Iron-Induced Accumulation of Lipofuscin-like Fluorescent Pigment in the Retinal Pigment Epithelium

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Purpose. One of the most prominent changes that occurs in the retinal pigment epithelium during senescence is the progressive accumulation of the autofluorescent pigment lipofuscin. Experiments were conducted to evaluate the role of nonenzymatic oxidation of photoreceptor outer segments in retinal pigment epithelium lipofuscin formation.

Methods. Albino Fischer rats were given intravitreal injections of ferrous sulfate, a catalyst that promotes nonenzymatic lipid oxidation. At 2 hours, 24 hours, and 7 days after ferrous sulfate administration, the retinas were examined with fluorescence microscopy to assess the formation of fluorescent products. At these same time intervals, organic solvent extracts of the retinas and retinal pigment epithelium-choroid complexes were prepared. The extracts were analyzed with thin layer chromatography to assay for the presence of soluble fluorophores. The ultrastructural appearances of the retinas were examined at the same time points.

Results. At both 2 hours and 24 hours after the ferrous sulfate treatment, the photoreceptor outer segments displayed a yellow-green fluorescence emission that was not present in untreated eyes. Associated with this in situ fluorescence were a number of blue-green emitting fluorophores in organic solvent extracts that did not correspond to any of the fluorophores extracted from the retinal pigment epithelium of old animals. One week after the ferrous sulfate treatment, the photoreceptor cells had degenerated and the retinal pigment epithelium contained large amounts of an autofluorescent pigment with a golden-yellow emission typical of lipofuscin. The iron-induced fluorophores could not be extracted from this pigment into either chloroform or dichloromethane.

Conclusions. The initial fluorophores that were formed as a result of nonenzymatic oxidation of outer segment components did not appear to be the same as those responsible for retinal pigment epithelium lipofuscin fluorescence. However, after the oxidized outer segments were phagocytosed by the retinal pigment epithelium, the latter cells became filled with a yellow-emitting fluorescent pigment that was similar in its fluorescence properties to lipofuscin. These observations suggest that lipofuscin fluorophores are not direct products of nonenzymatic lipid oxidation. However, some of these oxidation products may be modified after uptake by the retinal pigment epithelium to form insoluble lipofuscin fluorophores. Invest Ophthalmol Vis Sci. 1993;34:3161–3171.

Membrane-bound inclusions with characteristic fluorescence properties accumulate in numerous types of postmitotic cells during senescence. These inclusions have been designated lipofuscin, or age pigment. It has been proposed that lipofuscin fluorophores are generated by reactions between aldehyde products of nonenzymatic lipid oxidation and tissue amines.[1–6] This hypothesis is based primarily on two lines of evidence. First, substantial numbers of lipofuscin-like autofluorescent inclusions accumulate in various cell types of animals that are fed diets deficient in antioxidant nutrients or subjected to pro-oxidative stress.[7–9] Evidence purporting to link autoxidation directly to lipofuscin fluorophore formation has been provided primarily by work with in vitro model systems.[1,2,6] In vitro oxidation of lipids, either alone or in the presence of various other cellular constituents, was found to generate chloroform-soluble fluorescent products

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with uncorrected fluorescence excitation and emission spectra similar to the spectra of extracts from lipofuscin.

The role of lipid autoxidation in lipofuscin fluorophore formation has been called into question by the demonstration that the true fluorescence spectral properties of lipofuscin, lipofuscin extracts, and individual lipofuscin fluorophores differ substantially from the chloroform-soluble fluorescent products generated by oxidation of cellular constituents in vitro.[10-12] The use of uncorrected spectrofluorometers that have very low sensitivities at longer emission wavelengths apparently masked the spectral differences in emission between lipofuscin or lipofuscin extracts and the direct products of lipid autoxidation. When the fluorescence spectra were corrected for instrumental bias, substantial differences in the fluorescence spectral properties of lipofuscin extracts and soluble products of in vitro oxidation of tissue components were observed.[13] Extractable lipofuscin fluorophores had peak emissions at significantly longer wavelengths than did the fluorescent products of lipid or tissue autoxidation. Based on these in vitro oxidation experiments, chloroform-soluble lipofuscin fluorophores do not appear to be direct products of lipid oxidative breakdown. The relationship between the fluorescent products generated by oxidation of tissue constituents and the compounds responsible for lipofuscin fluorescence remains to be determined.

The retinal pigment epithelium (RPE) of the eye is an ideal tissue in which to study the association of lipid autoxidation with lipofuscin fluorophore formation. During senescence, the RPE shows a progressive accumulation of lipofuscin.[14-16] A substantial body of evidence indicates that the precursors for RPE lipofuscin are components of the adjacent photoreceptor outer segments,[17-24] which are phagocytosed by the RPE as part of the normal turnover process. The photoreceptor outer segments are very rich in the highly unsaturated fatty acid docosahexaenoic acid, a 22-carbon fatty acid with six double bonds.[25] The more unsaturated a fatty acid, the more easily it is autoxidized.[26] Thus, the outer segments should be highly susceptible to oxidative damage. Experiments were conducted to characterize the fluorescent products generated by outer segment oxidation in vivo, and to evaluate the possible relationship between these fluorescent oxidation products and RPE lipofuscin fluorophores.

MATERIALS AND METHODS

Animals and Treatments

Male Fischer albino rats were obtained at 3 months of age from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). Animals were housed three per cage in the University facility for 1 to 3 weeks before treatment. The animals were all kept in the same room under a 12-hour:12-hour light/dark cycle. Illumination was provided by 75-W incandescent bulbs. Light levels were determined with a Lutron model LX-101 light meter (Markson Science, Phoenix, AZ) with the probe placed face-up on the bottoms of the animal cages. During the light phase of the cycle, average illuminance measured at the cage bottoms was 10 to 15 lux. Total darkness was maintained during each 12-hour dark cycle. The room in which the animals were housed was maintained at a constant temperature of 20°C. All investigations were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Each animal was given a single intravitreal injection of ferrous sulfate in one eye, and either no injection or an injection of saline vehicle in the other eye. The rats were anesthetized with intramuscular injections of a combination of ketamine (80 mg/kg/body weight) and xylazine (12 mg/kg/body weight). One eye of each animal was then injected with 3 ml of freshly prepared 20 mmol/l $FeSO_4$ in 150 mmol/l NaCl. In some rats, the other eye was injected with the same volume of 150 mmol/l NaCl. All injections were made into the vitreous humor behind the lens.

Fluorescence Microscopy

At various time intervals after the intravitreal injections, rats were killed with CO_2 and their eyes were enucleated. Those eyes to be examined in cross-section were placed immediately in a fixative consisting of 1% paraformaldehyde, 1.25% glutaraldehyde, 130 mmol/l sodium cacodylate, and 130 μmol/l $CaCl_2$ at a pH of 7.4. The corneas, irises, and lenses were removed and the remaining portions of the eyes were gently agitated in the fixative at room temperature for 1 hour. The eyecups were then bisected in a plane parallel to the super-inferior meridian approximately 1 mm to one side of the optic nerve head. The larger portion of each tissue was washed for 10 minutes in 0.17 mol sodium cacodylate, pH 7.4, and then for 10 minutes in 1:1 cacodylate buffer:Tissue-Tek embedding medium (Miles Laboratories, Naperville, IL), and finally was embedded in Tissue-Tek embedding medium and frozen. Sections of the frozen tissues were cut with a Bright cryostat (Bright Instrument Co., Huntington, UK) along the super-inferior meridian at a thickness of 10 mm. The sections were mounted on glass slides in a solution consisting of 33% glycerol in 0.17 mol/l sodium cacodylate, pH 7.40. The sections were examined and photographed with a Zeiss Photomicroscope I (Carl Zeiss, Ober Kochen, Germany). equipped for epi-illumination. Fluorescent emissions were stimulated by light from a 100-W high-pressure mercury vapor source. The light employed for excita-

tion was passed through Zeiss filter set 487705, which consisted of a 400 to 440 nm band-pass excitation filter, an FT 460 dichromatic beam splitter and an LP 470 barrier. A 515 nm barrier filter was also placed in the emission path. Samples were photographed with Kodak EL 135 400 film (Eastman Kodak, Rochester, NY).

Flat preparations of the RPE were made from the eyes of some animals 1 week after the intravitreal injections. The rats were killed with CO_2 and the eyes were enucleated. The corneas, irises, lenses, and retinas were removed, leaving the eyecups lined by the RPE.[27] A series of radial cuts were made in the eyecups to enable them to lie fairly flat. The eyecups were mounted, RPE-side up, on glass slides in 0.17 mol/l sodium cacodylate buffer, pH 7.4. These preparations were then examined and photographed as described above for the sectioned tissues.

Fluorophore Extraction and Chromatography

At various times after treatment, analyses were performed to characterize the fluorophores that could be extracted from the retinas and RPE of treated and control eyes. Rats were killed with CO_2 and their eyes were enucleated immediately. The corneas, irises, and lenses were removed and discarded. The neural retinas were then dissected from the remainders of the eyecups. Thereafter, the neural retina and RPE eyecup samples were treated identically.

The retinas from two eyes were pooled in a glass homogenization sleeve containing 400 μl of ice-cold buffer of the following composition: 10 mmol/l HEPES; 150 mmol/l NaCl; 1 mmol/l Na_2EDTA; pH 7.40. The RPE-eyecups from the same eyes were pooled in another homogenization sleeve containing the same amount of buffer. The tissues were homogenized with approximately ten passes of a motor-driven Teflon pestle run at moderate speed. The homogenates were then each transferred to an 8-ml vial with a Teflon-lined cap. Soluble nonpolar components of the homogenates were extracted with dichloromethane. Each homogenate was vortex mixed for 60 seconds with 3.2 ml of 1:1 dichloromethane:methanol (v/v). Subsequently, 1.6 ml of dichloromethane was added to each sample and the vials were vortex mixed for another 60 seconds. Finally, 0.8 ml of water was added to each vial, the samples were vortex mixed again for 60 seconds, and the aqueous and organic phases of the extracts were separated by centrifuging the samples at 500*g* for 5 minutes. The dichloromethane-rich organic phase was removed and transferred to another vial. The aqueous phase of each sample was washed with 0.5 ml of dichloromethane, and the dichloromethane wash was removed and pooled with the first dichloromethane fraction. The fluorophore contents of both the organic and aqueous phases of each extract were analyzed with thin layer chromatography (TLC). Some samples were extracted using the chloroform–methanol procedure described by Folch and colleagues.[28] No differences in the fluorophore compositions of the extracts were observed between the two extraction procedures. Therefore, all analyses conducted for this study were performed using extracts obtained as described above.

The dichloromethane fraction of each extract was dried down under a stream of argon, and the residue was redissolved in 50 μl of 1:1 dichloromethane:methanol. The entire sample was spotted on a silica gel G TLC plate with a preabsorbent zone (Analtech, Newark, DE). The TLC plates were developed for 20 minutes in a mobile phase consisting of dichloromethane: methanol:water:acetic acid (5:1:0.1:0.03). After development, the plates were dried and photographed under ultraviolet illumination with maximum output at 366 nm.

The aqueous phase of each extract was lyophilized and the residue was resuspended in 50 μl of butanol:acetic acid:water (4:1:1). A 20-μl aliquot of each sample was spotted on a silica gel G TLC plate with a preadsorbent zone. The chromatograms were developed in butanol:acetic acid:water (4:1:1) for 100 minutes. After development, the plates were dried and photographed as described earlier.

Morphological Analyses

The effects of the $FeSO_4$ treatments on retinal morphology were examined at various times after iron administration. Rats were killed with CO_2 and their eyes were enucleated and placed in the mixed aldehyde fixative described earlier. The corneas, irises, and lenses were removed and discarded. The remainder of the eyecups were kept in the fixative at room temperature for 24 hours with gentle agitation. After fixation, a region of each retina just superior to the optic nerve head along the super-inferior meridian was dissected from each eye. This tissue was then postfixed with OsO_4 and embedded in epoxy resin.[16] Sections of the embedded tissue were cut at a thickness of 600 nm, stained with toluidine blue, and examined with Zeiss Axiophot microscope. Ultrathin sections were cut from the same blocks and were stained with uranyl acetate and lead citrate. The ultrathin sections were examined and photographed with a JEOL 1200 EX electron microscope (JEOL Ltd., Tokyo, Japan).

RESULTS

In Situ Iron-Induced Fluorescence in the Retina

As early as 2 hours after injection, $FeSO_4$ produced a pronounced fluorescence in the photoreceptor outer segments (Fig. 1). The outer segment fluorescence was

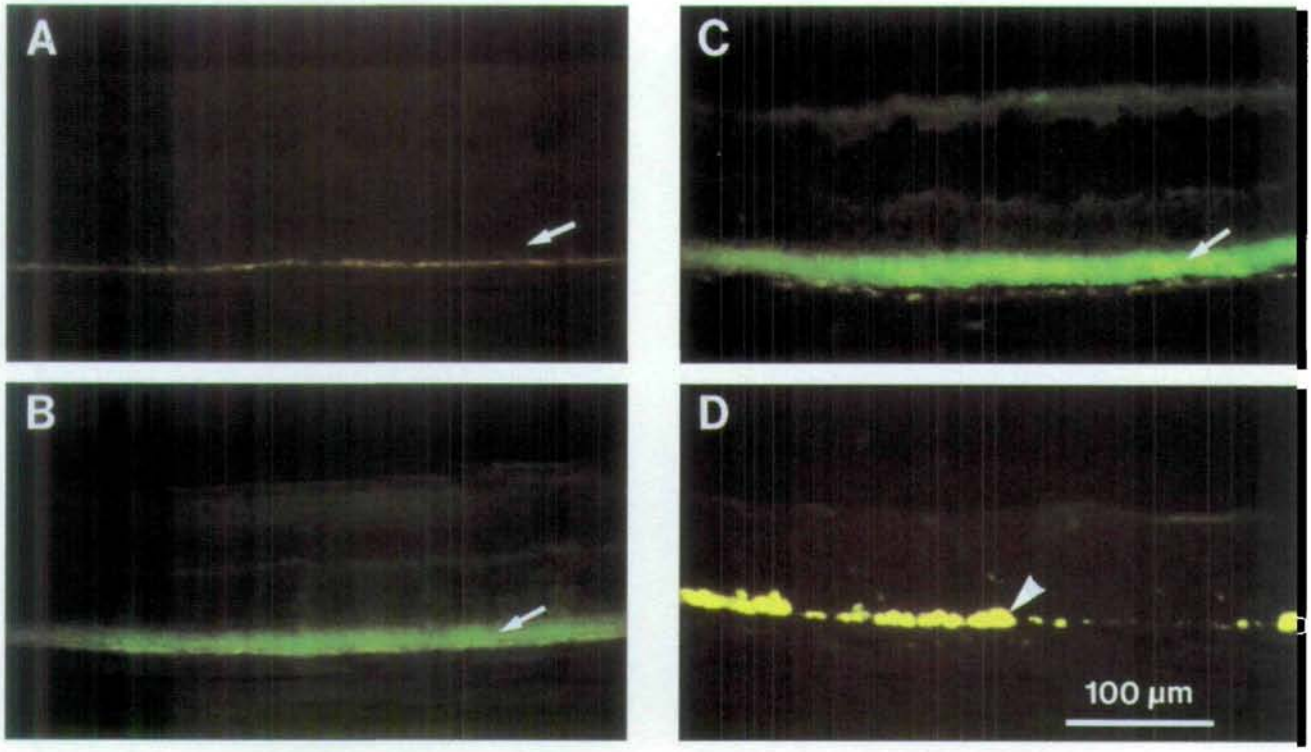

FIGURE 1. Fluorescence micrographs of cryostat sections of an untreated retina (**A**), and of retinas from iron-injected eyes at 2 hours (**B**), 24 hours (**C**), and 7 days (**D**) after injection. The photoreceptor outer segments are indicated by arrows, and the retinal pigment epithelium by an arrowhead.

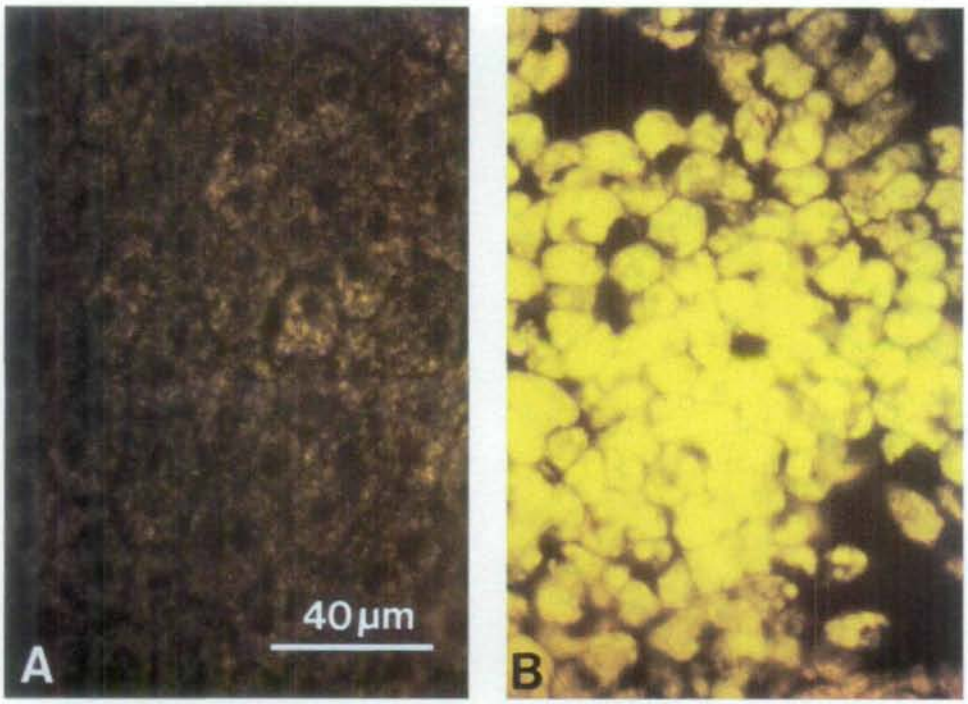

FIGURE 2. Fluorescence micrographs of whole-mount flat preparations of the retinal pigment epithelium from an untreated eye (**A**) and from an eye 1 week after it had been injected with $FeSO_4$ (**B**).

characterized by a green emission that was distinctly different from the gold-yellow emission of RPE lipofuscin. With the dose given, the iron-induced fluorescence was restricted to the outer segments and did not occur in other parts of the retina. The intensity of the outer segment fluorescence increased significantly by 1 day after treatment (Fig. 1). However, by 1 week after injection the outer segment fluorescence had disappeared from the retina. The RPE developed a substantial amount of a gold-yellow lipofuscin-like fluorescence 1 week after the $FeSO_4$ injections (Figs. 1, 2). The RPE-associated fluorescence was not uniformly distributed over the choroid, but was interspersed with nonfluorescent regions (Figs. 1, 2).

Effect of Iron on Retinal Morphology

Significant alterations in outer segment morphology were associated with the iron-induced outer segment fluorescence at both 2 and 24 hours after injection (Figs. 3, 4). The apical halves of the outer segments of treated eyes often appeared to have folded back on themselves and broken off from the basal portions of the outer segments. The resulting ball-like structures usually contained areas of disorganized and vesiculated disc membranes (Figs. 3, 4). One day after the $FeSO_4$ injection, many discs appeared to be in the process of swelling into tubular structures.

At 2 hours after injection, the RPE apical microvilli were disorganized, the cytoplasm contained numerous vacuoles, and the phagosome content of the RPE was increased (Fig. 3). After 24 hours, many RPE cells appeared to be undergoing severe degenerative changes (Fig. 3), whereas other cells appeared relatively normal except for the presence of large numbers of phagosomes and dark-staining inclusion bodies.

One week after the iron injections, the photoreceptor cells had almost completely disappeared from the retina. In addition, large areas of the choroid were denuded of RPE cells. Most of the remaining cells of the RPE were filled with abnormal inclusion bodies (Figs. 3D, 5). Many of these inclusions had banding patterns suggesting that they had been derived from phagocytosed rod outer segments (Fig. 5). The RPE cells remaining after 1 week had lost their normal hexagonal shape, and cell height was greatly increased in cells filled with autofluorescent inclusions. The morphology of the inner retina was affected much more moderately by the iron treatment, with a modest loss of cells that had nuclei in the inner nuclear layer.

Soluble Iron-Induced Fluorophores

Analyses were conducted to evaluate the chromatographic and spectral properties of the iron-induced fluorophores that could be extracted from the retina and RPE into dichloromethane. At 1 day after injection, several prominent blue-emitting bands were present on chromatograms of dichloromethane extracts of the neural retinas from the treated eyes (Fig. 6). These same fluorophores were sometimes present 2 hours after the iron treatments, but their intensity was less than that observed after 24 hours. The blue-emitting fluorophores were not present in the extracts from retinas that had not been exposed to $FeSO_4$ (Fig. 6). One week after the iron injections, when the autofluorescent outer segments had disappeared, the blue-emitting fluorophores were no longer present in extracts of the neural retinas. TLC of extracts from the control retinas contained a relatively nonpolar yellow-green–emitting component (Fig. 6), previously identified as retinol.[12] The retinol band was reduced in intensity at both 2 hours and 24 hours after $FeSO_4$ treatment, and was almost undetectable in the extracts of retinas 1 week after treatment with $FeSO_4$ (Fig. 6).

Dichloromethane extracts of the RPE-eyecups of untreated eyes contained a number of orange-emitting constituents that were separated from one another on TLC (Figs. 6, 7). These fluorophores appear to be components of RPE lipofuscin.[12] The amounts of these compounds that were extracted with dichloromethane increased with the ages of the animals from which the eyes were obtained (Fig. 7). In addition, the extracts contained a yellow-green emitting component that migrated near the solvent front on TLC. This component has been identified as a mixture of the retinyl esters that are stored in the RPE.[12] Iron treatment did not result in an increase in the amounts of any of the age-related orange-emitting fluorophores extracted from the RPE. In fact, the intensities of these fluorescent bands on TLC appeared to be slightly diminished after the iron treatment (Fig. 6). The intensity of the retinyl ester-specific fluorescence declined progressively with time after the iron treatment. The blue-emitting fluorophores observed in extracts of iron-treated neural retinas after 1 day were not present in extracts of the RPE-choroid at any time.

TLC analysis was also performed to characterize any soluble polar fluorophores that may have been present in the aqueous phases of the extracts from iron-treated and control eyes. The aqueous phases of the extracts from the neural retinas of all the treatment groups each had a single yellow-green emitting component that had a very low chromatographic mobility. There was no difference in the fluorescence intensity of this component between any of the treatment groups. The chromatograms of the aqueous phases of the RPE-eyecup extracts each had a single yellow-emitting fluorescent component that also had a very low chromatographic mobility. Iron treatment did not appear to affect the amount of this component that was extracted.

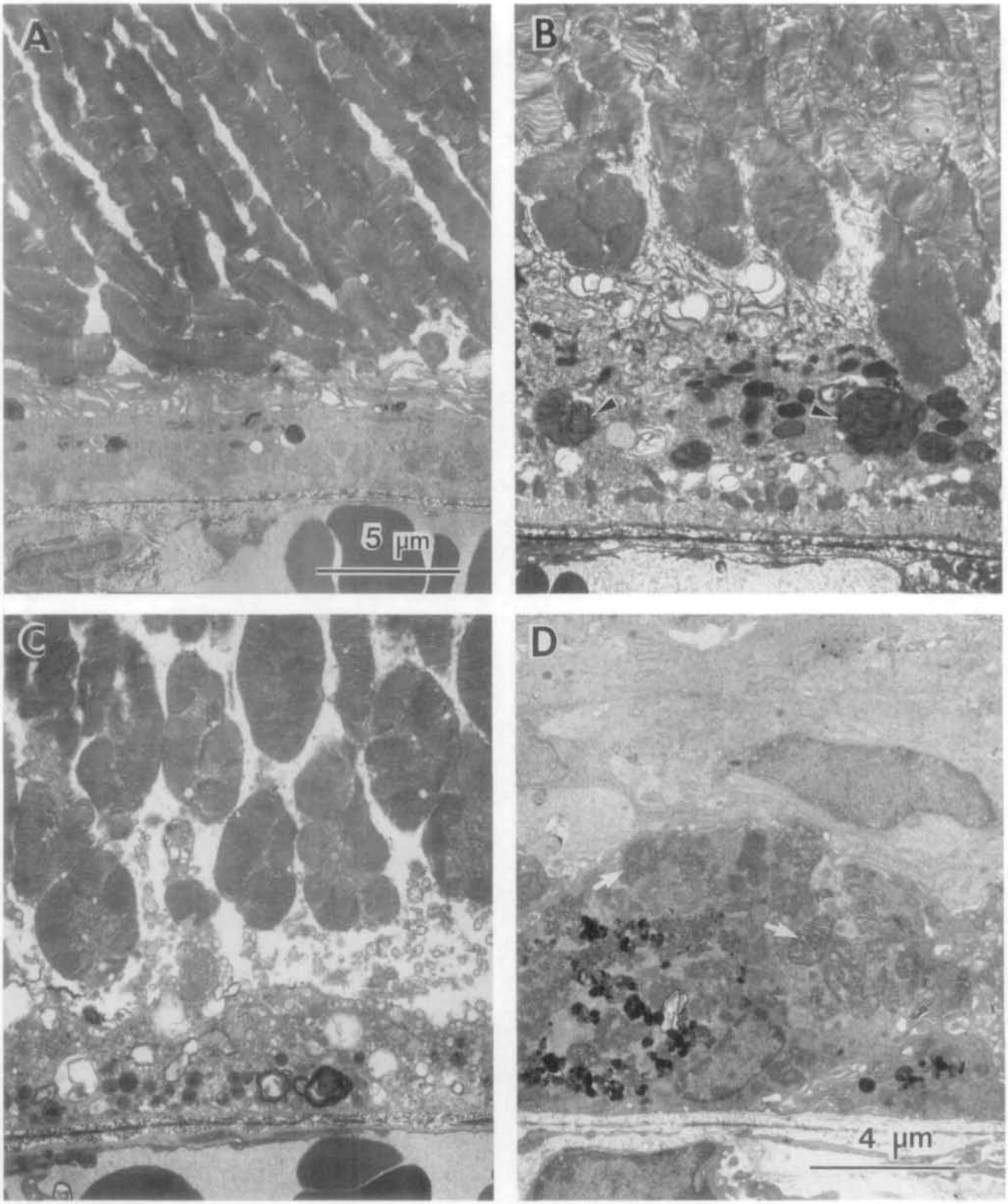

FIGURE 3. Electron micrographs of the retinal pigment epithelium and adjacent neural retina from an untreated eye (**A**) and from eyes 2 hours (**B**), 24 hours (**C**), and 7 days (**D**) after they had been injected with $FeSO_4$. Arrowheads in (**B**) indicate phagosomes. Arrows in (**D**) indicate iron-induced inclusions bodies with which the retinal pigment epithelium became filled. Bar in (**A**) indicates the magnification of all micrographs except (**D**).

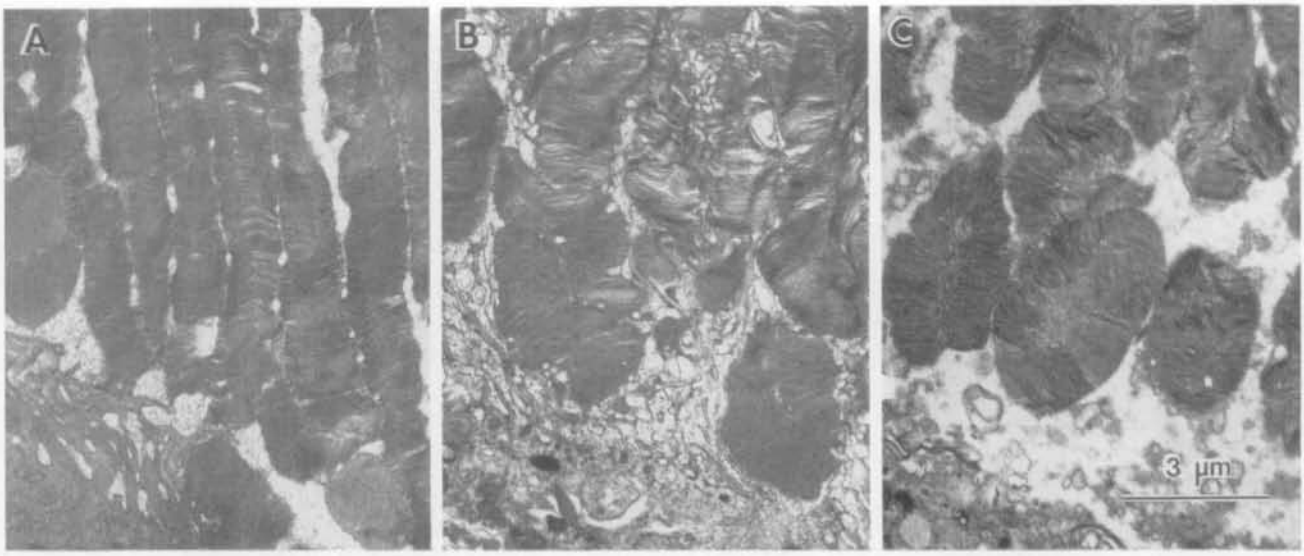

FIGURE 4. Electron micrographs of the apical outer segment regions of retinas from an untreated eye (**A**) and from eyes 2 hours (**B**) and 24 hours (**C**) after they had been injected with $FeSO_4$.

DISCUSSION

None of the compounds responsible for the autofluorescence of lipofuscin from the RPE or any other tissue have yet been definitively identified. Experiments with in vitro model systems have led to the hypothesis that at least some of the lipofuscin fluorophores are either direct products of lipid autoxidation, or are generated by the reaction of these products with proteins.[2,4–6] In particular, it was proposed that the major lipofuscin fluorophore is a conjugated Schiff base formed by reaction between amines such as those on proteins, and malonaldehyde, a product of lipid autoxidation.[1,2] The primary evidence supporting this conclusion is that the fluorescence properties of organic solvent extracts from tissues containing large amounts of lipofuscin are similar to the fluorescence properties of autoxidation products of tissue homogenates or of purified lipids or lipid-amino acid mixtures.[2,4–6] In a previous study, it was shown that the conclusions drawn from this evidence are incorrect, at least for retinal tissues. The

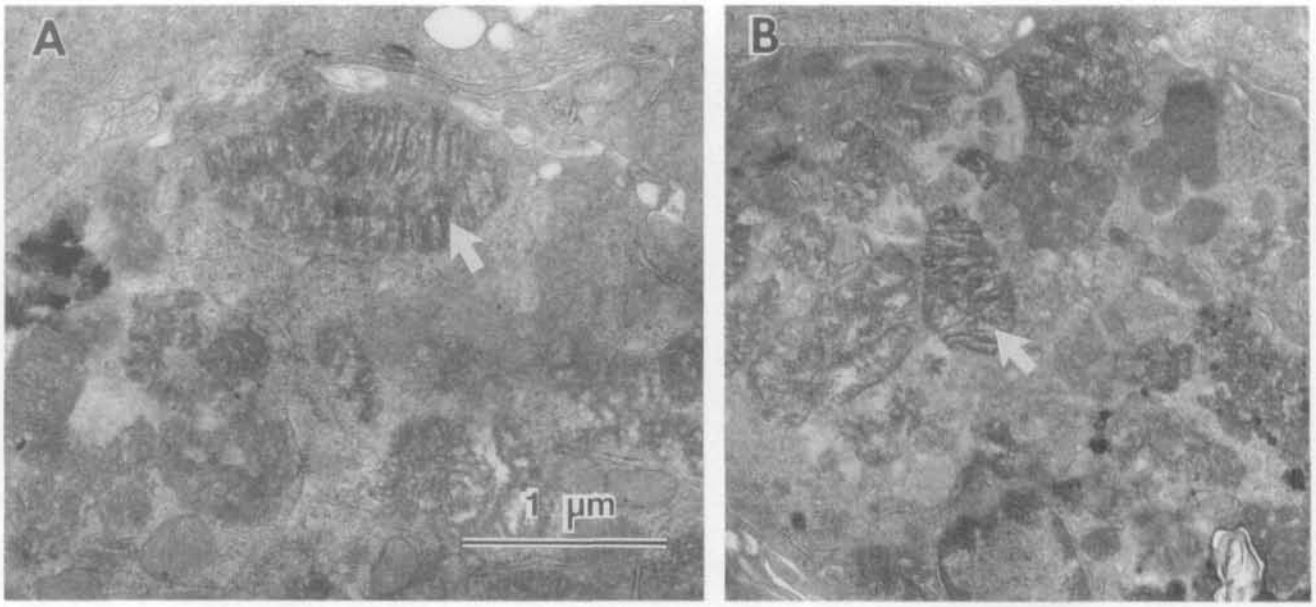

FIGURE 5. Electron micrographs of inclusion bodies (*arrows*) that were abundant in the retinal pigment epithelium 1 week after $FeSO_4$ treatments. These inclusions displayed striations suggestive of a photoreceptor outer segment origin.

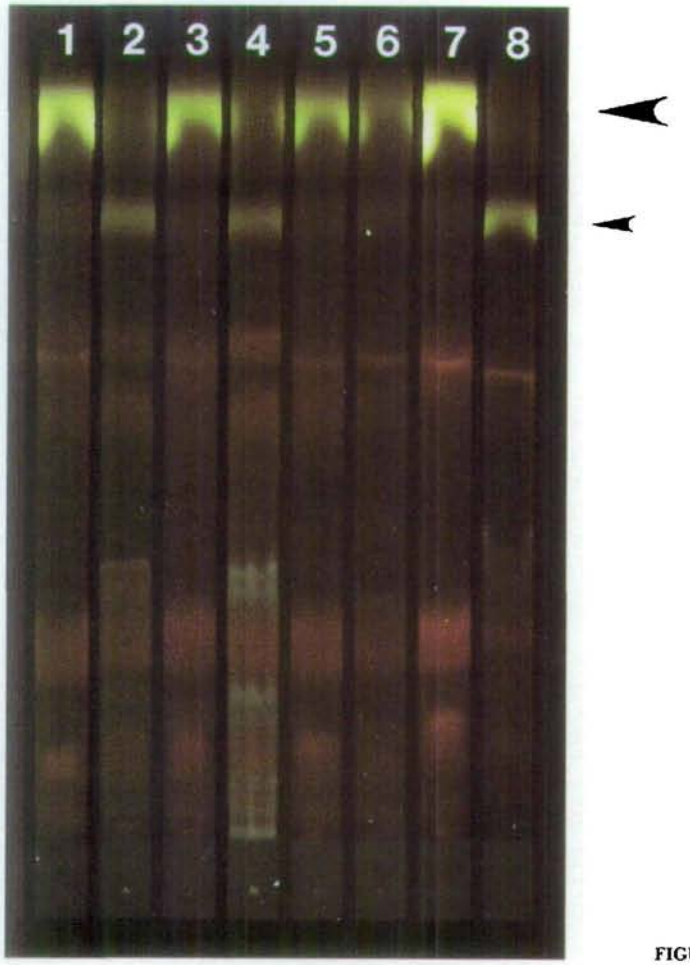

FIGURE 6. Thin layer chromatograms of dichloromethane extracts from untreated eyes and from eyes that had been injected with $FeSO_4$. The chromatograms were photographed under ultraviolet illumination. The lanes contain extracts from the following samples: (1) retinal pigment epithelium from Fe-injected eye 2 hours postinjection; (2) neural retina from Fe-injected eye 2 hours postinjection; (3) retinal pigment epithelium from Fe-injected eye 24 hours postinjection; (4) neural retina from Fe-injected eye 24 hours postinjection; (5) retinal pigment epithelium from Fe-injected eye 7 days postinjection; (6) neural retina from FE-injected eye 7 days postinjection; (7) retinal pigment epithelium from uninjected eye of a 4-month-old rat; and (8) neural retina from uninjected eye of a 4-month-old rat. The position of retinol is indicated by the smaller arrowhead, and that of the retinyl esters is indicated by the larger arrowhead.

FIGURE 7. Thin layer chromatograms of dichloromethane extracts from untreated eyes illustrating the effect of age on extractable fluorophores. The lanes contain extracts from the following samples: (1) retinal pigment epithelium from 4-month-old rat; (2) neural retina from 4-month-old rat; (3) retinal pigment epithelium from 26-month-old rat; and (4) neural retina from 24-month-old animal.

extractable fluorophores produced as a result of in vitro oxidation of neural retina or RPE preparations were blue-emitting compounds.[13] Their emission spectra were similar to those reported for the conjugated Schiff bases formed by reactions between malonaldehyde and amino acids (reported emission maximum 450 nm^2, which is clearly blue). These fluorescent oxidation products were not present in extracts of RPE rich in lipofuscin,[13] or in extracts of lipofuscin granules isolated from the human RPE.[12] Lipofuscin from all tissues has a peak fluorescence emission in the yellow to orange region of the spectrum,[10,11,29,30] and at least some of the yellow- and orange-emitting fluorophores can be extracted into organic solvents.[10,12,13] These soluble orange- and yellow-emitting compounds were not produced by in vitro oxidation of retinal or RPE homogenates.[13]

Although only blue-emitting extractable fluorophores are produced by in vitro oxidation of retinal tissues, dietary deficiency in antioxidant nutrients has been found to promote the accumulation of inclusions in the RPE that have a corrected fluorescence emission spectrum indistinguishable from that of lipofuscin.[11] This suggests that lipid autoxidation may yet be involved in the formation of yellow-emitting lipofuscin fluorophores, despite the fact that these fluorophores are not the initial products of lipid autoxidation. To evaluate this possibility, experiments were conducted

to characterize the fluorophores formed in the retina and RPE in situ in response to iron-catalyzed lipid autoxidation.

It is well established that ferrous iron promotes autoxidation of tissue lipids both in vitro and in vivo.[9,31] The more highly unsaturated a lipid is, the more susceptible it is to nonenzymatic oxidative degradation.[26] Because the outer segment disc membranes are rich in docosahexaenoic acid, they are particularly susceptible to autoxidation. Products of lipid autoxidation have been demonstrated in rod outer segments incubated in the presence of ferrous sulfate.[31]

At both 2 hours and 1 day after the ferrous sulfate treatments, the iron-induced fluorescence in the neural retina was restricted exclusively to the photoreceptor outer segments. Examination of retinal morphology at these two times also indicated that the early effects of iron-induced oxidative damage occurred primarily in the outer segments. Thus, the treatment-related fluorophores extracted from the retinas with dichloromethane at these times are likely to be oxidation products of the outer segment lipids, or at least to be derived from such products. The fluorophores extracted into dichloromethane displayed a blue emission when excited with ultraviolet light and were relatively polar, as judged by their chromatographic mobility. In these respects, they are similar to the fluorophores generated by incubation of retinal homogenates with ferrous sulfate in vitro.[13] None of the iron-induced extractable fluorophores were similar in both chromatographic mobility and fluorescence emission properties to any of the age-related fluorophores that could be extracted from the RPE. Thus, the initial dichloromethane-extractable fluorescent products of outer segment lipid autoxidation are clearly different in chemical composition from the fluorophores that can be extracted from lipofuscin.

No iron-induced fluorophores were detected in the aqueous phases of the extracts from the neural retinas that displayed iron-induced outer segment fluorescence. Thus, all of the soluble iron-induced fluorophores partitioned into the dichloromethane-rich phases of the extracts. It is possible that some of the iron-induced fluorophores in the outer segments were not solubilized by the extraction procedure that was used.[32]

One week after the ferrous sulfate treatments, the RPE cells remaining in the retina were filled with a yellow-emitting autofluorescent pigment, similar in its fluorescence properties to lipofuscin. This lipofuscin-like pigment is likely to have been derived from the oxidized outer segments phagocytosed by the RPE during the iron-induced photoreceptor degeneration. The blue-emitting fluorophores associated with the outer segments shortly after the iron treatment were not extracted from the RPE at 1 week after injection. This suggests that the blue fluorophores may have been modified after uptake by the RPE in such a way as to alter their fluorescence emission properties or extractability. It is possible that the blue-emitting fluorophores formed initially in the outer segments were converted into the yellow-emitting lipofuscin-like fluorophores on uptake by the RPE. Alternatively, the blue fluorophores may have been rapidly degraded or eliminated by the RPE, and the yellow-emitting compounds may have been formed through a pathway that differed from that by which the blue-emitting compounds were generated. It has been demonstrated that vitamin A plays a central role in RPE lipofuscin fluorophore formation.[17,19,33] It is possible that iron-induced oxidation of outer segment lipids promotes the incorporation of vitamin A into lipofuscin-like fluorophores that accumulate in the RPE. This possibility is consistent with previous experiments in which it was discovered that the accumulation of lipofuscin-like fluorophores in the RPE of vitamin E-deficient rats could be substantially reduced if the animals lacked the retinoids involved in the visual cycle.[17,33]

Although iron treatment resulted in the massive accumulation of a pigment in the RPE that had a lipofuscin-like fluorescence, there were no increases in the amounts of the dichloromethane-extractable orange fluorophores associated with RPE age pigment. Similarly, it has been reported that the lipofuscin-like fluorophores that accumulate in tissues in vivo as a result of vitamin E deficiency are largely insoluble in chloroform.[34] Like the iron-induced fluorescent pigment, the fluorophores from the RPE pigment resulting from vitamin E deficiency in animals fed normal levels of vitamin A were not extractable with chloroform-methanol.[35] It is clear that only a fraction of normal age pigment fluorophores are extractable with organic solvents,[12,32] but the proportion of the total lipofuscin fluorophores that cannot be extracted from the RPE is not known. Insoluble fluorophores are, however, significant components of normal RPE age pigment.[12] The relationship between the soluble and insoluble fluorophores is not currently known.

If the iron-induced RPE fluorophores are formed by a mechanism similar to that involved in age pigment formation, the chemical properties of these fluorescent compounds, including their solubilities, should be similar. Based on their fluorescence properties and their apparent derivation from components of the outer segments, it is likely that the insoluble yellow-emitting RPE fluorophores that form during normal aging and as a result of iron-induced autoxidation are similar. However, the iron-induced fluorescent pigment that accumulated in the RPE differs from normal RPE age pigment in ultrastructure and in the composition and content of extractable fluorophores; neither the orange-yellow chloroform-soluble fluoro-

phores nor the blue fluorophores in the aqueous phases of human RPE age pigment extracts[12] were present in the extracts of the RPE after iron treatment. Experiments with in vitro model systems suggest that the insoluble lipofuscin fluorophores consist primarily of proteins containing fluorescent adducts derived from oxidized lipids.[32] After long residence in secondary lysosomes in the RPE of aged rats, a fraction of these modified proteins may be partially degraded to yield autofluorescent fragments that are soluble in organic solvents. It will not be possible to determine with certainty whether the insoluble yellow-emitting fluorophores formed during aging and as a result of outer segment oxidation are identical until a means is found by which to solubilize these fluorophores so they can be purified and subjected to chemical structural analysis.

It is now clear that soluble fluorophores generated by the in vivo autoxidation of tissue lipids are not the compounds responsible for lipofuscin fluorescence. Thus, soluble lipofuscin fluorophores are probably not direct products of lipid autoxidation. However, oxidative damage to the outer segments does result in the accumulation of inclusions in the RPE with lipofuscin-like fluorescence. Therefore, it appears that autoxidation is indirectly involved in the formation of at least some RPE lipofuscin fluorophores. The precise mechanism by which iron-induced autoxidation promotes the accumulation of insoluble lipofuscin-like fluorophores in the RPE remains to be determined. Based on existing evidence, it appears that nonenzymatic oxidation of lipids generates a variety of products, some of which react with tissue proteins to form fluorescent adducts. The modified proteins are largely insoluble.[32] The presence of the oxidation-derived adducts probably impairs lysosomal degradation of the modified proteins, which accumulate in secondary lysosomes. During a long period, these stored proteins are probably partially degraded, and some of the fluorescent degradation products may become soluble. The proposed central role for proteins in the formation of lipofuscin fluorophores is supported by the fact that treatment of tissues with protease inhibitors in vivo results in the rapid accumulation of lipofuscin-like autofluorescent inclusions in the treated cells.[21,23,36]

That the iron treatments substantially reduced the amounts of retinoids in the retina and RPE (as determined by the fluorescence of retinoids on TLC) suggests that vitamin A oxidation may be involved in the formation of insoluble yellow-emitting fluorophores in the RPE. This is particularly likely in light of the fact that vitamin A deprivation significantly reduces both age-related lipofuscin fluorophore accumulation in the RPE, and the accumulation of insoluble lipofuscin-like fluorophores in the RPE of vitamin E-deficient rats.[17,33] One retinol oxidation product, vitamin A aldehyde, can spontaneously form adducts to protein lysine residues, and these adducts may be modified to render them fluorescent.

Key Words

autoxidation, photoreceptors, lipids, fluorophores, retina, aging

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