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LABORATORY INVESTIGATION

Accumulation of A2-E in mitochondrial membranes of cultured RPE cells

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Abstract
Background Lipofuscin occurs in association with various blinding diseases, including ARMD. Formation of lipofuscin is considered to be initiated by the inability of the RPE lysosome to degrade constituents of phagocytosed material resulting in its intralysosomal accumulation. Thus, the deposition of abnormal retinoid adducts causing the autofluorescent properties of RPE lipofuscin originates from abnormal products of the retinoid cycle contained in phagocytosed photoreceptor outer segments. The major lipofuscin retinoid conjugate A2-E was previously shown to exert toxic effects on RPE cells by directly damaging lysosomal function and structure. However, A2-E was also proposed to severely harm extralysosomal RPE cell structures during the pathogenesis of ARMD. This would require release or leakage of A2-E from the lysosomal compartment with subsequent targeting of other cellular compartments.
Methods We therefore now investigated intralysosomal accumulation, possible biodegradation, release from the lysosomal compartment and intracellular spreading of ^{14}C-labelled A2-E in cultured human RPE cells. We specifically loaded lysosomes of cultured human RPE cells with [^{14}C]A2-E.
Results A linear increase of intracellular radioactivity was observed during the 4-week loading period. Cell fractionation experiments indicated that more than 90% of loaded A2-E was specifically accumulating in the lysosomes. After loading, the fate of the radioactive label was chased over a period of an additional 4 weeks. No metabolism or secretion of A2-E to the medium was detectable. Subcellular fractionation revealed that during the chase period, about 13% were shifted from the lysosomes to mitochondrial fractions. This effect was strikingly intensified when after loading the cells with the labeled retinoid, its intralysosomal concentration was boosted by an additional load with non-labeled A2-E. Thus about 44% of the label were located in mitochondria at the end of the chase period. No significant spreading to other cell compartments was detectable.
Conclusions Since A2-E was suggested to act as a proapoptotic molecule via a mitochondrial pathway, we postulate that upon reaching a critical intralysosomal concentration, A2-E is released from the lysosome and then specifically targets the outer mitochondrial membrane thereby initiating apoptosis of the RPE cell. This may also apply correspondingly to other lipofuscin-associated molecules that cause leakage of the lysosomal membrane.

Keywords Lipofuscin · A2-E · Age-related macular degeneration · Mitochondria

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Introduction

Age-related macular degeneration (ARMD) is now the leading cause of registerable blindness in the Western

nations beyond 50 years of age [15]. The molecular basis of the disease process is still incompletely understood. Although ARMD is considered to have a multifactorial pathogenesis, several lines of evidence suggest that ageing changes of the retinal pigment epithelium (RPE) and Bruch's membrane play a key role during development of the disease [14]. Thus, early stage disease presents symptomatically with (i) a build-up of abnormal material in the lysosomal compartment of macular RPE cells, finally forming lipofuscin granules, and (ii) formation of drusen, that is extracellular deposits accumulating below the RPE monolayer in the inner portion of Bruch's membrane [13, 14]. In postmitotic RPE cells, autofluorescent lipofuscin granules accumulate with age as a byproduct of constant phagocytosis of membranous discs shed from distal photoreceptor outer segments (POS) [3], and clinical findings confirm that lipofuscin accumulations precede the development and enlargement of outer retinal atrophy in eyes with ARMD [13]. Formation of lipofuscin is considered to be caused by the inability of the RPE lysosome to degrade constituents of phagocytosed material. Thus lipofuscinogenesis may be initiated by phagocytosed compounds that are either stable towards hydrolytic attack of lysosomal enzymes, or affect the proper function of the lysosomal apparatus [16]. Stabilization of phagocytosed proteins towards lysosomal attack is most likely caused by postranslational modification and subsequent inter- or intramolecular crosslinking [25], whereas non-degradable lipoid-like compounds may originate as inadvertent products of the retinoid cycle [9]. The resulting abnormal retinoid adducts cause the bright autofluorescent properties of RPE lipofuscin [35]. The major fluorophore was identified as a pyridinium bis-retinoid, 2-[2,6-dimethyl-8-82,6,6-trimethyl-1-cyclohexen-1-yl)-1*E*, 3*E*,5*E*,7*E*-octatetraenyl]-1-(2-hydroxyethyl)-4-[4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1*E*,3*E*,5*E*-hexatrienyl]-pyridinium, which is derived from two molecules of vitamin A aldehyde and one molecule of ethanolamine and therefore named A2-E [7, 21]. Various A2-E derived photoisomers have been identified to date [17]. A2-E is considered a "prototype" RPE-damaging lipofuscin compound, since it is a marker of lipofuscin accumulation and exerts various toxic effects on RPE cells [16, 17, 29]. Thus, the compound impairs degradative lysosomal hydrolytic capacities for major catabolic pathways [12], acts as a photoinducible free radical generator [23] and lysosome damaging detergent [24], impairs phagocytosis and autophagy [2], and damages DNA [30]. Apoptotic cell death of RPE induced by A2-E action on mitochondrial membranes was also suggested to be involved in the pathogenesis of ARMD [27, 33].

Early studies suggested that biosynthesis of A2-E probably takes place in the acidic environment of the lysosome after phagocytosis of outer segment disks containing all-trans-retinal and phospatidyl ethanolamine [7]. More recent investigations proposed biosynthetic pathways of A2-E not occurring in the lysosome but in the photoreceptor outer segments [1, 18, 19, 21]. Nevertheless, irrespective of being formed in the RPE lysosome from phagocytosed precursors or being synthesized in the POS and subsequently delivered to the RPE lysosome by phagocytosis, in any case the lysosomal compartment represents the primary site of A2-E deposition in the RPE.

Once deposited, A2-E is presumed to be unable to exit the lysosome and the lysosome would not be capable of degrading A2-E, so it must accumulate in the lysosome [8]. Recent studies have shown an age-dependent build-up of A2-E in the lysosomal compartment [29, 36]. However, some of the damaging effects described for A2-E require the compound`s action on other cellular compartments, including mitochondria and the plasma membrane [26, 28, 33]. This would presuppose release of the compound from the lysosome and its subsequent accumulation in the target compartments. In deed, studies on the effects of A2-E on the latency of isolated lysosomes suggest that A2-E upon reaching a critical concentration can disintegrate the lysosomal membrane, thereby causing its leakage to the cytoplasm [24]. Nevertheless, direct experimental evidence for such intracellular spreading in intact cells is still missing.

We now investigated intralysosomal accumulation, possible biodegradation, release from the lysosomal compartment and intracellular spreading of ^{14}C-labelled A2-E in cultured human RPE cells.

Materials and methods

A2-E and [^{14}C]-A2-E synthesis and cell culture

A2-E was synthesised by coupling *all-trans* retinaldehyde (Sigma, Steinheim, Germany) to ethanolamine (Fluka, Buchs, Switzerland) (ratio 2:1) according to the procedure of Parish et al. [21]. [^{14}C]A2-E was analogously prepared with [^{14}C]-ethanolamine (Hartmann, Braunschweig, Germany) and *all-trans* retinaldehyde as starting material. Specific radioactivity of the compound applied in the loading experiments was 17 MBq/nmol.

Cell culture

Primary cultures of human RPE cells were isolated and maintained according to published standard procedures [12]. For [^{14}C]-A2-E-loading the compound was complexed with LDL-particles and loaded to cultured RPE cells by LDL-receptor-mediated endocytosis exactly as described previously for the unlabeled retinoid [12, 23]. This procedure provides efficient and highly specific loading of

A2-E to the lysosomal compartment [12]. Cell viability was monitored using the tetrazolium dye-reduction assay (MTT; 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). The test was performed as described by Mosmann [20]. Absorption was measured by a scanning multiwell spectrophotometer at 550 nm (Rainbow, Tecan Spectra, Crailsheim, Germany).

Cell fractionation studies

For fractionation into subcellular organelles RPE cells from a single culture flask (75 cm^2) were harvested by trypsinization, suspended in 5 mM HEPES, pH 7.6/0.25 M sucrose/0.2 mM EDTA, and disrupted by nitrogen cavitation (10 min, 20 bar). The homogenate was centrifuged at 1000 g for 10 min and the resulting postnuclear supernatant of 2 ml fractionated on 35 ml of self-generating gradient of 30% (vol/vol) Percoll (Pharmacia, Freiburg, Germany) in 5 mM HEPES, pH 7.6/0.25 M sucrose/0.2 mM EDTA in a Sorvall rotor T-865 (25,000 rpm for 40 min) at 4°C [32]. Fractions (0.8 ml) were collected from the top of the gradient and analysed by marker enzyme activity assays and by radioactivity measurements.

Marker enzyme assays

Alkaline phosphodiesterase (plasma membrane marker) was assayed with the substrate thymidine 5′ monophosphate p-nitrophenylester [32] and β-hexosaminidase (lysosomal marker) was measured fluorimetrically with the substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside [32]. UDP-galactosyltransferase (marker of ER and Golgi) and succinate dehydrogenase (mitochondrial marker) were determined according to Graham [10], lactate dehydrogenase (cytosol marker) acording to Storrie and Madden [32].

A2-E extraction and thin layer chromatography

For extraction of [^{14}C]A2-E RPE-cells were solubilised in 0.2 M ammonium hydroxide and the resulting solution lyophilised. The remainder was extracted twice with chloroform/methanol (1:1, v/v) and the extracts dried in a stream of nitrogen. Then the samples were redissolved in methanol and analysed by thin layer chromatography on silica 60 plates using the 11 componenent mobile phase system exactly as described by Eldred and Lasky [7].

Radioactivity measurements

Radioactive bands on thin layer chromatograms were detected by phosphoimaging (Cyclon Phospoimager, Packard, Merriden). For quantitation, the bands were scraped from the plates, extracted with methanol and radioactivity determined in a liquid scintillation counter (Tricarb 2900; Packard, Merriden). For measurement of radioactivity in cells or cell fractions the samples were disolved in 0.2 M NaOH, mixed with Ultima Gold Cocktail (Packard) and measured in the liquid scintillation counter. Quench correction was always by the tSIE-method.

Results

Pulse-chase experiments with radioactively labeled A2-E were conducted in cultured RPE cells to track its intracellular accumulation, its possible degradation or release to the medium, as well as its subcellular distribution during the chase. A linear accumulation of A2-E during the pulse was observed (Fig. 1). When [^{14}C]A2-E was extracted from the cells after 2 weeks of chase and analysed by thin layer chromatography all radioactivity was associated with a band corresponding to A2-E, indicating that no A2-E degradation had occured (data not shown). During the chase, only a minor decrease in the intracellular radioactivity was observed. This loss of radioactivity from viable cells results from cell death of about 10% of the cells during the chase phase as quantitated by MTT tests in parallel cultures treated equally (data not shown). Thus, no active release of A2-E or of metabolites resulting from lysosomal degradation was detectable.

During the 4 weeks loading period A2-E was efficiently targeted to the lysosomes, since more than 90% of radioactive A2-E was found in lysosomal fractions, as shown by cell fractionation experiments subsequent to loading of A2-E (Fig. 2). Only minor amounts were detectable in fractions representing mitochondria and plasma membranes.

Throughout the 4-week chase period after loading, radioactivity completely disappeared from the plasma membranes, whereas a progressive increase in mitochondria associated radioactivity was found (Fig. 2b and c). All other cell fractions contained no detectable A2-E, either immediately after loading (Fig. 2a) or through the chase (Fig. 2b and c). The partial relocation of A2-E from lysosomes to mitochondria might be explained by the detergent-like properties of A2-E, which upon reaching a critical intralysosomal concentration would harm the lysosomal membrane and cause leakage of A2-E to the cytoplasm, where it then becomes available for trapping by other organelles. In order to test this, a second set of experiments was conducted. After the 4-week period of [^{14}C]-A2-E-loading intralysosomal A2-E concentration was boosted by additional loading with a high concentration of unlabeled A2-E and possible translocation of radioactive A2-E from lysosomes to other cellular compartments was monitored by cell fractionation. A striking shift of radioactive A2-E from lysosomes to the

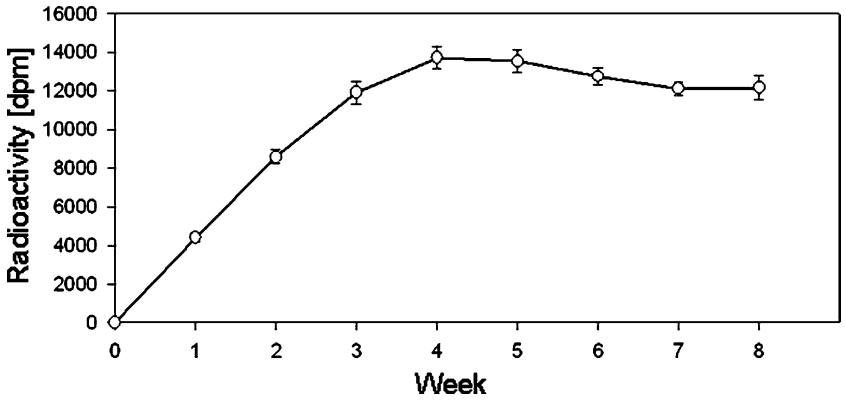

Fig. 1 *Intracellular accumulation of[^{14}C]A2-E* RPE cells were fed with the [^{14}C]A2-E-LDL complex for 4 weeks (medium change weekly with 2 kBq/ml medium). Cells were harvested at the indicated time points of loading (pulse period) as well as during the following 4 weeks of chase in normal medium (chase period). Harvested cells were counted for intracellular radioactivity as described in material and methods. Results are the mean of three determinations ±SD

mitochondrial fraction was observed, whereas radioactivity disappeared from the plasma membranes (Fig. 3a–c).

Discussion

In the course of photoreceptor renewal, photoreceptor outer segment membranes are shed, phagocytosed by the RPE and degraded enzymatically in the lysosomal compartment. Due to the task of digesting POS every day, the postmitotic RPE cell in the macular region, compared with all other cells in the body, has the largest amount of material to digest and dispose. Although the lysosome is equipped with a vast array of hydrolases capable of degrading all regular macromolecules constituting the POS, abnormal compounds contained in the phagocytosed material may be resistant to lysosomal attack, resulting in incomplete degradation and intralysosomal accumulation of these macromolecules. In postmitotic cells, ongoing delivery of even low amounts of undegradable material over time results in a build-up of stored material as a function of time, finally resulting in the deposition of complex heteropolymeric aggregates, termed lipofuscin [3, 34]. Besides acting as precursors for lipofuscin formation, altered or unprecedented POS compounds may also directly damage the RPE cells [31]. This concept is largely supported by investigations of the cellular actions of A2-E. On the one hand this compound was discovered as a major retinoidconstituent of lipofuscin accumulations, on the other hand it exerts various direct toxic effects on RPE cells [16].

Evidence that A2-E, due to its unprecedented structure, cannot be eliminated from the RPE cell, came from investigations with abcr-/-mice which upon light exposure accumulate A2-E in RPE. A2-E levels remained unchanged when the mice were subsequently transferred to darkness [19]. We now used a previously established test system, where the lysosomes of cultured RPE cells can be specifically loaded with A2-E by complexing the water-insoluble compound with low-density lipoprotein [12], which is efficiently transported to the lysosomes via receptor-mediated endocytosis, to directly follow up the compound's intracellular fate. Thus, we used this method to load radioactively labeled A2-E to the lysosomes of cultured RPE cells, allowing to trace the compound by sensitive radioactivity measurements.

The linear increase in cellular A2-E during the 4 weeks loading period demonstrates the efficiency of the method and cell fractionation immediately after loading proves specific targeting to the lysosomes. No secretion of A2-E to the culture medium was detectable and no radioactive metabolites were found, indicating that once accumulated, A2-E can neither be discarded nor be metabolized.

A minor portion of A2-E was located in the plasma membrane fraction immediately after loading. If leakage of A2-E from the lysosome would be causative for this plasma membrane targeting, an increase of radioactivity in plasma membrane fractions during ongoing chase would be expected. However radioactivity totally disappeared from this cell fraction, while lysosomal A2-E remained constant. Thus contamination of the plasma membrane by a minor accidental release of A2-E from LDL-particles during the phagocytosis process may have occured. This contamination is presumably eliminated by normal membrane turnover. Even after boosting the intralysosamal A2-E concentrations as conducted in the second set of loading experiments, no relocation of [^{14}C]A2-E from the lysosomal compartment to plasma membranes was detectable. Thus plasma membranes are not specifically targeted by A2-E after its leakage from lysosomes, and consequently direct A2-E-induced damage of the plasma membrane appears unlikely.

A progressive increase in mitochondria-associated radioactivity was observed during the 4 weeks chase following

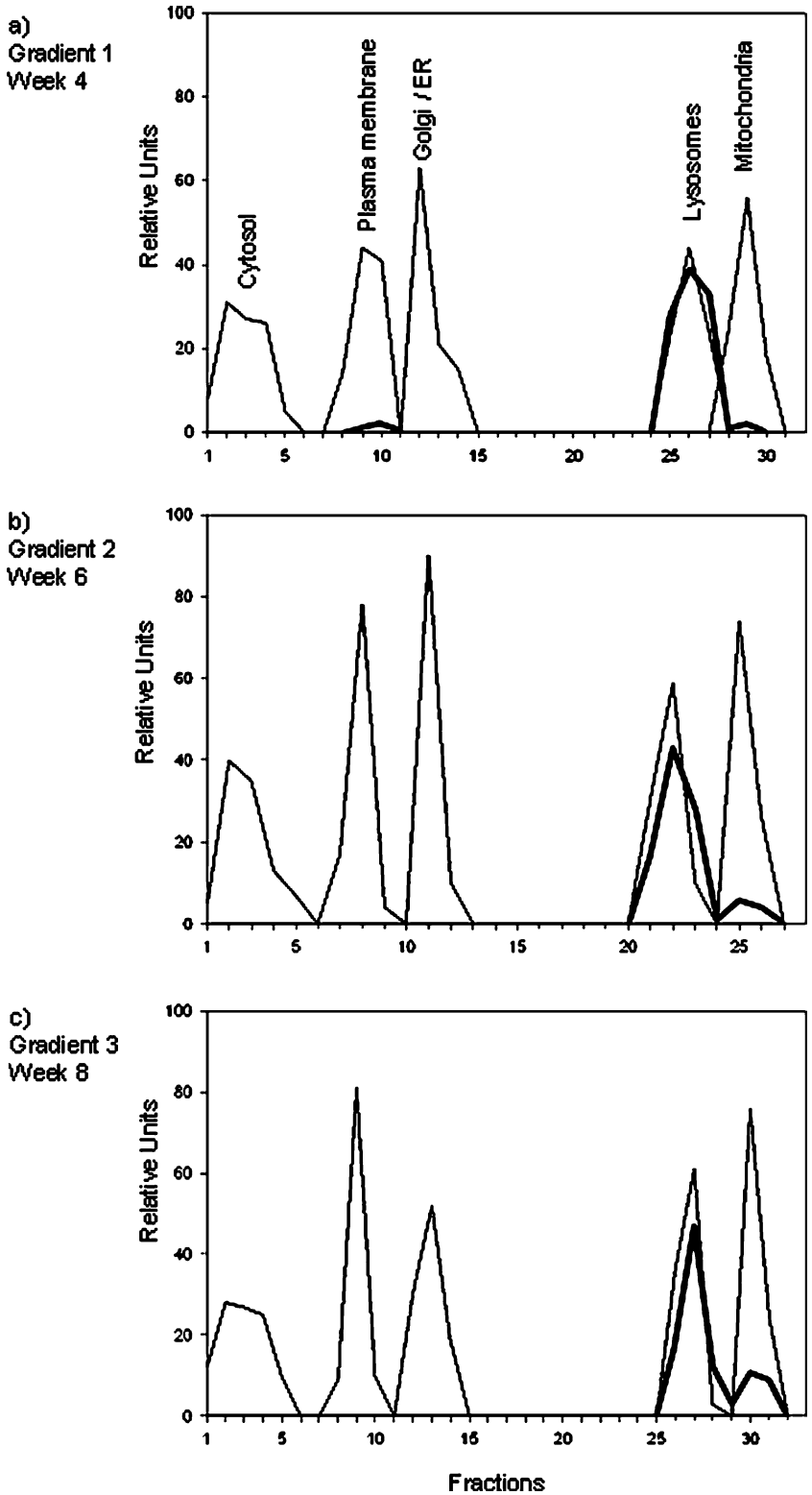

Fig. 2 Subcellular distribution of labelled [^{14}C]A2-E. Cells were loaded with [^{14}C]A2-E for 4 weeks as described above. Then subcellular distribution of radioactivity was chased during the following 4 weeks of culture in medium without [^{14}C]A2-E. Cells were harvested immediately after loading (**a**), after 2 weeks of chase (**b**) and after 4 weeks of chase (**c**). After harvesting, the cells were disrupted by nitrogen cavitation and subcellular fractions isolated by density gradient centrifugations. Radioactivity and activity of marker enzymes were measured as described in materials and methods. Total activities were set to a relative value of 100. The bold line represents distribution of [^{14}C] A2-E related radioactivity recovered cell-associated radioactivity at the end of pulse was 14,800 dpm) with regard to the different cellular compartments and their enzyme marker activities. Experiments were repeated twice

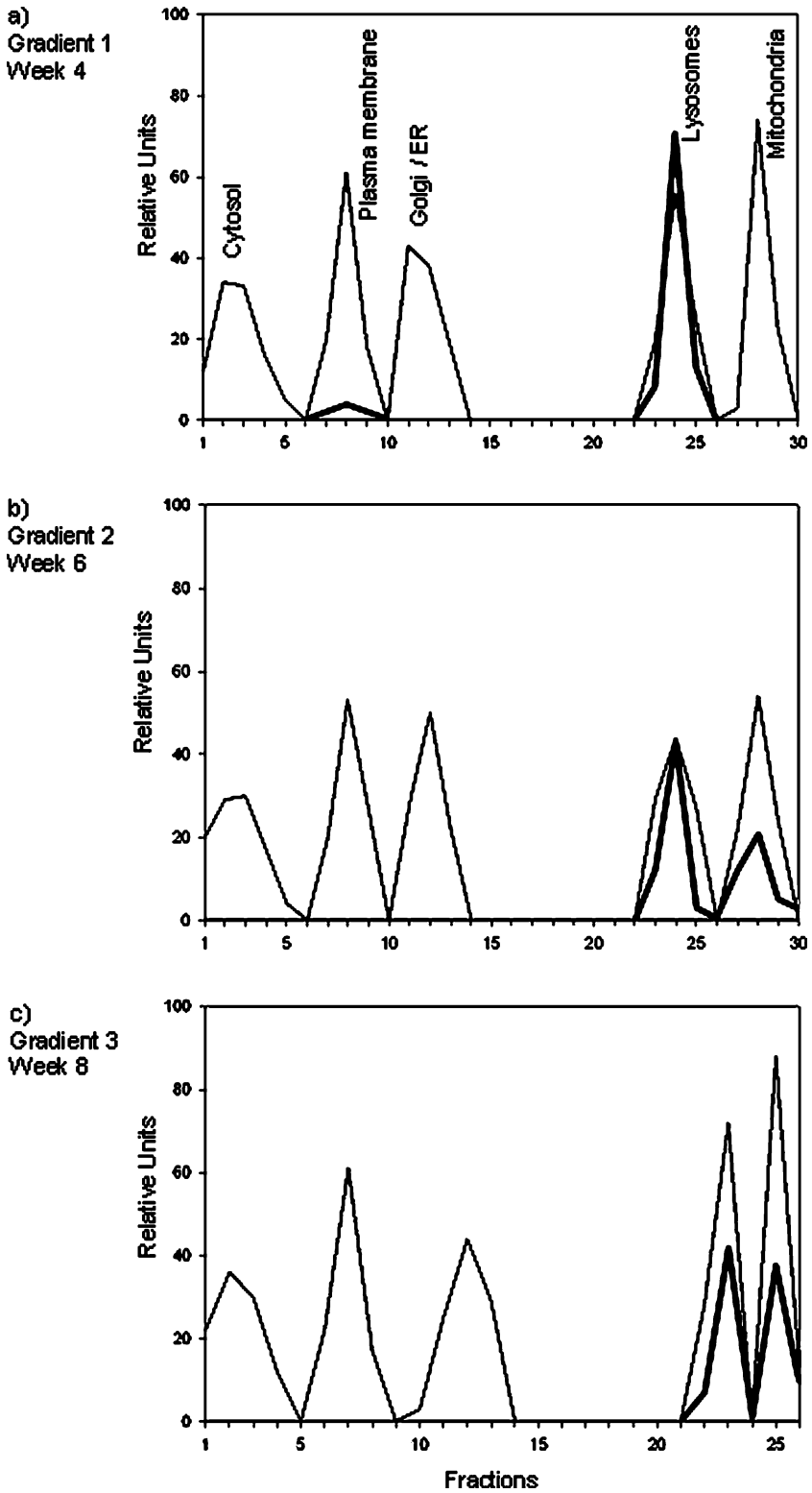

Fig. 3 *Effect of A2-E boost on subcellular distribution of labelled [^{14}C]A2-E* The experiment was conducted as described in the legend of Fig. 2 (total recovered cell-associated radioactivity at the end of pulse was 15,600 dpm) except that at the beginning of the chase period the cells were charged with an additional load of unlabelled A2-E (7 nmol A2-E in 1 ml medium for 1 week). Experiments were repeated twice

[^{14}C]A2-E loading of the lysosomal compartment. The shift of the radioactive label from lysosomes to mitochondria was strikingly enhanced when immediately after [^{14}C]A2-E loading, total intralysosomal A2-E concentration was boosted by an additional load with non-labeled A2-E. The structure of A2-E represents a cationic detergent and it was an early suggestion that it may act as an surfactant on cellular membranes [7]. Such action on the membrane of isolated RPE lysosomes was previously shown in latency experiments [24]. Our present results are in line with the idea that A2-E, due to its detergent-like properties, accumulates in the lysosomal membrane and thereby continually destabilizes the membrane. As a result upon reaching a critical concentration A2-E would leak from the lysosome. In consequence of its pronounced hydrophobicity, the released retinoid adduct will have to attain another hydrophobic environment, i.e. other intracellular membranes. Our results indicate that released A2-E is efficiently targeted to the mitochondrial membranes. The molecular basis underlying the pronounced affinity of A2-E to mitochondria is unclear, but is likely to relate to the unique membrane characteristics of mitochondria as compared to other cellular organelles. In particular mitochondrial membranes differ from other biomembranes by their lipid composition and by their very different set of membrane proteins [22]. In the mitochondrial pathway of apoptosis, caspase activation is closely linked to mitochondrial outer membrane permeabilization. Numerous pro-apoptotic signal-transducing molecules and pathological stimuli converge on mitochondria to induce mitochondrial outer membrane permeabilization, which is considered a key event for apoptosis induction [11]. In vitro experiments with isolated cell organelles indicated that the integrity of mitochondrial outer membrane is affected at significantly lower concentrations of A2-E as compared with lysosomes and other cellular membranes [24]. This implies that in vivo cytoplasmic A2-E concentrations capable of affecting the mitochondrial outer membrane may be reached after lysosomal rupture. A2-E was already suggested to act as a proapoptotic molecule via a mitochondrial pathway, in particular by detaching proapoptotic proteins from mitochondria [26, 27, 33]. Therefore, we propose the following sequence of cellular events finally resulting in A2-E- induced apoptosis of RPE cells. A2-E deposits first in the lysosomal compartment, accumulates there without further metabolism, due to its detergent-like properties damages the lysosomal membrane upon reaching a critical concentration, leaks from the lysosomal compartment and then specifically targets the outer mitochondrial membrane, thereby causing release of proapoptotic proteins. The ability to induce such molecular order of events may be a common characteristic of compounds that induce lysosome-initiated apoptosis [4–6]. Since the A2-E effects on lysosomal and mitochondrial membranes are mainly based on its detergent-like properties which are unlikely to be unique to A2-E, various still unidentified lipofuscin-associated molecules may share these properties, thereby contributing to apoptotic death of RPE. The chloroform extractable material representing mainly lipids and lipoid-like substances constitutes about 20% of the entire dry weight of lipofuscin. The formation of occular lipofuscin lipoids are considered to result from the phagocytosis of POS that are rich vitamin A. Thus ocular lipofuscin seems to represent a unique class of lipofuscin [16], which partly contains vitamin A derivatives, in particular various derivatives of retinoids and carotenoids [35]. Retinoid adducts that are characteristically composed of nonaromatic ring structures, polyprenoid side chains and hydrophilic end groups have detergent-like properties. Unfortunately, besides A2-E and its derivatives, only very few other lipofuscin chloroform extractable molecules, including other all-trans-retinal dimer conjugates and retinylpalmitate, have been characterized yet [9, 17, 31]. The identification of such compounds and a detailed elucidation of their mode of action are nescessary to determine the role of lysosome-induced apoptosis of RPE in the complex pathophysiology of ARMD.

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