Oxidation Causes Melanin Fluorescence

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**Purpose.** The goal of this study is the characterization of the strong yellow fluorescence of oxidized melanin in the retinal pigment epithelium (RPE) and the choroid.

**Methods.** Naturally occurring melanin in the human retina and choroid was oxidized by exposing fixed and plastic-embedded sections of a human eye to light and hydrogen peroxide. Synthetic melanin was also oxidized in vitro by exposure to light and hydrogen peroxide. The fluorescence of oxidized melanin was examined by absorption spectroscopy, fluorescence spectroscopy, and fluorescence microscopy.

**Results.** Naturally occurring melanin oxidized in situ exhibited a lipofuscin-like yellow fluorescence. Oxidation of melanin in vitro degraded the melanin polymer, resulting in a fluorescent solution. Fluorescence spectroscopy gave an excitation maximum at approximately 470 nm and an emission maximum at approximately 540 nm for both natural and synthetic melanin. Increasing the time of exposure to light or hydrogen peroxide increased melanin fluorescence.

**Conclusions.** The results indicate that the strong yellow fluorescence of melanin in the RPE and choroid in situ is a property of oxidized melanin and is not due to contamination of the melanin by proteinaceous or lipid materials. The data presented allow a reinterpretation of the results obtained from fluorescence investigations of melanin-containing tissue and suggest a link between melanin degradation and lipofuscin formation. (Invest Ophthalmol Vis Sci. 2001;42:241–246)

The retinal pigment epithelium (RPE) of normal adult human eyes contains two populations of pigment granules: melanin and lipofuscin. Lipofuscin is a highly fluorescent mixture of different fluorophores, which reflects the heterogenous nature of this cellular pigment. It is derived, at least in part, from product of phagocytosed and degraded photoreceptor outer segments (POSs). Emphasis has been put on the fluorescence properties of lipofuscin as a means to identify these granules in situ and to understand the mechanisms of lipofuscin formation. Particularly, autofluorescence is used to distinguish lipofuscin from melanin in situ.

In contrast to lipofuscin autofluorescence, only a very faint fluorescent signal has been thoroughly investigated and differentially characterized for melanin isolated from human RPE from donors of different ages. Additionally, statements can be found in published reports that even deny melanin fluorescence. The large increase of melanin fluorescence after oxidative conditions is generally ignored in ophthalmic research.

Because in clinical ophthalmology retinal fluorescence, in particular the autofluorescence associated with the RPE and Bruch’s membrane, is used for diagnostic purposes, it is essential to understand whether natural and/or artificial conditions can lead to the development of fluorescence. In this study we characterized the fluorescence of melanin in situ after oxidative conditions were established and compared it with the fluorescence of synthetic melanin subjected to the same oxidative conditions.

**Methods**

**Fluorescence of Oxidized Melanin Embedded in Spurr’s Resin**

An eye of a 52-year-old healthy male human donor was fixed in glutaraldehyde; as previously described. After fixation, the vitreous body was removed, and specimens from the macular area were excised, washed in 0.1 M cacodylate buffer (pH 7.4) dehydrated in a graded series of ethanol and embedded in Spurr’s resin.

Additionally, two types of melanin were embedded directly into Spurr’s resin: synthetic melanin prepared by oxidation of tyrosine with hydrogen peroxide purchased from Sigma (Deisenhofen, Germany) and isolated bovine melanosomes from RPE and choroid, prepared as previously described (referred to as bovine melanosomes).

Samples of the two types of melanin were embedded into Spurr’s resin without previous incubation with glutaraldehyde to test the influence of glutaraldehyde incubation, the presence of associated proteins and lipids in natural melanin, and the cytoplasmic environment of the tissue on the fluorescence properties of the oxidized melanin.

Semithin sections (approximately 700 nm) were prepared without staining. Oxidation of semithin sections of specimens embedded in Spurr’s resin was performed using a solution of hydrogen peroxide dissolved in polypyrrole glycol-2000 (Fluka, Buchs, Switzerland), a minimally hydrophilic medium, so that only traces of hydrogen peroxide were dissolved in the medium. A volume of 500 μl polypyrrole glycol-2000 was mixed thoroughly with 100 μl of 30% hydrogen peroxide centrifuged to separate the phases, and the aqueous phase was discarded. Semithin sections were placed on a microscope slide; 5 to 10 μl hydrogen peroxide dissolved in polypyrrole glycol was added to the section, and the slide was coveredslipped. Fluorescence observations were begun immediately (before any significant oxidation had occurred) and continued for 8 minutes under a fluorescence microscope (400-nm excitation, 520-nm barrier; Axioplan; Zeiss, Oberkochen, Germany) at a magnification of ×63 in combination with a microscope camera (MC 100; Zeiss). After 8 minutes, the microscope was switched to bright-field illumination, the illuminated field diaphragm was opened maximally, and maximum brightness was achieved by selecting 12 V (100-W halogen lamp), resulting in a fluorescent illumination of approximately 500,000 lux, as measured with a photometer (Colormaster 3F; Gossen, Erlangen, Germany). The illumination of the specimens was continued for an additional 8 minutes after oxidation and repeatedly examined by fluorescence microscopy.

**Fluorescence of In Vitro Oxidized Melanin**

Synthetic melanin (2.5 mg/ml) and of isolated bovine melanosomes (2.5 mg/ml) were incubated with the organic oxidizing medium de-
A comparison of (C) and (D) reveals that the melanin granules (arrows) were nonfluorescent (or only slightly) at the beginning of the oxidation (C) and became intensely fluorescent after 8 minutes of oxidation (D). Autofluorescence in the basal part of the RPE was due to the oxidized melanin (compare C and D). Autofluorescence of lipofuscin and oxidized melanin was almost indistinguishable in color and intensity (D). Lipofuscin autofluorescence decreased slightly with illumination (compare B and D). All micrographs show the same area of the same section. Magnification, ×630.

RESULTS

Fluorescence of Oxidized Melanin Embedded in Spurr’s Resin

Human Ocular Tissue In Situ. Untreated specimens from the macular area of a human donor eye showed an accumulation of lipofuscin in the RPE, identified by its strong autofluorescence, whereas the choroid was free of lipofuscin-like autofluorescence (Fig. 1B). Nonoxidized melanin (Fig. 1A) did not fluoresce before oxidation (Fig. 1B); however, after 8 minutes of oxidation by hydrogen peroxide, melanin granules in the RPE and choroid exhibited a strong fluorescence reminiscent of lipofuscin in apparent color and intensity (Fig. 1D). Double exposure of phase contrast and fluorescence allowed the simultaneous localization of lipofuscin granules (yellow) and melanin granules (black). A slight fluorescence of melanin in RPE and choroid resulted from initial oxidation. (D) After 8 minutes of oxidation, melanin granules in the choroid displayed an intense lipofuscin-like fluorescence.

To analyze the large increase in fluorescence by the oxidized melanin, 2.5 mg/ml synthetic melanin was incubated with a 50% aqueous hydrogen peroxide solution for 4 hours.

Time-Response Study of Fluorescence

To analyze the large increase in fluorescence by the oxidized melanin, 2.5 mg/ml synthetic melanin was incubated with a 50% aqueous solution of hydrogen peroxide and sonicated for rapid dissolution. Aliquots of 1.5 ml were added to each of six petri dishes (diameter, 3.5 cm) and the increase in fluorescence (450-nm excitation, 580-nm emission) was measured using a fluorescence multimode plate reader (CytoFluor Series 4000; PerSeptive Biosystems–Applied Biosystems, Foster City, CA) for up to 90 minutes. A solution of 30% hydrogen peroxide was used as control. The first time point measured was at 2.5 minutes from the beginning of the oxidation process. Fluorescence of nonoxidized melanin was measured against a background of water.

Spectroscopy

Absorption Spectroscopy. Absorption spectroscopy (300–800 nm) of the resultant yellow solution was performed using a spectrophotometer (DU-6; Beckman, Irvine, CA) at a scanning speed of 150 nm/min against air. The absorption spectrum of the oxidized melanin was compared with the spectrum of a 2.5 mg/ml solution of nonoxidized synthetic melanin in water.

Fluorescence Spectroscopy. Fluorescence spectroscopy of the oxidized melanin was performed using a spectrophotometer (F-2000; Hitachi, San Jose, CA), at a scanning speed of 240 nm/min, a bandwidth of 10 nm, a photomultiplier (PM) voltage of 400 V and a list interval of 5 nm. The scanning data were corrected for the Raman signal of water. Excitation and emission maxima were determined by the spectrophotometer’s autoscan function. The excitation wavelength for the emission spectrum was 470 nm, and the emission wavelength for the excitation spectra was 540 nm.

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Fluorescence of In Vitro Oxidized Melanin

To exclude the possibility that embedding in Spurr’s resin causes melanin fluorescence after oxidation, synthetic and isolated bovine melanosomes were oxidized in vitro and examined for fluorescence.

Oxidation of both synthetic and isolated bovine melanosomes by 30% hydrogen peroxide (according to Frangioni and Borgioli11) resulted in a yellow-ocher hydrophilic liquid. The solid black oxidized melanin liquefied almost completely on oxidation, creating an aqueous phase underneath the organic phase of the oxidizing medium (data not shown).

Oxidized synthetic melanin appeared in phase-contrast microscopy as a clear aqueous liquid with no apparent impurities (Fig. 3A). However, oxidized isolated bovine melanosomes formed a heterogeneous mixture of oxidized liquid melanin and solid residual particles (Fig. 3C).

By fluorescence microscopy nonfixed, nonembedded, oxidized synthetic melanin or isolated melanosomes fluorescence was green (Figs. 5B, 3D), whereas the fluorescence of oxidized melanin in situ (Fig. 1) and embedded directly into Spurr’s resin was yellow (Fig. 2). The impurities of the oxidized bovine melanosomes did not appear to be fluorescent or were only weakly fluorescent (Fig. 3D).

Spectroscopy

Absorption Spectroscopy. The absorbance spectrum of nonoxidized melanin is characterized by uniform absorbance from 800 to 350 nm, followed by a steep increase at 350 nm. In contrast, the absorbance of the yellow solution of oxidized melanin was also uniform but lower than in nonoxidized melanin from 800 to 600 nm than increase, reached the level of nonoxidized melanin at 425 nm, and remained at this level until the steep increase in absorbance at 350 nm that was observed for nonoxidized melanin (Fig. 4).

Fluorescence Spectroscopy. Examination of the oxidized melanin species by fluorescence spectroscopy resulted in an excitation peak at 471 nm for synthetic melanin and at 469 for bovine melanin. The emission peak of synthetic melanin was at 548 nm and for bovine melanosomes at 543 nm (Fig. 5). Excitation and emission spectra of oxidized synthetic and bovine melanin were similar, except for the presence of two additional excitation peaks for oxidized bovine melanosomes at 400 and 420 nm, forming a broad excitation plateau (Fig. 5).
slower and partial physiological oxidation of melanin that occurs during a lifetime. Melanins and RPE cells have been shown to produce hydrogen peroxide and hydroxyl radicals when exposed to light, suggesting the fluorescence of the RPE melanin in vivo may be the result of oxidation resulting from the constant irradiation by light during a lifetime. Melanin also undergoes spontaneous autooxidation without the addition of exogenous hydrogen peroxide. Additionally, some diagnostics and research devices such as fundus photometry involve artificial light exposure. Comparison of the fluorescence intensities of oxidized melanin and the autofluorescence of lipofuscin (Fig. 1) justifies the attribution of strong autofluorescence of oxidized melanin.

It has been suggested that the fluorescence induced by oxidative stress in the melanin of human, rat, and bovine RPE cells and melanocytes in situ and in vitro is the result of contaminating proteins and lipids. The results of our studies, which show that synthetic melanin, which does not contain contaminating proteins or lipids, becomes fluorescent after oxidation, suggests that these impurities do not contribute, or may only partially contribute to the fluorescence observed in vivo. In the present study, this explanation was ruled out by comparing the fluorescence properties of natural melanin with the fluorescence properties of synthetic melanin. The melanoproteins tended to reduce the photoreactivity of melanin toward oxidizable substrates, such as reduced nicotinamide adenine dinucleotide phosphate (NADPH). When these are removed or disrupted, the melanosomes are much more photoreactive.

The excitation and emission maxima (approximately 470 and 540, respectively) were the same for synthetic and isolated bovine melanosomes (Fig. 5). Differences in the spectra related only to the excitation spectra. Bovine melanosomes showed an additional excitation plateau at approximately 400 nm that was missing in the spectrum of synthetic melanin and probably is evidence of contamination in the phase-contrast micrograph (Fig. 3C). However, the possibility that the excitation spectrum of pure natural melanin without contamination differs from the one of synthetic melanin cannot be excluded.

The apparent color and intensity of the fluorescence induced in melanin by oxidation as observed in histologic sections (Fig. 1) was very similar to the intensity and color of lipofuscin (Figs. 1B, 1D). In fact, it is difficult to distinguish between RPE melanin and RPE lipofuscin in Figure 1D after oxidation. However, comparing Figure 1C (RPE and choroid at the beginning of oxidation process) and Figure 1D (same view of the RPE and choroid after 8 minutes of oxidation) makes the clear differentiation of the two types of pigment granules obvious.

Fluorescence spectroscopy of melanin oxidized in vitro reveals an emission maximum at 540 nm, and this wavelength appears as greenish yellow. Similarly, a greenish fluorescence was observed by fluorescence microscopy for melanin oxidized in vitro (Fig. 3). However, oxidized isolated bovine melanosomes and synthetic melanin embedded in Spurr’s resin (Fig. 2) exhibited an orange-yellow fluorescence similar to that of melanin oxidized in situ in histologic sections (Fig. 1).

The difference in the apparent color of in situ oxidized melanin (Fig. 1) and melanin embedded in Spurr’s resin (Fig. 2; orange-yellow) and of in vitro oxidized melanin (greenish yellow; Figs. 3, 5) may be due to the different physicochemical environments or the different concentration of the fluorophore. Gallas and Eisner observed that melanin samples irradiated with UV laser light exhibit a blue-green luminescence, whereas when solid samples of similar melanin are irradiated by the same light, the luminescence appears yellow. Additionally, Boulton et al. reported different emission spectra for melanin samples suspended in saline and melanin prepared for microscopy.

The blue shift observed for liquid samples of melanin analyzed by spectrophotometry is also observed for lipofuscin. In situ lipofuscin analyzed by microfluorometry generally shows yellowish autofluorescence; however, the fluorescence maxima of lipofuscin extracts analyzed by spectrophotometry are reported to be in the blue region (400–500 nm).
In this study the organic hydrophobic oxidizing medium of Frangioni and Borgioli was used to oxidize melanin in vitro, and it dissolved the melanin and formed a yellow, highly fluorescent hydrophilic liquid. Oxidation of eumelanins by hydrogen peroxide also results in a yellow aqueous liquid. The organic hydrophobic oxidizing medium of Frangioni and Borgioli was originally designed to eliminate melanin from heavily pigmented specimens prepared for histologic examination. (The term “bleaching” has been used in publications for at least three different concepts: 1) decreasing fluorescence; 2) decreasing light absorbance; and 3) complete elimination of pigment. In the present article we avoid the term “bleaching” by actually describing what we mean.)

By phase-contrast microscopy it is possible to observe the dissolution of melanin in situ exposed to oxidative conditions. Single melanin granules, for example, in the RPE, become translucent and finally merge with adjacent melanin drops (data not shown).

In 1943, Sachs was the first to report that pigments of various origin become fluorescent after oxidation with hydrogen peroxide. He reported the appearance of a yellow fluorescence in human RPE cells after incubation with hydrogen peroxide. In contrast to our findings, Sachs could not demonstrate the same effect for choroidal melanin. He termed the choroidal melanin “real melanin” and RPE melanin “fuscin,” because “real melanin” did not show any fluorescence, even after incubation with hydrogen peroxide. Contrary to Sachs, our data presented in Figure 1 demonstrate the strong autofluorescence of choroidal melanin after incubation with hydrogen peroxide. Fluorescence of neuromelanin after incubation with hydrogen peroxide was described by Barden in 1969, but the spectroscopic characterization remained controversial. Katz et al. reported the appearance of yellow-orange fluorescence in melanocytes of the choroid after incubation with permanganate and speculated that the choroidal fluorescence was due to melanin.

The fluorescence spectra (Fig. 5) of melanin fluorescence we observed after exposure to oxidative stress are similar to the data for the weak autofluorescence of nonoxidized melanin described by Gallas and Eisner, which suggests that the weak autofluorescence of melanin may simply reflect the beginning of its oxidation. This hypothesis is supported by the observation of the same authors that fluorescence of melanin is associated with structural defects of the melanin polymer. Incubation of melanin with hydrogen peroxide leads to an oxidative degradation of the melanin polymer. Thus, detection of melanin autofluorescence in skin, hair, and blood may also be explained by oxidation of melanin in these tissues. We also observed photoinduced melanin fluorescence of histologic sections without the addition of exogenous hydrogen peroxide, but this was an infrequent observation (data not shown). Similar observations have been reported by the groups of Cathy K. Dorey (personal communication, May 1999) and Jan Borovansky.

Our findings have significant implications for a wide range of investigations dealing with the detection and quantification of lipofuscin-like autofluorescence in melanin-containing tissue. Because RPE cells, as well as other melanin-containing cells are frequently exposed to laboratory light and variation in oxygen concentration in culture, the appearance of fluorescent material can be explained by the oxidation of melanin due to nonphysiological conditions during culture.

Another important issue is the potential role of oxidized reactive melanosomes in producing graphophotic stress to ocular tissues. Because of its low oxidation potential damage to melanosomes would likely lead to melanin oxidation, increase its fluorescent signal (possibly being confounded with lipofuscin), and increase graphic–oxidative stress to RPE cells through the photochemical reactions of melanin.

Boulton et al. report single excitation and emission peaks for human fetal melanin granules and the appearance of a second excitation and emission peak in melanin granules in adult donor eyes, with a progressive increase of melanin fluorescence intensity with increasing age. These additional excitation and emission peaks are similar to those of oxidized melanin we report herein and may be due to the accumulation of oxidized melanin in the adult eye, which is exposed to a high intracellular oxygen concentration and light over a lifetime.

The melanin fluorescence observed after oxidative stress and irradiation with light also affects investigations of in vivo fluorescence of the ocular fundus, as performed by Delori et al. The authors report that intrinsic fundus fluorescence results from at least two fluorophores: a dominant fluorophore with peak emission at 630 nm and a minor fluorophore with peak emission at 540 nm when excited at 470 nm. The spectral properties of this minor fluorophore (emission peak at 540 nm) correspond exactly to the spectral properties of oxidized melanin (Fig. 5). However, the authors rejected the interpretation that melanin was the minor fluorophore, because of the assumption that the melanin autofluorescence was too faint to cause this effect. However, our studies have shown that melanin autofluorescence is not weak but can be very intense.

Melanin autofluorescence in general may simply reflect a partial oxidation of the polymer. Therefore, the increasing melanin fluorescence after oxidative stress may be useful for designing a fluorescence spectroscopic method for determining or integrating oxidative stress in the ocular fundus, although it may be difficult to distinguish the fluorescence produced by lipofuscin versus the fluorescence induced by oxidized melanin.

Finally, the participation of fluorescent oxidized melanin compounds in the formation of lipofuscin should be considered. Lipofuscin is likely to be heterogeneous, because it is probably derived from autophagy and the degradation of POs. In fact, 10 different fluorescent fractions have been identified in lipofuscin extracted from RPE. Whether any of these fluorescent fractions are derived from melanin remains to be investigated. In RPE cells of elderly humans, pigment granules consist almost completely of melanin cores with lipofuscin margins and are called melanolipofuscin granules. The origin of these granules is still unknown; however, it is tempting to speculate that oxidized melanin contributes to their formation. Additionally, the hypothesis that degradation of melanin contributes to lipofuscin formation is supported by the pathologic course of diseases such as age-related macular degeneration (ARMD) in which there is a correlation between loss of melanin and the formation of lipofuscin and melanolipofuscins.

In conclusion, the results show that melanin fluorescence increases after oxidation and that the intense fluorescence of melanin subjected to oxidative stress is a characteristic of the oxidized melanin and is independent of the fixation protocol (embedding in Spurr’s resin) or proteinaceous or lipid contamination of the isolated melanin.

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References


