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Research Paper

Ultrastructure of Amelanotic Melanocytes from Human Hair Follicles☆

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Abstract

Objective: To investigate the ultra structure of amelanotic melanocytes (AMMC). **Methods**: The hair follicles obtained from normal human scalp by 0.50% collagenase type V treatment were washed with 0.1 mol/L phosphate buffer salt (PBS). Hair-follicle cell suspensions were prepared by trypsin treatment and cultured in melanocyte medium. Remaining keratinocytes were removed by differential trypsinization. 100μg/ml geneticin was used to eliminate the contaminating fibroblasts. At third passage, the cells were trypsinized, and then washed in phosphate-buffered saline and processed for transmission electron microscopy. **Results**: Under transmission electron microscope, the cultured cells showed round or oval shape, with single large nuclear and the karyotheca were double deck. There were obvious euchromosome within the nucleus, and sparse heterochromosome. There were various organelles in the cytoplasm, including plentiful melanosomes with nearly similar size, mitochondria, rough endoplasmic reticule (RER) and ribosome. The electron density granules in most of the melanosomes disposed along concentric circularities. Golgi apparatus in the cells was inconspicuous. **Conclusion**: The ultra structure of AMMC from human hair follicles is different from that of epidermal melanocytes, and these characteristics determine the functional immature of AMMC.

Keywords: amelanotic melanocyte; transmission electron microscope; melanosome

INTRODUCTION

In the clinic, with any kind of treatment for vitiligo, the repigmentation usually begins at the orifices of the hair follicles, then enlarges and coalesces to cover whole vitiliginous maculas [1]. Investigations have shown that amelanotic melanocytes (AMMC) located in the outer root sheaths (ORS) of hair follicles form the melanocyte reservoir in human skin[1-2]. Tobin et al [3] first reported the successful culture of AMMC in 1995, and demonstrated the cells by immunofluorescence staining. Based on their study, we successfully isolated and cultured AMMC in 2004[4]. But the ultra structure of AMMC was not described in detail.

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MATERIALS AND METHODS

Preparation of hair-follicle single-cell suspensions

Preparation of hair-follicle single-cell suspensions was performed according to Zhu et al [4]. In brief, scalp skin from plastic surgery was immediately immersed in Eagle's minimum essential medium (E-MEM) supplemented with 400 U/ml penicillin and 400 μg/ml streptomycin. The epidermis and the upper 1 mm of dermis were removed with a scalpel. The tissue was cut into 0.5 cm^2 pieces and placed in D-Hanks' balanced salt solution containing 0.50% dispase for 12-16 h at 4℃, and then immersed in E-MEM supplemented with 0.50% collagenase type V (Sigma) and 5% fetal bovine serum for 1 h at 37℃.

Individual hair follicles generated from the scalp skin were washed in 0.01 mol/L phosphate-buffered

salt (PBS) and treated with 0.05% trypsin and 0.53 mmol/L ethylenediamine tetraacetic acid(EDTA) for 5-10 min at 37℃. Individual cells were released from the single hair follicles by trypsin /EDTA treatment. The cell suspensions were filtered through a 200-pore steel sifter(Jinke Net Limited Company, Hebei province, China), which diameter of the pore was 76 μm, and centrifuged at 1000 r/min for ten minutes and then seeded into 6-well plates at a density of 4×10^5 cells per well with EMEM supplemented with 10% fetal bovine serum, 0.2 μg/ml cholera toxin (Sigma), 50 nmol/L 12-o-tetradecanoyl-phorbol-13-acetate (TPA), 0.05 mg/ml gentamicin, 2.5 μg/ml fungizone, and 10%-20% K-SFM (Gibco), including 50 μg/ml bovine pituitary extract (BPE) and 5 μg/ml epidermal growth factor (EGF) human recombinant protein. The cells were incubated at 37℃ in a humidified atmosphere with 5% CO2 in air and the medium was changed twice a week.

Twelve hours after plating, some cells attached to the substratum; Most of these attached cells were AMMC with smooth, bipolar, "neuronal" shapes, which were not pigmented melanocyte-like cells. Lots of keratinocytes identified by their cobblestone morphology were suspended in the media, and only a few had attached to the culture well. The fibroblasts were distinguished from AMMC on the basis of their size and their broad and flattened shape. Keratinocytes were removed by differential trypsinization. The contaminating fibroblasts were eliminated with 100μg/ml geneticin .

Transmission Electron microscopy (TEM)

AMMCs in culture were trypsinized, and then were washed in phosphate-buffered saline and processed for electron microscopy. Cell pellets were fixed for at least 18 h at 4℃ in 2.5% glutaraldehyde and 0.1 mol/L phosphate buffer(pH 7.2). Post fixation was performed in 1% osmium tetroxide and 0.1 mol/L phosphate buffers for 1 h followed by staining for 30 min in 1% uranyl acetate and 50% ethanol. The cell pellets were dehydrated using serial alcohol and acetone incubations and were embedded in spurr resin. A Sorvall MT-2B ultramicrotome was used to cut the cell pellets to 80 nm. Sections were stained with uranyl acetate and lead citrate. Grids were viewed in a Hitachi H-7000 transmission electron microscope at an accelerating voltage of 80 kV.

RESULTS

Under transmission electron microscope, the cells showed round or oval shape with few surface projection, possessed large and indented nucleus with obviously prominent double-membrane, which were not thickened. One single nucleolus located at center of nucleus(***Fig. 1***), frequently presented orbicular-ovate shape with shallow indentation,and accounted for 1/3 to 1/2 of the cell body. There were abundant euchromatin and a trifle of heterochromatin, which attached to the inner wall of nuclear membrane. Neither interchromatin granule nor perichromatin granule was observed.

In cytoplasm, endoplasmic reticules looked like lamella-split or tubuliform, and were accreted by a few of granular ribosomes. The rough endoplasmic reticulum(RER) cistern did not expand. There was a great deal of sporadic spherical or ellipse ribosomes surrounding RER.The ribosomes were scattered and not attached to endoplasmic reticulum. The number of mitochondria varied in cytoplasms. All in all, there were more stage Ⅲ-Ⅳ melanosomes in the cytoplasma, there were more mitochondria. The mitochondrias in AMMC presented oval shape with electron-dense double members. Within the cultured cells, Golgi apparatus was not obviously observed, and no centriole and coarse clumps of microfilament and microtube was observed.

The most specific performance in AMMC's cytoplasm was affluent melanosome wrapped by unit membranes (***Fig. 2***). These melanosomes surrounded the nucleus. In accordance with previous report[5], membrane vesicles containing no visible pigment but irregular internal membrane structures, which were defined as stage Ⅰ or premelanosomes. Stage Ⅱ melanosomes were characterized as elongation of vesicles, and ordered internal membranes into parallel structures. Stage Ⅲ melanosomes were characterized as deposition of melanin (sometimes appearing as beads on a string) on the internal fibers. Stage Ⅳ melanosomes filled with melanin pigment, without distinguishable luminal structures. In most of the cells, stage Ⅱ and Ⅲ melanosomes were abundant, and stage Ⅳ melanosomes were rare.

Most stage Ⅱ melanosomes were characterized as elongation of vesicles, with a long axis of 0.285-0.352 μm, and a minor axis of 0.183-0.203 μm, and ordering of internal membranes into parallel structures on which a small quantity of fine electron-dense grains disposed (***Fig. 3***). Minority of stage Ⅱ melanosomes presented as spherical with 0.159-0.215 μm in diameter. There were many intensive fine punctiform structures alined up and formed concentric circles in these cells.

Stage Ⅲ melanosomes had more melanin deposition on the internal fibers, sometimes appearing as beads on a string, so, were not clearly discerned stripes or lamellars as seen in stage Ⅱ melanosomes (***Fig. 4***). These electron density grains occupied almost spaces of the melanosomes. Lots of stage Ⅱ and Ⅲ melanosomes but a few stages Ⅰ and Ⅳ melanosomes were observed in cytoplasms.

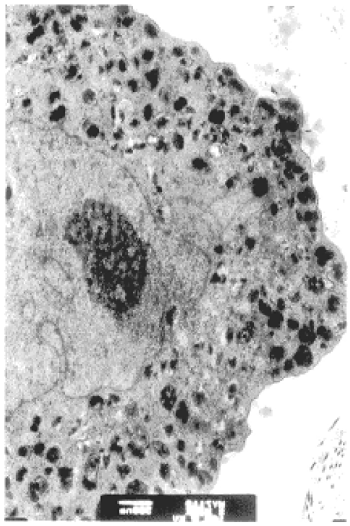

Fig. 1 The AMMC was oval shape with short and scarce surface projection, possessed a large, indented nucleus with double-membrane. Bar=500 nm

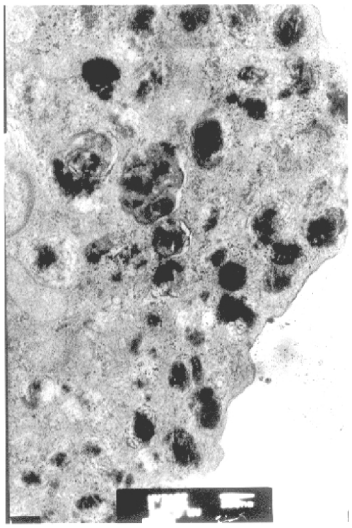

Fig. 2 There were affluent melanosomes wrapped by unit membranes in cytoplasm, most of them were stage Ⅱ and Ⅲ. Bar=200 nm

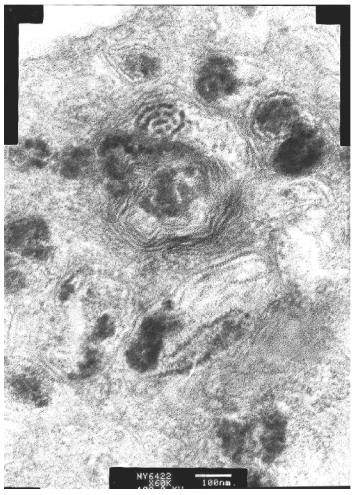

Fig. 3 Most stage Ⅱ melanosomes were characterized as elongation of vesicles and ordering the internal striation into parallel or concentric structures, on which a small quantity of fine electron-dense grain disposed. Bar=100 nm.

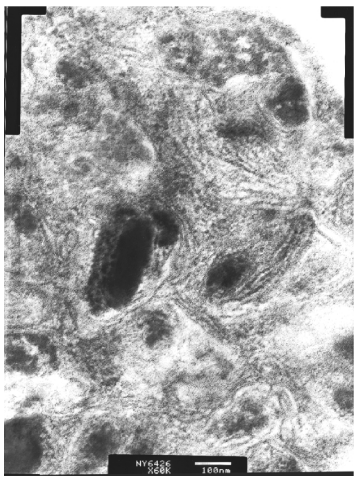

Fig. 4 Stage Ⅲ melanosomes presented more melanin depositing on the internal fibers, which almost could not be discerned clearly. Bar=100 nm.

DISCUSSION

Melanocyte-precursor cells have been described previously in telogen and early anagen hair germs by observation of epon sections using light microscopy. The existence of these precursor cells has not been generally accepted due to lack of ultrastructural evi dence[6]. Recentl investigations have shown that amelanotic melanocytes (AMMC) located in the middle and /or lower parts of the outer root sheaths (ORS) of hair follicles [7]. After different treatments for vitiligo, AMMC are stimulated to differentiate, proliferate, and migrate upward along the surface of the ORS to the nearby epidermis, where the melanocytes continue to migrate radially to form the

pigmented islands visible clinically in repigmented vitiligo lesions [8]. Therefore, the AMMCs in quiescent condition form are the melanocyte reservoir in human skin [9]. The extensive observation of AMMC showed its immature substructure. The ultrastrutual characteristics of AMMCs have not been found. The results of our study about AMMCs using transmission electron microscopy indicated as follows:

First, melaosomes transfer system in AMMC was unsound. In adherence condition, most of the AMMCs possessed bipolar, a few had three polar, otherwise, epidermal melanocytes(EM) exhibited round, oval, triangular or quadrangular perikarya from which arose more 5-10 long dendrites [10], and look like "overgrown root of tree". Under transmission electron microscope, our observation of AMMCs showed a few of short prominences on its surface. These findings illustrated that the AMMCs lacked dendrites,which actd as necessary structure for melanosomes transferring, however EM with mature function possessed aplenty dendrites. To our knowledge, microfilament, microtube and centriole, that regulate cell motion [11, 12], are abundant in EM. Endocytoplasmic reticulum (ER) that augmentates intracellular membrane areas, contribute to progression of various kinds of biochemical event in cells, acted as "workshop" of synthesizing organics. The principal function of ribosome is assembling amino acids into protein. However, the centriole, coarse clumps of microfilament or microtube were rare in our cultured cells observed by transmission electron microscopy. Rough endoplasmic reticulum (RER) in AMMC is less than that of EM [13], and without cisternal expansion. Ribosomes were scattered but not attached to ER in AMMCs that indicated lack of protein synthesis activity within AMMCs. So, we speculated that AMMCs lacked motion support frames and structures for protein synthesis.

Second, the quantity and morphology of melanosomes in AMMCs were different from those of MCs. Quantitatively, melanosomes surrounding cellular nucleus in an AMMC exceeded in a MC[13]. The majority of melanosomes of AMMC presented as spherical or oval, and approximately alike size. However most melanosomes in EM were characterized by appearance of fusiform and their size varied from one to another [12]. In cultured AMMCs, stage Ⅱ or Ⅲ melanosomes are abundant, few of stage I or Ⅳ melanosomes were seen. In the contrary, melanosomes of EM were in every stage. Among them, stage Ⅳ melanosomes located at the verge of cell body and had a tendency to creep. Previous investigations indicated that Golgi apparatus surrounded by some tiny vacuoles are abundant in EM. But no obvious Golgi appartus could be observed by transmission electron microscope in the present study. We concluded that the melanosomes of AMMC in quiescent condition did not complete melanonization and further form mature melanosomes and then transported from AMMC; these melanosomes were detained in stages Ⅱ and Ⅲ. We presumed that this stagnation prevented stage Ⅰ melanosomes from continuous production. Therefore, either Golgi's complex or stage Ⅰ melanosomes could not be perspicuously observed in AMMCs.

The size of stage Ⅰ and Ⅳ melanosomes varied considerably from each other, so we compared the dimension of stages Ⅱ and Ⅲ melanosomes in AMMC and EM respectively. The average diameter of stage Ⅱ melanosomes in AMMC was 0.256 μm. These were many punctiform granular, most of which arranged in concentric circle, a few of which aligned along thread. The diameter of melanosomes of EM was about 0.5 μm,in which fine electron dots were arranged in line. Stage Ⅲ-Ⅳ melanosomes were almost filled with electronic ally dense granulars in EM. These melanosomes appeared as fusiform with about 0.5 μm in average diameter, in which various amount of melanin deposited on parallel striation[14].

Analysis on ultra structure of AMMC may promote understanding why AMMC is function immature cell. It is suggestive that investigators pursuiting studies of AMMC's activation and migration identify the state and degree of activated AMMC through observing changes of its ultrastructure. Further studies about classifing melanosomes and deposition patterns of electron density granulars will be conducted.

References

[1] Falabella R. Grafting and transplantation of melanocytes for repigmenting vitiligo and other types of leukoderma. *Int J Dermatol* 1989; 28: 363-9.

[2] Cui J, Shen LY, Wang GC. Role of hair follicle in the repigmentation of vitiligo. *J Invest Dermatol* 1991; 97: 410-6.

[3] Tobin DJ, Colen SR, Bystryn JC. Isolation and long-term culture of hair follicle melanocytes. *J Invest Dermatol* 1995; 104: 86-9.

[4] Zhu WY, Zhang RZ, Ma HJ, Wang DG. Isolation and culture of amelanotic melanocytes from human hair follicles. *Pigment Cell Res* 2004; 17: 668-73.

[5] Watabe H, Valencia JC, Yasumoto KI, Yasumoto K, Kushimoto T,Ando H, *et al*.Regulation of tyrosinase processing and trafficking by organellar pH and by proteasome activity. *J Biol Chem* 2004;279:7971-81.

[6] Hirobe T. Origin of melanosome structures and cytochemical localizations of tyrosinase activity in differentiating epidermal melanocytes of newborn mouse skin. *J Exp Zool* 1982; 224:

355-63.

[7] Tobin DJ, Bystryn JC. Different populations of melanocytes are present in hair follicles and epidermal. *Pigment Cell Res* 1996; 9: 304-10.

[8] El Mofty AM. Vitiligo and psoralen. Pergamon Press. New York, 1968.164.

[9] Narisawa Y, Kohda H, Tanaka T. Three-dimensional demonstration of melanocytes distribution of human hair follicles: special reference to the bulge area. *Acta Derm Venereol* 1997; 77: 97-104.

[10] Zhang RZ, Zhu WY, Xia MY, Feng Y. Morphology of cultured human epidermal melanocytes observed by atomic force microscopy. *Pigment Cell Res* 2004; 17: 62-5.

[11] Okazaki K, Uzuka M, Morikawa F, Toda K, Seiji M. Transfer mechanism of melanosomes in epidermal cell culture. *J Invest dermatol* 1976; 67: 541-7.

[12] Vancoillie G, Lambert J, Mulder A, Koerten HK, Monmaas AM, Van Vostveldt P, *et al*. Cytoplasmic dynein colocalizes with melanosomes in normal human melanocytes. *Br J Dermatol* 2000; 143: 298-306.

[13] Xia MY. Ultra structure of human skin. In: textbook of dermatology and venerology, edited by Zhu wenyuan. First edition. Publishing company of the Southeast University. Nanjing, 1999.

[14] Setaluri V. The melanosome: dark pigment granule shines bright light on vesicle biogenesis and more. *J Invest Dermatol* 2003; 121: 650-60.