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Original Article

Effects of cathepsin B on proliferation, activation, and melanin synthesis of human hair follicle melanocytes

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Abstract: Objective: The aim of this study was to investigate the effects of cathepsin B on proliferation, activation, and melanin synthesis of human hair follicle melanocytes (HFM). Methods: HFM from normal people were cultured, *in vitro*, and its morphology, proliferation, tyrosinase activity, and melanin synthesis were observed in different concentrations of cathepsin B (0.3-1.2 ng/mL). Expression of pan-actin protein, after treatment, was detected by cell immunofluorescence assay while proliferation, tyrosinase activity, and melanin synthesis were detected via methyl thiazolyl tetrazolium method. Results: After treatment with cathepsin B for 7 days, the dendrites of HFM proliferated more in number and length compared to the control group. Expression of pan-actin and melanin was significantly higher than those in control group (both $P<0.01$), accompanied with increased tyrosinase activity, especially at a concentration of 0.6 ng/mL. Conclusion: Cathepsin B can induce proliferation of human HFM while increasing tyrosinase activity and melanin synthesis.

Keywords: Cathepsin B, human hair follicle melanocyte, proliferation, tyrosinase activity, melanin synthesis

Introduction

Reduction of pigmented melanocytes in hair follicles is the main cause of graying hair. Recovery of hair follicle melanocytes (HFM) is due to the migration of melanocytes with undamaged outer root sheaths from the middle and lower part of the hair follicle to the upper part, where cathepsins are located [1-4].

Cathepsin B, one of the cysteine proteases, is 80% homology of cathepsin Land and is found in hair follicles from outer root sheaths to inner root sheaths, as well as in HFM [4]. In addition, cathepsin B acts as a proapoptotic mediator in human epidermal melanocytes exposed to Ultraviolet A/B when it is released from lysosomes [5].

Whether cathepsin B plays a role in human HFM remains unknown. In this study, the effects of cathepsin B's overexpression or knock-down on morphology, proliferation, tyrosinase activity, and melanin content of HFM were observed.

Materials and methods

Main reagents and instruments

Goat anti-human pan-actin primary antibodies were obtained from ABM, Canada. Rabbit anti-goat secondary antibodies were horseradish peroxidase labeled and obtained from Keqing Biology, Suzhou, China. Minimum Eagle's medium (MEM) was from Gibco, USA. Fetal bovine serum (FBS) was from Hyclone, USA. Other reagents included 0.25-0.50% separase (Sigma, USA), methyl thiazolyl tetrazolium (MTT, Shanghai Huamei Biotechnology, China), cathepsin B (Sigma, USA), immunofluorescence kit (Boster Biological Technology, Wuhan, China), 4',6-diamidino-2-phenylindole (DAPI, Sigma, USA), and dimethyl sulfoxide (DMSO, Sigma, USA), as well as methoxsalen (Sigma, USA).

The low-temperature high-speed centrifuge, inverted microscope, micro-nucleic acid quantitative analyzer, microplate reader, and ultraviolet/visible spectrophotometer were all obtained from Thermo, Germany. Super clean bench was obtained from Suzhou Purification, China while

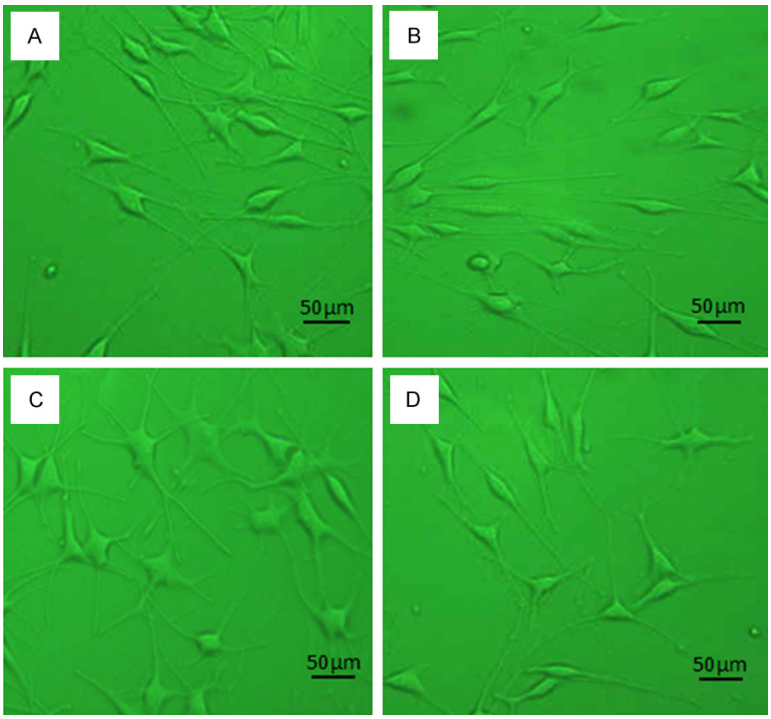

Figure 1. Morphologic changes of hair follicle melanocytes (400×); A. control group; B. 0.3 ng/mL cathepsin B group; C. 0.6 ng/mL cathepsin B group; D. 1.2 ng/mL cathepsin B group.

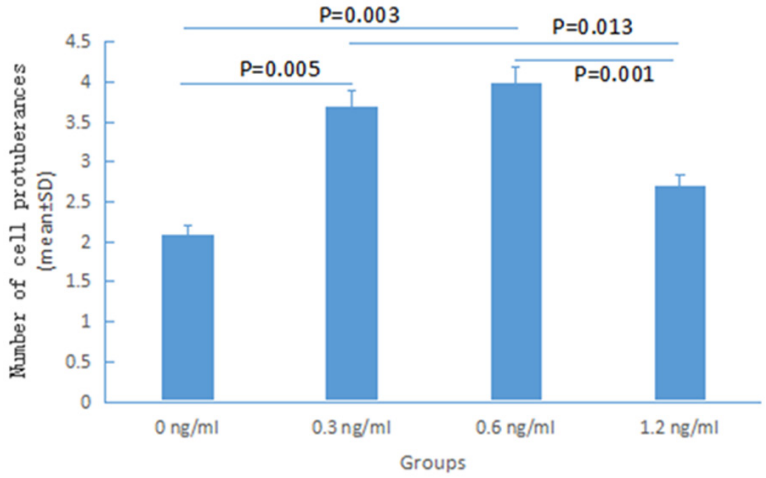

Figure 2. The number of hair follicle melanocytes dendrites in each group compared with the control group, HFM dendrites in the cathepsin B groups increased and the number of dendrites was highest in the 0.6 ng/mL cathepsin B group.

the immunofluorescence microscope was obtained from Beijing Liuyi, China.

Cell culture

Human HFMs were separated from a healthy donor's scalp via the separate method. They were cultured with MEM with penicillin 100 IU/mL, streptomycin 100 IU/mL, sodium bicarbonate 2 g/L, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid 2.98 g/L, sodium pyruvate 0.24 g/L, and 10% FBS. Cells were taken out from liquid nitrogen and warmed in a water bath at 37°C, immediately [6]. Next, cell suspension was transferred into the culture dish with RPMI1640 medium and cultured in an incubator containing 5% carbon dioxide. Afterward, the medium was changed every 2 days and regular subculture was carried out.

Morphology observation

Final concentrations of cathepsin B were set at 0.3 ng/mL, 0.6 ng/mL, and 1.2 ng/mL, respectively. In the control group, only phosphate buffer solution (PBS) was added in the culture medium. After culturing for a week, the morphology of HFM was observed by a high-powered microscope.

Effects of cathepsin B on expression of pan-actin protein

Cell immunofluorescence staining was used to detect expression of intracellular pan-actin (all forms of actin could be detected). First, melanocytes cultured with different concentrations of cathepsin B were fixed and then blocked with blocking buffer (PBS +5% goat serum +1% FBS) at room temperature for an hour. It was washed by PBS 3 times, 10 minutes each time. The primary antibody mouse anti-pan actin immunoglobulin G (diluted with 1% FBS PBS, 1:500) was added and incubated at room temperature for 16 hours. It was cleaned with 0.1% tween 20/PBS 4 times, 10 minutes each time. Goat anti-mouse secondary antibody con-

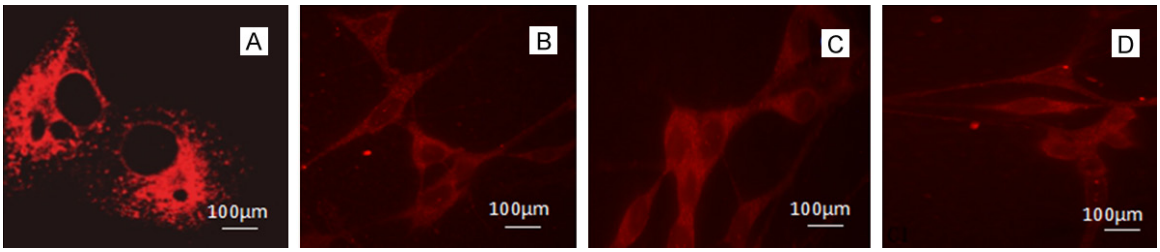

Figure 3. Expression of pan-actin protein detected by immunofluorescence (400×); A. Control group (0 ng/mL cathepsin B); B. 0.3 ng/mL cathepsin B group; C. 0.6 ng/mL cathepsin B group; D. 1.2 ng/mL cathepsin B group. The red immunofluorescence indicated pan-actin protein.

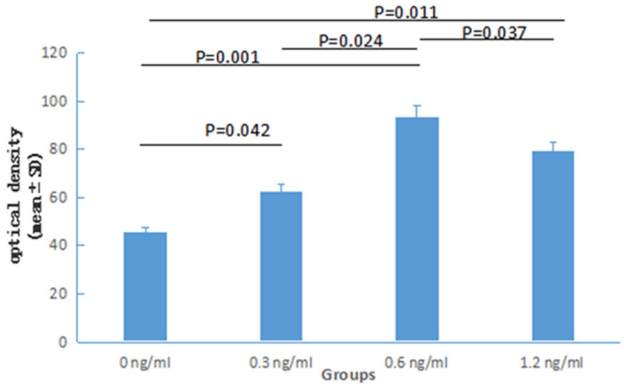

Figure 4. Optical density analysis of immunofluorescence in each group compared with control group (0 ng/mL cathepsin B), expression of protein in cathepsin B groups all increased and was the highest in 0.6 ng/mL cathepsin B group.

jugated fluorescein isothiocyanate (diluted with 1% FBS PBS, 1:250) was added and incubated for 2 hours at room temperature, then washed 3 times with TPBS, 10 minutes each time. Finally, it was cleaned 3 times with 0.9% sodium chloride solution, 10 minutes each time. Fluorescence intensity was analyzed by image J.

Detection of proliferation, activation, and melanin synthesis

HFM proliferation measurement: proliferation rate was calculated by MTT assay with the following formula [6]. Proliferation rate = (A490 with methoxsalen - A490 without methoxsalen)/(A490 without methoxsalen - A490 with medium) *100%.

Measuring method of tyrosinase activity referred to the report by Ando et al. [7]. Tyrosinase activity = A475 in cathepsin B group/A475 in control group *100%.

Measuring method of melanin content referred to reports by Ando et al. [7, 8]. Melanin content = A400 in cathepsin B group/A400 in control group * 100%.

Statistical processing

Observation results were statistically analyzed by SPSS23.0 software. All measurement data are expressed as mean ± standard deviation. One-way analysis of variance and Bonferroni's post hoc tests were used for comparison among groups. Enumeration data are expressed as number and rate and comparison among groups was tested by χ^2. Differences were statistically significant when $P<0.05$.

Results

HFM morphologic changes

After 1 week of culturing, the number of dendrites of HFM at each concentration of cathepsin B group was significantly increased. Cell bodies were also increased. Pigment granules were observed in the cytoplasm under a high-powered microscope, especially in the 0.6 ng/mL cathepsin B group. See **Figures 1**, **2**.

Pan-actin protein expression

Expression of pan-actin protein was detected by immunofluorescence and mainly expressed in the cell membrane. Compared with the control group, expression of pan-actin in cathepsin B groups increased, it was the highest at 0.6 ng/mL cathepsin B. See **Figures 3**, **4**.

HFM proliferation

Obvious proliferation of HFM could be observed at 3 days, 5 days, and 7 days (all $P<0.01$). Compared with the other three groups, effects reached the maximum in the 0.6 ng/mL cathepsin B group (compared with 0.3 ng/mL

Table 1. Proliferation of HFM treated by different concentrations of cathepsin B

Group	Proliferation rate			F value	*P* value
	3 d	5 d	7 d		
Control group	0.21±0.35	0.43±0.26	0.54±0.42	1.024	0.218
0.3 ng/mL	14.27±2.38	28.79±6.27	40.18±11.24	10.284	0.001
0.6 ng/mL	25.36±2.97	43.56±7.68	58.79±11.24	9.682	0.001
1.2 ng/mL	10.16±0.87	22.05±1.24	36.54±9.76	9.179	0.001
F value	8.932	10.835	9.631		
P value	0.008	0.001	0.003		

Table 2. Effects of different concentrations of cathepsin B on tyrosinase activity of hair follicle melanocytes

Group	Tyrosinase activity			F value	*P* value
	3 d	5 d	7 d		
Control group	101.64±1.26	106.74±3.94	111.28±4.57	1.237	0.304
0.3 ng/mL	122.53±10.36	166.85±20.32	182.94±20.35	6.273	0.029
0.6 ng/mL	144.38±19.06	227.53±45.72	271.86±39.05	9.305	0.003
1.2 ng/mL	112.05±15.83	133.43±14.65	148.09±22.34	7.316	0.011
F value	6.059	8.294	9.351		
P value	0.025	0.011	0.004		

Table 3. Effects of different concentrations of cathepsin B on melanin synthesis of hair follicle melanocytes

Group	Melanin synthesis			F value	*P* value
	3 d	5 d	7 d		
Control group	101.42±2.09	108.75±5.36	115.09±6.05	1.089	0.218
0.3 ng/mL	142.36±23.47	209.78±40.32	228.79±46.58	9.307	0.012
0.6 ng/mL	172.36±28.57	304.56±48.95	332.32±54.08	5.203	0.028
1.2 ng/mL	131.86±18.06	166.85±31.24	174.56±28.95	4.109	0.032
F value	7.834	8.018	9.657		
P value	0.018	0.012	0.002		

group, P = 0.003, compared with 1.2 ng/mL group, P = 0.001, and compared with the control group, P = 0.001). Proliferation rates in the 3 groups all evidently increased with prolongtion of time (all P = 0.001). See **Table 1**.

Tyrosinase activity

After treatment with cathepsin B for 3 days, 5 days, and 7 days, HFM showed significant increases in tyrosinase activity. Activity was the highest in the 0.6 ng/mL cathepsin B group (compared with 0.3 ng/mL group, P = 0.025; compared with 1.2 ng/mL group, P = 0.011; compared with the control group, P = 0.004). Effects of cathepsin B on promoting tyrosinase activity in the 3 groups all evidently increased with prolongation of time (P = 0.029, P = 0.003 and P = 0.011). See **Table 2**.

Melanin synthesis

After treatment with cathepsin B for 3 days, 5 days, and 7 days, HFM showed significant increases in melanin synthesis. Melanin content was the highest in the 0.6 ng/mL cathepsin B group (compared with the 0.3 ng/mL group, P = 0.018; compared with the 1.2 ng/mL group, P = 0.012; compared with the control group, P = 0.002). Effects of cathepsin B on melanin synthesis in the 3 groups all significantly increased with prolongation of time (P = 0.012, P=0.028 and P = 0.032). See **Table 3**.

Discussion

Melanocytes, in normal human skin, are mainly found in the basal layer of the epidermis and in hair follicles. In recent years, one study found that, due to HFM being different from epidermal melanocytes in morphological distribution, antigen expression, and functional characteristics, as well its role in the pathogenesis and treatment of the graying of hair, it may become a source of melanocytes for treatment of graying hair. Its regulation factors, however, are not clear yet [9]. Cathepsin B is a cysteine proteinase in lysosomes, playing a very extensive role and participating in many physiological and pathological processes [10]. One study has shown that expression of cathepsin B in photoaging skin and aging fibroblasts is decreased and time-dependent. This relates to the decline

of self-repair ability of photoaging skin [11]. The effects of cathepsin B on proliferation and activation of HFM requires further study.

This study indicated that cathepsin B showed obvious effects of promoting proliferation at 0.3 ng/mL cathepsin B, reaching the maximum at 0.6 ng/mL. Effects were decreased at 1.2 ng/mL compared with that at 0.3 ng/mL. Proliferation rates in the 3 cathepsin B groups increased in a time-dependent manner. Compared with the control group, tyrosinase activity and melanin synthesis increased significantly on the 5th day in the 0.3 ng/mL cathepsin B group, reaching the maximum in the 0.6 ng/mL group. Effects decreased in the 1.2 ng/mL group compared with the 0.3 ng/mL group but were still better than the control group. Therefore, cathepsin B can induce proliferation of human HFM, while increasing tyrosinase activity and melanin synthesis at suitable concentrations.

Additionally, after treatment with cathepsin B, HFM dendrites lengthened and increased and visible pigment granules appeared in the cytoplasm. Dopa staining turned to positive from negative and both cell bodies and dendrites were stained, suggesting that HFM was activated. This suggested that cathepsin B receptors might exist in HFM. Therefore, cathepsin B could directly stimulate proliferation and activation of HFM and participate in the pigment recovery of graying hair.

Cathepsin B can degrade myosin, troponin, myogenic protein, and actin. It plays an important role in extracellular matrix remodeling through the degradation of matrix, regulation of interstitial vascular proliferation, and assistance of cytokine regulation [12-14]. This study detected expression of pan-actin protein in melanocytes treated with cathepsin B by immunofluorescence. The results showed that pan-protein expression increased in 3 cathepsin B groups. It was the highest in 0.6 ng/mL cathepsin B group, indicating that cathepsin B could promote pan-actin protein expression in melanocytes. Melanosome transport includes two processes, the transport of melanosomes within melanocytes and transport of melanosomes from melanocytes to keratinocytes. Proteins involved in transport of melanosomes within melanocytes mainly including kinesin, dynein, and myosin. A large number of studies have shown that the above three proteins play an important role in the synthesis of melanin [15-19]. This study found that cathepsin B promoted the synthesis of pan-actin proteins as well as the synthesis of melanin. The mechanism might be that cathepsin B continuously cuts dipeptides from the desmin C-terminal via dipeptidase and its cleavage conditions do not depend on the existence of hydrophobic free amino acids at cleavage position [20]. The specific mechanisms, however, still require further research.

Previously, a study by Gopinathan et al. found that cathepsin B can promote proliferation and differentiation of pancreatic ductal carcinoma cells through mitogen-activated protein kinase pathways [21]. A study by Rajah et al. has suggested that cathepsin B inhibitor z-FAFMK can decrease levels of caspase-8, glutathione, and reactive oxygen species, thus inhibiting proliferation of T-cells [22]. In short, the mechanism by which cathepsin B activates and proliferates cells may involve a variety of channel proteins but specific mechanisms are not yet clear.

In conclusion, cathepsin B can induce proliferation of human HFM, while increasing tyrosinase activity and melanin synthesis. The specific mechanisms still require further study.

Disclosure of conflict of interest

None.

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