Autophagocytosis of melanosomes in cultured embryonic retinal pigment cells[1]

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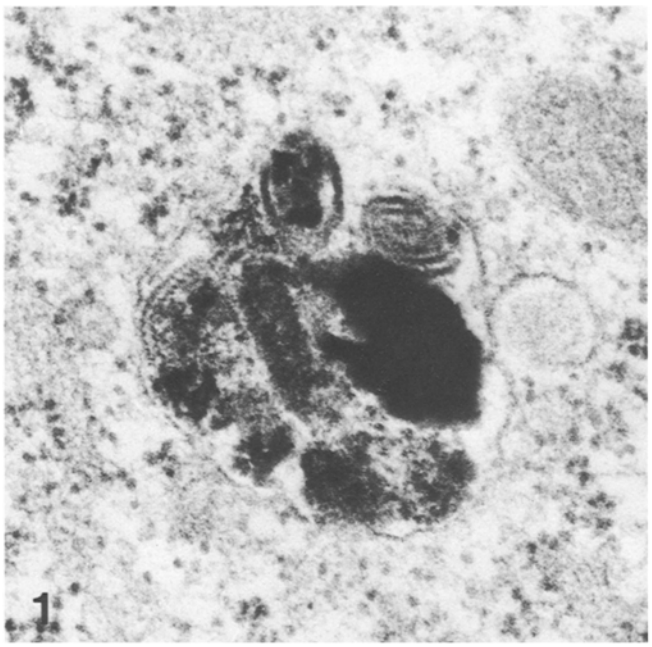
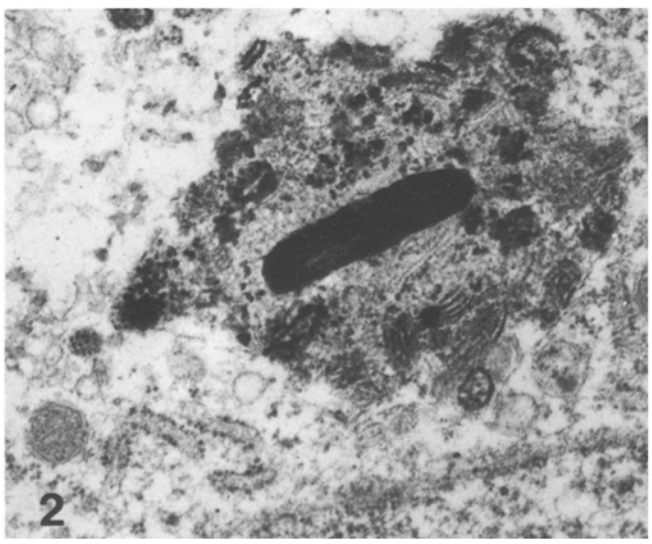
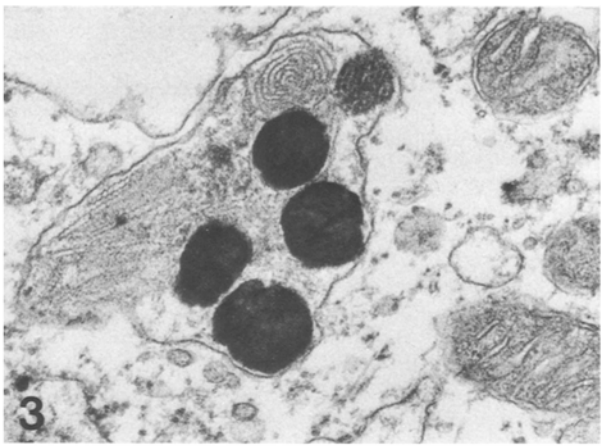
Summary. Autophagocytosis not only accounts for the early pigment loss found in proliferating cultured retinal pigment cells, but also occurs in slowly growing and in non-proliferating cells. Both melanosome synthesis and destruction may take place concurrently in cells. Autophagosomes may contain both fully formed melanosomes and also premelanosomes in various stages of formation, are positive for lysosomal enzyme activity, and likely represent secondary lysosomes.







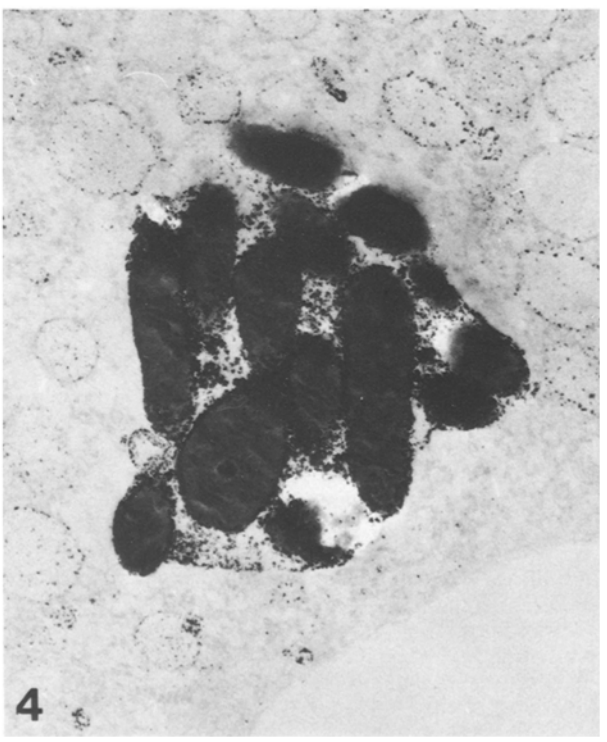


Figure 1. Autophagosome containing melanosomes in different stages of formation in cell from confluent colony 18 days in vitro. Medium supplemented with 0.625 µg/ml prostaglandin E_1. × 62,500.

Figure 2. Autophagosome containing a fully formed melanosome in a cultured retinal pigment cell 26 days in vitro. Culture was confluent and was maintained in medium containing serum and embryo extract. × 30,000.

Figure 3. Autophagosome containing fully formed melanosomes together with numerous premelanosome filaments. Cultures maintained in defined media without serum and embryo extract still contain autophagosomes, even after 3 weeks in vitro. × 30,000.

Figure 4. Large autophagosome from cell cultured 14 days in vitro in an unstained preparation showing the electron-dense acid phosphatase-positive reaction product. × 30,000.

Cultured retinal pigment cells undergo a process of dedifferentiation, or modulation of differentiation, in which tyrosinase activity and melanin synthesis become markedly reduced during an early proliferative phase in vitro. Following culture confluence, melanogenesis resumes and cells repigment[2,3]. Depigmentation of young cultures is accentuated by dilution of pre-existing melanosomes due to cell divisions and by an active melanolytic process involving autophagocytosis of melanosomes. It was found that autophagocytosis was inhibited by actinomycin D but not by colcemid, suggesting that cell proliferation was not a prerequisite for this modulation of differentiated function[4]. We have investigated the process of autophagocytosis in embryonic retinal pigment cells in both early and late phase cultures, maintained in a variety of media, in proliferating and in non-proliferating cells, and have used an electron microscopic cytochemical assay for acid phosphatase activity to characterize the autophagosomes in cultured cells.

Materials and methods. Retinal pigment cell cultures were established using 8-day White Leghorn chicken embryos, as previously described[2,5]. Briefly, eyes were trypsinized, the sclera and choroid were removed, and the retinal pigment epithelium was separated from the neural retina. A single cell suspension (2.5×10^4 cells/ml) was obtained by trituration. 4 ml was plated onto 60 mm plastic Falcon dishes and cultures were maintained at 37 °C in 5% CO_2 in air for up to 28 days in vitro. The medium was changed every 2 days. The media used included: 1. Puck's N-16 (GIBCO), 10% fetal bovine serum, 1% chick embryo extract; 2. above media without embryo extract; 3. media supplemented with 0.625 µg/ml or 6.25 µg/ml prostaglandins E_1 or E_2 (Upjohn); and 4. a serum-free chemically defined medium consisting of Ham's F-12 (GIBCO), glucose (2 mg/ml), $CaCl_2$ (0.12 mg/ml) and sodium bicarbonate (0.45 mg/ml). After various time periods in vitro, cultures were processed for electron microscopy; they were fixed in situ with phosphate buffered 2% glutaraldehyde at 25 °C for 15 min, rinsed with buffer, postfixed with 1% OsO_4 for 30 min, dehydrated with graded ethanols, and embedded in situ in Epon. The plastic-embedded cell monolayers were sectioned en face with a diamond knife using a Porter-Blum MT-2 ultramicrotome. Thin sections were stained with uranyl acetate and lead citrate and examined in an AEI Corinth 275 electron microscope. For the determination of acid phosphatase[6], cultures were fixed in situ in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 4 °C, rinsed in cacodylate buffer, rinsed in 0.1 M Tris buffer (pH 5), incubated for 15, 30 or 60 min at 37 °C in a solution of sodium beta-glycerophosphate and lead nitrate in Tris buffer, rinsed, osmicated and further processed as above. Thin sections were first examined without uranyl and lead staining for identification of electron-dense acid phosphatase-positive reaction product in cells.

Results and discussion. Cultures grown in medium containing serum and embryo extract proliferate rapidly for the 1st week in vitro. High growth rate is accompanied by depigmentation, involving reduced melanin synthesis and autophagocytosis of melanosomes. In confluent cultures, after 2–3 weeks in vitro, melanogenesis resumes but autophagocytosis of melanosomes is still found, even in hyperpigmented cultures treated with prostaglandins[5]. Large, membrane-bound aggregates containing melanosomes at different stages of development are commonly found. Autophagocytosis of melanosomes takes place in non-proliferating as well as in proliferating cells. Confluent cells maintained in defined media without embryo extract and serum do not proliferate and have also been found to maintain melanogenic activity in slowly proliferating, early phase cultures[7]. Autophagocytosis of melanosomes still takes place in these cells, demonstrating that this modulation of differentiated function is not dependent upon an early depigmenting, dedifferentiative phase in vitro.

Ultrastructurally, the cultured cells contain numerous melanosomes in different stages of formation and also within autophagosomes. We have found that melanosome-containing autophagosomes have acid phosphatase activity and likely represent lysosomal bodies engaged in melanosome lysis. The presence of melanosomes at different stages of formation within autophagosomes indicates that melanosome synthesis and destruction may take place concurrently in cells. Furthermore, the phenomenon of melanosome autophagocytosis is not restricted to proliferating dedifferentiated cells, as it occurs in both early (3 days) and late (4 weeks) phases of culture, in non-proliferating cells in serum-free medium, and in prostaglandin-stimulated cells with increased melanogenic activity.

The presence of melanosomes within phagolysosomal complexes in melanin-producing cells has been previously described, although usually found associated with alteration of normal function[8–11]. However, White Leghorn chicken feather germ melanocytes undergo a programmed cell death including melanosome autophagocytosis that is part of the normal differentiation of melanin pigmentation in this breed[6]. The normal daily turnover of rod outer segment membranes by White Leghorn retinal pigment cells has also been found to include melanosomes in phagolysosomal vesicles[12]. Autophagocytosis of melanosomes in bovine retinal pigment epithelium has also been shown to take place as part of the normal development of amelanotic epithelium in eyes with a tapetum lucidum[13]. In this case, similar to our findings, it was shown that depigmentation involved not only removal by autophagocytosis of pre-existing, fully-formed melanosomes, but also the removal of newly assembled premelanosomes.

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