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総説

網膜・硝子体のプロテオーム解析

岩田　岳

〔要　約〕

これまで「遺伝子」「DNA」という言葉をよく見聞きするようになったが，このDNA転写物であるRNAから生成される蛋白質（プロテオーム）の解析が，近年分析技術の向上とともに進歩をみせている。プロテオームはDNAとは異なり，加齢や疾患などの生体の状態によって変化するために，疾患の病態を解明するためだけではなく，体内の微量なプロテオームの変化が疾患の検査・早期診断に応用される可能性が期待されている。プロテオーム解析による網膜硝子体研究への応用と課題について紹介したい。

はじめに

ヒトゲノムプロジェクトが終了し，約2万3千個の遺伝子が発見された。この遺伝子から転写されるRNA（トランスクリプトーム）から10万種類以上のタンパク質が生成されると推察されている（図1）。近年，タンパク質のイオン化技術や質量分析計の精度が向上し，さらにそれを制御・解析ソフトウエアの改良によって，質量分析計の専門技術者でなくても細胞，組織，体液などのタンパク質（プロテオーム）を網羅的に測定し，データを解析することがある程度可能になってきた。健常と病気のプロテオームを比較し，その違いを明らかにすることは，疾患の発症機序を解明するために必要な情報をもたらすだけでなく，疾患バイオマーカーとして早期診断法への応用が期待される。硝子体プロテオームは疾患網膜の状態を反映してダイナミックに変化していると考えられる。網膜疾患によっては脈絡膜毛細血管から網膜成分が漏出し，血漿成分の変化として捉えられる可能性がある。本編ではここ数年間の質量分析計を用いた網膜・硝子体の網羅的タンパク質解析に焦点を絞り，その利用方法と臨床応用への可能性について紹介したい。

Ⅰ．硝子体のプロテオーム

硝子体は眼球内で最も体積を占める透明なゼリー状の組織であり，網膜と接しているために網膜疾患によってその組成は大きく変化していると考え

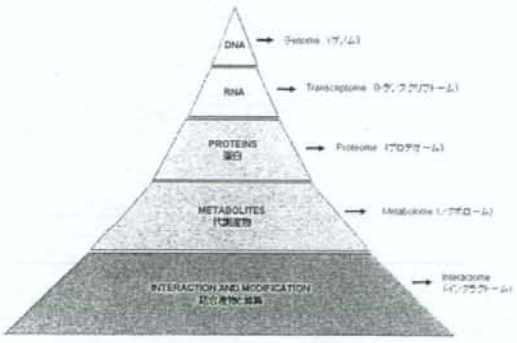

図1　生命現象の研究を総称してフェノミックスという

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られる。硝子体，房水，血漿の蛋白量をそろえて1次元電気泳動を行うと見分けがつかないほど泳動パターンは類似している。これは房水も硝子体も血漿由来の体液であり，血漿を構成する蛋白が硝子体や房水にも多く含まれていることを意味している。これまで我々が扱った房水や硝子体検体の蛋白濃度は出血の有無などによって0.1-1.0 mg/mlであるのに対し，血漿は50-70 mg/mlと50倍以上の蛋白濃度が測定されている。高蛋白濃度の血漿はプロテオミクス（プロテオーム研究）の分野で最も解析が先行しており，1万種類のタンパク質がすでに同定されている。血漿は22種類の蛋白が99%を占めており，微量蛋白は残り1%に含まれている（図2）。質量分析計の性質上，高い濃度で存在する蛋白から検出されるので，血漿を無分画のまま測定するとアルブミン，免疫グロブリン，トランスフェリンなどが検出され，微量タンパク質は検出されにくい。この数年間に22種類の蛋白を除去するための前処理技術として，各タンパク質に特異的な抗体を用いたアフィニティークロマトグラフィーカラムなどが開発されているが，我々は東レ株式会社が開発中の中空繊維（ホロファイバー）を用いた低分子分画装置を利用して，低分子量領域に絞って，微量成分の分画を試みている（図3）。硝子体プロテオームは血漿プロテオームで蓄積されたノウハウを応用して微量タンパク質の同定が今後盛んに行われると予想される。

日本は硝子体プロテオームでこれまで世界をリードしており，これまでに報告された4つのパイオニア的な硝子体プロテオーム研究をご紹介したい。2002年，中西等は糖尿病網膜症（3検体）と黄斑円孔（2検体）の患者の硝子体についてそれぞれ2次元電気泳動，質量分析計を用いて分析した結果，50種類の蛋白を同定し，この内の30は血漿には含まれていないことを明らかにした。IgG, α-antitrypsin, α-2-HS glycoprotein, complement C4断片が糖尿病の硝子体で増加していることを報告している[1]。2003年小山等は糖尿病網膜症患者の硝子体を1次元電気泳動後，質量分析計で分析した結果，84種類の蛋白の同定に成功し，前年に行った2次元電気泳動と合わせて121種類のタンパク質の同定に成功している。4種類の血管促進因子と3種類の抑制因子，PEDF, endostatin, thrombospondinが検出されている[2]。また同年，山根等は黄斑円孔（26検体）の硝子体を2次元電気泳動で分画し，400スポットを確認，78を同定している。同定されたペプチドは18の蛋白に由来しており，この中にはPEDF, prostaglanding-D2 synthase, IRBPが含まれていた。増殖性糖尿病網膜症（33検体）も同様に解析した結果，600スポットを確認し，121を同定した結果，38の蛋白が同定された。EnolaseとCatalaseが糖尿病の硝子体で顕著に増加しており，黄斑円孔の硝子体や糖尿病の血清中には検出されなかった[3]。2005年には大内等が，

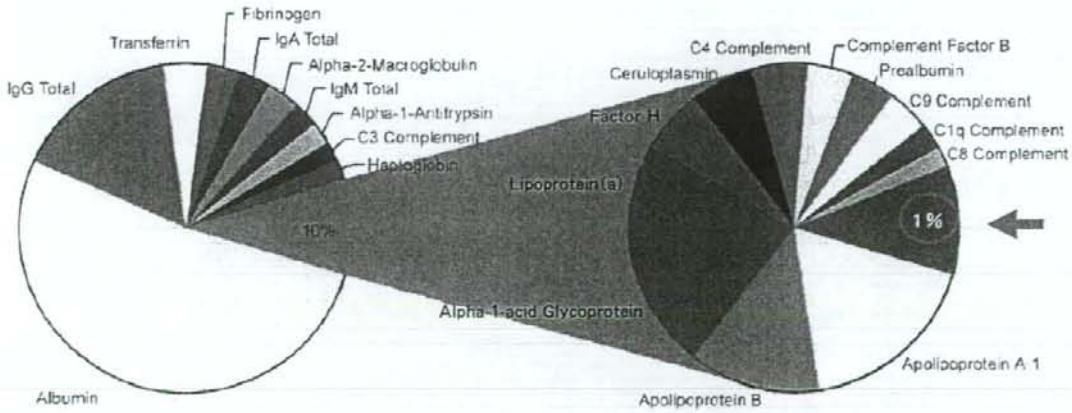

図2　血漿に含まれる蛋白とその割合。22種類のタンパク質が99%を占める

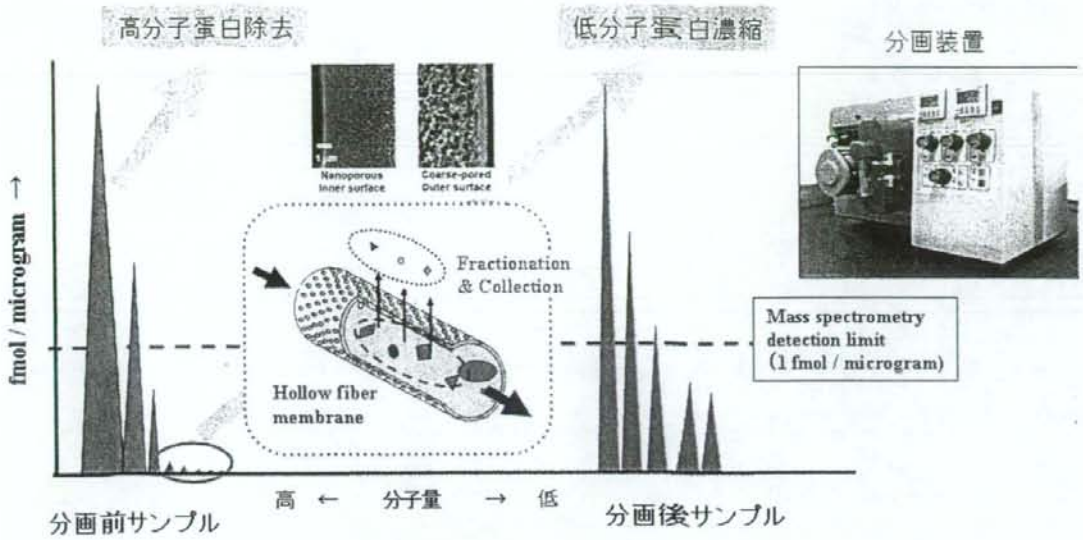

図3　中空紙を使った蛋白分画装置（東レ株式会社製造）による低分子量蛋白の濃縮

増殖性糖尿病初期（Pre-proliferative）で黄斑浮腫（DME）の有無（16：4検体）の硝子体を2次元電気泳動で分画，質量分析計した結果，DMEのグループから14種類の蛋白，non-DMEのグループから15種類の蛋白を同定した。特に8スポットは顕著にDMEグループで増加しており，そのうちの6つのスポットはPEDF, ApoA-4, Trip-11, RPBP, VDBPと同定された[4]。

硝子体サンプルは手術中に破棄されるものを倫理委員会の承認と文書による本人の了解を得て集められているが，検体の多くは病態末期のものが多く，これらの検体を解析しても疾患初期の硝子体の様子を知ることができない。また，日本では健常者眼球の硝子体を集めることができないために，ベースとなるデータが不足している。

II．網膜疾患を早期発見するための血漿バイオマーカーの探索

近年，全ゲノム配列が解読された結果，ゲノム上には平均で1,000塩基に1つの割合で異なる配列が存在することが明らかになった。この遺伝子多型(SNP：Single Nucleotide Polymorphism)の生理作用への影響についてはまだ明らかにされていないが，その利用方法については注目されている。ゲノム上の1つのSNPあるいは隣接する複数のSNPを組み合わせてブロックにし，これらと疾患の関連を調べる。同染色体に位置するSNPの組み合わせをハプロタイプと呼ぶが，全てのハプロタイプを明らかにするために国際ハップマッププロジェクト（http://www.hapmap.org/）が進行中である。3×10^9塩基から構成されるゲノム上には1千万個のSNPが存在すると計算されるが，これだけのSNP数を安価に効率よく解析することは技術的に困難であった。しかし，最近，シリコンベースのDNAチップによって数十万個のSNPを同時に検出することができるようになってきた。この方法によって，すでに加齢黄斑変性の2つのリスク遺伝子（*CFH*, *Htra1*）が同定されており，発症前にリスクの高い人を選別することが可能になってきた。しかし遺伝子情報だけでは発症の時期まで予測することは困難であり，発症前の蛋白の量的・質的変化を捉えて発症を予測する方法が検討されている。発症前に健常者の硝子体を検査目的で採取することはきわめて困難であり，これを血漿や尿で代行できるかが課題となっている。網膜・脈絡膜から漏れた蛋白が全身への循環によって希釈されることになり，この微量な変化を検出する精度が求められる。

我々は東京医科大学の西村俊秀客員教授との共

同研究によって血漿蛋白の微量変化を質量分析計によって検出できるか研究している。年齢65～88歳の加齢黄斑変性患者6名と白内障患者6名の血液を採血後3分以内に遠心分離によって得た血漿を用いている。血漿からアルブミンと免疫グロブリンを分離して，これを1次元電気泳動でさらに分画しても2疾患の泳動パターンに差は観察されないが，質量分析のマスクロマトグラムとマススペクトルを擬似的な2次表示にすると微量蛋白の変化が観察されるようになった。我々は同様な研究によって，発症前後の血漿サンプルによって疾患バイオマーカーの存在を検証したいと考えている。

Ⅲ．質量分析計を用いたドルーゼンのプロテオーム

最近の研究によって加齢黄斑変性に関係が深いとされてきたドルーゼンの構成蛋白が明らかになり，疾患と補体との関係が注目されている。Hagemanや Anderson 等は糸球体腎炎の患者で眼底にドルーゼンが観察されることから，糸球体の炎症に関わる補体の活性化が網膜下でも起こっていると推測し，免疫染色法によってこれを証明した[5,6,7]。さらに，Hollyfield 等もドルーゼンを抽出して，質量分析計によって蛋白組成を解析した結果，補体活性分子の存在を確認した[8]。ドルーゼン内で発見された蛋白にはアミロイドβや酸化ストレス関連分子など，補体活性化の原因になりうる蛋白が確認されている。

ヒトと同様に黄斑が発達している霊長類において，1970年代から加齢黄斑変性モデルの探索が行われてきた。独立行政法人医薬基盤研究所霊長類医科学研究センターにおいて生後2年でドルーゼンを発症するカニクイザルが社団法人予防衛生協会の鈴木通弘等によって発見され[9]，1頭の疾患個体から交配実験によって大家系に繁殖することに成功した（図4）。我々は厚生労働科学研究難治性疾患克服研究事業として研究班を組織して，ヒトと同様な方法によって疾患個体のドルーゼンを抽出し，プロテオーム解析と免疫染色法によって蛋白組成を解析した。その結果，補体活性分子，抑制分子，クリスタリンなど，ヒトに類似する蛋白組成が含まれることが明らかになった。疾患サルはヒトが50年以上かけて蓄積するドルーゼンと同成分のドルーゼンをわずか2年で生成していることになる。疾患は家系内で優性遺伝することから，単一遺伝子の変異によって発症していると考えられ，この原因遺伝子の同定はドルーゼン生成のメカニズムに関わる重要な情報をもたらすと期待している。

Ⅳ．黄斑のプロテオーム

霊長類と一部の鳥類などで発達している黄斑は視力を決定する重要な部位である。黄斑は錐体細胞が高密度に存在し，周辺網膜に比べて特徴ある構造をしていることから黄斑と周辺網膜との差を分子レベルで解明するために，転写産物（トランスクリプトーム）の解析が行われ，黄斑特異的な遺伝子発現も報告されている。この研究の延長線上には黄斑のプロテオーム解析が考えられるが，今日まで報告されていない。その理由には1）一般的な実験動物であるマウスやラットには黄斑が存在しないこと，2）黄斑部組織が微量であること，3）新鮮な黄斑を多数に手に入れることが困難であることなどが考えられる。我々は霊長類医科学研究センターにおいて研究目的に安楽死された13～19歳の正常なオスザル8頭から黄斑部と網膜周辺部を3mm径の円柱でくり抜き，これを16眼球について採取し，黄斑と周辺網膜の蛋白抽出液を準備した。これを2次元電気泳動によって分画し，黄斑と周辺網膜の泳動パターンから，それぞれに特異的なスポットを複数発見した。これらのスポットをゲルからくり抜き，ペプチドに分解した後に，質量分析計した結果，これまで発見されなかった複数のタンパク質が同定されてきた。黄斑疾患とこれらのタンパク質との関係については研究が続けられている。

Ⅴ．網膜・硝子体プロテオーム解析の今後の課題

日進月歩の質量分析計の技術革新はこれまで不可能であった蛋白の網羅的解析を可能にし，電気泳動で分画されたスポットを容易に同定できるようになった。眼科分野でもヒトの硝子体，房水，

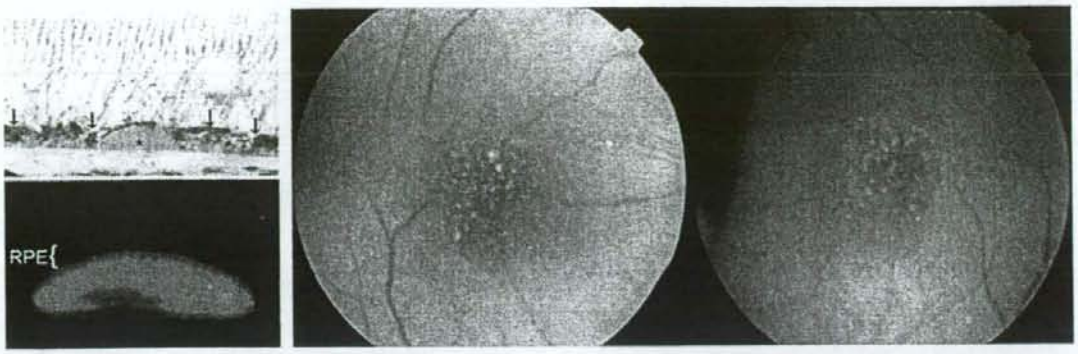

図4　独立行政法人医薬基盤研究所霊長類医科学研究センターで発見された若年性黄斑変性カニクイザルの眼底写真（右）とドルーゼン（左上）およびドルーゼン中の補体活性化（左下）

涙液の網羅的プロテオーム解析，トランスジェニックやノックアウトマウスの眼組織のプロテオーム解析などが行われている。また，最新機器と解析ソフトを利用するとリン酸化，糖，脂質の修飾を受けた蛋白の同定や修飾されたアミノ酸の特定も可能になった。我々は黄斑に特異的な蛋白を探索する過程で，リン酸化蛋白の量的変化を観察している。蛋白修飾の生理的な意味や疾患との関係は今後明らかにされていくと期待される。このような網羅的修飾蛋白の解析はプロテオミクスによって初めて可能になった技術である。

質量分析計にも克服されなければならない弱点が存在する。その一つに質量分析計には定量性が欠けることである。サンプル間に含まれる特定の蛋白について量的変化を高い精度で測定することができない。これまでは電気泳動後のバンドやスポットの濃さとして測定されたり，比較するサンプルを異なる同位体元素でそれぞれラベルして，質量分析計によってラベル化されたペプチドの検出回数として測定されたりする方法が行われてきた。同定した蛋白の定量性について今後の技術開発が望まれている。

おわりに

これまで利用されてきたDNAチップを使った網羅的なトランスクリプトーム解析に加え，これにプロテオーム解析を組み合わせることによって，解析する領域を遺伝子発現から蛋白発現までに広げることが可能になった。DNAチップによって23,000遺伝子の変化を観察し，同時に蛋白の有無を観察することができる。我々も同様な方法によって網膜色素上皮細胞を特異的に増殖させる因子（REF-1/TFPI-2）の作用機序を研究している[10]。この研究では発現量が変化する蛋白間の関係を裏付けるデータとして，遺伝子発現データを利用した。今後はここへ蛋白修飾のデータも加わり，フェノミックスの世界へ一歩近づき，疾患の理解，診断法の開発，さらには治療へと発展するものと期待される。

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HTRA1 promoter polymorphism predisposes Japanese to age-related macular degeneration

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Purpose: To study the effect of candidate single nucleotide polymorphisms (SNPs) on chromosome 10q26, recently shown to be associated with wet age-related macular degeneration (AMD) in Chinese and Caucasian cohorts, in a Japanese cohort.

Methods: Using genomic DNA isolated from peripheral blood of wet AMD cases and age-matched controls, we genotyped two SNPs, rs10490924, and rs11200638, on chromosome 10q26, 6.6 kb and 512 bp upstream of the *HTRA1* gene, respectively, using temperature gradient capillary electrophoresis (TGCE) and direct sequencing. Association tests were performed for individual SNPs and jointly with SNP complement factor H (CFH) Y402H.

Results: The two SNPs, rs10490924 and rs11200638, are in complete linkage disequilibrium (D'=1). Previous sequence comparisons among seventeen species revealed that the genomic region containing rs11200638 was highly conserved while the region surrounding rs10490924 was not. The allelic association test for rs11200638 yielded a p-value $<10^{-11}$. SNP rs11200638 conferred disease risk in an autosomal recessive fashion: Odds ratio was 10.1 (95% CI 4.36, 23.06), adjusted for SNP CFH 402, for those carrying two copies of the risk allele, whereas indistinguishable from unity if carrying only one risk allele.

Conclusions: The *HTRA1* promoter polymorphism, rs11200638, is a strong candidate with a functional consequence that predisposes Japanese to develop neovascular AMD.

Japanese patients are predominantly affected with vascular or "wet" AMD with little or no drusen deposition, in contrast to the Caucasian population which has a higher prevalence of drusen formation and the dry form of the disease. Association between the complement factor H (CFH) Y402H polymorphism (CFH 402) and age-related macular degeneration (AMD) has been shown in twelve or so different Caucasian populations [1,2]. However, that association failed to be replicated in Japanese populations, in which no control individual was found to be homozygous for the risk allele [3,4].

HTRA1 is a member of the heat shock serine proteases and is up-regulated by cellular stress. *HTRA1* is expressed in both the human and mouse retina [5,6]. Recently a promoter single nucleotide polymorphism (SNP) rs11200638 in *HTRA1* was shown to be highly associated with wet AMD [6,7]. Furthermore, *HTRA1* resides in a region of chromosome 10q26 that has been implicated as the "top" candidate region for AMD. Here we test two SNPs, rs10490924 (6.6 kb upstream of *HTRA1*), and rs11200638, for their association to wet AMD in a Japanese population.

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METHODS

We genotyped 88 neovascular AMD cases and 97 AMD-free age-matched controls for SNPs rs10490924 and rs11200638. Case and control individuals were the same as our previous CFH association study [3] with all cases being characterized as AMD grade 5B [1]. Among cases the mean age was 74.8 years (standard deviation: s.d. 8.8 years) and 70.5% male; among controls the mean age was 71.1 years (s.d. 9.1 years), and 38.1% male. Informed consent was obtained from all participants, and the procedures used conformed to the tenets of the Declaration of Helsinki. Genotyping was performed as described previously [3]. Briefly, PCR was performed using primers designed to amplify the genomic region containing each SNP (rs10490924 forward: 5'-GGT GGT TCC TGT GTC CTT CA-3', reverse: 5'-GGG GTA AGG CCT GAT CAT CT-3'; rs11200638 forward: 5'-CGG ATG CAC CAA AGA TTC TCC-3', reverse: 5'-TTC GCG TCC TTC AAA CTA ATG G-3'). Following amplification, genotype determination was performed on the PCR products using either temperature gradient capillary electrophoresis (TGCE; Reveal SpectruMedix,

State College, PA) or through direct sequencing using CEQ2000XL DNA analysis system (Beckman Coulter, Fullerton, CA).

Hardy Weinberg equilibrium (HWE) χ^2 values in the entire sample and controls only were calculated to identify possible genotyping errors. No extreme deviations (χ^2>50) were observed (Table 1). Linkage disequilibrium (LD) was measured by the D' value. For each SNP, Pearson's χ^2 tests with one degree of freedom for association were performed. Odds ratios (OR), population attributable risks (PAR), and their respective confidence intervals were calculated, formula in [8].

Previous functional data lead us to focus further analyses on rs11200638 [6,7]. Joint ORs for two SNPs (rs11200638 and CFH 402, previously genotyped) were calculated using standard methods [9]. Marginal ORs and their confidence intervals for the two SNP were calculated using logistic regression with SNP CFH 402 and rs11200638 as independent variables [9]. PARs were calculated using standard methods [9]. Confidence intervals around the PARs were constructed using 999 bootstrap replicates. To control for confounding, the Mantel-Hanzel test for association with two variables was used [9]. Four genotypic models were considered (Full, Recessive, Multiplicative, and Dominant) and the Aikake information criterion (AIC) was utilized to assess the fit of each model. All R scripts used in the analysis are available upon request.

RESULTS

SNP rs11200638, approximately 6.1 kb downstream of the surrogate SNP rs10490924, resides in the promoter of the *HTRA1* serine protease gene (512 base pairs upstream of transcriptional start site). These two SNPs were in almost complete linkage disequilibrium (LD) and showed strong association with AMD in the Hong Kong study [6] and in a Caucasian population from Utah [7].

In our cohort, the two SNPs were also in complete LD, from which only two major (frequency >5%) haplotypes, one predominant in cases and one in controls, were observed. Disease association tests yielded p-values of 4.74×10^{-11} and 1.79×10^{-12} for rs10490924 and rs11200638, respectively (Table 1). Given the previous evidence of higher conservation across species [6] and the functional consequence of rs11200638 on *HTRA1* expression [6,7], additional analyses focused on this SNP.

Reanalyzing the original CFH genotype data, we found the OR covered unity (Table 2) and all interval estimates of PAR for CFH 402 variants under the four genotypic models included zero (Table 3). Of the four models, the best fit to the *HTRA1* SNP genotypic effects, as assessed by Akaike's information criterion, was the recessive model, from which the risk genotype was AA and non-risk was GG and GA (Table 3). Under the framework of recessive rs11200638 and the two observed genotypes for CFH 402, no interaction was detected between the two SNPs based on the likelihood ratio test (Table 3). Odds ratios for different genotypes of rs11200638 do not vary a great deal depending on the CFH 402 genotypes, and vice versa (Table 2). In fact, the OR curves shown in Figure 1 indicate a "removable" interaction between the two SNPs, in which the original two OR curves become parallel (i.e. no

Table 1. Association of chromosome 10q26 single nucleotide polymorphisms with age-related macular degeneration

Attribute	rs10490924 (G/T)	rs11200638 (G/A)
HWE χ^2-combined	5.4	7.6
-controls only	0.98	0.88
Risk allele	T	A
Frequency in case	0.68	0.69
Frequency in control	0.33	0.32
Allelic association χ^2 nominal p-value	4.74E-11	1.79E-12

To examine genotyping errors, Hardy Weinberg Equilibrium (HWE) χ^2 values are computed with cases and controls combined and controls alone. The age range is 51 to 90 years old with mean 74.8 and standard deviation (s.d.) 8.81 in cases, and 50 to 88 years old with mean 71.1 and s.d. 9.08 in controls.

Table 2. Odds ratios for the joint and marginal effects of single nucleotide polymorphisms complement factor H 402 and rs11200638 on age-related macular degeneration

	rs11200638		
CFH 402	GG/GA	AA	CFH 402 risk (adjusted for rs11200638)
TT	1	7.92	1
CT	1.11	30.52	1.41 (95% CI: 0.54,3.74)
rs11200638 risk adjusted for CFH	1	10.02; 95% CI: 4.36,23.06	

CFH indicates complement factor H. Joint odds ratios were calculated from standard formulae. Marginal odds ratios and 95% confidence intervals were calculated using logistic regression (see Methods) with each SNP was adjusted for the other.

interaction after transformation with a logarithmic function). Overall, after adjusting for the CFH 402 SNP, individuals carrying the risk homozygote AA of rs11200638 are greater than 10 times more likely to have AMD than those with the other genotypes (Table 2).

DISCUSSION

These data reconfirm the association of the *HTRA1* promoter SNP rs11200638, independent of the CFH 402 polymorphism, with wet AMD. The present study genotyped two previously identified disease associated SNPs in the chromosome 10q26 region. Both SNPs showed similar significance levels. The first SNP, rs10490924, resides in the hypothetical locus, LOC387715. Several studies have found significant association between AMD and this SNP [10-12]. So far only one transcript from this hypothetical locus has been identified in one experiment. No study has identified the transcript or protein in the retina, much less identified a change in the protein as a result of the SNP. Additionally, sequence comparisons of seventeen species presented in DeWan et al. show higher sequence

Table 3. Two-way analyses of complement factor H 402 and rs11200638

Model for rs11200638	PAR%		(95% CI)		M-H test: p-value LRT p-value	AIC value
	CFH 402	rs11200638	CFH 402	rs11200638		
Full	3.4 (0, 9.7)	58.3 (50.5, 64.1)	0.07	8.30E-08	0.03	221.8
Recessive	4.6 (0, 10.7)	44 (40.5, 54.0)	0.23	6.20E-09	0.12	221.5
Multiplicative	1.7 (0, 7.8)	79.8 (73.0, 88.1)	*	*	0.02	225.7
Dominant	2.2 (0, 13.7)	58.6 (43.9, 78.9)	0.91	5.80E-04	0.1	246.9

Four genotypic models for rs11200638 are considered: Let r0, r1, and r2 be the marginal relative risks for genotypes GG, GA, and AA. Then, recessive model implies r0=r1; multiplicative model implies r1=r0r2; dominant model implies r2=r1; full model does not have any restriction on relative risks except that r0, r1, r2>0. The 95% confidence intervals (CI) of population attributable risk (PAR) were obtained via a bootstrap re-sampling method with 999 replicates. Mantel-Hanzel (M-H) tests are conducted for one SNP association adjusted for the other SNP; likelihood ratio tests (LRT) for joint single nucleotide polymorphism (SNP) association under a two-way multiplicative model: the relative risk (or OR) for any genotype pair (A, B) relative to the baseline pair (A0, B0) is the product of relative risk (or OR) of A relative to A0 and that of B relative to B0. AIC denotes the Akaike's information criterion to access goodness-of-fit for the rs11200638 model.

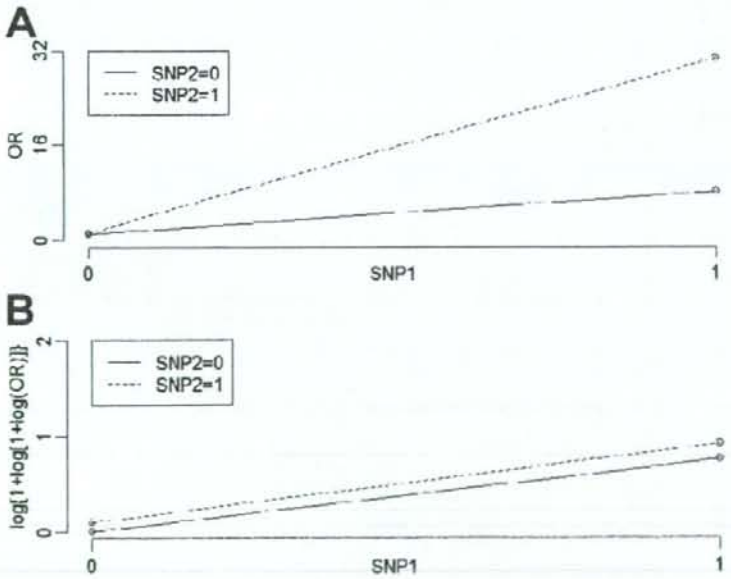

Figure 1. Odds ratio plots for two single nucleotide polymorphisms. Joint odds ratio plots for the single nucleotide polymorphisms (SNPs), complement factor H (CFH) 402, and rs11200638 before and after log transformation showing that the apparent interaction is a "removable" effect. SNP1=CFH 402: 0 is for TT and 1 is for CT; SNP2=rs11200638: 0 is for GG/GA and 1 is for AA. **A**: Original odds ratio (OR) curves: Height difference on the left is 1.11-1=0.11; height difference on the right is 30.52-7.92=22.60; slope for SNP2=0 is 7.92-1=6.92; slope for SNP2=1 is 30.52-1.11=29.41. **B**: Log(1+log(1+log)) transformation of the original OR.

conservation surrounding rs11200638 compared to that around rs10490924 [6]. *HTRA1* is expressed in the retina in humans [5] and mouse [6]. Computational analysis of the *HTRA1* promoter indicate that this SNP resides in a CpG island and may result in a change in the binding site for transcription factors AP2 and SRF [6]. Preliminary functional data suggest that individuals homozygous for the risk-allele at rs11200638 exhibit increased expression of *HTRA1* [6,7]. Therefore, given the existing functional data, it appears as if the *HTRA1* promoter polymorphism, rs11200638, is likely the underlying functional polymorphism in the 10q26 region. However, the mechanism to neovascularization is yet to be understood and will require intense investigation to uncover its link to the wet form of AMD.

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31 Animal Models for Eye Diseases and Therapeutics

TAKESHI IWATA AND STANISLAV TOMAREV

ABSTRACT

It is believed that more than 80% of the information our brain receives comes from the visual system. Dysfunction of the visual system can significantly lower the quality of life. The most prevalent causes of visual impairment are cataracts, glaucoma, and age-related macular degeneration (AMD), which is responsible for 69% of blindness globally. In spite of the high incidence of AMD and glaucoma, a limited amount of information is available on the underlying pathological mechanisms causing these diseases. Because experimental studies of AMD and glaucoma are limited in humans, the availability of animal models is very valuable to investigate molecular mechanisms and to test new therapeutic interventions. Appropriate animal models, such as monkey, mouse, rat, and zebrafish, facilitate the identification of new genes involved in the pathology and elucidate the genetic relationships between causative and modifier genes. In this chapter the advantages and difficulties of using animal models for vision research will be discussed. Several animal models including a primate model with defined macula for AMD research and genetically modified mice models for glaucoma research will be introduced.

Key Words: Vision, Age-related macular degeneration, Retina, Macula, Drusen, Glaucoma, Retinal ganglion cells, Optic nerve.

VISUAL IMPAIRMENT AND IMPORTANCE OF ANIMAL MODELS FOR EYE DISEASES

It is believed that more than 80% of the information our brain receives comes from the visual system. Dysfunction of the visual system can alter the normal human life style and significantly lower the quality of life. The causes of visual impairments and blindness vary among ethnic groups and the global regions where they live. There are many causes of visual impairments including diabetic complications, infections, and trauma; however the most prevalent causes of visual impairment are cataracts, glaucoma, and age-related macular degeneration (AMD). According to the World Health Organization, there were more than 161 million visually impaired individuals in 2002; 124 million of this group had low vision and 37 million were blind (http://www.who.int/mediacentre/factsheets/fs282/en/index.html) (Figure 31–1).

Cataract, glaucoma, and AMD are responsible for 69% of blindness globally. Although cataracts are the leading cause of blindness worldwide, recent advances in cataract surgery have significantly reduced the visual impairments caused by cataracts, especially in developed countries. Glaucoma, an optic neuropathy, is often associated with elevated intraocular pressure and is responsible for blindness in 6.7 million people across the world. Glaucoma is more common in individuals of African ancestry, and the incidence of glaucoma increases with age.

The most prevalent eye disease for elderly Europeans and Americans is AMD. This degenerative disease progresses from retinal deposits called drusen to neovascularization and retinal hemorrhages resulting in irreversible loss of central vision.

In spite of the high incidence of AMD and glaucoma, a limited amount of information is available on the underlying pathological mechanisms causing these diseases. Obtaining tissues for any disease is often difficult, and even when obtained, they may not be informative because the tissues are usually collected many hours or even days after death. Because experimental studies of AMD and glaucoma are limited in humans, the availability of animal models is very valuable because they can be used to investigate the molecular mechanisms causing these diseases and to test new therapeutic interventions. Animal models, compared to other experimental methods, e.g., cell and organ cultures or postmortem models, allow the study of different pathological factors and therapeutic treatments under *in vivo* conditions, i.e., with the visual and other systems of the body intact. Appropriate animal models, e.g., monkey, mouse, and zebrafish, also facilitate the identification of new genes involved in the pathology as well as elucidate the genetic relationships between causative and modifier genes. Equally important, specific genes can be altered in these models. Thus, it is possible to induce mutations in animals, and then search for specific phenotypes, e.g., abnormal intraocular pressure (IOP) and retinal ganglion cell degeneration. Subsequently, the affected genes can be identified by standard genetic procedures.

Many animal models of AMD and glaucoma have been developed in different mammalian and nonmammalian species. None of these models is a perfect reproduction of the human disease, and when choosing the animal model for research, the investigator should evaluate the following: (1) the similarity of the visual system of the model to that of humans, especially the eye; (2) the similarity of the time course of pathological changes in the model and in human eyes; (3) the ability to perform genetic manipulations; (4) the training required to produce affected animals; (5) the size of the eye; (6) the availability and difficulties in the

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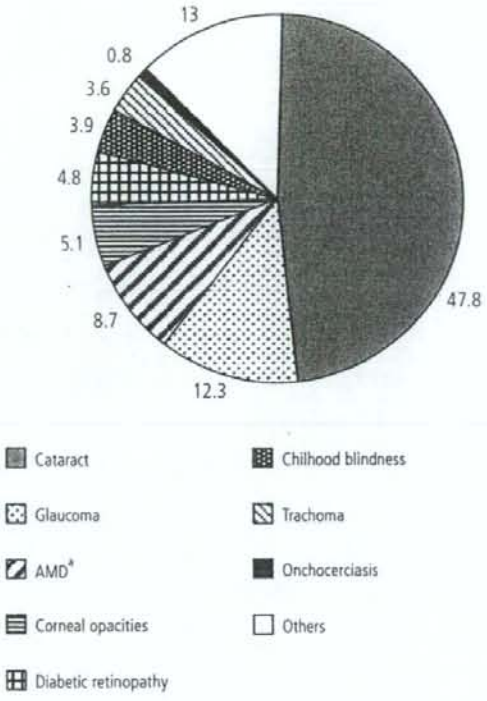

Figure 31–1. Global causes of blindness as a proportion of total blindness in the year 2002 (WHO). AMD, age-related macular degeneration.

methods of analysis; (7) the availability of animals; and (8) the cost.

AGE-RELATED MACULAR DEGENERATION

INTRODUCTION The retina is composed of nine layers of neural and glial cells that are arranged concentrically at the posterior pole of the eye. Incoming light is focused on the central area of the retina called the fovea, which is located in the center of the macular area (Figure 31–2). In humans, the size of the macula is approximately 6 mm in diameter (Figure 31–3). The outer (posterior) surface of the retina is covered by a monolayer of retinal pigment epithelial (RPE) cells that forms a diffusion barrier between the neural retina and the choroidal blood supply. The RPE regulates the transport of proteins to the retina and controls the hydration and ionic composition of the subretinal space. The physiological condition of the RPE is closely associated with the pathogenesis of AMD.

AMD is a blinding disorder characterized by a marked decrease in central vision associated with RPE atrophy with or without choroidal neovascularization (CNV). Many factors, including genetic, behavioral, and environmental, are involved in this disease. AMD is characterized by the degeneration of cone photoreceptors in the foveal region of the retina resulting in a decrease of central visual acuity. The progressive impairment of the RPE cells and damage to Brüch's membrane and choriocapillaris results in retinal atrophy and photoreceptor dysfunction. In some cases, CNV develops, and the new vessels penetrate Bruch's membrane and pass into the subretinal space.

Two types of AMD are recognized: the nonneovascular type is called the dry-type AMD and includes more than 80% of the cases; the neovascular type is called the wet-type AMD and it is progressive with a higher probability of blindness. The prevalence of AMD differs considerably among the different ethnic groups, but the incidence increases with age in all groups. A lower prevalence of AMD has been reported in individuals of African ancestry than of Anglo-Saxon ancestry. Other risk factors for AMD are cigarette smoking, obesity, hypertension, and atherosclerosis.

EPIDEMIOLOGY AND GENETICS Extensive epidemiological studies have shown a genetic component for AMD. Thus, twin studies have shown a higher concordance for AMD in monozygotic twins than in dizygotic twins.[1–3] In addition, first-degree relatives of individuals with AMD have a 2- to 4-fold higher incidence of AMD than individuals without a family history of AMD. Genetic segregation studies have also shown a genetic effect that accounts for approximately 60% of AMD with a single major gene accounting for about 55% of the risk of developing

AMD. Overall, the data have suggested that the etiology of AMD has a significant genetic component. Only a small proportion of the families with AMD shows Mendelian inheritance, and the majority of the individuals inherit AMD in a complex multigene pattern.

There have been a number of attempts to identify the genes that cause AMD. With the help of the haplotype marker project (HapMap Project), genome-wide scanning has identified at least 13 loci linked to AMD on different chromosomes.[4–6]

Recently, a polymorphism of complement factor H gene (*Y402H*) was shown to be associated with an increased risk for AMD.[7–10] These results were confirmed in many of the countries with large white populations but not in Japan.[11,12] This gene is located on chromosome 1q25-31 where one of the candidate loci was identified by linkage studies. Another recent study reported that a haplotype association of tandemly located complement 2 and factor B was protective for AMD.[13]

PATHOLOGY AND BIOCHEMISTRY The early stage of the dry-type AMD is characterized by a thickening of Bruch's membrane, aggregation of pigment granules, and increasing numbers of drusen. The thickening of Bruch's membrane obstructs its function as a "barrier" between the choroid and the RPE that protects the neural retina from the choriocapillary. Drusen are small yellowish-white deposits that are composed of lipids, proteins, glycoproteins, and glycosaminoglycans. They accumulate in the extracellular space and the inner aspects of Bruch's membrane (Figure 31–3). Drusen are not directly associated with visual loss but represent a risk factor for both the dry-type and wet-type AMD. The classification of hard and soft drusen is based on their size, shape, and color; hard drusen are yellowish with diameters <50 μm and are found in eyes that are less likely to progress to advanced stages of the disease, while soft drusen are darker yellow and larger in size, and are found in eyes more likely to progress to more advanced stages of AMD. A small percentage of dry-type AMD patients progress to the late stage of the wet-type AMD that is characterized by geographic atrophy or detachment of RPE and the development of CNV in the macular region. The presence of a CNV is the factor that most damages the neural retina because the newly developed vessels grow from the choriocapillaris through Bruch's membrane and extend laterally through the RPE cell layer (classic CNV) or extend between the inner Bruch's membrane and RPE (occult CNV). In advanced stages of AMD, the CNV and fluid leaked into the subretinal or intraretinal regions lead to cell death and retinal detachment.

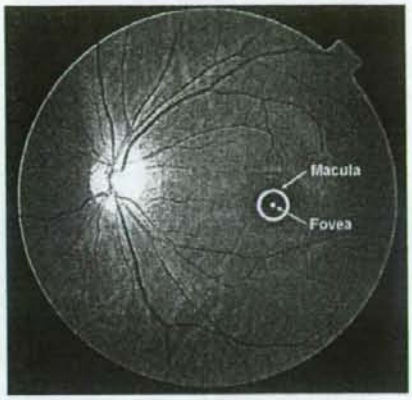

Figure 31–3. Fundus photograph of a normal human retina showing the location of the macula and the fovea.

Recent analyses of the progression of drusen have provided important clues that help understand the molecular pathology of AMD. Using both immunohistochemistry and proteomic techniques, the materials in drusen were found to be composed of molecules that mediate inflammatory and immune processes.[14,15] These molecules include components of the complement pathway and modulators of complement activation, viz. vitronectin, clusterin, membrane cofactor protein, and complement receptor-1. In addition, molecules triggering inflammation, viz. amyloid P component, α_1-antitrypsin, and apolipoprotein E, were identified in drusen. Cellular debris from macrophages, RPE cells, and choroidal dendritic cells has also been identified in drusen. On the other hand, crystallins, EEFMP1, and amyloid-β have been found at

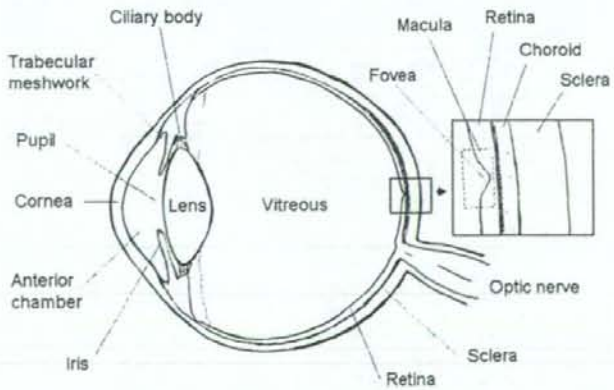

Figure 31–2. Schematic diagram of the human eye.

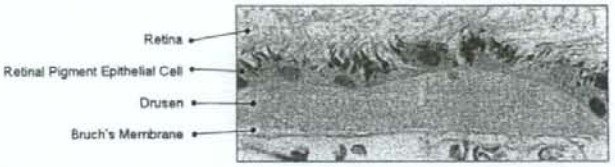

Figure 31–4. Retinal histological section showing the accumulation of drusen between the retinal pigment epithelium and Bruch's membrane in a primate model of AMD.

higher levels in drusen from individuals unaffected by AMD. The presence of immunoreactive proteins and the oxidative modifications of many proteins in drusen imply that both oxidation and immune functions are involved in the pathogenesis of AMD.

All of these findings suggest that complement activation triggers innate immune responses in the subretinal space. The codistribution of immunoglobulin G (IgG) and terminal complement complexes in drusen indicate that immune responses that directly target antigens in retinal cells might also be occurring. Antiretinal autoantibodies have been reported in a number of ocular disorders, e.g., macular degeneration in an aged monkey model.

ANIMAL MODELS Access to appropriate biological materials from affected donors at different stages of a disease is an absolute necessity for the study of mechanisms underlying the disease process. However, because it is nearly impossible to obtain retinal tissues from patients or controls, the development of animal models becomes crucial for investigating the biological pathways involved in the progression of the disease and for the development of therapeutic strategies.

Over the past few years, genetic engineering techniques have generated a number of animal models of AMD in mice, rats, rabbits, pigs, and dogs.[16] However, in mammals, a well-defined fovea is found only in primates (humans and monkeys), and a search for a monkey line affected with macular degeneration has been persistent for a long time.

A monkey with macular degeneration was first described by Stafford *et al.* in 1974. They reported that 6.6% of the elderly monkeys they examined showed pigmentary disorders and drusen-like spots.[17] El-Mofty *et al.* reported that the incidence of maculopathy was 50% in a colony of rhesus monkeys at the Caribbean Primate Research Center of the University of Puerto Rico.[18] At the Tsukuba Primate Research Center (Tsukuba City, Japan), Suzuki *et al.* found a single cynomolgus monkey (*Macaca fascicularis*) in 1986 with a large number of small drusen around the macular region (Figure 31–4).[19–21] This single affected monkey has multiplied to a large pedigree of more than 65 affected and 210 unaffected monkeys (Figure 31–5). Drusen were observed in the macular region as early as 1 year after birth, and the numbers increased and spread toward the peripheral retina throughout life. No histological abnormalities have been found in the retina, retinal vessels, or choroidal vasculatures of the eyes with drusen. Immunohistochemical and proteomic analyses of the drusen from these monkeys showed that the drusen were very similar to those in other monkeys with aged macular degeneration sporadically found in older monkeys and also with human drusen.[22,23] These observations by Umeda *et al.* have shown that the Tsukuba monkeys produce drusen that are biochemically similar to those in human AMD patients, but the development of the drusen occurs at an accelerated rate of over 25 times. Currently, 240 loci of the cynomolgus monkey are being investigated to try to identify the disease-causing gene and to understand the biological pathways leading to complement activation.

The eyes of monkey are structurally similar to human eyes, which make them extremely valuable for macular degeneration studies. However, there are limitations in using this species over other laboratory animals. Monkeys have a relatively longer life span, have a longer gestation period, have lower birth numbers resulting in a slower rate of expanding the pedigree, and are more difficult to genetically manipulate; in addition, the cost of maintenance is high. In other laboratory animals, the differences in the eye structure, lack of a fovea, and a low cone-to-rod ratio compared to humans have been considered to be a disadvantage for using them as AMD models. However, they are easier to manipulate genetically and easier and less expensive to maintain. This has made the development of a mouse model of AMD very attractive, and a number of mouse AMD models have been reported recently.

The mouse model described by Ambati *et al.* is deficient either in monocyte chemoattractant protein-1 or its cognate C–C chemokine receptor-2. These mice were found to develop the cardinal

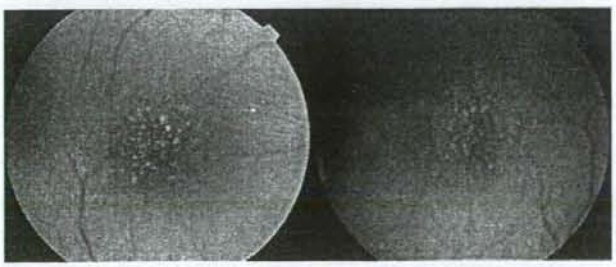

Figure 31–5. Photographs of the fundus of two monkeys with AMD in the Tsukuba Primate Research Center showing drusen.

features of AMD including accumulation of lipofuscin in drusen beneath the RPE, photoreceptor atrophy, and CNV.[24] An impairment of macrophage recruitment allowed the accumulation of C5a and IgG, which leads to the production of vascular endothelial growth factor by the RPE cells and the development of CNVs.

Another mouse model that has three known AMD risk factors—age, high-fat cholesterol-rich diet, and expression of human apolipoprotein E (apoE2, apoE3, andapoE4)—has been developed.[25] ApoE4-deficient mice are severely affected showing diffuse subretinal pigment epithelial deposits, drusen, thickened Bruch's membrane, and atrophy, hypopigmentation, and hyperpigmentation of the RPE.

Oxidative stress has long been linked to the pathogenesis of AMD. Imamura, *et al.* reported Cu, Zn-superoxide dismutase (SOD1)-deficient mice that had features typical of AMD in humans. Senescent $Sod1^{-/-}$ mice had drusen, thickened Bruch's membrane, and choroidal neovascularization.[26] The number of drusen increased with age and also after exposure of young $Sod1^{-/-}$ mice to excess light. The retinal pigment epithelial cells of $Sod1^{-/-}$ mice showed oxidative damage, and their β-catenin-mediated cellular integrity was disrupted. These findings suggested that oxidative stress may affect the junctional proteins necessary for the barrier integrity of the RPE. These observations strongly suggested that oxidative stress may play a major role in AMD.

The complement components, C3a and C5a, are present in drusen, and were observed in Bruch's membrane of a laser-induced CNV mice model. Neutralization of C3a or C5a by antibody or by blockade of their receptors by a complement inhibitor significantly reduced the CNV. These observations revealed a role of immunological mechanisms for angiogenesis and provided evidence for future therapeutic strategies for AMD.

Although the pathology of AMD is pronounced in the macula area, it is not confined to this region. Characteristics of human AMD such as thickening of Bruch's membrane, accumulation of drusen, and CNV have been observed in mouse models. Nevertheless, the primate model will still be the choice for AMD studies, especially at the stage when new therapeutic methods are tested and evaluated for the first time. However, it would be wise and more productive to study both primate and mouse models in AMD research. This will be necessary to determine the mechanisms underlying the disease and to identify clinical and molecular markers for the early stages of AMD. The findings from these studies will provide critical information needed to develop therapies for AMD.

GLAUCOMA

OVERVIEW Glaucoma is a heterogeneous group of complex neurodegenerative disorders that is characterized by the constriction of the visual field, death of retinal ganglion cells (RGCs), and a pathognomonic deformation of the optic nerve head (ONH) known as glaucomatous cupping. Glaucomas are classified into three main types: open-angle, closed-angle, and congenital glaucoma. Each of these types is subdivided into primary and secondary types.

EPIDEMIOLOGY AND GENETICS Primary open-angle glaucoma (POAG) is the most common form of glaucoma; it occurs in about 4.5 million people worldwide and accounts for 12% of all global blindness. By the year 2020, over 11 million people will be blind from primary glaucoma.[28] POAG is often, but not always, associated with elevated IOP, which is one of the main risk factors in glaucoma. However, about a third of all patients with POAG develop the disease without an IOP elevation, and in these patients, the IOP is continuously below 21 mm Hg. This form of POAG is called normal tension or low tension glaucoma (NTG). A reduction of the IOP, even in cases of NTG, is the main, clinically proven, treatment for glaucoma.

ANIMAL MODELS Among the different animal models of glaucoma, the monkey models are superior because of the anatomical similarity of the monkey eyes to human eyes and the phylogenetic similarities of these two species. At the same time, monkeys are extremely expensive and experiments on them require a highly skilled team of investigators.

Most of the existing animal models of POAG, including the monkey models, are based on the elevation of the IOP. An elevation of the IOP develops from an imbalance between aqueous humor production and outflow. Aqueous humor, a fluid produced by the ciliary body of the eye, drains out of the eye and into the blood circulatory system. The eye's outflow system consists of a series of endothelial cell-lined structures that include the trabecular meshwork (TM), Schlemm's canal (SC), which serves as a collector vessel, and the episcleral venous system (Figure 31–6). In most glaucoma models, the IOP is elevated as a result of a reduction or blockage of the aqueous humor outflow. In monkeys, an elevation of IOP is commonly induced by laser photocoagulation of the TM.[29,30] Several days after the laser treatment, the IOP increases and this elevation may last for more than a year, although more than one laser session is usually required to achieve a sustained elevation of the IOP. The IOP in treated eyes is usually between 25 and 60 mm Hg.

Other methods to elevate the IOP in monkey eyes include the anterior chamber injection of ghost red cells,[31] latex microspheres,[32] cross-linked polyacrylamide gels,[33] and enzymes.[34] Topical steroids have also been shown to elevate the IOP.[35] These latter treatments produce less consistent elevations than laser photocoagulation.[36]

Monkey glaucoma models have been shown to have changes in the optic disk, optic nerve, RGC, and nerve fiber layers similar to those observed in glaucomatous human eyes. Apoptosis was shown to be the cause of the RGCs death in a monkey photocoagulation model,[37] and apoptosis was later confirmed to be the cause in other animal models and in humans with glaucoma. Monkey glaucoma models have also been successfully used to study changes in the retinal gene expression pattern 30 days after laser photocoagulation of the TM,[38] and to test the effectiveness of new classes of hypotensive drugs.[36]

Rodent became the animal of choice when large numbers of animals were required, e.g., when examining the mechanism of RGC degeneration and neuroprotection. Several rat models of elevated pressure-induced optic nerve damage have been developed during the past decade, and they have been used to study changes in the retina and the optic nerve. Rats are easy to handle and the relatively large size of their eyes allows multiple, awake measurements of the IOP with commercially available equipment.[39] This latter is important because it is well documented that general anesthesia induces a rapid decrease in the IOP. Although there are certain differences in the structure of the rat and human eyes, all of the eye structures affected in glaucomatous human eyes exist in the rat eye.

In rat models, the IOP elevation is achieved by injection of concentrated saline solution into the episcleral veins,[40] laser

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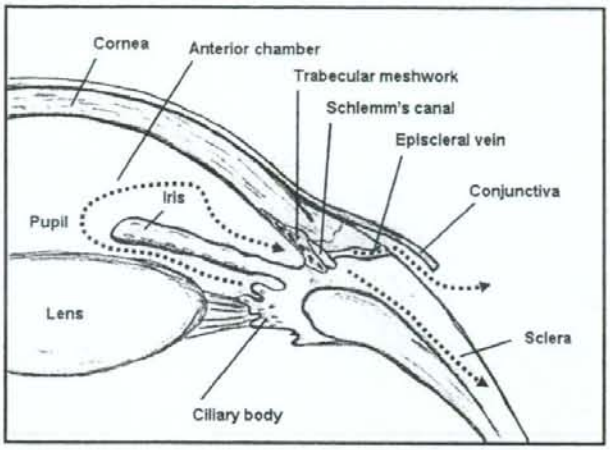

Figure 31–6. Schematic diagram of the anterior segment of the eye showing the trabecular meshwork and uveoscleral outflow pathways of aqueous humor.

photocoagulation of the TM after an injection of Indian ink into the anterior chamber,[41] laser photocoagulation of the TM,[42] and laser cauterization of episcleral veins.[43] All of these methods that lead to an elevation of the IOP require special training of the investigators.

Successful treatment of the eye leads to a rapid elevation of the IOP, although the level of elevation varies from eye to eye. Saline injection generally produces a wide range of IOP elevation from a very minimal rise to a 2-fold increase over the IOP in control eyes. The elevation of the IOP generally lasts for several weeks, and a second laser treatment is often required in the photocoagulation method to maintain an elevated IOP for more than 3 weeks.

A chronic elevation of the IOP in rats leads to apoptosis of the RGCs, degeneration of the optic nerve fibers, and remodeling of the ONH similar to those observed in human glaucomatous eyes.[40,44–45] Rat models of glaucoma have been used to study the effects of elevated IOP on the electroretinogram,[46] neuroprotective drugs,[47] and molecular changes in the retina and optic nerve using the candidate gene approach and array hybridization.[48]

A mutant rat strain was reported to have unilateral or bilateral enlargement of the eyes with an IOP ranging from 25 to 45 mm Hg. In this strain of rat, cupping of the optic nerve head was detected by funduscopic examination, and the cupping was more pronounced in older animals. The number of RGCs also declined with age.[49] Unfortunately, this strain was obtained from the Royal College of Surgeons colony that has a mutation in the receptor tyrosine kinase gene, leading to degeneration of the photoreceptors. Therefore, this strain can hardly be considered a good glaucoma model.

The construction of mouse models of glaucoma has lagged behind rat glaucoma models for a long time despite the advantages of mice over rats and other mammalian species for cost-effective genetic manipulations, availability of a wide spectrum of methods, and the existence of many genetically modified strains. However, it should be remembered that mouse and human eyes have certain important differences including the arterial blood supply to the optic nerve head and the absence of a lamina cribrosa.[50] The lamina cribrosa, a collagenous scaffold supporting the optic nerve, plays a critical role in the damage/protection of the human optic nerve.

One of the main difficulties working with mice is that their eyes are much smaller than the eyes of humans and rats, and new methods had to be developed to measure the IOPs in mice. To date, several invasive and noninvasive methods of IOP measurements have been developed for mice. The first remains one of the most reliable and accurate methods and does not depend upon the mechanical properties of the cornea. It involves the insertion of a glass microneedle connected to a pressure transducer into the anterior chamber of the eye. Using this method, it was shown that common mouse strains have different IOP between 10 and 20 mm Hg.[51] Other methods of IOP measurements in mice were later developed including noninvasive techniques. Noninvasive methods allow multiple IOP measurements to be completed in a short period of time, but the results of these measurements may depend upon mechanical properties of the cornea. To obtain reliable IOP readings, all of the described techniques require training.

Transgenic and gene-targeted knockout approaches have been used to develop several mouse models of glaucoma. The main advantage of these models is that the animals with the mutated gene provide a more uniform elevation of the IOP and damage to the retina and optic nerve similar to that found with surgically induced elevated IOPs. A large number of animals can be produced, and once a mutant mouse line is obtained, no special training is needed to produce more affected mice.

Several lines of transgenic mice have been developed that contained BAC DNAs with a mouse Tyr423His point mutation and Tyr437His point mutation of the human myocilin (Myoc) genes. The Tyr437His mutation in the Myoc gene leads to severe glaucoma in humans, and the mouse Tyr423His mutation corresponds to this human mutation. Expression of mutated mouse Myoc in the ocular drainage structures led to moderate (about 2 mm Hg during the day and 4 mm Hg at night) elevation of the

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IOP and progressive degenerative changes in the peripheral RGC layer and optic nerve that resembled glaucomatous changes in human eyes.[52] In 1-year-old animals, the peripheral retina of transgenic mice had approximately 20% fewer RGCs than the peripheral retina of control littermates.

Transgenic mice with a targeted mutation in the gene for the α_1-subunit of collagen type I have also been constructed. This mutation blocks the cleavage of collagen by matrix metalloproteinase-1. Transgenic mice expressing mutated collagen had elevated IOPs. The difference in the IOP between control and transgenic mice gradually increased to a maximum of 4.8 mm Hg at 36 weeks. Because these mice had progressive optic nerve axon loss with normal organization of the drainage structures, it has been suggested that they may be used as a mouse model of POAG.[53]

Recent data have demonstrated that transgenic mice expressing mutated optineurin under the control of the ubiquitous ROSA26 promoter develop optic nerve cupping and death of the RGCs without elevation of the IOP.[54] These transgenic mice may represent the first animal model of NTG.

The surgical methods used to produce rat glaucoma models have also been used in mice. However, performing surgery on the mouse eye is even more challenging than on rat eyes because of the difference in size. A significant elevation of the IOP was found in the eyes of C57BL/6J mice that had an injection of indocyanine green dye into the anterior chamber and diode laser treatment of the TM and episcleral vein region.[55] At 10 days after the surgery, the mean IOP in the operated eyes was 33.6 ± 1.5 mm Hg versus 15.2 ± 0.6 mm Hg in the control eyes. However, the IOP returned to normal 60 days after the surgery. Histological examination of the treated eyes 65 day after the surgery revealed anterior synechia, a decrease in the number of RGCs, thinning of all retinal layers, and damage to the optic nerve structures without evidence of prominent cupping.[55] A reduction in the function of the outer retinal layers, confirmed by electroretinographic studies, may indicate that this model produces more extensive changes in the retina compared to the glaucoma in humans.

Similar to the above model, an elevation of IOP was induced by argon laser photocoagulation of the episcleral and limbal veins in C57BL/6J mouse eyes[56] or by cauterization of three episcleral veins in CD1 mouse eyes.[57] During the first 4 weeks following laser treatment, the mean IOP in the treated eyes was about 1.5 times higher than in control eyes. The number of RGCs had decreased by $22.4 \pm 7.5\%$ of that in the controls at 4 weeks after treatment. Most of the TUNEL-positive apoptotic cells were detected in the peripheral retina.[56]

Cauterization of the episcleral veins led to a maximum IOP elevation within 2–9 days, and the IOP decreased progressively thereafter to reach more or less normal values after 24–33 days. There was a 20% decrease in the number of RGCs 2 weeks after the surgery.[57]

The DBA/2J strain has high IOP and has become a popular mouse model to study secondary angle-closure glaucoma. This mouse strain has mutations in two genes, *Tyrp1* and *Gpnmb*.[58] DBA/2J mice develop pigment dispersion, iris transillumination, iris atrophy, and anterior synechia. At the age of 9 months, the IOP was elevated in most mice and the elevation was accompanied by the death of the RGCs, optic nerve atrophy, and optic nerve cupping. Although no group of the RGCs was especially vulnerable or resistant to degeneration, fan-shaped sectors of dead or surviving RGC radiated from the ONH.[59] It has been suggested that axon damage at the ONH might be a primary lesion in this model.[59]

Several important observations were made from the studies on the DBA/2J model. It was shown that the proapoptotic protein BAX is required for the survival of RGCs but not for RGC axon degeneration, suggesting that BAX may be a candidate human glaucoma susceptibility gene.[60] Unexpectedly, a high dose of γ-irradiation accompanied by syngeneic bone marrow transfer protected the RGCs in DBA/2J mice.[61] Similar to the results obtained with rat and monkey models, genes involved in the glial activation and immune response were activated in DBA/2J retina as shown by array hybridization.[62]

Complement component, 1Q, was upregulated in the retina of several animal models of glaucoma as it is in human glaucoma with the timing suggesting that complement activation plays a significant role in the pathogenesis of glaucoma.[63]

Taken together, these findings confirm that animal models might be used to look for a molecular mechanism involved in glaucoma in humans.

The modulation of the activity of genes involved in the development of the anterior segment of the eye may lead to relatively rare developmental glaucomas that account for less than 1% of all glaucoma cases. Several genes have been implicated in congenital glaucoma and anterior segment disgenesis. They include *Cyp1b1*, *Foxc1*, *Foxc2*, *Pitx2*, *Lmx1b*, and *Pax6*. Several lines of mice with defects in these genes have been studied with glaucoma in mind (see Gould *et al.*[64] for a review). For example, mutation in the *CYP1B1* gene (cytochrome P450, family 1, subfamily b, polypeptide 1) may lead to primary congenital glaucoma (PCG) in humans. Although Cyp1b1 knockout mice did not develop elevated IOP, they had ocular abnormalities similar to the defects in humans with PCG, viz. small or absent Schlemm's canal, defects in the TM, and attachment of the iris to the TM and anterior synechia.

Mutations in the *FOXC1* gene, which encodes a transcription factor with a forkhead-winged-helix DNA binding domain, cause a range of eye abnormalities associated with glaucoma, e.g., iris hypoplasia, Axenfeld and Rieger anomaly, and Rieger syndrome. Foxc1$^{-/-}$ mice die at birth, while Foxc1$^{+/-}$ animals are viable but have defects in the eye drainage structures without changes in IOP. Similar eye defects were observed in Foxc2$^{+/-}$ mice. It has been suggested that *Foxc1*$^{+/-}$ and *Foxc2*$^{+/-}$ mice are useful models for studying anterior segment development and anomalies, and may allow the identification of genes that interact with *Foxc1* and *Foxc2* (or *FKHL7* and *FKHL14*) to produce a phenotype with elevated IOP and glaucoma.

Other animals, including rabbit, pig, and bovine, have also been used to develop animal models of glaucoma, but none of them is widely used for different reasons. Zebrafish became a powerful model for advanced genetic studies in vertebrates, especially in the case of complex diseases, and was proposed as a model for identification of modifier genes for glaucoma.[65]

CONCLUSIONS

In summary, animal models of glaucoma, including the most widely used rodent and monkey models, have already provided interesting new information about mechanisms of glaucoma in humans. However, it should be remembered that even in monkey models the time course of changes in the glaucomatous eyes may

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be significantly accelerated compared to that in human glaucomatous eyes, and all discussed models are indeed just models of glaucoma in humans. Results obtained with these models should not be automatically applied to human glaucoma and should be confirmed by testing in humans whenever possible. It has become clear that reaction to the same insult, e.g., elevated IOP, may be somewhat different in different animal models. Glaucoma studies in animals may help us identify the molecular mechanisms involved in the development of glaucoma in each particular model. By comparing these mechanisms, it may be possible to find some common mechanism that might be involved in glaucoma formation in humans. This will be extremely valuable for the development of new therapeutic approaches for the treatment and prevention of glaucoma in humans.

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