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Association of Nonfibrillar Amyloid Oligomers with Drusen of Aging and Age-related Macular Degeneration

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Meinen Eltern und Brüdern

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Background of the study

Age-related macular degeneration (AMD) is the leading cause of irreversible vision loss among people over 65 years in the USA and Europe. Symptomatically, AMD varies widely in severity ranging from slight distortions in mild cases to a complete loss of the central vision in the worst cases. So far treatment is limited and a promising strategy for a complete cure does not exist, mainly because the underlying cause is still unclear and remains a source of controversy.

Significant similarities between AMD and so called protein misfolding disorders (PMDs) or amyloid diseases, such as Alzheimer disease (AD), diabetes type II or Parkinson disease, led to the assumption that AMD could follow a similar pathogenic pathway that is believed to be common in all PMDs. Firstly, there is strong correlation with advancing age and secondly, but most importantly AMD and PMDs are unique in their pathological feature in respect to the abnormal formation and accumulation of deposits that compromise a mixture of numerous proteins. Since these deposits can be observed in a variety, but only in disease affected tissues, it is believed that characteristic PMD-associated protein deposits are in a cause-effect relationship or at least contribute to the irreversible degenerative process that can be seen in the disease affected tissues. For instance, the extracellular formation of protein deposits in

cortical and cerebrovascular regions in AD- patients or the intracellular formation of protein deposits in the pancreatic islets of diabetes type II-patients are strongly associated with the development of each particular disease. Similar to AD and diabetes type II, AMD is characterized through extracellular formations of protein deposits in the neural retina with increasing age and disease progress. In AMD these protein deposits are called drusen and their similarity with protein deposits of PMDs is striking in respect to their protein composition. Furthermore the close proximity of drusen to degenerated photoreceptor- and retinal-pigmented epithelium cells (RPE) indicates their significance in the pathogenesis of AMD in a similar way as it has been proposed for protein deposits in PMDs.

Recent data from biochemical and pathological studies indicated that the underlying cause for PMDs could be the result of an in vivo occurring protein misfolding process. In vitro studies demonstrated that this process could be described in a simplifying form as a multi-step cascade in which a specific disease-related protein (e.g. Aβ-peptide in AD, IAPP-peptide in diabetes type II) undergoes a conformational reorganization from a monomeric into an oligomeric structure that ultimately converts into an fibrillar structure. Eventually these fibrils are observed in form of 6- to 10 nm filaments as the essential element within the PMD-associated protein deposits and therefore represent the common hallmark of all PMDs. For that reason amyloid fibrils have been so far the focus of research for many years concerning their contribution to the pathogenesis of PMDs. However, recent compelling pathological and biochemical data led to a

paradigm shift supporting the hypothesis that not the soluble monomers or insoluble fibrils but their oligomeric cascade predecessors are the primary underlying cause for tissue degeneration in PMDs. In vitro and in vivo studies demonstrated that amyloid oligomers, which are described as 3 to 10 nm spherical particles, have the striking ability to permeabilize cell membranes, penetrate into cells, disrupt intracellular membranes and eventually lead to cell death. So far the toxic mechanism of oligomers is unknown and under further investigation, but recent animal models support the idea of oligomers as an underlying cause in the pathogenesis of PMDs, since their occurrence in disease-affected tissues correlates much better with emerging clinical symptoms and degenerative features than with amyloid fibrils, which can be seen mostly post-symptomatically excluding their possibility to be a degenerative causing factor.

Despite all similarities with PMDs, AMD has been so far not accepted as a "classical" PMD, mainly because drusen do not exhibit amyloid fibrils and amyloid fibrils are, as mentioned above, the common hallmark of PMDs and are the only accepted indication for the presence of a protein misfolding process. However, recent in vitro studies demonstrated that a protein misfolding process does not inevitable lead to the formation of fibrils suggesting that pathogenic oligomers might be present within the disease-affected tissue even without accumulations of PMD characteristic amyloid fibrils. Recent evidence suggests that desmin-related cardiomyopathy may be such a disease. These observations raised the questions if AMD might be such a "non-classical" PMD in which toxic

oligomers are present in the absence of non-toxic fibrils. Therefore the goal of this study was to investigate: (i) the presence and the morphology of oligomers in the neural retina of AMD affected and non-AMD affected eyes (ii) the presence of oligomers in respect to drusen, RPE – and photoreceptor cells that are the main affected cells in AMD and (iii) the toxic effect of a variety of oligomers towards RPE cells in vitro.

For this purpose we took advantage of a recently developed conformation dependent "anti-oligomer" antibody that recognizes specifically oligomers of numerous disease-associated proteins, such as the aβ-peptide of AD, α-synuclein protein of PD or the IAPP peptide of diabetes type II. Furthermore this antibody inhibits the toxic effect of oligomers, suggesting that the "anti-oligomer" antibody indeed recognizes toxic oligomers.

Through a variety of methods, such as confocal immunofluorescence microscopy, electron microscopy, enzyme-linked immunosorbent assays and MTT based toxicity assays we made following observations: (i) oligomers are present in form of 100 nm small vesicles in the neural retina of AMD affected donor-eyes, but not in AMD unaffected eyes (ii) oligomers are located as well within drusen as in close proximity to RPE cells, but not to photoreceptor-cells (iii) oligomers of numerous disease associated proteins are indeed toxic to RPE cells and their toxicity can be inhibited by the anti-oligomer antibody.

In summary our study suggests the significance of oligomers in the pathogenesis of AMD and indicates that AMD shares commonalities with other PMDs in respect to protein missfolding.

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Drusen deposits associated with aging and age-related macular degeneration contain nonfibrillar amyloid oligomers

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Nonstandard abbreviations used:

Aß, amyloid ß; AD, Alzheimer disease; AMD, age-related macular degeneration; Bm, Bruch membrane; IAPP, islet amyloid polypeptide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; PD, Parkinson disease; RPE, retinal pigmented epithelium.

Introduction

The formation of insoluble extracellular deposits consisting of misfolded, aggregated protein is a hallmark of many neurodegenerative diseases. Extracellular deposits are also present in aging human eyes and in eyes afflicted by age-related macular degeneration (AMD). These deposits, called drusen, are found beneath the basement membrane of the retinal pigmented epithelium (RPE) and the inner collagenous layer of the Bruch membrane (Bm). Despite the well-established correlation between the presence of drusen and AMD, the underlying cause of drusen formation and its role in RPE and photoreceptor cell degeneration are not fully understood (1-3).

Recent evidence suggests that drusen formation and AMD share some similarities with amyloid diseases such as Alzheimer disease (AD) and Parkinson disease (PD). Like AMD, amyloid diseases are strongly correlated with advancing age and the formation of deposits. Moreover, these amyloid deposits contain a wide range of lipids and proteins, many of which are also present in drusen. Shared components of amyloid deposits and drusen include proteins such as vitronectin, amyloid P, apolipoprotein E, and even the amyloid ß (Aß) peptide that is associated with amyloid plaques in AD (4-6). In humans, the *APOE*4* allele shows a strong positive association with AD. Interestingly, expression of the *APOE*4*

allele in transgenic mice leads to ocular changes that mimic the pathology associated with human AMD (7). In addition, acute phase reactants, complement components, immune modulators, and other inflammatory mediators are present in amyloid deposits as well as in drusen, suggesting a possible common role for the inflammatory pathway in AMD and amyloid diseases (8-10). It is particularly noteworthy that the presence of complement components such as C5, C5b9 and C3 fragments had been observed in drusen of varying sizes and shapes, from small, "hard" drusen to large, "soft" drusen, in aging eyes as well as in AMD eyes (9-11). These observations are consistent with the idea that complement activation may be involved in drusen biogenesis. Together with the recent discovery that a polymorphism in complement factor H increases the risk factor of AMD (12-15), substantial attention is now focused on the role of inflammation in the pathogenesis of this disease.

Despite the shared similarities mentioned above, AMD has thus far not been classified as an amyloid disease. Among the principal differences is the fact that classical amyloid diseases typically exhibit large amounts of amyloid fibrils (16). For example, in the case of AD, the characteristic plaques consist primarily of fibrillar Alzheimer Aß peptide, while the Lewy bodies found in PD are abundant in α-synuclein fibrils. These amyloid fibrils are elongated, 6- to 15-nm-wide rod-like structures of indeterminate length that are characterized by a common cross ß structure (17). In addition to their related structural features, amyloid fibrils display

characteristic tinctorial properties, such as thioflavin T and congo red staining (18-20). Though drusen do stain with thioflavin T and congo red, the characteristic apple-green birefringence often seen in congo red–stained amyloid fibrils is not present (5, 6). Although amyloid proteins such as the Aß peptide, transthyretin, immunoglobulin light chains, and amyloid A are found in drusen and sub-RPE deposits (4-6, 9, 21), electron microscopy studies have yielded sparse evidence of the presence of bona fide amyloid fibrils. These observations have precluded AMD from being viewed as a classical amyloid disease.

Amyloid fibril formation is a multistep protein misfolding cascade of molecular events, wherein a monomeric protein undergoes a conformational reorganization into a number of different oligomeric, ß sheet–containing structures that ultimately convert into amyloid fibrils (22, 23). Numerous studies of various amyloid diseases have led to the perception that nonfibrillar oligomers, rather than amyloid fibrils, might be the primary toxic agents (23-30). This notion has been supported by animal models demonstrating that amyloid fibrils do not seem to be required for the pathogenesis of amyloid diseases (30-34). These results suggest that additional amyloid diseases might be identified wherein pathogenic nonfibrillar oligomers are present without considerable accumulations of amyloid fibrils. Recent evidence suggests that desmin-related cardiomyopathy may be such a disease (35). The central goal of the present study was to investigate whether nonfibrillar amyloid oligomers

are present in drusen. To address this question, we took advantage of a recently developed A11 "anti-oligomer" antibody that specifically recognizes nonfibrillar oligomers (24). Although this antibody was initially raised against a molecular mimic of nonfibrillar oligomer made from the Alzheimer Aß peptide, it has been shown to also detect toxic oligomers made from a variety of other amyloidogenic proteins (24) such as α-synuclein, islet amyloid polypeptide (IAPP), prion 106–126 peptide, polyglutamine, lysozyme, human insulin, and, as recently demonstrated, the yeast prion protein (36). Remarkably, this antibody does not show reactivity (as demonstrated by dot blots and ELISA) with soluble monomeric protein, soluble low–molecular weight oligomers, or fibrils (24). It has been suggested, therefore, that the nonfibrillar amyloid oligomers from different proteins exhibit common structural features (24). Notably, the antibody also exhibits a strongly protective effect against oligomer-induced toxicity, indicating that oligomers do indeed represent a toxic species (24).

The utility of this generic anti–amyloid oligomer antibody has been established in immunocytochemical studies as well. For example, through this antibody, the presence of nonfibrillar oligomers has been demonstrated in AD-affected brains (24). These toxic oligomers were found to be in close proximity to senile plaques, yet they have shown a distinct localization from the fibrillar plaque region, perhaps indicating the initial stage of amyloid fibril deposition (24). Furthermore, the same antibody

was used in immunocytochemical studies to identify amyloid oligomers in the above-mentioned study on desmin-related cardiomyopathy (35).

In the present study, by using the anti-oligomer antibody, we detected the toxic oligomers within drusen-containing donor eyes but not in control eyes without drusen. The presence of amyloid oligomers suggests that the underlying pathogenesis in AMD could be related to that of amyloid diseases.

Results

Immunofluorescent microscopy revealed the presence of amyloid oligomers in distinct areas of eyes that contained drusen. Antibody reactivity was most frequently observed centrally within drusen, wherein the fluorescent signal typically accumulated in close proximity to the inner collagenous layer of the Bm and formed a distinct subdomain. These structures, which we refer to as amyloid oligomer cores, did not vary appreciably in size, even as the size of the drusen varied. In the smallest drusen (<20 μm), the amyloid oligomer cores comprised the majority of the drusen content (Figure 1, A and B). In larger drusen, the cores remained at approximately 15 μm in diameter and retained the same spatial

relationship, abutting the Bm whether they had the appearance of hard drusen (Figure 1, C–F) or macular soft drusen (Figure 1, G and H). While many of the cores were spherical (e.g., Figure 1, B and D), others appeared more elongated in shape (e.g., Figure 1, F and H). These data suggest that the oligomer cores may occur at an early point during drusen biogenesis but do not appear to grow as drusen become larger.

Although the size of the amyloid oligomer cores appeared to be restricted, the number of cores per druse did vary. Larger drusen in particular were sometimes observed to contain several amyloid oligomer cores (Figure 2, A, B, and I), suggesting that these drusen may have formed from a coalescence of smaller drusen. While not all drusen were observed to contain amyloid oligomer cores, given the small size of the cores relative to the larger drusen, many were likely to be out of the plane of section and thus not detectable. Table 1 summarizes the results obtained from the eyes of 19 individuals. Strikingly, anti-oligomer antibody reactivity was observed in all eyes that contained drusen, but was not observed in eyes from age-matched controls or from those of young donors that did not contain drusen. These data establish a direct correlation between the presence of amyloid oligomers and drusen and suggest a possible role for oligomer cores in drusen biogenesis.

Besides the presence of amyloid oligomer cores within drusen, oligomer staining was also observed at the Bm in some cases, especially where it

appeared to be thickened (Figure 2, C and D) and below the Bm in basal linear deposits (Figure 2, E–H). Occasional staining within the RPE was also observed (Figure 2H). Staining was not observed in the neural retina (data not shown). Thus, antibody reactivity is also associated with additional pathological changes that are characteristic of AMD.

Specificity of the anti-oligomer antibody is exhibited in Figure 2, I and J, which depict serial sections obtained from the same druse. This druse contained several foci of anti-oligomer reactivity (Figure 2I). Staining was not seen when the section was incubated without the primary antibody (data not shown), nor when the antibody was preincubated with nonfibrillar oligomers made from the $Aß_{1–40}$ peptide (Figure 2J). It should be noted that the nonfibrillar oligomers made from $Aß_{1–40}$ and $Aß_{1–42}$ are structurally similar with regard to their immunoreactivity with the anti-oligomer antibody and their cellular toxicity (24). The difference lies in the kinetics of their formation: $Aß_{1–42}$ forms oligomers on a faster time scale than does $Aß_{1–40}$ (24, 37). We performed ELISA in order to further test the specificity of the anti-oligomer antibody in tissue homogenates prepared from the neural retina or from the underlying tissue containing drusen/RPE/Bm. In vitro synthesized nonfibrillar $Aß_{1–40}$ oligomers served as a positive control. As shown in Figure 3A, a dose-dependent reactivity to increasing amounts of $Aß_{1–40}$ oligomers was observed in the presence of the oligomer-specific antibody, but not when the antibody was omitted. Comparisons of antibody reactivity were also made between extracts

prepared from the neural retina and from the tissue containing drusen/RPE/Bm (Figure 3B). A dose-dependent reactivity was observed with extracts prepared from drusen-containing tissue, whereas no reactivity was observed in the absence of the primary antibody. Little reactivity was observed with extracts prepared from the neural retina of the same donor eye. Thus the positive signal seen in drusen appears to be highly specific for amyloid oligomers.

The data presented thus far support the notion that amyloid oligomers are present in drusen. Further inspections of the sections at higher magnifications using laser scanning confocal microscopy revealed a punctate pattern of small vesicular structures that increased in density toward the center of the amyloid oligomer core (Figure 4A). Occasionally, a decreasing gradation of the punctate pattern appeared to extend toward the RPE (Figure 4B). In other instances, the vesicular structures were observed to penetrate through the layers of the Bm (Figure 4C). Together, these data suggest that the amyloid oligomers could be trafficked between the RPE cells, the drusen, and the choroid.

In order to confirm the vesicular nature of the cores, we first used indirect immunofluorescence to identify a druse that contained an amyloid oligomer core. An adjacent serial section was then prepared for immunogold labeling, followed by electron microscopy (Figure 4D). As judged by distribution of the gold particles, these studies did indeed verify

that the amyloid oligomers were associated with vesicular structures. Again, these vesicular structures appeared to be more concentrated near the Bm, although similar structures were occasionally labeled within the apical aspect of the druse closer to the RPE as well (data not shown).

The structures of the amyloid oligomer cores appear to be similar in some respects to dendritic cell processes, as reported by Hageman et al. (8). Double staining with the anti-oligomer antibody (green, FITC) was performed in conjunction with anti–HLA-DR (red, Texas red) in order to determine whether the immunoreactivities colocalized (Figure 5, A–D). Strong HLA-DR reactivity was seen within the drusen (Figure 5B) or beneath the Bm, where it appeared to penetrate into the druse and come into close proximity to the oligomer core (Figure 5C) and even completely surround it (Figure 5D). In some instances, HLA-DR reactivity appeared to be in close proximity to the amyloid oligomer core (Figure 5B). Upon closer inspection, it was clear that the immunofluorescent signals did not overlap (Figure 5, C and D). Thus the amyloid oligomer cores are distinct structures from the HLA-DR–positive dendritic cell processes described above.

Double staining was also performed on drusen sections to visualize oligomer cores and vitronectin, an acute phase protein that is a major component of drusen (38) (Figure 5, E–H). All drusen stained positively for vitronectin, whereas oligomer cores were present only in a subset of drusen (e.g., Figure 5F). Vitronectin tends to have heterogeneous labeling

patterns. In drusen that reacted positively for both oligomer cores and vitronectin, no overlap in their signals was observed. To ascertain whether the oligomer cores are assembled from Aß, sections containing drusen were costained for these 2 components (Figure 5, I–L). Most drusen contained either the Aß assemblies or oligomer cores, but not both. Consistent with previous reports, Aß reactivity was associated with vesicular structures within drusen (Figure 5, K and L). In 1 druse that reacted with both antibodies, the fluorescent signals did not colocalize: the amyloid oligomer reactivity was associated with the RPE, whereas the Aß reactivity decorated spherical structures within the druse (Figure 5L). Thus amyloid oligomers do not appear to colocalize with many of the known drusen components.

Different tissue or cultured cell types show varying susceptibility to the toxicity of amyloid aggregates (39). We sought to examine whether amyloid oligomers are toxic to RPE cells, given their close proximity to each other in eyes that contain drusen. As expected, oligomeric, but not soluble monomeric, IAPP and α-synuclein exerted toxicity toward human primary RPE cells and SH-SY5Y human neuroblastoma cells (Figure 6A). Similarly, oligomers made from $Aß_{1–40}$ were toxic to human primary RPE cells in culture. This toxicity was largely blocked when the A11 anti-oligomer antibody was included in the incubation mixture (Figure 6B). These results confirm that nonfibrillar oligomers made from amyloidogenic protein and peptides are toxic to human primary RPE cells. Thus, the presence of amyloid oligomers in close proximity to RPE cells

may have a negative impact on the physiology of these cells during drusen biogenesis and in AMD.

Discussion

Nonfibrillar oligomeric structures made from amyloidogenic proteins or peptides are thought to contribute to the pathogenesis of amyloid diseases. Such structures can be detected in tissue sections in situ by a recently developed conformation-specific, but not sequence-specific, antibody (24). Through the use of this antibody, we demonstrated the presence of amyloid oligomers in drusen-containing eyes and eyes that have been clinically diagnosed with atrophic AMD. Importantly, no reactivity was observed in control eyes without drusen, which suggests that the formation of amyloid oligomers is a disease-specific process. Since nonfibrillar amyloid oligomers demonstrated toxicity toward cultured primary human RPE cells, they may contribute to their demise during the disease process. Thus, AMD and amyloid diseases appear to share similar protein misfolding events and may share common pathogenic pathways as well. One commonality is the discovery that spherical Aß assemblies as well as other proinflammatory proteins commonly seen in AD plaques are also present in drusen (9, 10). In particular, Anderson et al. showed that a single druse may contain no Aß structures or a large number of them, ranging in diameter from 0.25 to 10 μm and displaying highly organized concentric

layers when viewed under an electron microscope (5). Thus, the Aß assemblies reported by Anderson et al. are structurally distinct from the oligomer-associated vesicles due to differences in their size, shape, and distribution. Indeed, our data show that they did not colocalize in drusen. It is important to note, however, that the epitope for Aß may have been masked within the oligomeric structure, as is the case when Aß monomers are transformed into amyloid fibrils (40). Therefore, we cannot preclude the possibility that the oligomeric cores in drusen are made up of Aß. Another drusen subdomain, described by Mullins and Hageman (41), is composed of core-like structures that exhibit *Arachea hypogea* agglutinin reactivity. Although these structures to some extent resemble the oligomeric structures described herein, they range in diameter from 5 to 38 µm, whereas the amyloid oligomer cores are typically 10–15 µm. It appears then that the oligomeric structures discussed here differ distinctively from the substructures within drusen that had been described previously. Composition of the oligomeric structures within drusen has yet to be determined and is under investigation. Our data have yielded further evidence that AMD and amyloid diseases share common pathogenic pathways, although amyloid fibrils have not been observed in drusen. Amyloid protein–related toxicity in the absence of fibrils had been observed in the past. For example, in a transgenic mouse model for AD, overexpression of the human wild-type ß-amyloid precursor protein leads to learning deficits and Aß deposition without amyloid plaque formation (42). In the case of a mouse model for PD, it has been shown that

overexpression of wild-type α-synuclein results in motor abnormalities and the formation of α-synuclein–containing, nonfibrillar inclusions (43). In transgenic rats expressing human IAPP that served as an animal model for type 2 diabetes, apoptosis of pancreatic islet cells did not correlate with amyloid formation (33). These results indicate that the presence of amyloid fibrils is not a prerequisite for pathogenesis and implicate the toxic nonfibrillar oligomers as an underlying cause of cell loss (30). Thus, AMD and desmin-related cardiomyopathy (35) might be just 2 of several diseases that are related to amyloid diseases, yet do not exhibit noticeable amyloid fibril deposition. Although it is not obvious why amyloid fibrils are difficult to detect in drusen, the rate at which oligomers and fibrils are turned over are likely to be of importance. As mentioned above, amyloid fibril formation is a stepwise process, and the overall yield of oligomers and fibrils depends upon the underlying kinetics of each step. Thus, 2 possible explanations for the low degree of fibril deposition are slow rates of fibril formation or fast rates of clearance. It is known that rates of amyloid fibril formation are largely dictated by experimental conditions, and biochemical data suggest that under appropriate conditions the stability of oligomers can be maintained for extended periods of time (44, 45). The stability of the oligomers in the drusen may also be stabilized through interacting with other proteins. In addition, it is conceivable that oligomers might be cleared out of drusen before they can be converted into fibrils. Although the present study provides no direct evidence of such clearance, the ability of oligomeric structures to penetrate through the Bm suggests

this possibility.

Recent immunocytochemical data on HLA-DR reactivity in drusen suggest the presence of dendritic cell processes in drusen (8). Dendritic cells are antigen-presenting cells that take up foreign substances and, in principle, may facilitate the clearance of amyloid oligomers. Indeed, our studies show the presence of HLA-DR–reactive structures in drusen similar to that reported by Hageman et al. (8). These putative dendritic cell processes appear more frequently in and around the drusen than the amyloid oligomers, and they were sometimes found in close proximity to the amyloid oligomer cores. They do not, however, appear to colocalize. Future studies should enable us to determine whether a relationship exists between the dendritic cells and the amyloid oligomer cores. Our data add to the growing list of evidence that reveals similarities between AMD and amyloid diseases (4-7). It is particularly noteworthy that proinflammatory proteins have been identified in the extracellular deposits associated with these diseases. Evidence of complement activation has been observed within certain RPE cells, small drusen, and large, soft drusen that are present in aging eyes as well as in AMD eyes (10). This observation has led to the hypothesis that aberrant immune reactions may play a role in drusen biogenesis. Notably, a polymorphism in complement factor H, a key regulator of complement activation, has recently been identified as a major risk factor for AMD (12-15). This finding has placed a significant focus on the role of complement activation in the pathogenesis of AMD: what are the factors that lead to the activation of the immune response?

Here we report the presence of amyloid oligomers in a similar distribution of drusen, RPE cells, and basal deposits. It is noteworthy that these oligomers have been implicated in the pathogenesis of amyloid diseases due to their demonstrated toxicity toward cells. It is possible that the presence of oligomers in close proximity to RPE cells may compromise their function, leading to activation of the complement cascade and formation of drusen.

In summary, the presence of amyloid oligomers in drusen suggests that AMD and amyloid diseases share commonalities with respect to protein misfolding and pathogenesis. AMD and desmin-related cardiomyopathy (35) may well come to represent the first examples of a new class of amyloid disease in which oligomeric intermediates, rather than mature amyloid fibrils, accumulate.

Methods

Human tissue. Intact human donor eyes were obtained from the Oregon Lions Sight and Hearing Foundation and the Alzheimer Disease Research Center (ADRC) Neuropathology Core of the University of Southern California. Eyes from 19 individuals were examined, 4 of which had documented clinical histories of AMD (Table 1). All eyes were kept at 4°C and processed at less than 24 hours postmortem. Fixation was avoided

since it would have interfered with antigen detection using the anti-oligomer–specific antibody. After removing the anterior pole, the retina was peeled off and the posterior pole of the eyeball was examined under a dissecting microscope (MZ125; Leica Microsystems) for the presence of drusen. All areas containing drusen were included. Tissue was cut into 1-cm x 0.5-cm rectangles, using a coated stainless steel razor blade, and embedded in OCT (Tissue-Tek; Sakura Finetech).

Confocal immunofluorescence microscopy. Frozen embedded tissue was sectioned on a cryostat (CM 3050S; Leica Microsystems) at –20°C. Frozen sections 8–10 μm thick were collected on precleaned Superfrost plus-slides (VWR International), air dried for 30 minutes, and stored at –20°C. Immunocytochemical studies using the anti-oligomer–specific antibody were performed as described previously (24). Briefly, sections were blocked overnight at 4°C in blocking solution (phosphate-buffered saline containing 2% BSA and 2% goat serum) and incubated the following day with affinity-purified anti-oligomer–specific antibody (1.6 mg/ml) for 1 hour at room temperature. Sections were then washed and incubated with a fluorescein-conjugated goat anti-rabbit antibody (1:100; Vector Laboratories) for 1 hour at room temperature. In order to detect oligomers and HLA-DR or drusen components such as vitronectin and Aß, sections were processed as described above and incubated with mouse anti-human HLA-DR antibody (0.5 mg/ml, BD Biosciences — Pharmingen), mouse anti-vitronectin antibody (1:200, BioSource International), or mouse anti-

Aß antibody (1:100, 4G8; Signet Laboratories Inc.), which is directed against residues 17–24 of the Aß peptide. Digital images of immunostained sections were acquired on an LSM 510 Zeiss laser scanning confocal microscope.

Electron microscopy. Prefibrillar oligomers were first identified in frozen sections using immunofluorescence. Adjacent serial sections known to contain oligomers were incubated with the anti-oligomer antibody, and subsequently with 5 nm gold-conjugated goat anti-rabbit antibody (Ted Pella Inc.). The sections were washed and pre-embedded in 4% agarose. Agarose-embedded sections were then briefly fixed in OsO_4, dehydrated in increasing concentrations of ethanol, infiltrated with epoxy resin, and sectioned at 70 nm using an ultramicrotome (Ultracut UCT; Leica Microsystems) for electron microscopy. Images were obtained using a transmission electron microscope (EM10; Zeiss).

Preparation of soluble A ß oligomers. Aß oligomers were prepared as described previously (26). Briefly, 1.0 mg Aß was dissolved in 400 µl 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) for 10 minutes at room temperature. Aliquots (100 µl) of the solution were added to 900 µl double-distilled H_2O in siliconized Eppendorf tubes. After 10 minutes of incubation the samples were centrifuged for 15 minutes at 14,000 *g*, and the supernatant fraction was transferred to a new siliconized tube. The HFIP was evaporated by blowing under an N_2 stream for 5–10 minutes.

The samples were then stirred at 500 rpm using a Teflon coated micro stir bar for 24–48 hours at room temperature. Aliquots were taken at 6- to 12-hour intervals to check for the presence of spherical oligomers.

ELISA. ELISA was performed with tissue homogenates and oligomers made from the Aß peptide. To process eye tissue samples, neural retina was peeled off the underlying RPE/Bm/choroid complex at the posterior eye pole. Isolated tissues (neural retina or the underlying RPE/Bm/choroid complex) were homogenized using a tip sonicator (Microson) in double-distilled H_2O and centrifuged, and supernatant was collected. ELISA was performed using the anti-oligomer–specific antibody as described by Kayed and colleagues (24). Briefly, samples were diluted in coating buffer (0.1 M sodium bicarbonate) and added to wells of a 96-well microplate (BD). After 2 hours of incubation at 37°C, samples were blocked for 2 hours at 37°C with 3% BSA tris-buffered saline with 0.1% Triton X-100. Anti-oligomer antibody (100 µl, 1:2500) was added and incubated at 37°C for 1 hour, prior to incubation with 100 µl of horseradish peroxidase–conjugated anti-rabbit IgG for 1 hour at 37°C. Subsequent to development with 3,3',5,5'-tetramethylbenzidine, the reaction was stopped with 100 µl 1 M HCl, and plates were read at 450 nm (Benchmark Plus; Bio-Rad Laboratories).

Cell viability assay. Cell viability was assessed spectrophotometrically using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide–

based (MTT-based) assay (Sigma-Aldrich). RPE cells isolated from human fetal eyes were obtained from Advanced Bioscience Resources Inc. The cells were maintained in DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum at 37°C. Third to fourth passage cells were seeded at 10,000 cells per well in a 96-well plate and grown for 3–4 days to approximately 90% confluence. SH-SY5Y human neuroblastoma cells were maintained in DMEM with 10 nM HEPES, 10% fetal bovine serum, 4 mM glutamine, penicillin (200 U/ml), and streptomycin (200 µg/ml) in 5% CO_2 at 37°C. They were differentiated and plated as previously described (24). Prior to the toxicity assay, media was replaced with indicated concentrations of Aß oligomers alone or with equal molars of the A11 anti-oligomer antibody dissolved in phenol red–free DMEM. The conditions were carried out in triplicate. After 4 hours, MTT dissolved in DMEM was added to the cells and incubated for an additional 4 hours. Insoluble crystals were dissolved by adding MTT solubilization solution (10% Triton X-100, 0.1 N HCl in anhydrous isopropanol), and absorbance was measured at 570 nm.

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Table and Figures

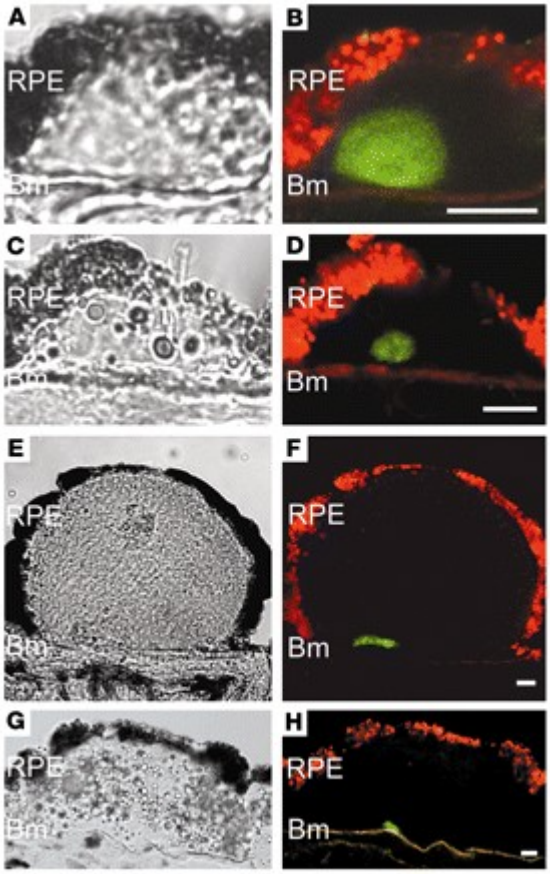
Table 1

Oligomer reactivity is specific for drusen-containing tissue

Age	Sex	Ocular history	Drusen	Oligomers
Eyes with drusen				
87	M	No data	+	+
86	M	Unremarkable	+	+
88	F	AMD	+	+
96	F	No data	+	+
87	F	No data	+	+
94	F	No data	+	+
92	M	Unremarkable	+	+
92	F	Pseudophakia	+	+
75	F	AMD	+	+
83	M	Intraocular lens surgery	+	+
82	F	AMD	+	+
98	M	AMD	+	+
88	F	Cataract	+	+
77	M	Unremarkable	+	+
Eyes without drusen				
87	M	Unremarkable	–	–
81	M	Cataract	–	–
56	F	Unremarkable	–	–
21	F	Unremarkable	–	–
Fetus		No data	–	–

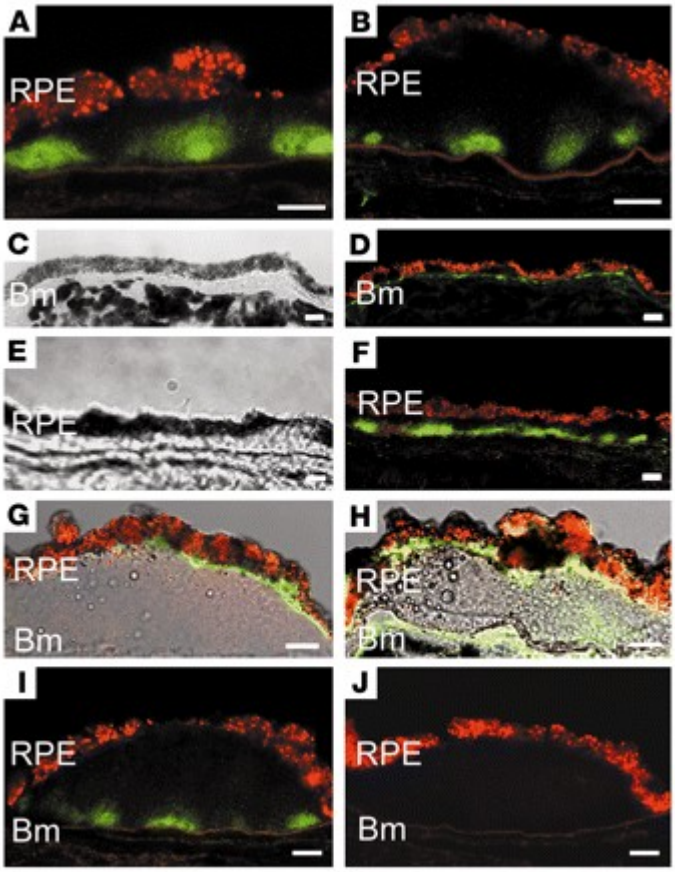
Whole eyes from 19 donors were screened by confocal microscopy for the presence of amyloid oligomers. Oligomer reactivity was observed only when drusen were present. No reactivity was observed in age-matched control eyes without drusen or in eyes from young donors that did not contain drusen.

Figure 1



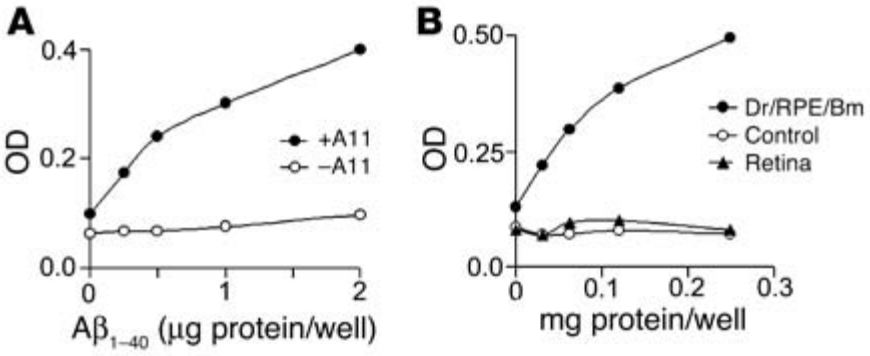
Immunolocalization of amyloidogenic oligomers in drusen by confocal laser microscopy. (**A**, **C**, **E**, and **G**) Differential interference contrast images. (**B**, **D**, **F**, and **H**) Confocal fluorescence images of amyloid oligomer cores (green, FITC channel). Drusen exhibit amyloid oligomer reactivity in the form of a core-like structure that accumulates centrally within drusen and in close proximity to the Bm. Autofluorescence of lipofuscin granules in the RPE cytoplasm is shown in red (Cy3 channel). (**A** and **B**) Anti-oligomer–specific antibody recognizes a spherical structure (~15 μm) in a small druse (~30 μm). (**C**–**F**) Two larger drusen with centrally located core structure. (**G** and **H**) A very large macular soft druse from an 81-year-old female donor. Despite the difference in sizes and shapes of the drusen, the amyloid oligomer cores remain 10–15 μm in size. Scale bars: 10 μm.

Figure 2



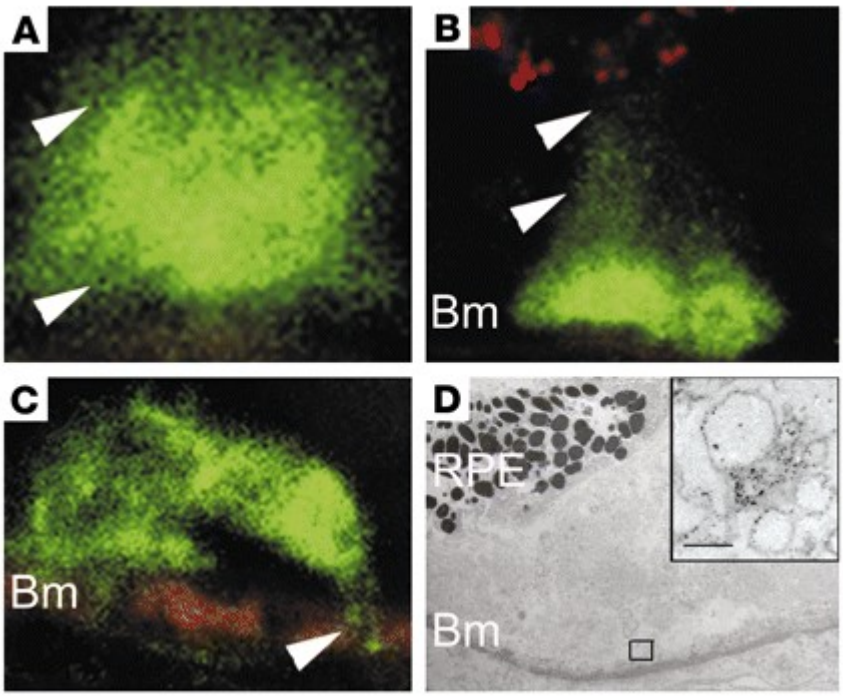
Presence of amyloid oligomers in drusen and thickened Bm. Amyloid oligomer reactivity was visualized with fluorescein (green), and lipofuscin autofluorescence was visualized using the Cy3 channel (red). Multiple amyloid oligomer cores were sometimes observed in large drusen (**A** and **B**), as if a large druse may have formed from the fusion of several smaller drusen. The amyloid oligomer cores retained their size and relative positions within the druse and in proximity to the Bm. Within eyes that contained drusen, the oligomers occasionally accumulated above the Bm, in the form of basal linear (**C** and **D**) or basal laminar (**E**–**H**) deposits, particularly in instances where the Bm appeared to be thickened. (**H**) Staining within RPE cells was also observed. **C** and **D** are differential interference contrast images of **D** and **F**, respectively. (**I** and **J**) Specificity of the antibody in cryosections is demonstrated in adjacent sections of a large druse. (**I**) Multiple amyloid oligomer cores were visualized through use of the anti-oligomer antibody. (**J**) Reactivity was eliminated when the primary antibody was preincubated with amyloid oligomers synthesized from the $A\beta_{1-40}$ peptide. Scale bars: 10 μm.

Figure 3



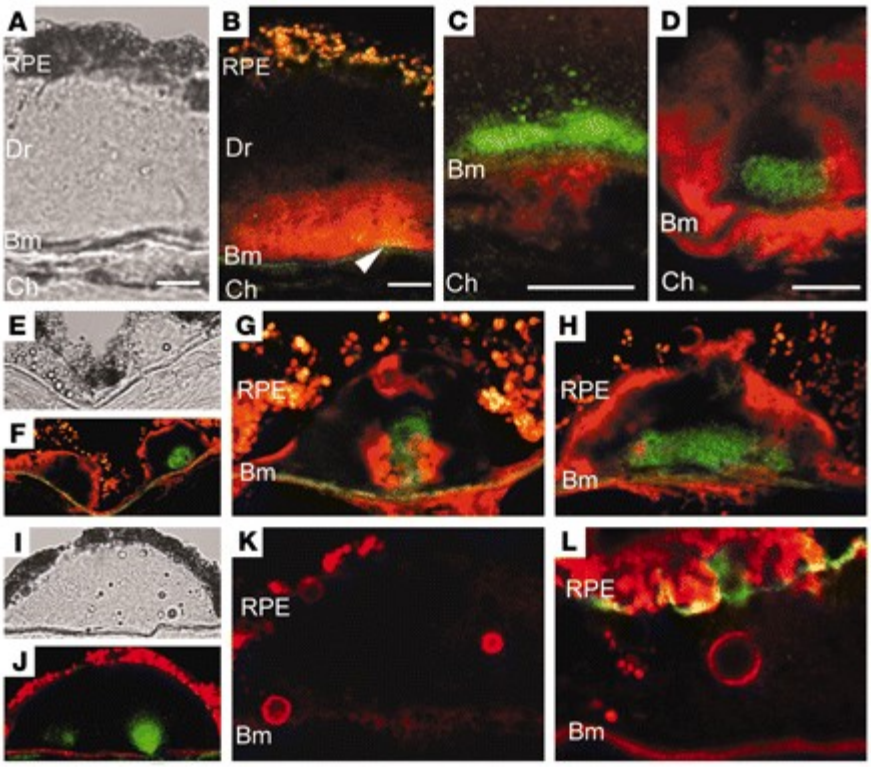
ELISA of retinal extracts using the A11 anti-oligomer antibody. (**A**) Increasing amounts of oligomers made from the $A\beta_{1–40}$ peptide resulted in a dose-dependent response when incubated with the A11 anti-oligomer–specific antibody (filled circles). Little or no reactivity was observed when the $A\beta_{1–40}$ oligomers were incubated without the primary antibody (open circles). (**B**) Dose-dependent reactivity was observed when the A11 antibody was incubated with increasing amounts of extract prepared from dissected drusen/RPE/Bm tissue from a 76-year-old male donor (filled circles). Little or no reactivity was observed when the primary antibody was omitted (open circles). Extracts prepared from the neural retina (filled triangles) of the same donor eye did not show a dose-dependent response when incubated with the A11 antibody. Dr, drusen.

Figure 4



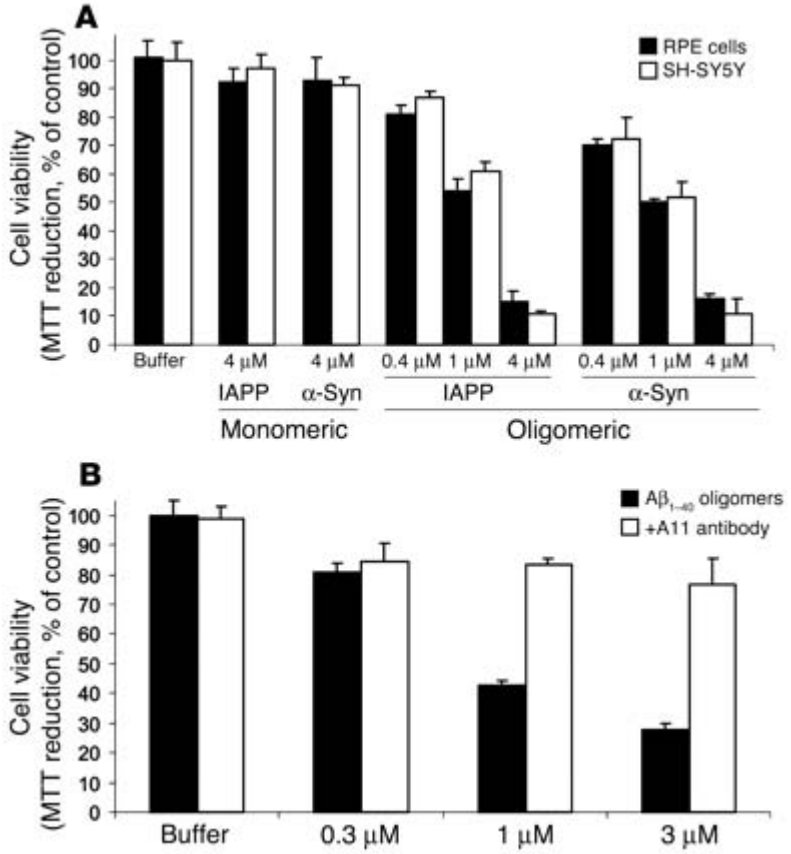
Morphology of amyloid oligomer cores in drusen at higher magnification. (**A**–**C**) Confocal micrographs of drusen. Amyloid oligomer cores are labeled with fluorescein (green), and lipofuscin autofluorescence in the RPE is visualized in red (Cy3 channel). (**A**) Amyloid oligomer cores seemed to consist of an aggregate of small vesicular structures (arrowheads) that increased in density toward the center. (**B**) Some of these vesicular structures appeared to extend toward the RPE with diminishing density (arrowheads). (**C**) Occasionally, the amyloid oligomer cores penetrated through the Bm and extended toward the choroid (arrowhead). (**D**) Ultrastructure of an amyloid oligomer core is depicted in an immunogold-labeled electron micrograph (inset), wherein gold particles decorate vesicular structures that are heterogeneous in size. The highest density of gold particles seen in **D** was from the region above the Bm (rectangle). Scale bars: 100 nm (**D**, inset). Magnification, x3,000 (**A**), x2,000 (**B**–**D**).

Figure 5



Codistribution of amyloid oligomer cores and other known drusen components. (**A**, **E**, and **I**) Differential interference contrast images. (**B**–**D**, **F**–**H**, and **J**–**L**) Confocal fluorescence images; amyloid oligomer cores were labeled with fluorescein (green). (**A** and **B**) Both antigens were present in a large druse, wherein the amyloid oligomer core was enveloped within the HLA-DR reactive region (labeled with Texas red). (**C** and **D**) At higher magnification, it is clear that the amyloid oligomer core and HLA-DR reactive subdomain did not colocalize in these drusen. (**C**) In one instance, the HLA-DR reactive region, perhaps reflecting a dendritic cell process, was observed as originating from the choroid (Ch), coming in close proximity to the Bm, and contacting the condensation of vesicular structures that represent the amyloid oligomer core. (**D**) In another instance, HLA-DR reactivity was observed as encompassing the choroid, the Bm, and the druse. Within the druse, HLA-DR reactivity appeared to surround the oligomer core, with no indication of colocalization. Similarly, no colocalization was observed with vitronectin (**F**–**H**) or Aß (**J**–**L**), both labeled with Texas red (red). Lipofuscin autofluorescence within RPE is also visualized in the Cy3 channel (red). Scale bars: 10 µm. Magnification, x250 (**E**, **F**, **I**, and **J**), x1,500 (**G**), x1,000 (**H**), and x2,000 (**K** and **L**).

Figure 6



Toxicity of nonfibrillar amyloid oligomers to human primary RPE cells. (**A**) Amyloid oligomers were toxic to cultured SH-SY5Y human neuroblastoma cells (white bars) and human primary RPE cells (black bars). Oligomeric forms of IAPP and α-synuclein (α-Syn), but not soluble monomers, demonstrated toxicity. Cell viability was assessed by MTT reduction. (**B**) Increasing amounts of amyloid oligomers made from $A\beta_{1–40}$ also showed a dose-dependent toxicity to cultured human primary RPE cells (black bars). This toxicity was largely blocked by adding equal molars of the A11 anti-oligomer antibody (white bars). Error bars represent SD; $n = 3$.

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Future Prospects

This study demonstrates a direct correlation between the appearance ofdrusen and the existence of toxic oligomers in eyes with Age-related Macular Degeneration (AMD). Nineteen donor eyes were tested for the existence of toxic oligomers in the neural retina through use of a conformational dependent anti-oligomer antibody (1). Toxic oligomers were found in all eyes with drusen (fourteen eyes) as well within drusen as in close proximity to retinal-pigmented epithelial cells (RPE) that are known to degenerate in the process of AMD. Contrary, eyes without drusen (five eyes) had no signs of oligomer existence. As well eyes of comparable donor age (two eyes) as eyes of younger donors (three eyes) were oligomer negative, which conclusively eliminates the possibility of age-correlation and emphasizes the possible importancy of toxic oligomers in the pathogenesis of AMD. In order to evaluate the results of this study it is important to note, that

- the underlying cause of AMD is still unknown, even if new studies indicate an inflammatory process as a crucial part of AMD pathogenesis (2),
- drusen, dependent on size and frequency, are seen as the first indication of AMD-development (3),
- the discussed oligomers represent a specific protein structure that are a result of a protein missfolding process and are seen as a primary pathogen for clinical symptoms (4) and degenerative tissue processes in protein missfolding disorders (PMDs) (5).

Therefore is the indication of a direct correlation between toxic oligomers and drusen of highest interest in double respect and will be following discusssed on the basis of our results.

1. Indication of a possible protein missfolding process in the pathogenesis of AMD

and therefore possible transfer of knowdledge from in vitro and in vivo PMDs-studies to AMD:

A) Protein-Idendity
B) RPE cells as a source of toxic oligomers
C) RPE cells represent a target for toxic oligomers
D) Oligomers and the immunesystem in respect to the pathogenesis of PMDs and AMD

2. Indication of a primary pathogen for a degenerative process in AMD

and therefore new therapeutic and prophylactic strategies for AMD with toxic oligomers as a primary target

1. Indication of a possible protein missfolding process in the pathogenesis of AMD

A) Protein-Idendity

The fact that each PMD seems to be based on the missfolding of one

particular protein (for example the aβ-peptide in Alzheimers, the IAPP peptide in Diabetes Typ II or the α-synuclein protein in Parkinsons leads to the question of the protein-idendity of oligomers in drusen (6). This study could not answer this question, since the specific anti-oligomer antibody recognizes exclusively a oligomeric structure of numerous PMDs-related proteins, but not the aminoacid sequence of a particular protein (1).

The approach of double-labeling with the conformational-dependent antioligomer antibody and different aminoacidsequence-dependent antibodies that are directed against the aminoacidsequence of the prominent drusen protein vitronectin or the Alzheimers-related aβ-peptide was without success.

Of course one possibility cannot be excluded. The change of conformation from a monomeric to a oligomeric structure may mask the epitope of the proteins and inhibits therefore the recognition through aminoacidsequence-dependent antibodies (7). Furthermore, in vitro studies demonstrate that even proteins, which are so far not to be known to be disease-related, could underlie a similar protein missfolding process (8, 9) that is observed with proteins of PMDs. Therfore all drusen proteins (10) may be considered as a possible candidate. The idendity of the protein is of highest interest, since most neurodegenerative diseases have a genetic background with mutations of the missfolded protein (prion-mutations in the Gerstmann-Straussler syndrome or hereditary Creutzfeldt-Jakob disease, taumutations in autosomal dominant dementia and alpha-synuclein-mutations in autosomal dominant Parkinsons) or mutations of their

precursor protein (APP in Alzheimers) (11). Further studies with laser capture microscpy or immuno- precipitation and mass spectometry may help to answer this central question of the protein-idendity of the oligomeric structures within drusen.

B) RPE cells as a source of toxic oligomers

A recent Prion-disease study suggested, that the missfolded protein is released through vesicles (exosomes) into the extracellular space after fusion with the cell membrane (12). Therefore someone can only speculate if RPE cells could be the source of the monomeric or oligomeric protein in the case of AMD, but the massive existence of oligomers in close relationship to RPE cells may emphasize this suspicion. Furthermore, immunofluorescence microscopy demonstrates oligomers in vesicular form or bound to vesicles in drusen. Interestingly, these vesicles can be followed back to specific RPE cells and exhibit an average diameter of 100 nm (electron microscopy result), a size which has been also observed of „prion-exosomes“ (12).

Further studies should show, if observed vesicles represent oligomers in vesicular form or oligomers that are bound to vesicles (exosomes). The size of the observed vesicles in drusen indicates the latter, because oligomers are described in the literature in a much smaller size of 3-10 nm (13). Furtherbapproaches may include vesicle markers (MHC II, anti-ganglioside antibodies)

C) RPE cells represent a target for toxic oligomers

Our study demonstrates that oligomers are in close proximity to RPE cells, but not to photoreceptor cells. Therefore only RPE cells may be a primary target for oligomer-induced toxicity. Furthermore we could demonstrate that RPE cells are successible for oligomer-induced toxicity. This is of interest, because new studies demonstrated that oligomer-induced toxicity may be a cell dependent process relating to their membran composition, such as cholesterol (14). Membrane composition may change through age, diet, genetics, disease or development. Therefore it is important to investigate if the membran composition of RPE cells may differ in age or disease, particular in AMD, which could affect the oligomer-induced toxicity in one or the other way (data not shown).

D) Oligomers and the immunesystem in respect to the pathogenesis of AMD

New studies demonstrate that a chronic inflammatory process may be an important key player as well in AMD as in Parkinsons (15), Alzheimers (16) and other PMDs (17). It has been shown that specific polymorphismn of particular mediators of the inflammatory cascade are risk factors in Alzheimers (16) and Parkinsons (15). Interestingly, a similar polymorphism regarding the factor H (HF1) has been recently also observed in AMD (18). Factor H inhibits the complement cascade and

therefore protects tissue from uncontrolled action of the complement cascade. But the question remains open what factor actually triggers or activates the complement cascade. It is important to note, that HF1 colocolizes with amyloid vesicles in drusen. Even if these vesicles seem to differ from oligomeric vesicles described in this study, toxic oligomers should be considered as an activating factor of the complement cascade (19, 20). Therefore someone can speculate that toxic oligomers are not only responsible for cell death trough direct toxicity, but also exhibit the capability to activate the complement cascade, which might lead to increased tissue damage in HF1 polymorphism. Furthermore, a continously forming process of oligomers without elimination (the rate of forming is faster than the rate of eliminating) may result in a continously activation of the immune system.

2.: Oligomers as a target for new therapeutic und prophylactic strategies

So far the underlying cause for AMD is unknown. Therefore therapeutic strategies are limited and directed against particular symptoms, such as choroidal neovascularization, and are applied in specific cases. Prophylactic strategies are generell and include advices regarding a healthy diet (nuts) or environmental behaviour (smoking).
Therefore, the proof of toxic oligomers in AMD-eyes may lead to new therapeutic strategies with oligomers as a primary target, including drugs that

• inhibit the formation of oligomers (such as the curry-pigment curcumin, 21)
• inhibit the interaction with membrane components, such as cholesterol as described above
• neutralize the oligomer-induced toxicity.

Interestingly, passive or active vaccination could already demonstrate some promising success in animal modells of PMDs. Application of small doses of oligomers of the abeta-peptide into AD-mice resulted in the production of antibodies that neuralize the toxicity of oligomers in cell cultures (22). A similar effect could be observed trough vaccination with oligomerspecific abeta-antibodies (23). Unfortuanetly, the idendity of the oligomeric protein in AMD is so far unknown. But with the use of the anti-oligomer antibody, the idendity of the protein might not even be of interest, because this antibody is directed against a toxic structure and might limit the risk of the development of antibodies that react against functional proteins. A use of this antibody might be a silver bullet for therapy in all PMDs with oligomers as a primary target. Our results indicate that AMD might be a candidate for this therapeutic approach.

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Korrelation zwischen amyloidalen oligomerischen Proteinstrukturen und Drusen der Alters-bedingten Makuladegeneration

Summary

Protein misfolding and aggregation are thought to underlie the pathogenesis of many „amyloid diseases“, such as Alzheimers and Parkinsons, whereby a stepwise protein misfolding process begins with the conversion of soluble protein monomers to prefibrillar oligomers and progresses to the formation of insoluble amyloid fibrils. Drusen are extracellular deposits found in eyes afflicted with age-related macular degeneration (AMD). Recent characterizations of drusen have revealed protein components that are shared with amyloid deposits. However, characteristic amyloid fibrils have thus far not been identified in drusen. In this study, we tested the hypothesis that nonfibrillar oligomers may be a common link in amyloid diseases. Oligomers consisting of distinct amyloidogenic proteins and peptides can be detected by a recently developed antibody that is thought to recognize a common structure. Notably, oligomers exhibit cellular toxicity, which suggests that they play a role in the pathogenesis of neurodegenerative diseases. Through use of the anti-oligomer antibody, we came to observe the presence of nonfibrillar, toxic oligomers in drusen. Conversely, no reactivity was observed in age-matched control eyes without drusen. These results suggest that amyloid oligomers may be involved in drusen biogenesis and that similar protein misfolding processes may occur in AMD and amyloid diseases.

Zusammenfassung

Protein-Missfaltung und Aggregation können als zugrunde liegende Ursache in der Pathogenese von „Amyloidalen Erkrankungen“, wie z.B. die Alzheimersche und Parkinsonsche Krankheit, betrachtet werden. Bei diesen ändert sich die Konformation eines Proteins schrittweise von einer anfänglichen monomerischen über eine pre-fibrilläre oligomerische bis hin zu unlöslichen amyloidalen fibrillären Struktur ändert. Drusen sind extrazelluläre Proteinablagerungen, die sich in von AMD-betroffenen Augen finden lassen. Neuere Drusenstudien beschreiben eine Reihe von Proteinen, die sich auch in amyloidalen Ablagerungen finden lassen. Allerdings sind bis heute keine amyloidalen Fibrillen als Bestandteil von Drusen entdeckt worden. In dieser Studie testen wir die die Hypothese, daβ amyloidale oligomerische Strukturen eine Gemeinsamkeit in amyloidalen Erkrankungen darstellen. Interessanter Weise besitzen oligomerische Proteinstrukturen eine Zelltoxizität, was deren Bedeutung in der Pathogenese von neurodegenerativen Erkrankungen unterstreicht. Die oligomerische Struktur von verschiedenen amyloidalen Proteinen und Peptiden kann von einem erst kürzlich entwickelten Antikörper erkannt werden. Durch die Benützung dieses anti-oligomer spezifischen Antikörpers konnten wir nichtfibrilläre, toxische Oligomere in Drusen entdecken. Im Gegensatz dazu waren keine Oligomere in Kontrollaugen ohne Drusen zu finden. Dieses Ergebnis schlägt vor, daβ amyloidale Oligomere in der Enstehung von Drusen involviert sind und daβ ein ähnlicher Proteinmissfaltungsprozeβ in AMD und amyloidalen Erkrankungen stattfindet.

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