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% CFH 402	PAR% rs11200638	(95% CI) CFH 402	(95% CI) rs11200638	M-H test: p-value LRT p-value	M-H test: p-value AIC value
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.

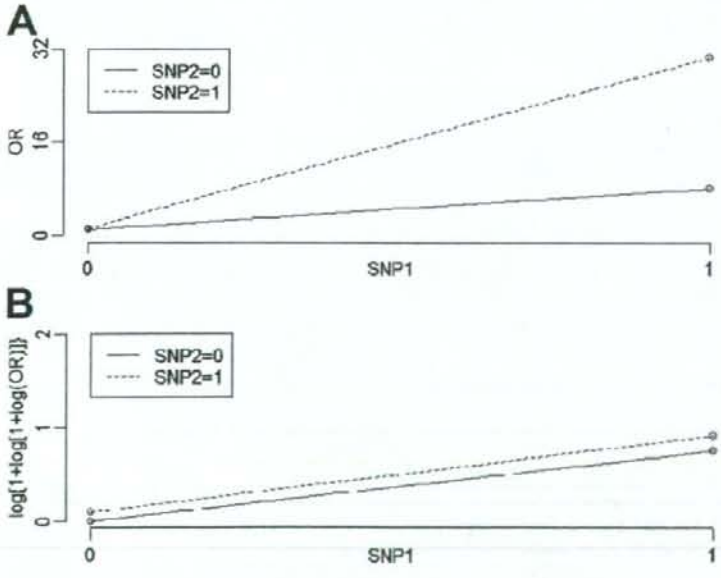

Figure 1. Odds ratio plots for two single nucleotide polymorphisms. Joint odds ratio plots for the single nucleotide polymorphisms (SNPs), complement factor H (CFH) 402, and rs11200638 before and after log transformation showing that the apparent interaction is a "removable" effect. SNP1=CFH 402: 0 is 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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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

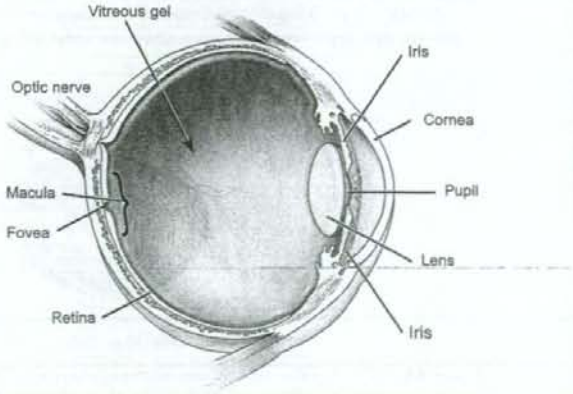

Figure 68.1 Diagram of the eye with principal structures of the anterior segment, retina, and optic nerve indicated. Courtesy of the National Eye Institute, National Institutes of Health.

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 α-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.

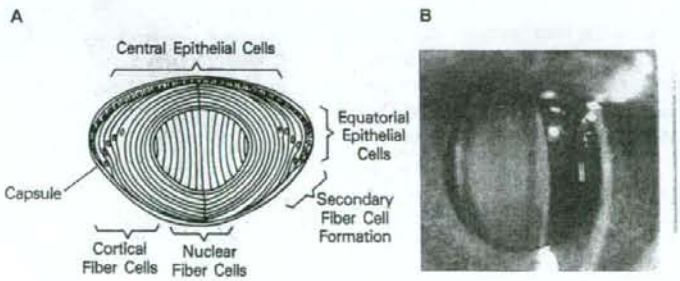

Figure 68.2 A. A diagram showing lens structure including the anterior epithelial cells; the cortical fiber cells, which elongate and loosen their nuclei and mitochondria; and the nuclear cells, in which this process has been completed. The ends of the nuclear fiber cells abut each other in a complex 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 α-crystallins, but also in the β- and γ-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. αA-Crystallin is cleaved nonenzymatically, particularly between Asn 101 and Glu 102. An aspartate residue in αA-crystallin appears especially susceptible because it easily forms a succinimide intermediate. Phosphorylation of lens proteins also occurs. Nonenzymatic glycosylation (glycation) occurs, especially of the ε-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. γ-Crystallins, and especially γS-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 β- and γ-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 α-crystallins, which have a chaperone-like activity. Binding by α-crystallins maintains the solubility of $\beta\gamma$-crystallins and reduces light scattering, but the α-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 α-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 α-crystallins, MnSOD, catalase, glutathione peroxidase, and γ-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 βB2-crystallin polypeptide. The βB2-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 α- and β-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 γ-crystallins and their mRNAs. However, the gene causing this cataract segregates independently of the γ-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 γ-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,

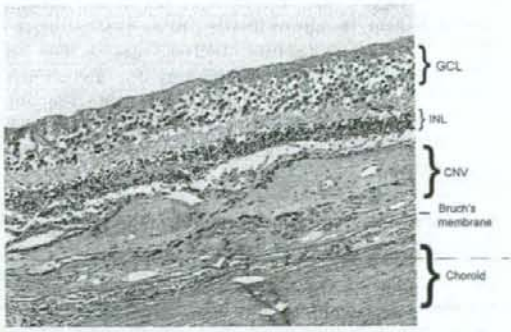

Figure 68.3 Histological section of the retina showing macular degeneration. Although the ganglion cell layer (GCL), inner nuclear layer (INL) and choroid are well preserved, the outer nuclear layer, which should appear similar to the INL, has been in large part replaced by fibrovascular choroidal neovascularization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

smaller (with diameters of less than 50 μm), and less likely to progress to later stages of the disease. Soft drusen are larger, dark yellowish in color, and more likely to be associated with more advanced stages of the disease. In later stages of AMD, choroidal neovascularization and leakage of serous fluid into the subretinal (occult CNV) or intraretinal (classical CNV) regions leads to cell death and detachment of the RPE. Visual acuity is significantly affected when geographic atrophy of the RPE takes place in the fovea.

Epidemiology of macular degeneration

AMD has a multifactorial (or complex) etiology with contributions from a combination of environmental and genetic factors and a strong age effect. The prevalence of AMD increases dramatically with age, although the prevalence cited in various reports is highly dependent on the definition used for AMD. Overall, AMD increases from less than 1 to 2% at 50 years of age to as high as 15% at 90 years old. It has been suggested that increased skin pigmentation tends to protect from AMD, and this correlates to lower prevalence of AMD in African derived populations than Caucasians in some, but not all, studies. Various other risk factors may predispose to AMD including systemic hypertension and atherosclerosis, as well as cigarette smoking. Both photo-oxidation and inflammation have been suggested as possible pathogenic mechanisms for AMD, although the precise mechanism through which these result in disease has not been delineated.

Genetic factors have been implicated in AMD by epidemiological studies including twin studies and formal segregation analyses (Heiba *et al.*, 1994; Hammond *et al.*, 2002; Seddon, Ajani, and Mitchell, 1997). First degree relatives of individuals with AMD appear to have a two- to four-fold increased incidence of AMD over control individuals without a family history of AMD. Twin studies suggest that concordance for AMD in monozygotic twins is approximately twice that in dizygotic twins. Formal segregation analysis suggests that there is a major gene effect accounting for approximately 60% of AMD with a single major gene accounting for about 55% of AMD risk. Overall, these data suggest that the etiology of AMD has a significant genetic component.

HUMAN STUDIES OF MACULAR DEGENERATION

Mendelian linkage and association studies

In addition to ARMD, several Mendelian forms of macular degenerations have been described. The age of onset, pattern of inheritance, and clinical characteristics of these diseases vary widely. To date, about 17 human Mendelian macular degeneration genes have been mapped (Tuo, Bojanowski, and Chan, 2004). So far genes for nine different forms of human Mendelian macular degenerations have been identified using a positional cloning approach. These genes can be broadly classified into two groups: genes that are expressed in photoreceptors (ELOVL4, RDS/peripherin, RPGR, and ABCA4) and genes expressed in RPE (Bestrophin, EFEMP1, TIMP3, Hemicentin-1, and CTRP5). The genes ELOVL4, RDS, RPGR, and ABCA4 are expressed in both rod and cone photoreceptors. Except for the ELOVL4 gene, mutations in the remaining three genes were shown to be associated with retinitis pigmentosa (RP) in addition to macular degeneration. Mutations in TIMP3, EFEMP1, Hemicentin-1, CTRP5, and Bestrophin have not been implicated in RP. Of the genes involved in causing macular degeneration, all four photoreceptor-expressed genes are associated with an atrophic phenotype, whereas the RPE-expressed genes are associated with subretinal deposits and drusen in the early stages of the disease, which then progresses to neovascularization at later stages. The genes EFEMP1, TIMP3, Hemicentin-1, and CTRP5 share structural homology and are components of the extracellular matrix, and Bestrophin was reported to be a membrane channel. Recently, Fibulin-5, which also belongs to the fibulin family of extracellular proteins and shares homology with the EFEMP1 and CTRP5 proteins, was shown to be associated with AMD.

Linkage and association studies of AMD

Although some families show Mendelian inheritance of AMD, the disease in the general population is inherited in a complex or multifactorial fashion. In attempts to identify the genes that contribute to AMD risk in the population at large, investigators have looked at inheritance of AMD in small families or even pairs of affected siblings. A number of studies have examined families in which more than one member is affected with AMD, to determine whether polymorphic genetic markers at known positions in the human genome are co-inherited with the disease. These genome-wide scans have identified at least 21 linked regions on multiple chromosomes, including most consistently regions on chromosomes 1q, 9q, 10q, 12q, and 16q. However, AMD has not been associated with mutations in genes in any of these regions except complement factor H.

Three genes, ATP binding cassette subfamily A member 4 (ABCA4), apolipoprotein E (APOE), and complement factor H (HF1), have been reported to be associated with susceptibility to AMD in the general population (Tuo, Bojanowski, and Chan, 2004). However, the role of ABCA4 is somewhat controversial, and it probably is responsible for a few percent of AMD cases at most. Involvement of APOE in AMD seems to be more solid, with most studies showing a risk ratio of individuals carrying at least one APOE-ε4 allele reduced to about 40 to 50% of control values, although some studies could not replicate this finding. Recently, a Y402H polymorphism in the complement factor H protein has been shown to be associated with a two- to seven-fold increase in risk for AMD in two studies of unrelated individuals.

The gene encoding complement factor H lies in the chromosome 1q25-31 region implicated in linkage studies of both a large single family and of multiple small families and sibling pairs. One study suggested that this gene might account for as much as 50% of the hereditary tendency of AMD in the general population (Edwards *et al.*, 2005). In addition, the biochemical activities of both APOE and HF1 are consistent with the proposed atherosclerotic and inflammatory associations of AMD and the histological and biochemical analysis of the subretinal deposits. Thus, significant progress is being made in understanding the biological nature of the genes associated with macular degenerations and their roles in the disease. However, despite these advances little is understood about the overall mechanism underlying the disease process.

BIOCHEMISTRY AND PATHOLOGY OF MACULAR DEGENERATION

Histological changes

Among the early hallmarks of AMD are drusen, which are complex deposits of lipids, proteins, glycoproteins, and glycosaminoglycans that accumulate in the extracellular and inner aspects of Bruch's membrane (Anderson *et al.*, 2002). These subretinal deposits, accompanied by a diffuse thickening of Bruch's membrane, have been speculated to form a physical barrier between the RPE and choroid, obstructing the flow of nutrients from choroid to RPE, or possibly resulting in loss of cell-cell contact between RPE and Bruch's membrane and causing degeneration of retinal tissue. The RPE cells are responsible for phagocytosis and degradation of outer segment disks shed by photoreceptors. As they age and undergo oxidative stress, lipofuscin accumulates in the lysosomal compartment and leads to cellular damage and further impaired function. Though the origin of drusen remains controversial, current opinions are beginning to favor the vasculature of the choriocapillaris as a primary source rather than an intracellular source from the RPE. It is possible that the presence of lipofuscin and cellular debris excites an immune reaction and leads to the formation of drusen. This is reflected by the presence of immune components in drusen (Anderson *et al.*, 2002; see later).

Chorioretinal neovascularization (CNV) is the most common cause of vision loss in AMD. New vessels from the choriocapillaris grow through Bruch's membrane and branch horizontally through the RPE cell layer (termed classic CNV) or between the inner Bruch's membrane and RPE (termed occult CNV because it doesn't show up on angiography). Although the impetus for CNV has not been definitively determined, there are suggestions that imbalances in growth factors include pigment epithelial derived factor (PEDF, which inhibits vascular outgrowth) and vascular endothelial growth factor (VEGF, which stimulates vascular growth), possibly as a result of hypoxia and inflammation of the RPE. Even in the absence of CNV, the changes to the RPE Bruch's membrane and the outer plexiform layer of the retina result in scar formation at that level with concomitant damage to the neurosensory outer retina, termed geographic atrophy, which can also result in loss of central vision.

Composition of drusen and its implications

Understanding the composition of drusen provides important clues to the molecular pathology of the disease. In addition to classical immunohistochemical techniques, several advanced proteome analysis tools have begun to provide detailed information about the nature and composition of drusen. Perhaps the most significant of the new findings is that drusen contain protein molecules that mediate inflammatory and immune processes. These include immunoglobulins, components of complement pathway, and modulators for complement activation (e.g., vitronectin, clusterin, membrane cofactor protein, and complement receptor-1), molecules involved in the acute-phase response to inflammation (e.g., amyloid P component, α1-antitrypsin, and apolipoprotein E), major histocompatibility complex class II antigens, and HLA-DR antigens (Crabb *et al.*, 2002). Cellular components also have been identified in drusen, including RPE debris, lipofuscin, and melanin, as well as processes of choroidal dendritic cells, which are felt to contribute to the inflammatory response (Mullins *et al.*, 2000).

In addition to immune components, a number of other proteins occur in drusen, some of them also found in atherosclerotic plaques and other age-related diseases in which protein deposits occur. The most common of these appear to be TIMP-3, clusterin, vitronectin, and serum albumin. Other proteins found in drusen include serum amyloid P component, apolipoprotein E, IgG, Factor X, and some complement proteins (Mullins *et al.*, 2000). A number of proteins are found exclusively or in increased amounts in drusen associated with AMD than in drusen from individuals unaffected by AMD. These include some crystallins, EEFMP1, and amyloid-beta. In addition, the presence of immunoreactive proteins and oxidative modifications of many proteins found in drusen implicate both oxidation and immune functions in the pathogenesis of AMD.

Immune aspects

These findings have led to the suggestion that immune complex-mediated inflammation damages RPE cells, and choroidal dendritic cells are activated and recruited by injured RPE, whereas RPE cells respond to control dendritic cell activation by secreting proteins that modulate the immune response. Shed or phagocytosed cell membranes of injured RPE or dendritic cells are postulated to function as cores for these secreted components to accumulate and form extracellular deposits.

Furthermore, the codistribution of IgG and terminal complement complexes in drusen implicates an immune response directed against retinal antigens, and the immune complex formation might be taking place at the site of drusen formation. This hypothesis is supported by the presence of putative anti-retinal autoantibodies in the sera of patients with ARMD. Anti-retinal autoantibodies previously have been reported in a number of ocular disorders, including retinitis pigmentosa, paraneoplastic retinopathies, and retinal vasculitis (Anderson *et al.*, 2002). In addition, patients with membranoproliferative glomerulonephritis, in which complement activation and immune complex deposition cause glomerular injury, develop drusen deposits resembling those in ARMD in ultrastructure and composition including C5 and IgG. However, the role of antiretinal autoantibodies in the pathogenesis of ARMD has not been examined in detail. It remains unknown whether the initiation of chronic inflammation and subsequent drusen formation requires autoimmune-mediated events as a primary factor. To clarify the role of autoimmunity, immunogenic molecules for circulating antiretinal autoantibodies in patients need to be identified.

Oxidative aspects

Oxidative damage is implicated in the pathogenesis of AMD by both theoretical considerations and experimental data (Roth, Bindewald, and Holz, 2004). The retina has a highly active metabolism with a resultant high oxygen demand, and is exposed to light and polyunsaturated fatty acids, all of which tend to increase its susceptibility to photo-oxidative damage. In a fashion somewhat analogous to that seen in the lens, as the retina ages its antioxidant defenses begin to decline, here including both antioxidant enzymes and antioxidants such as lutein, and macular pigment density. As the RPE age oxidation of lipids and other cellular components result in accumulation of nonmetabolizable material as lipofuscin in the lysosomes, leading to their enlargement and formation of lipofuscin granules. These closely parallel drusen formation in time and distribution in the retina. In addition, epidemiological correlation of AMD with light exposure, age, and light pigmentation as well as the prevention or delay of AMD by antioxidant vitamins in the AREDS trial also support an oxidative role in AMD.

ANIMAL MODELS OF MACULAR DEGENERATION

Overview

Limited access to appropriate biological materials, especially eye samples from affected donors at different stages of the disease, are an absolute necessity to study mechanisms underlying the macular degenerations. Because it is nearly impossible to obtain these human retinal tissues from patients or from normal controls, animal models play a crucial role for investigating the biological pathway of disease development and for testing therapeutic strategies. Because age-related macular degeneration shares phenotypic similarities with monogenic macular degenerations, manipulation of these genes associated with monogenic macular degenerations to develop transgenic mouse models has been popular. Over the past few years, genetic engineering technologies has allowed the generation of a rapidly growing number of animal models for retinal diseases (Chader, 2002). Animal models have been used to investigate potentially protective therapeutic agents to treat photoreceptor degeneration, stem cell technology, or to test somatic gene therapy strategies (Ali *et al.*, 2000). They are also valuable for studying environmental effects like diet or light on the degeneration process. The animals that have been used to evaluate therapeutic strategies involve rodents, rabbits, pigs, and dogs. However, macula is found only in primates and birds; a monkey model with macular degeneration would be extremely valuable as they not only have a defined macula, but they are also evolutionarily close to humans.

Macular degeneration in monkeys was first described by Stafford in 1974 (Stafford, Anness, and Fine, 1984). He reported that 6.6% of elderly monkeys showed pigmentary disorders and/or drusen-like spots. El-Mofty and colleagues reported 50% incidence of maculopathy in a rhesus monkey colony at the Caribbean Primate Research Center of the University of Puerto Rico in 1978. The following report from the center indicated that specific maternal lineages had a statistically significant higher prevalence of drusen. Researchers have described a cynomolgus monkey (*Macaca fascicularis*) colony at the Tsukuba Primate Research Center (Tsukuba city, Japan) with a high incidence of macular degeneration and its pattern of inheritance (Umeda *et al.*, 2005).

Several other naturally occurring animal models have been described for retinal diseases. Rodents, mainly mice, are the most popular animal models as maintenance is less expensive compared to larger animals. However, a low cone:rod ratio and lack of a macula make mice less suitable for studying cone diseases and macular degenerations. Although the pathology in human ARMD is pronounced in the macula area, it is not confined to this central region .alone. Abnormal accumulation of drusen and progressive degeneration of the retina, RPE, and underlying choroid characteristics were observed in mouse models generated by candidate gene manipulation or senescence acceleration (Ambati *et al.*, 2003). Choroidal neovascularization also has been described in naturally occurring mouse models. These observations suggest that the lack of a macula in mice may not be a disadvantage when considering the advantages of using the mouse as a model for studying macular degenerations with drusen.

Although monkey models are extremely important for macular degeneration study, there are limitations

using nonhuman primates as animal models, such as longer gestation and life span, slow rate of expanding the pedigree, and cost of maintenance. These limitations can be overcome only by utilizing the mouse model parallel to the monkey model. One such model is a mouse line expressing an inactive form of cathepsin D. The impaired enzymatic activity affects phagocytosis of photoreceptor outer segments in the RPE cells, and the mice demonstrate basal laminar and linear deposits.

Animal model of early and late onset macular degeneration monkey

In 1986, a single cynomolgus monkey (*Macaca fascicularis*) with heavy drusen was found in the Tsukuba Primate Research Center. After 19 years of mating experiments, that single pedigree has grown to having 57 affected and 182 unaffected monkeys. Macular changes are observed as early as two years after birth, with basal laminar deposits first appearing in the macular region and progressing toward the peripheral retina throughout the lifetime (see Figure 68.4). In all the cases examined no abnormalities were found in the optic disc, retinal blood vessels, or choroidal vasculatures. The affected monkeys share phenotypic similarities with the early stages of ARMD, such as drusen and accumulation of lipofuscin. The immunohistochemical and proteome analysis of drusen in these monkeys share significant similarity with composition of age-related macular degeneration monkeys and also with previously reported human drusen composition. The meaning of this observation is that early onset monkeys produce the same drusen as ARMD patients at an accelerated rate of 25 times. Thirteen human candidate gene loci have been excluded by linkage and haplotype analysis. Therefore, the gene associated with macular degeneration in these monkeys is likely to be novel and the genes involved in causing drusen phenotype in humans and monkeys could be either the same or belong to the same biological pathway.

Studies involving early-onset and late-onset macular degeneration monkeys present a unique opportunity to study two independent target points in the biological pathway of retinal tissue that lead to degeneration of the macula at different stages of life. The gene associated with monkey macular degeneration is likely to be a novel gene as we have excluded most of the known macular degeneration loci. Cloning of the monkey macular degeneration gene will allow us to study the biological processes causing degeneration of retina. Due to high conservation between human and macaque genomes, genes associated with macular degeneration in monkeys should possibly play a key role in maintaining the normal function of retina in humans and is likely to be associated with macular degeneration in humans. Although some of the monogenic macular degeneration genes are not associated with ARMD, the phenotype observed in monkeys strongly suggests that this gene may play a role in human ARMD, and this cannot be established until validated by screening patients with ARMD. Understanding the mechanism underlying macular degeneration in these monkeys will enhance our understanding of the disease, identify clinical or molecular markers for early detection, and provide critical information needed to develop therapies for these diseases.

Progressive Open Angle Glaucoma (POAG)

BRIEF OVERVIEW

Epidemiology of POAG

Primary open angle glaucoma is a major cause of blindness throughout the world, affecting between 1 and 2% of individuals over 40 years of age (Klein *et al.*, 1992). The greatest risk factor for the development of POAG is ocular hypertension, to the extent that an elevated intraocular pressure (IOP) is often incorporated into the definition of glaucoma. In addition, the evidence implicating a genetic influence in glaucoma is very strong, and has been borne out in both model-based and model-free linkage studies. Finally, diabetes and myopia have been suggested to be related to development of POAG, but the evidence for this is inconsistent, although it seems likely that high myopia might contribute to development of POAG.

Pathology and physiology of POAG

Although the etiology and even the pathophysiology of glaucoma are still poorly understood, risk factors for glaucoma can be thought of as including both those in the anterior chamber, which tend to increase intraocular

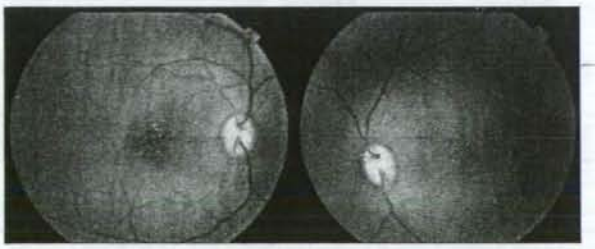

Figure 68.4 Funduscopic view of the retina in Tsukuba primate model of macular degeneration showing drusen and macular changes.

pressure, and those in the retina and optic nerve, which tend to increase susceptibility to damage from elevated or even normal intraocular pressure. Clinically, glaucoma generally is characterized by excavation of the optic disc as seen on funduscopic examination and visual field defects with elevated intraocular pressure included either as a part of the disease or a risk factor. In a simplified schema, one might think of increased resistance of the trabecular meshwork or Schlemm's canal to outflow of the aqueous humor causing an increase in intraocular pressure, which then acts upon sensitive retinal ganglion cells. These cells then degenerate, resulting in both the increased depth and width of the optic cup and the visual field defects. If the increased pressure is acute as it usually is in juvenile onset glaucoma, this process can be painful, but generally POAG is an insidious disease in which the first recognized symptom may be irreversible visual field changes.

Although primary open angle glaucoma (POAG) is characterized by visual field loss corresponding to the excavation of the optic disc (see Figure 68.5), it is usually associated with an elevation of the intraocular pressure (IOP) over 21 mmHg. Although the pathogenesis of glaucomatous optic neuropathy is poorly understood, it is generally accepted that the IOP is a major risk factor. By definition, there is no increase in IOP over 21 mmHg at any time in eyes with normal-tension glaucoma (NTG), although it is difficult to rule out fleeting or previous elevations of IOP. IOP is heavily influenced by the inflow and outflow of aqueous humor, a plasma filtrate actively generated at stroma of ciliary body and filtered across the blood-aqueous barrier. The aqueous flows from the posterior chamber to the anterior chamber via the pupil and is released through two routes, the trabecular route and uveoscleral route. Any disturbance in the flow can cause abnormal IOP leading to a death of retinal ganglion cells (RGC), and damage to the surrounding structure of the optic nerve head where optic nerve fibers leave the eye for visual cortex.

HUMAN STUDIES OF POAG

Linkage studies

At least six loci for autosomal dominant POAG have been mapped through linkage studies, termed GLC1A-F, on chromosomes 1q23, 2cen-q13, 3q21-q24, 8q23, 10p15-p14, and 7q35-q36. A genome-wide scan in multiple small families from an Afro-Caribbean population provided significant evidence for linkage to regions on chromosomes 2q (but separate from the Mendelian POAG locus GLC2B and the infantile glaucoma locus GLC3A on chromosome 2) and 10p. Presumably, these represent loci for glaucoma risk factors common in the general population, as do the loci on chromosomes 2, 14, 17, and 19, identified by examining siblings in an American population of European descent. It is particularly important to note that few of these studies have been confirmed; especially the technically more difficult and laborious studies of POAG in the general population.

Association studies

In addition to the identification of myocilin as a causative gene in glaucoma described earlier, which was carried out by linkage studies primarily in families with juvenile glaucoma and very elevated intraocular pressure, association studies have identified sequence changes in myocilin as a risk factor in a small percentage of POAG cases. Two additional genes have been shown to be involved in glaucoma by demonstrating an association between sequence changes in those genes and glaucoma in population studies. One of these genes is optineurin, for which the strongest associations have been obtained with normal tension glaucoma, but which also might be associated with POAG in some populations. A second is the OPA1 gene, which is known primarily as a cause of optic atrophy, but is also associated with normal tension glaucoma, though not with high tension primary open angle glaucoma in most studies. Association of both these genes with normal tension glaucoma suggests that

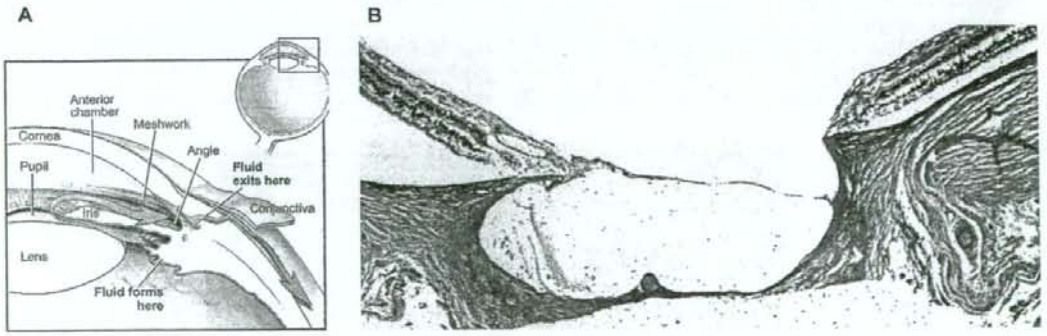

Figure 68.5 A. Diagram depicting the flow of aqueous humor from synthesis in the ciliary body to exit from the anterior chamber through the trabecular meshwork and Schlem's canal. **B.** Histological section showing an excavated optic cup in an individual with glaucoma. Courtesy of Dr. Chi Chao Chan, National Eye Institute, National Institutes of Health, from the collection of Dr. W. R. Green.

there may be some relationship between normal tension glaucoma and optic atrophy, and also emphasizes the importance of genetic changes that sensitize the retina and optic nerve to minor elevations of even normal intraocular pressure.

BIOCHEMISTRY AND PATHOLOGY OF POAG

Histological changes

It is estimated that roughly 20 to 50% of the large retinal ganglion cells (RGC) are lost in POAG. Although the reduction of RGC density occurs equally throughout the retina, visual sensitivity is first lost in areas where the initial RGC density is low, especially in the peripheral regions of the retina. As the disease progresses, atrophy of the nerve fiber layer is usually observed as additional RGC is lost. Typically, vertical collapse of the optic nerve head (ONH), loss of the neural rim at the ONH, rearrangement of central blood vessels, and loss of supporting tissue occur. Scanning electron microscopy of retinas with early stages of glaucoma shows evidence of initial collapse of the anterior lamina cribrosa, primarily in the vertical poles of the optic nerve head. Based on primate studies, optic cups with larger diameters are more susceptible to high ocular pressure and thus to glaucoma.

Role of the trabecular meshwork

Trabecular meshwork (TM) is a lamellated sheet of complex tissue that covers the inner wall of Schlemm's canal. TM has uniquely developed at the angle of primates, filtering the aqueous humor out of the eye. TM consists of two parts: the nonfiltering portion mainly occupied by trabecular cells and the filtering portion. Trabecular cells are highly phagocytic cells removing particles, cell debris, and protein from the aqueous humor. The first glaucoma locus, the *trabecular meshwork inducible glucocorticoid response* (TIGR), also known as myocilin, initially was identified by looking at genes whose transcription is highly induced by steroids in these cells. The filtering portion consists of three tissues: the cribriform layer, the corneoscleral meshwork, and the uveal meshwork. These trabecular beams or strands are intertwiningly connected to each other, forming a complex filtering mesh surrounding Schlemm's canal. The trabecular beams are thickened by accumulation of extracellular materials and decrease of cell density within the corneoscleral and uveal meshwork in aged eyes.

ANIMAL MODELS OF POAG

Overview: Difficulty of modeling the human eye

Limited access to appropriate biological materials, especially eye samples from affected donors at different stages of the POAG, is an impediment to the study of mechanisms underlying the disease. Because of the extreme difficulty in obtaining such diseased eyes from both patients and normal controls, animal models play a crucial role in investigating the biological pathway of disease development and in testing therapeutic strategies.

Different types of animal models for POAG have been found or created to mimic the optic nerve damage to resemble POAG phenotypes in humans. The greatest difficulty in constructing an animal model for POAG lies in the diversity of the anterior structures of the eye among different species (Tripathi and Tripathi, 1972, 1973). These structural differences include different iridocorneal angles or absence of specific quadrants from the TM. Nevertheless, within the limited areas in which interpretation of the data from a specific animal model parallels that in the human, various animals including the cow, dog, cat, horse, rabbit, chicken, and monkey can be used to observe POAG under various experimental conditions.

Animal models of POAG

Various animal models for inducible glaucoma have been reported. Argon laser photocoagulation of the TM in rhesus monkeys results in sustained elevation of IOP and has been used extensively to study early damage to the optic nerve head (May *et al.*, 1997). Corticosteroids such as betamethasone and dexamethasone have been used to treat rabbits, dogs, and cats to develop ocular hypertension (Bonomi *et al.*, 1978). Steroid treatment generally produces progressive glaucoma, but this process is reversed after about two months after cessation of the steroid. Trabecular blockage caused by inflammation after α-chymotrypsin treatment also has been used to produce elevated IOP in rabbit and monkey eyes (Vareilles *et al.*, 1977). Some types of avian species (chicken, quail, and turkey) have been known to develop elevated IOP as a consequence of continuous exposure to light.

Mouse models of glaucoma

Naturally occurring inherited animal glaucoma models are rare. However, extensive classification of IOP in mouse strains and molecular biological techniques to manipulate certain genes to produce transgenic or knockout/knockin mice recently have resulted in the development of a number of animal models with definitely known genetic causes for their disease (Chang *et al.*, 1999). As discussed earlier, four genes, myocilin (MYOC, TIGR), cytochrome P4501B1 (CYP1B1), optineurin (OPTN), and WDR36, currently are associated with glaucoma. OPTN, mutations of which are responsible for 16.7% of families with hereditary human NTG, is homologous to an inhibitory regulatory subunit of the high molecular kinase complex for the phosphorylation of NF-κB. Some of its known functions include inhibition of the tumor necrosis factor-alpha pathway, interaction with transcription factor IIIA, and mediation of the Huntington and Rab8 interaction for regulation of

membrane trafficking and cellular morphogenesis. OPTN is induced by TNF-α and binds to an inhibitor of TNF-α and the adenovirus E3-14.7 kDa protein. To determine the effects of human glaucoma mutations in a transgenic mouse system, mice over-expressing wild type OPTN, OPTN carrying the glaucoma associated mutation E50K, and OPTN with exon5 deleted were constructed. Although wild-type OPTN do not show any abnormalities and the exon 5 deleted construction was found to be lethal prenatally, mice transgenic for the E50K mutant OPTN show steep optic nerve cupping with rearrangement of supporting tissue and blood vessels 18 weeks after birth (see Figure 68.6). The RGC and astrocyte loss observed is similar to the end phase changes seen in human glaucoma patients. Understanding the mechanism underlying normal tension glaucoma in these transgenic mice will enhance our understanding of each step leading to optic nerve cupping and how to prevent it. Based on the success of the mouse model, use of larger animals such as transgenic rabbits or pigs, in which more precise measurement of IOP and trials of surgical procedures suitable for therapy in humans are possible are currently being investigated.

Other glaucoma mouse models have been made through genetic manipulation. Knockout and transgenic mouse models of myocilin were made to answer the question whether elevated expression of the myocilin protein can influence the IOP (Gould *et al.*, 2004). Up to a fifteen-fold increase in myocilin expression failed to result in elevation of the IOP, any abnormality of retinal ganglion cells, or cupping of the optic nerve head. Mice lacking the cytochrome P450 1B1 (CYP1B1) gene were generated on B6 and 129X1/SvJ mouse stains (Libby *et al.*, 2003). Both strains were affected by the CYP1B1 deficiency with focal angle abnormalities, but 129X1/SvJ albino strains lacking tyrosinase were more severely affected, suggesting the presence of tyrosinase as an important developmental molecule.

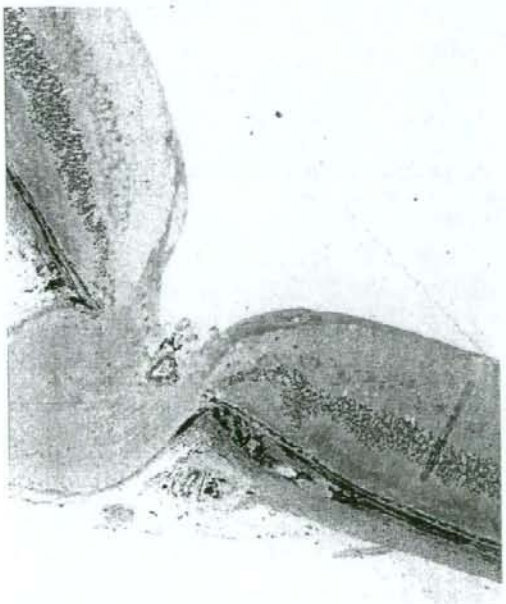

Figure 68.6 Histological section demonstrating excavation of the optic disc in an 18-week-old E50K mutant OPTN transgenic mouse.

Conclusion

In this chapter we have provided a brief overview of age-related eye diseases and the current state of knowledge and research on three of these. Age-related cataracts, age-related macular degeneration, and progressive open angle glaucoma account for much of the population burden imposed by age-related eye diseases. Although no perfect system to study these diseases exists today, an increasing number of experimental models are being developed. Although none is an exact replica of the clinical disease and should not be applied indiscriminately, each of these can provide useful information on some aspects of the disease in humans. They promise to accelerate the pace of research and provide mechanistic and therapeutic insights into the diseases that threaten sight in our aging population.

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Proteomic and Transcriptomic Analyses of Retinal Pigment Epithelial Cells Exposed to REF-1/TFPI-2

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Purpose. The authors previously reported a growth-promoting factor, REF-1/TFPI-2, that is specific to retinal pigment epithelial (RPE) cells. The purpose of this study was to determine the genes and proteins of human RPE cells that are altered by exposure to TFPI-2.

Methods. Human primary RPE cells were cultured with or without TFPI-2. Cell extracts and isolated RNA were subjected to proteomic and transcriptomic analyses, respectively. Proteins were separated by two-dimensional gel electrophoresis followed by gel staining and ion spray tandem mass spectrometry analyses. Transcriptomic analysis was performed using a DNA microarray to detect 27,868 gene expressions.

Results. Proteomic analysis revealed c-Myc binding proteins and ribosomal proteins L11 preferentially induced by TFPI-2 in human RPE cells. Transcriptomic analysis detected 10,773 of 33,096 probes in the TFPI-2 treated samples, whereas only 2186 probes were detected in the nontreated samples. Among the genes up-regulated by TFPI-2 at the protein level were c-*myc*, Mdm2, transcription factor E2F3, retinoblastoma binding protein, and the *p21* gene, which is associated with the c-*myc* binding protein and ribosomal protein L11.

Conclusions. The mechanisms by which TFPI-2 promotes the proliferation of RPE cells may be associated with augmented c-*myc* synthesis and the activation of E2F in the retinoblastoma protein (Rb)/E2F pathway at the G1 phase of the RPE cells. Activation of ribosomal protein L11 and the Mdm2 complex of the p53 pathway may be counterbalanced by the hyperproliferative conditions. (*Invest Ophthalmol Vis Sci.* 2007;48: 516–521) DOI:10.1167/iovs.06-0434

Retinal pigment epithelial (RPE) cells play important roles in maintaining the homeostasis of the retina. RPE cells, located between the sensory retina and the choroidal blood supply, form a diffusion barrier controlling access to the subretinal space, with the RPE membrane regulating the transport of proteins and controlling the hydration and ionic composition of the subretinal space. The sensitivity and viability of the photoreceptors thus depend on RPE-catalyzed transport activity. Proteins in the RPE cells that function in ionic, sugar, peptide, and water transport have been identified.[1] Damage to RPE cells generally leads to degeneration of the neural retina, as occurs in retinitis pigmentosa and age-related macular degeneration. Transplantation of the healthy retinal pigment cells or embryonic stem cells differentiating into RPE cells would be an ideal therapeutic approach to treat such diseases, and such attempts have been made.[2]

An alternative approach to treat these retinal diseases would be the use of a growth factor that promotes proliferation of the remaining RPE cells in a damaged retina or one that stimulates the regeneration of damaged RPE cells. To find such factor(s), the proteins expressed in human fibroblast cells were fractionated and assayed, leading to the isolation of RPE cell factor-1 (REF-1), which selectively promoted the proliferation of primary human RPE cells.[3]

Subsequently, the cDNA of REF-1 was cloned using information from the N-terminal amino acid sequences, which was identical with the tissue factor pathway inhibitor-2 (TFPI-2).[3] Earlier studies have shown that TFPI-2 is a Kunitz-type serine protease inhibitor[4–6] involved in the regulation of extrinsic blood coagulation[4,7] and in the proliferation, invasion, and metastasis of various types of malignant cells.[4,8–15] Extensive studies on the physiological roles of TEPI-2 have revealed that the ERK/MAPK pathway[13] may be associated with the up-regulation of the *TFPI-2* gene and that DNA methylation[9,10] in certain tumor cell lines may be related to the downregulation of the *TEPI-2* gene. When TFPI-2 is added to the culture medium of vascular smooth muscle cells, it promotes cell proliferation.[14]

Our initial finding that TFPI-2 enhanced RPE proliferation prompted us to question how this was achieved. We applied proteomic and transcriptomic analyses to screen the changes in the expression of the RNAs and proteins in RPE cells and will show that the proliferation promoting activity of TFPI-2 on RPE cells is associated with the regulation of an oncogene product, c-*myc*, and representative cancer repressor proteins retinoblastoma protein (Rb)/E2F and p53.

Materials and Methods

TFPI-2 Treatment of Human RPE Cell Culture

Human primary RPE cells (passage 5) were seeded at a density of 2.5×10^4 cells/0.5 mL per well in 24-well plastic plates (BD Biosciences, Franklin Lakes, NJ) with Dulbecco modified MEM (DMEM; Invitrogen Japan, Tokyo, Japan) containing 15% fetal calf serum (FCS, Invitrogen). TFPI-2 was added to 20 wells with the RPE cells at 10 ng/mL concentrations and was incubated at 37°C for 24 hours for the proteomic samples, and for 6 hours, 12 hours, and 24 hours for the transcriptomic samples. An equal amount of saline was added to 20 wells containing RPE cells for controls. TFPI-2 was donated by Toray Industries, Inc., Tokyo, Japan.

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