厚生労働科学研究費補助金感覚器障害研究事業

# 緑内障の危険因子の解明による診断法の開発、緑内障マウスを用いた視神経保護薬の開発と予防・治療法への応用

平成１８年度研究報告書

平成１９年３月

主任研究者　岩田　岳

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厚生省科学研究費補助金（感覚器障害研究事業）

# 緑内障の危険因子の解明による診断法の開発、緑内障マウスを用いた視神経保護薬の開発と予防・治療法への応用

## 総括研究報告書


| | | |
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研究要旨：緑内障の危険因子として遺伝因子の関与が考えられる。我々は緑内障患者に特有の遺伝子多型を網羅的に解析した結果、統計学的に優位なリスク遺伝子を複数発見した。何れもまだ緑内障との関連が報告されていない遺伝子群である。また、これまでに先天性緑内障遺伝子として発見されたオプチニュリンとWDR36について遺伝子改変マウスを作製したところ、緑内障の特徴を発現するマウスが誕生した。緑内障研究において発症の始点となるリスク遺伝子の解明とその結末である動物モデルの開発に成功したこととにより、今後はその2点の中間にあたる発症機序の解明に力を注ぎたい。


### A.　研究目的

緑内障は遺伝子、環境、習慣、加齢など複数の因子によって発症する多因子疾患である。特に緑内障患者の遺伝的な背景を把握することは早期診断を可能とし、早期に予防を始めることができる。これまでの遺伝学的研究から欧米人と日本人の遺伝的背景は大きく異なっており、欧米人のデータを日本人にそのまま当てはまらないことが明らかにされている。すなわち、日本人の緑内障については日本人の患者から得られたDNA検体を解析する必要がある。今回我々は多施設共同研究によって収集した緑内障患者DNAを低眼圧緑内障及び高眼圧緑内障に分類し、患者特有の遺伝子多型を選別することを目的に個々の患者１００名に対し５０万個の遺伝子多型（SNP）を解析し、２００名の対照群と比較した。

複数の疾患について血漿組成が微量変化することが報告されており、我々は緑内障早期診断法として遺伝子多型解析に加えて血漿プロテオーム解析を行う。

また、緑内障研究においてこれまで困難とされてきた開放隅角緑内障（正常眼圧）マウスの作製に世界で初めて成功した。このマウスは緑内障患者と同様な神経乳頭の陥凹や神経節細胞死が観察されている。このマウスモデルは正常眼圧緑内障の発症機序の解明に役立つだけでなく、視神経保護薬や治療薬をスクリーニングするためのバイオアッセイ系として利用することができる。本研究事業ではすでに発見されている先天性緑内障遺伝子の改変マウスの作製だけでなく、リスク遺伝子の改変マウスについても作製を試みる。

これらのマウスについて視神経保護薬として可能性のある神経栄養因子やヒストン・デ・アセチレースの阻害剤、フリーラジカル関連薬剤、免疫抑制剤、グルタチオン亢進薬などによる視神経保護薬の効果を検討する。動物モデルにおける薬の効果を数値化するための評価系の確立に取り組む。また、より動物実験に適する大型眼球のラ

ットや霊長類を用いたドラッグデリバリー法の開発行う。

B. 研究方法

１）緑内障危険因子（遺伝子多型）に早期診断法の確立：

正常眼圧緑内障と高眼圧緑内障患者のDNA検体をそれぞれ２５０検体収集する。この多施設共同研究には国立病院機構、岐阜大学医学部眼科教室（山本　哲也)、順天堂大学医学部眼科教室（村上　晶)、順天堂大学医学部浦安病院眼科（溝田　淳）が参加した。収集DNA検体は個々にAffymetrix社の５０万SNPチップあるいはIllumina社の５０万 SNP チップを用いて検出を行った。SNPチップのシグナルは患者（１００人）－健常者（１００人）間で比較された後、数ヶ月にわたる統計処理が行われ、緑内障に固有の遺伝子多型が選別された。これらの遺伝子多型（SNP)をさらにグループ化し、ハプロタイプブロックとして疾患との連鎖を解析した。さらに SNP チップ解析に含まれなかった患者 DNA についても解析が継続されている。本研究では日本人の緑内障患者における遺伝子背景を調査することにより、欧米人とは異なるリスクの高い遺伝子変異や遺伝子多型が発見される可能性がある。

２）緑内障早期診断のための血漿プロテオーム解析：

全ての疾患において血漿蛋白組成に何らかの影響があると考えられ、遺伝子多型解析に加えて緑内障の早期診断を目的とした血漿バイオマーカーの探索を行っている。開放隅角緑内障患者の血漿50検体と健常者の 50 検体について４種類の分画法を使い、イオントラップ型質量分析計（LC-MS/MS）によって測定する。血漿中には分子量 5 万ダルトンを越える22種類の蛋白が重量比で９９％を占めており、これらの主蛋白を東レ株式会社が開発中の血漿低分子蛋白分画装置を使って除去した。低分子分画は逆相クロマトグラフィーでさらに分画され、トリプシン処理を行った後にイオン交クロマトグラフィー、逆相クロマトグラフィーで２次元的に分画され、質量分析計で蛋白質の同定を行った。質量分析スペクトグラムは２種類の蛋白同定ソフトウエアーによって解析し、両ソフトでリストされた蛋白を緑内障患者と健常者間で比較検討している。

３）．４つの既知緑内障遺伝子の詳細な機能解析と遺伝子変異による機能への影響の解明：

単一の遺伝子の変異による緑内障の原因遺伝子として MYOC、CYP1B1、OPTN、WDR36 の４遺伝子が発見されており、本研究ではこの中でも最も発症頻度の高い MYOC、正常眼圧力緑内障に関係する OPTN、そして最近発見された WDR36 の機能解析研究をおこなった。MYOC は毛様体から房水中に分泌されることが知られているが、その機能は明らかにされていない。MYOC の発現・精製は難しく、きわめて不安定であるために、COS-7細胞で強制発現を行い、培養液中に分泌されたMYOC分子を複数の培養細胞に加えて細胞膜蛋白との相互作用を検討した。OPTN については抗体を作製し、細胞内局在を調べる。また、緑内障患者で発見された遺伝子変異によってOPTNと蛋白相互作用への影響を調べる。WDR36 は現在も仮想遺伝子としてその機能は未知のままである。抗体作製などにより、網膜内での局在を調べる。

４）正常眼圧緑内障マウスを用いた発症機序の解明と神経保護薬の開発：

我々はこれまで困難とされてきた進行性の開放隅角緑内障マウスの作製に世界で初めて成功した。正常眼圧緑内障患者の一部に観察される OPTN 変異体（E５０K)を強制発現しており、緑内障の特徴である視神経乳頭の陥凹や神経節細胞死が観察された。接触型と非接触型の眼圧測定法で疾患マウスを測定した結果、何れも正常な14mmHgの眼圧を維持している。このマウスモデルは正常眼圧緑内障の発症機序の解明に役立つだけでなく、予防薬や治療薬を試験するためのバイオアッセイ系として利用することができる。

本研究では OPTN 遺伝子にとって E50K よりも大きな障害となる３アミノ酸欠損やモチーフ欠損マウスを作製中で、より重篤な

緑内障マウスモデルをめざす。さらに、WDR36 遺伝子改変マウスも同様な手法で作製し、OPTN マウスとの表現型の比較を行う。

これらのマウスは視神経保護薬として可能性のある神経栄養因子やヒストン・デ・アセチレース、フリーラジカル関連薬剤、免疫抑制剤、グルタチオン亢進薬などの薬効についてその評価系として利用する計画である。主に神経節細胞の生死を基準にした数値化が可能な評価系の確立をめざす。

さらに、眼球の大きなラットや霊長類モデルへと移行して、マウスでは困難な眼球内へのドラッグデリバリー法についても検討を行いたい。

C. 研究結果

1）緑内障危険因子（遺伝子多型）に早期診断法の確立：

多施設共同研究で収集された開放隅角緑内障患者の DNA は Affymetrix 社の５０万 SNP チップあるいは Illumina 社の２５万 SNP チップを用いて遺伝子多型解析が行われ、検出シグナルを患者と健常者間で比較された。この結果、緑内障患者に特有の遺伝子多型が複数発見され、統計学的計算を行ったところ緑内障のリスク遺伝子を複数同定することに成功した。これらの遺伝子は緑内障の早期診断に利用できるだけでなく、緑内障の発症に関する重要な情報をもたらす発見につながると考えられる。何れの遺伝子もこれまでに緑内障との関係は明らかにされておらず、今後の機能解析に期待される。これら遺伝子群については特許出願中である。

2）緑内障早期診断のための血漿プロテオーム解析：

遺伝子多型解析によるリスク遺伝子の発見に加え、患者血漿蛋白の微量変化を疾患バイオマーカーとして利用するための分析を開始した。東レ低分子量分画装置を用いて個々の患者と健常者の血漿検体について５０KDa 以下の蛋白を分離し、逆相クロマトグラフィーによって分画を行った。クロマトグラムは白内障患者やその他の眼疾患患者とは異なっており、質量分析計によるプロテオーム解析の結果が期待される。

3）４つの既知緑内障遺伝子の詳細な機能解析と遺伝子変異による機能への影響の解明：

緑内障原因遺伝子ミオシリン（MYOC）を COS-7 細胞で強制発現し、細胞外の培養液中に分泌された MYOC 分子を複数の培養細胞に加えて細胞膜蛋白との相互作用を検討した。その結果、コンフルエントな状態の NIH3T3 細胞膜に結合することを発見した。その他の細胞には結合せず、疎に培養されている NIH３T３細胞に対しても結合しなかった。蛋白架橋剤によって MYOC と結合する蛋白を捕らえる実験を行っているが、まだ同定にはいたっていない。

MYOC の蛋白構造を明らかにすることは、機能解明や阻害薬開発のための貴重な情報をもたらす。我々は MYOC の結晶化を行うために発現法と精製法を確立したが、結晶化条件を検討中で、まだ結晶を得ていない。結晶化が実現されれば北海道大学の稲垣先生等と共同で X-線結晶解析装置によって蛋白構造を明らかにする予定である。

正常眼圧緑内障の原因遺伝子として発見されたオプチニュリン（OPTN）は複数の蛋白質と相互作用することが明らかにされており、重篤な E50K 変異が Rab8 蛋白との結合部位であるとから、我々は水晶発振子などをつかって OPTN－Rab8 の蛋白相互作用を計測してきた。E50K 変体は Rab8 との相互作用ができないために細胞内での小胞体輸送系が障害されると考えられる。抗 OPTN 抗体による免疫染色法によって Rab8 との局在は一致する場合が観察されている。網膜神経節細胞株（RGC5）を使った E50K 変異体の強制発現によって細胞内オルガネラの変化が期待されたが、光学顕微鏡レベルでは異常や細胞死は観察されなかった。

最も新しい緑内障遺伝子 WDR36 は WD ドメインを蛋白中央に持つ WDR ファミリーの一つである。WDR は仮想遺伝子としてその機能は未知のままである。抗 WDR36 抗体を作製し、眼球内での局在を調査中である。

4）正常眼圧緑内障マウスを用いた発症機序の解明と神経保護薬の開発：

OPTN の変異体（E５０K）を全身で強制発現

したマウスを１６系頭作製した。このマウスは緑内障の特徴である視神経乳頭の陥凹や神経節細胞が観察された。また、E50K 変異体が網膜全体を含む全身で発現しているにもかかわらず、網膜神経節細胞の特異的な細胞死が観察できた。２種類の眼圧測定計を使ってマウスの眼圧を測定した結果、全てのマウスでほぼ14mmHg を維持していた。眼圧の測定は午前９－正午の間で行われた。逆走蛍光標識によって生存する神経節細胞数を測定した結果、E50K 変異体マウスは生後１年間で細胞数が約５％減少することが明らかにされた。

D. 考察

緑内障の有病率は岐阜県多治見市と日本緑内障学会が中心となって行った疫学調査（多治見スタディー）によって、４０歳以上で約5.0％に、７０歳以上では14％と高い発症率であることが明らかにされた。さらに開放隅角緑内障患者の約９割が正常眼圧緑内障と報告され、アメリカ人の26％と比べても大差であることが明らかとなった。このことは日本人緑内障患者が欧米人患者と異なる遺伝背景であると考えられる。日本人の緑内障に関係する危険因子を発見し、日本人のための発症前診断が可能になれば、早期に予防が開始できて、発症を未然に防ぐことや、発症の時期を遅らせることが可能となる。

我々は緑内障患者に特徴的な遺伝子と蛋白が存在するか調べるために遺伝子多型解析及び血漿プロテオーム解析を行った。この結果、緑内障患者に優位に現れる遺伝子多型が発見された。さらにこれらの遺伝子多型が遺伝子中に複数あることから、緑内障リスク遺伝子であると考えられる。何れの遺伝子もこれまでに報告されてきた単一緑内障原因遺伝子とは異なり、多因子緑内障遺伝子として遺伝子間の関係を明らかにする必要がある。隅角や網膜における局在や遺伝子欠損マウスの作製が次の実験目標である。

単一緑内障遺伝子としてすでに報告されているミオシリン（MYOC）、オプチニュリン（OPTN）、WDR36 の３遺伝子についてはその正常機能な疾患こと関係が不明である。我々はMYOC が毛様体から分泌されてどのように機能するのか明らかにすることによって、MYOC が眼圧調整に関連する分子であるか明らかにしたい。今回の実験から MYOC は密なNIH3T3 細胞表明に結合できることを発見し、結合相手の同定を急いでいる。この蛋白を明らかにし、隅角内での局在が明らかになれば、MYOC の変異によって眼圧が上昇するメカニズムが明らかにされる可能性がある。

正常眼圧緑内障の原因遺伝子として発見されたオプチニュリン（OPTN）は複数の蛋白質と相互作用することが明らかにされており、重篤な E50K 変異が Rab8 蛋白との結合部位であるとから、我々は水晶発振子などをつかってOPTN－Rab8 の蛋白相互作用を検討してきた。Rab8 は細胞内の小胞体輸送系に関与しており、神経節細胞死との関係を明らかにしたい。

新しい緑内障遺伝子 WDR36 は WD ドメインを蛋白中央に持つ WDR ファミリーの一つで仮想遺伝子としてその機能は未知のままである。我々は他の WDR 蛋白について蛋白構造を調べた結果、明らかにされているのは蛋白中央部の WD ドメインだけであることを知った。蛋白構造解析プログラム（日立 BioPackage）をつかって WDR36 の WD ドメインを構造計算のみできれいに構築することができた。しかし、N 末端や C 末端の構築には失敗している。両末端については部分発現と精製によって北海道大学稲垣研究室で NMR による構造解析を予定している。

我々はオプチニュリンとWDR36遺伝子改変マウスを作製し、神経節細胞保護薬のアッセイ系を確立することによって薬効評価を数値化できるように研究している。OPTNとWDR36については１アミノ酸置換に加えてより障害された遺伝子を発現することによって、より重篤な緑内障を早い時期に発症するマウスの作製を試みている。

作製されたマウスについて視神経保護薬として可能性のある神経栄養因子、ヒストン・デ・アセチレース、フリーラジカル関連薬剤、免疫抑制剤、グルタチオン亢進薬などの投与によって神経節細胞の保護が可能であるか検討する。さらに、正常ラット・マウスを使った神経線維の切断・クランプに対しても薬効を評価したい。効果のあった薬についはより眼球がよりヒトに類似する霊長類

（独立行政法人医薬基盤研究所筑波霊長類医科学研究センター）を用いて同様に検討を行う。

E. 結論

我々は緑内障患者に特有の遺伝子多型を網羅的に解析した結果、統計学的に優位なリスク遺伝子を複数発見した。何れもまだ緑内障との関連が報告されていない遺伝子群であり、今後の機能解析が期待される。また、これまでに先天性緑内障遺伝子として発見されたオプチニュリンと WDR36 について遺伝子改変マウスを作製したところ、緑内障の特徴を発現するマウスが誕生した。緑内障研究において発症の始点となるリスク遺伝子の解明とその結末である動物モデルの開発に成功したこととにより、今後はその 2 点の中間にあたる発症機序の解明に力を注ぎたい。

F. 健康危険情報

特になし

G. 研究発表

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シンポジウム１「房水流出路の不思議に迫る　-基礎と臨床-」
岩田岳　「緑内障マウスの分子生物学的解析」

第４５回網膜硝子体学会（東京、２００６）
シンポジウム１「眼内液・組織から得られる情報」
岩田岳　「硝子体：硝子体液、蛋白」

4th International Conference on Innate

Immunity（コルフ島、ギリシャ、２００６）
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H. 知的所有権の出願・取得状況

| | |
|---|---|
| 1 特許取得 | 出願中 |
| 2 実用新案登録 | なし |
| 3 その他 | なし |

# II. 研究成果

# 68

# Models of Age-Related Vision Problems

J. Fielding Hejtmancik, Marc Kantorow, and Takeshi Iwata

*The visual system provides unique opportunities to study the aging process, as well as challenges in understanding and developing therapies for age-related eye diseases. Exposure of the lens to high levels of photo-oxidative stress and the lack of protein turnover in the lens nucleus make it an optimal system in which to study protein modifications in aging. Similarly, the high level of metabolic activity in the retina and the necessity for turning over large amounts of lipids provide particular research opportunities as well. Finally, visual diseases associated with aging are among the most common threats to the quality of life in the elderly. Of age-related visual diseases, three result in a particularly high burden on the population: age-related cataracts, age-related macular degeneration, and progressive open angle glaucoma. Thus, these are dealt with in some detail in this brief review. Because of space and formatting limitations, much work described in this review could not be cited directly. The citations for most of these can be found in the references and general sources given in the chapter, and we apologize to those authors whose work is not cited directly. In addition, parts of this review draw from previous work by the three authors, reflecting their continuing preferences in style and arrangement.*

## Overview of the Visual System

### BASIC ANATOMY/PHYSIOLOGY/BRIEF BIOCHEMISTRY

Components of the visual system include the optical components of the anterior eye (cornea, aqueous humor, lens, and vitreous body), retina, optic nerves, optic tracts, optic radiations, visual cortex, and a variety of nuclei (see Figure 68.1). The optical components of the eye focus light on the retina, which transduces the light signal into neural signals, and passes these neural signals through the optic nerves and tracts to central structures that perform more elaborate processing, integrating their information with that of the other senses. Any disease that interferes with the function of these components will cause loss of vision and blindness, and each part of the visual system has specific susceptibilities to age-related diseases or damage.

### TYPES OF AGE-RELATED VISUAL DISEASES AND THEIR IMPACT ON SOCIETY

The predominant causes of age-related visual impairment and blindness vary between the developed and developing countries, and even within various demographic and ethnic groups within single countries (Thylefors *et al.*, 1995). There are many causes of visual loss in elderly patients, including diabetic retinopathy, stroke, and retinal vascular occlusive disease, along with other age-related visual diseases including pterygia and presbyopia. However, in most populations the greatest causes of blindness and vision loss in the elderly include cataracts, glaucoma, and age-related macular degeneration (Congdon, Friedman, and Lietman, 2003; Buch *et al.*, 2004).

Cataracts are the leading cause of blindness across the world, blinding 17 million persons worldwide. Cataracts are usually correctable by surgery in developed countries, with about 5% of the American population over 40 years old having undergone cataract surgery. However, they remain a significant cause of visual disability even in developed countries, being the leading cause of low vision in the United States (Congdon, Friedman, and Lietman, 2003). Glaucoma is an optic neuropathy, often related to elevated intraocular pressure, which is responsible for blindness in 6.7 million people across the world. Glaucoma is more common in

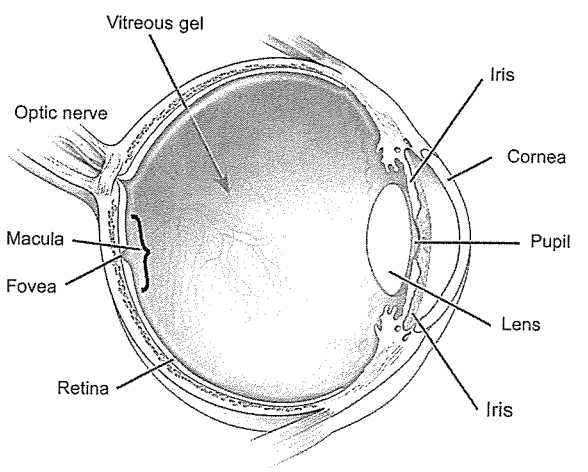


**Figure 68.1** Diagram of the eye with principal structures of the anterior segment, retina, and optic nerve indicated. Courtesy of the National Eye Institute, National Institutes of Health.

African-derived populations, and increases with age. Finally, the greatest age-related cause of blindness in European-derived populations of developed countries is age-related macular degeneration (AMD). This degenerative disease progresses from fatty retinal deposits called drusen to neovascularization and retinal hemorrhage, resulting in irreversible loss of central vision.

## Lens and Cataracts

The eye lens (see Figure 68.2), which contains perhaps the highest concentration of proteins found in any tissue, transmits and focuses light onto the retina. It is formed of a single cell type that differentiates from an anterior layer of cuboidal epithelia and migrates posteriorly to form elongated lens fiber cells that make up the lens nucleus. In this process, the developing fiber cells synthesize high levels of lens crystallins before losing their nuclei and mitochondria. Thus, the lens fiber cells lack aerobic metabolism and contain high concentrations of $\alpha$-crystallins, which are members of the small heat shock protein family and have chaperone activity; and $\beta\gamma$-crystallins, which are related to prokaryotic structural proteins.

### BRIEF OVERVIEW

The lens is susceptible to damage with aging since its cells cannot be replaced in this encapsulated tissue and its proteins cannot turn over in the nonnucleated fiber cells. Not only does this result in a decrease in function of the normal aged lens, but it also sets the stage for development of senescent cataract in individuals with additional environmental insult or genetic proclivity. As the lens ages, vacuoles and multilamellar bodies appear between fiber cells, and occasionally the fiber plasma membrane is disrupted. Most of the elaborate cytoskeletal structure of the lens cells disappears with aging, and by the fifth decade the ability to accommodate is essentially lost. There is a decrease in transparency of the normal lens with aging so that the intensity of light reaching the retina is reduced by about ten-fold by 80 years of age.

Cataracts which can be defined as any opacity of the crystalline lens, result when the refractive index of the lens varies significantly over distances approximating the wavelength of the transmitted light. Variation in the refractive index over these distances can result from changes in lens cell structure, changes in lens protein constituents, or both (Hejtmancik, Kaiser-Kupfer, and Piatigorsky, 2001). Cataracts are generally associated with breakdown of the lens micro-architecture. Vacuole formation can cause large fluctuations in optical density, resulting in light scattering. Light scattering and opacity also can occur if there are significant high molecular weight protein aggregates roughly 1000 Å or more in size. The short-range ordered packing of the crystallins, which make up over 90% of soluble lens proteins, is important in this regard; to achieve and maintain lens transparency crystallins must exist in a homogeneous phase.

A variety of biochemical or physical insults can cause phase separation of crystallins into protein-rich and protein-poor regions within the lens fibers. The proteins either remain in solution or form insoluble aggregates or even crystals, any of which can result in light scattering. When mutations in crystallins are sufficient in and of themselves to cause aggregation, they usually result in congenital cataracts, but if they merely increase susceptibility to environmental insults such as light, hyperglycemic, or oxidative damage, they might contribute to age-related cataracts (Hejtmancik and Smaoui, 2003). Thus, congenital cataracts tend to be inherited in a Mendelian fashion with high penetrance, whereas age-related cataracts tend to be multifactorial, with both multiple genes and environmental factors influencing the phenotype. This makes them significantly less amenable to genetic and biochemical study. Finally, although the young human lens is colorless, a gradual increase in yellow pigmentation occurs with age. As this pigmentation increases, it can result in brunescent or brown cataracts.

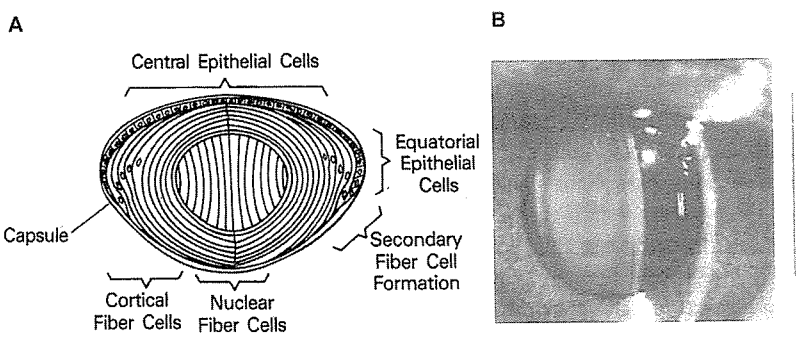


**Figure 68.2** A. A diagram showing lens structure including the anterior epithelial cells; the cortical fiber cells, which elongate and loosen their nuclei and mitochondria; and the nuclear cells, in which this process has been completed. The ends of the nuclear fiber cells abut each other in a complex pattern to form the lens sutures. **B.** Slit lamp photograph of a nuclear cataract, the most common type of age-related cataract in European populations. Courtesy of Dr. Manuel Datiles, National Eye Institute, National Institutes of Health.

*Lens proteins and their age-related modifications*

Enzymatic activity in the lens tends to decrease with age and to be lower in the central cells of the lens nucleus than in the cortical and anterior epithelial cells. As the lens ages, the $Na^+$ and $Ca^{2+}$ concentrations rise, reflecting an increase in lens permeability or a decrease in pumping efficiency. With aging, both the N- and C-terminal arms of half of the intrinsic membrane protein (MP26) molecules undergo proteolysis to form MP22. The lens contains neutral proteinase, also called the *multicatalytic-proteinase complex*, which preferentially degrades oxidized proteins, leucine aminopeptidase, calpains, and the protease cofactor ubiquitin, whose activation increases after oxidative stress. The activity of these proteinases is controlled by inhibitors, which appear to be concentrated at the periphery of the lens.

Aging also leads to an increase in high-molecular-weight aggregates and water-insoluble protein between 10 and 50 years of age, especially in the $\alpha$-crystallins, but also in the $\beta$- and $\gamma$-crystallins. There is also partial degradation of crystallins and covalent modifications of crystallins and other lens proteins, including an increase in disulfide bridges, deamidation of asparagine and glutamine residues, and racemization of aspartic acid residues. $\alpha$A-Crystallin is cleaved nonenzymatically, particularly between Asn 101 and Glu 102. An aspartate residue in $\alpha$A-crystallin appears especially susceptible because it easily forms a succinimide intermediate. Phosphorylation of lens proteins also occurs. Nonenzymatic glycosylation (glycation) occurs, especially of the $\varepsilon$-amino groups of lysine. Through the Maillard reaction, the glycation products can result in increased pigmentation, nontryptophan fluorescence, and nondisulfide covalent crosslinks. Lens proteins can also undergo carbamylation, which can induce cataracts, and may be the mechanism for the association of cataracts with chronic diarrhea and uremia. $\gamma$-Crystallins, and especially $\gamma$S-crystallin, are particularly susceptible to degradation and modification in age-dependent and other cataracts, largely being degraded to low-molecular-weight peptides by increased proteolysis in the cataractous lens.

In age-related cataracts the lens presumably develops reasonably normally during infancy and remains clear in childhood. Then, by somewhat arbitrary definition, at some time after 40 years of age, progressive opacities begin to form in the lens. As mentioned earlier, these opacities almost certainly result at least in part from the cumulative damage of environmental insults on lens proteins and cells. Many of the age-related changes seen in crystallins are accelerated in the presence of oxidative, photo-oxidative, osmotic, or other stresses, which are known to be associated with cataracts. Susceptibility to these alterations may be exacerbated by barriers to movement of small molecules between the central lens nucleus and the metabolically more active epithelium. Many of these changes can be induced *in vitro* or in model systems by the same stresses epidemiologically associated with cataracts (Davies and Truscott, 2001; Spector, 1995). In contrast, some changes do not appear to be implicated in cataractogenesis and may even serve to protect crystallins from harmful modifications.

The lens crystallins form one obvious target for this accumulated damage, although they are certainly not the only one. Thus, as the $\beta$- and $\gamma$-crystallins slowly accumulate damage over the lifetime of an individual, they lose the ability to participate in appropriate intermolecular interactions, and even to remain in solution. As these crystallins begin to denature and precipitate, they are bound by the $\alpha$-crystallins, which have a chaperone-like activity. Binding by $\alpha$-crystallins maintains the solubility of $\beta\gamma$-crystallins and reduces light scattering, but the $\alpha$-crystallins appear not to renature their target proteins and release them into the cytoplasm, as do true chaperones. Rather, they hold them in complexes that, though soluble, increase in size as additional damaged protein is bound over time until they themselves begin to approach sizes sufficient to scatter light. Eventually, it seems likely that the available $\alpha$-crystallin is overwhelmed by increasing amounts of modified $\beta\gamma$-crystallin and the complexes precipitate within the lens cell, forming the insoluble protein fraction that is known to increase with age and in cataractous lenses.

*Brief epidemiology of age-related cataracts*

Age-related cataracts are associated with a number of environmental risk factors, including cigarette smoking or chronic exposure to wood smoke, obesity or elevated blood glucose levels, poor infantile growth, exposure to ultraviolet light, and alcohol consumption (The Italian-American Cataract Study Group, 1991). Conversely, antioxidant vitamins seem to have a protective effect, although this has not been borne out by all studies.

There is increasing epidemiological evidence that genetic factors are important in the pathogenesis of age-related cataracts (McCarty and Taylor, 2001). In 1991, the Lens Opacity Case Control Study indicated that a positive family history was a risk factor for mixed nuclear and cortical cataracts, and the Italian–American cataract study group supported a similar role for family history as a risk factor in cortical, mixed nuclear and cortical, and posterior subcapsular cataracts. In 1994, the Framingham Offspring Eye Study showed that individuals with an affected sibling had three times the likelihood of also having a cataract. The Beaver Dam Eye Study examined nuclear sclerotic cataracts using sibling correlations and segregation analysis. Although a random environmental major effect was rejected by this study, Mendelian transmission was not rejected, and the results suggested that a single major gene could account for as much as 35% of nuclear and up to 75% of cortical cataract variability. Most recently, the twin eye study demonstrated significant genetic influence of age-related cortical cataracts,

with heritability accounting for 53 to 58% of the liability for age-related cortical cataracts. This hereditary tendency was consistent with a combination of additive and dominant genes, with dominant genes accounting for 38 to 53% of the genetic effect, depending on whether cataracts were scored using the Oxford or Wilmer grading systems. Similarly, genetic factors were found to account for approximately 48% of the risk for nuclear cataracts.

## HUMAN STUDIES ON AGE-RELATED CATARACTS

### *Linkage studies*

In addition to epidemiological evidence implicating genetic factors in age-related cataracts, a number of inherited cataracts with post-infantile age of onset or progression of the opacity throughout life have been described. Mutations in beaded filament specific protein 2 (BFSP2) can cause juvenile cataracts, the Marner and Volkmann cataracts can be progressive, mutations in aquaporin 0 (MIP) and γC-crystallin can cause progressive cataracts, and the CAAR locus is linked to familial adult onset pulverulent cataracts. These all suggest that for at least some genes, a mutation that severely disrupts the protein or inhibits its function might result in congenital cataracts inherited in a highly penetrant Mendelian fashion, whereas a mutation that causes less severe damage to the same protein or impairs its function only mildly might contribute to age-related cataracts in a more complex multifactorial fashion. Similarly, mutations that severely disrupt the lens cell architecture or environment might produce congenital cataracts, whereas others that cause relatively mild disruption of lens cell homeostasis might contribute to age-related cataracts.

### *Association studies*

The hyperferritinemia-cataract syndrome is a recently described disorder in which cataracts are associated with hyperferritinemia without iron overload. Ferritin L levels in the lens can increase dramatically. The molecular pathology lies in the ferritin L iron responsive element, a stem loop structure in the 5′ untranslated region of the ferritin mRNA. Normally, this structure binds a cytoplasmic protein, the iron regulatory protein, which then inhibits translation of ferritin mRNA, which may exist in the lens at levels approaching that of a lens crystallin. Mutation of this structure and overexpression of ferritin by loss of translational control in the hyperferritinemia-cataract syndrome results in crystallization of ferritin in the lens, and other tissues as well. Ferritin crystals appear as breadcrumb-like opacities in the cortex and nucleus. Ferritin cataracts serve as an example that the presence of crystallin proteins at such high levels in the protein-rich lens cytoplasm requires that they must be exceptionally soluble. This is emphasized by the occurrence of cataracts resulting from single base changes decreasing crystallin solubility but not stability.

Lamellar and polymorphic cataracts have been associated with missense mutations in the MIP gene. One mutation, E134G, is associated with a nonprogressive congenital lamellar cataract, and the second T138R is associated with multifocal opacities that increase in severity throughout life. When expressed in *Xenopus laevis* oocytes, both of these mutations appear to act by interfering with normal trafficking of MIP to the plasma membrane and thus with water channel activity. In addition, both mutant proteins appear to interfere with water channel activity by normal MIP, consistent with the autosomal dominant inheritance of the cataracts.

Galactosemic cataracts provide an interesting example of mutations that severely affect a gene causing congenital cataracts, and of milder mutations that contribute to age-related cataracts. Deficiencies of galactokinase, galactose-1-phosphate uridyl transferase, and severe deficiencies of uridine diphosphate 1-4 epimerase cause cataracts as a result of galactitol accumulation and subsequent osmotic swelling. The latter two are also associated with vomiting, failure to thrive, liver disease, and mental retardation if untreated, whereas the cataracts in galactokinase deficiency are isolated. Interestingly, galactosemic cataracts initially are reversible both in human patients and in animal models. In 2001, a novel variant of galactokinase, the Osaka variant with an A198V substitution, was shown to be associated with a significant increase in bilateral cataracts in adults (Okano *et al.*, 2001). It results in instability of the mutant protein and is responsible for mild galactokinase deficiency, leaving about 20% of normal levels. This variant allele frequency occurs in 4.1% in Japanese overall and 7.1% of Japanese with cataracts. The allele was also present in 2.8% of Koreans but had a lower incidence in Chinese and was not seen in blacks or whites from the United States. This and other GALK1 variants appeared to be absent from Northern Italians with age-related cataracts, suggesting that the genetic contributions cataract might vary in different populations.

The GALK1 results fit in well with the known influence of hyperglycemia on age-related cataracts. That these cataracts result from polyol accumulation is suggested by work in galactosemic dogs and transgenic and knockout mice. Dogs have aldose reductase levels similar to those in humans and when stressed readily develop sugar cataracts that are prevented by aldose reductase inhibitors. Mice, which have very low aldose reductase activity in the lens, are naturally resistant to sugar cataracts, either galactosemic or hyperglycemic. However, upon transgenic expression of aldose reductase, mice readily develop cataracts, especially when the galactokinase or sorbitol dehydrogenase gene is deleted. Consistent with these animal data are the recent findings that susceptibility to cataracts as a diabetic complication in humans is associated with specific allele Z of the

microsatellite polymorphism at 5′ of the aldose reductase gene.

## BIOCHEMICAL STUDIES OF AGE-RELATED CATARACTS

*Crystallin modifications associated with cataracts*

The lens crystallins are a major potential target for accumulating damage associated with age-related cataracts, although there are certainly others. Thus, as the crystallins accumulate modifications and damage over the lifetime of an individual, their ability to participate in appropriate intermolecular interactions, and even to remain in solution, decreases. Whether proteins in age-related cataracts become insoluble as a result of complete or partial denaturation, or whether they simply become less soluble due to modifications that leave their protein folds largely intact or both, is not currently known. However, it seems clear that modifications to crystallin proteins accumulate with aging and accelerate during cataractogenesis, and the combination of crystallin modification, disulfide-crosslinking, denaturation, and aggregation results in loss of lens transparency and cataract formation (Hanson *et al.*, 2000). The protein modifications involved in this process include, but are not limited to, proteolysis, racemation, oxidative changes, and glycation. The many factors believed to induce these modifications include free radicals and superoxides, along with a loss of the lens' reducing state causing oxidation and disulfide-crosslinking, sugar accumulation causing glycation, and cyanate causing carbamylation.

Protein modifications in age-related cataracts are believed to arise from a combination of environmental and endogenous factors. For instance, considerable evidence suggests that oxidative modifications are a hallmark of age-related cataracts and oxidation of crystallins and other lens proteins likely results from reactive oxygen species that are produced by both UV-light exposure and are also a byproduct of mitochondrial respiration during which as much as 2% of respiratory oxygen is converted to reactive oxygen species. A major result of oxidation is conversion of methionine to methionine sulfoxide, which increases with age in the human lens and reaches levels as high as 60% in age-related cataracts relative to clear lenses.

Multiple identified and yet unidentified proteases are present in the lens and proteolyzed crystallins are a predominate feature of age-related cataracts. Among multiple lens proteases that have been identified to act on crystallin proteins, calcium-activated proteases are believed to play major roles. Proteolysis of specific crystallins is believed to result in protein aggregation and cataracts.

Proteins in age-related cataracts become insoluble as a result of complete or partial denaturation or by becoming less soluble due to modifications that leave their protein folds largely intact, or perhaps by a combination of these processes. Many highly studied Mendelian congenital cataract models support both denaturation, as is seen in the association of some severe crystallin mutations with cataracts, and simple insolubility with maintained protein folds as is seen in other cataracts. Many classical studies have demonstrated that lens proteins become insoluble because they are denatured as the lens ages. Insoluble protein in the aged cataractous lens not only is denatured and crosslinked, but a fraction exists as relatively short peptides cleaved from larger proteins. It seems likely that the presence of large amounts of unstable or precipitated crystallin, or other protein, does damage to the lens cell and its proteins and eventually contributes to cataracts not only directly through light scattering by protein aggregates but eventually also through disruption of cellular metabolism and damage to the cellular architecture. This is clear from numerous mouse models of cataracts resulting from crystallin mutations (Graw and Loster, 2003).

*Gene expression changes in cataract*

In addition to crystallin modifications, age-related cataracts are also associated with changes in gene expression detected at the level of increased or decreased mRNA in the lens epithelium (Hejtmancik and Kantorow, 2004). Since the lens epithelial cells cover the anterior surface of the lens, whereas in age-related cataracts the opacities tend to occur in the nuclear or cortical fiber cells, these gene expression changes likely reflect responses of lens epithelial cells to the presence of underlying cataracts and/or altered epithelial function in the presence of cataracts. These gene expression changes nevertheless point to altered lens pathways associated with this disease. For instance, the mRNAs encoding metallothionein and osteonectin (also known as SPARC, secreted acidic protein rich in cysteines) are increased in cataracts, whereas those for protein phosphatase 2A regulatory subunit and some ribosomal proteins including L21, L15, L13a, and L7a are decreased. These alterations suggest that increased binding of toxic metals and Ca++ with a concomitant decrease in growth pathways and protein synthesis are features of cataract.

In addition to the identification of individual alterations in gene expression, more recent studies have sought to identify the full range of gene expression changes that occur in the lens epithelium upon cataract formation using DNA microarrays. Although literally thousands of genes whose expression is altered in cataract have been identified in these studies, some specific examples of genes increased in cataract include SP1 required cofactor for transcriptional regulation, osteomodulin, chloride channel 3, Na+K+ transporting polypeptide beta 1, and Ca++ transporting ATPase, whereas genes decreased include αA-crystallin, multiple glutathione peroxidases, multiple ribosomal subunits, HSP 27, Na+/K+ ATPase and transketolase. The majority of the identified genes are decreased in cataract, suggesting loss of gene expression

as a consequence of lens damage. Functional clustering of the identified genes suggests that the genes increased in cataract tend to be associated with transcriptional control, ionic and cytoplasmic transport, protein salvaging pathways, and extracellular matrix components; transcripts decreased in cataract tend to be associated with protein synthesis, defense against oxidative stress, heat shock/chaperone activity, structural components of the lens, and cell cycle control (Hejtmancik and Kantorow, 2004).

*Enzyme changes associated with cataracts*

In addition to the protein modification and gene expression changes noted earlier, numerous metabolic and enzyme activity changes are also associated with age-related cataracts. These changes include decreased reduced glutathione content, decreased NADPH levels, increased free $Ca^{++}$ levels, increased activity of specific proteases, and decreased ionic balance, among others. Considerable evidence suggests that many of these changes, other metabolic changes, and loss of lens protein function results from loss of the activities of specific lens protective and repair enzymes and other homeostatic systems. Although the evidence for these changes has been almost exclusively derived from animal, cell, and organ culture experimental systems, loss of the activities of multiple protective systems including $\alpha$-crystallins, MnSOD, catalase, glutathione peroxidase, and $\gamma$-glutamylcysteine synthetase among many others are believed to contribute to loss of lens function and ultimately cataract formation. In addition to the loss of lens protective and homeostatic systems, the loss of key repair systems including thioltransferase and methionine sulfoxide reductases are also believed to be key events in cataract formation.

## ANIMAL MODELS OF AGE-RELATED CATARACTS

*Overview*

Since cataractogenesis is a complex process accompanied by numerous secondary changes, animal models may provide useful information for delineating the causes of senescent and other cataracts. Hereditary cataracts in rodents have been especially useful in this regard (Graw and Loster, 2003). One example is the Philly mouse, which displays an autosomal dominant cataract in which there is a deficiency of $\beta$B2-crystallin polypeptide. The $\beta$B2-crystallin mRNA has a deletion of 12 nucleotides, resulting in a four-amino-acid deletion in the encoded protein. It has been hypothesized that this causes aberrant folding of the protein and that cataract formation occurs as a result of the molecular instability of this crystallin and is therefore a good model to examine the roles of crystallin proteolysis and aggregation in age-related cataract formation. Other models suggest that some metabolic lesions can also cause cataracts. The Nakano mouse, which has autosomal recessive cataracts mapping to chromosome 16, shows reduced synthesis of $\alpha$- and $\beta$-crystallins. This is probably due to an increase in the Na+/K+ ratio occurring because of inhibition of the sodium-potassium pump. The Fraser mouse, which displays an autosomal dominant cataract, shows preferential loss of $\gamma$-crystallins and their mRNAs. However, the gene causing this cataract segregates independently of the $\gamma$-crystallin gene cluster, suggesting that changes in crystallin expression must be secondary in this cataract. It resides on chromosome 10 and has been suggested to be allelic with the mouse lens opacity gene (LOP).

*Emory mouse*

Unlike the animal cataract models eariler, the Emory mouse is an interesting model for age-related cataracts that has been phenotypically but not molecularly or genetically well-characterized (Kuck, 1990). Two substrains of Emory mice in which cataracts develop at five to six months (early cataract strain) and six to eight months (late-cataract strain) are known. Emory mouse cataracts increase in severity with age and are initiated in the lens superficial cortex. They eventually progress into the deep anterior cortex and ultimately result in complete opacification. Emory mouse cataracts exhibit multiple changes that appear to mimic accelerated aging including abnormal lens growth, decreased protein accumulation, conversion of soluble to insoluble protein, decreased reduced glutathione, decreased protein sulfhydryl levels, decreased superoxide dismutase activities, decreased catalase activity, decreased glutathione peroxidase activity, decreased $\gamma$-glutamylcysteine synthetase activity, and accelerated conversion of MP26 to MP24. The Emory mouse is also associated with changes in gene expression including decreased synthesis of crystallins and increased expression of ARK tyrosine kinase, which is believed to be a major upstream activator of the stress response in many cell types.

*In vivo hyperbaric oxygen treatment*

Many of the modifications undergone by lens proteins in aging and cataractous lenses are consistent with those seen in photo-oxidative stress, and oxidative stress is known to be a risk factor in age-related cataracts (Giblin *et al.*, 1995). Thus, exposing animals to increased oxygen tension to simulate the more prolonged oxidative stress associated with aging is an attractive and logical model system for understanding human cataract. In these studies, animals are exposed to 100% oxygen at increased pressure several times weekly for two to three months, and lens opacities are monitored by imaging with a slit lamp. Molecular and biochemical changes in the treated animals subsequently are correlated with lens opacity and oxygen treatment. Hyperbaric oxygen treatment *in vivo* accelerates lens opacity in the nuclear region of the guinea pig lens including loss of water soluble and cytoskeletal proteins, formation of protein disulfides, and

degradation of MIP26. Such modifications are similar to modifications reported to occur in the nuclei of aging and cataractous human lenses, confirming that hyperbaric oxygen treatment is an excellent model to study those processes occurring in human cataracts.

*Other*

In addition to the preceding models, cell culture, organ culture, and transgenic mice provide powerful tools for the study of lens transparency. Multiple lens epithelial cell lines have been used to identify and functionally analyze those enzymes and other proteins important for resistance to oxidative stress, chaperone function, and other processes associated with cataractogenesis. For instance, the importance of specific enzymes such as methionine sulfoxide reductase and MnSOD for maintaining lens cell viability and resistance to oxidative stress have been identified through the over-expression or silencing of these enzymes in lens cells, which are subsequently treated with H2O2 and/or other oxidants associated with cataracts. Other approaches include similar experiments using lens cells cultured from animal knockouts deleted for specific lens proteins such as αA-crystallin. In addition to cultured lens cells, cultured whole lenses also have been employed to monitor multiple biological events associated with cataracts.

In practice, creation of cataractous transgenic mouse lines is facilitated by the lens being readily examined for transparency, providing a rapid and efficient means to screen for phenotypic effects of transgenic insertions. Most cataracts in transgenic mice are associated with abnormalities of lens development, especially uncontrolled growth, toxic ablation of specific lens cells, or immune destruction of the lens. Lens abnormalities have been caused in transgenic mice using a variety of strategies. Expression of diphtheria toxin or ricin under the control of a lens-specific α-crystallin or γ-crystallin promoter, respectively, has caused ablations within the lens.

In addition to transgenic expression of normal or modified proteins, disrupted expression of a protein normally found in the lens has been shown to cause cataracts. Lack of αA-crystallin expression causes cataracts with inclusion bodies in central lens fiber cells (Brady *et al.*, 1997). Other knockouts associated with cataracts include osteonectin, connexins, and glutathione peroxidase. Collectively, these engineered cataract models emphasize the importance of the crystallins, cytoskeleton, and intercellular matrix for lens transparency.

## Macular Degeneration

### BRIEF OVERVIEW

Macular degenerations are a phenotypically and genotypically heterogeneous group of blinding disorders characterized by central vision loss associated with RPE atrophy with or without choroidal neovascularization. Of these, age-related macular degeneration (AMD) is a degenerative disorder of the cone-rich macular and perimacular regions of the retina with resulting loss of central visual acuity. Although AMD principally affects the supporting and metabolic structures of the retina including the retinal pigment epithelial (RPE) cells, the choriocapillaris, and Bruch's membrane, vision loss comes from the resulting retinal atrophy and its associated photoreceptor dysfunction (see Figure 68.3). Visual dysfunction is made worse by neovascularization, the ingrowth of choroidal vessels through defects in Bruch's membrane, with secondary hemorrhage, and retinal detachment that characterize the "wet" form of AMD. This is contrasted to the "dry" or nonneovascular form, which comprises 80% of the disease but results in only roughly 20% of its associated blindness. Drusen, small yellow-white deposits below the retina, are increased in individuals with AMD. Although they do not cause visual loss by themselves, drusen represent a risk factor for development of both the geographical atrophy (dry) and neovascularization (wet) types of AMD, especially when they are soft or indistinct. Recent results from the Age-Related Eye Disease Study suggest that the incidence of AMD could be lowered significantly by diet supplementation with high-dose antioxidant vitamins and zinc.

The clinical terms *dry* and *wet* typically are used to refer to different forms of AMD, with the dry form sometimes progressing to the wet form. Early stages of the dry form are characterized by focal pigmentation and accumulation of drusen between the RPE and Bruch's membrane. In later stages, the wet form is characterized by choroidal neovascularization, detachment of the RPE, and geographic atrophy of the RPE in the macular region. Drusen are classified as hard and soft, based on their shape, diameter, and color. Hard drusen are yellowish,

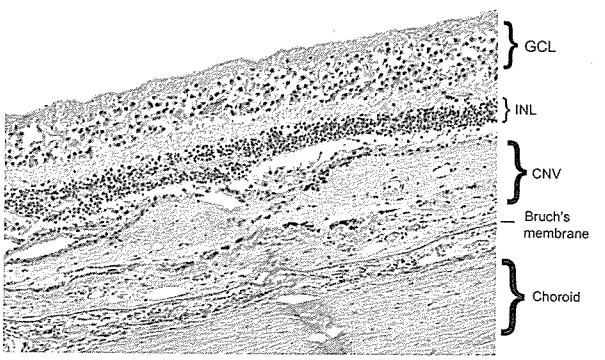


**Figure 68.3** Histological section of the retina showing macular degeneration. Although the ganglion cell layer (GCL), inner nuclear layer (INL) and choroid are well preserved, the outer nuclear layer, which should appear similar to the INL, has been in large part replaced by fibrovascular choroidal neovascularization (CNV). Courtesy of Dr. Chi Chao Chan, National Eye Institute, National Institutes of Health.