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Melanosome degradation in epidermal keratinocytes related to lysosomal protease cathepsin V

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ABSTRACT

The cause of hyperpigmentation, such as solar lentigo and seborrheic keratosis, is the excessive accumulation of melanin pigments in the epidermal basal layer. Melanin pigments are synthesized in the melanosomes, which are specific organelles produced by melanocytes in the basal layer. Melanosomes containing melanin pigments are transported to the neighboring keratinocytes. However, the behavior of melanosomes after being transported to the keratinocytes has been poorly understood.

In this study, we focused on a lysosomal protease cathepsin V (CTSV) to clarify the mechanism underlying melanosome degradation in the keratinocytes. Using immunohistochemical observation, we found that CTSV was highly expressed across the entire epidermis in normal skin; however, CTSV expression levels were lower in the basal layer than those in the stratum corneum side in the hyperpigmented region. Moreover, we found that melanosome degradation was suppressed in CTSV knockdown cells. These results indicated that CTSV is involved in melanosome degradation.

1. Introduction

The main factor that determines the human skin color is the amount of melanin. Melanin pigments are synthesized in the melanosomes, which are lysosome-related organelles produced by melanocytes in the basal layer. Melanosomes are transported from the melanocytes to the neighboring keratinocytes, where they form an umbrella-like structure called “melanin cap” and play the role of protecting the cell nucleus from harmful ultraviolet radiation [1].

In hyperpigmentation disorders of the skin, such as seborrheic keratosis and solar lentigo (age spots), excessive melanosomes accumulate at the basal layer of the epidermis [2]. It is considered that melanosome degradation is stagnated in the epidermal basal cells of hyperpigmented skin. However, the final destination of melanosomes after melanin cap formation has been poorly understood.

Examination of melanosome distribution in each layer of the epidermis showed that the majority is in the basal layer, and melanosome number decreases toward the stratum corneum [3]. Therefore, melanosomes are assumed to undergo digestion and degradation along with differentiation of epidermal cells.

Lysosomes contain various enzymes to break down biomolecules engulfed by a cell. In recent years, the expression of cathepsin V (CTSV), a cysteine protease in lysosomes, has been reported to be higher in light-colored skin than in dark skin [4].

Moreover, melanosomes in keratinocytes derived from light-colored skin were observed in the vicinity of colloidal gold-labeled CTSV by immune electron microscopy [5].

In that study, differential expression of six lysosomal hydrolases was confirmed by microarray analysis of the epidermis from light-colored and dark skin. Interestingly, only the protein expression and activity of CTSV were confirmed. Based on these previous studies, CTSV is assumed to be involved in melanosome degradation and one of the factors regulating pigmentation.

2. Materials and methods

2.1. Human skin/immunohistochemical staining

Skin with hyperpigmentation (from a 46-year-old male) and normal skin (from a 32-year-old female) were obtained from Japanese volunteer subjects. A physician collected the skins after obtaining informed consent. The collected skin tissues were fixed in

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phosphate-buffered with 4% paraformaldehyde, embedded in paraffin, and sliced into 5-μm sections. After deparaffinization and blocking processes, the tissues were subjected to fluorescence immunohistochemistry using anti-cathepsin V (Abcam; ab166894) and Alexa Fluor® 647 goat anti-rabbit IgG (Life Technologies) as the primary and secondary antibodies, respectively. Melanin pigments were visualized using the Fontana-Masson stain. Slides were observed under a fluorescence microscope (BZ-X700, KEYENCE Corporation).

2.2. Cell culture

The human melanoma cell line, HMV-II (RCB0777), was provided by the RIKEN BRC. HMV-II cells were cultured in DMEM/F12, GlutaMAX medium containing 10% fetal bovine serum (GIBCO). Normal human epidermal keratinocytes (NHEKs) (Life Technologies) were cultured in EpiLife medium supplemented with human keratinocyte growth supplement (Life Technologies). HaCaT keratinocytes were provided by the German Cancer Research Center ("DKFZ", Germany) under a Material Transfer Agreement. HaCaT cells were cultured in DMEM medium containing 10% fetal bovine serum (GIBCO). All types of cells were grown at 37 °C under 5% CO_2.

2.3. Melanosome incorporation into keratinocytes

The isolation of melanosomes was performed as previously described [6], with a few modifications. Briefly, HMV-II human melanoma cells were cultured in HYPERFlasks (Corning). After seven days of culture, the cells were harvested and stored in a freezer until used. Lysis buffer (0.1 M Tris-HCl pH 7.5, 1% Igepal CA-630, 0.01% SDS) was added to the cells (1.6×10^8 cells/4 mL lysis buffer) and stored on ice for 20 min, with mixing every 10 min. After centrifugation (1000 × g for 10 min at 4 °C), the supernatants were transferred to new tubes and centrifuged again in the same manner. The supernatants were further centrifuged (20,000 × g for 10 min at 4 °C); the precipitates were washed twice by PBS with brief and gentle mixing to avoid dispersion and were centrifuged again (20 000 × g for 10 min at 4 °C). Finally, 200 μl of PBS was added to the pellets and mixed by pipetting. The isolated melanosomes from HMV-II melanoma cells were added as suspensions to NHEKs or HaCaT keratinocytes. After 24 h of incubation, keratinocytes were washed with PBS to remove non-incorporated melanosomes.

2.4. Immunofluorescence staining and measurement of melanosomes in keratinocytes

Keratinocytes were cultured on chamber slides (Iwaki Science). After melanosome uptake, the cells were fixed with 4% paraformaldehyde/PBS and were permeabilized with 0.2% Triton X-100/PBS. The cells were then incubated in 0.2% BSA/PBS, followed by treatment with anti-gp100 antibody (Ab34165, 1:100) and anti-cytokeratin antibody (Ab80826, 1:100). Cells were then incubated with Alexa Fluor® 488- or 647-labeled secondary antibodies (Life Technologies; 1:250) and Hochest33342 for nuclear staining. Finally, slides were mounted in ProLong® Diamond Antifade Reagent (Life Technologies) and observed under a fluorescence microscope (BZ-X700, KEYENCE Corporation).

Melanosome content in the cells was analyzed using a flow cytometer (BD FACS Calibur™). Collected cells were fixed by phosphate-buffered 4% paraformaldehyde, followed by membrane permeation using 0.2% Triton X-100. After blocking with 1% BSA/PBS, the cells were incubated with primary antibody for gp100, NKI/beteb (Ab34165) overnight at 4 °C, and then Alexa Fluor® 488-labeled secondary antibody (Life Technologies) for 1 h at room temperature. Intracellular labeled-gp100 was quantified using the value of mean fluorescence intensity. All flow cytometry data were analyzed by the FlowJo® software.

2.5. siRNA transfection

CTSV-specific siRNA (Silencer® Select, ID s3758) and control siRNA (Silencer® Select Negative Control #1 siRNA) were purchased from Life Technologies. HaCaT keratinocytes were transfected with either 10 nM CTSV siRNA or control siRNA, using Lipofectamine® RNAiMAX (Life Technologies).

2.6. Immunoblotting

Cells were lysed in protein extraction reagent (0.2 M phosphate buffer, pH 6.8; 1% Triton X-100; Protease Inhibitor Cocktail) and analyzed on 10% SDS-PAGE. Proteins were transferred to PVDF membranes and blocked with 1% BSA in PBS. The membranes were incubated with CTSV primary antibody overnight at 4 °C and then with HRP-conjugated secondary antibody (GE Healthcare) for 1 h at room temperature. Immunoreactivity was detected using Super-Signal® West Pico chemiluminescence substrate (Pierce), and β-actin (A5441, Sigma-Aldrich) was used as an internal loading control standard.

2.7. Statistics

Comparisons between two groups were performed using Student's *t*-test. Data values were expressed as mean ± S.D. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. CTSV expression decreases in the hyperpigmented region of human skin

Firstly, we investigated CTSV expression in the hyperpigmented region of human skin tissue. In histological images, the accumulation of intense melanosomes in the basal layer was observed in hyperpigmented skin compared with the normal skin (Fig. 1a and b). Immunofluorescence staining of CTSV in normal skin showed that CTSV is strongly expressed across the entire epidermis (Fig. 1c). Conversely, in the hyperpigmented region, CTSV levels were lower in the basal layer than in the stratum corneum (Fig. 1d). These results indicated that decreased CTSV levels were associated with excessive accumulation of melanosomes in basal keratinocytes and suggested that CTSV reduction may be linked to lower degradation of melanosomes.

3.2. Melanosomes are degraded in the lysosomes in keratinocytes

CTSV is a lysosomal protease. To examine whether melanosome degradation in keratinocytes occurs in the lysosomes, we prepared melanosome-incorporated keratinocytes (cf. "Materials and method 2.3"). We measured gp100 (melanosome specific marker) [7,8], and flow cytometry was used for the quantitative analysis of melanosome content. In this model, the number of melanosomes decreased over time in the cultured keratinocytes. (Supplemental Fig. S1).

By adding leupeptin (a cysteine/serine protease inhibitor) [9], the decrease of melanosome content in keratinocytes was inhibited (Fig. 2a and b). This indicated that melanosome degradation is mediated by lysosomes proteases, including CTSV.