A R T I C L E S

Modulation of Sub-RPE Deposits In Vitro: A Potential Model for Age-Related Macular Degeneration

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PURPOSE. Sub-RPE deposits form in a variety of conditions most notably in age-related macular degeneration. The purpose of this study was to generate sub-RPE deposits in vitro and to test the hypotheses that high protein concentrations or retinal homogenate increase deposit formation and that a challenge with tumor necrosis factor (TNF)-α or metalloproteinase (MMP)-2 decreases such deposits.

METHODS. ARPE-19 cells were grown on plastic and on collagen type I–coated membrane inserts in media containing various concentrations of fetal calf serum (FCS), bovine serum albumin, or porcine retinal homogenate. In addition, cells grown on membrane inserts were treated with TNF-α or MMP-2. Sub-RPE deposits were assessed by electron microscopy and classified into fibrillar, condensed, banded, and membranous subtypes. The area of the micrograph occupied by each type was estimated with a point-counting technique. MMP-2 activity was assessed in tissue culture supernatants by zymography.

RESULTS. With increasing time in culture, total deposit formation did not change, but the amount of condensed material deposited by ARPE-19 cells increased while the fibrillar component decreased. Albumin challenge resulted in an increased amount of deposit, predominantly of the membranous type. Challenge with retinal homogenate led to a greater net deposit formation with significant increases in the condensed and banded forms. Cells treated with TNF-α or MMP-2 showed a dramatic reduction in all types of sub-RPE deposit. Zymography demonstrated that unchallenged cells produced predominantly MMP-2. Retinal homogenate challenge reduced the total amount of active MMP-2 produced, and TNF-α stimulated MMP-9 production.

CONCLUSIONS. Sub-RPE deposits formed in vitro share ultrastructural features with those seen in vivo. Deposit formation can be modulated by challenge with retinal homogenate, TNF-α, or MMP-2. Significantly, the results provide proof of the principle that sub-RPE deposits can be formed and modified in vitro. (*Invest Ophthalmol Vis Sci.* 2004;45:1281–1288) DOI: 10.1167/iovs.03-0671

Age-related macular degeneration (AMD) is the most common cause of untreatable blindness in the developed world.[1] Although the pathogenesis of AMD is poorly understood, it is widely considered that disease of the retinal pigment epithelium (RPE) and Bruch's membrane, the extracellular matrix (ECM) structure on which the RPE rests, is of central importance. Both with aging and in AMD, extracellular deposits accumulate beneath the RPE and within Bruch's membrane. The variation in the appearance of these deposits can be marked, and these differences have led to numerous attempts to classify sub-RPE deposits both clinically and histopathologically. Although no universally accepted scheme has been adopted, focal deposits, known as drusen, and diffuse deposits have been described. Ultrastructurally, five types of drusen have been defined,[2] all of which, by definition, occur beneath the basement membrane of the RPE cell. Their biogenesis has been reviewed recently by Hageman et al.[3] The two principal types of diffuse deposit described are basal laminar deposit (BLamD) and basal linear deposit (BLinD).[4] BLamDs are composed of granular and amorphous material, usually with embedded banded structures. These banded assemblies are composed of a series of double bands (30 nm apart) that are repeated axially approximately every 100 nm. Axial filaments traversing these bands are also present. The precise composition and origin of BLamD has not been elucidated, although it has been suggested that it is a form of fibrous long-spacing collagen.[5,6] BLamD normally forms under the RPE plasma membrane and internal to its basement membrane, although similar material, including banded assemblies, appear throughout the layers of Bruch's membrane. BLinD is located just external to the basement membrane of the RPE, in the inner collagenous zone of Bruch's membrane and consists primarily of membrane-bound structures and vesicular material. It has been suggested that BLinD is a relatively specific indicator of AMD and not simply a nonspecific manifestation of aging.[7] BLinD contains esterified and unesterified cholesterol, neutral lipids, and phospholipids.[8]

Sub-RPE deposits have been implicated as either the cause or a consequence of AMD.[9–16] It may be that sub-RPE deposits are best regarded as an aging phenomena that is exaggerated in AMD,[5] but even so it remains possible that prevention or modification of these deposits may provide a novel therapeutic approach to AMD. Certainly, there is a need to develop therapies that act to prevent the development of the sight-threatening complications of choroidal neovascularization and geographic atrophy.

Although animal models exist for retinal degenerations and some aspects of AMD, such as subretinal neovascularization,[17] few such models exist for sub-RPE deposit formation (see Note Added in Proof). We therefore sought to develop an in vitro

system in which to study the pathogenesis of sub-RPE deposit formation. We also sought to begin an investigation of the mechanisms of deposit formation and breakdown by challenging the RPE cells in a variety of ways. Our first approach evolved from the observation that sub-RPE deposits are seen in long-standing exudative retinal detachment, where the RPE cells are bathed in serum-rich fluid.[18,19] Subretinal fluid is known to contain approximately 16 g/L total protein that is predominantly albumin and immunoglobulin.[20]

Furthermore, sub-RPE deposits appear after retinal degenerations,[21] such as Sorsby's fundus dystrophy and Doyne's honeycomb dystrophy (Malattia leventinine). Finally, we have observed similar sub-RPE deposits in a young donor eye with extensive retinal destruction due to toxoplasmosis infection (Luthert PJ, unpublished observations, 1998). Therefore, we explored the effects of treating the cells with medium containing retinal homogenate.

There is increasing evidence to support the hypothesis that matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPS) play an important role in the pathogenesis in AMD (reviewed in Ref. 22). Immunohistochemistry, Western blot analysis, and reverse zymography have shown an increase in TIMP-3 protein in Bruch's membrane with age and in AMD.[23] Sorsby's fundus dystrophy is a disease caused by mutations in the TIMP-3 gene. In particular, this disease causes patients to produce BLamD in large quantities.[24] Further evidence for a potential role of MMPs was from a report of an increase in MMP-2 and MMP-9 in Bruch's membrane-choroid with age.[25] Also choroidal neovascular membrane formation in AMD is associated with an accumulation of MMP-2 and 9.[26] In this study, we altered the MMP activity by directly adding MMP-2 to the culture medium.

If formation of sub-RPE deposits is a key process in the pathogenesis of AMD, it is possible that suppression of formation or enhanced clearance of deposit may be of therapeutic benefit. It is very difficult to assess diffuse deposits clinically, but drusen are more readily seen and have been shown to regress after laser treatment.[27–29] Drusen regression occurs even remote to the site of the laser burns. The mechanism of laser-induced regression of drusen is not known, but one possibility is that it provokes a low-grade inflammatory response. As laser treatment has been shown to cause increased levels of TNF-α production[30] (Morimura Y, et al. *IOVS* 2001;42:ARVO Abstract 1218), a reduction in drusen may be associated with increased TNF-α. In addition, TNF-α is a proinflammatory cytokine and has been reported to activate MMP-2.[31,32] In the present study, we developed an in vitro RPE system that can be challenged with increased protein load, MMPs, and TNF-α to establish the effect on sub-RPE deposits with time. It is envisaged that this model will be beneficial for the investigations of the mechanisms of the formation of RPE deposits and potential strategies for inhibiting production.

METHODS

Cell Culture

All experiments were performed in triplicate, in independent assays. ARPE-19, a spontaneously arising human RPE cell line (American Type Culture Collection, Manassas, VA), was grown in DMEM F12 medium (Sigma-Aldrich, Poole, UK) supplemented with 10% (vol/vol) fetal calf serum (FCS; Invitrogen-Gibco, Paisley, UK), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen-Gibco), and 2 mM L-glutamine (Invitrogen-Gibco),[33] on collagen type I supports (ICN, Thame, UK) and on 24-well plates. Cells were counted in a hemocytometer, and each membrane insert or well was seeded with 1×10^5 cells. The culture medium was changed two times per week. The culture model was initially characterized in separate experiments, at all time periods examined and with the various treatments, according to the number of cells and cell morphology. Studies were performed after 5, 7, and 11 weeks of culture to ensure a stable number of cells. Confluence was assessed in simultaneous cultures by counting the number of cells after fixation and nuclear staining with propidium iodide (50 μg/mL; Sigma-Aldrich).

Treatment of RPE with Protein and Retinal Homogenate

To test the hypothesis that increasing protein concentration within the culture medium promotes formation of deposits, we grew cells in 3%, 10%, or 20% (vol/vol) FCS (Sigma-Aldrich) or with bovine serum albumin (0.42% wt/vol 10% normal culture medium; Sigma-Aldrich). For the retinal homogenate experiments, porcine retina was isolated fresh on the day of slaughter. After homogenization by trituration for 2 minutes using a 10-mL disposable pipette (Falcon, Liverpool, New South Wales, Australia), the homogenate was snap frozen in penicillin-streptomycin solution and stored at −70°C. Each retina was suspended in 20 mL of medium with 10% (vol/vol) FCS. Cells were treated for 5 days before analysis.

Treatment of RPE with TNF-α and MMP-2

Cells that had been grown on membrane supports and exposed to 5 days of treatment with retinal homogenate were then cultured in serum-free medium for 48 hours. RPE monolayers were then treated with TNF-α (10 or 40 ng/mL; R&D Systems, Minneapolis, MN) or MMP-2 (1 or 70 ng/mL; R&D Systems) for a period of 48 hours in serum-free medium. Control samples were treated with serum-free medium alone. The cells were fixed in situ and processed for electron microscopy (EM). The tissue culture supernatant was collected for zymography.

Electron Microscopy

Cells were fixed with 2.5% (vol/vol) glutaraldehyde and 0.5% (wt/vol) tannic acid (Sigma-Aldrich), buffered to pH 6.9 with 0.07 M sodium cacodylate-HCl. After 12 hours, the cells were washed three times with

TABLE 1. Morphological Classification of Sub-RPE Deposits Formed In Vitro

Deposit Type	Abbreviation	Description
Fibrillar	Fib	Electron loose, the most frequently found deposit, composed mainly of filamentous material
Condensed	Con	Dense granular or closely knit fibrillar material with no space component
Banded	Band	Clusters of banded material with approximately 100-nm periodicity
Membranous	Mem	Bilayered membrane-like circular or oval vesicular structures
Space/no deposit	Space	Any area without deposit between the cell plasma membrane and the substrate

Abbreviations coincide with those used in Figures 2–6.

PBS, osmicated for 1 hour with a 1% (vol/vol) aqueous solution of osmium tetroxide and dehydrated through ascending concentrations of alcohol (50%–100%, 10 minutes per step). After four changes of 100% ethanol, wells containing the cells were filled with Araldite resin, which was cured overnight at 60°C. Random semithin and ultrathin sections were cut with a microtome (Ultracut S; Leica, Cambridge, UK) fitted with a diamond knife. After contrasting with 1% uranyl acetate and lead citrate, thin sections were viewed and photographed at ×10,000 magnification (1010 TEM; JEOL, Tokyo, Japan). Images were selected at low magnification where three grid-squares in sequence were adequately visible and the RPE cell well was apposed to the substrate. Three images were obtained of each ultrathin section. Negatives were developed on paper electron microscopy film (8.3 × 10.2-cm; 4489; Eastman Kodak, Rochester, NY; printed on 10 × 8-in. multigrade paper; Ilford, Basildon, UK). Tilting of the microscope stage was not routinely performed on specimens examined for morphometry; however, two examples of condensed deposits with a suspicion of banding were tilted to demonstrate the relationship between the condensed deposits and banded material (1200 EX microscope; JEOL). All chemicals were purchased from Agar Scientific Ltd. (Stansted, UK) unless stated otherwise.

Ultrastructural Assessment of Sub-RPE Deposits

Sub-RPE deposits were assessed in photomicrographs, using a systematic random sampling scheme, validated by analysis of intraobserver and interobserver variability (data not shown). The deposits were classified into a number of subtypes, as described in Table 1 and illustrated in Figure 1.

Three micrographs were analyzed per membrane and the area fraction of deposit type was estimated by using a point-counting technique[34–36] with a 5 × 5-mm square sampling grid. Taking area estimates and dividing by the length of the sub-RPE space sampled generated an estimate of equivalent deposit thickness in nanometers. The total amount of deposit formed was calculated by summing the fibrillar, condensed, banded, and membranous components.

Gelatin Zymography

Supernatants from the cultures, grown on membrane inserts, were denatured with an equal volume (15 μL) of dissociating buffer (70 mM Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 2% [wt/vol] sodium dodecyl sulfate, 0.0025% [vol/vol] bromophenol blue; Novex; Invitrogen-Gibco) for 10 minutes at room temperature. Standardization was achieved by using identical volumes of medium through all steps. The samples were then resolved on a 10% (vol/vol) Tris-glycine polyacrylamide gel (Novex; Invitrogen-Gibco) containing 0.1% (wt/vol) gelatin for 90 minutes with constant 125 V voltage and 40 mA current, within running buffer (25 mM Tris base, 192 mM glycine, 0.1% [wt/vol] sodium dodecyl sulfate, pH 8.3; Novex; Invitrogen-Gibco). Prestained molecular weight markers (marker range, 7,200 to 208,000; Bio-Rad, Hemel, UK) were also run with the samples. The gels were then placed in renaturing buffer (2.5% [vol/vol]) Triton X-100; Novex; Invitrogen-Gibco) with gentle agitation for 30 minutes. The renaturing buffer was removed and replaced with developing buffer (50 mM Tris base, 200 mM sodium chloride, 5 mM calcium chloride, 0.2% Brij 35; Novex; Invitrogen-Gibco) for 30 minutes, which was replaced with fresh developing buffer and incubated overnight at 37°C. The gel was stained with Coomassie blue (0.5% [wt/vol]; Bio-Rad in 45% (vol/vol) methanol, 45% (vol/vol) distilled water, and 5% (vol/vol) glacial acetic acid for 2 hours. The gel was destained (45% [vol/vol] methanol, 45% [vol/vol] distilled water, and 5% [vol/vol] glacial acetic acid) to visualize the clear bands of protease activity against the blue background.

Statistical Analysis

Data were analyzed initially with three-way ANOVA, followed by individual analysis using Student's *t*-test when two groups were compared and the Kruskal-Wallis test when three groups were compared (S-Plus ver. 4.5; statistical software; StatSci, Seattle, WA).
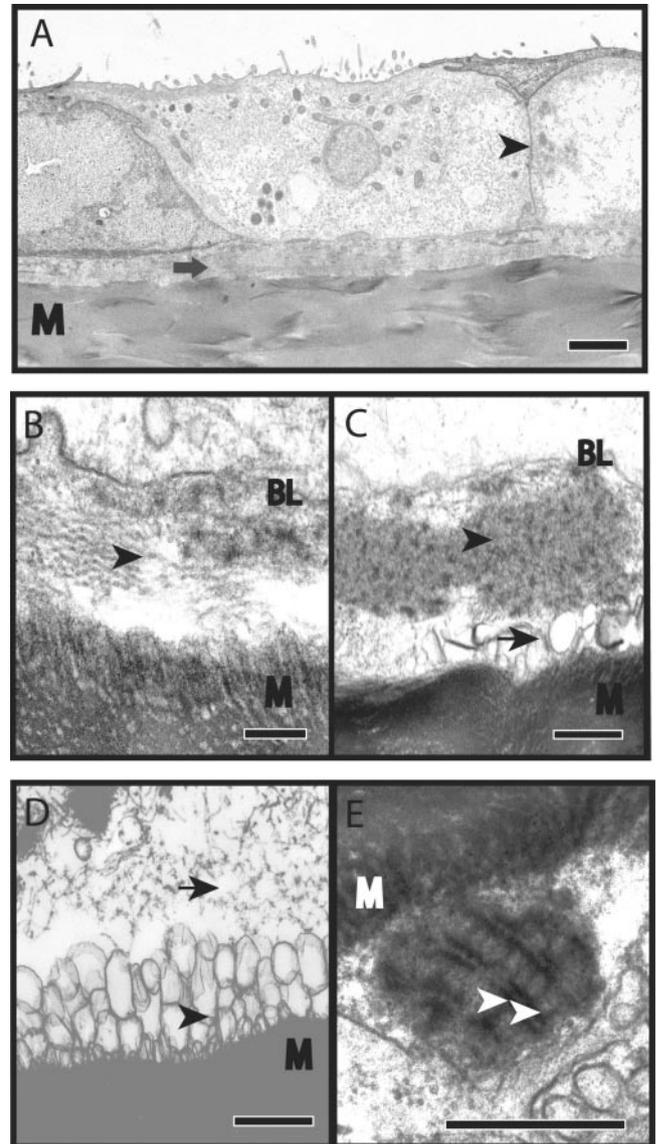

FIGURE 1. Sub-RPE deposits formed in culture (**A**) shows The RPE cell monolayer with apical microvilli and junctions (*arrowhead*). *Arrow*: ECM region between the cell basal lamina and the membrane insert. (**B**) Fibrillar deposits formed in an orderly fashion parallel to the membrane support; *arrowhead*: fibrils seen on end. (**C**) Condensed deposits with probable banding material (*arrowhead*), which became visible on rotation of the grid. (**D**) Membranous deposits (*arrowhead*); similar deposits are visible in (**C**, *arrow*). Also shown are fibrillar deposits (*arrow*). (**E**) Definite banding deposits (*arrowheads*). M, membrane support; BL, basal lamina of ARPE-19 cell. Scale bars, 500 nm.

RESULTS

Time Course of Deposit Formation

To evaluate the effects of increasing duration in culture, cells were assessed 5, 7, and 11 weeks after seeding. Ultrastructural analysis revealed that there was no change in the amount of total sub-RPE deposit present with increasing time ($P = 0.832$). However, the amount of condensed deposit increased with time in culture ($P < 0.001$). The fibrillar component also altered with time in culture, showing an increase between 5 and 7 weeks followed by a decrease from 7 to 11 weeks of culture ($P = 0.004$). The abundance of the remaining deposit
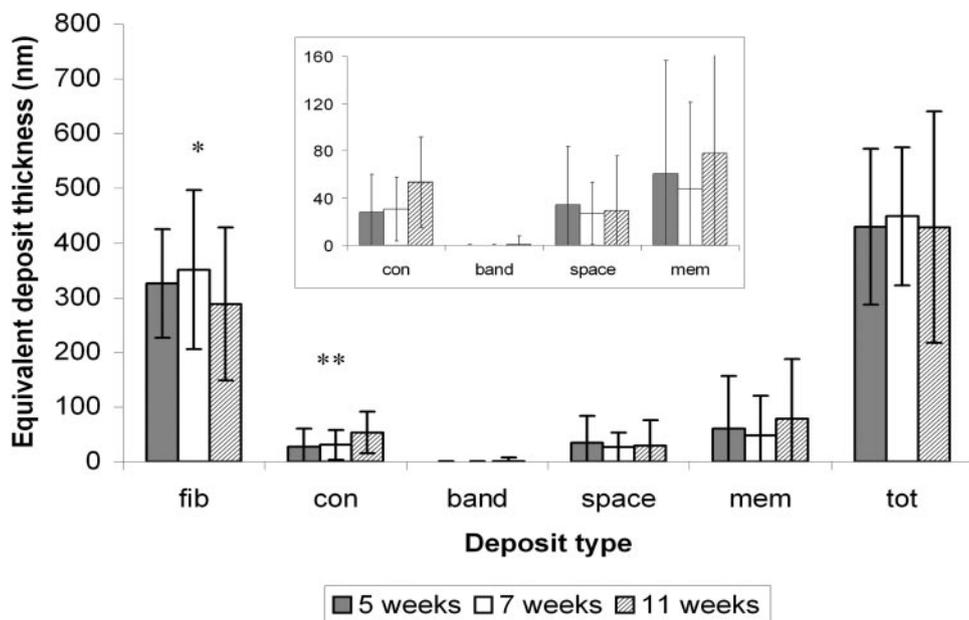
FIGURE 2. Different deposit types are compared with time in culture. Note the increasing amount of condensed deposits with time and the increase and decrease of the fibrillar deposit with time. *Inset*: condensed, band, space, and membranous components at higher magnification. Error bars, mean ± SD. Fib, fibrillar deposit; con, condensed deposit; band, banded; space, space component; mem, membranous; tot, total deposit (*P < 0.005; **P < 0.001).

types did not alter with time in culture (Fig. 2; Kruskal-Wallis test). Two examples of condensed deposit were tilted in the electron microscope, demonstrating a definitive banding pattern with a periodicity equivalent to that in vivo.

Effect of Challenging ARPE-19 Cells with FCS, Albumin, and Retinal Homogenate

Cells incubated with 3%, 10%, and 20% (vol/vol) FCS at 5, 7, and 11 weeks of culture showed no change in the total deposit formation ($P = 0.205$), or in amounts of individual subtypes of deposit (Fig. 3; Kruskal-Wallis test). In contrast, albumin challenge at the same time points resulted in a significant increase in the total amount of deposit formed ($P = 0.019$), which was due to a significant increase in the membranous deposits ($P = 0.001$). The other deposit types were not significantly altered with the addition of albumin to the media (Fig. 4; Student's

t-test). As with albumin, the addition of retinal homogenate to cultures grown for 5, 7, and 11 weeks, resulted in an increased amount of total deposit ($P = 0.024$). This increase was due to significant increases in the condensed ($P < 0.001$) and banded ($P = 0.015$) types. Fibrillar and membranous deposits were not altered significantly. The amount of space within the sub-RPE region was also found to increase significantly ($P < 0.001$; Fig. 5; Student's *t*-test).

Effect of Tissue Culture Substrate

Comparison of sub-RPE deposits between cells grown on a permeable membrane insert or tissue culture plastic revealed a significantly different pattern of deposition for total deposit ($P < 0.001$), with more condensed ($P < 0.001$) and membranous deposits ($P < 0.001$) forming in cultures grown on the membrane inserts (Fig. 6; Student's *t*-test).
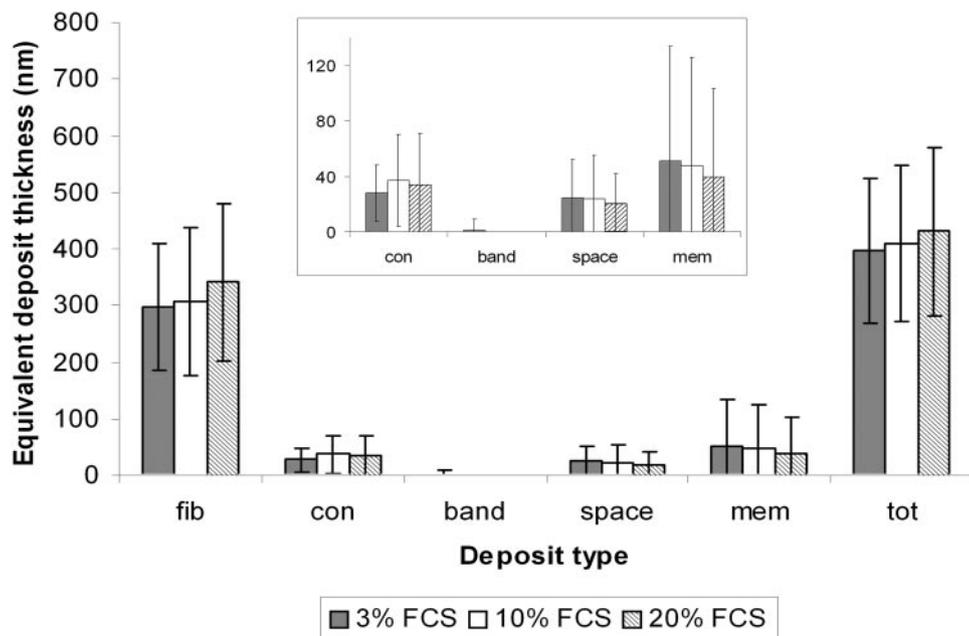

FIGURE 3. The effect of alterations in the concentration of FCS used in the medium. Cultures were grown for 5, 7, and 11 weeks and averaged. Changes in the amount of FCS used in the medium caused no significant difference in the thicknesses of any of the deposit types. Error bars, mean ± SD. The *inset* and abbreviations are as described in Figure 2.
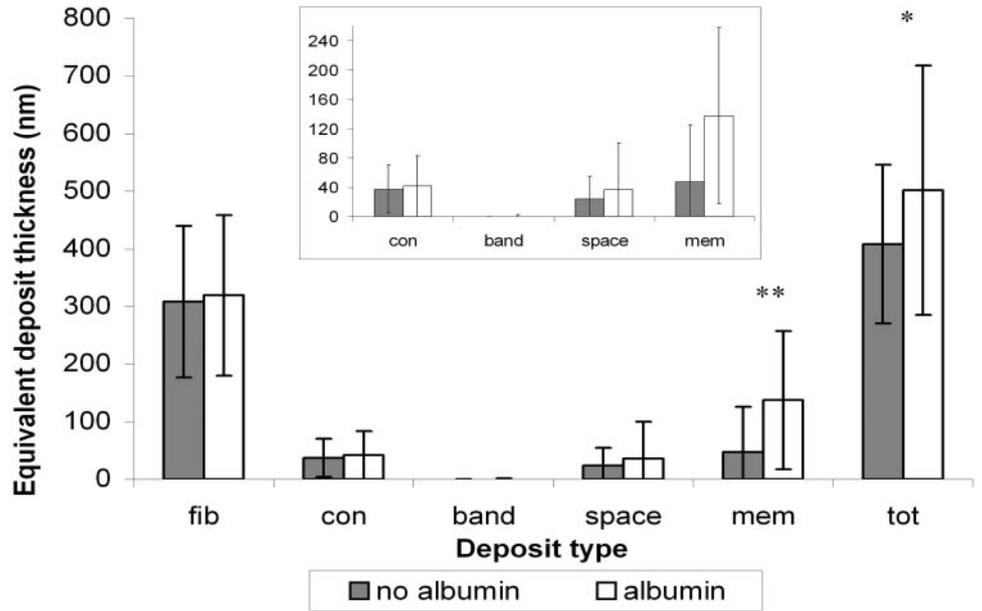
FIGURE 4. The effect of albumin on deposit formation in cultures grown for 5, 7, and 11 weeks and averaged. Error bars, mean ± SD. The *inset* and abbreviations are as described in Figure 2 (**$P < 0.005$; *$P < 0.025$).

Effect of TNF-α or MMP-2

Cells treated with TNF-α or MMP-2 showed a dramatic reduction in the amount of sub-RPE deposit ($P = 0.004$ and 0.035, respectively; ANOVA), involving all the various deposit subtypes (data not shown). There was an apparent dose–response relationship with TNF-α. Low doses of MMP-2 (1 ng/mL) reduced sub-RPE deposit in 7- and 11-week cultures but not in 5-week cultures (Fig. 7). The higher dose of MMP-2 (70 ng/mL) reduced the deposits at all time points examined.

MMP Production

Unchallenged ARPE-19 cells grown for 7 weeks predominantly produced MMP-2, which was reduced after treatment with retinal homogenate. TNF-α challenge caused a similar reduction in MMP-2 production but also significantly increased MMP-9 production. Adding MMP-2 did not markedly alter the balance of active MMP at either dose used (Fig. 8).

DISCUSSION

In this study we explored the formation of sub-RPE deposits in a tissue culture system. The simplicity of such a system makes it possible to demonstrate that RPE cells are capable of forming many of the components of diffuse deposits under the RPE and in Bruch's membrane that occur with aging and in AMD. It also makes it possible to manipulate the formation of deposits with a view toward understanding their pathogenesis. Such an understanding may lead to new strategies for the treatment of AMD in its early stages, before sight-threatening complications such as geographic atrophy and choroidal neovascularization develop.

A particularly striking component of the sub-RPE deposits, both in vivo and in the study reported herein, is the material with electron-dense banding at a periodicity of approximately 100 nm. It remains a matter of debate whether this banded material is central to the pathogenesis of AMD, but several
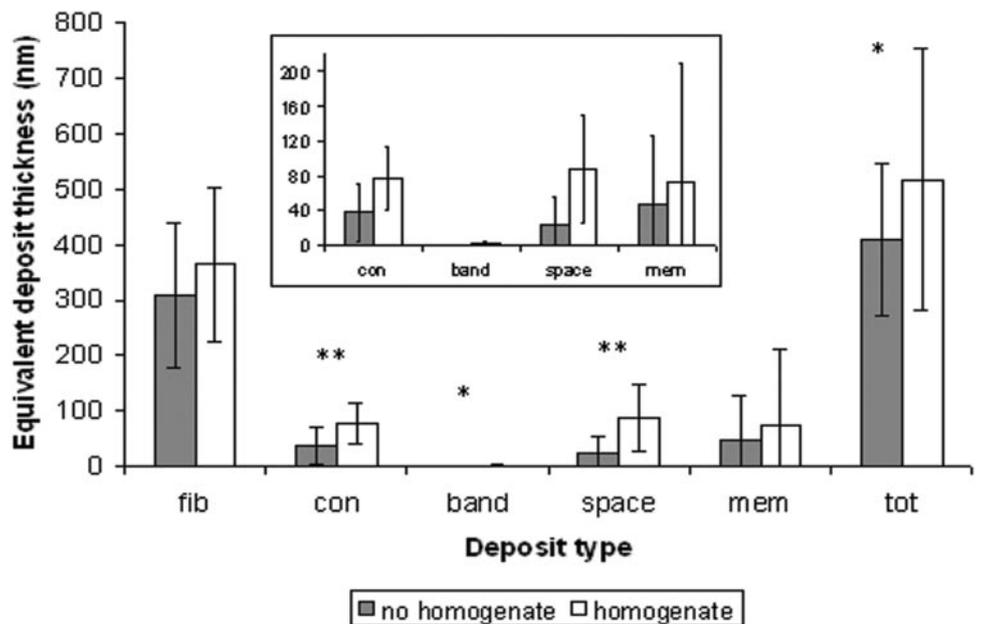

FIGURE 5. Porcine retinal homogenate challenge increased thicknesses in all types of deposits described at all time points examined (average of 5, 7, and 11 weeks). Error bars, mean ± SD. The *inset* and abbreviations are as described in Figure 2 (*$P < 0.025$, **$P < 0.001$).
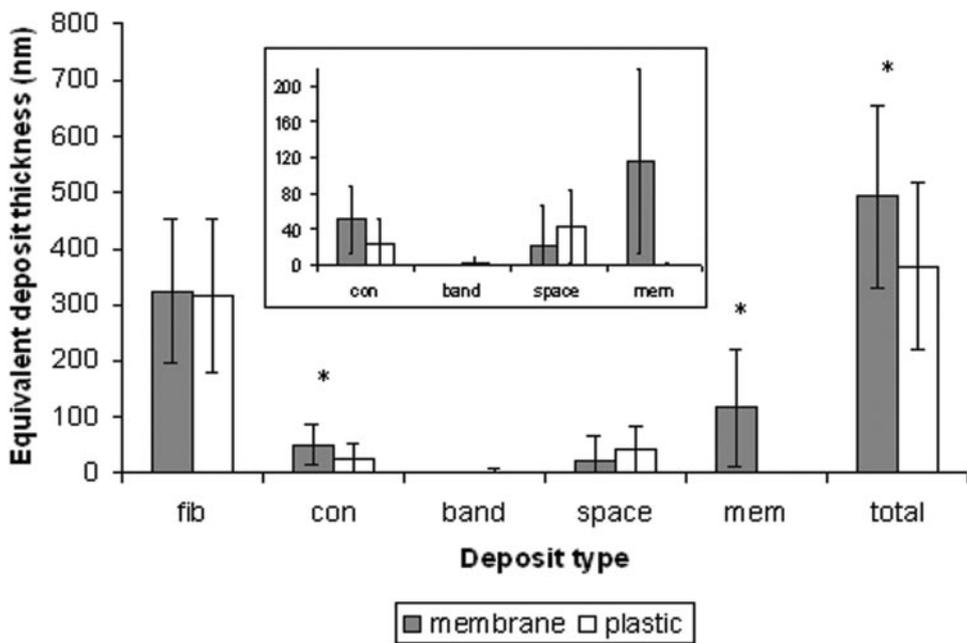
FIGURE 6. Comparison of thicknesses of deposit types when cells were grown on collagen membranes versus tissue culture plastic at all time points examined (an average of 5, 7, and 11 weeks). Error bars, mean ± SD. The *inset* and abbreviations are as described in Figure 2 (*$*P < 0.001$).

research groups have noted its association with disease.[10–13,15,16] In some areas the banded material was closely associated with amorphous looking electron-dense material, which we call condensed deposit, and from the results in the current study, it is clear that such material viewed with the correct orientation may also contain banded structures. It seems likely, therefore, that condensed and banded materials are related. This possibility is strengthened by the observation that condensed material can appear banded under certain culture conditions in cultures of rat skin and mouse corneal and trabecular meshwork cultures.[37,38] Of note, from 7 to 11 weeks, the amount of fibrillar material decreased, whereas condensed material increased and banded material was visible without tilting the specimen. This observation is consistent with the notion that fibrillar material coalesces to form a condensed deposit, although further studies are needed for clarification. At present, the composition of banded material is

unclear. It has recently been shown that the banding pattern periodicity is the same as that of polymerized type VI collagen,[39] and similar analysis of the in vitro deposits shows the same pattern (Knupp C, Amin S, unpublished observations, 2002). Various studies have shown that RPE cells can form collagen types I through IV,[40–43] but there appears to be no clear demonstration by immunochemical or biochemical techniques of type VI collagen in the sub-RPE space. It is important to note that type VI collagen has been demonstrated in vivo to be present on the outer aspect of the choriocapillaris, with a possible role in anchoring the choriocapillaris to the larger choroidal vessels.[44]

The other distinct form of deposit consisted of circular membranous profiles that lay in a relatively ordered fashion adjacent to the RPE plasma membrane and in many ways were similar to the BLinD noted in studies of the ultrastructure of aging and AMD-affected eyes. This evidence suggests that cul-
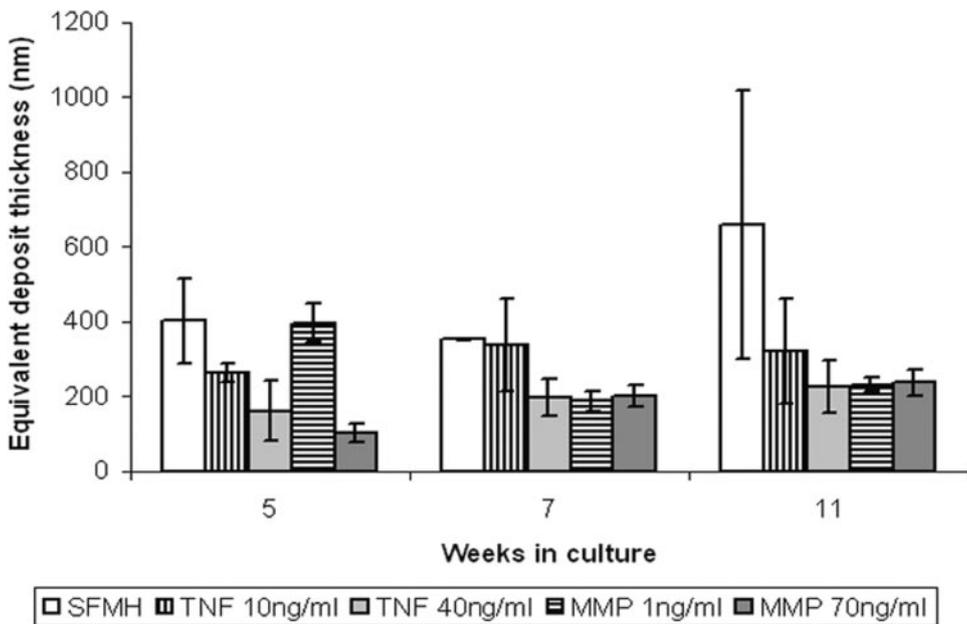

FIGURE 7. Effect of TNF-α and MMP-2 on the total thickness of deposit compared with the effect of no treatment and serum-free medium with retinal homogenate (SFMH). A deposit subtype analysis demonstrated that all deposit types were affected (data not shown). MMP-2, at a concentration of 70 ng/mL, reduced the total amount of deposit formed at all time points, whereas at the lower dose of 1 ng/mL it affected 7- and 11-week cultures only. TNF-α also reduced the total amount of deposit formed at all time points, but the lower dose had a reduced effect ($P = 0.004$ for TNF-α and 0.035 for MMP-2; ANOVA). Error bars, mean ± SD.

FIGURE 8. Zymography of tissue culture supernatants (grown for 7 weeks) after treatment with TNF-α or MMP-2. T40, TNF-α 40 ng/mL; T10, TNF-α 10 ng/mL; SFH, serum-free medium with retinal homogenate; SF, serum-free medium; M1, MMP-2 1 ng/mL; and MMP-70, MMP-2 70 ng/mL.

tured RPE cells, in isolation, are capable of making the major categories of deposit in AMD. What was not observed was any structures reminiscent of drusen. This may be due to the culture conditions, the relatively short duration of cell cultures and an absent basement membrane. More likely, however, is that drusen formation requires coordinated interaction with other cell types. For instance, Hageman et al. have demonstrated that many drusen have dendritic cell processes within their cores,[3] and such cells were not included in our study.

Exposing the ARPE-19 cells to different culture conditions modulated the abundance of different deposit types. Although altering the concentration of FCS had little effect, the inclusion of a relatively high concentration of albumin in the culture medium dramatically increased the membranous material. The mechanism by which this occurred is unclear, and further studies are needed to clarify the precise composition of the membranous debris.

Treating the cells with retinal homogenate was intended to recreate the situation that occasionally occurs in eyes in which retinal destruction is associated with sub-RPE deposit formation. After challenge with retinal homogenate, condensed and banded deposits increased, and this was also reflected in the total amount of deposit. Although the absolute amount of extra deposit formed was relatively small, the duration of exposure was only 5 days, a very short time in relation to the rate of evolution of early disease in AMD. An increased amount of deposit could arise from increased production, decreased degradation, or a combination of the two. Our studies also show that homogenate treatment can lower the activity of MMP-2 and that MMP-2 added to the culture medium enhances removal of the sub-RPE deposit, suggesting that it is a substrate for MMP-2. It is therefore possible that challenge with retinal homogenate leads to an increase in deposit by reducing MMP-2 activity, although, clearly, other factors may also be involved. Retinal homogenate treatment also significantly increased the area under the sub-RPE where no deposit was seen (space component). In addition, it was consistently observed that cultures challenged with retinal homogenate detached from their substrate more readily than did the cultures not challenged with retinal homogenate. Preliminary experiments to quantify this relative difference in attachment corroborated the initial observation (data not shown). Pigment epithelial detachments are a common manifestation of AMD and may represent changes in attachment of the RPE to Bruch's membrane.[45] Further investigation of this reduction of adhesion in the model system on treatment with retinal homogenate may come to represent a forme fruste of serous RPE detachments.

To explore directly the role of MMP-2, this metalloproteinase was added to the culture medium. We found that the amount of deposit present was reduced, and from the zymography data it was clear that the addition of MMP-2 increased the amount of active MMP-2 in the culture system, independent of the dose. The level of active MMP-2 production was not substantially above the level recorded in cells treated with serum-

free medium alone. This implies that there is endogenous activation of MMP-2 in the culture system used; however, this may not be the case in vivo. In addition, MMP-2 activity was not increased after TNF-α treatment, and so there appears to be at least two pathways by which sub-RPE deposits may be cleared.

It is of interest that TNF-α application led to the induction of active MMP-9 and a reduction in the amount of deposit. It is not known how laser treatment leads to the clearance of drusen, but given that laser therapy has been reported to increase TNF-α expression, it now appears that downstream increased production of active MMP-9 is one possibility. A recognized complication of laser treatment is the formation of a choroidal neovascular membrane,[46] presumably due to damage to Bruch's membrane. Pharmacological approaches would be expected to avoid this complication.

In conclusion, it is possible to create sub-RPE deposits in vitro and to use this model system to manipulate the amount of deposit present to gain insights into the pathogenesis of deposit formation. From the findings in the present study, it appears that MMP-2 and MMP-9 expression or function may be of importance. It is possible that subtle modulation of MMP activity in vivo will provide an opportunity to promote deposit clearance and prevent disease progression.

Note Added in Proof

Since the original preparation of this manuscript we have become aware of several animal models of sub-RPE deposit formation.

Cousins SW, Marin-Castano ME, Espinosa-Heidmann DG, Alexandridou A, Striker L, Elliot S. Female gender, estrogen loss, and Sub-RPE deposit formation in aged mice. *Invest Ophthalmol Vis Sci.* 2003;44:1221–1229.

Espinosa-Heidmann DG, Sall J, Hernandez EP, Cousins SW. Basal laminar deposit formation in APO B100 transgenic mice: complex interactions between dietary fat, blue light, and vitamin E. *Invest Ophthalmol Vis Sci.* 2004;45:260–266.

Kliffen M, Lutgens E, Daemen MJ, de Muinck ED, Mooy CM, de Jong PT. The APO(*)E3-Leiden mouse as an animal model for basal laminar deposit. *Br J Ophthalmol.* 2000;84:1415–1419.

Marneros AG, Keene DR, Hansen U, et al. Collagen XVIII/endostatin is essential for vision and retinal pigment epithelial function. *EMBO J.* 2004;23:89–99.

Rakoczy PE, Zhang D, Robertson T, et al. Progressive age-related changes similar to age-related macular degeneration in a transgenic mouse model. *Am J Pathol.* 2002;161:1515–1524.

References

1. Evans J, Wormald R. Is the incidence of registrable age-related macular degeneration increasing? *Br J Ophthalmol.* 1996;80:9–14.
2. Hageman GS, Mullins RF. Molecular composition of drusen as related to substructural phenotype. *Mol Vis.* 1999;5:28.
3. Hageman GS, Luthert PJ, Chong NHV, Johnson LV, Anderson DH, Mullins RF. An integrated hypothesis that considers drusen as biomarkers of immune- mediated processes at the RPE-Bruch's membrane interface in aging and age-related macular degeneration. *Prog Retin Eye Res.* 2001;20:705–732.
4. Loeffler KU, Lee WR. Terminology of sub-RPE deposits: do we all speak the same language? *Br J Ophthalmol.* 1998;82:1104–1105.
5. van der Schaft TL, de Bruijn WC, Mooy CM, Ketelaars DA, de Jong PT. Is basal laminar deposit unique for age-related macular degeneration? *Arch Ophthalmol.* 1991;109:420–425.

6. van der Schaft TL, Mooy CM, de Bruijn WC, Bosman FT, de Jong PT. Immunohistochemical light and electron microscopy of basal laminar deposit. *Graefes Arch Clin Exp Ophthalmol*. 1994;232:40–46.

7. Curcio CA, Millican CL. Basal linear deposit and large drusen are specific for early age- related maculopathy. *Arch Ophthalmol*. 1999;117:329–339.

8. Pauleikhoff D, Sheraidah G, Marshall J, Bird AC, Wessing A. Biochemical and histochemical analysis of age related lipid deposits in Bruch's membrane. *Ophthalmologe*. 1994;91:730–734.

9. Vinding T. Occurrence of drusen, pigmentary changes and exudative changes in the macula with reference to age-related macular degeneration: an epidemiological study of 1000 aged individuals. *Acta Ophthalmol (Copenh)*. 1990;68:410–414.

10. Green WR, Enger C. Age-related macular degeneration histopathologic studies. The 1992 Lorenz E. Zimmerman Lecture. *Ophthalmology*. 1993;100:1519–1535.

11. Kliffen M, van der Schaft TL, Mooy CM, de Jong PT. Morphologic changes in age-related maculopathy. *Microsc Res Tech*. 1997;36:106–122.

12. Loffler KU, Lee WR. Basal linear deposit in the human macula. *Graefes Arch Clin Exp Ophthalmol*. 1986;224:493–501.

13. Sarks SH. Ageing and degeneration in the macular region: a clinicopathological study. *Br J Ophthalmol*. 1976;60:324–341.

14. Spraul CW, Lang GE, Grossniklaus HE, Lang GK. Histologic and morphometric analysis of the choroid, Bruch's membrane, and retinal pigment epithelium in postmortem eyes with age-related macular degeneration and histologic examination of surgically excised choroidal neovascular membranes. *Surv Ophthalmol*. 1999;44(suppl 1):S10–S32.

15. Spraul CW, Grossniklaus HE. Characteristics of Drusen and Bruch's membrane in postmortem eyes with age-related macular degeneration. *Arch Ophthalmol*. 1997;115:267–273.

16. Spraul CW, Lang GE, Grossniklaus HE, Lang GK. Characteristics of drusen and changes in Bruch's membrane in eyes with age-related macular degeneration: histological study. *Ophthalmologe*. 1998;95:73–79.

17. Chader GJ. Animal models in research on retinal degenerations: past progress and future hope. *Vision Res*. 2002;42:393–399.

18. Damato BE, Foulds WS. Tumour-associated retinal pigment epitheliopathy. *Eye*. 1990;4:382–387.

19. Ulbig MR, Riordan-Eva P, Holz FG, Rees HC, Hamilton PA. Membranoproliferative glomerulonephritis type II associated with central serous retinopathy. *Am J Ophthalmol*. 1993;116:410–413.

20. Berrod JP, Kayl P, Rozot P, Raspiller A. Proteins in the subretinal fluid. *Eur J Ophthalmol*. 1993;3:132–137.

21. Kuntz CA, Jacobson SG, Cideciyan AV, et al. Sub-retinal pigment epithelial deposits in a dominant late-onset retinal degeneration. *Invest Ophthalmol Vis Sci*. 1996;37:1772–1782.

22. Sethi CS, Bailey TA, Luthert PJ, Chong NH. Matrix metalloproteinase biology applied to vitreoretinal disorders. *Br J Ophthalmol*. 2000;84:654–666.

23. Kamei M, Hollyfield JG. TIMP-3 in Bruch's membrane: changes during aging and in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 1999;40:2367–2375.

24. Weber BH, Vogt G, Pruett RC, Stohr H, Felbor U. Mutations in the tissue inhibitor of metalloproteinases-3 (TIMP3) in patients with Sorsby's fundus dystrophy. *Nat Genet*. 1994;8:352–356.

25. Guo L, Hussain AA, Limb GA, Marshall J. Age-dependent variation in metalloproteinase activity of isolated human Bruch's membrane and choroid. *Invest Ophthalmol Vis Sci*. 1999;40:2676–2682.

26. Steen B, Sejersen S, Berglin L, Seregard S, Kvanta A. Matrix metalloproteinases and metalloproteinase inhibitors in choroidal neovascular membranes. *Invest Ophthalmol Vis Sci*. 1998;39:2194–2200.

27. Choroidal Neovascularization Prevention Trial Research Group. Laser treatment in eyes with large drusen: short-term effects seen in a pilot randomized clinical trial. *Ophthalmology*. 1998;105:11–23.

28. Friberg TR. Laser photocoagulation of eyes with drusen: will it help? *Semin Ophthalmol*. 1999;14:45–50.

29. Folk JC, Russell SR. Can laser photocoagulation of eyes with high-risk drusen prevent vision loss from age-related macular degeneration? *Ophthalmology*. 1999;106:1241–1242.

30. Bradley JM, Anderssohn AM, Colvis CM, et al. Mediation of laser trabeculoplasty-induced matrix metalloproteinase expression by IL-1beta and TNFalpha. *Invest Ophthalmol Vis Sci*. 2000;41:422–430.

31. Han YP, Tuan TL, Wu H, Hughes M, Garner WL. TNF-alpha stimulates activation of pro-MMP2 in human skin through NF-(kappa)B mediated induction of MT1-MMP. *J Cell Sci*. 2001;114:131–139.

32. Gearing AJ, Beckett P, Christodoulou M, et al. Processing of tumour necrosis factor-alpha precursor by metalloproteinases. *Nature*. 1994;370:555–557.

33. Dunn KC, Aotaki-Keen AE, Putkey FR, Hjelmeland LM. ARPE-19, a human retinal pigment epithelial cell line with differentiated properties. *Exp Eye Res*. 1996;62:155–169.

34. Dithmar S, Curcio CA, Le NA, Brown S, Grossniklaus HE. Ultrastructural changes in Bruch's membrane of apolipoprotein E-deficient mice. *Invest Ophthalmol Vis Sci*. 2000;41:2035–2042.

35. Yucel YH, Zhang Q, Weinreb RN, Kaufman PL, Gupta N. Atrophy of relay neurons in magno- and parvocellular layers in the lateral geniculate nucleus in experimental glaucoma. *Invest Ophthalmol Vis Sci*. 2001;42:3216–3222.

36. Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the dissector. *J Microsc*. 1984;134:127–136.

37. Kajikawa K, Nakanishi I, Yamamura T. The effect of collagenase on the formation of fibrous long spacing collagen aggregates. *Lab Invest*. 1980;43:410–417.

38. Hirano K, Kobayashi M, Kobayashi K, Hoshino T, Awaya S. Experimental formation of 100 nm periodic fibrils in the mouse corneal stroma and trabecular meshwork. *Invest Ophthalmol Vis Sci*. 1989;30:869–874.

39. Knupp C, Amin SZ, Munro PM, Luthert PJ, Squire JM. Collagen VI assemblies in age-related macular degeneration. *J Struct Biol*. 2002;139:181–189.

40. Li W, Stramm LE, Aguirre GD, Rockey JH. Extracellular matrix production by cat retinal pigment epithelium in vitro: characterization of type IV collagen synthesis. *Exp Eye Res*. 1984;38:291–304.

41. Campochiaro PA, Jerdon JA, Glaser BM. The extracellular matrix of human retinal pigment epithelial cells in vivo and its synthesis in vitro. *Invest Ophthalmol Vis Sci*. 1986;27:1615–1621.

42. Newsome DA, Pfeffer BA, Hewitt AT, Robey PG, Hassell JR. Detection of extracellular matrix molecules synthesized in vitro by monkey and human retinal pigment epithelium: influence of donor age and multiple passages. *Exp Eye Res*. 1988;46:305–321.

43. Kigasawa K, Ishikawa H, Obazawa H, Minamoto T, Nagai Y, Tanaka Y. Collagen production by cultured human retinal pigment epithelial cells. *Tokai J Exp Clin Med*. 1998;23:147–151.

44. Marshall GE, Konstas AG, Reid GG, Edwards JG, Lee WR. Collagens in the aged human macula. *Graefes Arch Clin Exp Ophthalmol*. 1994;232:133–140.

45. Bird AC, Bressler NM, Bressler SB, et al. An international classification and grading system for age-related maculopathy and age-related macular degeneration. The International ARM Epidemiological Study Group. *Surv Ophthalmol*. 1995;39:367–374.

46. Choroidal Neovascularization Prevention Trial. Laser treatment in fellow eyes with large drusen: updated findings from a pilot randomized clinical trial. *Ophthalmology*. 2003;110:971–978.