Effects of diethylstilbestrol on the proliferation and tyrosinase activity of cultured human melanocytes

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Abstract. The aim of the present study was to observe the effects of different exogenous estrogen diethylstilbestrol (DES) concentrations on the human melanocyte proliferation and tyrosinase activity. Skin specimens were obtained following blepharoplasty, and the melanocytes were primary cultured and passaged to the third generation. The melanocytes were seeded in 96-well plates, each well had $5x10^3$ cells. The medium was changed after 24 h, and contained 10^{-4}-10^{-8} M DES. After the melanocytes were incubated, the proliferation and tyrosinase activity were detected by the MTT assay and L-DOPA reaction. DES (10^{-8}-10^{-6} M) enhanced the proliferation of cultured melanocytes. The intensity was positively correlated with the concentration of drug. DES, >10^{-5} M, inhibited the melanocytes proliferation or even produced the toxicity effect. Following the addition of 10^{-6} M DES to the medium, the tyrosinase activity of melanocytes was significantly increased, with $P<0.05$. In conclusion, a certain concentration of DES promoted the proliferation of melanocytes, enhanced the activity of tyrosinase and promoted pigment synthesis of melanocytes, with the optimal concentration of 10^{-6} M.

Introduction

Certain pigmented diseases, such as melasma, often occur in females. These hyperpigmentations result from an increase in melanin and its treatment remains a challenge. The epidermal pigmentation and skin malignant melanoma may be associated with estrogen. The pigmented spot becomes more severe and malignant melanoma proceeds rapidly during an abnormal menstruation or pregnancy. There are changes in the estrogen level in these physiological processes. Therefore, estrogen is considered an important factor that affects pigmented diseases (1). Diethylstilbestrol (DES) is a synthetic nonsteroidal estrogen that can produce the same pharmacological effects as natural estrogen (2). In order to explore the mechanism, the excess skin following eyelid blepharoplasty was collected for melanocyte culture and different DES concentrations were used to detect the effect on the proliferation and tyrosinase activity of melanocytes.

Materials and methods

Reagent. DES (D4628; Sigma, St. Louis, MO, USA), M254 melanocytes culture medium (M-254-500; Invitrogen Life Technologies, Carlsbad, CA, USA), Human Melanocytes Growth Supplement (HMGS, S-002-5; Invitrogen Life Technologies), 0.25% neutral proteinase (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), 0.25% trypsin mixed with 0.02% EDTA, newborn calf serum (Sijiqing Biotechnology Co., Hangzhou, China), 0.1% L-DOPA (D9628; Sigma) and S-100 monoclonal antibody (BMO120; Wuhan Boster Biological Technology, Ltd., Wuhan, China) were used.

Drug preparation. An electronic balance was used to weigh 26.8 mg DES. It was dissolved in 10 ml dehydrated ethanol. A 50-μl solution was removed and diluted with 5 ml M254 culture medium; 10^{-4} M DES was acquired. Subsequently, it was diluted into 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} M as final concentrations. The solutions were preserved at 4˚C in darkness.

Melanocytes cultured. The excess skin following eyelid blepharoplasty was collected for cell culture. Informed consent was obtained from all the patients and it was approved by the Institutional Review Board. The specimen was soaked with povidone-iodine solution for 5 min and was subsequently flushed with saline. The subcutaneous tissue and part of the dermis was cut off. The specimen was cut into 0.5-mm strips. The tissue was soaked in 0.25% neutral protease and preserved overnight at 4˚C. The epidermis was removed and digested by 0.25% trypsin and 0.02% EDTA at 37˚C for 5 min. The

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digestion was stopped by serum. A single-cell suspension could be acquired after being blown by pipette. Following this it was filtered by 200-mesh and centrifuged at 400 x g for l6 min, twice. The M254 medium mixed with HMGS was added to the cell suspension. The cell was seeded with a density of $5x10^5$ in a 25-cm^2 culture bottle, which was incubated at 37˚C and 5% CO_2. The medium was changed after 48 h. When melanocytes grew to 70-80% fusions, 0.25% trypsin mixed with 0.02% EDTA was added for digestion. The cells were gently blown, and serum was added to stop the digestion. Centrifugation was performed at 400 x g for 6 min, twice, and the supernatant was discarded. The fresh M254 medium and HMGS was added for passage, and cultured with a density of $5x10^5$ cells in a 25-cm^2 culture bottle. The medium was changed firstly after 24 h, and then every 3-4 days.

Melanocyte identification. The second generation of cells were seeded on a slide, and subsequently fixed by cold acetone. L-DOPA (0.1%) was added and incubated for 4 h at 37˚C for DOPA staining. Both DOPA and immunohistochemical staining of S-100 were used for identification.

Effect of different concentrations of exogenous DES on melanocyte proliferation by the MTT method. The third-generation melanocytes in the logarithmic growth phase were used. Following trypsin digestion, the cell concentration was adjusted to $2.5x10^4$/ml and inoculated in a 96-well culture plate. There were 6 groups in total; 3 wells for each group and 200 μl of cell suspension for each well. After incubation for 24 h, the medium was changed and different DES concentrations in the medium were added to wells in different plates, with blank medium and medium containing 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M DES. Each group was cultured for 5 days, and subsequently 20 μl MTT (5 mg/ml) was added to each well. The cells were incubated for 4 h, and the supernatant was discarded. To each well 150 μl dimethylsulfoxide was added. The cells were oscillated for 10 min, and the absorbance value [optical density (OD)] was measured with the enzyme mark instrument (Bio-Cell Ltd., Ramat Gan, Israel), with an excitation wavelength of 490 nm.

Effects of DES on melanocyte tyrosinase activity by the L-DOPA reaction. When the melanocytes had reached 70-80% confluence, the cells were digested and seeded according to the method for the MTT assay. A total of 7 groups (group 1 contained medium with no cells, group 2 contained no DES and groups 3-7 contained medium with 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} or 10^{-4} M DES), with 3 wells for each group. The melanocytes were inoculated for 72 h. Subsequently, the supernatant was discarded and the cells were washed with phosphate-buffered saline twice. Triton X-100 (100 μl; 10 ml/l) was added to each well and the cells were agitated for 5 min, before 100 μl L-DOPA (0.1%) was added and incubated for 2 h at 37˚C. The absorbance value A was measured by the enzyme mark instrument with 490 nm wavelength. The activity of tyrosinase = $(OD_{group\ X} - OD_{group\ 1})/(OD_{group\ 2} - OD_{group\ 1}) \times 100\%$, (group X refers to groups 3-7).

Statistical analysis. Results are expressed as mean ± standard deviation. Statistical analysis was performed with the SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) software. All the data were analyzed by analysis of variance followed by the post hoc Bonferroni/Dunnet multiple comparison test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Melanocytes culture. Primary culture of cells from the skin samples began to adhere after 24 h, with some multi-dendritic cells scattered among the epidermal cells under inverted microscopy. The multidendritic cells had ≥2 dendrites and an orbicular-ovate nucleus. Epidermal cells did not grow well and appeared to be slightly adherent in the melanocyte culture medium, which was discarded following medium exchange (Figs. 1 and 2).

Cell identification. Following DOPA staining, black particles could be observed in the cytoplasm and dendrites after light microscopy analysis (Fig. 3). Anti-S-100 protein immunohistochemistry showed yellow staining in the cytoplasm and dendrites, which was strong positive (Fig. 4).

MTT assay. The OD value changes reflect the effect of different concentrations of exogenous estrogen DES on the proliferation of human melanocytes. The proliferation of cultured melanocytes *in vitro* was induced by 10^{-8}-10^{-6} M DES. The intensity was positively correlated with the drug concentration. Concentrations of $>10^{-5}$ M DES presented inhibition or toxicity effects on the melanocytes growth (Figs. 5 and 6). The variance analysis showed that 10^{-8}, 10^{-7} and 10^{-6} M concentration groups compared with the control groups had significant differences ($P<0.05$) (Fig. 7).

L-DOPA reaction for tyrosinase activity. As shown in Fig. 8, different concentrations of exogenous estrogen DES had different results for the tyrosinase activity of melanocytes. The tyrosinase activity was significantly induced by 10^{-8}-10^{-6} M DES, compared with the control group ($P<0.05$). The maximum effect was for 10^{-6} M DES incubation, whereas 10^{-4} M DES inhibited the tyrosinase activity.

Discussion

In humans, estrogen may be involved in numerous physiological processes of skin and hair follicles, such as skin aging, hair loss (3), wound healing and scar formation (4). Hyperpigmentation has been documented during pregnancy (melasma), in women ingesting oral contraceptives containing estrogens and in female and male infants treated with ointments containing estrogens (5). Particular regions of the body appear to be affected, including the genitals, abdomen, face and mammary areola. These clinical observations suggest that melanocytes can respond to estrogens by increasing their levels of pigmentation (6). The association between the changes of estrogen and morbidity of melasma has been the focus of previous studies (7).

DES is a synthetic nonsteroidal estrogen, which can produce the same pharmacological effects as natural estrogen (2). Using physiological concentrations of DES, the effects of different concentrations of exogenous DES on cultured melanocyte

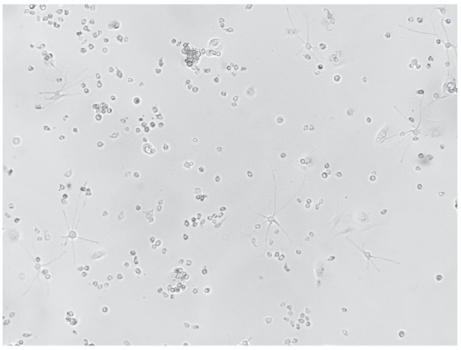

Figure 1. Primary culture of skin melanocytes. The gradual exfoliation of the epidermal cells (magnification, x200).

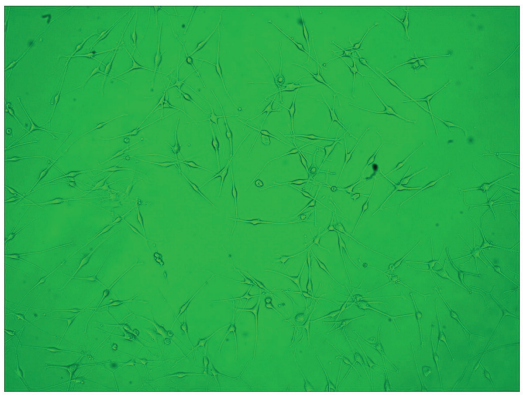

Figure 2. Melanocytes grew well after culture for 10 days (magnification, x200).

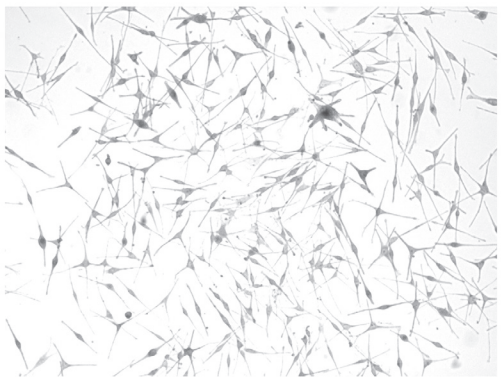

Figure 3. L-DOPA staining showing cytoplasmic staining [black particles (magnification, x200)].

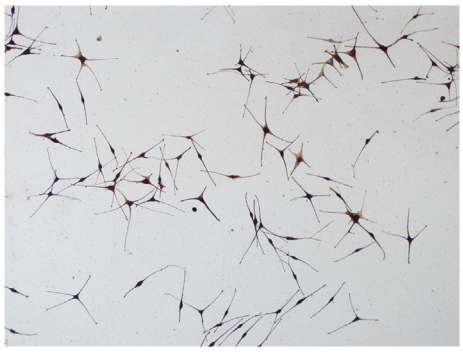

Figure 4. S-100 positive staining in the cytoplasm (magnification, x200).

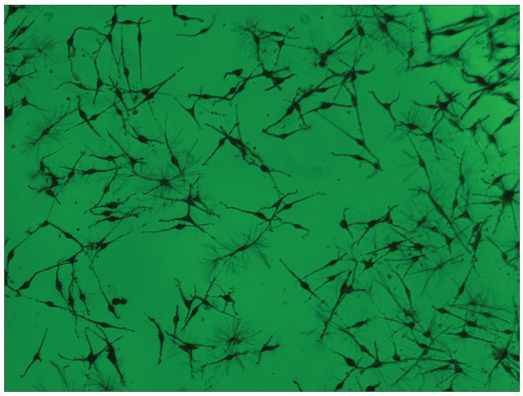

Figure 5. MTT staining when cultured in 10^{-7} M diethylstilbestrol (magnification, x200).

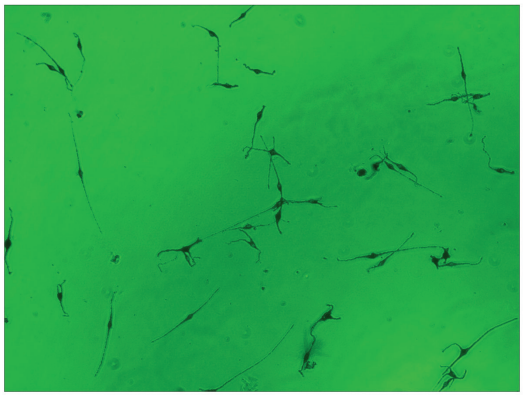

Figure 6. MTT staining when cultured in 10^{-5} M diethylstilbestrol (magnification, x200).

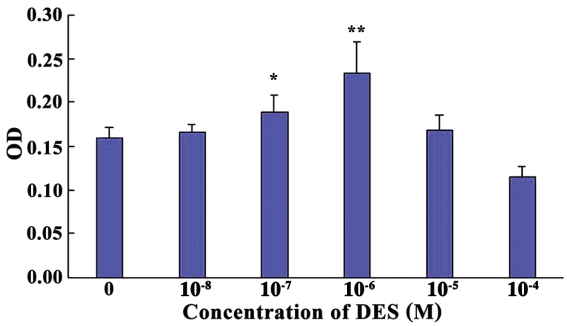

Figure 7. Effect of different diethylstilbestrol (DES) concentrations on human melanocytes proliferation. OD, optical density. *P<0.05 and **P<0.01.

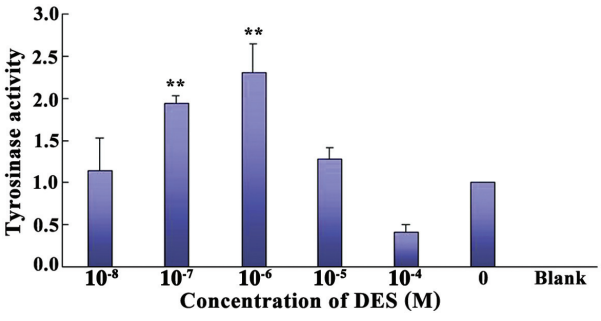

Figure 8. Effect of different diethylstilbestrol (DES) concentrations on the tyrosinase activity of melanocytes.**P<0.01.

proliferation were assessed using the MTT assay and L-DOPA reaction. The results showed that the 10^{-8}-10^{-6} M concentrations of DES promoted the proliferation of cultured melanocytes, and the intensity of the effect was positively correlated with the drug concentration. Up to 10^{-5} M DES, the dendritics of cells were less, the shape became rounded, the cells grew slowly and the expansion ability decreased, which indicated that $>10^{-5}$ M DES produced inhibitory or toxic effects on the growth of melanocytes and inhibited the tyrosinase activity. The results suggest that by estrogen adjustment, the occurring estrogen-related pigmentation disorders may be reduced or improved.

Estrogen usually plays a role in combination with the estrogen receptor (ER). Despite strong clinical indications of an association between pregnancy and melanocytic responsiveness, no studies have successfully established a linkage between hormone responsiveness and nevi during pregnancy. The absence of ERα in melanocytes and melanoma discouraged further investigation. A second form of the receptor, ERβ, was also discovered (8). ERα is mainly expressed in the sebaceous gland cells, while it is weakly expressed in the epidermis. ERβ has a stronger expression in keratinocytes, including the epidermal basal layer and prickle cell layer (9,10).

Estrogens can signal in a multifaceted manner involving diverse receptors that modulate genomic or non-genomic pathways, which in turn may have independent, synergistic or opposing actions. In addition, cell specific co-factors are also required and ligands that exhibit estrogenic activity in certain cells, paradoxically exhibit estrogen antagonism in others. Other studies have shown that DES could promote the generation of pro-opiomelanocortin and α melanocyte-stimulating hormone in melanocytes (11,12), which promotes the proliferation of melanocytes and pigment synthesis.

Estrogen deficiency following menopause results in atrophic skin changes and acceleration of skin aging. Estrogen administration has positive effects on human skin by delaying or preventing skin aging manifestations. However, the use of estrogen replacement is a risk factor in breast and uterine cancer (13). Through the present experiment, we further verified the role of estrogen in the human skin pigment formation. How to establish the balance of estrogen to prevent estrogen-related skin aging and pigmentation disorder disease is a challenge.

Acknowledgements

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