. One of the late-stages of ARM, neovascular AMD, involves the sprouting of new blood vessels from the

choriocapillaris into the subretinal space. The trigger for this neo-angiogenesis has not been elucidated, but the development of neovascular AMD appears to be correlated with extracellular deposits accumulating in ARM (Green and Enger, 1993; Sarks, et al., 1994; Sarks, 1980). Therefore, it is reasonable to assume that hypoxia plays a role in the pathogenesis of this disease. In other tissues where angiogenesis is involved, e.g. in wound healing, a broad range of molecules, including angiogenic growth factors is involved. Thus, we investigated the role of the most important angiogenic growth factors in ARM. The possible relationship between these angiogenic growth factors and the development of neovascular AMD is discussed in chapter 7. Finally, concluding remarks and suggestions for future research are given in chapter 8.

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CHAPTER 2

Morphologic Changes in Age-Related Maculopathy

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2.1 Introduction

In this review the morphologic changes in the aging human macula will be discussed, and the appearance by funduscopic examination will be shown where appropriate. Although the causal relationship between subsequent stages of ARM has not been established, we describe the changes in ARM that usually appear in life in the different structures of the human macula. Each of these changes will be described separately and in the following order: a brief introduction of the structure, the appearance on funduscopic examination, the (immuno)histopathology, and the ultrastructure (not all of these data are available for each structure). Thus, successively, age-related alterations in the neuro-retina, the retinal pigment epithelium (RPE), Bruch's membrane with drusen and basal laminar deposit (BLD), the choriocapillaris, and the choroid, will be reviewed. For a better understanding of the terminology a brief description of the most important structures of the normal human macula will be given in the first paragraph.

2.2 Anatomy of the normal human macula

The macula lutea, also called the yellow spot due to deposition of yellow pigment (xantochrome granules), is located in the posterior pole of the human eye starting 0.5 mm from the optic nerve head (blind spot). Although its diameter is only 6 mm, the macula is the most important part of the eye for contrast discrimination due to the high concentration of cones in this area. The innermost part of the macula is the fovea (1.85 mm in diameter), with at its center the foveola (diameter 0.35 mm). Outside the fovea is the parafovea (radius 0.5 mm), and the perifovea (radius 1.5 mm). The retina can be divided (starting from the vitreous) in the neuro-retina and the RPE. The neuro-retina is composed of the inner limiting membrane, nerve fiber layer, ganglion cells, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, and photoreceptor layer (Figure 2.1). External to the RPE lies Bruch's membrane that forms a barrier between the retina and the choriocapillaris (Figure 2.1). The choriocapillaris is a thin capillary network closely related to Bruch's membrane, and is the internal part of the choroid, a layer of loose connective tissue with larger blood vessels and melanocytes (Figure 2.1). The choriocapillaris is essential for the nutrition of the retina. The outermost layer of the eye is the sclera. Although the architecture of the retina outside the macular region is similar, there are many differences in the individual layers due to the specialized functions of the macula. Whereas the nerve fiber layer in the macula is only a thin layer on top of the ganglion cells, in the peripheral retina it is more prominent. On the other hand the ganglion cell layer is more conspicuous in the macula. A more extensive description can be found in "Histology of the human eye" (Hogan, et al., 1971).

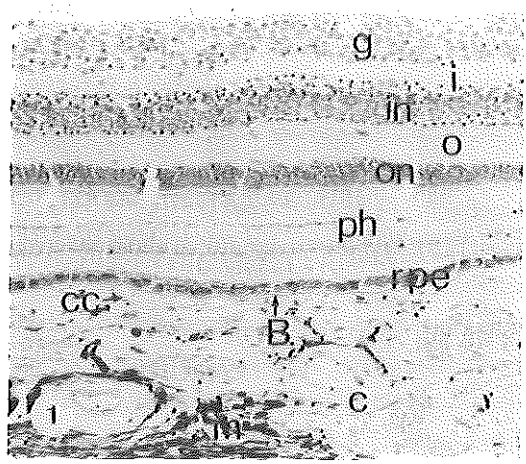


Figure 2.1. Light microscopic image of a normal macula. Mayer's hematoxylin, magnification 200x. g = ganglion layer; i = inner plexiform layer; in = inner nuclear layer; o = outer plexiform layer; on = outer nuclear layer; ph = photoreceptor layer; rpe = retinal pigment epithelium; B = Bruch's membrane; cc = choriocapillaris; c = choroid; m = melanocytes.

2.3 Aging Changes in the Neuro-Retina and Retinal Pigment Epithelium

Like all cells of neural origin, the cells in the retina are post-mitotic and differentiated (Friedman and Tso, 1968). This means that, under normal circumstances, they are unable to regenerate new cells after the loss of old or damaged cells. Therefore, the thickness of the retina decreases with advanced age, due to loss of photoreceptor and ganglion cells (Dorey, et al., 1989). The photoreceptor outer segments contain stacks of photoreceptor discs that are continuously shedded during the visual cycle.

During life the RPE cells continuously phagocytize the shedded discs. Each RPE cell is in contact with 20-30 photoreceptors, and each photoreceptor discards 30-100 discs/day (Grindle and Marshall, 1978). This results on average, in a turnover rate of 2000 discs in the parafovea, 3500 in the perifovea and 4000 discs in the peripheral retina per day, with a photoreceptor/RPE cell ratio of 24, 44, and 42, respectively (Hogan, 1972). Photoreceptors are highly susceptible to phototoxic effects of visible and ultraviolet light, due to the large amount of polyunsaturated fatty acids in their densely stacked phospholipid membranes, and it has been suggested recently that rods are more vulnerable to aging changes than are cones (Curcio, et al., 1993). Lipid peroxidation in the membranes occurs by free radicals, which are formed by the absorbed radiant energy. These modified lipids cannot be digested by the lysosomal enzymes of the RPE cells (Lerman, 1988; Yurchenco and Furthmayr, 1984). In principle, they could be involved in the development of ARM. The combined action of superoxide dismutase, catalase, and glutathione peroxidase in the RPE cells is one metabolic pathway for protection against oxidative damage. Although superoxide dismutase activity shows no correlation with aging and ARM, the activity of catalase decreases significantly

with aging and ARM (Liles, et al., 1991). This information supports the idea that oxidative damage is of importance in the development of ARM.

When the digestion of the shedded discs is incomplete or diminishes, lipofuscin granules (Figure 2.2) are formed (Feeney Burns, et al., 1980; Grindle and Marshall, 1978). These granules are considered as residual bodies, which accumulate in the RPE cytoplasm. Lipofuscin is a heterogeneous aggregation of damaged molecules rather than a native product (Yurchenco and Furthmayr, 1984) and is present in all aging species and aging tissues. Like melanin, lipofuscin absorbs radiation from the infrared through the visible and far into the ultraviolet band, with an increasing efficiency as photon energy increases (Yurchenco and Furthmayr, 1984). There is no evidence that these lipofuscin granules can be extruded from the cytoplasm (Feeney-Burns, et al., 1990). The number of lipofuscin granules increases rapidly after birth during the first two decades of life (Wells, et al., 1989) especially in the macula, although in the fovea less lipofuscin has been found (Feeney Burns, et al., 1980). Between the ages of 20 and 60 years the amount of lipofuscin is relatively stable, but after the age of 60 years the number of lipofuscin granules increases again (Wells, et al., 1989). There is an inverse relationship between the number of lipofuscin granules and the number of melanin granules in the RPE cells of the macula (Feeney Burns, et al., 1980; Feeney-Burns and Ellersieck, 1985; Weiter, et al., 1986). The increase in lipofuscin causes a detectable enlargement of the cells during normal aging (Friedman and Tso, 1968; Sarks, et al., 1988; Wells, et al., 1989). It is hypothetical that the cell metabolism is gradually affected as the cytoplasmic space is reduced (Sarks, et al., 1988). The decrease of the number of RPE cells is correlated with the accumulation of lipofuscin (Dorey, et al., 1989). The phagocytizing capacity of lipofuscin loaded cells has to be taken over by neighboring RPE cells. Subsequently, these cells are flooded by the photoreceptor discs, which have to be digested. Because the RPE cells cannot divide under normal conditions, they must "renew" themselves by autophagy and the subsequent synthesis of new cell organelles (Feeney-Burns and Ellersieck, 1985). Although the enzyme activity of acid phosphatase, and cathepsin D, two lysosomal enzymes involved in the degradation of ingested photoreceptor outer segment material, increases with increasing age (Boulton, et al., 1994), there is a rapid accumulation of lipofuscin in the remaining RPE cells. Consequently, the cell metabolism gets even more affected, resulting in RPE atrophy that might finally lead to AMD (Dorey, et al., 1989). No correlation has been found between the amount of lipofuscin and the development of AMD, although Weiter et al. (1986) found an increased amount of lipofuscin in two patients with AMD, in comparison with age-matched controls.

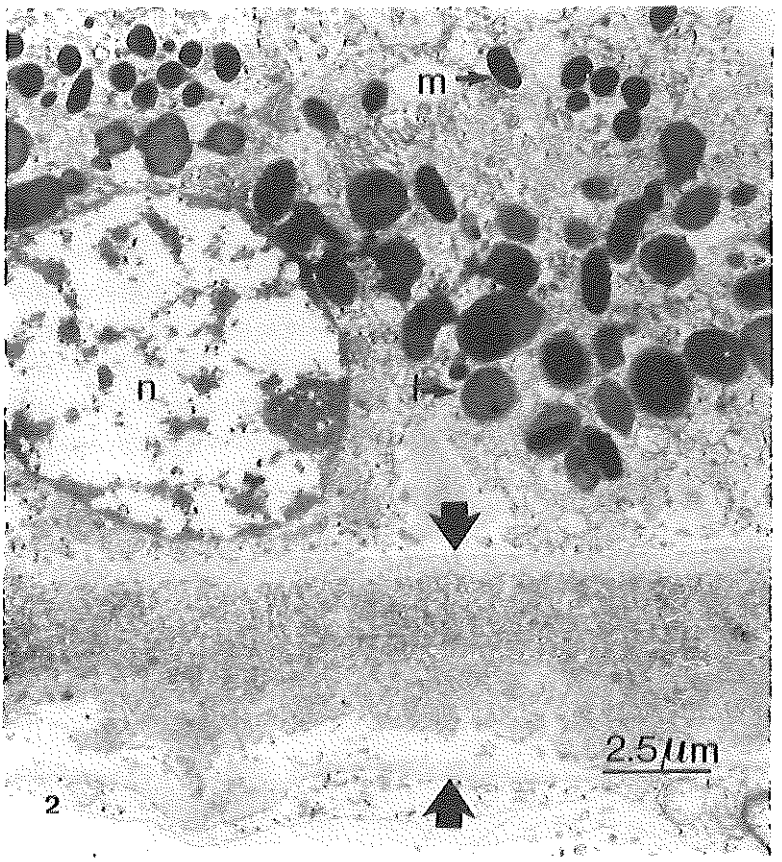


Figure 2.2. Electron micrograph of lipofuscin (l) accumulation in a retinal pigment epithelium (RPE) cell. Bruch's membrane (between large arrows) is filled with debris. m = melanin granule; n = nucleus of RPE cell.

2.4 Aging Changes in Bruch's Membrane

Bruch's membrane is composed of five layers; the basement membrane of the RPE, the inner collagenous zone (ICZ), the elastic layer, the outer collagenous zone (OCZ) and the basement membrane of the choriocapillaris. With advancing age, the thickness of Bruch's membrane increases in almost all eyes (Ramrattan, et al., 1994; Sarks, et al., 1988; Sarks, 1976; van der Schaft, et al., 1992b). Thus the "normal" structure of Bruch's membrane in older individuals appears to be debris-filled (Feeney-Burns and Ellersieck, 1985).

In histological sections Bruch's membrane increases from a thin line in children to a thick membrane in the elderly (Figure 2.3 and 2.4) (Hewitt, et al., 1989; Ramrattan, et al., 1994; Sarks, 1976; van der Schaft, et al., 1992b). In the human macula this thickening is

mainly seen in the OCZ, especially between the capillaries of the choriocapillaris, where the so-called intercapillary pillars (Figure 2.4) are formed (Killingsworth, 1987; van der Schaft, et al., 1992b). In the peripheral retina the age-related thickening is located predominantly in the ICZ (Hewitt, et al., 1989). With advancing age, an increasing amount of calcium phosphate is deposited, initially in the elastic lamina of Bruch's membrane, that can easily be demonstrated with the von Kossa stain (Figure 2.5) (Davis, 1981; Feeney-Burns and Ellersieck, 1985; van der Schaft, et al., 1992b). With special lipid stains (Oil red O and Sudan Black), it has been revealed that above the age of 30 years the amounts of neutral lipids and phospholipids increase, which makes the membrane hydrophobic. This may form a barrier for the fluid transport from the retina to the choriocapillaris, and vice versa (Bird, 1991; Pauleikhoff, et al., 1990c; Sheraidah, et al., 1993). However, it has recently been demonstrated that the permeability of Bruch's membrane decreases in the first 4 decades of life, during which time no increased lipid deposition is established (Moore, et al., 1995).

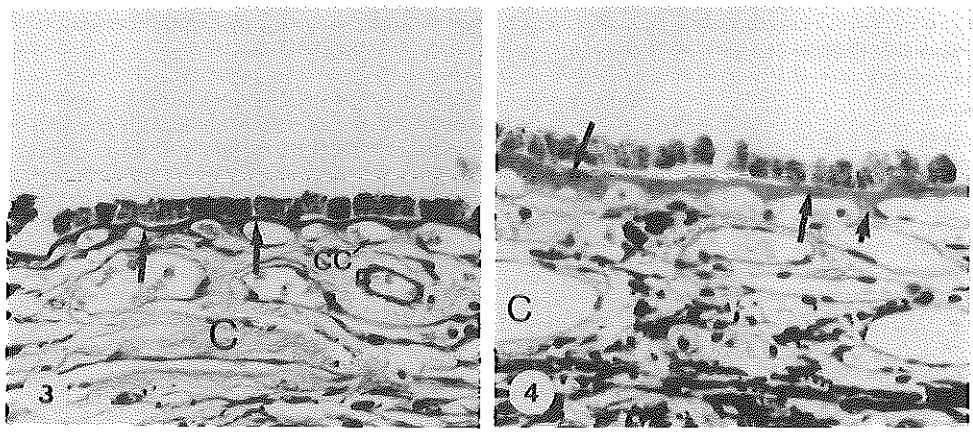


Figure 2.3. Light microscopic image of normal Bruch's membrane (arrows). Periodic acid-Schiff reaction, magnification 400x. C = choroid; cc = choriocapillaris.

Figure 2.4. Light microscopic image of diffuse thickening of Bruch's membrane (long arrows). Note the intercapillary pillars (short arrow). Periodic acid-Schiff reaction, magnification 400x. C = choroid.

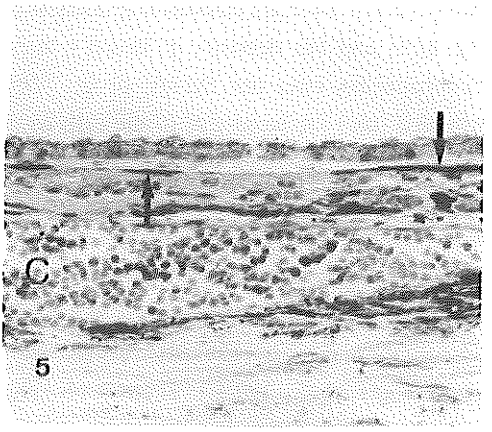


Figure 2.5. Light microscopic image of calcifications in Bruch's membrane (arrows). Von Kossa stain, magnification 400x. C = large choroidal blood vessel.

By electron microscopic evaluation the age-related thickening of Bruch's membrane is mainly due to an accumulation of waste products of cellular origin (Figure 2.2 and 2.6), consisting of small vesicles, dense granules surrounded by a double membrane, curly membranes and membranous bags, filled with smaller vesicles, and banded material with a periodicity between 100 and 120 nm, which is called long-spacing collagen (LSC, Figure 2.2 and 2.6) (Löffler and Lee, 1986; Nakaizumi, 1964; van der Schaft, et al., 1991). These structures might be derived from the RPE cells (Feeney-Burns and Ellersieck, 1985; Killingsworth, 1987; Killingsworth, et al., 1990; Pauleikhoff, et al., 1990c; Sarks, 1976).

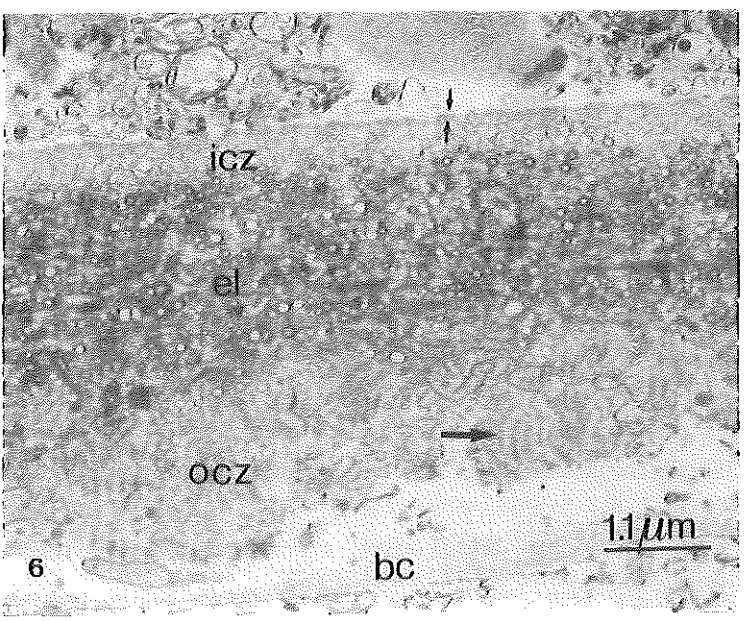


Figure 2.6. Electron micrograph of cellular debris in Bruch's membrane. Higher magnification of Figure 2.2. Note the long spacing collagen (large arrow) in the outer collagenous zone (ocz). The basement membrane of the retinal pigment epithelium is between small arrows. icz = inner collagenous zone; el = elastic lamina; bc = basement membrane of the choriocapillaris.

Nevertheless, there are considerable differences in the number of chemical elements between the RPE and the deposits in Bruch's membrane, as revealed by electron probe X-ray microanalysis (van der Schaft, et al., 1992a). Some subcellular structures seem to be extruded from the basal side of the cells as membranous bags, instead of being digested by the lysosomal enzymes of the RPE cells, although physical separation of bodies from the RPE has not been shown until degeneration of these cells was in an advanced stage (Sarks, et al., 1988). One hypothesis is, that due to age-related atrophy of the choriocapillaris, this debris cannot be cleared away in due time and accumulates in Bruch's membrane (Sarks, 1976). It has also been suggested that a prolonged choroidal filling phase on fluorescein angiography may be causally related with a diffuse thickening of Bruch's membrane (Pauleikhoff, et al., 1990b). There is no indication that the cellular debris is a post mortem artifact (Feeney-Burns and Ellersieck, 1985).

The accumulation of abnormal material in Bruch's membrane might be the cause of the cellular response, which is sometimes seen in ARM (Killingsworth, et al., 1990). Leucocytes and macrophages can be found near breaks in Bruch's membrane on the choriocapillaris side (Figure 2.7), but are not a consistent finding (Killingsworth, et al., 1990; van der Schaft, et al., 1993). However, it is unknown why these cells are attracted to Bruch's membrane. Possible attractants such as immune complexes, complement and fibrinogen could neither be detected in Bruch's membrane nor in deposits like BLD and drusen (see next two paragraphs) in human maculae (van der Schaft, et al., 1993).

2.5 Drusen

Drusen are located between the RPE basement membrane and the ICZ of Bruch's membrane. They have been described predominantly in the periphery of the eye (Hewitt, et al., 1989), especially in the equatorial region (Sarks, 1970), but later investigation has been focussed on drusen in the posterior pole (Green, 1985; Sarks, et al., 1980).

The origin of drusen has been a source of speculation. It has been postulated that drusen consist of exudative material from the choriocapillaris (Sarks, 1980). Others think that they are comprised of cellular debris from the RPE (Farkas, et al., 1971; Green, et al., 1985; Ishibashi, et al., 1986; Killingsworth, et al., 1990). Their element composition on electron-probe X-ray microanalysis gives no clues on the molecular nature (van der Schaft, et al., 1992a). The cellular structures, which can be observed within the drusen, are believed to be derived from macrophages (Grindle and Marshall, 1978; Sarks, 1980) or RPE-derived cells (Burns and Feeney Burns, 1980), although others do not agree with this hypothesis (Ishibashi, et al., 1986).

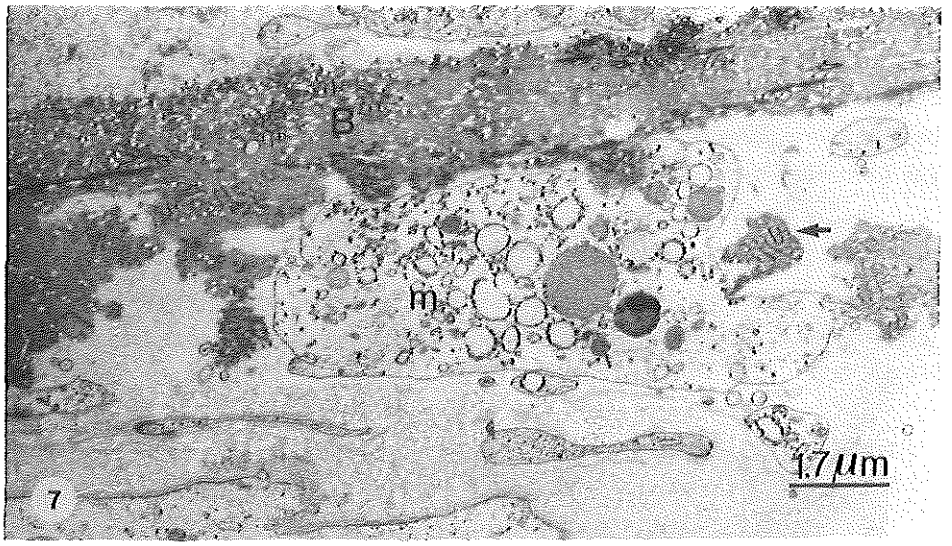


Figure 2.7. Electron micrograph of a macrophage (m) beneath Bruch's membrane (B). The macrophage is engulfing debris from Bruch's membrane including long spacing collagen (arrow).

At present there is no satisfactory classification for drusen, which covers all the different clinical and histological features of drusen (Green, et al., 1985). Several types of drusen are distinguished in the literature (Sarks, et al., 1988). These have been given several -often confusing- names for each type of drusen, due to separate lines of approach by clinicians, pathologists and biochemists. A description of the terminology will be given here.

Small hard drusen are, both on funduscopic and histologic examination, the predominant type of drusen. Funduscopically such drusen appear as small, yellow-white spots surrounded by a slightly darker rim (Figure 2.8).

By light microscopic evaluation these drusen are smooth surfaced, globular or dome-shaped structures between the RPE and Bruch's membrane (Figure 2.9). Small hard drusen consist of hyalin material and often contain multiple globular calcifications, lipids and mucopolysaccharides (Farkas, et al., 1971; Ulshafer, et al., 1990). That is why they are sometimes also called "crystalline" drusen.

On electron microscopic examination most hard drusen mainly consist of amorphous, finely granular material with the same electron-density as the RPE basement membrane (Figure 2.10). Curly membranes and different kinds of vesicles as well as tubular structures

can also be found within these drusen (Green, et al., 1985). The RPE cells overlying the drusen often seem to be attenuated and hypopigmented, while the laterally located RPE cells are hypertrophic and hyperpigmented (Burns and Feeney Burns, 1980). This arrangement of the RPE cells explains why these drusen may appear on fundoscopic pictures as small yellow dots surrounded by a slightly darker rim.

Small "hard" drusen are assumed to be low-risk drusen with respect to the development of AMD (Coffey and Brownstein, 1986; Green and Key, 1977; Green, et al., 1985; Sarks, 1976) and with respect to a decrease in visual acuity (Hewitt, et al., 1989; Leibowitz, et al., 1980). They appear in 6.2 % of all eyes with ARM (Green and Enger, 1993).

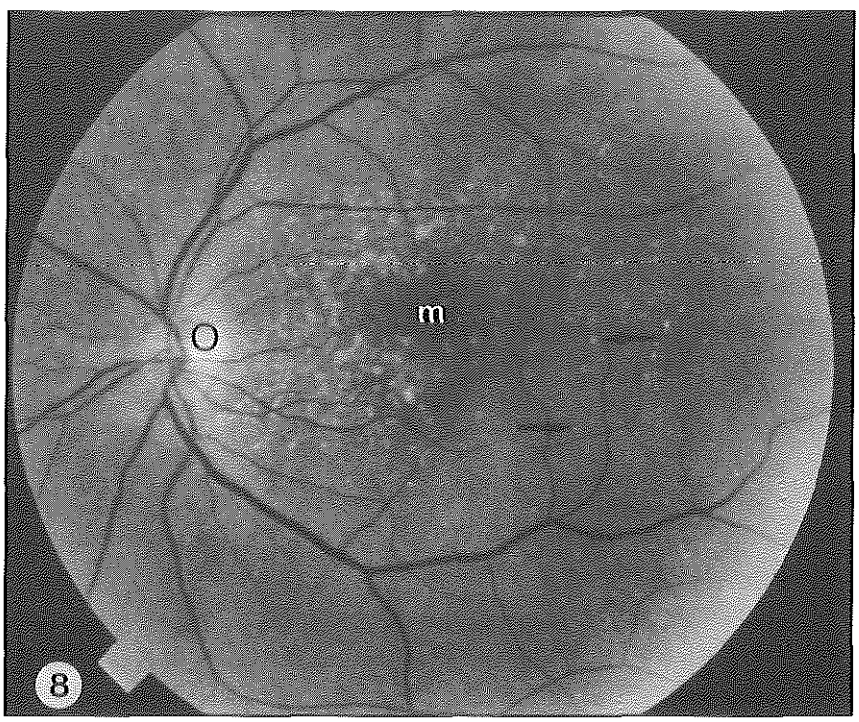


Figure 2.8. Multiple small hard drusen (arrows) seen on funduscopic examination as small yellow-white spots. The slightly darker region in the middle of the figure is the macula (m). Note that many of the drusen are melting into each other. O = optic disc.

Figure 2.9. Light microscopic image of two small hard drusen (arrows) between the basement membrane of the RPE and the inner collagenous zone of Bruch's membrane. Note the hypopigmented RPE cells overlying the biggest drusen. Periodic acid-Schiff reaction, magnification 400x. C = choroid.

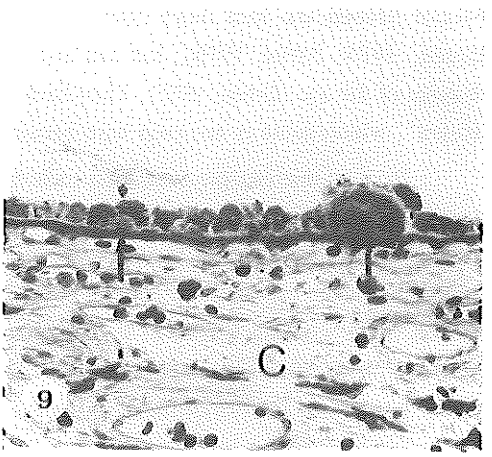
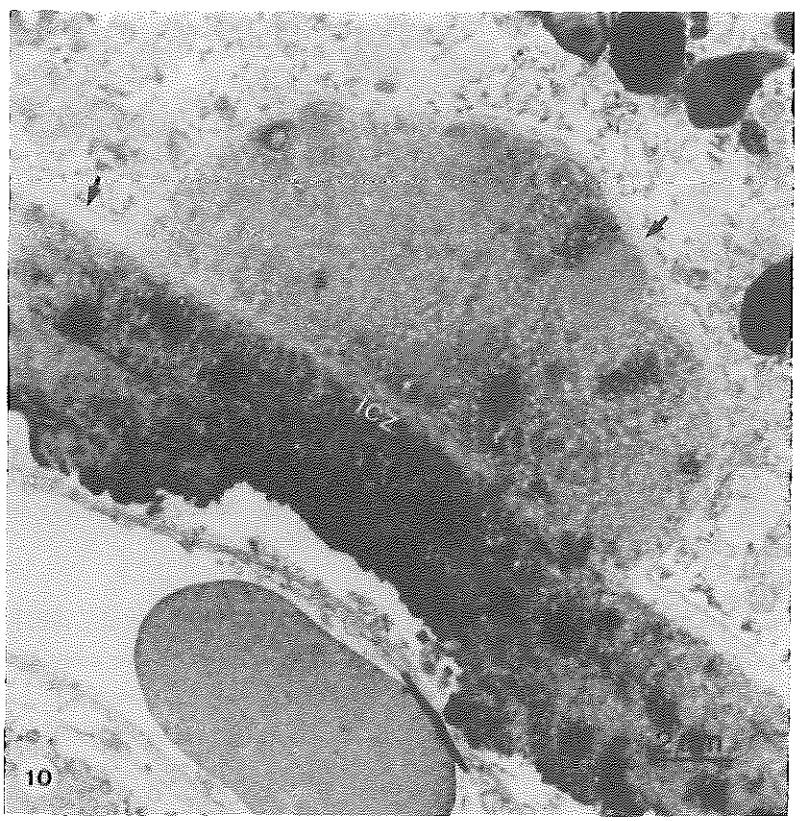


Figure 2.10. Electron micrograph of a hard drusen between the basement membrane of the retinal pigment epithelium (arrows) and the inner collagenous zone (icz) of Bruch's membrane.



Mixed drusen are seen more often with increasing age (Coffey and Brownstein, 1986; Green, 1985; Sarks, 1976), but are also secondary to trauma, inflammatory and other diseases of the eye (Green, 1985). They have, in a confusing way, been called "degenerative drusen", which name is not used here because in our opinion all drusen are degenerative.

By light microscopic evaluation such drusen are generally larger than the typical hard drusen, and consist partly of hyalin material and partly of coarse granular material. Such drusen often contain many calcium phosphate globules (Figure 2.11), although all types of drusen may show calcifications (Green, et al., 1985). Mixed drusen are assumed to be "hard" drusen filled with discharges of membranous debris from degenerating RPE, which have passed the RPE basement membrane (Sarks, 1982; Sarks, et al., 1980). In this situation the lateral borders of the drusen have disappeared, but the contents still seem to be separated from each other by an invisible membrane.

Ultrastructurally these drusen are composed of small amounts of amorphous, finely granular material and relatively large amounts of membranous, tubular and vesicular material (Kenyon, et al., 1985).

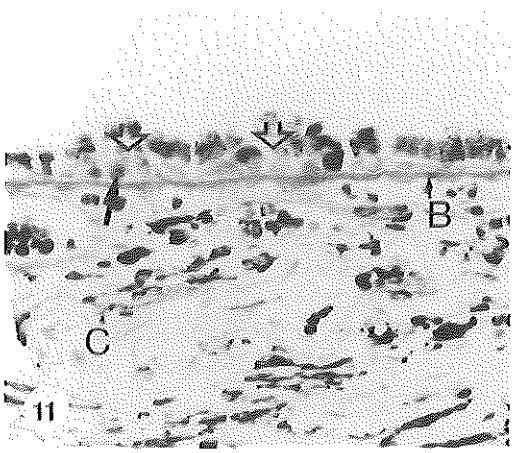


Figure 2.11. Light microscopic image of mixed drusen (open arrows). Note the calcifications within the drusen (long arrow). Mallory staining, magnification 400x. B = Bruch's membrane; C = choroid.

Soft drusen can develop through the softening of hard drusen (Green, et al., 1985), or more commonly by the formation of soft drusen de novo. Soft drusen evolve more rapidly than hard drusen. They tend to become confluent, and separate the RPE basement membrane from the rest of Bruch's membrane over relatively long distances (Green, et al., 1985). Probably, soft drusen are a result of RPE dysfunction (Green, et al., 1985; Sarks, 1976).

On funduscopic examination such drusen usually appear as large yellow-white spots

with indistinct or "soft" borders (Figure 2.12). They are preferentially located within the fovea (Sarks, et al., 1988) so that RPE and photoreceptor atrophy, developing in relation to soft drusen, will start closer to the foveola. Due to their sloping edges, soft drusen often cannot be distinguished from small serous RPE detachments on funduscopic pictures (Green, et al., 1985; Kenyon, et al., 1985), and this may create difficulties in classification and treatment of these detachments.

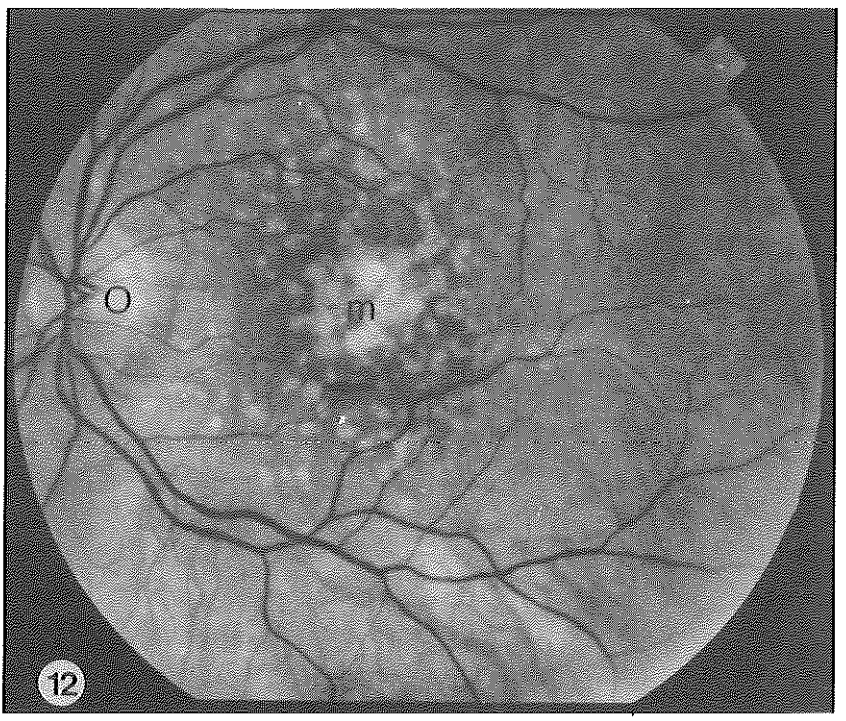


Figure 2.12. Multiple soft drusen seen by funduscopic evaluation as large yellow-white spots with indistinct or "soft" borders (arrow). Note the confluent drusen in the macula (m). O = optic disc.

On light microscopic examination soft drusen appear as large drusen, with sloping edges, and often seem to be empty or contain pale staining membranous or fibrillar material (Figure 2.13) (Sarks, 1980). The overlying RPE is attenuated or atrophic (Figure 2.13).

Ultrastructurally soft drusen are composed of double-layered coiled membranes, with some amorphous material and calcifications (Figure 2.14) (Killingsworth, et al., 1990). In most eyes with soft drusen, BLD is also found in the same area (Green, et al., 1985; Lewis, et al., 1986).

Clinically, eyes with confluent soft drusen are at high risk for subretinal neovascularization and geographic atrophy. Therefore, these drusen are called "high-risk" drusen (Bressler, et al., 1989; Green, et al., 1985; Sarks, 1980; Sarks, 1982).

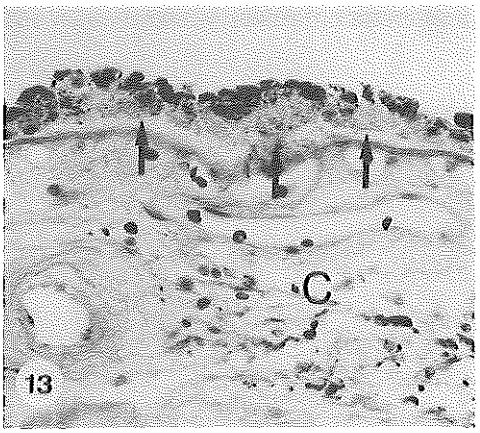
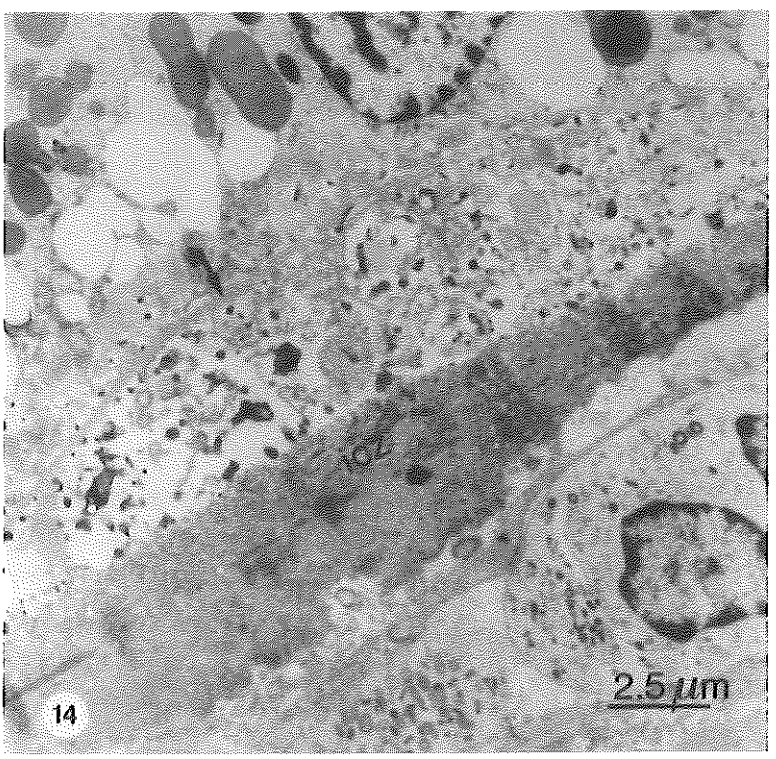


Figure 2.13. Light microscopic image of a soft drusen (arrows) between the RPE and Bruch's membrane. The RPE is atrophic. Note the indistinct borders of the drusen. Mallory staining, magnification 400x. C = choroid.

Figure 2.14. Electron micrograph of a soft drusen between the basement membrane of the RPE (arrow) and the inner collagenous zone (icz) of Bruch's membrane. Note the difference to the hard drusen in Figure 2.10.



Diffuse drusen is a histopathologic designation for a diffuse thickening of the inner aspect of Bruch's membrane, and is commonly observed in eyes of older persons (Figure 2.15) (Kenyon, et al., 1985). On ophthalmoscopic examination these drusen cannot be differentiated from continuous soft drusen, or small RPE detachments.

Ultrastructural studies have revealed the presence of vesicles, electron-dense particles, fibrils and clusters of LSC (Coffey and Brownstein, 1986; Green, 1985; Green and Key, 1977; Green, et al., 1985; Tso, 1985).

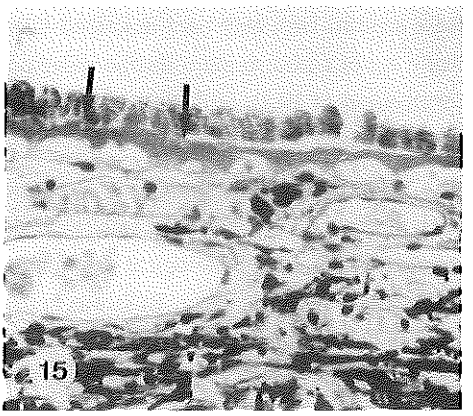


Figure 2.15. Light microscopic image of diffuse drusen. Note the small vesicles often seen in this type of drusen (arrows). Periodic acid-Schiff reaction, magnification 400x.

The chemical composition of drusen, especially the lipid content, is assumed to be important in the development of ARM (Bird, 1992; Sheraidah, et al., 1993). Drusen contain phospholipids or neutral lipids, which are hydrophilic and hydrophobic, respectively (Pauleikhoff, et al., 1992a). If drusen consist of neutral lipids, they confer hydrophobic properties, which could hypothetically hamper the diffusion of water and water-soluble molecules through Bruch's membrane (Bird and Marshall, 1986). This hydrophobicity causes hypofluorescence on fluorescein angiography, and is correlated with RPE detachment (Pauleikhoff, et al., 1990a; Pauleikhoff, et al., 1992b).

Drusen can be seen in the eyes of 26% to 85% of people above the age of 30 years (Bressler, et al., 1989; Coffey and Brownstein, 1986; Leibowitz, et al., 1980; van der Schaft, et al., 1992b; Vingerling, et al., 1995). There is no preference for the left or right eye (Coffey and Brownstein, 1986; Lewis, et al., 1986; Vinding, 1989) and there is no predilection for males or females (Lewis, et al., 1986), but drusen are seen more often in whites than in blacks on funduscopic pictures. Commonly, drusen are thought to be associated with ARM. However, only 0. 5% - 2% of the population will finally develop AMD, despite the high prevalence of drusen (Green, et al., 1985; Vingerling, et al., 1995).

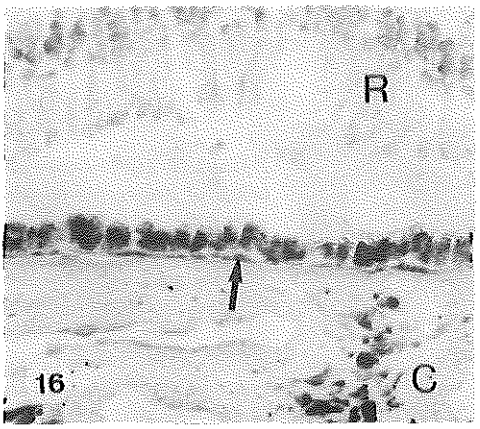
2.6 Basal Laminar Deposit

In 55% of the eyes of the elderly (Green and Enger, 1993), extracellular deposits have been found between the RPE and its basement membrane (Figure 2.16), which have been called "basal laminar deposit(s)" (BLD) (Green and Enger, 1993; Sarks, et al., 1988; van der Schaft, et al., 1992b), "basal linear deposit(s)" (Löffler and Lee, 1986; Sarks, 1976) or "linear basal deposits" (Feeney-Burns and Ellersieck, 1985). BLD has been found predominantly in the posterior pole of the eye (Lewis, et al., 1986; Sarks, 1973). Although these deposits have only been described histologically in enucleated eyes, it has been proven that it is not a post mortem artifact (Garron, et al., 1958; Weiter, et al., 1985). Due to the localization of BLD, and the basement membrane materials continuously produced by the RPE, it seems likely that BLD is derived from excessive basement membrane materials.

By funduscopic evaluation the presence of BLD is not recognizable as such. In the past, a case report has been published describing a patient with multiple confluent soft drusen, which after histological examination were determined as not being drusen (Frank, et al., 1973). Unfortunately, BLD was not recognized as an entity, by that time. Like drusen, BLD might be indirectly visible due to the atrophy and degeneration of the overlying RPE. Large amounts of BLD may be recognizable as a fine mottling of the RPE at an early stage of geographic atrophy. In our opinion BLD is probably recognized as soft drusen with fluorescein angiography, because of its extensive spread and indistinct borders.

The development of BLD beneath the RPE appears to be a reliable histological criterion for the degree of degeneration of the overlying RPE cells (Sarks, 1976). Consequently, maculae with early stages of ARM are sometimes histologically classified, in class 0 to class 3 BLD (corresponding with group I to IV by Sarks, 1976), according to the appearance of this deposit (van der Schaft, et al., 1992b).

In BLD class 0, no BLD is present. Small patches of BLD (class 1, Figure 2.17) under a few RPE cells will probably not disturb fundus pigmentation. The photoreceptors are unaffected. Class 1 BLD is not considered to be ARM. With increasing age, however, these deposits become larger and form a thin continuous layer (BLD class 2, Figure 2.16). Loss of photoreceptors is mild, and on ophthalmoscopic examination hypopigmentation or hyperpigmentation might be seen, due to accompanying RPE changes. In a more advanced stage (BLD class 3, Figure 2.18) a thick continuous layer of BLD is seen, which separates the RPE from the inner layer of Bruch's membrane. The overlying RPE becomes elevated, and more irregular in shape. There is sometimes a migration of solitary, hypertrophied RPE cells between the photoreceptors. Loss of photoreceptors becomes more apparent.



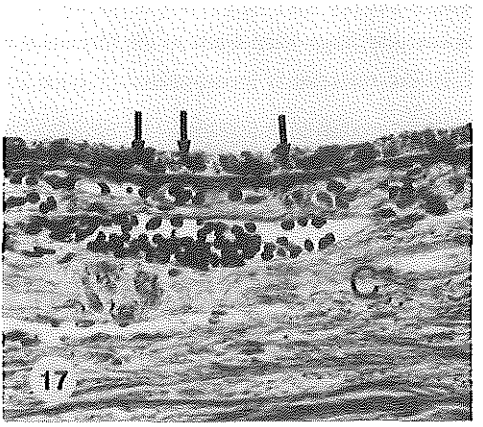


Figure 2.16. Light microscopic image of a thin continuous layer (class 2) of basal laminar deposit (BLD, arrow), between the RPE and Bruch's membrane. Stained with dolichos bifloris agglutinin, magnification 400x. R = photoreceptors of the outer-retina; C = choroid.

Figure 2.17. Light microscopic image of small patches of basal laminar deposit (class 1 BLD, arrows). Mallory staining, magnification 400x. C = choroid.

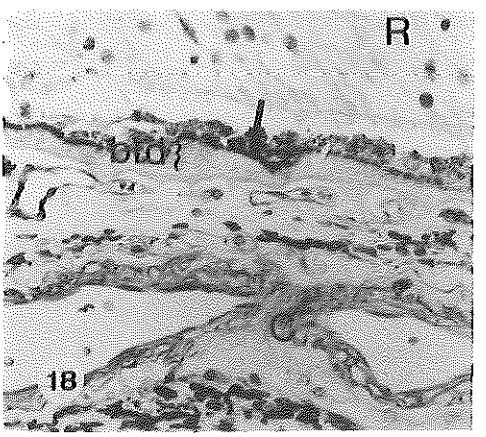


Figure 2.18. Light microscopic image of basal laminar deposit (BLD) class 3. A thick layer of BLD, of at least half the height of the RPE cells, is present (brace), and the RPE on top of the BLD is partially degenerated. A small part of the BLD adjacent to the RPE is stained with polyclonal antibodies against laminin (arrow). Magnification 400x. C = choroid; R = atrophic outer-retina.

At a later stage, in eyes with a thick layer of BLD, the RPE cells become depigmented and atrophic, finally resulting in the disappearance of the RPE cells and the accompanying photoreceptors. The BLD generally remains present after the RPE has disappeared (Sarks, 1976). This is called the atrophic type of AMD, or geographic atrophy (Figure 2.19). The fall out of photoreceptors seems secondary to changes in, and beneath the RPE. In eyes with the neovascular type of AMD, capillaries are described coursing from the choriocapillaris through breaks in Bruch's membrane and the BLD, without a previous

detachment of the RPE, which is commonly associated with neovascularization. This neovascular stage often leads to the formation of a fibrovascular scar (Junius-Kuhnt) in which BLD-like material is incorporated (Figure 2.20A +2.20B).

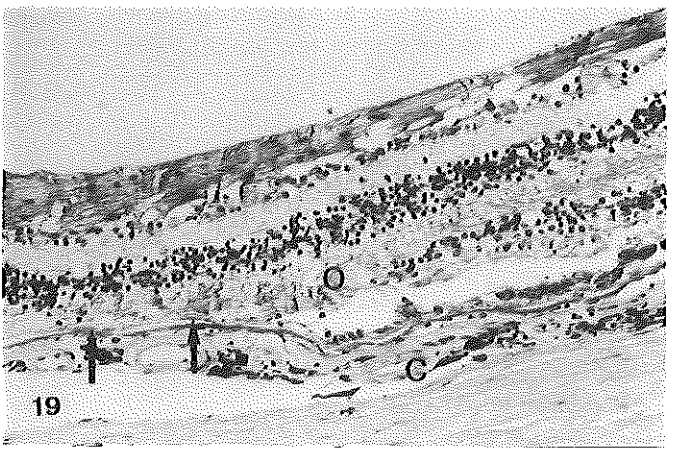
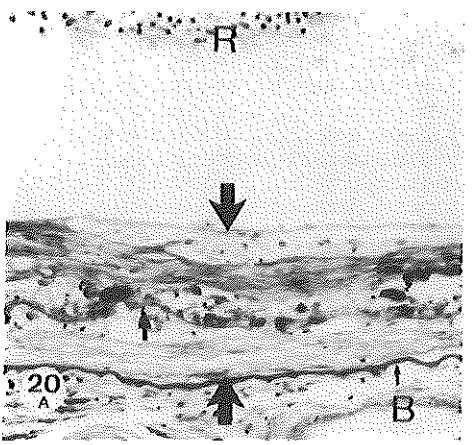


Figure 2.19. Light microscopic image of geographic atrophy with RPE and photoreceptor cell degeneration increasing from the right to the left side of figure. On the right part the RPE and outer plexiform layer are still visible; on the left the outer plexiform layer is in direct contact with the persisting BLD (arrows). Mallory staining, magnification 200x. O = outer plexiform layer; C = chorold.



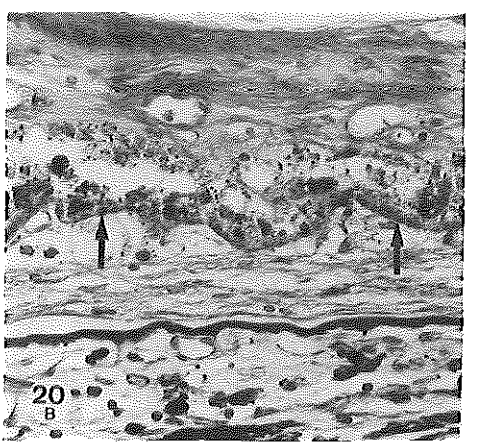


Figure 2.20A. Light microscopic image of disciform macular degeneration (the disciform scar between thick arrows). The retina is totally disorganized. In the middle of the scar are the remnants of the RPE and a layer of BLD-like material (thin arrow). Mallory staining, magnification 200x. B = Bruch's membrane. R = remnants of neuro-retina.

Figure 2.20B. Same macula with higher magnification (400x). Note the BLD-like material in the middle of the scar (arrows). The staining properties and density of this material are the same as those of BLD.

It has been proven by immunohistochemistry that a small part of BLD adjacent to the RPE contains collagen type-IV and laminin (Figure 2.18) (Marshall, et al., 1992; van der Schaft, et al., 1994). However, the main part of BLD consists of carbohydrate structures, most likely part of glycoproteins or proteoglycans, which differ from the carbohydrate structures on laminin and collagen type IV (Figure 2.22) (Kliffen, et al., 1994). Glycosaminoglycans could not be demonstrated in BLD (Löffler and Lee, 1986). A specific carbohydrate (GalNAc) that binds to the lectin Dolichos Bifloris Agglutinin, and that normally is not present in the human macula was also found in BLD (Figure 2.22) (Kliffen, et al., 1994). Because drusen do not stain with Dolichos Bifloris Agglutinin, this lectin can be used for discrimination between BLD and drusen on light microscopic examination (Kliffen, et al., 1994).

By electron microscopic evaluation, BLD shows similarity with LSC, and a homogeneous material with the same electron density as basement membranes (Figure 2.21) (Löffler and Lee, 1986; van der Schaft, et al., 1991). Interestingly, LSC is only rarely found within drusen (Kenyon, et al., 1985). However, LSC has been found in other parts of the human eye, including the outer and inner collagenous zone of Bruch's membrane, as well as in other tissues, e.g. heart and skin (van der Schaft, et al., 1991).

The exact pathogenesis of BLD is unknown, and the importance of BLD in the development of ARM is uncertain (Bird, 1991; Löffler and Lee, 1986), although some authors are convinced of the fact that the presence of BLD is positively correlated with the development of ARM (Feeney-Burns and Ellersieck, 1985; Sarks, 1976). BLD class 1 will probably not disturb fundus pigmentation, nor does it influence visual acuity (Sarks, 1976). In BLD class 2 the most common visual acuity measured, was between 5/10 and 6/10 (Sarks, et al., 1988; Sarks, 1976). In eyes with BLD class 3 vision was markedly decreased with an average visual acuity of 3/10 (Sarks, 1976).

2.7 Aging Changes in the Choriocapillaris

In eyes with ARM, changes in the structure of the capillary network (Fryczkowski, et al., 1989) and atrophy of the choriocapillaris and choroid can be found (Feeney-Burns, et al., 1990; Ramrattan, et al., 1994; Tso and Friedman, 1967). Recently, a significant decrease of the capillary density in ARM has been reported (Ramrattan, et al., 1994). Once the lumina of the choriocapillaris are obliterated, the intercapillary pillars become eroded by cellular activity (Sarks, et al., 1988). The relative ischemia might explain the development of chorioretinal vascular anastomoses in AMD (Fryczkowski, et al., 1989), but the exact

mechanism of this, and of the centripetal growth of the neovascular subretinal membranes towards the fovea is unknown.

The RPE cells are known to produce vascular stimulating as well as inhibiting factors, which can act on the choriocapillaris (Campochiaro, et al., 1986; Glaser, et al., 1985; Korte, et al., 1984). Experimental damage of the RPE, induced atrophy of the choriocapillaris (Korte, et al., 1984). In other experiments, in which a slow degeneration of the RPE was induced, neovascularization was seen (Zrenner, 1990). It is thought that in ARM the interaction between the RPE and the choriocapillaris is disturbed (Hogan, 1972). However, it is still uncertain whether the changes in the choriocapillaris are secondary to other changes in the macula, or whether this is one of the initiating factors of ARM (Delaney and Oates, 1982; Tso and Friedman, 1967).

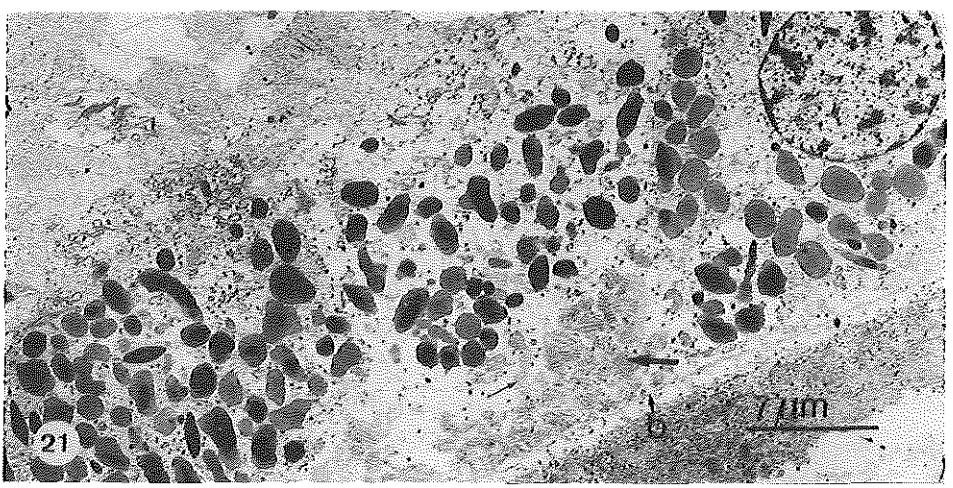


Figure 2.21. Electron micrograph of BLD between the RPE and the basement membrane of the RPE (b). The BLD consists of long spacing collagen (thick arrow) and a homogeneous material (thin arrow) with the same electron density as basement membranes.

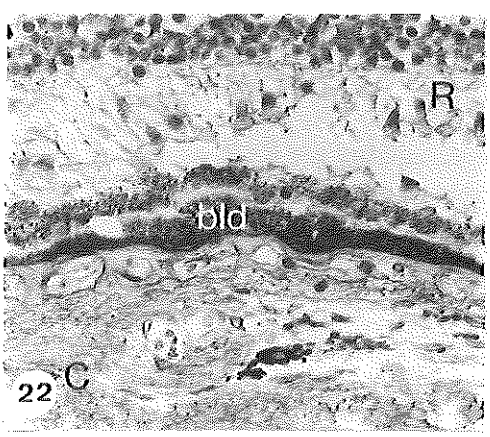


Figure 2.22. Light microscopic image of class 3 basal laminar deposit (BLD) stained with the lectin dolichos bifloris agglutinin. Note that approximately 90% of the BLD is stained. Magnification 400x. C = choroid; R = outer-retina.

2.8 Geographic Atrophy

In geographic or areolar atrophy, the RPE disappears and the choriocapillaris gets markedly atrophied. Consequently, the vessels of the choroid become visible by funduscopic evaluation (Figure 2.23). In this condition, photoreceptor cell loss is seen, because the photoreceptors are metabolically dependent on the RPE (Green, et al., 1985). This is best indicated on histological examination by a gradual loss of the outer nuclear layer of the retina, which results in a direct and firm contact between Bruch's membrane, and the outer plexiform layer of the retina (Figure 2.19). The loss of the RPE and photoreceptors occurs in advance of the choriocapillaris atrophy. The firm contact of the outer plexiform layer with Bruch's membrane, is one of the reasons why geographic atrophy prevents the development of a serous or hemorrhagic detachment of the retina in the same area (Sarks, et al., 1988). It has also been postulated that new vessel formation is dependent on viable RPE, and thus can only occur outside the area of atrophy (Sarks, et al., 1988). The edge of the atrophic area is called the junctional zone. This zone is marked by several layers of hypertrophic RPE cells, and the frequent occurrence of BLD (Figure 2.19). Neovascularization, if present, tends to occur in this zone, sometimes accompanied by giant cells and macrophages (Green and Key, 1977; Sarks, et al., 1988; Schatz and McDonald, 1989; van der Schaft, et al., 1993). The remaining visual acuity is dependent on the site and the extent of the geographic atrophy. If it is in the foveola, the visual acuity will (most often) decrease to the level of finger counting.

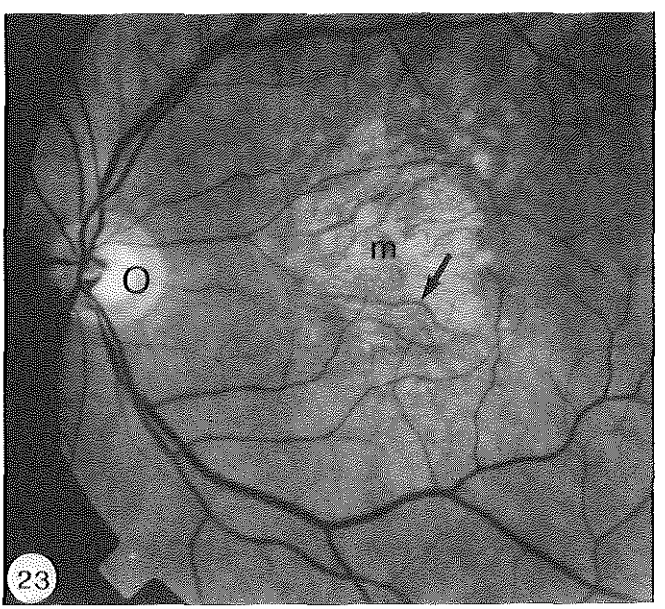


Figure 2.23. Funduscopical image of geographic atrophy in the macular area. The vessels of the choroid (arrow) become visible because of the atrophy of the retinal pigment epithelium and neuro-retina. Note the large number of soft drusen surrounding the macula, where the choroidal vessels are invisible. O = optic disc; m = macula.

2.9 Neovascular or Disciform Macular Degeneration

In some aged eyes, new subretinal vessels have been found between the RPE and Bruch's membrane, which can be seen on funduscopic examination (Figure 2.24) and more markedly on fluorescein angiography. This process takes place, preferentially, in the presence of a thick layer of BLD (Blumenkranz, et al., 1986; Sarks, 1975; Sarks, 1976; Sarks, et al., 1980) and soft drusen (Green, et al., 1985). These abnormal vessels originate from the choriocapillaris, and penetrate Bruch's membrane by pre-existing, or newly formed breaks. The new vessels tend to exudate serous fluid, proteins, lipids or blood under the RPE, often leading to a RPE detachment (Green, 1985; Green, et al., 1985). This fluid might even break through the RPE monolayer, and cause an overlying neurosensory detachment. This type of AMD is, therefore, often called the neovascular, exudative or "wet" type of AMD (Figure 2.25) and because of the disc shaped RPE detachment, and the resulting fibrovascular scar, it is also called the "disciform" type of AMD.

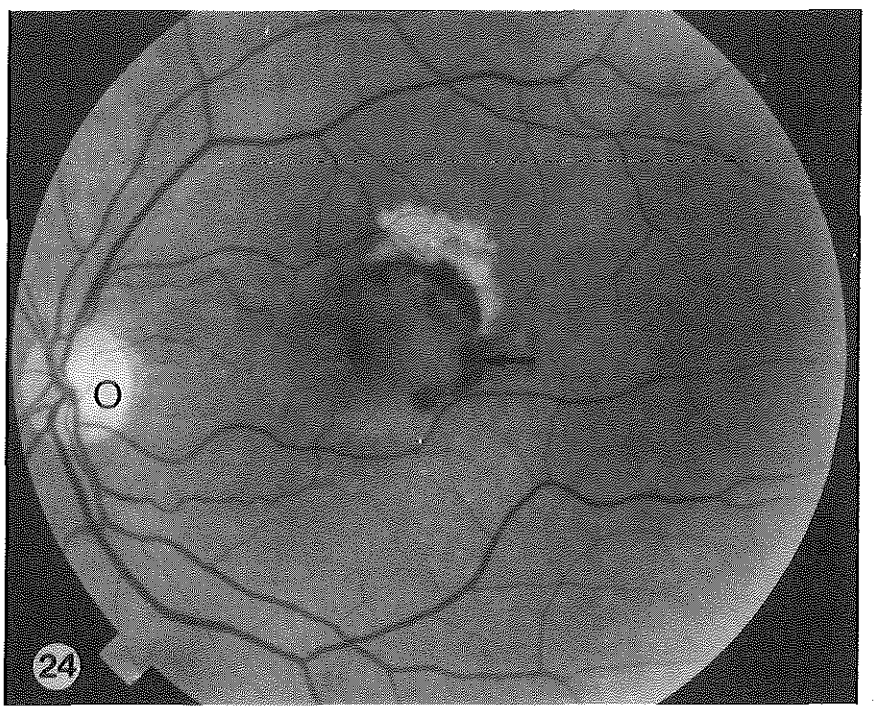


Figure 2.24. Funduscopical image of hemorrhage (arrow) from neovascular membrane in the macula. The white rim adjacent to the hemorrhage is either a hard exudate or old blood. O = optic disc.

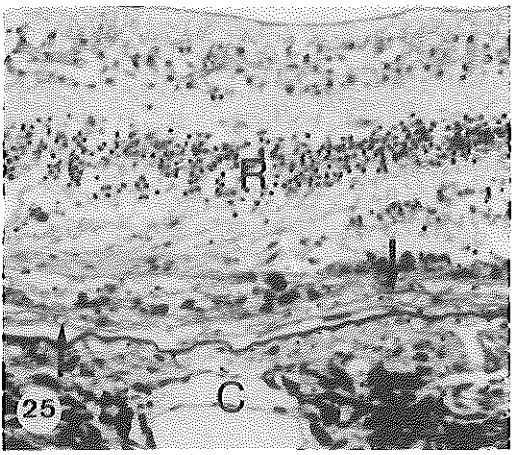


Figure 2.25. Light microscopic image of subretinal neovascularization. Note the blood vessels (arrows) between the remains of the RPE and Bruch's membrane. Mallory staining, magnification 200x. C = choroid; R = neuroretina.

The induction of angiogenesis in AMD has been suggested to be caused by macrophages (Penfold, et al., 1990; van der Schaft, et al., 1993). These macrophages are seen at the choroidal side of Bruch's membrane in eyes with a disciform reaction. In these maculae, Bruch's membrane is thinner than normal for that age, and is sometimes even discontinuous (Sarks, 1970; van der Schaft, et al., 1993). Although it seems likely that growth factors such as transforming growth factors and fibroblast growth factors are involved in the process of neovascularization (Amin, et al., 1994), there is no proof that they are the initiating factors. Subretinal neovascularization is often accompanied by a low-grade chronic inflammatory reaction, as seen in histological sections (Sarks, et al., 1988). A subsequent fibrovascular, disciform scar, is formed by fibroblasts that invade and organize the hemorrhage (Green, et al., 1985). The overlying RPE cells and photoreceptors are separated from their supply of nutrients from the choriocapillaris, by the scar. The RPE cells are shifted laterally and start to clump, which often results in clusters of pigmented cells, buried in a thick fibrotic scar. They become atrophic and finally disappear. The result is a disciform scar in the macula (Figure 2.20A and 2.20B). Since the histological abnormalities, leading to neovascularization, extend over several disc diameters, the disciform response can be multifocal, and particularly endanger the fovea, where the changes are generally most severe (Figure 2.26).

The neovascular form of AMD has been found in 0. 5% of the population (Bressler, et al., 1989). Between 55 and 64 years it is 0.1%, but over 85 years the prevalence is 7.4% (Vingerling, et al., 1995). Disciform lesions account for 80%-90% of blindness due to AMD (Hyman, et al., 1983; Young, 1988). AMD is often bilateral, but an interval of several years

between fellow eyes in the development of AMD is usually seen (Lavin, et al., 1991). Patients with the exudative form of AMD have a yearly risk of 12% of developing an exudative lesion in the contralateral eye (Ferris, 1983). Of the patients with this form of AMD, 70% will develop a seriously diminished visual acuity (less than 1/10) within 24 months (Blumenkranz, et al., 1986).

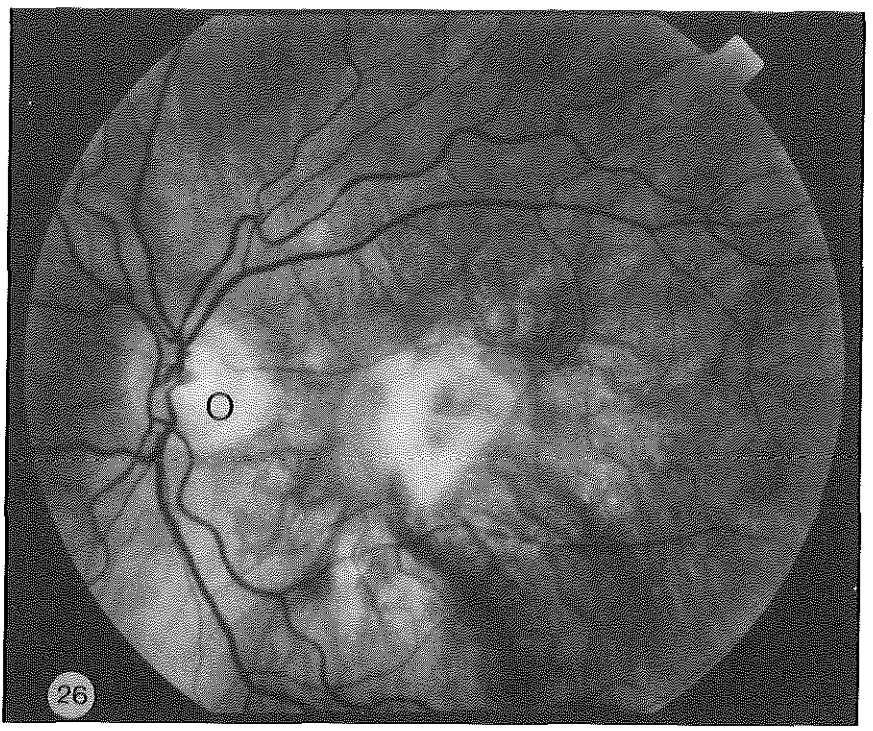


Figure 2.26. Funduscopical image of a large disciform scar in the macular area. In contrast with geographic atrophy no choroidal vessels are visible within the scar. O = optic disc.

2.10 Conclusions

The different morphologic changes described in the previous sections are related to the development of ARM. However, there are several variations in the evolution of ARM to AMD. The most prominent morphologic changes supposedly leading to AMD, are soft drusen and BLD. Soft drusen can be seen in vivo by ophthalmoscopic examination, but BLD is only histologically visible, and can not be clinically recognized. The amount of the deposits seems to affect the pathogenesis and severity of ARM. A considerable amount of

histopathological research has been done on these deposits, but their chemical composition, which might be important in the development of AMD (Bird, 1992; Sheraidah, et al., 1993; van der Schaft, et al., 1992a), has only been partly resolved. This information is necessary for further study on the source of the deposits, and the natural course of ARM. Since there is still no adequate therapy for the severely invalidating AMD, and because of the aging population resulting in even more patients with this disease, it is necessary to intensify the research on ARM in order to prevent AMD, or find a sufficient therapy for it.

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CHAPTER 3

Biochemistry of the Extracellular Deposits in Age-Related Maculopathy

3.1 Bruch's Membrane

Bruch's membrane has a high collagen component. There are now 16 known subtypes of collagens, that are widely spread through the body. Collagen types I, II, and III account for 80-90% of all the collagens in the body and are the classical fibril forming collagens (Ayad, et al., 1994). In Bruch's membrane collagen type I, III, IV, V, VI, VII, and VIII have been identified (Marshall, et al., 1994; Marshall, et al., 1992; Marshall, et al., 1993). Aging of collagens occurs throughout the body and is associated with increased cross-linking. The collagens in Bruch's membrane are ordered in a matrix and also show more cross-linking with increasing age (Karwatowski, et al., 1991). This cross-linking causes decreased solubility, increased stiffness and increased resistance of the collagen to enzymatic digestion (Karwatowski, et al., 1991; Schnider and Kohn, 1982). In Bruch's membrane, this process could hamper the transport of vesicular and membranous debris, which in turn may lead to the accumulation of this kind of material in the aging Bruch's membrane (Karwatowski, et al., 1991). In addition, these cross-linked collagens are a perfect substrate for ingrowing capillaries through Bruch's membrane (Ingber and Folkman, 1988; Vernon and Sage, 1995).

Next to collagens, Bruch's membrane also contains proteoglycans. They are large molecules, that consist of a few to several hundreds glycosaminoglycan side chains covalently bound to a core protein (Figure 3.1). Glycosaminoglycans (GAGs) are large carbohydrate molecules that consist of repeating disaccharide units (Table 1). There are five kinds of GAGs present in the human body: chondroitin sulfate, heparan sulfate, keratan sulfate, dermatan sulfate, and hyaluronic acid (Table 1). Hyaluronic acid is the only one that is not bound to a core protein. Configurational variation in the disaccharide bonds, the length of the side chains, and the different positions of sulfate all lead to increased diversity in the chemical and physical properties of the GAGs. The proteoglycans in Bruch's membrane are of importance in the filtration properties of Bruch's membrane due to their high content of anionic sites (Call and Hollyfield, 1990; Hewitt, et al., 1989). Age-related alterations in the composition of these proteoglycans could therefore influence permeability. The proteoglycans

composition of these proteoglycans could therefore influence permeability. The proteoglycans in Bruch's membrane consist of 40% chondroitin sulfate and 60% heparan sulfate. This rate does not change with increasing age. Newly synthesized GAGs are normally present in Bruch's membrane as 75% chondroitin sulfate and 25% heparan sulfate, whereas this ratio alters to 45% and 55%, respectively, in eyes with signs of ARM (Hewitt, et al., 1989). This means that the turn-over rate of heparan sulfate relatively increases in comparison with chondroitin sulfate.

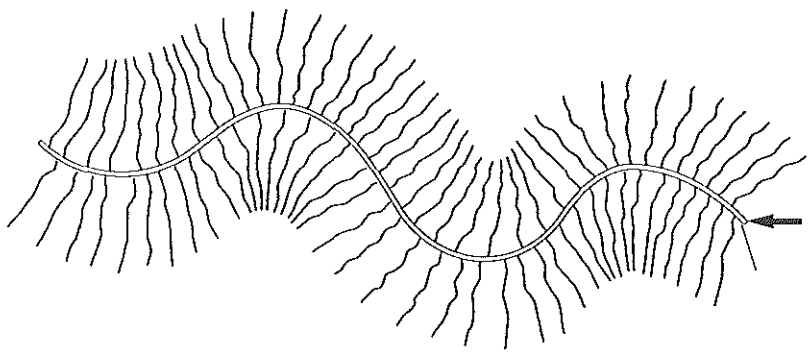

Figure 3.1. Model of a proteoglycan. The glycosaminoglycan side chains are covalently bound to one core protein (arrow).

Table 1. Components of Glycosaminoglycan Disaccharide Units

Chondroitin sulfate:	[glucuronic acid -(β1-3)- N-acetylated galactosamine]$_n$
Keratan sulfate:	[galactose -(β1-4)- N-acetylated glucosamine]$_n$
Dermatan sulfate:	[iduronic acid -(α1-3)- N-acetylated galactosamine]$_n$
and	[glucuronic acid -(α1-3)- N-acetylated galactosamine]$_n$
Heparan sulfate:	[glucuronic acid -(β1-4)- N-acetylated glucosamine]$_n$
and	[iduronic acid -(β1-4)- N-acetylated glucosamine]$_n$
Hyaluronic acid:	[glucuronic acid -(β1-3)- N-acetylated glucosamine]$_n$

The amount of neutral lipids and phospholipids increases in Bruch's membrane above the age of 30 years, which makes the membrane hydrophobic, and thus may form a barrier for the fluid transport from the retina to the choriocapillaris, and vice versa (Bird, 1991; Pauleikhoff, et al., 1990b; Sheraidah, et al., 1993). The increased amount of lipids is much more prominent in the macula than in the equatorial part of the aging Bruch's membrane (Holz, et al., 1994). In addition, it has recently been demonstrated that the hydraulic conductivity of Bruch's membrane already decreases in the first four decades of life, during which time no increased lipid deposition is established (Moore, et al., 1995).

3.2 Drusen

The chemical composition of drusen is assumed to be important in the development of ARM (Bird, 1992; Sheraidah, et al., 1993). Although it seems likely that diffuse drusen have more impact on the pathogenesis of ARM than solitary drusen, it appears that they have the same constitution of lipids (Pauleikhoff, et al., 1990b). Drusen contain phospholipids or neutral lipids, which are hydrophilic and hydrophobic, respectively (Pauleikhoff, et al., 1992a). If drusen consist of neutral lipids, they confer hydrophobic properties, which could hypothetically hamper the diffusion of water and water-soluble molecules through Bruch's membrane (Bird and Marshall, 1986). This hydrophobicity causes hypofluorescence on fluorescein angiography, and is correlated with RPE detachment (Pauleikhoff, et al., 1990a; Pauleikhoff, et al., 1992b). The accumulation of hydrophilic phospholipids in drusen, on the other hand, is associated with neovascular AMD (Pauleikhoff, et al., 1990a).

Glycoproteins or proteoglycans in the several sub-types of drusen have so far not been investigated.

3.3 Basal Laminar Deposit

Little is known on the chemical composition of BLD. Ultrastructurally, it has been demonstrated that a part of BLD resembles basement membrane material (Löffler and Lee, 1986; van der Schaft, et al., 1991). Furthermore, it has been proven by immunohistochemistry that a small part of BLD adjacent to the RPE contains collagen type-IV and laminin (Marshall, et al., 1992; van der Schaft, et al., 1994). Although the lipid components of drusen and Bruch's membrane have been studied, lipids in BLD have not been investigated. Because BLD stains with the PAS-reaction on paraffin sections, it is likely that BLD contains glycoproteins or complex carbohydrate structures like proteoglycans. Therefore, it was one of the aims of this thesis to investigate which specific carbohydrate

structures are present in BLD and whether these carbohydrates are part of glycoproteins or proteoglycans.

The accumulation of hydrophilic extracellular matrix material in BLD in the macular area could be a causal factor in the development of subretinal neovascularization, because this material is favorable for cell migration, and an important substance in angiogenesis (Berger, et al., 1982; Roden and Horowitz, 1978; Vernon and Sage, 1995). Furthermore, it is hypothesized that the extracellular matrix plays a role in the formation of the three-dimensional vascular structure (Vernon and Sage, 1995). This hypothesis is in accordance with the observed association of hydrophilic drusen and neovascular AMD (Pauleikhoff, et al., 1990a).

More knowledge on the chemical composition of drusen and BLD, both associated with the development of AMD, could give a clue to the initiating events leading to this severely invalidating disease.

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CHAPTER 4

Analysis of Carbohydrate Structures in Basal Laminar Deposit in Aging Human Maculae

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ABSTRACT

Purpose. To analyze carbohydrate structures in Basal Laminar Deposit (BLD), an extracellular material, which accumulates between the retinal pigment epithelium (RPE) and Bruch's membrane. BLD has been shown to correlate positively with visual loss in age-related macular degeneration.

Methods. Thirteen post-mortem human maculae with BLD were histochemically examined by light-microscopy using the monoclonal antibody HNK-1 and seven lectins: Canavalia Ensiformis (ConA), Soybean Agglutinin (SBA), Wheat germ Agglutinin (WGA), Dolichos Bifloris (DBA), Ulex Europaeus (UEA-I), Ricinius Communis Agglutinin I (RCA-I) and Peanut Agglutinin (PNA). Three maculae were stained with polyclonal antibodies against laminin and collagen type-IV.

Results. BLD was exclusively stained by DBA and SBA whereas Con A, WGA, UEA-I, RCA-I and HNK-1 stained various other structures in the human macula as well. The main part of the BLD adjacent to Bruch's membrane stained with these lectins and the monoclonal antibody HNK-1, whereas only a small part of the BLD adjoining to the RPE stained with antibodies against laminin and collagen type-IV. Drusen did neither stain with any lectin nor with any antibody.

Conclusions. DBA and SBA, which bind specifically to an α-D-GalNAc moiety, are specific markers for the light-microscopical detection of BLD in human macular tissue. Furthermore, we conclude that BLD contains several carbohydrate structures other than the carbohydrate moieties on laminin and collagen type-IV. If drusen contain carbohydrate structures, these must be different from those in BLD.

Introduction

Age-related macular degeneration was first described by Haab in 1888 as an age-related abnormality of the macula lutea, affecting central vision. The aging human macula shows varying degenerative histopathological changes, e.g. the presence of drusen (Green, et al., 1985; Sarks, 1976; van der Schaft, et al., 1992), thickening of Bruch's membrane (Green, et al., 1985; Sarks, 1976; van der Schaft, et al., 1992; Young, 1987), accumulation of lipofuchsin in the retinal pigment epithelium (RPE) (Young, 1987), and basal laminar deposit (BLD) (Löffler and Lee, 1986; Sarks, 1976; van der Schaft, et al., 1992; van der Schaft, et al., 1994). The amount of histologically detectable BLD, an extracellular material, which accumulates between the RPE and Bruch's membrane, is considered to be an indicator of the degree of photoreceptor degeneration and has been shown to correlate positively with visual loss (Sarks, 1976). Little is known about the origin or the composition of BLD (Green, et al., 1985; Löffler and Lee, 1986; Sarks, 1976; van der Schaft, et al., 1992; van der Schaft, et al., 1994; Young, 1987). It has been assumed that BLD consists mainly of basement membrane material such as the glycoproteins laminin and collagen type-IV, although no formal proof has been given (Marshall, et al., 1992; Sarks, et al., 1988; Sarks, 1976; van der Schaft, et al., 1992; van der Schaft, et al., 1994; Young, 1987). Besides the carbohydrate structures on laminin and collagen type-IV, basement membranes contain many other glycoconjugates (Kefalides, 1973).

The aim of this investigation was to analyze carbohydrate structures in BLD and to find a specific marker for BLD, using histochemical and immunohistochemical techniques. Accordingly, we tested if BLD reacts with lectins with varying carbohydrate-affinities like α-D-GalNAc (DBA and SBA), α-D-Mannose (CON A), ß-D-GlcNAc (WGA), NeuNAc (WGA), ß-D-Gal(1-3)-D-GalNAc (PNA), α-L-Fucose (UEA-I) and ß-D-Gal > α-D-Gal (RCA-I). We also checked the monoclonal antibody HNK-1, which reacts with GlcUAβ1-3Galβ1-4GlcNAc moieties (Chou, et al., 1985; Kruse, et al., 1984; Pesheva, et al., 1987).

Materials and Methods

Preparation of Macular Tissue

From a large series of human eyebank eyes, used in previous studies on age-related macular degeneration (van der Schaft, et al., 1992; van der Schaft, et al., 1994), we selected 13 maculae of 10 patients aged between 85 and 98 years (mean 91) on the presence of relatively large amounts of BLD, class 2 and 3. Class 2 resembles a thin continuous layer and class 3 a thick layer of at least half the height of the RPE (van der Schaft, et al., 1992). We didn't

use class 1 (small solitary patches of BLD), because the amount of BLD in class 1 is too less to adequately illustrate the staining of BLD with the lectins and HNK-1. Although the specimens were also examined by electron microscopy in previous studies, we did not use these data to confirm the presence of BLD, but made a new series of consecutive paraffin sections to be sure of the occurrence of BLD in all section. BLD was histologically demonstrated by Mallory staining. Time between death and fixation ranged from 4.5 to 12 hours. After enucleation the macula was dissected from the globe and hemisectioned in the direction of the optic nerve. Both halves were fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.4, for 24 hrs at room temperature) and embedded in paraffin for histochemical and immunohistochemical studies. In the authors' opinion, methods for securing human tissue were humane, included proper consent and approval, and complied with the Declaration of Helsinki.

Light Microscopy

Paraffin sections (5 μm) were mounted on 3-amino-propyl-tri-oxy-silane (AAS) (Sigma, St. Louis, USA) coated glass slides. After deparaffinization and rehydration they were rinsed with water and phosphate-buffered saline (PBS). The slides were placed in a Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor Rancorn Cheshire, England), and incubated for one hour with either the monoclonal antibody HNK-1 (American Tissue Type Culture Collection, 1:10 dilution) (Abo and Balch, 1981), or one of the seven biotinylated lectins mentioned below. These lectins were Canavalia Ensiformis (ConA, Concanavalin A, Lot 80718), Glycine Max (SBA, Soybean Agglutinin, Lot 70326), Triticum Vulgaris (WGA, Wheat Germ Agglutinin, Lot A0201), Dolichos Bifloris (DBA, Lot 80705), Ulex Europaeus (UEA-I, Agglutinin I, Lot 80713), Ricinius Communis Agglutinin I (RCA-I, Lot 80721) and Arachis Hypogaea (PNA, Peanut Agglutinin, Lot 80629). All were commercially obtained (Vector-laboratories, Burlingame, USA) and were used at a concentration of 20 μg/ml PBS. After washing with PBS and incubation for 30 minutes with alkaline phosphatase (AP) conjugated streptavidin (Biogenex, San Ramon, USA, dilution 1:50), the slides were rinsed with PBS and finally with 0.2 M Tris-HCl pH 8.0 before staining for 30 minutes with 0.3% New Fuchsin/Tris-HCl (Sigma, St. Louis, USA). The sections were counterstained with Mayer's hematoxylin for 15 seconds, rinsed for 10 minutes with water and air-dried. In case of incubation with the HNK-1 monoclonal antibody, the slides were incubated for 30 minutes with biotinylated secondary antibodies (Multilink, 1:100 dilution, from Biogenex, San Ramon, USA), before incubation with AP-conjugated streptavidin. Other steps were identical as described for lectin staining. In negative controls the lectins were

replaced by PBS or incubated with a specific blocking sugar (0.2 M α-D-GalNAc for DBA and SBA) for 15 minutes before application to the tissue sections. The monoclonal antibody HNK-1 was replaced by normal mouse serum (DAKO, Glostrup, Denmark, dilution 1:10). We also used macular parts with no BLD as internal negative control. RPE served as internal positive control for ConA and WGA, blood vessels of the choroid were positive controls for UEA-I and RCA-I and ganglion cells of the human retina for HNK-1. Human kidney-tissue, which is known to contain epitopes for all the used lectins (Laitinen, et al., 1987; Leathem and Atkins, 1983), was used as external positive control for SBA, DBA and PNA.

Consecutive slides, from three maculae with BLD class 3, were also stained with antibodies against laminin and collagen type-IV according to a previous study (van der Schaft, et al., 1994).

Results

The binding of a series of lectins and the monoclonal antibody HNK-1 with BLD in thirteen human maculae is shown in Table 1. BLD stained positive with WGA and DBA in all specimens. In twelve out of the thirteen maculae BLD stained with HNK-1. The main part of the BLD strongly stained near Bruch's Membrane and the coloring fainted toward the RPE (Figure 4.1); this was the case with all lectins used (except for PNA) and also with the monoclonal antibody HNK-1. We did not find any difference between the binding of BLD class 2 or 3. PNA neither stained BLD nor any other structure in the macula.

Of the structures in the posterior part of the human eye, BLD stained exclusively positive with DBA and SBA (Table 2, Figures 1-3). However, in three maculae the media of the blood vessels, e.g. in the retina and choroid, also stained with DBA and SBA. These blood vessels in the same eyes also stained with HNK-1. Besides BLD, Con A, WGA, UEA-I, RCA-I and HNK-1 stained also varying structures of the retina (e.g. basal membranes, photoreceptors), choriocapillaris, optic nerve and/or RPE (Table 2, Figure 4.4).

Hard and soft drusen, which were found in 5 of 13 specimens, did neither stain with any of the lectins nor with the monoclonal antibody HNK-1. All positive controls were affirmative. Negative controls revealed no specific staining. No change in staining properties were seen in parts of the maculae were no BLD was present.

Corresponding to the results of Van der Schaft et al (van der Schaft, et al., 1994) only a small part of the BLD near the RPE in one macula (out of 3) stained positive with antibodies against laminin (Figure 4.5A) and collagen type-IV (not shown, but almost identical with Figure 4.5A).

Table 1. Staining Characteristics of Basal Laminar Deposit in Thirteen Human Maculae.

	Macula no.	1	2	3	4	5	6	7	8	9	10	11	12	13
	BLD class	2	3	2	2	2	3	2	2	2	2	2	3	3
	Specificity													
CON A	α-D-mannose	+	+	+	+	-	±	-	+	+	+	+	-	+
SBA	α-D-GalNAc	±	+	+	+	-	-	+	-	+	-	+	±	±
WGA	ß-D-GlcNAc and NeuNAc	+	+	+	+	+	+	+	+	+	+	±	+	±
DBA	α-D-GalNAc	±	+	+	+	+	+	+	+	+	+	+	+	+
UEA-I	α-L-Fucose	-	+	+	-	-	-	-	+	-	-	-	-	-
RCA-I	ß-D-Gal > α-D-Gal	-	±	+	-	-	-	-	-	-	-	-	-	±
PNA	ß-D-Gal(1-3)-D-GalNAc	-	-	-	-	-	-	-	-	-	-	-	-	-
HNK-1	GlcUAβ1-3-Galβ1-4GlcNAc	+	+	-	+	+	+	+	+	+	+	+	+	+

Staining: + = positive, ± = faintly positive, - = negative.

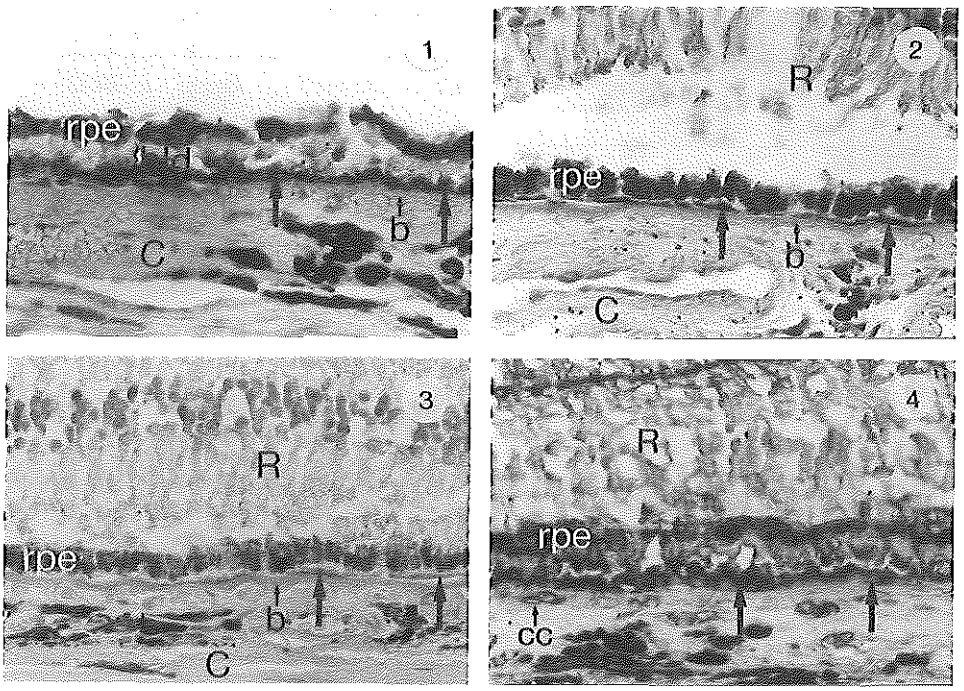

Figure 4.1. Section of macula with BLD class 3 stained with Dolichos biflorís (DBA) (magnification x400). Note the diminishing staining (large arrows) of the BLD toward the retinal pigment epithelium (rpe). C = choroid, b = Bruch's membrane.

Figure 4.2. Section of macula with BLD class 2 stained with Dolichos biflorís (DBA) (magnification x400). The BLD is stained exclusively (large arrows) in the human macula. C = choroid, R = retina, b = Bruch's membrane, rpe = retinal pigment epithelium.

Figure 4.3. Section of macula with BLD class 2 stained with Soybean agglutinin (SBA) (magnification x400). The BLD is stained exclusively (large arrows) in the human macula. C = choroid, R = retina, b = Bruch's membrane, rpe = retinal pigment epithelium.

Figure 4.4. Section of macula with BLD class 3 stained with Wheat germ agglutinin (WGA) (magnification x400). Besides the BLD (large arrows), some parts of the retinal pigment epithelium (rpe), retina (R) and choriocapillaris (cc) also stain positively. C = choroid.

Table 2. Lectin and HNK-1 Binding in the Human Eye (N=13)

Lectin	Bruch's Membrane	Choroid*	RPE*	Retina*	Optic Nerve*	BLD
CON A	-	-	+ (13)	+ (13)	+ (13)	+ (10)
SBA	-	-	-	-	-	+ (9)
WGA	-	+ (13)	+ (13)	+ (13)	+ (13)	+ (13)
DBA	-	-	-	-	-	+ (13)
UEA-I	-	+ (13)	-	-	-	+ (3)
RCA-I	-	+ (9)	+ (7)	+ (4)	-	+ (3)
PNA	-	-	-	-	-	-
HNK-1	-	-	+ (13)	+ (13)	+ (13)	+ (12)

*Staining: + = positive, - = negative. Between parentheses: number of positive specimens. RPE = retinal pigment epithelium. BLD = basal laminar deposit. See text for lectin abbreviations. *: Varying parts in this structure stained.*

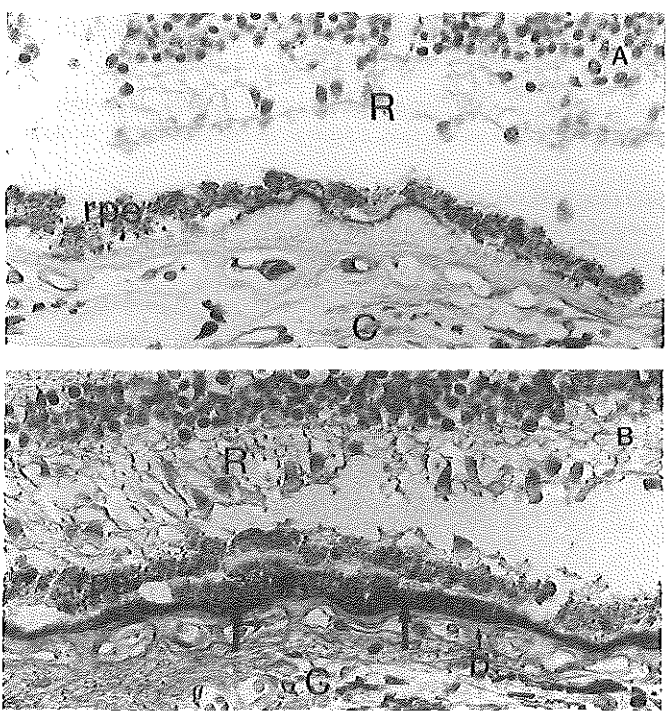

Figure 4.5. Section of macula with BLD class 3. (A) Stained with antibodies against laminin (magnification x400). About 10% of the BLD adjacent to the retinal pigment epithelium (rpe) stained (large arrows). (B) Section of same macula stained with Dolichos biflorus (DBA) (magnification x400). About 90% of the BLD (large arrows) adjacent to Bruch's membrane (b) is stained. R = retina, C = choroid, cc = choriocapillaris.

Discussion

Our results indicate that BLD contains different carbohydrate moieties with varying expression. The most consistent accessible carbohydrate residues in BLD were α-D-GalNAc, ß-D-GlcNAc, NeuNAc and the HNK-1 epitope. Variability in lectin binding is probably due to other carbohydrate epitopes on the same or adjacent glycoconjugates, which can enhance or inhibit the binding of lectins (Kivelä and Tarkkanen, 1987; Wu, 1984). It is also possible that carbohydrate structures in BLD are slightly different from one patient to another, due to enzymatic digestion or modification.

BLD was exclusively stained by DBA and SBA. The positive staining of the blood vessels in three specimens can be related to blood group A as reported by Kivelä and Tarkkanen (Kivelä and Tarkkanen, 1987). In our study these clinical data were not available. None of the hard and soft drusen seen in our specimens stained with either the lectins or the monoclonal antibody HNK-1. Other studies on BLD using antibodies against laminin, collagen type-IV and heparan sulphate proteoglycan also reported no staining of drusen with these antibodies (Marshall, et al., 1992; van der Schaft, et al., 1994).

Ultrastructurally the main components of BLD show similarity with fibrous long-spacing collagen (FLSC) and a homogeneous material with the same electron density as basement membranes (Löffler and Lee, 1986; van der Schaft, et al., 1992). FLSC is a polymer of collagen, with a banded ultrastructure and a periodicity between 100 and 120 nm, which in vitro precipitates in the presence of chondroitin sulphate (Chapman and Armitage, 1972). It has been demonstrated that BLD reacts with antibodies against basement membrane elements, like collagen type-IV, heparan sulphate proteoglycans and laminin (Marshall, et al., 1992; van der Schaft, et al., 1994). The basal extracellular matrix of the normal RPE in vivo and in vitro is also positive for antibodies against basal membrane elements (Campochiaro, et al., 1986). However, the positive staining with antibodies against laminin and collagen type-IV occurred only in the homogeneous part of the BLD in a small rim adjacent to the RPE cell surface (Figure 4.5A) (van der Schaft, et al., 1994). The FLSC did not react with any of the above mentioned antibodies against basement membranes (van der Schaft, et al., 1994). In contrast the lectins and the monoclonal antibody HNK-1 in our study were bound to the main part of the BLD, and this was most prominent near Bruch's membrane (Figure 4.5B). Unfortunately, it is impossible to distinguish between the FLSC and the homogeneous part of BLD with light-microscopy. However, the relatively thick layer of positive staining with the lectins and the monoclonal antibody HNK-1 seems to be suggestive for the FLSC component of BLD. Further electron microscopical investigation is required to support this concept.

It has been demonstrated that lipids accumulate in Bruch's membrane in the aging human macula (Pauleikhoff, et al., 1990). However, it is unlikely that BLD consists of glycolipids because processing to wax dissolves out carbohydrates linked to fat (Leathem and Atkins, 1983; Wirbel, et al., 1984). Therefore, we assume that BLD contains carbohydrate structures that are most likely part of glycoproteins or part of complex carbohydrate molecules. We also conclude that the main part of BLD contains several carbohydrate structures different from carbohydrate structures on laminin and collagen type-IV.

In conclusion we found that BLD contains α-D-GalNAc, which in our study was not detectable in other structures of the macular tissue. Therefore DBA or SBA can be used as a specific marker for the detection of BLD. Further analysis of the composition of BLD associated carbohydrate structures may provide information about the structure and origin of BLD in age-related macular degeneration.

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CHAPTER 5

Protein Analysis of Human Maculae in Relation to Age-Related Maculopathy

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ABSTRACT

BACKGROUND: Age-related maculopathy (ARM) is the most common cause of blindness in the elderly in the western world. Its early stage is characterized by many histopathologic changes, including two extracellular deposits, basal laminar deposit (BLD) and drusen. Since the origin and chemical composition of BLD and drusen are unknown and are considered to be important in the development of ARM, we analyzed proteins in human macular tissue associated with ARM.

EXPERIMENTAL DESIGN: Homogenized macular extracts of 15 human eyes with ARM, and 10 age-matched control eyes, were examined by two-dimensional electrophoresis. The proteins in the gels were silver stained and the obtained protein-patterns were analyzed by a computer-imaging system.

RESULTS: Five glycoproteins were specifically present in human maculae with ARM ($p=0.0009$). One of the spots was characterized by sequence analysis as haptoglobin β-chain, another had a high homology with a part of the interphotoreceptor retinoid-binding protein precursor. However, the 100 % matching of the latter was not statistically significant, because we could only sequence eight amino acids of this protein.

CONCLUSIONS: The known association between haptoglobin β-chain and atherosclerosis and the increase of this glycoprotein in human maculae with ARM supports the recently described relationship between atherosclerosis and ARM found in an epidemiologic study. Furthermore, the neovascular growth stimulating properties of haptoglobin warrant further research into haptoglobin as a possible inducing agent of late stages of ARM.

Introduction

Age-related maculopathy (ARM) embraces many different histopathologic and morphometric changes in the central part of the human retina such as the accumulation of lipofuscin in the retinal pigment epithelium (RPE), atrophy of the choriocapillaris, thickening and calcification of Bruch's membrane, drusen, and basal laminar deposit (BLD) (Feeney Burns, et al., 1980; Killingsworth, 1987; Kliffen, et al., 1995; Löffler and Lee, 1986; Ramrattan, et al., 1994; Sarks, et al., 1988; Sarks, 1976; van der Schaft, et al., 1992b). Drusen and BLD are both extracellular deposits, which are easily observed by light-microscopy. The former are located within Bruch's membrane, the latter between Bruch's membrane and the RPE. The late stages of ARM, called age-related macular degeneration (AMD), appear in two forms, geographic atrophy and neovascular AMD. The RPE and consequently the photoreceptor cells disappear in geographic atrophy (Bressler, et al., 1988; Kliffen, et al., 1995; Maguire and Vine, 1986; Sarks, et al., 1988). In neovascular or "exudative" AMD, new vessel ingrowth from the choriocapillaris into the subretinal space is observed (Bressler, et al., 1988; Green, et al., 1985; Kliffen, et al., 1995; Young, 1987). Both forms of AMD can severely affect central vision and are the main cause of blindness in the elderly in the western society (Klein and Klein, 1982; Leibowitz, et al., 1980; Vingerling, et al., 1995b). The histopathology of this disease is well described, but the information on its pathogenesis is still incomplete (Bressler, et al., 1988; Green, 1985; Green, et al., 1985; Kliffen, et al., 1995; Sarks, et al., 1988).

Large drusen and BLD (and the chemical composition of these deposits) are assumed to be important determinants in the pathogenesis of AMD (Bird, 1992; Gass, 1973; Green, et al., 1985; Pauleikhoff, et al., 1990a; Sarks, 1976; Sheraidah, et al., 1993; van der Schaft, et al., 1992a). It is known that drusen contain lipids (Grossniklaus, et al., 1992; Killingsworth, et al., 1990; Pauleikhoff, et al., 1992) and probably also proteins (Bird, 1992). In a previous study we proved that BLD contains carbohydrate structures most likely part of glycoproteins (Kliffen, et al., 1994). To the best of our knowledge, there is no information on the protein components of age-related structures in human maculae. Therefore, we compared in this study the protein composition of ARM maculae with those of healthy (age-matched) human maculae.

Experimental Design

Preparation of Human Macular Tissue

We obtained 25 human eye bank eyes from 16 subjects aged between 46 and 91 years of age (mean age 78 years), with post-mortem times from 5 to 15 hours. From these eyes one cm^2 with the macula in the middle was cut out. This piece of tissue was horizontally divided through the fovea in the direction of the optic nerve head in two equal parts. From one half the choroid and retina together were separated from the sclera and vitreous and subsequently frozen and stored in liquid nitrogen until further use. The other half was fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.4, for 24 hrs at room temperature). The formalin fixed part was embedded in paraffin for histochemical classification (van der Schaft, et al., 1992b). Serial paraffin sections (5 μm) were stained with HE, PAS, and Mallory staining. The maculae were classified according to their histological gradation of BLD (class 0 to 3) and drusen (class 0 to 3) (van der Schaft, et al., 1992b). In brief: BLD class 0, resembled no BLD; class 1, small solitary patches of BLD; class 2, a thin continuous layer of BLD; class 3, a thick layer of BLD of at least half the height of the RPE cells. Drusen class 0, resembled no drusen; class 1, 1 to 3 drusen; class 2, 4 to 10 drusen; class 3, many or confluent drusen (van der Schaft, et al., 1992b). Since no great disturbances in the architecture of the maculae are present in maculae with both BLD class 0 or class 1, and drusen class 0, these eyes were categorized as control samples. All other maculae were classified as samples with ARM. The histological classification of ARM maculae is consistent throughout a macula (van der Schaft, et al., 1992b). Therefore, it was assumed that one half of a macula is representative of a whole macula. Since there was no clinical data available on the donor tissues, the classification was fully based on the histological sections.

Two-dimensional electrophoresis

The choroid and retina, frozen in liquid nitrogen, were defrosted and homogenized together. Two-dimensional electrophoresis of this material was performed, and the proteins in the gels were subsequently visualized with a silver staining technique. The M_r and iso-electric point (pI) values of the proteins were calculated by a computer-imaging system. Finally, N-terminal amino-acid sequences of the proteins of interest were determined.

Results

Fifteen eyes of nine subjects aged between 46 and 91 years (mean age 79 years; standard deviation 13 years) were histologically classified as having ARM (subjects no. 8 to 16, Table

1A and 1B). There were no late stages of ARM included in this study. Ten eyes of seven subjects aged between 62 and 83 years (mean age 77 years; standard deviation 8 years) were classified as healthy (subjects no. 1 to 7, Table 1A and 1B). The mean age of both groups was not significantly different (p=0.74).

Table 1A. Proteins (P1 to P5) Related To ARM (Left Eyes of Subjects).

Subject	pm[a]	age	drusen[b]	BLD[c]	P1	P2	P3	P4	P5
1	15	68	0	0	±	±	±	-	-
2	5	82	0	0	±	±	+	-	-
3	9.5	62	0	0	±	±	±	-	-
4	8	81	0	0	-	-	-	-	-
5	6.5	83	0	1	±	±	±	-	-
6	8	79	0	1	+	±	±	-	-
7	7	82	0	1	±	+	±	-	-
8	14	83	0	2	+	++	+	±	-
9	5	86	0	3	+	++	++	++	±
10	12	74	1	0	++	+++	+++	++	+
11	6	46	1	0	+++	+++	+++	++	+
12	10	82	2	1	+	++	+++	+++	+++
13	9	79	2	2	±	+	+	+	±
14	10	91	3	3	+++	+++	+++	++	++
15	9	79	3	3	++	+++	+++	++	+
16	14	88	3	3	++	+++	+++	++	±

The first seven subjects were, prior to the electrophoresis, histologically classified as healthy, the other nine subjects were classified as having ARM. The relative amounts of P1 to P5 in the individual maculae are expressed in this table.

Staining was determined in comparison to an internal control (albumin) and total protein content. - = absent, ± = just visible, + = distinct staining, ++ = strong staining, +++ = very strong staining.

[a] *Post-mortem time (pm) in hours.*

[b] *Drusen class 0 to 3. See materials and methods for explanation.*

[c] *Basal laminar deposit (BLD) class 0 to 3. See materials and methods for explanation.*

Table 1B. Proteins (P1 to P5) Related To ARM (Right Eyes of Subjects).

Subject	pm[a]	age	drusen[b]	BLD[c]	P1	P2	P3	P4	P5
2	5	82	0	0	±	±	±	-	-
3	9.5	62	0	0	+	+	+	+	-
5	6.5	83	0	1	±	±	±	-	-
8	14	83	0	2	++	+++	++	±	-
11	6	46	1	0	++	+++	+++	++	+
12	10	82	2	0	+	+++	+++	+++	++
14	10	91	3	3	+++	+++	+++	++	++
15	9	79	3	3	+++	+++	+++	+++	++
16	14	88	3	3	+	++	++	+	±

Subject 2, 3, and 5 were classified as healthy, subject 8, 11, 12, 14, 15, and 16 were classified as having ARM. The relative amounts of P1 to P5 in the individual maculae are expressed in this table.

Staining was determined in comparison to an internal control (albumin) and total protein content. - = absent, ± = just visible, + = distinct staining, ++ = strong staining, +++ = very strong staining.

[a] *Post-mortem time (pm) in hours.*

[b] *Drusen class 0 to 3. See materials and methods for explanation.*

[c] *Basal laminar deposit (BLD) class 0 to 3. See materials and methods for explanation.*

For comparison of the protein composition of ARM maculae and control maculae we used only one eye of each subject (n=16, Table 1A). This was always the left eye in case we had utilized two eyes of one subject in our experiments.

There was a substantial amount of five proteins (P1 to P5) in human maculae with BLD (class 2 and 3) and/or drusen (Figure 5.1A, Table 1A). These five proteins were scarce or absent in all control maculae (Figure 5.1B, Table 1A; p=0.0009). We could not find any differences in the amount and the composition of the proteins between the left and right eye of one subject (n=9, r=0.74, p=0.02). The amounts of protein P1 to P5 in the right maculae of these donors are listed in Table 1B. The use of albumin (Figure 5.1A) as an internal standard for blood content shows that the increase of the five proteins was not due to small differences in composition of the macular samples, for instance caused by varying amounts of blood in the choroid.

The five proteins were characterized by two-dimensional electrophoresis as follows:

Protein spot 1 (P1) with M_r 38900 and pI 6.25; protein spot 2 (P2) with M_r 39200 and pI 6.14; protein spot 3 (P3) with M_r 40200 and pI 6.02; protein spot 4 (P4) with M_r 41800 and pI 5.82; and protein spot 5 (P5) with M_r 42600 and pI 5.61. No difference in the amount of these proteins was found between maculae with either BLD (class 2 and 3) or drusen nor in maculae with both these deposits.

With sequence analysis we found 93% (14/15) identity in the first 15 N-terminal amino-acids of P3 (Table 2) compared to first 15 N-terminal amino-acids of the β-chain of haptoglobin with a p-value of 10^{-5}. Furthermore, we found 88% (7/8) identity in 8 N-terminal amino-acids of P2 (Table 2) compared to the IRBP precursor (amino-acids 942 to 949) with a p-value of 0.93. We could not detect the mature IRBP (M_r 145000, pI 4.4 to 4.8) in any of the silver-stained gels. The first 21 N-terminal amino-acids of protein spot 6 (P6, Figure 5.1A, Table 2) showed a 90% (19/21) correspondence with the N-terminal amino-acid sequence of the β-chain of creatine kinase with a p-value of 10^{-5}. This protein was present in all maculae and its M_r (43800) was used as an internal molecular weight marker. P1, P4, and P5 could not be sequenced (Table 2). All five protein spots were N-linked glycoproteins as revealed with N-glycanase digestion of the macular samples (Figure 5.2).

Apart from the mentioned five proteins, the protein patterns of extracts of healthy maculae and maculae with ARM were highly reproducible and almost identical to each other (Fig 1A, 1B). Also the inter-assay and intra-assay results were highly reproducible for one sample. The protein patterns from maculae with higher post-mortem times of 10 to 15 hours (6 samples) and maculae with post-mortem times up to 10 hours (19 samples) appeared to be similar; thus, we included all samples in our results (Table 1A and 1B). These patterns were assessed both visually and by computer-assisted scanning of the gels.

Staining of tissue sections with antibodies against haptoglobin (both α-chain and β-chain) revealed specific staining in the lumina and the innermost layer of the blood vessels in both maculae with ARM and control maculae. There was no difference in the anti-haptoglobin histopathologic staining pattern between control maculae and maculae with ARM. However, a quantification of haptoglobin content is not possible with this technique. Neither drusen nor BLD stained with antibodies against haptoglobin.

Staining with antibodies against IRBP showed specific staining of the inter-photoreceptor matrix in all samples, but no specific staining was observed in drusen or BLD.

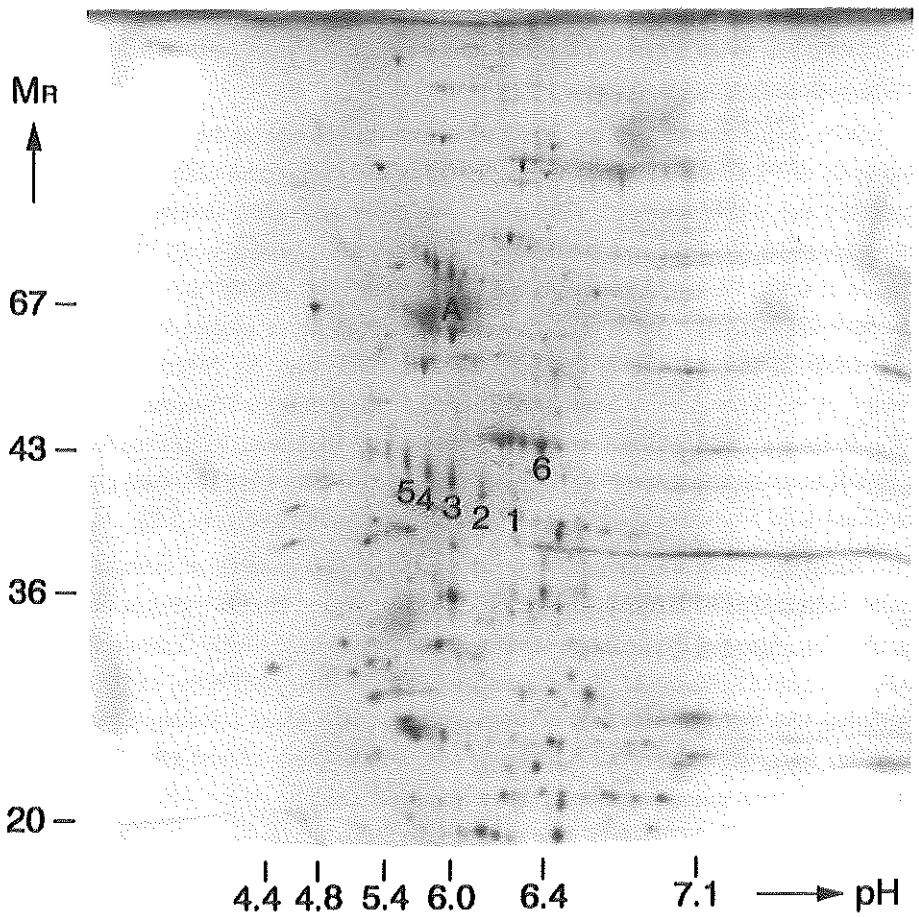

Figure 5.1A. Two-dimensional electrophoresis of proteins in human macular tissue with age-related maculopathy. Note the presence of five protein spots (P1 to P5) that do not (or slightly) appear in healthy human maculae (Figure 5.1B). Protein spot 6 (Creatine-kinase, Table 2) and A (Albumin, Table 2) were used as internal calibration markers. Macular tissue from subject number 12 (Table 1A) was used for this figure.

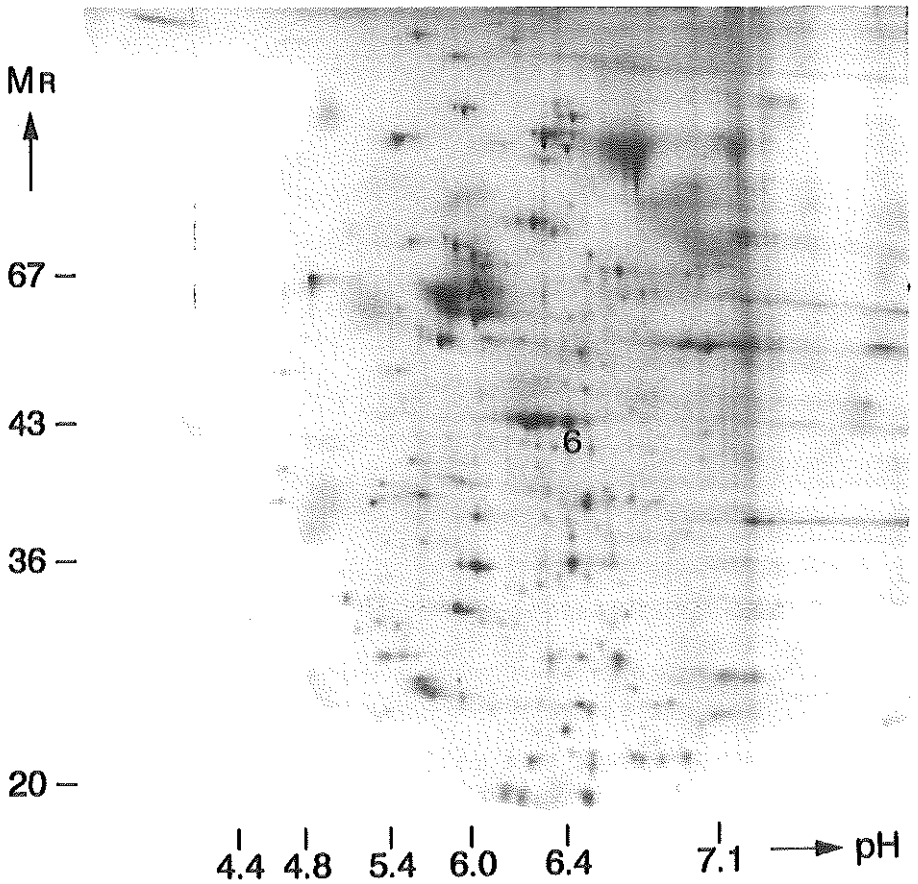

Figure 5.1B. Two-dimensional electrophoresis of proteins in healthy human macular tissue. Proteins P1 to P5 are not expressed at all, whereas all the other protein spots are comparable with those in ARM maculae (Figure 5.1A). Macular tissue from subject number 4 (Table 1A) was used for this figure.

Table 2. Sequence Analysis of N-Terminal Amino-Acids.

Protein	M_r (x 10^3)[a]	pI[a]	Description	N-terminal sequence
P1	38.9	6.3	?	-
P2	39.2	6.1	IRBPP	PHRQxPRA
P3	40.2	6.0	Hp β-chain	ILGGHLDAKGxFPWQ
P4	41.8	5.8	Hp β-chain[b]	-
P5	42.6	5.6	Hp β-chain[b]	-
P6	43.8	6.4	Creatine-kinase	PFxNxHNALKLR-FPAEDEFPD
A	63.0	6.0	Albumin[b]	-

Glycoproteins P1 to P5 were specifically present in human maculae with ARM. Protein P6 (creatine-kinase) was used as internal calibration marker for molecular weight and protein A (albumin) was used as internal calibration marker for protein content.

Hp = haptoglobin.

IRBPP = part of interphotoreceptor retinoid-binding protein precursor

x = Non determinable amino-acid (most likely serine).

[a] *Compared to M_r of molecular weight standards and iso-electric point (pI) of GAD3PDH carbamalytes.*

[b] *Concluded after comparison with plasma and red blood cell protein maps (Golaz, et al., 1993).*

Discussion

In this study the protein composition of human maculae was determined. Since the total protein amount of one human macula is only about 1 mg and fresh human donor eyes are hard to obtain, it is obvious that techniques with a high resolution capacity had to be used. With the silver staining technique nanograms of proteins can be detected. This implies that we can only detect the main proteins in the tissue with this technique and any change in minor proteins can not be detected. About 500 proteins were analyzed by the computer-imaging system.

A significant increase in the expression of five proteins in human maculae with either BLD (class 2 and 3) or drusen was observed compared to control maculae (Figure 5.1A and 5.1B, Table 1A). Consequently, these proteins are suspect to be related to ARM. However, it could not be determined whether any of these five proteins were significant components of drusen or BLD. They might have originated from histologically less obvious changes that

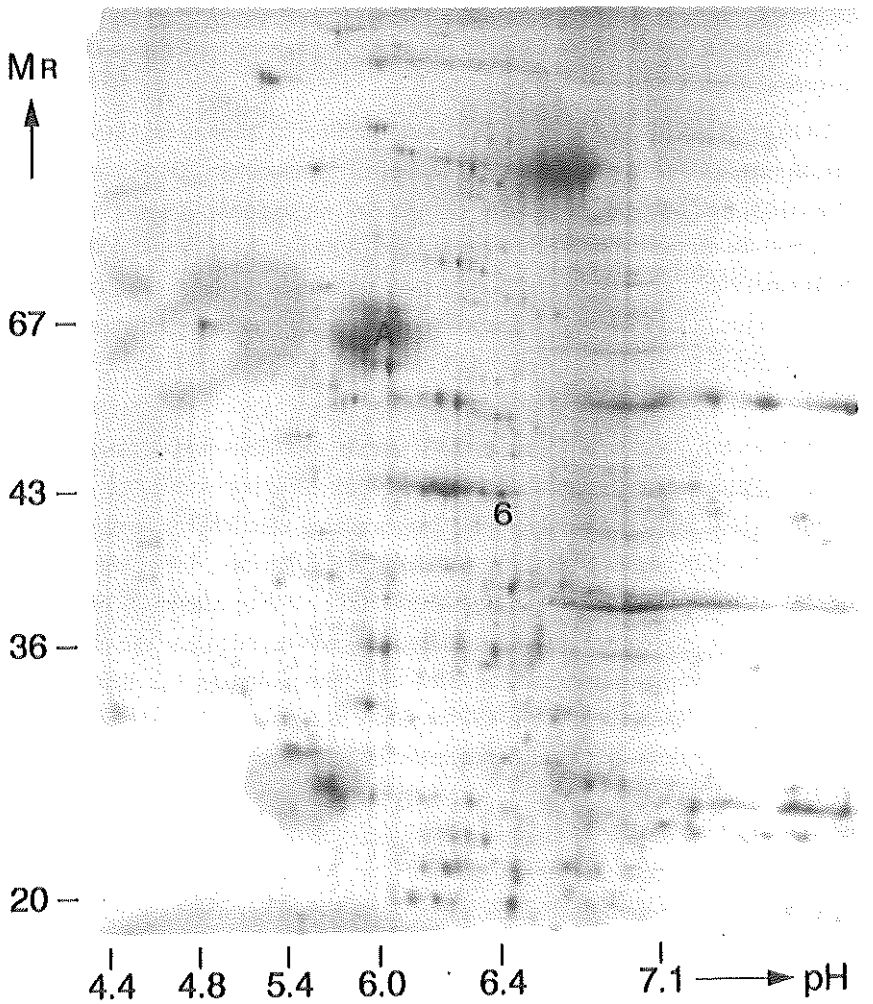

Figure 5.2. Two-dimensional electrophoresis of proteins in human macular tissue with age-related maculopathy after treatment with N-Glycanase. The same macular tissue was used as in Figure 5.1A. N-Glycanase removes the asparagine linked carbohydrate side-chains from glycoproteins P1 to P5. Consequently, the M_r and pI of the glycoproteins were changed. Note that the five proteins specifically present in human maculae with ARM (P1 to P5, Figure 5.1A) totally have disappeared.

coincide with ARM, e.g. in the choriocapillaris or in the outer retina. Because the staining intensity of P1 to P5 was stronger in maculae with drusen class 1 and no BLD (subjects 10 and 11, Table 1A) than in maculae with BLD class 2 or class 3 (subjects 8 and 9) and no drusen, it could be speculated that drusen are of more importance than BLD in respect of these proteins (Figure 5.2).

Glycoprotein P3 had a 93% identity with the first 15 N-terminal amino-acids of the β-chain of haptoglobin ($p=10^{-5}$). The M_r and pI of glycoprotein P3 (Table 2) were both identical with the M_r and pI of the β-chain of haptoglobin (Golaz, et al., 1993). Consequently, we characterized this protein as haptoglobin β-chain. Haptoglobin is a carrier for free hemoglobin in the plasma and prevents the heme to be oxidative and the hemoglobin to be wasted through the kidneys. Haptoglobin is known to be anti-oxidative and is also one of the acute phase proteins (Halliwell and Gutteridge, 1990). Furthermore, haptoglobin is assumed to be angiogenic (Cid, et al., 1993).

Haptoglobin β-chain is found in fatty streaks and fibro-fatty lesions in the aortic intima in atherosclerosis (Stastny and Fosslien, 1992). So it is possible that the increase in haptoglobin β-chain found in this study might be correlated with atherosclerosis. With aging, there is an increase in calcifications (Feeney-Burns and Ellersieck, 1985; van der Schaft, et al., 1992b) and lipids (Bird, 1991; Pauleikhoff, et al., 1990b; Sheraidah, et al., 1993) in Bruch's membrane, the morphologic boundary between the retina and the choriocapillaris. Recently, also a relationship between ARM and atherosclerosis has been found in an epidemiologic study (Vingerling, et al., 1995a). These authors found an increased percentage of atherosclerotic plaques, but no differences in vascular thickness, in the aorta bifurcation and carotid artery in patients with ARM (personal communications). Furthermore, a relationship between ARM and sclerosis of the choriocapillaris has been found in a clinico-pathologic study (Sarks, 1976). In neovascular AMD new vessels grow from the choriocapillaris into the subretinal space. Since haptoglobin has a potential angiogenic role, and we have found haptoglobin in the blood vessels of the choroid, it might be that haptoglobin β-chain is one of the angiogenic factors in this process.

Glycoprotein P2 had a 88% identity with eight amino-acids of the interphotoreceptor retinoid-binding-protein (IRBP) precursor (Table 2). Normally, IRBP is only found at the apical side of the RPE in the interphotoreceptor matrix and is produced by the photoreceptor cells (Adler and Spencer, 1991; Liou, et al., 1991). Since the amino-acid sequence of P2 was not statistically significant when compared to the IRBP precursor, we could not conclude homology. Nevertheless, it could be feasible that there is an increased or mal-production, or a mal-metabolism of IRBP in ARM. A possible reason for an increased production could be

that much of the IRBP is bound to accumulating derivatives of retinol in the RPE (e.g. in lipofuscin granules) and is not available for its normal transport function. As a result of both hypotheses the photoreceptors will presumably produce more IRBP to preserve a sufficient concentration of IRBP in the interphotoreceptor matrix.

Haptoglobin β-chain and IRBP are both N-linked glycoproteins. With N-Glycanase treatment of the macula extracts, we proved that P1 to P5 are N-linked glycoproteins as well (Figure 5.2).

Glycoproteins P4 and P5 were either N-terminally blocked or the amounts were too small to investigate by sequence analysis. However, they are likely to be iso-forms of haptoglobin β-chain, because haptoglobin β-chain normally appears as three to eight closely related protein spots on two-dimensional electrophoresis (Golaz, et al., 1993). We were also unable to obtain a sequence of glycoprotein P1 for the same reasons, but have no clue to the identity of this protein.

In conclusion, we found a higher amount of five glycoproteins in human macular tissue with ARM than in age-matched control eyes. Little is known about the significance of these proteins in regard to ARM. However, the positive identification of one of these proteins as the haptoglobin β-chain and its relation with angiogenesis and neovascular growth, might fit in the concept about the pathophysiology of ARM.

Methods

Chemicals

All equipment, chemicals, and buffers were purchased from Millipore Corporation (Etten-Leur, The Netherlands), unless otherwise described.

Two-dimensional electrophoresis

The choroid and retina together frozen in liquid nitrogen were defrosted and homogenized on ice in 0.5 ml of 1 mM phenylmethylsulfonylfluoride/Milli-Q water (Sigma, St. Louis, MO), aliquoted in 25 μl and stored in a -80°C freezer.

Homogenized material ($\pm$ 50 μg of protein in 25 μl) was precipitated with 25 μl acetone for 20 minutes at -20 °C and centrifuged at 12,000 rpm for 5 minutes in an Eppendorf microcentrifuge at 4 °C. The pellet was solubilized with 25 μl of sample buffer A (0.3% SDS, 200 mM dithiotreitol, 28 mM Tris-HCL, and 22 mM Tris-base) and heated for 4 minutes at 90°C. Two-dimensional electrophoresis was performed with the Millipore

Investigator system. We used ampholytes with a pH range from 3-10 for the first dimension. For the second dimension 10 % Duracryl gels were used. Glyceraldehyde-3-phosphate dehydrogenase (GAD3PDH) carbamalytes (Pharmacia, Woerden, the Netherlands) and molecular weight standards (Gibco, Gaithersburg, USA) were used as iso-electric focussing and molecular-weight markers, respectively. Albumin was used as an internal reference for protein content. Proteins in two-dimensional gels were visualized with a silver staining technique. The M_r and iso-electric point (pI) values of the proteins were calculated by a Millipore computer-imaging system. All steps were according to the manufacturers' instructions.

Preparative two-dimensional electrophoresis

Preparative two-dimensional electrophoresis was performed to obtain enough purified protein for N-terminal amino-acid sequence analysis. Therefore, 250 μl of homogenized material ($\pm$ 500 μg of protein) was precipitated with cold acetone for 20 minutes and centrifuged at 12,000 rpm for 5 minutes in an Eppendorf microcentrifuge. The pellet was solubilized with 250 μl of sample buffer A and heated for 15 minutes at 60°C. First and second-dimension electrophoresis were performed as described above, according to the manufacturers' description. Additionally, we added 0.1 mM natriumthioglycolaat to the upper running buffer in the second dimension. After electrophoresis the gels were blotted on a PVDF membrane (Immobilon-P) and blots were subsequently stained with filtered (0.45 μm) Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO). Protein spots of interest were cut out and analyzed.

Amino-acid sequence analysis

N-terminal amino-acid sequences of proteins were determined on a protein-sequencing system (Model 473A, Applied Biosystems, Foster City CA, USA). A computer-assisted comparison of the N-terminal amino-acid sequences with known sequences in the Swiss Protein Data bank was made. To verify if the found matches were statistically significant, calculations of the p-values were made according to Karlin et al. (Karlin and Altschul, 1990).

Detection of N-linked Glycoproteins

To determine asparagine-linked glycoproteins, samples were treated with N-Glycanase (Genzyme Corporation, MA) prior to loading, according to Tarentino et al. (Tarentino, et al., 1985).

Immunohistochemistry

Because, results pointed to increased levels of haptoglobin and inter-photoreceptor retinoid binding protein (IRBP), paraffin sections of the maculae were examined for the presence of these proteins.

Paraffin sections (5 μm) were mounted on 3-amino-propyl-tri-oxy-silane (AAS; Sigma, St. Louis, MO) coated glass slides. After deparaffinization and rehydration they were placed in 3% H_2O_2/methanol for 25 minutes to eliminate endogenous peroxidase activity. The slides were placed in a Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor Rancorn Cheshire, England) and incubated for one hour with rabbit-polyclonal antibodies against human IRBP (gift from the Department of Ophthalmo-immunology of the Netherlands Ophthalmic Research Institute, Amsterdam) or with goat-polyclonal antibodies against human haptoglobin (Beckman, Fullerton, CA). Subsequently, the slides were incubated for 30 minutes with peroxidase-labeled swine-anti-rabbit or rabbit-anti-goat secondary antibodies (Dako, Glostrup, Denmark), respectively. The slides were rinsed with phosphate-buffered saline (PBS, pH 7,6) between all incubation steps. Finally, the slides were stained for 7 minutes with 3,3 diaminobenzidine (Fluka Chemica, Oud-Beijerland, The Netherlands) and counterstained with Mayer's hematoxylin.

Statistical analysis

The Student's t-test was used to compare the differences in age between the cases with ARM (n=9) and controls (n=7) in order to rule out any change in protein composition correlated with age.

Differences in amount of a specific protein between maculae with ARM and control maculae were analyzed by subdividing the amount of protein in two groups. Albumin was taken as an internal control for total protein content. No or just visible staining was arbitrarily called low concentration. The group with higher protein concentration was defined as those with distinct staining. Differences between these two groups were tested using Fisher's exact test.

The correlation of the protein composition between the left and right eye of the individual cases (n = 9) was tested with Spearman's rank correlation tests.

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CHAPTER 6

Identification of Glycosaminoglycans in Age-Related Macular Deposits

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ABSTRACT

Objective. To investigate the presence and localization of glycosaminoglycans (GAGs) in basal laminar deposit (BLD) and drusen in age-related maculopathy (ARM).

Methods. Conventional histological staining techniques, and monoclonal antibodies specific for several GAGs, were used on paraffin embedded human maculae. Furthermore, macular homogenates were analyzed with two-dimensional electrophoresis. Quantitative analysis of GAGs was done spectrophotometrically using dimethylmethylene blue.

Results. Immunohistochemically, all BLD stained positive for chondroitin-4-sulfate, and focally positive for heparan sulfate proteoglycan. Drusen did not stain with any of the monoclonal antibodies. With two-dimensional electrophoresis it was demonstrated that macular extracts, with and without ARM, contained chondroitin sulfate. Heparan sulfate was only expressed in maculae with ARM. The total amount of GAGs in macular extracts with BLD was significantly higher than in maculae without BLD ($p=0.001$).

Conclusions. Significant differences in amount and composition of GAGs, between maculae with and without ARM were demonstrated with these techniques. The possible consequences for the pathogenesis of ARM are discussed.

Introduction

The late stages of age-related maculopathy (ARM) can severely affect central vision and are the main cause of blindness in the elderly in the western world (Klein, et al., 1992; Leibowitz, et al., 1980; Vingerling, et al., 1995). Information on the pathogenesis of this disease is still incomplete (Bressler, et al., 1988; Green, 1985; Green, et al., 1985; Kliffen, et al., 1995; Sarks, et al., 1988). Recently, a classification system for ARM has been proposed for use in epidemiologic studies (Bird, et al., 1995). However, this classification does not cover the many histopathologic changes in the aging human macula (Feeney-Burns, et al., 1990; Green and Enger, 1993; Kliffen, et al., 1995; Pauleikhoff, et al., 1990b; van der Schaft, et al., 1992).

Drusen and basal laminar deposit (BLD) are both extracellular deposits accumulating in ARM. Hard nodular drusen are located external to the basement membrane of the retinal pigment epithelium (RPE). Soft drusen, and confluent drusen are located between the basement membrane of the RPE and the remainder of Bruch's membrane, or external to a diffuse thickening of the inner aspect of Bruch's membrane. Basal laminar deposit is located between the cell membrane of the RPE and its basement membrane (Green and Enger, 1993; Kliffen, et al., 1995). These macular deposits are assumed to be important determinants in the pathogenesis of the late stages of ARM, but their origin and chemical composition are mostly unknown (Bird, 1992; Green, et al., 1985; Kliffen, et al., 1995; Pauleikhoff, et al., 1990a; Sarks, 1976; Sheraidah, et al., 1993).

In previous studies we have demonstrated, that BLD consists partly of basement membrane material adjacent to the RPE (van der Schaft, et al., 1994), and contains carbohydrate structures most likely as components of glycoproteins or complex carbohydrate molecules (Kliffen, et al., 1995; Kliffen, et al., 1994). Glycosaminoglycans (GAGs) are large carbohydrate molecules of repeating disaccharides, mostly linked to proteins to form a proteoglycan. Proteoglycans occur in the extracellular matrix and on cell surfaces. In the latter situation they function in cell adhesion. In the extracellular matrix an important function of the hydrophilic proteoglycans is to retain water molecules and thus to provide tissue turgor. Proteoglycans function in that capacity in the maintenance of the vitreous (Berman, 1991). Proteoglycans provide an environment favorable for cell migration, as e.g. in angiogenesis (Berger, et al., 1982; Roden and Horowitz, 1978). Matrix growth factors as fibroblast growth factor, platelet derived growth factor, and transforming growth factor are bound to proteoglycans, especially heparan sulphate proteoglycan. Therefore, GAGs may play a role in the development of neovascular growth in the late stages of ARM.

The purpose of this study was to investigate the presence and localization of GAGs

in human maculae with and without early stages of ARM, in particular in eyes with drusen and BLD.

Materials and Methods

Preparation of Human Macular Tissue

We used 25 fresh human eye bank eyes from 17 subjects aged between 56 and 94 years of age (mean age 82 years), with post-mortem intervals between 5 and 14 hours (mean 9 hours). Each macular specimen (1 cm^2) was divided into two equal parts by cutting along its horizontal diameter. From one half the choroid and retina together were separated from the sclera and vitreous, subsequently frozen, and stored in liquid nitrogen until further use. The other half was fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.4), and embedded in paraffin for histopathological classification and (immuno-) histochemistry.

Histopathological Classification of Human Macular Tissue

Paraffin sections (5 μm) were stained with hematoxylin-eosin, the periodic-acid-Schiff reaction, and Mallory staining. The maculae were histologically classified according to their gradation of BLD and drusen as follows. No BLD (class 0); small solitary patches of BLD (class 1); a thin continuous layer of BLD (class 2); a thick layer of BLD of at least half the height of the RPE cells (class 3). No drusen (class 0); 1 to 3 drusen (class 1); 4 to 10 drusen (class 2); many or confluent drusen (class 3) (van der Schaft, et al., 1992). Since limited histologic changes are present in maculae with both BLD class 0 or class 1, and drusen class 0 or class 1, these eyes were categorized as control samples. All other maculae were classified as samples with ARM. No eyes with histologic geographic atrophy or neovascularization were included.

Histochemistry

Alcian blue at different acid concentrations (pH 0.2, 1.0, 2.5, and 3.2), and Hale's colloidal iron staining in the modification according to Müller were used to identify GAGs. In order to obtain more specific staining of the distinct GAGs with their different critical electrolyte concentrations (Scott and Dorling, 1965), Alcian blue staining was performed at pH 5.8 with increasing $MgCl_2$ concentrations (0.06, 0.1, 0.3, 0.7, and 1.0 M).

Immunohistochemistry

Paraffin sections (5 μm) of all maculae were mounted on 3-amino-propyl-tri-oxy-silane (Sigma, St. Louis, MO) coated glass slides. After deparaffinization and rehydration they were rinsed with water and phosphate-buffered saline. The slides were placed in a Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor Rancorn Cheshire, England), and incubated for 30 minutes with either a mouse-monoclonal antibody directed against chondroitin-4-sulfate, basement membrane heparan sulfate proteoglycan, or keratan sulfate disaccharides. These antibodies were commercially obtained (Chemicon International, Temecula, CA) and were used at 1:400, 1:10, and 1:3200 dilutions, respectively. After washing with phosphate-buffered saline, the slides were incubated for 30 minutes with biotinylated secondary antibodies (Multilink, 1:100 dilution, Biogenex, San Ramon, MO). The slides were washed again and incubated for 30 minutes with alkaline-phosphatase-conjugated streptavidin (Biogenex, San Ramon, MO, dilution 1:50). Finally the slides were rinsed with 0.2 M Tris-HCl pH 8.0, and stained for 30 minutes with 0.3% New Fuchsin/Tris-HCl (Sigma, St. Louis, MO). The sections were counterstained with Mayer's hematoxylin. Prior to the incubation with anti-chondroitin-4-sulfate, and anti-heparan sulfate proteoglycan antibodies, the sections were treated with 0.1 U/ml chondroitinase ABC (Sigma, St. Louis, MO) for 2 hours at 37 °C, and 5.5 mU/ml pronase E (Sigma, St. Louis, MO) for 10 minutes at 37 °C, respectively. In negative controls the primary antibodies were replaced by normal mouse serum (DAKO, Glostrup, Denmark, dilution 1:10). As a second negative control for anti-chondroitin-4-sulfate antibodies, chondroitinase ABC digestion was omitted prior to the incubation with the primary antibody. Photoreceptors and perineurium were used as internal positive controls for chondroitin-4-sulfate and keratan sulfate, respectively. Basement membranes in human skin tissue were used as a positive control for heparan sulfate proteoglycan monoclonal antibodies.

Two-Dimensional Electrophoresis and Quantitative Analysis of GAGs

The choroid/retina preparations frozen in liquid nitrogen were defrosted and homogenized on ice in 0.5 ml Milli-Q water. The main part (450 μl) of these homogenates was acidified with one drop of 1N HCl and subsequently added to 40 ml of 0.05% Alcian blue in 0.05 M sodiumacetate buffer (pH 5.8). Subsequently, GAGs were isolated according to Mossman and Patrick (1982) (Mossman and Patrick, 1982). These isolated GAGS were used for two-dimensional electrophoresis on cellulose acetate membranes according to Abeling et al. (1974) (Abeling, et al., 1974). Quantitative analysis of GAGs was done on the homogenates, using dimethylmethylene blue as has been described previously for measuring

glycosaminoglycans in urine (de Jong, et al., 1992). Furthermore, a protein-assay (Bicinchoninic acid, Pierce, Rockford, IL) was performed.

The Student's t-test was used to compare the amounts of GAGs/mg protein between ARM maculae and control maculae. In order to avoid bias only one eye of a single donor was used in this analysis.

Results

Histopathological Classification of Human Macular Tissue

Twenty maculae were classified as having ARM. Five maculae had no ARM and were used as controls.

Histochemistry

Alcian blue at pH 0.2 stained BLD blue (Figure 6.1, page 98). Increase of the pH to 1.0, and 2.0 decreased the staining gradually, while Alcian blue staining was negative at pH 3.2. With increasing $MgCl_2$ concentrations, alcian blue staining of the BLD showed a reduced intensity at a critical electrolyte concentration of 0.7 M $MgCl_2$. However, a weak staining persisted at a 1.0 M $MgCl_2$ concentration. Hale's colloidal iron stained BLD blue to purple (Figure 6.2A, page 98). Hyaluronidase treatment markedly reduced the staining intensity of BLD, although some staining persisted. In contrast with BLD, drusen were negative for alcian blue staining, but stained with Hale's colloidal iron (Figure 6.2B). The staining of drusen disappeared totally after treatment with hyaluronidase.

Immunohistochemistry

Specific staining with a monoclonal antibody against chondroitin-4-sulfate was found diffusely in BLD (Figure 6.3A, page 99). The choroid, the outer collagenous zone of Bruch's membrane (focally), the apical side of the RPE, and the photoreceptor outer segments stained with this antibody as well. With heparan sulfate proteoglycan immunostaining, BLD stained focally positive adjacent to the RPE in two of four maculae with BLD class 3 (Figure 6.3B). Since the BLD in these two maculae stained only at places overlying a soft drusen, it might be basal linear deposit (diffuse thickening of the inner aspect of Bruch's membrane) at these places as well, because the discrimination between BLD and basal linear deposit is hard to make (Green and Enger, 1993). The monoclonal antibody against keratan sulfate revealed specific staining in the outer collagenous zone of Bruch's membrane, but not in BLD (Figure

6.3C). Drusen did not stain with these immunohistochemical techniques, except for a large confluent drusen in one macula that reacted with the antibody directed against keratan sulfate (Figure 6.3D). These immunohistochemical staining patterns are summarized in Table 1. The several sub-types of drusen are not specified in this table, because only one confluent drusen showed positive staining.

Negative controls showed no staining except for a weak to moderate staining in the choroid after incubation with the monoclonal antibody directed against chondroitin sulfate without prior treatment with chondroitinase ABC. The internal and external positive controls were affirmative.

Table 1. Immunohistochemical Staining Pattern of Human Maculae.

GAGs		BLD†	drusen‡	BM (OCZ)	BM (ICZ)	RPE	Choroid
CS	ARM	+ (19/19)	-	-	-	+ (20/20)	+ (20/20)
	control	+ (3/3)	-	-	-	+ (3/5)	+ (5/5)
KS	ARM	-	+ (1/8)*	+ (20/20)	+ (2/20)	+ (3/20)	+ (16/20)
	control	-	-	+ (5/5)	-	+ (2/5)	+ (4/5)
HS	ARM	+ (2/19)	-	-	-	-	-
	control	-	-	-	-	-	

*GAGs = glycosaminoglycans; BLD = basal laminar deposit; BM = Bruch's membrane; OCZ = outer collagenous zone; ICZ = inner collagenous zone; RPE = retinal pigment epithelium; CS = chondroitin-4-sulfate; KS = keratan sulfate; HS = heparan sulfate; ARM = age-related maculopathy; + = moderate to strong staining; - = no staining; † = 19 of 20 maculae with ARM had BLD, and 3 of 5 controls had BLD class 1; ‡ = 8 of 20 maculae with ARM had drusen, and 1 of 5 controls had drusen class 1; * = one confluent drusen stained.*

Two-Dimensional Electrophoresis and Quantitative Analysis of GAGs

Seven human maculae with ARM (mean age 83 years) and 4 controls (mean age 71 years) were used for the two-dimensional electrophoresis and quantitative analysis of GAGs (Table 2). Chondroitin sulfate was demonstrated in all these (n=11) maculae (Figure 6.4A, and 6.4B, page 100). In five of seven maculae with ARM, heparan sulfate was detected additionally (Figure 6.4B). In two controls without BLD or drusen no heparan sulfate was detected at all. The two other controls with BLD class 1 (case 10 and 11, Table 2) contained

relatively small amounts of heparan sulfate as well. On the other hand, one of the two maculae with ARM, that showed no heparan sulfate had drusen class 3 (case 1, Table 2), but no BLD. Other GAGs (hyaluronic acid, dermatan sulfate, and keratan sulfate) could not be detected in the macular extracts with two-dimensional electrophoresis. A discrimination between chondroitin-4-sulfate and chondroitin-6-sulfate is not possible with this technique.

Table 2. Classification of Human Maculae and Results of Quantitative Analysis of GAGs.

case*	age	drusen class†	BLD class†	classification	μg GAGs/ mg protein
1	79	3	0	ARM	12.5
2	75	3	1	ARM	12.2
3	86	0	2	ARM	15.0
4	81	0	2	ARM	17.2
5	81	2	2	ARM	16.3
6	94	0	3	ARM	14.1
7	86	2	3	ARM	18.2
8	71	0	0	control	10.5
9	56	0	0	control	12.4
10	70	0	1	control	13.2
11	86	1	1	control	13.6

BLD = basal laminar deposit; GAGs = glycosaminoglycans;
ARM = age-related maculopathy.
** = all eleven cases were used for two-dimensional electrophoresis.*
† = for histopathological classification see materials and methods.

The results of quantitative analysis of the human macular homogenates are summarized in Table 2. The average amount was 15.1 μg GAGs/mg protein in macular extracts with ARM, compared to 12.4 μg GAGs/mg protein in extracts of control maculae. This increase in maculae with ARM was not statistically significant ($p=0.06$). However, the average amount of GAGs/mg protein in macular extracts with BLD class 2 and class 3 (16.2 μg/mg, $n=5$), compared to the average amount of GAGs/mg protein in extracts with BLD class 0 and class 1 (12.4 μg/mg, $n=6$) was highly statistically significant ($p=0.001$,

S.E.M.=0.41, Figure 6.5, page 101). There was no association between the total amount of GAGs/mg protein and drusen.

Discussion

(Immuno)histochemistry

Chondroitin-4-sulfate was immunohistochemically demonstrated in BLD. This was supported by the disappearing alcian blue staining at a critical electrolyte concentration of 0.7 M $MgCl_2$. It is possible that deposition of chondroitin-4-sulfate in BLD, and the outer collagenous zone of Bruch's membrane is one of the reasons for the reduced hydraulic conductivity of Bruch's membrane reported in aging eyes (Bird, 1992; Moore, et al., 1995; Pauleikhoff, et al., 1990b), because of the hydrophilic nature and negative charges of chondroitin sulfates. On the other hand, chondroitin-4-sulfate deposition at these sites could have been caused by the reduced hydraulic conductivity in Bruch's membrane. Since there is no BLD in normal eyes, and chondroitin sulfates are not broken down in the extracellular matrix (Poole, 1986), it is possible that normally these GAGs are transported through Bruch's membrane to the choriocapillaris.

Nodular drusen, confluent drusen (but not soft drusen), and the intercapillary pillars of thickened Bruch's membranes stained strongly with Hale's colloidal iron staining, which means that they contain GAGs. This staining totally disappeared after treatment with hyaluronidase. However, they did not stain with any of the monoclonal antibodies nor with alcian blue (except one confluent drusen, that stained with antibodies directed to keratan sulfate). These data suggest that nodular drusen and confluent drusen, as well as a thickened Bruch's membrane, contain hyaluronic acid. Hyaluronic acid is also a GAG and normally present in the extracellular matrix.

The purple color of the BLD with Hale's colloidal iron staining, and the persisting weak staining of BLD after alcian blue with 1.0 M $MgCl_2$, could be due to the presence of a collagenous component (Scott and Dorling, 1965) in BLD, e.g. long spacing collagen. This would also explain the remaining weak staining of the BLD after hyaluronidase digestion. Except for a small rim of collagen type IV adjacent to the RPE, no special collagen types have been demonstrated in BLD yet (Marshall, et al., 1994; van der Schaft, et al., 1994).

Two-Dimensional Electrophoresis and Quantitative Analysis of GAGs

With two-dimensional electrophoresis we found chondroitin sulfate and heparan sulfate in extracts of human maculae with BLD. The significant quantitative increase of total GAGs in

these macular extracts could be attributed to the presence of chondroitin sulfate and heparan sulfate in BLD. However, GAGs (in particular chondroitin sulfate) are also abundantly present in the normal extracellular matrix, and in the interphotoreceptor matrix (Berman, 1991). Since the amount of BLD in the macular extracts was small in comparison with the total amount of tissue, it is possible that these GAGs in extracts of maculae with BLD are also derived from another part in the macula related to BLD. It has been demonstrated that synthesis of heparan sulfate, and chondroitin sulfate in the inner retinal cells is increased following photoreceptor degeneration (Landers, et al., 1994), a pathologic feature also occurring in ARM. Furthermore, an increased proportion of heparan sulfate proteoglycan has also been shown in Bruch's membrane in several retinal pathologies, including ARM (Hewitt, et al., 1989). In contrast to others we could neither demonstrate heparan sulfate proteoglycan in Bruch's membrane nor in drusen (Newsome, et al., 1987). This can be attributed to methodological differences. These authors have used a different antibody, and cryostat sections instead of the paraffin sections we used. Although formaldehyde fixation alters the molecules, the antibodies we used in this study were specifically made for these fixed molecules. However, a long fixation time of our tissue samples (upto two weeks) could cause loss of the heparan sulfate antigen, which could also explain the absence of reaction in the RPE basement membrane.

It has been demonstrated, that chondroitin sulfates induce epiretinal membranes (Russell and Hageman, 1992). They may also be involved in the pathogenesis of intravitreal membranes in proliferative vitreoretinopathy (Hagedorn, et al., 1993). In the same way GAGs could play a role in the development of subretinal neovascular membranes occurring in the late stages of ARM, as GAGs are very suitable for new blood vessels to grow in, and GAGs enhance the biological activity of growth factors (Berger, et al., 1982; Hagedorn, et al., 1993; Roden and Horowitz, 1978). A higher level of fibroblast growth factor and transforming growth factor has been reported in choroidal neovascular membranes (Amin, et al., 1994). Therefore, the accumulation of GAGs in BLD might play a role in the development of subretinal neovascularization. This could also be an explanation for the clinical observation that subretinal neovascular growth in ARM is mostly directed towards the macula, where BLD is most prominent (Green and Enger, 1993).

In conclusion we demonstrated that chondroitin-4-sulfate and heparan sulfate proteoglycan are present in BLD, and that human macular extracts with BLD contain a significantly higher amount of GAGs than macular extracts without BLD. The deposition of these GAGs in BLD could play a role in the pathogenesis of subretinal neovascularization in the late stages of ARM.

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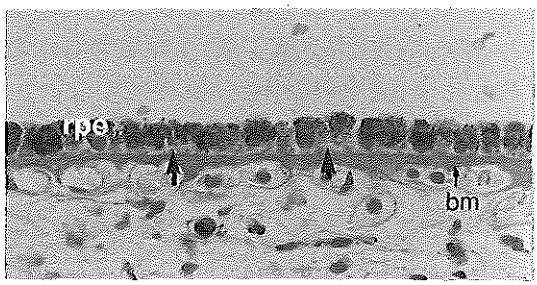

Figure 6.1. Section of a human macula with ARM (age 86 years) stained with Alcian blue. There is a slight blue staining in the basal laminar deposit (arrows). Original magnification 400x. rpe = retinal pigment epithelium, bm = Bruch's membrane.

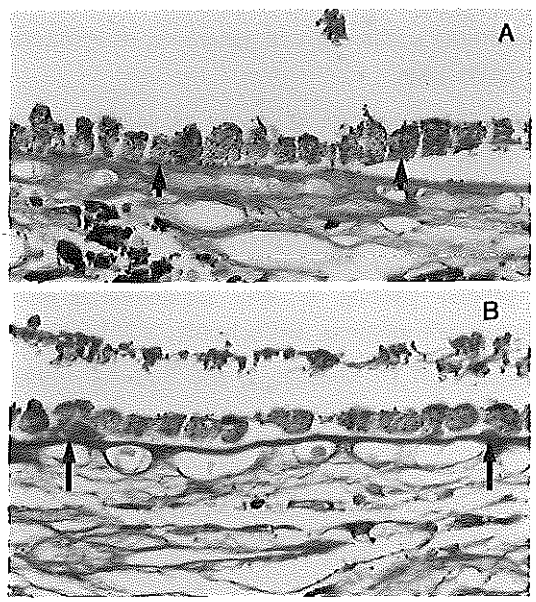

Figure 6.2. Sections of human maculae with ARM (age 81 years) stained with Hale's colloidal iron staining. (A) The basal laminar deposit (arrows) is stained blue. (B) Both the small nodular drusen and the larger confluent drusen stained blue (arrows). Original magnification 400x.

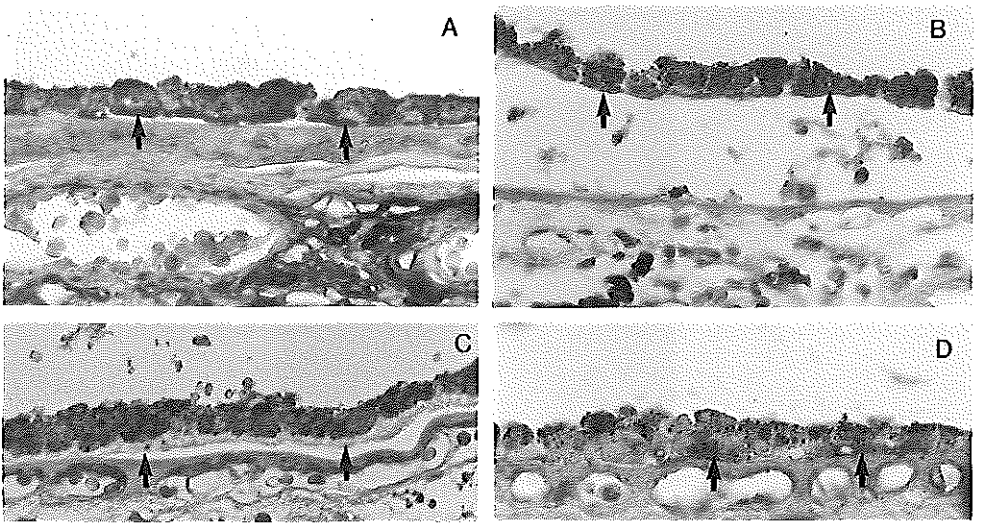

Figure 6.3. Sections of human maculae with ARM (age 94, 81, 83, and 88 years, respectively) stained with monoclonal antibodies. A. directed against chondroitin-4-sulfate; the basal laminar deposit (arrows) stains positive. B. directed against heparan sulfate; the basal laminar deposit (arrows) stains positive. C. directed against keratan sulfate; the outer collagenous zone of Bruch's membrane stains positive. Note that BLD (arrows) does not stain. D. directed against keratan sulfate; a larger confluent drusen (arrows) stains positive (D). Positive staining is red (new Fucsine), original magnification 400x.

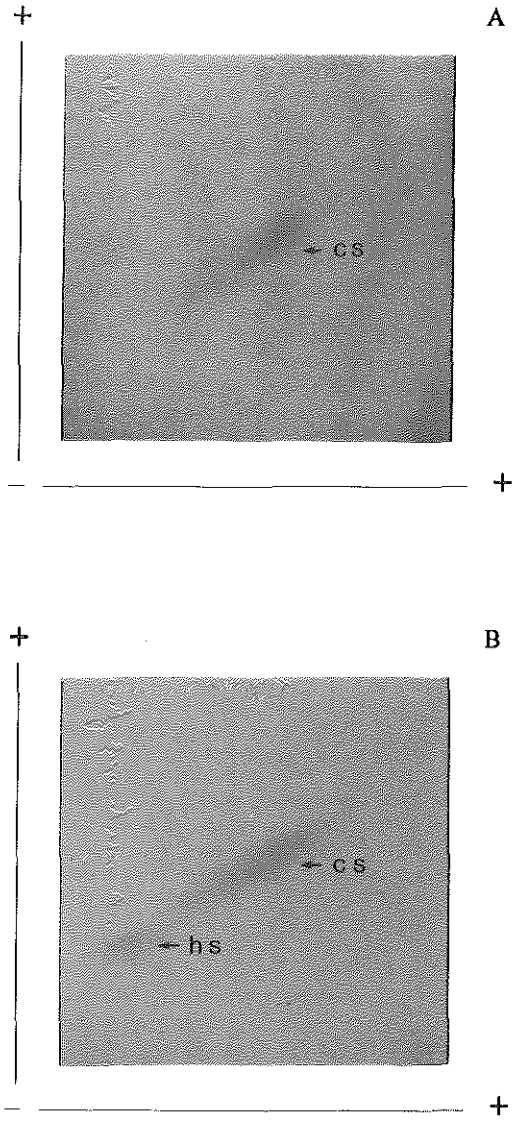

Figure 6.4. Photographs of cellulose acetate plates after two-dimensional electrophoresis with (A) a control human macular homogenate (age 71 years), and (B) a human macular homogenate with age-related maculopathy (ARM, age 81 years). Note that there is a second spot (heparan sulfate, hs) under the large spot of chondroitin sulfate (cs) on the plate with the human macula with ARM.

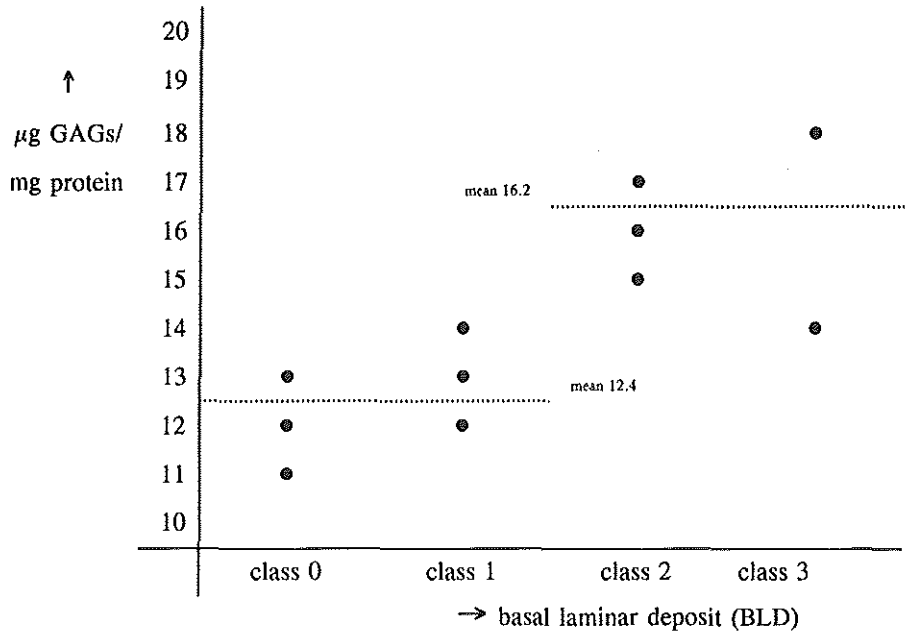

Figure 6.5. Relationship between the quantitatively measured glycosaminoglycans (GAGs) per mg protein and the class of basal laminar deposit (BLD, see materials and methods). The increase of the amount of GAGs between maculae with BLD class 0 and class 1 (12.4 µg), and BLD class 2 and class 3 (16.2 µg), is statistically significant ($p=0.001$; $n=11$; S.E.M. $=0.41$).

CHAPTER 7

Increased Expression of Angiogenic Growth Factors in Age-Related Maculopathy

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Submitted

ABSTRACT

Purpose. The late stages of age-related maculopathy (ARM), especially neovascular macular degeneration (AMD), can severely affect central vision and are the main cause of blindness in the elderly in the western world. It has been shown that angiogenic growth factors are present in neovascular membranes in AMD. However, it is not known if angiogenic growth factors play a role in the onset of neovascularization.

Methods. In order to elucidate the involvement of angiogenic growth factors in the initiation of neovascularization in early stages of ARM, we investigated immunohistochemically the expression patterns of VEGF, TGF-β, b-FGF, and PDGF-AA on 18 human maculae with ARM, and on 11 control specimens.

Results. We found a significantly increased expression of VEGF ($P=0.00001$) and TGF-β ($P=0.019$) in the retinal pigment epithelium (RPE) of maculae with ARM compared to control maculae. Furthermore, an increased expression of VEGF and PDGF was found in the outer nuclear layer of maculae with ARM.

Conclusions. Our results indicate that increased expression of VEGF in the RPE, and in the outer nuclear layer in maculae with ARM could be involved in the pathogenesis of neovascular macular degeneration, and is possibly induced by hypoxia. Furthermore, enhanced TGF-β expression in the RPE-cells of maculae with early stages of ARM could, in addition to its indirect angiogenic role, induce the accumulation of extracellular deposits like basal laminar deposit and drusen.

Introduction

Age-related maculopathy (ARM) is a disorder of the macular area of the eye. Many histopathological changes, e.g. drusen and basal laminar deposit (BLD) can be seen in early types of ARM (Green and Enger, 1993; Kliffen, et al., 1996b). The late stages of ARM are nowadays called age-related macular degeneration (AMD) and are subdivided in dry AMD (geographic atrophy), and wet AMD (neovascular AMD) (Bird, et al., 1995). AMD is the main cause of blindness in the elderly population in the western world, and the prevalence of neovascular AMD is twice as high as dry AMD (Klein, et al., 1992; Vingerling, et al., 1995b). During neovascular AMD, the sub-RPE space is invaded by growing new blood vessels (angiogenesis) sprouting from the underlying choriocapillaris (Bressler, et al., 1988; Green and Enger, 1993; Kliffen, et al., 1996b; Sarks, et al., 1988). These neovascular blood vessels can cause hemorrhages, leading to the formation of a disciform scar with rapid visual impairment. Despite several studies on the morphology of ARM, the molecular mechanisms and factors contributing to the pathogenesis of neovascular AMD remain to be characterized.

Angiogenesis, being a complex phenomenon comprising of several distinct processes that include endothelial cell migration and proliferation, extracellular proteolysis, and vessel wall remodeling, has been implicated in many pathophysiological processes. Several growth factors have been shown to contribute to the molecular events involved in the regulation of blood vessel growth (Ferrara, et al., 1995; Folkman and Shing, 1992). Likewise, it is assumed that angiogenic growth factors like vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β), basic-fibroblast growth factor (FGF), and platelet derived growth factor (PDGF), could play an important role in the pathogenesis of neovascular AMD. Recently, increased levels of b-FGF and TGF-β in surgically removed neovascular membranes from eyes with neovascular AMD have been demonstrated (Amin, et al., 1994; Reddy, et al., 1995). However, it is not yet established whether these growth factors play a role in the initiation of the process or they are secondary to the new blood vessel formation.

VEGF, also referred to as vascular permeability factor or vasculotropin is a heparin-binding peptide mitogen with narrow target cell specificity, apparently limited to endothelial cells derived from small and large blood vessels. It promotes vascular permeability and angiogenesis in vivo (Ferrara, et al., 1995). TGF-β is a cytokine that has been implicated in cell proliferation, angiogenesis, and deposition of extracellular matrix (Sporn, et al., 1987). Another angiogenic heparin binding growth factor, b-FGF, has been suggested to contribute to the process of ocular neovascularization (Amin, et al., 1994; Sivalingam, et al., 1990). PDGF is highly chemotactic to the RPE cells (Yoshida, et al., 1992), and is known to induce

intraocular vascular proliferation (Leschey, et al., 1990). Cultured RPE cells have been shown to produce all above mentioned growth factors (Adamis, et al., 1993; Campochiaro, et al., 1989; Ishigooka, et al., 1992; Ocrant, et al., 1991; Tanihara, et al., 1993).

The purpose of this study was to investigate the possible presence of angiogenic growth factors (VEGF, TGF-β, b-FGF, and PDGF-AA) in the early stages of ARM, and in maculae with established neovascular AMD, in order to elucidate the possible role of these growth factors in the initiation of the angiogenesis in progressing ARM.

Materials and Methods

Preparation of Human Macular Tissue

We used 29 human eye bank eyes from 25 subjects aged between 38 and 96 years (mean age 78 years), with post-mortem time from 1 to 11 hours (mean 7 hours). The macular area (about one $cm^{2)}$ was dissected from the ocular tissue. The macula was further horizontally divided through the fovea in the direction of the optic nerve head in two equal parts. One half was fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.4), and embedded in paraffin for histopathological classification and immunohistochemistry.

Histopathological Classification of Human Macular Tissue

Paraffin sections (5 μm) were stained with hematoxylin-eosin, the periodic-acid-Schiff reaction, and Mallory staining. The maculae were histologically classified according to their gradation of drusen and BLD. Drusen and BLD are both extracellular deposits accumulating in ARM. Drusen are located external to the basement membrane of the RPE, i.e. within Bruch's membrane. BLD is located between the basal cell membrane and the basement membrane of the RPE, i.e. outside Bruch's membrane. Large drusen and BLD (and the chemical composition of these deposits) are assumed to be important determinants in the pathogenesis of ARM (Bird, 1992; Green, et al., 1985; Pauleikhoff, et al., 1990; Sarks, 1976; Sheraidah, et al., 1993; van der Schaft, et al., 1992a). For the classification of drusen and BLD see van der Schaft et al (1992) (van der Schaft, et al., 1992b). In brief: no BLD (class 0); small solitary patches of BLD (class 1); a thin continuous layer of BLD (class 2); a thick layer of BLD of at least half the height of the RPE cells (class 3). No drusen (class 0); 1 to 3 drusen (class 1); 4 to 10 drusen (class 2); many or confluent drusen (class 3) (van der Schaft, et al., 1992b).

Since no great changes in histology of the maculae are present in maculae with both BLD class 0 or class 1, and drusen class 0 or class 1, these eyes were categorized as control

samples. All other maculae were classified as samples with ARM. Two maculae with established neovascular AMD were also included for examination of growth factor expression.

Immunohistochemistry

Paraffin sections (5 μm) of all maculae were mounted on 3-amino-propyl-tri-oxy-silane (Sigma, St. Louis, MO) coated glass slides. After deparaffinization and rehydration they were rinsed with water and phosphate-buffered saline. The slides were placed in a Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmoor Rancorn Cheshire, England), and incubated for 20 minutes with normal goat serum. After this blocking step, slides were incubated for 30 minutes with either a rabbit polyclonal antibody directed against VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), TGF-β (R&D Systems Europe, Abingdon, UK), b-FGF (Serotec, Kidlington. Oxford, UK), or PDGF-AA (Genzyme, Cambridge, MA). These antibodies were characterized as described by the suppliers and were used at a dilution of 1:100, 1:1500, 1:400, and 1:150, respectively. The anti-TGF-β polyclonal antibody was pan-specific to the several subtypes of TGF-β. After washing with phosphate-buffered saline, the slides were incubated for 30 minutes with biotinylated secondary antibodies (Multilink, 1:75 dilution, Biogenex, San Ramon, MO). The slides were washed again and incubated for 30 minutes with alkaline-phosphatase-conjugated streptavidin (Biogenex, San Ramon, MO, dilution 1:50). Finally the slides were rinsed with 0.2 M Tris-HCl pH 8.0, and stained for 30 minutes with 0.3% New Fuchsin/Tris-HCl (Sigma, St. Louis, MO). The sections were counterstained with Mayer's hematoxylin for 15 seconds, rinsed for 10 minutes with water and air-dried. Prior to the incubation with anti-TGF-β and anti-b-FGF antibodies, the sections were treated with 5 μg/ml saponin for 30 minutes at room temperature, and with 5.5 mU/ml pronase E (Sigma, St. Louis, MO) for 7 minutes at 37 °C, respectively. In negative controls, the primary antibodies were replaced by normal rabbit serum (DAKO, Glostrup, Denmark, dilution 1:10). As a second negative control for VEGF immunostaining, the anti-VEGF antibodies were blocked with the VEGF-peptide (Santa Cruz Biotechnology) prior to the incubation of the tissue. We did not perform peptide blocking with the other antibodies, because the most important findings in this study concern VEGF. Furthermore, the anti-TGF-β antibody is pan-specific and the two other antibodies have been described in the literature. Blood vessels were used as internal positive controls for anti-VEGF, and anti-PDGF antibodies. Nerve fibers were used as internal positive controls for TGF-β, and b-FGF immunostaining. Growth factor expression was semi-quantified on the basis of percentage of positively stained tissue, and in a lesser extent the intensity of tissue staining: - = no

staining; ± = faint staining in less than 50% of the tissue; + = positive staining in less than 50% of the tissue; ++ = positive staining in more than 50% of the tissue. Sections were scored twice (by the first and third author) with a 3 weeks interval. Intra-observer variability was negligible. Inter-observer variability was only observed between ± and + scores in some cases. These cases were scored after mutual consultation. In our opinion, these four gradation groups are readily discriminated, especially because positive staining occurred either in less than 33% of the tissue or in more than 66% of the tissue. Therefore, we have chosen to leave out the 33% to 66% group in our gradation system. For the same reason we did not perform statistical analysis on the intra-observer, and inter-observer variability.

Statistical Analysis

To compare the protein expression of VEGF, TGF-β, b-FGF, and PDGF between ARM maculae and controls, we have used the Mann-Whitney test. The difference of the individual growth factor expression between ARM and control maculae was assumed to be statistically significant if $P < 0.05$. Because photoreceptors and inner-retinae were not present in all specimens, P-values could not be computed for these tissue layers.

Results

Histopathological Classification of Human Macular Tissue

Eleven maculae were classified as controls: 9 maculae had neither BLD nor drusen, 1 macula had BLD class 1, and 1 macula had BLD class 1 and drusen class 1. Sixteen specimens were classified as maculae with ARM; 6 maculae had only BLD (class 2 or 3), 1 macula had only drusen (class 3), and 9 maculae had both BLD (class 2 or 3) and drusen (class 2 or 3). Two maculae had neovascular AMD. The differences of growth factor expression, in the RPE and choroid, between ARM maculae and controls are visualized in Figure 7.1. The statistical analysis was performed on these data.

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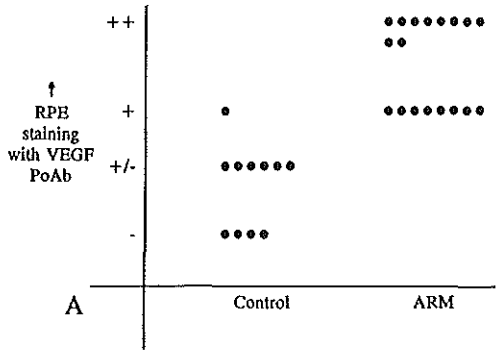
Figure 7.1. Comparison of the immunohistochemical expression of VEGF (A and B), TGF-β (C and D), b-FGF (E and F), and PDGF-AA (G and H) in RPE (A, C, E, G) and choroid (B, D, F, H) between control maculae and ARM maculae.


++
+
+/-
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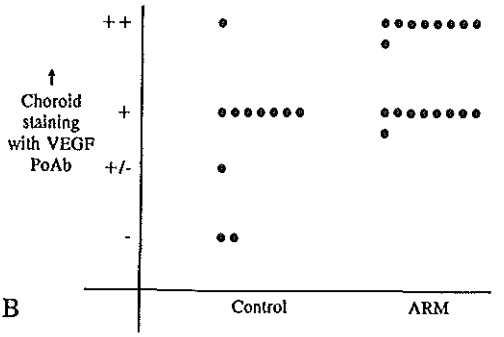
↑ RPE staining with VEGF PoAb
Control
ARM

A


++
+
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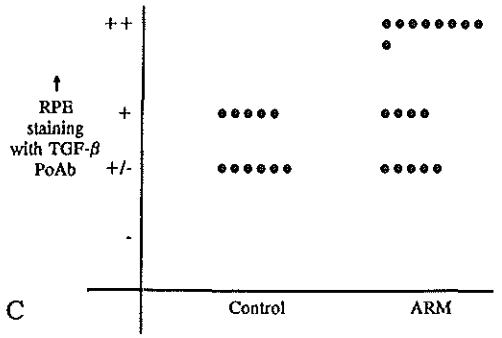
↑ Choroid staining with VEGF PoAb
Control
ARM

B


++
+
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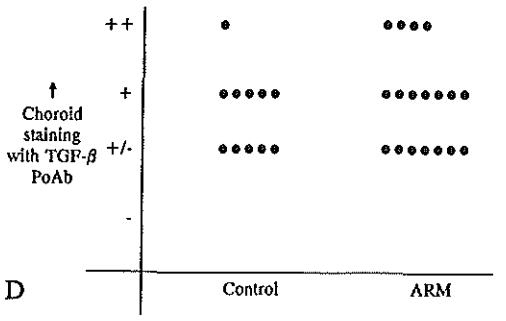
Control
ARM

C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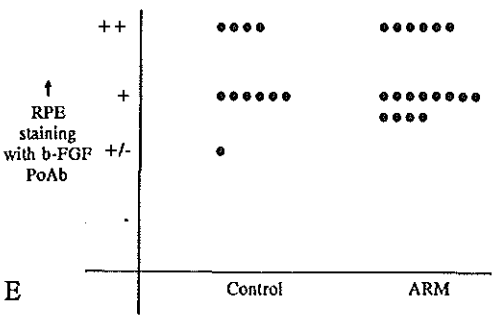
Control
ARM

D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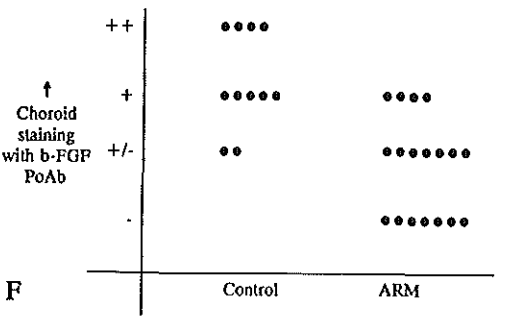
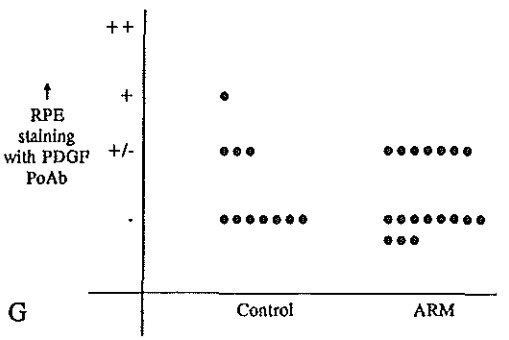
+
+/-
-
↑ RPE staining with b-FGF PoAb
Control
ARM

E


++
+
+/-
-
↑ Choroid staining with b-FGF PoAb
Control
ARM

F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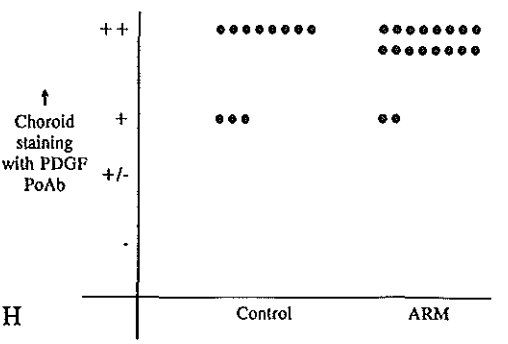
Control
ARM

G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Control
ARM

H

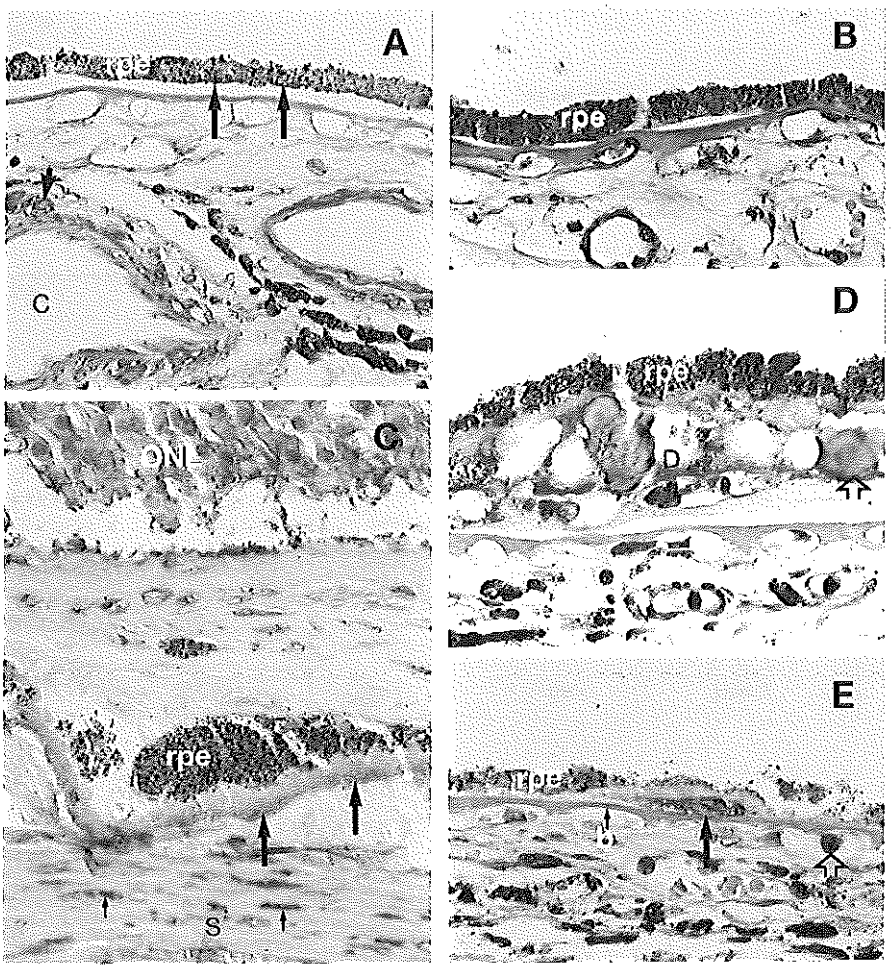

Figure 7.2. Light microscopic images of immunohistochemistry with anti-VEGF antibodies. a: Typical example of VEGF expression in a control macula; VEGF is present in smooth muscle cells (short arrows) of choroidal blood vessels (C) and in some (long arrows) of the retinal pigment epithelial cells (rpe). b: The expression of VEGF in the RPE is increased in ARM maculae in comparison with control maculae. c: The outer nuclear layer (ONL) and basal laminar deposit (long arrows) show expression of VEGF in this macula with neovascular macular degeneration. The myofibroblast cells (small arrows) in the scar tissue (S) contain VEGF as well. d: Expression of VEGF in a large drusen (D) with calcified bodies (open arrow) in an ARM macula. e: Macrophages (long arrow) that have infiltrated Bruch's membrane (b) in a ARM macula show intense staining for VEGF. A monocyte (open arrow) shows VEGF expression as well. Original magnifications 400X.

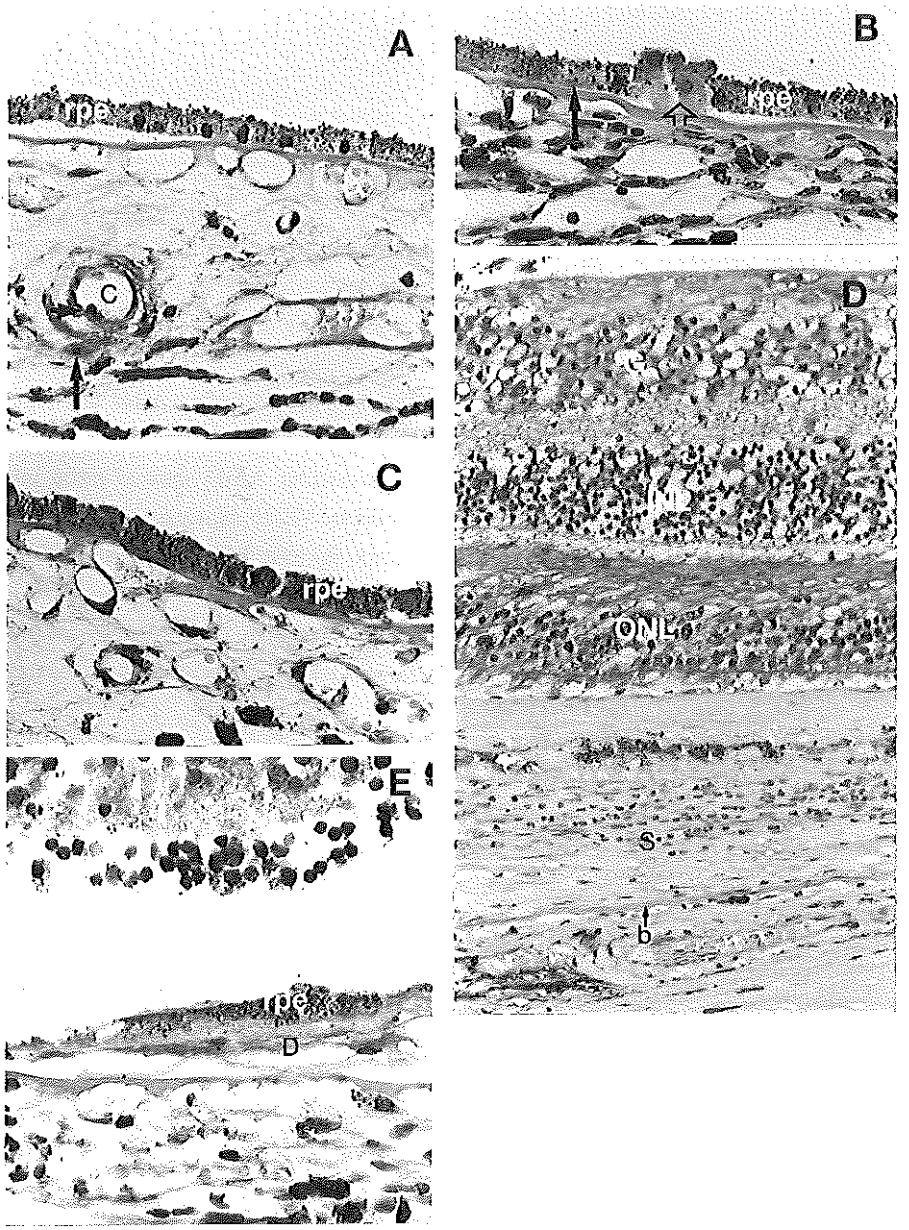

Figure 7.3. Light microscopic images of immunohistochemistry with anti-TGF-β (a and b), b-FGF (c), and PDGF-AA (d and e) antibodies. a: Control maculae show TGF-β expression in smooth muscle cells (long arrow) and endothelial cells (short arrow) of choroidal blood vessels (C). The RPE shows only faint staining in most of the control maculae. b: TGF-β expression is increased in the RPE of this typical ARM macula. Basal laminar deposit (long arrow) contains TGF-β as well. Note that the most prominent RPE staining is present on top of a drusen (open arrow) that itself shows no staining. c: A typical example of an ARM macula that shows b-FGF expression in the RPE. d: PDGF-AA expression in a macula with neovascular macular degeneration. The retina demonstrates PDGF-AA expression, whereas the retina in control maculae shows no staining at all. Note that the expression in the outer nuclear layer (ONL) is more prominent than in the inner nuclear layer (INL) and ganglioncell layer (G). e: PDGF expression in a large drusen in a macula with neovascular macular degeneration. S = scar tissue, b = Bruch's membrane. Original magnifications 400X.

Immunolocalization of VEGF

Immunohistochemically, VEGF was localized in the smooth muscle cells of the blood vessels of the choroid and in some of the RPE-cells in control maculae (Figure 7.2a). The outer nuclear layer, the inner nuclear layer, and the ganglion cell layer of the retina stained as well. A significant increase in VEGF expression was found in the RPE-cells ($P=0.00001$) and choroidal blood vessels ($P=0.0064$) of human maculae with early ARM and maculae with neovascular AMD as compared to controls (Figure 7.2b). Furthermore, the outer nuclear layer of human maculae with ARM and neovascular AMD showed more intense VEGF staining (Figure 7.2c) than those of control maculae. VEGF expression was also found in BLD (9 out of 17 maculae) (Figure 7.2c). Except for one large calcified drusen that showed positive staining for VEGF (Figure 7.2d), none of the drusen were stained. Additionally, a strong positive staining for VEGF was found in macrophages infiltrating through Bruch's membrane (Figure 7.2e). In negative controls, no staining was seen at all.

Immunolocalization of TGF-β

TGF-β was scarcely localized in some of the RPE cells, and in the walls of blood vessels (smooth muscle cells as well as endothelial cells) in the choroid of control human maculae (Figure 7.3a). A significantly increased expression of TGF-β was found in the RPE of human maculae with early ARM, and in maculae with neovascular AMD compared to control specimens ($P=0.019$). The expression in the choroid was the same in both groups. BLD stained with antibodies directed against TGF-β in 8 out of 17 maculae (Figure 7.3b). These were the same maculae that showed expression of VEGF in BLD. None of the drusen stained with the anti-TGF-β antibody (Figure 7.3b).

Immunolocalization of b-FGF

Antibodies directed against b-FGF, showed expression of this growth factor in all RPE-cells and in the blood vessels of the choroid in control human maculae. A significantly decreased expression of b-FGF was found in the choroidal blood vessels of ARM maculae ($P=0.0005$), but no difference in expression was found in the RPE, or in the photoreceptors. The distribution of b-FGF in ARM maculae is reflected in Figure 7.3c. BLD stained weakly with anti-b-FGF antibodies in 9 out of 17 maculae, 7 of these stained with anti-VEGF, and anti-TGF-β antibodies as well. The extracellular matrix surrounding the macrophages infiltrating through Bruch's membrane stained also weakly positive for b-FGF. None of the drusen stained with the anti-b-FGF antibody.

Immunolocalization of PDGF-AA

In control maculae PDGF was only localized in the smooth muscle cells of the blood vessels. The outer nuclear layer in 3 maculae with early stages of ARM, and both maculae with neovascular AMD stained strongly, whereas the inner nuclear layer, and ganglioncell layer stained positive as well (Figure 7.3d). None of the control specimens showed positive staining in the retinal layers. Some RPE cells stained weakly in 6 maculae with ARM and in 4 controls. BLD did not express PDGF. One large drusen involved in the edge of a neovascular membrane showed positive staining for PDGF-AA (Figure 7.3e). All other drusen were negative.

Discussion

We have demonstrated a significantly increased expression of VEGF and TGF-β in RPE-cells of human maculae with early stages of ARM compared to those of control specimens. The blood vessels in the choroid showed a significantly increased expression of VEGF in ARM maculae as well. No differences in RPE staining of ARM maculae compared to controls were found with anti-b-FGF, and anti-PDGF antibodies. About 50% of the human maculae with early ARM showed VEGF, TGF-β, and b-FGF staining in BLD. Localization of these angiogenic growth factors in BLD points for their involvement in the pathogenesis of subretinal neovascularization.

Recently, it has been shown that human RPE-cells in culture synthesize and secrete VEGF (Adamis, et al., 1993). In our study, we found enhanced immunoreactivity for VEGF in RPE-cells of ARM maculae. The expression of VEGF in vivo as well as in vitro is strongly induced by a number of factors including hypoxia and ischemia (Miller, et al., 1994; Minchenko, et al., 1994; Sharma and Schaper, 1993; Shweiki, et al., 1992). Atrophy of the choriocapillaris and atherosclerosis resulting in a relative ischemia of the outer retina are assumed to be involved in the development of neovascular AMD (Kliffen, et al., 1995a; Kliffen, et al., 1996b; Ramrattan, et al., 1994; Vingerling, et al., 1995a). In our study we found higher levels of VEGF in the RPE and choroid, as well as in the outer nuclear layer of maculae with ARM. Furthermore, the inner nuclear layer of the retina showed faint staining as well. In contrast, Miller et al. (1994) found an increase of VEGF expression in the inner nuclear layer, but not in the outer nuclear layer of ischemic retina, in a primate model for retinal neovascularization (Miller, et al., 1994). This difference could be attributed to the two separate vascular beds supplying the retina with oxygen and nutrients. A retinal capillary network that supplies the inner nuclear layer, and ganglion cell layer, and a network

of extra-retinal capillaries in the choroid supplying the RPE and the outer nuclear layer. The former is involved in diabetic retinopathy, which has been studied by Miller et al. (1994) (Miller, et al., 1994). The latter is involved in neovascular AMD (Green and Enger, 1993; Kliffen, et al., 1996b). Irrespectively, these results indicate that the differential increased VEGF expression in the outer and inner retinal layers in neovascular AMD and in proliferative diabetic retinopathy, respectively, could be due to ischemia caused by insufficiency of either of the capillary beds. Furthermore, recent findings of Pe'er et al. (1995), support the contention of differential expression of VEGF in the retina as they demonstrated spatial localization of VEGF mRNA in retinae of several other ischemic retinal disorders (Pe'er, et al., 1995).

In the present study, we demonstrated an intense expression of VEGF in infiltrating macrophages in ARM. The infiltration of macrophages through Bruch's membrane has been assumed to be involved in the process of neovascularization in ARM (Killingsworth, et al., 1990; Lopez, et al., 1991; Penfold, et al., 1985). This migration could well be promoted by the increased levels of VEGF in maculae with ARM (Clauss, et al., 1990).

TGF-β is an indirect angiogenic growth factor that stimulates angiogenesis by inducing the production of other angiogenic factors like VEGF (Pertovaara, et al., 1994). TGF-β plays an important role in the formation of extracellular matrix (Sporn, et al., 1987). It increases the production of matrix molecules and inhibits matrix degradation through stimulation of protease inhibitors synthesis, and inhibiting the synthesis of proteases (Sporn, et al., 1987). We demonstrated a significantly increased expression of TGF-β in the RPE-cells of maculae with early stages of ARM, strongly suggesting that TGF-β could be involved in the accumulation of extracellular deposits like BLD and drusen. BLD contains many extracellular matrix molecules like collagen type-IV, laminin, glycosaminoglycans and several other carbohydrate structures (Kliffen, et al., 1994; Kliffen, et al., 1996a; van der Schaft, et al., 1994). Furthermore, TGF-β is highly chemotactic to monocytes (Wahl, et al., 1987). The increased expression of this growth factor in the RPE-cells of early ARM maculae could also be attributed to its role in the infiltration of macrophages through Bruch's membrane.

Basic-FGF has been reported to be able to rescue photoreceptors from degeneration (Faktorovich, et al., 1992; LaVail, et al., 1992). Although, we have demonstrated b-FGF expression in BLD and the RPE, we could not demonstrate higher levels of this growth factor in the RPE-cells of maculae with early stages of ARM or with neovascular AMD (and subsequent degeneration of photoreceptors) compared to control specimens. Therefore, we can confirm the results of two recent papers, demonstrating expression of b-FGF in RPE-cells involved in surgically removed neovascular membranes (Amin, et al., 1994; Reddy et

al., 1995), but we can not point to a pathogenic role of b-FGF in early stages of ARM. Furthermore, we have found a significantly decreased expression of b-FGF in the choroidal blood vessels in maculae with early stages of ARM compared to control maculae, indicating that b-FGF may not be contributing to early stages of ARM where other potent angiogenic factors like VEGF act as endothelial cell mitogen.

We demonstrated an increased expression of PDGF in the outer nuclear layer of 3 out of 17 maculae with early ARM, and in all maculae with neovascular AMD, whereas none of the control maculae showed expression of PDGF in this layer. However, not all specimens contained all retinal layers, so it seemed unwise to perform statistical analysis on the staining characteristics of the retinal layers in this study. Similar to the results of Reddy et al. (1995), we did not see expression of PDGF in the RPE-cells. Since PDGF is highly growth promoting and chemotactic to the RPE-cells (Yoshida, et al., 1992), the increased expression of PDGF in the outer nuclear layer could be attributed to its participation in the migration of RPE-cells towards the inner retina, often seen during AMD.

In conclusion, we have demonstrated that a cascade of a number of angiogenic growth factors is involved in the early stages of ARM. Therefore, they may be involved in the pathogenesis of neovascular AMD. The increased levels of VEGF in RPE-cells, and the outer nuclear layer of maculae with ARM might be caused by a relative hypoxia of the outer retina. Increased TGF-β expression in the RPE-cells of maculae with early stages of ARM could, in addition to its indirect angiogenic role (e.g. stimulating the production of angiogenic growth factors like VEGF), induce the accumulation of extracellular deposits like BLD and drusen. It is imperative to emphasize here that in primates a specific inhibition of VEGF can prevent retinal neovascularization (Miller et al; presented at the ARVO annual meeting, 1995). Therefore, it might be speculated that the identification of changes in VEGF levels in human maculae with ARM can provide us with a molecular target for anti-angiogenic therapies in treating neovascular AMD (D'Amato and Adamis, 1995).

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CHAPTER 8

Concluding Remarks

Age-related maculopathy has been described for the first time in the 19th century, but little information on the chemical composition of the structures related to ARM has been obtained (Chapter 3). One of the major problems in studying ARM is the lack of fresh human tissue, that is necessary for most of the biochemical techniques. Furthermore, the macular area in the eye consists of only a small amount of tissue. A piece of macular tissue of one eye (± 0.1 cm^3) contains about 2 mg total protein. Highly specialized techniques, e.g. two-dimensional polyacrylamide gel electrophoresis, and two dimensional electrophoresis on cellulose acetate membranes had to be used in this study to cope with the very small amounts of human eye tissue. Most of these techniques require unfixed tissue samples. Since there is no animal model of ARM available, we had to use fresh post-mortem human eyes for this research. This thesis describes several investigations that have been performed to elucidate the chemical composition and the origin of the structures related to ARM, in particular drusen and BLD.

There are several possibilities regarding the pathogenesis of ARM. Among those are a genetic abnormality, a metabolic problem, free radical damage or perhaps a combination of these (Figure 8.1). If a genetic factor is assumed, there are two main ways to acquire more information on the pathogenesis of ARM. One way, as described in this thesis, is to investigate the biochemical contents of the deposits accumulating in ARM. This information could lead to more knowledge on the constitution, and possibly the cause of the deposits. One could think of enzyme deficiencies or abnormal configured molecules. With this information, the genes (if any) that are responsible for the accumulation of the deposits in ARM could be elucidated. The discovery of mutant genes that appear to be part of the pathology could be the start of revealing the whole disorder process. Conversely, the second way is to start with searching for any susceptibility genes. However, the difficulties that arise when investigating genes of a late onset disease are ample. The lack of large pedigrees, diagnostic specificity, disease heterogeneity and the unknown modifying effect of the environment are just few of the problems. Recent genetic epidemiologic studies support the opinion that ARM could have (in part) a genetic component (Heiba, et al., 1994; Klein, et al., 1994). I share the opinion that ARM is multifactorial (Figure 8.1) (Goldberg, et al., 1988; Leibowitz, et al., 1980; Tso,

1985; Young, 1987), and think that both, the biochemical and the genetical way have to be further explored in order to better understand the whole (complex) process of ARM.

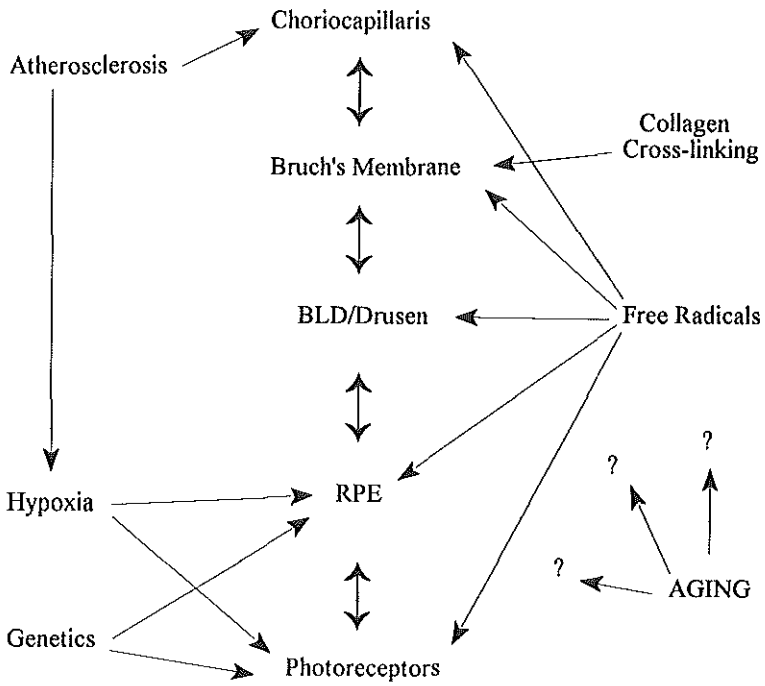

Figure 8.1. Possible etiologic factors in the pathogenesis of ARM. The structures in the center of the figure might all influence each other. This figure is not intended to be complete, but covers the factors discussed in this chapter. Metabolic abnormalities/insufficiencies within the cells (RPE and photoreceptors) are not displayed, but are possibly also involved in the pathogenesis of ARM.

Technical reasons did not allow us to sufficiently separate BLD and drusen from the rest of the tissue. Therefore, we used choroidal/retinal preparations from half of the macular area in the studies described in this thesis, involving biochemical techniques. The other halves of the maculae were used for histological classification of the degree of ARM. The

separation of the (macular) choroid/retina from the rest of the tissue (retina and vitreous) was reproducible, relatively fast, and the age-related deposits, located between the retina and the choroid, were not spilled. On the other hand, results might have been more specific when we could have used isolated extracellular deposits. The development of a technique to isolate the deposits from the rest of the tissue could be a tremendous advantage for future studies.

Since lipids dissolve during paraffin embedding, we were not able to detect any glycolipids in the paraffin embedded tissue (Chapter 4). Preliminary findings on frozen sections indicate that there are no lipids present in BLD (unpublished results). In contrast, drusen are packed with all sorts of lipids (Chapter 3). Therefore, it may well be possible that carbohydrate epitopes are present in drusen in the form of glycolipids. A preliminary report of Mullins et al. (1995) who were working with lectins on freshly isolated drusen is in favor of this suggestion. Whether the carbohydrate structures and the glycosaminoglycans, found in our studies, are part of one and the same molecule may be the subject of future studies (Chapter 4, and 6).

It is possible that molecules are present in eyes with ARM that do not appear in control eyes, or that the deposits consist of abundant molecules that are also produced by the RPE in normal situations. An inducer of the latter could be transforming growth factor beta (TGF-β). We demonstrated a significantly increased expression of TGF-β in the RPE-cells of maculae with early stages of ARM, suggesting that TGF-β could be involved in the accumulation of extracellular deposits like BLD and drusen (Chapter 7). If these deposits consist of abnormal molecules, they could be caused by a metabolic insufficiency of the RPE, e.g. a relative enzyme deficiency. Such a metabolic shortcoming could also be associated with hypoxia and/or atherosclerosis or can have a genetic origin. Another possibility is that the RPE-photoreceptor complex is damaged during life by free radicals that are constantly produced by the highly metabolic RPE-cells and are also induced by light. Molecules that are altered by these free radicals could give rise to the accumulation of intracellular deposits like lipofuscin, or to extracellular deposits like BLD and drusen. It has been assumed for a long time that lipofuscin granules consist mostly of lipid peroxidation products. However, it is now clear that other products (e.g. damaged proteins) must be present in lipofuscin granules, because the autofluorescence pattern of lipid peroxidation products differ from that of lipofuscin granules (Eldred and Katz, 1989; Eldred and Katz, 1991). It is possible, that some of the glycoproteins, we have found with two-dimensional electrophoresis in human maculae with ARM, are examples of these aberrant molecules (Chapter 5). A possible reason for an increased amount of IRBP-precursor could be that much of the IRBP is bound to accumulating derivatives of retinol in the RPE (e.g. in lipofuscin granules) and is not

available for its normal transport function. As a result the photoreceptors will presumably produce more IRBP to preserve a sufficient concentration of IRBP in the interphotoreceptor matrix. The accumulation process might ultimately deteriorate the RPE, resulting in AMD. Although changes in antioxidant levels like superoxide dismutase and gluthation are demonstrated in ARM (Cohen, et al., 1994; Prashar, et al., 1993), antioxidant therapies have not been proven successful yet (Newsome, et al., 1988; West, et al., 1994).

The higher level of vascular endothelial growth factor (VEGF) we have found in maculae with ARM, could well be induced by hypoxia and provides an argument in favor of the involvement of hypoxia in the pathogenesis of ARM (Chapter 7). It has been found by others that in primates a specific inhibition of VEGF can prevent retinal neovascularization in diabetes (Miller, et al., 1995). Therefore, it might be speculated that the identification of changes in VEGF levels in human maculae with ARM can provide us with a molecular target for anti-angiogenic therapies in treating neovascular AMD. Obviously, this kind of treatment does not act on the cause of the disease, but it may delay the progression of the neovascularization, which threatens visual acuity most. Further research is warranted to investigate the possible role of hypoxia of the RPE and photoreceptors in ARM.

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CHAPTER 9

Summary

Age-related maculopathy (ARM) is an age-related degenerative disorder of the central part of the retina. Its end-stages are called age-related macular degeneration (AMD) and are the most common cause of blindness in the elderly in the western world. In The Netherlands the prevalence is about 11% in people over the age of 85 years. The number of people affected by AMD will rise because of the aging population. Unfortunately, there is no good therapy available for this invalidating disease.

Age-related maculopathy is characterized by many histopathological changes, some of which can be seen clinically by ophthalmoscopy. The morphologic changes in ARM, and their correlations with clinical signs are reviewed in chapter 2 of this thesis. Histologically, the most prominent aging changes in early stages of ARM are drusen and basal laminar deposit (BLD). Both extracellular deposits are assumed to be important in the development of ARM. Clinically as well as histologically, several types of drusen are associated with ARM. Although, BLD can not be discriminated clinically by funduscopy, a relationship between the histologically classified amount of BLD and RPE/photoreceptor degeneration has been described already in 1976. It was known from the literature that drusen and BLD stain with the periodic-acid-Schiff reaction (PAS), a routine histological staining, that stains all kinds of carbohydrate structures. Therefore, we started with further characterization of these carbohydrate structures with lectins on formalin fixed post-mortem human eyes. Lectins are molecules that selectively bind to specific carbohydrate epitopes. With this study we demonstrated that BLD contains several carbohydrate structures, most likely glycoproteins and/or glycosaminoglycans. Furthermore, BLD contains almost exclusively in the macula a N-acetylated galactosamine. Drusen did not react with any of these lectins. This means that the lectin that specifically binds with N-acetylated galactosamine (Dolichos bifloris agglutinin), can be used as a tool to isolate BLD from the rest of the tissue.

With two-dimensional gel electrophoresis we compared the protein patterns of human maculae with and without AMD in order to check if any of the carbohydrate epitopes, found with the lectin study, are part of specific glycoproteins. With this study a significant increase

in the haptoglobin β-chain, a precursor-protein of the interphotoreceptor retinoid binding protein (IRBP), and three other (not identified) glycoproteins was found in human maculae with BLD and drusen, compared to controls. A homogenate of a whole macula (without sclera) was used for this research, because it appeared impossible to separate drusen and BLD from the rest of the tissue. Therefore, we can not conclude whether these glycoproteins are part of BLD and/or drusen, or from other ARM related changes. The identified glycoproteins did not react with the lectins used in the previous study. It is possible that the carbohydrate structures, found with the lectins, are located on very large molecules (e.g. proteoglycans), that could not be detected with this electrophoresis technique. The increase in haptoglobin β-chain in human maculae with drusen and BLD could be related to atherosclerosis, because this molecule accumulates in atherosclerotic plaques. Furthermore, evidence of an association between atherosclerosis and ARM has been found in epidemiologic studies.

To investigate the presence and localization of glycosaminoglycans (the carbohydrate components of proteoglycans; see Figure 3.1) in age-related macular deposits, we have used another specific two-dimensional electrophoresis technique. The same kind of macular material was used as in the glycoprotein study. Furthermore, the total amount of glycosaminoglycans was measured in these samples. For the localization of the specific glycosaminoglycans in macular tissue, paraffin sections were stained with several monoclonal antibodies. We found that maculae with ARM contain significantly more glycosaminoglycans per mg protein compared to controls, and that BLD contains chondroitin sulfate and heparan sulfate. The accumulation of glycosaminoglycans in BLD might play a role in the development of subretinal neovascularization. This could also be an explanation for the clinical observation that subretinal neovascular growth in ARM is mostly directed towards the macula, where BLD is most prominent.

It is assumed that neovascular AMD, one of the end-stages of ARM, could be induced by hypoxia of the outer retina. We have demonstrated a significantly increased expression of vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-β) in RPE-cells of human maculae with early stages of ARM compared to those of control specimens. The increased levels of VEGF in RPE-cells, and the outer nuclear layer of maculae with ARM might be caused by a relative hypoxia of the outer retina. It was concluded, that VEGF, and TGF-β could be involved in the development of neovascular AMD. Increased TGF-β expression in the RPE-cells of maculae with early stages of ARM could, in addition to its indirect angiogenic role (e.g. stimulating the production of angiogenic growth factors like VEGF), induce the accumulation of extracellular deposits like

BLD and drusen.

In future studies, it would be interesting to investigate whether the carbohydrate structures and the glycosaminoglycans are part of one big molecule. If this is the case, it is possible that molecules are present in eyes with ARM that do not appear in control eyes. Probably these molecules are caused by a metabolic shortcoming of the retinal pigment epithelium, that plays a crucial role in homeostasis of the retina. Such a metabolic insufficiency could be associated with hypoxia and atherosclerosis or can have a genetic origin. When the precise composition of BLD and drusen is revealed, we could get more insight in the pathogenesis of ARM in order to find a therapy or preventive measures for this severely invalidating disease.

CHAPTER 10

Samenvatting

Ouderdoms maculopathie (naar het engels afgekort met ARM) is een aan de leeftijd gerelateerde degeneratieve aandoening van het centrale deel van het netvlies. De eindstadia van deze aandoening zijn geografische atrofie en neovasculaire maculadegeneratie en worden ouderdoms maculadegeneratie (naar het engels afgekort met AMD) genoemd. Zij zijn de meest voorkomende oorzaak van sociale blindheid bij mensen boven de leeftijd van 65 jaar in de westerse wereld. De prevalentie loopt in Nederland op tot 11% bij mensen van 85 jaar en ouder. Aangezien deze populatie nog steeds toeneemt zal AMD een steeds groter probleem vormen. De ziekte werd een eeuw geleden voor het eerst beschreven door Haab (1888), maar tot op heden is er weinig bekend over de pathogenese. Helaas is er ook nog steeds geen goede therapie voor de meeste mensen met deze zeer invaliderende ziekte beschikbaar.

Tijdens het ouder worden van de humane macula in het centrum van het netvlies van het oog treden er een aantal goed omschreven histopathologische veranderingen op. Deze morfologische veranderingen staan beschreven in hoofdstuk 2. Deze veranderingen treden voornamelijk op in het retinale pigment epitheel, de membraan van Bruch en de choriocapillaris. Met name drusen en basale laminaire depositie (BLD), twee extracellulaire deposities, worden gezien als belangrijke factoren in de ontwikkeling van de eindstadia van ARM. Deze eindstadia zijn meestal precies in het centrum van de macula (de fovea) gelegen, waardoor het niet meer mogelijk is om details waar te nemen. Meestal worden beide ogen door deze ziekte getroffen en kunnen deze mensen bijvoorbeeld niet meer lezen, autorijden en televisie kijken.

De doelstelling van dit onderzoek was om meer van de chemische samenstelling van de extracellulaire deposities (drusen en BLD) te weten te komen en zo meer inzicht in de pathogenese van ARM te krijgen. Helaas is er voor ARM geen diermodel voorhanden en zijn de histologische beginstadia van deze ziekte klinisch vaak niet te onderscheiden. Daarom kan voor dit onderzoek alleen humaan materiaal worden gebruikt, dat vervolgens eerst histologisch geclassificeerd dient te worden.

Uit de literatuur is bekend dat drusen en BLD aankleuren met de periodic-acid-Schiff reaction (PAS), een routine histologische kleuring, die (aspecifiek) koolhydraat-verbindingen in weefsels aankleurt. Daarom zijn wij begonnen met het verder karakteriseren van deze koolhydraatgroepen door middel van het testen van lectines op formaline gefixeerd post-mortem materiaal. Lectines zijn moleculen die zeer selectief met bepaalde koolhydraat-moleculen kunnen binden. Uit dit onderzoek kwam naar voren dat BLD diverse koolhydraatmoleculen bevat waaronder, vrijwel exclusief in de macula, een N-geacetyleerde galactosamine. Drusen reageerden niet met de door ons gebruikte lectines. Door dit onderzoek is BLD nader gespecificeerd; het blijkt te bestaan uit glycoproteïnen en/of proteoglycanen. Bovendien kan de lectine, die exclusief het BLD aankleurt, als hulpmiddel worden gebruikt om de moleculen uit het BLD van de rest van de macula te scheiden.

Daarna hebben wij met behulp van een twee-dimensionale gel elektroforese techniek de eiwitpatronen van maculae met voorstadia van ARM vergeleken met die van controle maculae om te onderzoeken of de koolhydraten die wij met het lectine-onderzoek hadden gevonden onderdeel zijn van specifieke glycoproteïnen. Uit dit onderzoek bleek dat de β-keten van haptoglobine, een voorloper van het interfotoreceptor retinoid bindende eiwit (naar het engels afgekort met IRBP) en nog drie andere (niet geïdentificeerde) glycoproteïnen veel meer voorkomen in maculae met BLD en drusen dan in controle maculae. Voor dit onderzoek is echter het homogenaat van hele maculae gebruikt (behalve de sclera), omdat het niet mogelijk bleek om drusen en BLD van de andere structuren in de macula te scheiden. Daarom kan er geen uitspraak worden gedaan of de glycoproteïnen die wij hebben gevonden specifiek in drusen of BLD zitten of in andere hiermee gerelateerde afwijkingen in ARM. De gevonden glycoproteïnen reageerden niet met de lectines uit het vorige onderzoek. Het is mogelijk dat de koolhydraatgroepen in BLD, die met de lectines zijn aangekleurd, onderdeel uitmaken van zeer grote moleculen (bv. proteoglycanen), die met de gebruikte elektroforese techniek niet zijn te detecteren. De toename van de β-keten van haptoglobine in maculae met drusen en BLD zou gerelateerd kunnen zijn aan atherosclerose, omdat dit molecule in atherosclerotische vaatwandverdikkingen ophoopt. Er zijn overigens aanwijzingen uit epidemiologisch onderzoek dat er een relatie bestaat tussen ARM en atherosclerose.

Om te onderzoeken of de koolhydraatmoleculen in het BLD onderdeel zijn van glycosaminoglycanen (GAGs) hebben wij gebruik gemaakt van een elektroforese techniek om selectief GAGs te scheiden. Hiervoor is hetzelfde soort materiaal gebruikt als voor de analyse van de glycoproteïnen in de macula. De totale hoeveelheid GAGs in maculae met ARM en controles werd ook gemeten. Bovendien hebben wij paraffine coupes gekleurd met twee

histologische kleuringen om GAGs in het algemeen aan te kleuren. Tenslotte zijn er coupes gekleurd met monoclonale antilichamen die specifiek met diverse GAGs reageren. Hiermee hebben we gevonden dat maculae met ARM meer GAGs per mg eiwit bevatten dan controle maculae en dat BLD twee GAGs bevat, namelijk chondroitinesulfaat en heparansulfaat. De ophoping van GAGs in BLD zou een rol kunnen spelen in de ontwikkeling van neovasculaire AMD, aangezien deze moleculen nodig zijn voor de migratie en de groei van vaatwandcellen. Dit zou ook een verklaring kunnen zijn voor het feit dat neovasculaire AMD de neiging heeft om naar het centrum van de macula toe te groeien, waar de laag BLD meestal het dikst is.

Het wordt wel verondersteld dat neovasculaire AMD door hypoxie van het netvlies wordt veroorzaakt. We hebben een significante toename van vasculaire endotheliale groei factor (VEGF) en transformerende groei factor (TGF-β) gevonden in het retinale pigment epitheel van ARM maculae in vergelijking met controle maculae. De toename van VEGF zou veroorzaakt kunnen zijn door hypoxie van het netvlies en zo in relatie kunnen staan met de ontwikkeling van neovasculaire AMD. De toename van TGF-β in ARM maculae zou, naast een indirect angiogene rol, een rol kunnen spelen in de ophoping van extracellulaire deposities als drusen en BLD.

Het zou interessant zijn om in toekomstige studies uit te zoeken of de verschillende koolhydraten, die gevonden zijn met de lectinestudie, en de glycosaminoglycanen onderdeel zijn van één groot molecule. Als dit het geval is, kan dat betekenen dat er in ARM moleculen worden gevormd, die normaal niet voorkomen in het oog. Mogelijk is de vorming van dit molecule een gevolg van een stofwisselingsprobleem van het retinaal pigment epitheel, dat een cruciale rol vervult in de homeostase van het netvlies. Een dergelijk stofwisselingsprobleem kan bijvoorbeeld geassocieerd zijn met hypoxie en atherosclerose of kan een genetische achtergrond hebben. Wanneer de precieze samenstelling van BLD en drusen bekend is, kan men meer inzicht in de pathogenese van ARM verkrijgen teneinde een therapie of preventieve maatregel te vinden, die deze ziekte kan genezen of voorkomen.

DANKWOORD

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Rots in de branding, Lous Ruempol, is naast een kei in de organisatie en (financiële) administratie ook een enorm hartelijke en gezellige gesprekspartner.

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Een leven zonder Georgie kan ik mij niet meer voorstellen.

CURRICULUM VITAE

30 april 1967	geboren te Amsterdam
1973-1979	lager onderwijs aan de Hogewegschool te Amsterdam
1979-1981	V.W.O. aan de christelijke scholengemeenschap Buitenveldert te Amsterdam
1981-1985	V.W.O. aan de christelijke scholengemeenschap Noord-Kennemerland te Alkmaar
1985-1989	Doctoraal geneeskunde aan de Vrije Universiteit te Amsterdam
1989-1992	Artsexamen aan de Vrije Universiteit te Amsterdam
1993-heden	Assistent in opleiding bij het instituut Oogheelkunde aan de Erasmus Univeriteit te Rotterdam
1995	Lustrumprijs van de landelijke vereniging van arts-assistenten voor het onderzoek beschreven in dit proefschrift

Cursussen

Inspanningsfysiologie aan de faculteit der bewegingswetenschappen van de VU. Computer-programmeren aan de faculteit informatica van de VU. Oxford engels, filosofie, immunologie, klassieke statistische procedures en proefdierkunde aan de faculteit der geneeskunde van de EUR