Photodynamic therapy with 5-aminolevulinic acid suppresses IFN-γ-induced K17 expression in HaCaT cells via MAPK pathway

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Introduction

Psoriasis is a chronic inflammatory skin disease involving the epidermis, which is characterized by keratinocyte hyperproliferation and scaly erythematous plaques, showing a significant negative impact on quality of life. Psoriasis is estimated to affect 2% of the world’s population, with varying prevalence in different parts of the world. Psoriasis can occur at any age, but usually peaks at 20-30 and over 50 years of age. Trigger factors of psoriasis can be divided into two classes: exogenous and endogenous. The former includes physical and chemical factors, and seasonal variations, whereas the latter contains allergies, hormonal changes, and emotional stress. Recent studies have revealed that psoriasis is associated with many other diseases, such as obesity, dyslipidemia, diabetes mellitus, and cardiovascular disorders. Nonetheless, so far, there is still a lack of causative treatment for psoriasis. Therefore, effective treatment for psoriasis is urgently needed to improve the quality of life and reduce the possibility of psoriasis-triggering diseases.

Photodynamic therapy (PDT), which was first described by Oscar Raab in 1890, caused significant cell destruction by combining a photosensitizer with light and oxygen. The effect of PDT depends on the absorption of harmless visible light, which then produces reactive oxygen species (ROS), such as singlet oxygen, that destroy cancer cells, blood vessels, and pathogenic microorganisms. Various light sources can be applied for PDT, including blue lights, red lights, incoherent lamps, and light emitting diodes. The light source is directly related to the therapeutic effect on the specific disease. Photosensitizers used for PDT include 5-aminolevulinic acid (ALA), verteporfin, methylene blue, methyl-aminolevulinic acid (MAL), and hypericin. PDT is now a widely used treatment for various skin tumors and infectious or inflammatory skin disorders. PDT is mainly applied for plaque psoriasis, and in some cases for palmoplantar pustulosis, but has no effect on nail psoriasis. However, more biochemical studies are needed to confirm the effect of PDT on treating psoriasis and to investi-
igate the underlying mechanisms, to ensure maximum therapeutic effect while avoiding potential side effects.

One of the key characteristics of activated keratinocytes is the alteration of keratin (K) expression, including K1, K10, K6, K16, and K17. Of note, K17, as a hallmark, plays a crucial role in the pathogenesis of psoriasis and is the only keratin known to be induced by psoriasis-associated cytokines. Earlier studies have shown that interferon (IFN)-γ produced by natural killer (NK) cells and CD4+ T cells has pro-inflammatory effects on keratinocytes, and plays a central role in the overall pathogenesis of psoriasis. Also, the IFN-γ level in serum is correlated with clinical severity and activity of psoriasis. Thus, IFN-γ might be an inducer of keratinocyte hyperproliferation, and K17 could serve as a marker for evaluating the effect of psoriasis therapy.

In this study, the effect of PDT treatment, with ALA as a photosensitizer, on keratinocyte proliferation was investigated. The expression of the proliferative marker K17 was also evaluated. The therapy was also compared with the commonly used calcipotriol therapy. In summary, PDT plus ALA was investigated for use as an alternative medicine for psoriasis treatment.

**Materials and Methods**

**Cell Culture**

HaCaT cells, an immortalized human epidermal keratinocyte cell line, were purchased from Nanjing Keygen Biotech Co., LTD. HaCaT cells were cultured in dulbecco’s modified eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in a humidified atmosphere at 5% CO₂. HaCaT cells were stimulated with IFN-γ (250 U/ml) for 48 h, after which the cells were used for proliferation assays. For photodynamic treatment, ALA was used as a photosensitizer and was sustained for 4 h. Irradiation was performed with light at a wavelength of 635 nm at a dose of 70 J/cm².

**MTT Assay**

Cells of 80% confluence were treated with 0.25% Trypsin-EDTA (KeyGen Biotechnology Co., Ltd, Nanjing, Jiangsu, China) and the cell suspension was adjusted to a density of 5×10⁴ cells per ml. Cells were seeded at 100 µl/well into 96-well plates for MTT detection and incubated at 37°C for 24 h. Drugs were dissolved in complete culture medium, and 100 µl of the suspension was added per well of the 96-well plates. A negative control was set by adding only complete culture medium. The plates were then incubated at 37°C for 48 h; 20 µl of MTT stock solution (5 mg/ml) was added to each well and incubated for 4 h, allowing the formation of formazan product. After carefully removing the media, 150 µl of 100% DMSO was added to the wells to dissolve the formazan product. The supernatant was collected by centrifugation, and then transferred to a new 96-well plate. The absorbance of the reaction was measured using a spectrophotometer at 490 nm. The percentage rate of cell inhibition was calculated as previously described.

**Annexin V/7-AAD Staining**

Cells were seeded in 6-well plates and were allowed to adhere overnight at 37°C.

Cells were treated with the indicated drugs or the medium alone (negative control) for 48 h. Annexin V/7AAD (KeyGen, Biotechnology Co., Ltd, Nanjing, Jiangsu, China) staining was performed according to the manufacturer’s instructions. In brief, cells were digested and resuspended in binding buffer at 5×10⁶/ml. Five microlitres of APC-conjugated Annexin V and 5 μl of 7-aminoactinomycin D (7-AAD) were added to 100 μl of the cell suspension for 15 min at room temperature in the dark. Samples were analyzed by flow cytometry within 4 h, and were stored at 2-8°C in the dark.

**Real-time PCR**

Total RNA was extracted from HaCaT cells with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. RNA concentration and purity were determined by ultraviolet spectrophotometry. cDNA was synthesized with 2 µg of total RNA. β-actin was used as an internal control. The PCR reaction was performed in triplicate in a volume of 20 µl. Relative gene expression was evaluated using the ΔΔCT method.

**Immunoblotting**

Immunoblotting was performed as per standard methods, as previously described. Cells were homogenized in lysis buffer supplemented with protease inhibitor cocktail on the ice and boiled for 10 min. Protein concentration
was estimated using the BCA protein quantification kit (KeyGen, Biotechnology Co., Ltd, Nanjing, Jiangsu, China) according to the manufacturer’s instructions. Aliquots of samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp, Billerica, MA, USA). After washing with Tris buffered saline and Tween 20 (TBST), blots were incubated with specific primary antibodies overnight at 4°C and indicated secondary antibodies for 2 h at room temperature. The blots were further washed with TBST, and specific protein bands were visualized using enhanced chemiluminescence.

Statistical Analysis

All statistical calculations were performed using the SPSS10 statistical software (SPSS Inc., Chicago, IL, USA). Data is represented as mean ± SD. Significant effects between treatment and control groups were analyzed using the Student’s t-test. Statistical significance was considered when \( p \) was less than 0.05.

Results

Effect of IFN-\( \gamma \) on the Proliferation of HaCaT Cells and Keratin 17 Expression

To establish the psoriatic model, HaCaT cells were treated with 250 U/ml IFN-\( \gamma \) and proliferation rate was analyzed 48 h later. HaCaT cells that were treated with IFN-\( \gamma \) for 48 h showed a significant increase in proliferation as determined by MTT assay. To confirm that IFN-\( \gamma \) could induce the expression of K17, we performed immunoblotting and Real-time PCR to determine protein and mRNA levels of K17. IFN-\( \gamma \)-treated cells showed an approximate 4-fold increase in K17 protein level compared with that in control cells (Figure 1A). Consistent with these results, the mRNA level of K17 in HaCaT cells after IFN-\( \gamma \) treatment was also increased, showing an approximate 3-fold increase compared with that of control cells (Figure 1B). These results suggest that treatment of 250 U/ml IFN-\( \gamma \) for 48 h can successfully induce a psoriatic model in HaCaT cells, by triggering cell hyperproliferation and by increasing the expression of the psoriasis marker K17.

Figure 1. Establishment of psoriatic model in HaCaT cells. A, Immunoblotting analysis of K17 expression in HaCaT cells treated with vehicle or 250 U/ml IFN-\( \gamma \) for 48 h (left panel). Quantitative analysis of the intensity of K17 in different groups of cells and the intensity was normalized to the internal control, GAPDH (right panel). B, Real-time PCR analysis of mRNA levels of K17 in HaCaT cells treated with vehicle or 250 U/ml IFN-\( \gamma \) for 48 h. Data were normalized with GAPDH. Graphs show data (mean ± SD) of three independent experiments each performed in triplicate. \( * p < 0.05, ** p < 0.01 \) were compared to control.
The therapeutic effect of ALA-PDT on keratinocytes

Effect of ALA-PDT Treatment on HaCaT Cell Viability

To investigate the effect of PDT on psoriasis progression, we administrated ALA at different final concentrations (0, 0.1, 1, and 10 mM) as a photosensitizer in IFN-γ-treated HaCaT. The effect on cell viability was detected using the MTT assay. ALA-PDT treatment showed a dose-dependent effect on the decrease in HaCaT cell viability, with inhibitive rates of 20.28%, 34.87%, and 44.37% in the IFN-γ + 0.1 mM ALA-PDT, IFN-γ + 1 mM ALA-PDT, and IFN-γ + 10 mM ALA-PDT groups, respectively (Figure 2A). In addition, we attempted to determine if the inhibitory effect of ALA-PDT treatment was time-dependent. Towards this end, IFN-γ-treated HaCaT cells were challenged with 1 mM ALA and analyzed for viability at 6, 12, 24, and 48 h time points by MTT assay. Unsurprisingly, we found that ALA+PDT treatment showed a time-dependent inhibitory effect on IFN-γ-treated HaCaT cells. The inhibitory rates of ALA-PDT treatment on IFN-γ-treated HaCaT cell proliferation were 28.19%, 34.86%, 42.44%, and 50.88% at 6, 12, 24, and 48 h after ALA treatment, respectively (Figure 2B). These results indicate that ALA-PDT treatment can significantly inhibit the proliferation of IFN-γ-treated HaCaT cells both in a dose- and time-dependent manner, revealing the therapeutic potential for treating psoriasis.

Effect of ALA-PDT Treatment on K17 Expression

Studies have shown that K17 protein is over-expressed in psoriatic lesions and is essential to the pathogenesis of psoriasis; therefore, we determined the expression of K17 in IFN-γ-treated HaCaT cells in different treatment groups. As revealed in Figure 3A, ALA alone did not decrease protein levels of K17 in IFN-γ-treated HaCaT cells, whereas cells treated with PDT alone showed significantly decreased K17 protein levels compared with those in the control group. Furthermore, ALA could enhance the inhibitory effect of PDT on K17 expression (Figure 3A). The mRNA expression profiles of K17 were the same with respect to protein expression in all four groups (Figure 3B). Similar to the cell viability experiment, the expression pattern of K17 was also checked for dose and time dependency. ALA was added into the medium to obtain different final concentrations of 0, 0.1, 1, and 10 mM, after cells underwent IFN-γ stimulation and PDT treatment. Using immunoblotting analysis, we found that ALA could bring about the reduction of K17 in a dose-dependent manner for an incubation period of 4 h with 1 mM ALA treatment, which yielded the most significant reduction in K17 protein (Figure 3C). Next, we chose 1 mM as the final concentration of ALA and evaluated the expression of K17 at 6, 12, 24, and 48 h af-
ter ALA treatment. As expected, the reduction of K17 was more significant with the increase in incubation time of ALA together with PDT treatment in IFN-γ-treated HaCaT cells (Figure 3D). The normalized relative intensity of K17 reached 0.70 ± 0.02, 0.62 ± 0.04, 0.41 ± 0.03, 0.28 ± 0.03, and 0.11 ± 0.03 in IFN-γ Control, and 6 h, 12 h, 24 h, and 48 h of IFN-γ+ALA-PDT treatment, respectively (Figure 3D). Together, these data strongly support the inhibitory effect of ALA-PDT treatment on K17 expression in HaCaT cells.

**Figure 3.** Expression of K17 in IFN-γ-treated HaCaT cells after ALA-PDT treatment. A, IFN-γ-induced K17 expression was decreased when treated with ALA-PDT (working concentrations of ALA of 0, 0.1, 1, and 10 mM; left panel). Normalized intensity of K17 to GAPDH is shown (right panel). B, The effect of time-dependency of K17 was also determined and a representative image is shown. IFN-γ-treated HaCaT cells were treated with 1 mM ALA-PDT for 0, 6, 12, 24, 48 h, and K17 expression was detected by immunoblotting (left panel). Normalized values of K17 against GAPDH were as shown (right panel). C, IFN-γ-induced K17 expression was decreased with treatment of ALA, PDT, and ALA-PDT. Normalized intensity of K17 to GAPDH is shown (right panel). D, mRNA levels of K17 in different groups as in C. *p < 0.05, **p < 0.01 were compared to the model or the time 0.
Comparison of Therapeutic Effect of ALA-PDT with Calcipotriol Treatment

Topical calcipotriol, a vitamin D3 derivative, has been commonly used for the treatment of psoriasis. In this study, we compared the therapeutic effect of ALA-PDT with calcipotriol in HaCaT cells at both molecular and cellular levels. HaCaT cells were divided into the following three treatment groups: IFN-γ, IFN-γ+calcipotriol, and IFN-γ+1 mM ALA-PDT. The levels of total p38, c-Jun N-terminal (JNK), and ERK (T-p38, T-JNK, T-ERK), as well as their phosphorylated states (P-p38, P-JNK, P-ERK) in different groups, were assessed by immunoblotting. Results revealed that the levels of T-JNK, P-JNK, T-p38, and P-p38 were all significantly higher in the IFN-γ+1 mM ALA-PDT group compared to the other two groups.

Figure 4. Comparison of the therapeutic effect of ALA-PDT with calcipotriol. A, Representative images of immunoblotting results of HaCaT cells treated with IFN-γ, IFN-γ+calcipotriol, and IFN-γ+1 mM ALA-PDT. K17, T-p38, T-JNK, T-ERK, P-p38, P-JNK, P-ERK, PARP, and caspase 3 were detected. GAPDH was used as an internal control. B, Annexin V/PI staining of HaCaT cells treated with IFN-γ, IFN-γ+calcipotriol, and IFN-γ+1 mM ALA-PDT. Data were representative of three independent experiments with similar results.
increased with IFN-γ+1 mM ALA-PDT compared to those in the IFN-γ+calcipotriol group (Figure 4A). However, the levels of T-ERK and P-ERK were not altered in both IFN-γ+1 mM ALA-PDT and IFN-γ+calcipotriol groups (Figure 4A). Furthermore, the level of K17 expression was lower in IFN-γ+1 mM ALA-PDT than that in IFN-γ+calcipotriol group (Figure 4A). We analyzed the levels of the apoptosis-related genes PARP and caspase 3. As expected, the IFN-γ+1 mM ALA-PDT group showed an increased expression of PARP and caspase 3 compared to the IFN-γ+calcipotriol group, suggesting a better pro-apoptotic role of ALA-PDT than calcipotriol (Figure 4A). To confirm the role of ALA-PDT, we performed flow cytometry-based analysis of cell apoptosis. The percentage of apoptotic cells was calculated by adding the percentages of Annexin V+ PI- cells (early stage of apoptosis) and Annexin V+ PI+ cells (late stage of apoptosis). Flow cytometry data showed that the percentage of apoptotic cells in the IFN-γ+1 mM ALA-PDT group was approximately 1.5-fold that of the IFN-γ+calcipotriol group (Figure 4B). These data indicate that ALA-PDT treatment shows better therapeutic effects than the commonly used calcipotriol treatment in the IFN-γ-induced HaCaT cell psoriatic model.

**Discussion**

Previous studies have already shown that many kinds of cytokines are essential for the immune system. Among these cytokines, IFN-γ is secreted by T helper 1 cells (Th1), NK cells, CD8+ T cells, and antigen presenting cells (APCs), which play a critical role in both innate and adaptive immunity. In this study, we focused on IFN-γ as an inducer of the psoriatic model. In psoriatic lesions, alterations in keratinocyte function produce inflammatory factors that promote chronic, self-amplifying loops of immune activity such as IFN-γ. IFN-γ promotes K17 expression, an important marker in the pathogenesis of psoriasis, in activated keratinocytes through the JAK/STAT pathway. Therefore, evaluating the expression of K17 in IFN-γ-induced human keratinocyte hyperproliferation has become an important way to determine therapeutic effect for strategies of psoriasis therapy. In the present study, we established the cell model by using 250 U/ml of IFN-γ for 48 h in HaCaT cells and detected the effect of ALA-PDT by examining the level of K17.

ALA-PDT is a therapeutic technique that utilizes photosensitizing drugs to preferentially create ROS inside the tissue when exposed to light. ALA has a molecular weight of 167.6 Da and can be delivered to the cutaneous tissue by a topical delivery system. ALA is the precursor for the synthesis of endogenous protoporphyrin IX (PpIX), the most important intermediate for photosensitization during ALA-PDT, through the cellular heme biosynthetic pathway. In this study, PDT was performed using light with a wavelength of 635 nm at a dose of 70 J/cm². We evaluated the dose- and time-dependent effects of ALA-PDT treatment in IFN-γ-induced HaCaT cells. With the increased cellular uptake of ALA, cell viability was significantly decreased. When the concentration was fixed, cells showed decreased viability when exposed to ALA for longer. Our results show that ALA-PDT treatment is suitable for inhibiting hyperproliferation of keratinocytes in psoriasis.

One of the molecular mechanisms of PDT is the direct induction of cell death of keratinocytes, which includes apoptosis, necrosis, and autophagy. Many factors can affect the manner and extent of cell death from PDT, including the wavelength and energy density of the light source, concentration, physical and chemical properties, and subcellular levels of the photosensitizer, and oxygen concentration. Photosensitizers are categorized into two groups: first generation photosensitizers (such as the hematoporphyrin derivative, photofrin) and second-generation photosensitizers (porphyrin precursors such as ALA, and methyl aminolevulinic acid (MAL)). High levels of ROS can induce the necrosis of cells, whereas low levels ROS can induce cell apoptosis, and sublethal levels of ROS can change the receptors on cell membranes, and cause the release of cytokines. In the present study, we evaluated the time- and dose-dependent effect of ALA-PDT treatment on cell viability. Our results show that ALA-PDT treatment showed an increase in the inhibitory rate on HaCaT cell viability upon treatment with increasing concentrations of ALA. Furthermore, we found that ALA-PDT treatment showed an increase in the inhibitory rate with an increase in incubation time. Our data indicate that ALA-PDT treatment shows a dose- and time-dependent inhibitory effect on the proliferation of IFN-γ-treated HaCaT cells.

ALA-PDT can change the expression profile of keratins. K16 is not expressed in normal epidermal tissues but is expressed in the hyper-
proliferative cells of psoriasis lesions. K10 is a marker of normal differentiation of keratinocytes and is poorly expressed in psoriatic skin lesions. Studies have shown that ALA-PDT can decrease the expression of K16 and increase the expression of K10 in plaque psoriasis35. Accordingly, we measured the expression of another keratin, K17, which is the only keratin induced by psoriasis-associated cytokines. Consistent with the inhibitory effect of ALA-PDT on cell viability, the expression of K17 was downregulated in a dose- and time-dependent manner. The expression of K17 was decreased the most when the concentration of ALA was 1 mM, and this concentration was used for further experiments. Immunoblotting analysis indicated that ALA-PDT shows a therapeutic effect for psoriasis both at cellular and molecular levels.

The vitamin D analogue, calcipotriol, has been reported for its role in attenuating the abnormal differentiation and proliferation of psoriatic keratinocytes35. Given that calcipotriol shows adverse effects on the skin barrier, it is of great importance to seek an alternative treatment. MAP kinases constitute an important node of three signaling pathways, including p38, ERK1/2, and JNK, which control several important functions within the cell, such as cell proliferation, differentiation, gene expression, and apoptosis36. Therefore, we compared the effect of ALA-PDT with that of calcipotriol by evaluating the total and phosphorylated protein levels of p38, ERK1/2, and JNK. Levels of T-JNK, P-JNK, T-p38, and P-p38 were all significantly increased in the ALA-PDT group compared to the calcipotriol group. No differences in T-ERK and P-ERK were observed in both groups. Furthermore, apoptosis-related genes were examined. As expected, ALA-PDT showed higher levels of PARP and caspase 3 than calcipotriol. The apoptotic effect of ALA-PDT was further confirmed by flow cytometry-based analysis of cell apoptosis.

Conclusions

We suggest that ALA-PDT shows better therapeutic effects than calcipotriol and might work as an alternative to calcipotriol.

Conflict of interest

The authors declare no conflicts of interest.

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