The Role of TMEM45A in Molecular Mechanism of Cell Differentiation Induced by Retinoic Acid

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Abstract: **Objective:** To study the effect of all-trans retinoic acid on the growth cycle of human keratinocyte line HaCaT and the expression of transmembrane protein 45A at different differentiation stages of HaCaT, so as to clarify the regulatory role of TMEM45A in the proliferation and differentiation of epidermal keratinocytes induced by retinoic acid. **Methods:** HaCaT cells cultured in vitro was used as the research object. After exposed a certain concentration of ATRA, the changes of cell cycle were detected by flow cytometry, and the expression of TMEM45A at mRNA and protein levels were detected by RT-PCR and immunohistochemical method at different stages. **Results:** Compared with the control group, the rate of G1 phase was significant increased, while S phase were decreased. Moveover, it was related to the function of time. The expression levels of TMEM45A mRNA and protein decreased gradually with the prolongation of ATRA treatment time. **Conclusion:** ATRA can inhibit the proliferation of keratinocytes and decrease the expression of TMEM45A at both mRNA and protein levels, suggesting that TMEM45A may play an important roles in maintaining the proliferation of human normal skin.

Key words: All-Trans-Retinoic Acid; TMEM45A; HaCaT

Introduction

The epidermis is a stratified squamous epithelium, and keratinocytes are the main component of epidermis. The abnormal proliferation and differentiation of keratinocytes are closely related to the occurrence of a variety of skin diseases. Transmembrane protein 45A (TMEM45A) , a member of the TMEM protein family, is located in the Golgi apparatus and plays an important role in regulating proliferation and differentiation in the epidermis and various tumor tissues [1]. Retinoids can induce proliferation, differentiation and apoptosis of epidermal cell, thus maintaining the normal keratinization process of epidermal tissue. At present, it has been widely used in the treatment of skin diseases, such as psoriasis, acne, skin tumors, pigmented skin diseases and so on. So whether TMEM45A is involved in the mechanism of epidermal differentiation induced by retinoic acid has not been studied yet. In this study, we investigated whether all-trans retinoic acid could induce the changes of TMEM45A in keratinocyte line HaCat, and explored the regulatory role of TMEM45A in the proliferation and differentiation of epidermal keratinocytes induced by retinoic acid.

Materials & methods

Cell culture and treatment

HaCaT cells were cultured in RPMI-1640 complete medium which contained 10% fetal bovine serum, at 37 ℃ in a CO2 incubator. Change the fluid every other day. They were classified into two groups. The first group was called blank control group, i.e. the cells were cultured in regular medium. In the second group, ATRA at a concentration of 10-6mol was added into the medium and kept for 48h. Cells were collected for detection after 24h and 48h dosing.

Cell cycle determination

The cultured cells were routinely digested and pipetted to form a suspension which contained 75% ethanol and 3% fetal bovine serum. Fixed at 4°C overnight, incubated at 37°C for 30 min with 11×L of RNaseA solution. Then it was stained in a dark room for 30min, by adding with iodine Propidium solution. The cell cycle was measured by Flow cytometry and the DNA content of cell cycle was analyzed by Cellquest software.

RT-PCR

Total RNA was extracted using Trizol according to the manufacturer,s instructions. An amount of 5μg total RNA was reverse-transcripted to cDNA which was the amplification template. PCR was carried out by the following protocol: a 95 ℃ denaturation step for 35s followed by 40 cycles of denaturation at 95 ℃ for 5S, annealing temperature at 60 ℃ for 30s. and 60 ℃ for 1min. The PCR products were identified by 2% agarose gel electrophoresis.

Immunohistochemical detection

According to the operating instructions of SP kit, the cells collected both in the control group and the ATRA groups. They were added with chromogenic solution, and the expression of TMEM45A was observed by microscope and photographed. The staining intensity was analyzed by MoticMed 6.0 Digital Medical Image Analysis System (A). Under the same conditions, 400 × digital photos were taken, and each slide was taken with 10 visual fields. The yellow area in the picture is used as the gray value to measure the average gray level.

Statistical analysis

Data are expressed as mean ± SEM. T-tested was performed on the experimental results. Differences were considered significant at $P<0.05$.

Results

ATRA inhibited the proliferation of keratinocytes

The ability of ATRA to inhibit proliferation of HaCaT cells was examined by flow cytometry. HaCaT cells were collected respectively both in the control group and the ATRA groups which exposed a certain concentration of ATRA for 24 and 48 hours. The results showed that compared with the control group, ATRA could change the distribution of HaCaT cell cycle at different times. The rate of G1 phase was significant increased, while S phase was decreased. Moveover, it was related to the time of ATRA treatment with significant difference ($p<0.05$). It is suggested that retinoic acid can arrest cells in G1 phase, which may inhibit cell proliferation by blocking the process of cell cycle, and the inhibition of cell proliferation is more obvious with the extension of action time. The results are shown in Table 1-1 and figures 1-1 to 1-4.

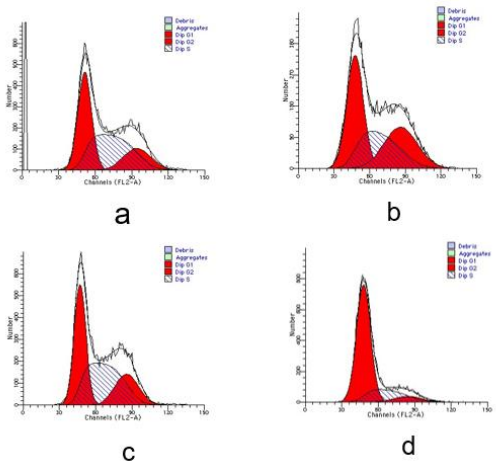

Figure 1-1 Cell cycle diagram after exposed ATRA (Note: a, b are 24h control group and ATRA group respectively; c, d are 48h control group and ATRA group respectively)

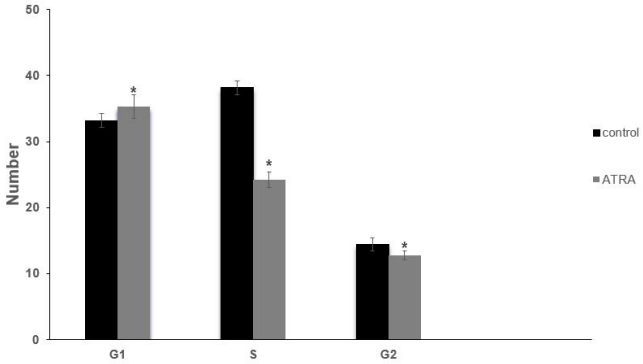

Figure 1-2 Cell cycle changes after treated by ATRA for 24 hours

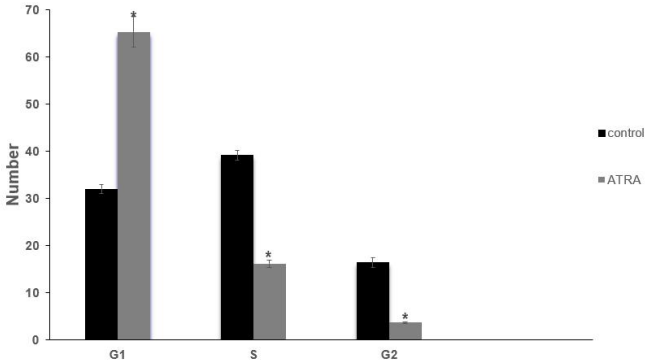

Figure 1-3 Cell cycle changes after treated by ATRA for 48 hours

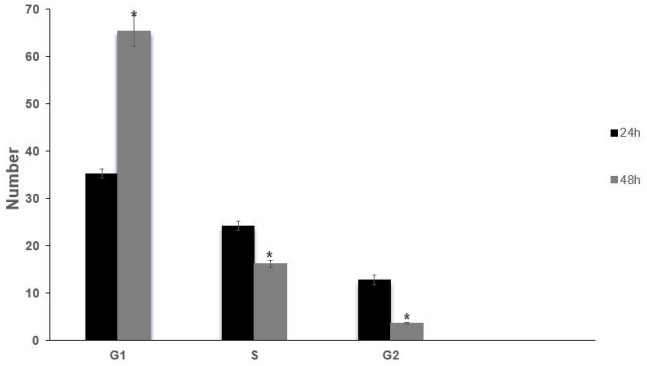

Figure 1-4 Changes of cell cycle after treated by ATRA for 24 and 48 hours

Table 1-1 Comparison of cell cycle after treated by ATRA

Group	24h		48h	
	Control group (%)	Experimental group (%)	Control group (%)	Experimental group (%)
S	38.16±1.37	24.24±1.23*	39.16±1.25	16.16±1.16*
G1	33.21±1.13	35.33±1.15*	32.05±1.19	65.36±1.02*
G2	14.42±1.03	12.76±1.07*	16.36±1.09	3.68±1.01*

Note: *: compared with the control group, $p<0.05$

ATRA down-regulated the expression of TMEM45A

The levels of TMEM45A mRNA and protein were examined by RT-PCR and immunohistochemical method in HaCaT cells grown with or without ATRA. Compared to β-actin control, the level of TMEM45A protein was significantly reduced in correspondence with an decrease in its mRNA after ATRA treatment.

The gray value of the β-actin band of the sample and the internal reference was measured by a multi-function image analyzer, and the expression level of TMEM45A mRNA was evaluated by the ratio of the sample value to the expression of the internal reference β-actin. The results showed that compared with the control group, the expression of TMEM45A mRNA decreased after retinoic acid treatment ($p<0.05$). With the prolongation of ATRA action time, the expression of TMEM45A mRNA gradually decreased. The results are shown in (Figure 2-1). The software measured the gray value of TMEM45A in HaCaT cells at different proliferation stages and made statistical analysis (Fig. 2-2, table 2-1).

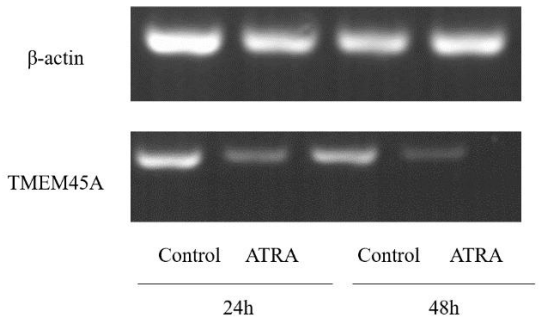

Figure 2-1 Expression of TMEM45A mRNA in HaCaT cells after treated by ATRA with 1 μ mol/L

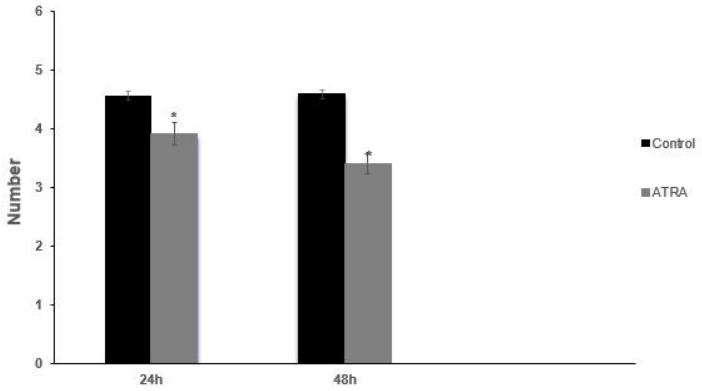

Figure 2-2 Gray scale rate of TMEM45A after treated by ATRA for 24 and 48 hours

Table 2-1 mRNA gray scale analysis and statistics result($\overline{x}$±s)

Group	Control group	Treatment group
24h	4.56±0.32	3.72±0.21*
48h	4.59±0.24	3.40±0.23*

Note: *: compared with the control group, $p<0.05$

The results showed that compared with the control group, the protein expression level of TMEM45A in the dosing group gradually decreased with the prolongation of ATRA treatment time. (Figure 3-1). The software measured the gray value of TMEM45A in HaCaT cells at different proliferation stages and made statistical analysis (Fig. 3-2, table 3).

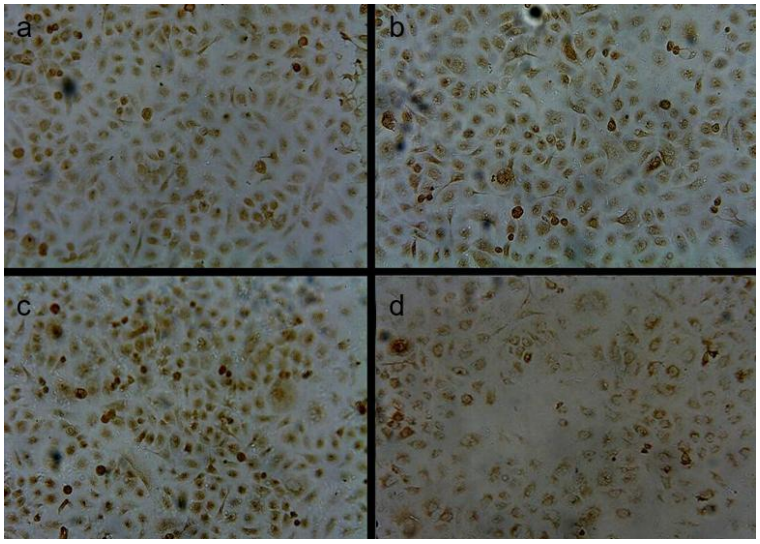

Figure 3-1 Expression of TMEM45A protein in HaCaT cells after treated by ATRA with 1 μ mol/L (Note: A and B are 24h control group and ATRA group respectively; C and D are 48h
control group and ATRA group respectively)

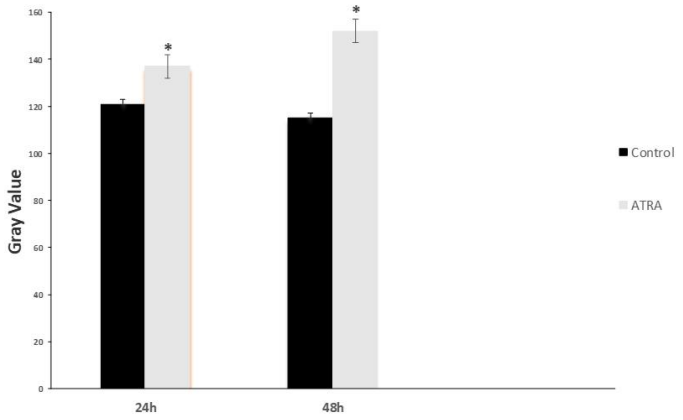

Fig. 3-2 Gray scale rate of TMEM45A after treated by ATRA for 24 and 48 hours

Table 3 Protein gray scale analysis and statistics result($\overline{X}$±s)

Group	Control group	Treatment group
24h	121±0.03	137±0.09*
48h	115±0.02	152±0.03*

Note: *: compared with the control group, $p<0.05$

Discussion

As the outermost layer of the skin, the epidermis is the first protective barrier of the human body and plays a vital role in maintaining the stability of the internal environment. Keratinocytes are the main component of the epidermis. They have a wide range of physical properties and have an important impact on the structure and function of the skin. The occurrence of a variety of skin diseases are closely related to its abnormal proliferation and differentiation, such as neurodermatitis, prurigo nodosa, psoriasis, pityriasis rosea, photoaging skin diseases, keratoderma, and premature skin failure syndrome. [2-4].

Transmembrane protein (TMEM) is a member of the large family of TMEM proteins, located on the Golgi apparatus, and is an important component of mitochondrial membrane, endoplasmic reticulum membrane, lysosomal membrane and Golgi apparatus membrane [1]. TMEM45A plays a key role in inducing proliferation and differentiation in epidermis and various tumor tissues. One study [5] found that the expression of TMEM45A was significantly increased in glioma tissue. Once the TMEM45A gene was silenced, the proliferation of glioma cells was significantly reduced. Thibodeau et al found that the expression of TMEM45A mRNA in high-grade renal clear cell carcinoma was significantly higher than that in low-grade renal clear cell carcinoma, normal renal tissue and benign renal disease tissue. Similarly, Guo et al [6] found that the expression of TMEM45A was also significantly increased in ovarian cancer. After silencing the expression of TMEM45A gene, cell proliferation was inhibited and the cell cycle was blocked in G1 phase, proving that TMEM45A gene can promote cell proliferation in ovarian cancer cells. Finally, It is important that TMEM45A is widely expressed in epidermal keratinocytes. Hayez a et al found that the expression of TMEM45A was increased in epidermal granular keratinocytes, which was related to the trans Golgi network and the granular layer in vivo. At the same time, its signal was also found at the anchor location of the intermediate filament of the stratum corneum and the keratinocyte, which was considered to be involved in epidermal keratinization. TMEM45A mRNA expression was up-regulated in both psoriasis and actinic keratosis, which was involved in epidermal proliferation and differentiation [7-10]. Retinoids are active derivatives of vitamin A. Since the 1920s, people have recognized that vitamin A plays a key role in the formation, proliferation, differentiation and maintenance of normal epithelium, retinoids began to attract extensive attention and research. Subsequent studies found that it plays an anti-proliferation and pro-differentiation effects in a variety of tumor cells [11-12]. For example, retinoic acid is often used to induce and differentiate malignant tumors, such as thyroid cancer, breast cancer, gastric cancer, lung cancer, bladder cancer, ovarian cancer, etc [12-13]. Further studies have found that retinoids play a key role in regulating

the normal keratosis of epidermal keratinocytes. They can induce the proliferation and differentiation of keratinocytes, promote normal keratosis, and correct hyperkeratosis and dyskeratosis [14-15]. At present, retinoids have been widely used in the treatment of skin diseases, such as psoriasis, skin tumors, acne and keratosis.

In this study, flow cytometry was used to detect cell cycle changes, and it was further confirmed that retinoic acid also inhibited HaCat cell differentiation and promoted epidermal keratinocyte HaCat cell cycle arrest in the early stage of DNA synthesis. RT-PCR and immunohistochemistry showed that ATRA treatment of HaCaT cells could reduce the gene and protein levels of TMEM45A. Thus, it is proved that ATRA can inhibit the proliferation of HaCaT cells, and the expression of TMEM45A which can be reduced at both mRNA and protein levels, suggesting that TMEM45A may play an important roles in maintaining the proliferation of human normal skin. The proliferation effect of ATRA-induced HaCaT cells may be achieved by down-regulating TMEM45A, and its more specific mechanism needs to be further studied.

Funding: This work was supported by the Science and Technology Projects for Social Development in Shaanxi Province (No. 2022SF-416).

Conflict of Interest: The authors declare that they do not have a conflict of interest.

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