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Mouse Fibroblast Expressing Human Tyrosinase with DHICA-Oxidase Activity Produces Predominantly Pheomelanin Deposit in Lysosome

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ABSTRACT—The melanogenic gene-transfected cell system serves as a useful tool for the study of the symphonic relation between melanin synthesis and intracellular organelles such as melanosomes in melanocytes. We constructed melanin-producing mouse fibroblasts by transfection of human tyrosinase cDNA to investigate the intracellular changes caused by tyrosinase expression. DHICA-oxidase (5,6-dihydroxyindole-2-carboxylic acid oxidase) activity without TRP-1 (Tyrosinase Related Protein-1) expression in the cells suggested that human tyrosinase also possesses a DHICA-oxidase activities different from mouse tyrosinase. Electron microscopic observation indicated that melanin-deposit organelles have some lysosomal features. These properties of melanin-deposit organelles in tyrosinase expressing fibroblasts provide one evidence for the hypothesis that melanosome is the specialized lysosome in melanocytes.

INTRODUCTION

Melanocytes in vertebrates can produce two types of melanin pigments, sulfur-containing pheomelanin and non-sulfur eumelanin. These pigments are biopolymer synthesized from tyrosine and deposited within specific subcellular organelles termed melanosomes in melanocytes.

Tyrosinase (monophenol monooxygenase, EC 1.14.18.1) is one of the key enzymes, especially related to the early process of melanogenesis. Tyrosinase catalyzes firstly the conversion of tyrosine to 3,4-dihydroxy-phenylalanine (DOPA), secondly DOPA to DOPAquinone. Pheomelanin is synthesized from DOPAquinone via 5-cystenyl DOPA. The pathway of eumelanogenesis has been thought to be that DOPAquinone is converted to DOPAchrome (2-carboxy-2,3-dihydroindole-5,6-quinone) and further to a melanin-monomer, dihydroxyindole (DHI) with spontaneous decarboxylation, then spontaneous or tyrosinase-mediated DHI-oxidation which results in eumelanin (Hearing *et al.*, 1987). However, recent investigations have shown that eumelanin consists of not only this DHI-melanin but also melanin from another pathway (Orlow *et al.*, 1992). DOPAchrome is converted to another type of melanin-monomer, 5,6-dihydroxyindole-2-carboxylic acid (DHICA) catalyzed by DOPAchrome tautomerase, further DHICA-melanin is produced from DHICA-oxidation with DHICA-oxidase.

Molecular biological studies of mouse coat color mutations have shown the relation between these melanogenic genes and enzymes. For example, tyrosinase related protein-2 (TRP-2) was mapped at *slaty* locus which encodes DOPAchrome tautomerase (Tsukamoto *et al.*, 1992; Jackson *et al.*, 1992) , and TRP-1 was mapped at *brown* locus in mice (Shibahara *et al.*, 1986; Jackson, 1988). In case of TRP-1, many kinds of enzymic activity have been reported including catalase (Halaban and Moellmann, 1990), DHICA-oxidase (Jiménez-Cervantes *et al.*, 1994), tyrosine hydroxylase (Zhao *et al.*, 1994) and DOPAchrome tautomerase (Winder *et al.*, 1993b). However the specific enzymic activity of TRP-1 has been unclear.

Furthermore the precise role of tyrosinase and these enzymes in the pathway of melanin biosynthesis have been inconclusive.

On the other hand, the subcellular organelle as the site of melanin-deposit, melanosome was termed by Seiji and co-workers (Seiji *et al.*, 1963). The maturation of the melanosomes has four successive stages (Quavedo *et al.*, 1987). Cytochemical staining for the DOPA-oxidase activity of tyrosinase indicated the presence of the enzyme in not only the melanosomes but also in the Trans-Golgi network (TGN), and in a population of coated vesicles. The maturation process involves a bipartite pathway for melanosomal biogenesis. Firstly "premelanosomes" with incompletly organized matrix bud from the TGN, and subsequently the matrix condense into recognizable form in melanosomes. Concurrently or

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subsequently, coated vesicles containing tyrosinase arise from the TGN. The fusion of these vesicles with the melanosome is believed to be the initiation of melanogenesis (Novikoff *et al.*, 1968; Chakraborty *et al.*, 1989).

In addition to these findings related to the maturation process of melanosomes, it appears that melanosomes have some lysosomal properties. The organelle, lysosome is defined by some kind of properties, substantially the presence of enzymes such as acid phosphatase and of some specific membrane proteins such as lysosome associated membrane protein-1 (LAMP-1), and functionally the incorporation of materials from extracellular to intracellular via endocytosis and phagosytosis (Kornfeld and Mellman, 1989). Recent studies have shown the presence of acid phophatase activity (Seiji and Kikuchi, 1969; Wolf and Shreiner, 1971) and LAMP-1 (Zhou *et al.*, 1993) in melanosomes. The phagocytotic activity of melanocytes was also reported (Le Poole *et al.*, 1993).

These findings demonstrated that melanosomes have lysosomal enzymes and membrane proteins, further melanosomes can fuse endosomes and phagosomes in similar to lysosomes. These lysosomal property of melanosomes led us to the hypothesis that melanosomes are the specialized lysosome (Mishima, 1994; Orlow, 1995).

Recently, it has been reported that tyrosinase cDNA was transfected into mouse fibroblasts. Mouse fibroblasts do not have melanosomes, do not express tyrosinase, and hence do not produce melanin. After the transfection, the transfected-fibroblasts produced melanin (Bouchard *et al.*, 1989; Winder, 1991; Winder *et al.*, 1993a; Mishima, 1993). However, enzyme activities, melanin composition and properties of melanin-deposit organelles in the transfected-cells have not been well investigated.

In this paper we report on the novel enzyme activity of DHICA-oxidase with human tyrosinase and the lysosomal properties of pheomelanin-deposit organelles in tyrosinase expressing mouse fibroblasts.

MATERIALS AND METHODS

Cell culture

The mouse fibroblast L929 (ATCC CCL1) derived from C3H mouse embryo was purchased from American Type Culture Collection. Cells were cultured routinely in Eagle's MEM supplemented with 10% fetal bovine serum (FBS) and antibiotics. Tyrosinase cDNA-transfected cells were cultured in the same medium with 200 μg/ml of Geneticin (Gibco).

For experiments cells in monolayer were harvested with 0.25% trypsin/0.02% EDTA in Ca^{2+}, Mg^{2+}-free phosphate buffered saline (PBS).

Transfection of human tyrosinase-cDNA

Human tyrosinase cDNA expression plasmid, BCMGS-NHT-2 (Takeda *et al.*, 1989; Karasuyama *et al.*, 1989; Ando *et al.*, 1993) was transfected into L929 cells by electroporation using Gene Pulser (Bio-Rad Laboratories). The transfection conditions were as follows, 20 μg of the plasmid, 10^7 cells in cold PBS, 1000 V, 25 μF, time constant for 0.4 msec. After cultivation for 48 hr, the transfected cells were selected in the 10% FBS supplemented Eagle's MEM with 800 μg/ml of Geneticin (Gibco BRL) for 2 weeks. Some of pigmented colonies were picked and analyzed. These pigmented clones gave similar results in the following assays. The results of most typical clone, LHT-2 are reported here.

Reverse transcription mediated PCR (RT-PCR)

Total RNA from cells were prepared by ISOGEN (Wako Pure Chemicals). The first strand cDNA synthesis from total RNA was performed by the oligo dT-priming method using First-Strand cDNA Synthesis kit (Pharmacia). Total RNA from 10^5 cells were used for cDNA synthesis and PCR.

The primer sets for detection of specific mRNAs by RT-PCR were as follows. Mouse glyceraldehyde 3-phosphate dehydrogenase (G3PDH) mRNA was used as the positive reference.

Human tyrosinase: 5'ATGGAGAAGGAATGCTGTCC3', 5'GACT-GATGGCTGTTGTACTC3'. Mouse tyrosinase: 5'CAGCTTTCAGGCA-GAGGTTC3', 5'ATGGCTATTATACTCTTCTG3'. Human and mouse tyrosinase cDNA were not cross-amplified by these two primer sets. Mouse TRP-1: 5'CTGTGATTGAGACT3', 5'AGGCTCCTGCAGCA3'. Mouse TRP-2: 5'CCAATGCCTTGCACACTCAG3', 5'GCTGAGACC-TGTCTCCATTA3'. Mouse G3PDH: 5'TGAAGGTCGGTGTGAACGG-ATTTGGC3', 5'CATGTAGGCCATGAGGTCCACCAC3'.

Amplification was performed for 35 cycles at 94°C for 30 sec, 54°C for 30 sec, and 72°C for 30 sec with a GeneAmp PCR system 9600 (Perkin-Elmer). Total RNA prepared by the same procedure from mouse B16 melanoma cells and L929 mouse fibroblast cells were used as a reference. The PCR products were analysed by agarose gel electrophresis.

Isolation of glycoproteins and enzyme activity assays

We assayed melanogenic enzyme activities in glycoprotein fraction of LHT-2 cell extract for enhancing the sensitivity of detection. The isolation of glycoprotein was performed as previously described (Wilczek and Mishima, 1995).

Melanogenic enzyme activities in glycoprotein fraction were determined by HPLC. Tyrosine hydroxylase activity was measured by simultaneous monitoring consumption of tyrosine and formation of DOPA. DOPA-oxidase activity was determined either by formation of DOPAchrome or consumption of DOPA. The activities of DHI- and DHICA-oxidases were measured as consumption of substrate. Dopachrome tautomerase activity was measured as formation of DHICA from 0.5 mM DOPAchrome. DOPAchrome was synthesized by oxidation of DOPA with periodate. In these assays heat inactivated samples were used as a reference. The amounts of substrate exhausted and/or product formed in the particular assay were calculated on the basis of calibration curves prepared using appropriate standards. These precise assay conditions were previously described (Wilczek and Mishima, 1993,1995).

Protein concentration measurement

Protein concentration was determined by Protein Assay kit (Bio-Rad) based on Bradford's method (Bradford, 1976). Bovine serum albumin was used as a reference standard.

Melanin assays

Total eumelanin content was measured by the spectrophotometric method (Ito *et al.*, 1993). Briefly, samples (100 μl cell suspension of 10^7 cells) were hydrolyzed with 500 ml of 57% HI and 30 μl of H_3PO_2 for 16 hr at 130°C. The eumelanin particles were precipitated by addition of 50% ethanol followed by centrifugation. The precipitated eumelanin was washed three times with ethanol and solubilized in hot alkali (1 N NaOH, 30 min at 100°C) after adding of H_2O_2. The mixtures were centrifuged and absorbance of supernatants was measured at 350 nm with a spectrophotometer and compared with a standard curve using sepia melanin (Sigma).

The DHICA-melanin content was analyzed by oxidation of samples with potassium permanganate under acidic pH and quantitation of the pyrrole-2,3,5-tricarboxylic acid (PTCA) with HPLC.

The oxidation and HPLC conditions were described (Ito and Fujita,1985).

Pheomelanin content was assayed by reductive degradation in hydriodic acid and quantitation of aminohydroxyphenylalanin (AHP) with HPLC as described (Ito and Fujita,1985).

The DHICA-melanin and pheomelanin contents were calculated on the basis that 1 µg of PTCA and 1 µg of AHP roughly correspond to 50 µg of DHICA-melanin and 5 µg of pheomelanin, respectively (Ito and Fujita,1985). DHICA-melanin content in total eumelanin was calculated by the method as described (Wilczek *et al.*, 1996).

Electron microscopy

The L929 cells and LHT-2 cells were washed with 0.1M sodium cacodylate buffer (pH7.2) (SC-buffer), fixed with 2% glutaraldehyde in SC-buffer for 1 hr at 4°C, and then washed with SC-buffer again. For DOPA-reaction to detect the intracellular localization of tyrosinase activity, the fixed cells were incubated with SC-buffer containing 0.1% of L-3,4-dihydroxyphenylalanine (DOPA) for 3 hr at 37°C. After the post fixation with 1% osmium tetroxide for 1 hr at 4°C, these DOPA-treated and non-treated cells were stained with 2% uranyl acetate in 50% ethanol, and dehydrated with ethanol and embedded in Epoxy resin. Thin sections were prepared with an ultramicrotome (Reichert-Jung, Austria) and were counterstained with lead citrate. These sections were examined with a 1200EX electron microscope (JEOL Ltd., Tokyo, Japan).

Acid phosphatase reaction for electron microscopy

Acid phosphatase activity was used as a marker for lysosomes according to Gomori's method (Gomori, 1952). Briefly, cells were washed with SC-buffer, fixed with 2% glutaraldehyde in SC-buffer for 1 hr at 4°C, and then washed with SC-buffer again. The fixed cells were incubated in the substrate buffer [45.5 mM acetate buffer, pH5.0, 8% sucrose, 3 mM Pb $(NO_3)_2$, 10 mM Na-β-glycerolphosphate (Merck)] at 37°C for 60 min, washed with SC-buffer and postfixed in osmium tetroxide. After postfixation cells were then processed for electron microscopy according to the procedures as described above except for the uranyl acetate staining.

Latex-particle phagocytosis experiment

After 24 hr from the seeding of 10^4 cells in 65 mm cell culture dish, twenty microliters of latex-particle suspension (average diameter is 95 nm, Sekisui) was added into the culture medium. Continuing the culture, and cells were served to the electronmicroscopy observation after 5-days from seeding.

RESULTS

Expression of melanogenic genes in LHT-2

LHT-2, one of stable pigmented human tyrosinase cDNA-transfected fibroblast clone, was analyzed by RT-PCR assay to confirm whether or not the expression of transfected-human tyrosinase cDNA induces intrinsic mouse melanogenic gene expression. As shown in Fig. 1, no mRNA of melanogenic genes were detected in L929, but PCR products of transfected-human tyrosinase mRNA was detected only in RNA from LHT-2. Further mouse tyrosinase, TRP-1, and TRP-2 mRNA were not detected in RNA from LHT-2. This result showed that the transfected human tyrosinase-cDNA was expressed and did not induce intrinsic mouse melanogenic gene expression in LHT-2.

Enzyme activities in glycoprotein fraction from LHT-2

As shown in Table 1, tyrosine hydroxylase, DOPA-oxidase, and DHI-oxidase activities were detected in glycoprotein fraction from LHT-2. These activities were derived from the human tyrosinase which was transfected into mouse fibroblasts from the results of RT-PCR assay. DOPAchrome tautomerase activity was not detected in either glycoprotein fraction or the result of RT-PCR assay. TRP-1 mRNA encoding DHICA-oxidase, was not detected, but the DHICA-oxidase activity was detected in glycoprotein-fraction of LHT-2. More precise enzymological analyses of these enzymes was reported in another of our papers (Wilczek *et al.*, 1995). In contrast to these enzyme activities in LHT-2 glycoprotein fraction, no enzyme activity was detected in glycoprotein fraction from L929 fibroblast cells.

Fig. 1. RT-PCR assay of LHT-2. The positive reference G3PDH mRNA was detected in RNA from L929, LHT-2, and B16 cells. The PCR product of human tyrosinase mRNA (arrow) was only detected in RNA from LHT-2 which was human tyrosinase cDNA-transfected L929. Mouse tyrosinase, TRP-1 and TRP-2 mRNA were detected only in RNA from B16, but no mRNA of these melanogenic genes were detected in RNA from L929 and LHT-2 cells. L, L929 mouse fibroblast. T, LHT-2. B, B16 mouse malanoma. G3PDH, PCR with mouse G3PDH primers. hTYR, PCR with human tyrosinase primers. mTYR, PCR with mouse tyrosinase primers. mTRP-1, PCR with mouse TRP-1 primers. mTRP-2, PCR with mouse TRP-2 primers.

Table 1. Enzyme activities in glycoprotein fraction from LHT-2

Assay	Enzyme activity[1] [nmol/min/mg protein]
Tyrosine-hydroxylase	4.0 ± 0.1
DOPA-oxidase	273.2 ± 4.1
DHI-oxidase	67.2 ± 3.5
DHICA-oxidase	4.6 ± 0.5
DOPAchrome tautomerase	0.0 ± 0.0

[1]Mean of triplicate assay ± SD. Concentration of substrate was 1 mM in each assay except tyrosine-hydroxylase which was measured with 0.1 mM L-tyrosine.

Composition of LHT-2 produced melanin

To determine the composition of melanin, we measured pheomelanin, total eumelanin and DHICA-melanin content in LHT-2.

As shown in Table 2, pheomelain content was 2.3 µg/10^6 cells, and total eumelanin content was 0.27 µg/10^6 cells. Pheomelanin content was about 8.5 times more than total eumelanin. And DHICA-melanin content was 0.24 µg/10^6 cells, 88.9% of eumelanin was DHICA-melanin.

Table 2. Melanin composition produced in LHT-2

Melanin	Amount of melanin[1] [μg/10^6 cells]
Pheomelanin	2.30 ± 0.42
Total eumelanin	0.27 ± 0.02
DHICA-melanin	0.24 ± 0.06
DHICA-melanin content in total eumelanin	88.9%

[1]Mean of triplicate assay ± SD.

These results showed that the expression of human tyrosinase in fibroblasts predominantly induced pheomelanin, and DHICA-melanin might be produced without DOPAchrome tautomerase activity.

Electron microscopic morphological observations

To investigate the details of subcellular compartments in the pigmented LHT-2 and parental fibroblast L929, electron microscopic observation was performed.

Electron microscopic observation without DOPA-reaction are shown in Fig. 2A, B, C. Many electron-dense, melanin-deposit vacuoles of about 1 μm diameter were observed in LHT-2 (Fig. 2A, C). These organelles showed various grades of melanin deposit such as the maturating melanosomes observed in pigment cells. And similar size vacuoles were also observed in parental fibroblast L929 (Fig. 2B).

Electron micrographs of LHT-2 with DOPA-reaction are shown in Fig. 2D. No DOPA-oxidase activity was detected in L929 cells . In LHT-2, TGN, coated vesicles and the melanin-deposit organelles acquired distinct DOPA-oxidase activity similar to that observed in pigment cells. These results

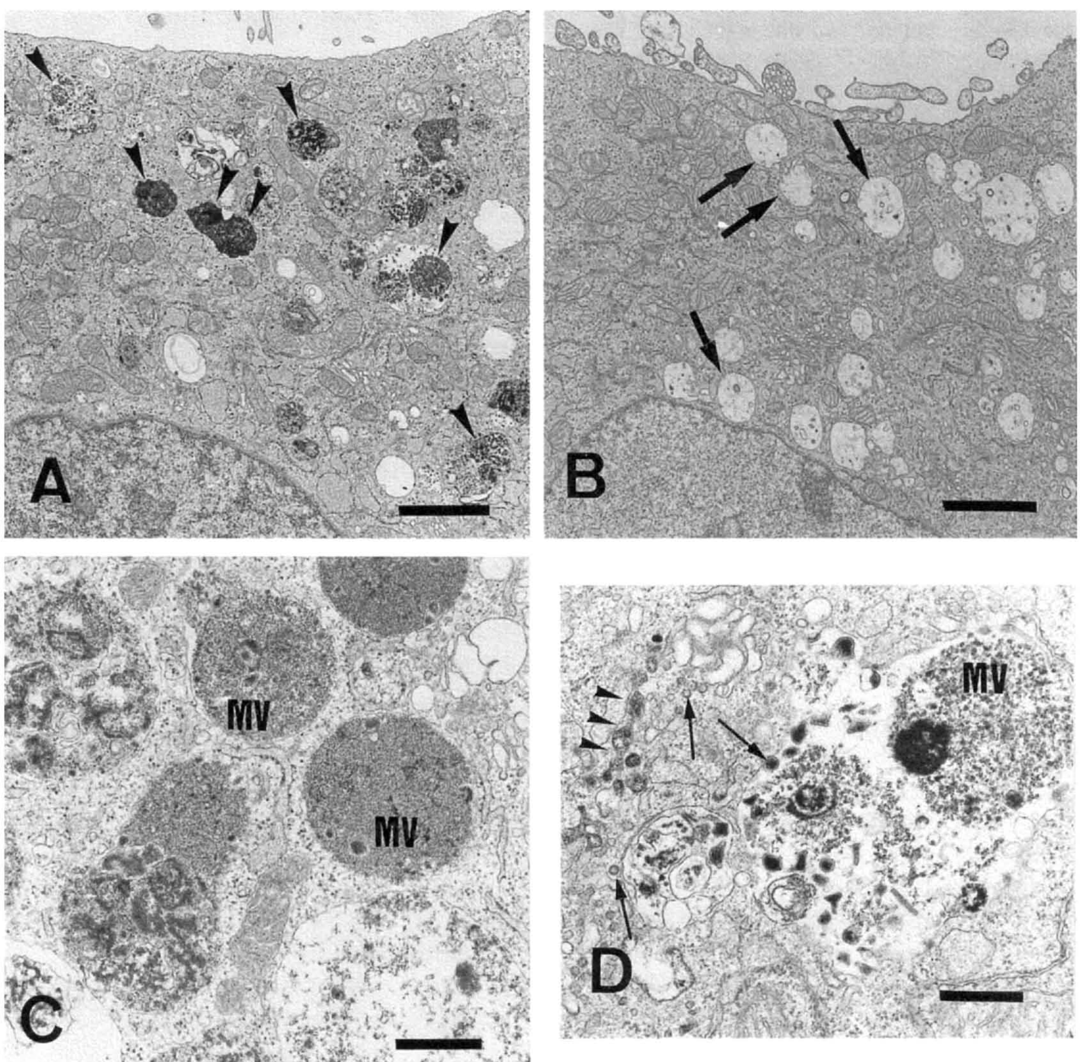

Fig. 2. Electron micrographs of LHT-2 (A, C) and L929 (B); DOPA-reaction of LHT-2 (D). (A) There were various grades of melanin-deposit vacuoles (arrowheads) in LHT-2. Bar: 2 μm. (B) There were many vacuole-shaped organelles (arrows). Bar: 2 μm. (C) Magnified view of LHT-2. Melanin-deposit vacuole (MV). Bar: 500 nm. (D) DOPA-reaction of LHT-2. No DOPA-oxidase activity was detected in L929. But in LHT-2, Trans-Golgi network (arrowhead), coated vesicles (arrow) and the melanin-deposit vacuoles (MV) acquired distinct DOPA-oxidase activity similar to that observed in pigment cells. Bar: 500 nm.

indicated that in LHT-2, human tyrosinase was synthesized on the ribosome at the rough ER, glycosylated at TGN, and transported to the melanin-deposit organelles by coated vesicles.

In addition, morphological observations suggested melanin-deposit organelles in LHT-2 to be lysosome, thus cytochemical and functional analyses were performed to investigate the feature of these organelles. The result of acid-phosphatase cytochemistry are presented in Fig. 3. Both melanin-deposit organelles in LHT-2 (Fig. 3A) and the vacuole in L929 (Fig. 3B) showed the activity of acid-phosphatase (small arrows). And the 100 nm latex particles were phagositized into both the melanin-deposit organelles in LHT-2 and the vacuoles with similar size in L929 as shown in Fig. 3C, D.

These observations indicated that the vacuoler organelles of 1 μm diameter in LHT-2 and L929 were homologous organelles and these possesed the lysosomal features.

DISCUSSION

The melanogenic gene-transfected cell system serves as a useful tool for the study of the melanin synthesis and intracellular organelles such as melanosomes in melanocytes.

To investigate the intracellular changes caused by tyrosinase expression, we transfected human tyrosinase cDNA expression vector into mouse L929 fibroblast which have no melanosomes, tyrosinase and melanin. After the transfection we obtained some melanin-producing clones.

The RT-PCR assay have shown that human tyrosinase mRNA was detected, but not mouse tyrosinase, TRP-1, and TRP-2 mRNAs in LHT-2, which is one of the typical pigmented

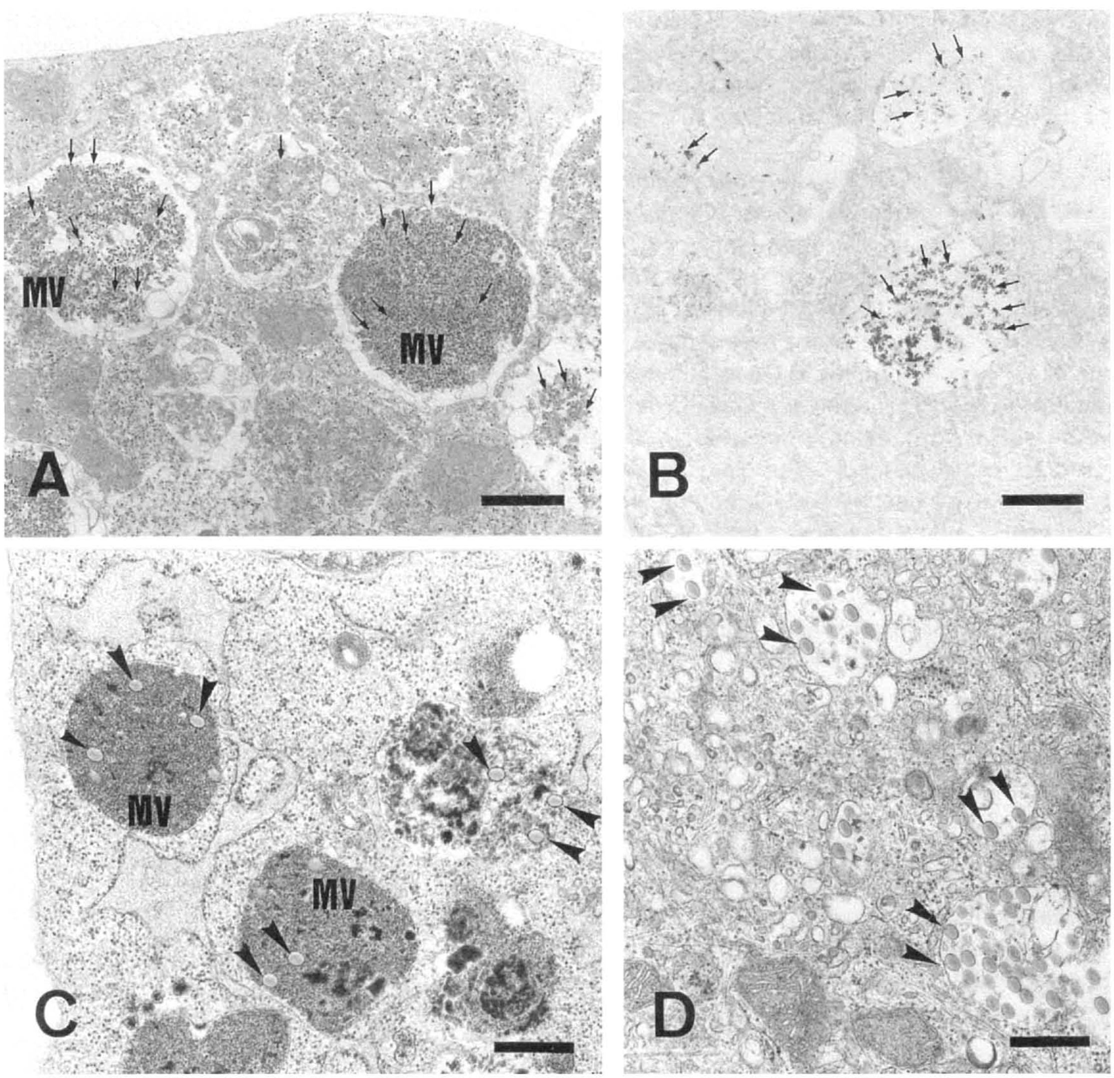

Fig. 3. Lysosomal feature of melanin-deposit organelle. Electron micrographs of LHT-2 (A) and L929 (B) with acid-phosphatase reaction. There were many fine acid-phosphatase reaction products (small arrow) at melanin-deposit vacuoles (MV) in LHT-2 and at vacuole-shape organelles in L929. Bar: 500 nm. Latex-particles were phagocytosed by organelles in LHT-2 (C) and L929 (D). Melanin-deposit vacuole (MV) in LHT-2 and vacule-shaped organelle in L929 phagocytosed latex particles (arrowheads). Bar: 500 nm.

clone. These results suggest that melanogenesis can be induced by the expression of transfected-human tyrosinase cDNA alone without intrinsic mouse TRPs expression.

In previous experiments of mouse tyrosinase cDNA-transfected mouse fibroblasts, it was reported that tyrosine hydroxylase and DOPA-oxidase activities derived from transfected tyrosinase cDNA (Bouchard *et al.*, 1989; Winder, 1991; Winder *et al.*, 1993a). We also detected tyrosine hydroxylase, DOPA-oxidase, DHI-oxidase in glycoprotein fraction of LHT-2.

DHICA-oxidase activity without expression of TRP-1 encoding DHICA-oxidase was detected. The DHICA-oxidase activity encoded by human tyrosinase cDNA is the first-reported measurement.

A recent report (Jiménez-Cervantes *et al.*, 1995) from another group using human melanoma cell line expressing tyrosinase but not TRP-1, supports our findings using human tyrosinase-expressing fibroblasts.

This evidence suggests that human tyrosinase posses not only tyrosine hydroxylase, DOPA-oxidase, DHI-oxidase, but also residual DHICA-oxidase activities different from mouse tyrosinase as previously reported (Jiménez-Cervantes *et al.*, 1994).

Nevertheless, the absence of DOPAchrome tautomerase activity in LHT-2 was observed, yet almost all produced eumelanin was DHICA-melanin. It is known that metal ions have the ability to convert DOPAchrome to DHICA at least *in vitro* (Prota, 1988). Therefore DHICA-melanin produced without DOPAchrome tautomerase can be explained that DOPAchrome might be converted to DHICA by intracellular metallic ions and DHICA-melanin is produced from DHICA by DHICA-oxidase activity of human tyrosinase. Thus the absence of DHICA-melanin reported as previously (Winder *et al.*, 1993a) might be explained by the fact that they used fibroblasts expressing mouse tyrosinase without DHICA-oxidase activity which is different from human tyrosinase. Mutant mouse which have normal tyrosinase, but deficient of TRP-1, produce pheomelanin predominantly and low content of DHICA-melanin in eumelanin (Ozeki *et al.*, 1995; Prota *et al.*, 1995). These evidences also suggest that mouse tyrosinase have no or very low activity of DHICA-oxidase.

Electron microscopic observation of LHT-2 and L929 fibroblasts implied the identification of the melanin-deposit organelles in LHT-2.

The melanin-deposit organelles in LHT-2 and the vacuoler organelles in L929 have similar size and spherical shape, and both of them have acid-phosphatase and phagocytosed latex-particles. These observations clearly suggest that melanin-deposit organelle in LHT-2 and the vacuole organelle in L929 are homologous organelle, and the origin of them may be lysosomes.

Recently the origin of melanosomes is under discussion. Transfection experiments of melanogenic genes have been expected not only to serve as a useful tool for biochemistry, but also to throw light on the discussion, however previous investigations have not had clear conclusions. The lysosomal property of melanin-deposit organelle in LHT-2 provides one evidence for the hypothesis that melanosome is the specialized lysosome in melanocyte.

Finally, the melanin analyses revealed that pheomelanin was predominantly produced in LHT-2. Also another group reported similar results (Winder *et al.*, 1993a). These results suggest that the expression of tyrosinase alone is insufficient for eumelanognesis. In addition, it may be that tyrosinase expressing fibroblasts avoid the cytotoxicity of melanin-intermediate DHI by producing pheomelanin-excess melanin, because the cells cannot avoid DHI-pathway without DOPAchrome tautomerase activity. Preliminary results of tyrosinase and TRP-2 cDNA double-transfection experiments indicated that only tyrosinase and DOPAchrome tautomerase activities were also insufficient to produce eumelanin (in preparation). These findings may indicate that some other factors, such as TRP-1 and/or melanosomal protein pMel-17 (Zhou *et al.*, 1994), are necessary for stable eumalnogenesis and residual DHICA-oxidase activity of human tyrosinase may be insufficient for complement of human TRP-1 deficiency.

To answer this question, various combinations of TRPs cDNA- transfected fibroblasts were constructed and these cells are under investigation.

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