LIPOFUSCIN FLUOROPHORE INHIBITS LYSOSOMAL PROTEIN DEGRADATION AND MAY CAUSE EARLY STAGES OF MACULAR DEGENERATION

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Key Words

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Abstract

One of the autofluorescent compounds that accumulates within the lipofuscin granules of the human retinal pigment epithelium (RPE) has now been identified as a quaternary nitrogen-containing cationic amphiphile (the bis-retinoid pyridinium salt, A2-E). Experimental evidence suggests that it may be responsible for lipofuscinogenesis in the RPE through its ability to inhibit lysosomal proteolysis. Furthermore, it may be involved in the events that trigger the changes leading to age-related macular degeneration (AMD), the leading cause of untreatable blindness in the elderly. It is suggested that if similar weakly basic nitrogenous compounds or cationic amphiphiles arise in reactions between amines and aldehydes in other tissues, a "self-assembling lysosomotropic amine" mechanism may provide an alternative explanation for lipofuscinogenesis those cell types as well.

Introduction

The lipofuscin pigments that appear with age in a variety of postmitotic cell types have several things in common: lysosomal origin, autofluorescence, lipophilicity [1-3]. However, there is also a great deal of variability: differences in morphology, buoyant density, extractability, etc. [4-6]. It is likely that the composition of the granules in each tissue is as individual as the cell types in which they arise. What is sought is some underlying unifying mechanism that can explain the gradual accumulation of undigested residues in the lysosomes of such a mix of cell types. Unlike the case for lysosomal storage diseases [7,8], no specific gene defect seems to be involved, and no specific substrate accumulates as a result. The retinal pigment epithelium (RPE) of human eyes is one of the specialized postmitotic cell-types in which lysosomal residues accumulate with age.

Role of the retinal pigment epithelium in the retina:

To understand the origin and consequences of lipofuscin granules in the RPE, a basic knowledge of retinal organization and function is required. In cross-section, the retina is seen to be a highly organized,

multilayered tissue. The innermost layers contain the nerve cells that collect and transmit nervous impulses from the photosensitive photoreceptor cells to the brain through the optic nerve.

The photoreceptor cells are elongated cells composed of inner and outer segments. The inner segment contains the cell nucleus and all of the metabolic machinery responsible for its function. Photoreceptor cells are one of the most metabolically active cell-types of the body, second only to embryonic and tumor tissues in metabolic demands [9]. The photoreceptor outer segments are highly specialized appendages on ciliary stalks giving the cells their familiar descriptive names: the rods and cones. These structures are composed of stacks of membrane disks (resembling stacks of coins) into which are inserted the molecules responsible for capturing photons: proteins linked covalently to molecules of vitamin A (rod rhodopsin and cone iodopsin) [10].

Below the rods and cones is a gap filled with a matrix material: the interphotoreceptor matrix [11]. Then there is a monolayer of epithelial cells: the retinal pigment epithelium. These cells are laid down at birth and remain without replacement throughout the life of the individual [12]. The RPE cells are joined together by tight junctions and represent an important component of the blood retinal barrier [13].

The RPE cells rest on a thick multilayered collagenous membrane called Bruch's membrane, and underlying this membrane is an extensive network of fenestrated capillaries called the choriocapillaris [14]. All nutrients required by the photoreceptor cells and all metabolic byproducts must pass to and from the circulatory system through Bruch's membrane and the RPE. Proper permeability characteristics of Bruch's membrane and active transport functions of the RPE are vital to the functioning of the retina. In addition, the RPE acts to re-isomerize vitamin A from its *trans-* to *cis-* configuration in the vitamin A cycle of the visual process [15]. Thus, the physical integrity of Bruch's membrane and functional capacity of the RPE are key factors in retinal function. Impairment of RPE function and disruption of Bruch's membrane permeability characteristics are believed to lead to age-related macular degeneration; the accumulation of RPE lipofuscin has long been suspected as the cause of this impairment [16].

Accumulation of lipofuscin granules in the RPE

The first age-related change in the retina occurs within the retinal pigment epithelium. Residual lysosomal storage bodies begin to appear in the RPE. The accumulation of lipofuscin begins in childhood with a steep increase until about 20 years of age, followed by a more gradual increase beyond 50 years of age. By about 50 years the lipofuscin granules take up about 20% of the cell volume [17]. Eventually, massive burdens of these granules accumulate within the RPE, especially in the central region of the retina referred to as the macula.

Source of lipofuscin granule contents

RPE age pigments arise largely as a consequence of the photoreceptor outer segment renewal process. The photoreceptors (the rods and cones) undergo a constant renewal of their light sensitive outer segments

[18], as well as portions of their cytoplasmic inner segments [19]. In the renewal process, the tips of the outer segments are shed, phagocytosed by the pigment epithelium cells and are dismantled by lysosomal degradation [18]. To balance this shedding, new disc membranes form in the rods at the base of their outer segments; the cone molecular membrane components are similarly renewed. A complete outer segment in humans and Rhesus monkeys turns over in about 10-12 days [18]. Within a 24 hour period, a single pigment epithelial cell of the Rhesus monkey digests about 2,000 disc membranes that come from rods alone. In addition, there come more, but not precisely known numbers of discs from the cones. The highest density of lipofuscin granules is found in the pigment epithelium of the central, perifoveal fundus, where there is also the highest photoreceptor density [20].

Within the RPE, the phagosomes coalesce with primary lysosomes and become dismantled through the action of lysosomal enzymes [21]. The new phagosomes consist of the outer segment discs: the phospholipid double membrane of the outer segments in which are inserted the visual pigments. For reasons not previously known, the dismantling is occasionally not complete and as a consequence, there arise residual bodies, that in the course of time accumulate as lipofuscin granules in the cytoplasm. Also, RPE autophagy contributes to the process as is evident from the presence of an amalgamation of RPE melanin and lipofuscin (melanolipofuscin) [22]. As RPE lipofuscin increases, the number of melanin granules decreases with a reciprocal increase in the number of melanolipofuscin granules [17]. Additionally, there appear to be changes in the composition of the granules over time. For example, there are changes in the fluorescence spectra of lipofuscin and also melanin with increasing age [23].

Nature of RPE lipofuscin storage products

The above chronology of events is based largely on morphological and histochemical studies. It still leaves unanswered the question of what molecular mechanisms can account for the slow accumulation of lipofuscin in the RPE.

Shortly after the histological description of these granules [24], work began on the analysis of their contents. It was soon seen that they were residual lysosomal storage bodies in that they contained the normal complement of lysosomal enzymes [25]. The granules contained a mixture of lipids and proteins [26]. Unlike the genetically inherited lysosomal storage diseases, no single storage component predominated, and no lysosomal enzyme deficiency or defect could be discovered. The cause of lipofuscin accumulation remained a mystery.

RPE age pigments fluoresce golden yellow upon ultraviolet illumination [22]. Such long wavelength emissions are very unusual for biochemical compounds of animal origin [27]. Since these residual lysosomal storage granules did not seem to fit the picture of a typical lysosomal storage disease, investigators soon turned to this unusual fluorescence as holding a potential key to the reason for lipofuscin formation and accumulation. The fluorescence of lipofuscin in the pigment epithelium was first

investitgated by Feeney [22], and different teams analyzed more temporal and spatial distributions [20]. Nutritional studies suggested that dietary vitamin A and nutritional antioxidants, esp. vitamin E, contributed to the formation of these fluorescent pigments [28, 29].

It was long unknown, which chemical components could be responsible for the observed fluorescence. Different hypotheses have been put forward. One appealing and long-standing hypothesis was that lipid peroxidation products arising in the photoreceptor outer segment membranes served to cross link any amine-containing compound rendering them both nondegradable and autofluorescent [30]. When investigated further, however, this hypothesis could not be verified. The autofluorescent products of lipid oxidation were quite different from those seen in the lipofuscin granules of human RPE [31-33], and the vitamin E deficiency pigments of the RPE differed in solubility characteristics and fluorophoric composition from the age pigment granules [34].

It has been argued that this long wavelength emission might be caused either by a concentration-dependent inner filter effect or by a metachromatic effect [35, 36]. In the case of RPE lipofuscin, this cannot be the case in that isolated diluted fluorophores still exhibit emission peaks ranging from yellow-green to orange-red in dilute solutions, consistent with the emissions from the *in situ* granules [37]. Fluorophores isolated from liver and heart age pigments also exhibited long-wavelength emissions, but differed from RPE fluorophores in their chromatographic mobilities [38].

The fluorescent components of human RPE age pigments have been extracted, separated, and spectrally analyzed [37]. We subsequently succeeded in the purification, characterization, and synthesis of two orange-emitting fluorophores, one found predominantly in the decaying outer segment debris of the RCS rat retina [39], and the other found exclusively in the RPE age pigments [40]. The compounds proved to be derivatives of vitamin A. Through a Schiff base reaction, two molecules of retinaldehyde bond with either one molecule of ethanolamine (A2-E) or phosphatidylethanolamine (A2-PE) [40-42]. Further work has established the structure of these compounds as pyridinium bisretinoids that have never before been encountered [43].

With this discovery, for the first time a marker molecule is known, which can be used in the study of the formation and fate of RPE lipofuscin. Its structure immediately suggested a mechanism for its initial formation, a molecular mechanism for the accumulation of lipofuscin granules, and a molecular explanation for the subsequent processes that could link RPE lipofuscin accumulation with AMD.

Mechanism of formation of A2-E

The formation of A2-E is envisioned as follows [41]: *trans*-retinaldehyde is released from rhodopsin upon capture of a photon of light. This retinaldehyde is normally converted to *trans*-retinol by a photoreceptor outer segment *trans*-specific retinol dehydrogenase [44]. This oxidoreductase utilizes NADPH as a source of reducing equivalents [44]. If for some reason, *trans*-retinaldehyde escapes reduction

(eg., low NADPH availability or bright light overwhelming the oxidoreductase system), it is then free to react further with available amines. In some circumstances, it may recombine with opsin [45]. Otherwise, it has been demonstrated that *trans*-retinaldehyde will preferrentially remain within the hydrophobic environment of the lipid bilayer. Only upon disruption of the bilayer is it seen to be capable of reacting with the ethanolamine of phosphatidylethanolamine [46]. Thus, the reaction is likely to proceed only under rare conditions in the POS if disc membrane disruption occurs (as in the decomposing photoreceptor outer segment debris of the RCS rat [39], or possibly in threshold light damage [47, 48]). More likely, the reaction occurs after phagocytosis and the membrane begins to be disassembled within the acidic environment of the RPE lysosome.

The reactions between retinaldehyde and ethanolamine are Schiff base reactions and are acid catalyzed [49]. The 1:1 retinaldehyde:ethanolamine reaction product is not detected in great abundance. Normally, Schiff base reaction products are quite unstable, especially in the presence of water, and either go on to dissociate or further react [50]. In this case, it can react with another retinaldehyde to form A2-E. Ethanolamine presumably derived from disc membrane phosphatidylethanolamine [51], appears to be the preferred reactive amine.

Once formed, A2-E is a charged quaternary amine and cannot readily leave the lysosome, and the normal contingent of lysosomal enzymes are not capable of degrading this compound. Therefore, it will remain within the lysosome until high concentrations are reached.

That it takes years for the age-pigments to form is not surprising. The reaction of retinaldehyde with the photoreceptor phospholipid must be a relatively rare incident. After absorption of a photon by rhodopsin, there is a distinct intermediate step in which the "bleached" chromophore is reduced to retinol. Only if a molecule of all-*trans* retinaldehyde escapes this reduction, could the fluorophore A2-E form. Normally there is sufficient oxidoreductase present to convert all retinaldehyde that is released to retinol. Even if a molecule of retinaldehyde remains unreduced, the membrane must be opened up or dusrupted for the aldehyde end of the molecule to gain access to the hydrophilic ethanolamine head group of the phospholipids. Then the reaction must proceed under acidic conditions. Once the 1:1 product is formed, conditions must be right for yet another unreduced retinaldehyde molecule to arrive and react before the 1:1 product has a chance to hydrolyze into its starting components. When the system is operating properly, these circumstances should rarely, if ever, arise.

Effect of A2-E on lysosomal proteolysis

The question remains as to whether A2-E can actually cause lysosome malfunction and residue accumulation. We synthesized A2-E, complexed it with LDL, and administered it to cultured fibroblasts [43]. In this manner the A2-E is delivered directly to the fibroblast lysosome via the LDL receptor mechanism. Lysosomal, but not extralysosomal protein degradation was significantly inhibited.

The mechanism of this inhibitory effect has yet to be determined. Primary, secondary and tertiary amines are weak bases. At neutral pH, they are able to diffuse throughout the cell. Upon reaching acidic compartments, these amines become protonated and trapped because of their charge. This proton trapping is capable of elevating the lysosomal pH outside the pH optimum of the proteolytic enzymes, and substrates accumulate. The ability of weakly basic amines to inhibit lysosomal function by such a mechanism was first envisioned and demonstrated by the discoverer of the lysosomes, Christian deDuve [52]. Since then hundreds of compounds have been classified as lysosomotropic amines and have become primary tools in the investigation of lysosomal function [53].

A2-E, however, is a quaternary nitrogen compound with a fixed positive charge. Because it is formed within the lysosome, it will remain there. If its counterion were a hydroxyl ion, it might be expected that it behaved as an even stronger base. However, hydroxyl anions are unlikely to exist under physiological conditions. The more likely counterion to A2-E is chloride ion in that this ion is transported as a counterion to the hydrogen ions by the lysosomal proton pump [54]. Thus, A2-E is more likely to exist as a neutral salt. Other quaternary ammonium compounds have been shown to inhibit lysosomal function [55]. The mechanism is not yet well-defined, but may involve substrate complexation [56] as opposed to specific enzyme inhibition.

We have also shown that A2-E and other retinaldehyde:ethanolamine reaction products are capable of photodynamic activation of oxygen [57, 58]. Other lysosomally targetted compounds used in photodynamic therapy of tumors have been shown to deactivate lysosomal enzymes upon photic stimulation [59]. In that light of the proper wavelength for A2-E activation reaches the RPE *in vivo* [60], this may be an additional mechanism of lysosomal inhibition specific to the RPE.

In short, we have now shown that A2-E will inhibit lysosomal enzyme function. This is consistent with the past inability to find any defective enzymes responsible for RPE lipofuscin buildup, and it is consistent with the lack of any one specific storage product accumulating in the residual bodies.

A2-E as a "lysosomotropic"-detergent

Another observation that is explicable by A2-E effect on lysosomes is the reported sigmoidal buildup of granules over time [17]. A2-E is not only a quaternary nitrogen compound, but it also possesses two hydrophobic side groups (the two vitamin A moieties) that in combination with the hydrophilic pyridinium group and ethanolic side chain, give the compound the amphiphilic structure of a cationic detergent (surfactant). One very characteristic feature of similar lysosomotropic detergents is that they display sigmoidal dose-response curves [61]. They accumulate within the lysosome over time and upon reaching an upper limit related to the critical micelle concentration of the detergent, they begin to disrupt the lysosomal membrane, causing leakage. This could readily explain why the lipofuscin granules in the RPE exhibit a sigmoidal curve in their rate of accumulation and seem to accumulate benignly over long periods of time.

Consequences of leakage

If at some point A2-E leaked from the lysosome, either through a detergent effect [61] or a photodynamic effect [62], it could be expected to redistribute throughout the cell. Among the sites that it could insert is the plasma membrane.

When charged amphiphilic compounds insert into plasma membranes, they cause it to bend and eventually vesiculate [63]. In tests of the effects of A2-E on model RBC membrane systems, we have demonstrated that it, too, will cause a predictable directional shedding of membrane vesicles [43]. This type of shedding behavior by RPE cells is precisely what is thought to be involved in the formation of the basal laminar and linear deposits in Bruch's membrane that can lead to drusen formation, the clinical harbinger of AMD [64, 65].

Potential for lysosomotropic amine involvement in lipofuscinogenesis in other tissues

Reaction of amines and aldehydes is already widely believed to be a cause of lipofuscinogenesis in other tissues [66, 67]. Yet no such products have been directly identified from any source of lipofuscin granule. Nor have comparisons between in vitro lipid oxidation products and tissue lipofuscin granule contents been as rigorous as they should be [33]. Therefore it is not yet possible to determine to what extent lipid oxidation reactions are responsible for age pigment formation in specific ageing tissues. Pigments generated *in vitro* under highly prooxidant conditions [67] or *in vivo* under severe dietary antioxidant deficiency [34] may be more akin to ceroid than to age pigments [68].

Nonetheless, aldehydes arising from lipid oxidation may still react with amines. Although Schiff base reaction products are unstable, they can further react to form stable endproducts. The work by Kikugawa and colleagues [69] demonstrates formation of stable heterocyclic nitrogen compounds that might be capable of acting as lysosomotropic amines or detergents. Potentially any primary amine should be reactive. For reasons that are not yet clear, ethanolamine derived from phosphatidylethanolamine seems to be the favored amine in retinal pigment epithelial lipofuscinogenesis. It is interesting to note that N-acylphosphatidyl-ethanolamines that are catabolized to free N-acylethanolamines, have been noted to arise in degenerative BHK cells [70], myocardial infarct tissues [71], and in cerebral ischemia [72]. It must be re-emphasized, however, that such weakly basic amines or cationic amphiphiles must be directly demonstrated in specific tissue lipofuscin granules before concluding that such a mechanism is at play in other tissues.

Conclusions

The work on RPE lipofuscin granules has resulted in several new findings. This is the first time that any autofluorescent compound has been isolated, purified and identified from any naturally occurring lipofuscin granule. This also is the first time that any inhibitor of lysosomal proteolysis has been identified from a naturally occurring lipofuscin granule. While lysosomotropic amines and detergents have been synthesized and utilized widely as research tools and pharmaceuticals, this is the first demonstration of a

naturally ocurring compound with similar properties. The structure of A2-E represents a new class of retinoid that has never before been described. It may be acting as a photosensitizer in the cell. Finally, this represents the first time that a potential direct biochemical link has been discovered between lipofuscin granule accumulation and an age-related disease process. Whether these findings hold potential for increasing our understanding of lipofuscinogenesis in other tissues remains to be seen.

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Inoue: Because Ito cells (fat storing stellate cells) in the liver are the major site for storage of carotenoid, similar fluorescent metabolites would also be found in the lysosomes of hepatocytes and/or Ito cells if photochemical reaction does not underlie the mechanism of its biosynthesis. Do you see similar reaction products in the liver?

Eldred: I have not looked at liver lipofuscin recently and do not know whether A2-E accumulates there. I suggest that it probably does not and that the lipofuscin composition will differ from one tissue to the next. Of all of the tissues other than the eye, that accumulate lipofuscin, I would be less surprised to find A2-E in hepatocytes because of their role in vitamin A metabolism. However, only the aldehyde form will be reactive, and I do not think that it should be present in adequate amounts in these cells to form A2-E.

Tatariunas: Can you show me a slide of the original intrinsic fluorescence spectrum of complex retinoid and ethanolamine?

Eldred: The full excitation and emission spectra of all of the fluorescent fractions that we have separated have been published (Exp. Eye Res., 47:71-84, 1988).

Brunk: Have you any evidence of apoptotic cell death of RPE cells in age related macular degeneration?

Eldred: Apoptotic cell death has been seen in the electron microscopic studies of Dr. Lynette Feeney-Burns. It occurs particularly in the RPE cells that overlie large hard drusen. These cells fragment apically and basally, and she has reported that the fragments are phagocytosed by the functional RPE surrounding the drusen. Dr. Charlotte Reme also observed apoptotic cell death (esp. chromatin condensation) after light damage, and RPE apoptosis lags photoreceptor cell apoptosis by about 24 hours. I do not think that apoptotic fragmentation accounts for the shedding of membranous vesicles by the basal surface of the RPE during basal laminar and basal linear deposit formation in Bruch's membrane. This is a much slower process that follows lipofuscin accumulation. I feel that the A2-E induced shedding mechanism may be a more likely mechanism in this case.

Kikugawa: Formation of the pyridinium salt A2-E by reaction of retinal and ethanolamine is very interesting. I think this reaction is common to alpha, ß-unsaturated aldehydes. How do you think about this?

Eldred: I believe that this is true. In the case of retinaldehyde the presence of an appropriately placed methyl branch promotes the cyclization reaction. A short chain alpha,ß-unsaturated

aldehyde or a branched aldehyde may be more likely to cyclize in this manner to form the pyridinium ring. The structure of the amine could also promote ring closure in some cases.

Zs.-Nagy: Can you tell us more details about the nature of the ESR signals you presented in your lecture?

Eldred: Yes. I am sorry that time did not allow more detailed discussion of these results. This work was done in collaboration with Dr. James Dillon at Columbia University in New York and his coworkers. The results are to be published in Photochemistry and Photobiology. The particular specra that I showed indicated the formation of carbon-centered radicals upon illumination of retinaldehyde-ethanolamine reaction products. In the absence of oxygen, hydrogen was abstracted from the solvent (methanol) resulting in free radical formation. In the presence of oxygen, other oxygen based radicals were generated as well. A variety of other studies have shown the photosensitizing abilities of certain components of RPE lipofuscin granules as well.

Nakano: I am very interested in the sigmoidal accumulation of the pigments. Do you have any speculation on this sigmoidal accumulation?

Eldred: When Dr. Feeney-Burns first described this phenomenon, it was a surprise. As shown, there is very little accumulation during the first 10 years of life. Then in the next 10 years, there is a dramatic rise in the amount of lipofuscin followed by a much slower rate of accumulation, thereafter. Why this occurs is a mystery. Dr. Fite in her work with Japanese quails has shown that RPE lipofuscin formation occurs to a greater extent in female than male quails, implying involvement of sex steroids. Perhaps puberty has an effect on human RPE lipofuscin formation, but I have no idea how this should be so. It is during the time of slow accumulation later that I feel may be an indication that A2-E could start to leak out and initiate basal shedding.