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 metalloprotease (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. 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.

ARTICLES

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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 longstanding exudative retinal detachment, where the RPE cells are bathed in serum-rich fluid. Subretinal fluid is known to contain approximately 16 g/L total protein that is predominantly albumin and immunoglobulin.

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sub-RPE deposits in a young donor eye with extensive retinal destruction due to toxoplasmic infection (Luthert PJ, unpublished observations, 1998).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

There is increasing evidence to support the hypothesis that matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) play an important role in the pathogenesis of 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. 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. 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. Also choroidal neovascular membrane formation in AMD is associated with an accumulation of MMP-2 and 9. 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. 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 (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. 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). 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 × 10⁵ 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 cell was 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 cacodylate-HCl buffered to pH 7.4. The cells were then fixed with 2.5% (vol/vol) glutaraldehyde and 2% (wt/vol) osmium tetroxide in cacodylate-HCl for 1 hour at room temperature. After washing, the cells were postfixed with 1% (vol/vol) osmium tetroxide and 2% (vol/vol) potassium ferrocyanide in cacodylate-HCl for 1 hour at room temperature. Cells were then washed three times with cacodylate-HCl and dehydrated in increasing concentrations of acetone. Prior to embedding in Spurr resin, the cells were infiltrated with 1:1 Spurr resin–acetone and then with Spurr resin. Sections were cut from each sample, stained with uranyl acetate and lead citrate, and examined using an electron microscope (Philips CM12; FEI Company, Hillsboro, OR). All sections were examined at ×10,000 magnification.

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

<table>
<thead>
<tr>
<th>Deposit Type</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibrillar</td>
<td>Fib</td>
<td>Electron loose, the most frequently found deposit, composed mainly of filamentous material with no space component</td>
</tr>
<tr>
<td>Condensed</td>
<td>Con</td>
<td>Dense granular or closely knit fibrillar material with no space component</td>
</tr>
<tr>
<td>Banded</td>
<td>Band</td>
<td>Clusters of banded material with approximately 100-nm periodicity</td>
</tr>
<tr>
<td>Membranous</td>
<td>Mem</td>
<td>Bilayered membrane-like circular or oval vesicular structures</td>
</tr>
<tr>
<td>Space/no deposit</td>
<td>Space</td>
<td>Any area without deposit between the cell plasma membrane and the substrate</td>
</tr>
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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 contrast with 1% uranyl acetate and lead citrate, thin sections were viewed and photographed at \( \times 10,000 \) 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 (8.5 \( \times \) 10.2-cm; 4489; Eastman Kodak, Rochester, NY; printed on 10 \( \times \) 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 with a 5 \( \times \) 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 \( \mu \)L) of dissociating buffer (70 mM Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 2% [wt/vol] sodium dodecyl sulfate, 0.025% [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).

**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...
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).
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
research groups have noted its association with disease.\textsuperscript{10-15,16} 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.\textsuperscript{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,\textsuperscript{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,\textsuperscript{40-45} but there appears to be no clear demonstration by immunohistochemical or biochemical routines 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.\textsuperscript{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-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{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).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure7.png}
\caption{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.}
\end{figure}
The role of MMP-2 in the formation of sub-RPE deposits in AMD has been investigated. Drusen, which are a hallmark of AMD, contain dendritic cell processes within a choroidal neovascular membrane. The addition of retinal homogenate to RPE cultures resulted in an increase in the amount of deposit formed, likely due to the reduction of MMP-2 activity. This reduction in MMP-2 was confirmed by zymography and zymographic data.

**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; SF, serum-free medium with retinal homogenate; SFH, serum-free medium with retinal homogenate; M1, MMP-2 1 ng/mL, and M1-70, MMP-2 70 ng/mL.

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, 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 I, Elliot S. Female gender, estrogen loss, and Sub-RPE deposit formation in aged mice. *Invest Ophthalmol Vis Sci. 2003;44:1221–1229.*


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### References


