Antifibrotic activity of Hypocrellin A united LED red light irradiation on Keloid Fibroblasts through counteracting TGF- β /Smad/autophagy/apoptosis signaling pathway

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Abstract

Keloid disease is characterized by abnormal proliferation of fibroblast and continuous deposition of extracellular matrix (ECM) components. More and more attention in dermatopathology is paid to photodynamic therapy (PDT) with visible light. The natural photosensitizer Hypocrellin A (HA) is identified to develop a splendid light induced anticancer, antimicrobial and antiviral activity. In this experiment, we investigated the impacts of HA united light-emitting diode (LED) red light irradiation on human keloid fibroblast cells (KFs). Our results showed that HA united red light irradiation treatment (HA-R-PDT) decreased KFs viability, reduced KFs collagen production and ECM accumulation, inhibited cell proliferation, suppressed cell invasion and induced cell apoptosis. Moreover, our observations demonstrated that TGF- β /Smad signaling pathway and autophagy were restrained by HA-R-PDT. TGF- β 1 could accommodate autophagy in KFs through both Smad and ERK pathway, while inhibiting autophagy could mediate TGF- β 1 level by the negative feedback. Therefore, HA-R-PDT could suppress cell hyper-proliferation, collagen synthesis and ECM accumulation in KFs via regulating TGF- β 1-ERK-autophagy-apoptosis signaling pathway. HA-R-PDT deserves systematic investigation as a potential therapeutic strategy for keloid treatment and autophagy might be a promising candidate in the therapy of KFs.

Introduction

Keloid, regarded as benign tumor that commonly seen in cosmetic surgery and dermatology, is characterized by the overabundance deposition of extracellular matrix (ECM)[1]. Keloid fibroblasts proliferate abnormally and produce excessive ECM proteins, such as collagen I, α -SMA and fibronectin, which is one of the most prominent factors involved in the development of keloid. Besides, the apoptosis of keloid fibroblasts is dysregulated[2]. The incidence of keloid is relatively high in people of Asian associated with a genetic susceptibility and have a high postoperative recurrence rate[3]. Albeit there are multiple standards of therapy for keloid, containing surgery, local drug injection, cryotherapy and radiotherapy, the clinical outcomes are not satisfactory[4]. Otherwise, removal of keloid with surgical excision alone results in a high recurrence about more than 50%, and inconvenience of the current process lead to poor compliance with medication, manifesting that treatment of keloid is a knotty problem[5]. Accordingly, further study on the pathogenesis of keloid generation and development of effective therapeutic tactics for treatment of keloid are essential and imperative.

Recently, phototherapy united visible light has absorbed increasingly attention in dermatological treatment due to its safety and efficacy, which is known as a crucial technique in cosmetic dermatology [6, 7]. Photosensitizer, visible light and oxygen are essential element of photodynamic therapy. Because of their structure diversity and low biological toxicity, pure natural products of photosensitizers are getting more and more attention in the medical field. Hypocrellin A (HA), an effective photosensitizer, is extracted from the traditional Chinese herbal medicine *Hypocrella bambusae* Sacc [8]. HA has the merits of proper irradiation derivative anticancer and antivirus activities, low biological toxicity and quick metabolism in normal tissue; hence it has been extensively used in treating skin diseases[9]. Red light, whose wavelength ranges from 620 nm to 770 nm, has been extensively applied in photodynamic therapy on

account of its profound ability to deep into the skin layer but hardly cause any damage. Many clinical applications suggested that the effect of HA was boosted when united with visible light because of the enhanced light energy [10]. For instance, photodynamic therapy with HA could execute antitumor activity through reducing cell activity and inducing cell apoptosis. Whereas, the exact molecular mechanism of HA united visible light has still not fully determined. Since keloid possesses some characteristics of tumors with an unlimited expansion of dermal fibrosis, we carried out research by combining HA with LED red light to evaluate the effect on cell apoptosis, invasion, proliferation and the underlying anticancer mechanism.

The molecular basis of keloid is perplexing, involving genetic and environmental factors, such as inflammatory reaction, abnormal expression of cytokine, and cutaneous injury [11]. Despite the pathogenetic mechanism underlying keloid generation and development remains unclear, the histopathological features of this disease are in relation to an overabundance of ECM deposition, which owing to unlimited hyperplasia of keloid fibroblasts [4, 12]. Thus, the pertinent treatments may be aimed at inhibiting multiplication or increasing apoptosis of KFs. Research reported that keloid fibroblasts are similar to benign tumors, with vigorously continuous generation of collagens and growth factors and a high ability for cell proliferation and invasion [1]. Several regulatory factors have been found to participate in the pathogenesis of keloids. Among these mediators, transforming growth factor- β 1 (TGF- β 1), a polyfunctional cytokine that involved in the regulation of a variety of cells' proliferation, differentiation and migration, is deemed as a main risk factor for keloid occurrence[13]. Notably, TGF- β 1 is essential for cell growth and collagen synthesis, and the expression of TGF- β 1 is significantly increased in KFs[14, 15]. What is more, a number of cytokine are disturbed in keloid pathogenesis and recurrence, for instance, extracellular matrix syntheses genes collagen I, α -SMA and matrix metalloproteinases (MMPs) [16, 17]. These cytokines are involved in the mediation of a series of reactions in KFs, including fibroblast growth, ECM generation and accumulation, inflammatory infiltration and matrix remodeling. Thus, either trying to lessen the production of related cytokines or shutting off their signal interaction might offer a potential method for keloid treatment.

Autophagy is a highly conserved intracellular catabolic process that mediates the degradation and recycling of redundant cytoplasmic components at constitutive levels to preserve the biological homeostasis [18]. As a result, autophagy is linked to several human pathophysiology, such as aging, cancer and inflammatory response [19]. There has been plenty of researches focused on the importance of autophagy in cellular homeostasis, whereas, few studies follow with interest the function of autophagy in pathological scars, such as keloid generation and invasion. Considering that autophagy is vital for cell proliferation and cellular homeostasis under various adverse circumstances, we assume that abnormal proliferation of fibroblast is interrelated with the activity of autophagy. Moreover, previous researches suggested that autophagy could regulate TGF- β expression[20] and enhance TGF- β 1 mediated fibrosis in human lung fibroblasts[21]. So, we attempt to clarify the impact of TGF- β 1 on KFs from the perspective of autophagy, simultaneously.

Our previous results exhibited the potential role of HA-R-PDT in the suppression of proliferation in squamous carcinoma A431 cell, which delivered a more synergistic effect than HA alone [22]. In the present study, we verified that HA-R-PDT suppressed cell proliferation, invasion and down-regulates collagen synthesis and ECM accumulation. Furthermore, HA-R-PDT induced mitochondrial-mediated apoptosis. Meanwhile, we estimated the interaction between TGF- β 1 and autophagy pathway via inhibiting TGF- β pathway and interfering autophagy process respectively. Our results demonstrated that declined TGF- β 1 could inhibit autophagy in KFs through both Smad and ERK pathway, and inhibiting autophagy could adjust TGF- β 1 expression level by the negative feedback. Therefore, HA-R-PDT may be a promising therapeutic method for treating keloids.

Materials And Methods

Reagents and antibodies

The specific primary antibodies for cleaved caspase-3, ERK, phospho-ERK (Thr²⁰²/Tyr²⁰⁴), Beclin-1, Smad2, Smad3, phospho-Smad2 (Ser^{465/467}) and phospho-Smad3 (Ser^{423/425}) were purchased from Cell Signaling Technology (Boston, MA, USA); antibody against LC3B, Rapamycin, 3-methyladenine (3-MA), Mitochondrial Membrane Potential Assay Kit (II), Autophagy assay kit (Sigma-Aldrich, St. Louis, MO, USA); anti-p62/SQSTM1 MBL International (Woburn, MA, USA); β -actin antibody and peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, USA); pre-stained protein standards (Fermentas, Lithuania); CCK-8 assay kit (Dojindo, Kumamoto, Japan); Click-iT™ EdU Alexa Fluor Imaging kit was bought from Invitrogen (Carlsbad, CA, USA); transfection reagents Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA); Human AnnexinV-FITC (AV) and propidium iodide (PI) detection kit and Cycle test Plus DNA Reagent kit were from BD Bioscience (San Jose, CA, USA); Reverse transcription kit and SYBR Green I Master mix and Chemiluminescence (ECL) detection kit were purchased from Thermo Fisher Scientific Inc (MA, USA); HA was purchased from the Institute of Chemistry, Chinese Academy of Sciences, China. Hypocrellin (HA) was dissolved with dimethyl sulfoxide (DMSO) and stored at the temperature of -20 °C. The real-time fluorescence quantitative PCR was from ABI 7500 (USA). Micro-plate reader was Spectra Max 190 produced by Molecular Devices (Sunnyvale, CA), Flow cytometer was from Becton Dickinson (CA, USA) and fluorescence Microscope was from Zeiss (Carl Zeiss AG, Germany).

LED source and irradiation

Considering the skin penetration of visible light and HA absorption, we selected LED red light at a wavelength of 630 nm. The output power of LED light source was inspected by the Chinese National Institute of Metrology (Supplementary Fig. 1). The irradiation intensity is 5.68 mW/cm². Irradiation dose = irradiation intensity $\times$ time. In our experiment, the irradiation dosage of 3J/cm² was selected based on pre-tests. KFs were pre-treated with HA for 4 hours followed by irradiation with LED red light at a distance about 20 mm. Prior to irradiation, the medium was replaced by PBS to avoid the formation of photochemical compounds. Controls groups were incubated in PBS, placed outside incubator, and kept light-protected for an equivalent time to ensure the consistency of the experiments. All the tests were

classified into four groups: non-treated control group, HA treated alone, LED red light irradiation alone and HA-R-PDT group, where the HA concentration used in our study is 0.25 μ M.

Cell culture

Primary human KFs were excised from chest keloid tissues of a male patient after having obtained informed consent. In brief, tissues were made a 5-minute wash in PBS for 3 times, then the epidermis was eliminated by scalpels and the rest dermis was cut into minor blocks followed by 0.25% trypsinase-EDTA digestion at 37°C for 4 hours. After digestion, the tissue/cell suspension was passed through a 100 μ m filter to get rid of the remaining tissue residuals. Then collected cells were washed with PBS and centrifuged at 1000 rpm for 5 min. Finally, re-suspended cells in FibroLife Basel medium supplemented with 2.5 μ g rh Insulin, 7.5mM L-Glutamine, 25 μ g Ascorbic Acid, 5ng rh FGF- β , 2% FBS (LifeFactor), 5 μ g Hydrocortisone Hemisuccinate and 100 U/ml penicillin (Gibco). KFs were maintained at 37 °C in a saturated humidity atmosphere with 5% CO₂. Cells of third and sixth passages were utilized in the present research.

Cell viability assay

The KFs were seeded in 96-well plates at 6×10^3 cells/well and cultured for 20 hours. Then replaced with fresh medium with or without Hypocrellin (0–1 μ M) and continually cultured for 4 hours. After pre-procedure, KFs were irradiated with red light for 9 min. Twenty hours later, cell growth was detected by the CCK-8 assay kit. In brief, aspirated the medium from the plates and added 10 μ L/well CCK-8 (5 mg/ml) solution in medium to plated at 100 μ L/well and incubated at 37 °C for 2 hours. The optical density was surveyed using a microplate reader at 450 nm (Spectra Max 190; Molecular Devices, Sunnyvale, CA). All the assays were performed in triplicate and three replicate wells were performed for each group.

Hoechst staining

Cell apoptosis was evaluated by dyeing with Hoechst 33258. KFs were treated as mentioned earlier. After being fixed with 4% paraformaldehyde, cells were incubated with 10 μ M Hoechst 33258 for 20 min at room temperature. Then cells were monitored using a fluorescence microscope (Zeiss, Germany). Apoptosis was stipulated as the ratio of nucleus condensation and the assessment was performed by counting about 200 cells of each group.

Mitochondrial membrane potential measurement

Mitochondrial membrane potential was detected through the Mitochondrial Membrane Potential Assay Kit (II) (Sigma-Aldrich, MO, USA) according to the product instruction. TMRE (tetramethylrhodamine ethyl ester perchlorate), a cell membrane penetrable fluorescence dye, can assemble at intact mitochondria. In normal cells, TMRE accumulates in the mitochondria and emits an orange-red fluorescence with a maximum at 575 nm; while in cells with damaged mitochondria or have lost mitochondria activity cannot

accumulate TMRE. After KFs were treated as described above, discarded the medium and incubation with 200 nm TMRE in 1 X PBS at 37°C for 15 min. Then cells were washed with 1 X PBS for 3 times. In the end, fluorescence intensity was measured under the fluorescence microscope (Zeiss, Germany).

Apoptosis assay

Cell apoptosis was monitored by Human AnnexinV-FITC (AV) and propidium iodide (PI) detection kit. In brief, KFs were treated as described above. After harvested and washed with PBS, cells were re-suspended in 100 μ l 1 $\times$ Annexin V-FITC binding buffer. Afterwards, cells were incubated with 5 μ L/tube Annexin V-FITC in the dark for 15 min at room temperature and stained with 5 μ L/tube propidium iodide (PI) dye. Apoptotic cells were evaluated by a fluorescence-activated cell-sorting (FACS) flow cytometer (Becton-Dickinson, CA, USA). The results were estimated by the Cell Quest software on flow cytometry. All experiments were repeated three times.

Cell cycle analysis

For cell cycle analysis, KFs were harvested after being treated as previously mentioned and washed with PBS. The quantification of cell cycle was analyzed by Cycletest Plus DNA Reagent kit according to the product's manual. In brief, cells were first incubated in solution A for 10 min at room temperature and light-protected, then sequentially added solution B and solution C. Analyses were performed using the FACS Calibur system and data were dissected with Cell Quest software (BD Biosciences, San Jose, CA, USA).

EdU incorporation assay

The EdU incorporation was performed using the ClickiT™ EdU Alexa Fluor Imaging kit according to its manufacturer's instruction. After noted treatment, KFs were incubated with 25 μ M 5-ethynyl-2'deoxyuridine in the culture medium for additional 4 hours. Then cells were fixed with 4% formaldehyde at room temperature for 30 min and neutralized the residual fixing solution with Glycine for 5 min. Afterwards, cells were washed with 3% BSA for 3 times and then permeabilized via exposing to 0.5 % Triton X-100 in PBS for 20 min. Ultimately, cells were stained with reaction cocktail light-protected for 30 min, followed by dyeing with Hoechst 33342 for 20 min at the concentration of 5 μ g/ml. The Hoechst positive cells (blue) and EdU positive cells (green) were photographed under a fluorescence microscope (Zeiss, Germany). These results were repeated in three independent experiments and observed in three randomly selected regions.

Transient transfection

The si-ATG5 and si-Smad 3 shRNA-expressing vector was obtained from Invitrogen (Shanghai, China). GFP-LC3 and GFP-vector were kindly donated by doctor Lin. About 5×10^4 cells were seeded in 6 well plates 20 hours before transfection. KFs were transfected with lipofectamine 3000 according to the manufacturer's protocol.

Identification of cellular autophagy

KFs were seeded in 6 well plate at a density of 2×10^5 cells. After reaching 40-50% confluence, cells were transfected with 1 $\mu\text{g}/\mu\text{l}$ GFP-LC3 or GFP-vector DNA with lipofectamine 3000 according to the product instructions. After that, cells were treated as stated above. Autophagy was inspected by analyzing the dispersion of fluorescent LC3 spots of autophagosome under a fluorescence microscope (Zeiss, Germany). The number of LC3 dots was counted not less than 100 cells per well.

Autophagy level detection

The autophagy level in KFs was inspected using an autophagy assay kit (Sigma, USA). KFs were seeded in 96-well plates at 6×10^3 cells/well and cultured for 20 hours. Then replaced the culture medium with fresh medium with or without Hypocrellin ($0.25 \mu\text{M}$) and continually cultured for 4 hours. After pre-progression, KFs were irradiated with red light for 9 min. 20 hours later, cells were incubated with 100 μl of the autophagosome detection working solution and incubated at 37°C for 30 min. Then cells were washed with wash buffer for 3 times. The autophagy signal was detected by fluorescence microscope (Zeiss, Germany).

ELISA assay

About 8×10^3 KFs were seeded in 96-well plates in triplicate 20 hours before treatment. KFs were treated as described above. Then the supernatants were collected and measured by human TGF- β 1 ELISA kit according to the manufacturer's protocol (KeyGEN Biotech, CHN). In brief, incubated the samples in the plates coated with anti-TGF- β 1 antibody for 90 min at 37°C . After washing with wash buffer, the plate was then incubated with antibody conjugated to horseradish peroxidase for 60 min at 37°C . Afterwards, added developer solution to each well and incubated for 15 min, and then added stop solution to each well to stop enzyme reaction. Ultimately, the plate was detected using a microplate reader at 450 nm (Spectra Max 190; Molecular Devices, Sunnyvale, CA). Each assay was performed in triplicate and three replicate wells were performed for each group.

In vitro migration assay

The migration potential was monitored by both wound healing assay and trans-well assay. For the wound healing assay, cells were pre-seeded in 35 mm plates. When reaching 90% confluence, the single cell layer was scratched with a sterile 200- μl pipette tip. Then we treated the KFs with HA and irradiated with red light as described above and continued to culture. The speed of scratch healing was surveyed at different time points under microscope. Results were presented as the ratio of scratching filled by the KFs. Besides, the invasion capability was tested by Trans-well assay (8.0 μm pore membranes, Corning). In short, after KFs were treated as described earlier, harvested cells and seeded 2×10^3 cells in the above compartment of the cubicle with 0.15 ml of serum-free medium in 24-well plates, and the downward well was appended 0.5 ml of medium plus 10% FBS. Then cells were incubated overnight, in 5% CO_2 at 37°C . After that, discarded the medium in the chambers, and used a sterilized cotton bud to erase the non-migrated cells

on the top surface of the membrane. After that, we fixed the filter membrane in 4% paraformaldehyde for 25 min and stained with 0.1% crystalviolet for 5 min. Micrographs were captured by fluorescence microscope (Zeiss, Germany). The numbers of the migrated cells were obtained from five stochastically selected fields under the microscope.

RNA reversed transcription and quantitative real-time

Total RNA extraction was performed using the Trizol RNA isolation reagent (Invitrogen) and reverse transcription were using Reverse Transcription Kit (Promega, USA). The mRNA levels were detected using SYBR Green PCR Master Mix (Promega, USA) in a real-time thermal cycler (Bio-Rad, USA). The primer sequences used in each reaction were listed in table 1. Briefly, reaction conditions include: initial denaturation at 95°C for 2 min; then 95°C for 15s, 60°C for 45s and total of 40 cycles; meltingtemperature:60degrees centigradeto90degrees centigrade consuming 30min. The relative gene expression levels were normalized to the endogenous control gene β -actin and calculated by the comparative Ct ($\Delta\Delta C_t$) method. Each data was presented as the mean standard deviation from three independent experiments.

Western Blotting

After having been treated as previously described, cells were washed with PBS and lysed using ice-cold RIPA buffer, and measured by BCA Protein Assay Kit (Beyotime, Nanjing, China). The equivalent proteins were separated on 12% SDS-PAGE gels and then transferred onto PVDF membranes (Millipore, Billerica, MA, USA). After that, membranes were blocked in 5% milk for 45 min at room temperature and then incubated with specific primary antibodies at 4°C overnight, followed by probed with secondary anti-rabbit horseradish peroxidase-conjugated antibody. The expression of related protein was detected by an enhanced chemiluminescent ECL reagent (Millipore, Billerica, MA, USA). Bands were quantified by Image J software (National Institutes of Health, Maryland, USA).

Statistical analysis.

Statistical analysis was carried out using the Student's paired t-test or two-way ANOVA with GraphPad Prism software (Version 8.0, La Jolla, CA, USA). All values were shown as means $\pm$ standard deviation (SD) of three individual experiments. Value of * $p < 0.05$ and ** $p < 0.01$ were considered statistically significant.

Results

Effect of HA-R-PDT on cell proliferation in KFs

To evaluate the best effect of HA for mediating KFs proliferation, we performed CCK-8 assay to determine the concentration of HA-R-PDT on the cell viability at first. Based on our preliminary experiments, 3J/cm² of red light irradiation dosage with different concentration of HA (0–1 μ M) were chosen for KFs

treatment. The results showed that in comparison to the non-treated control group (Ctrl), KFs treated with HA-R-PDT exhibited a significantly decreased cell viability ($p < 0.05$), and the cell mortality rate was near 50 percent when KFs were treated at 0.25 μM HA. When KFs treated with HA or red light irradiation alone, only tiny decrease in cell viability was observed (Fig. 1A). These results provided the basis for subsequent experiments. Therefore, we used the concentration of 0.25 μM HA in the experiments. To have a better awareness of the inhibitory effect of HA-R-PDT on KFs, we investigated the efficacy on cell proliferation using the EdU incorporation assay kit, where EdU is used to detect the proportion of cells in S phase, which can be inserted into the duplicated DNA molecules when cell is growing. As shown in Fig. 1B, DNA synthesis was obviously decreased by HA-R-PDT, as fewer KFs were found to be capable of incorporating EdU in comparison with the non-treated control group ($p < 0.01$); additionally, there were no noticeable differences between the non-treated control group and HA alone treated group or red light irradiated group. As a consequence, we further assessed the cell cycle via flow cytometry. The exposure of KFs to HA-R-PDT gave rise to a retardation at the G2/M transition, resulting in an apparent augment in the G2/M phase from about 4.1% to 17.5% ($p < 0.05$) (Fig. 1C). While no obvious changes were seen between the control group and HA alone treated group or red light irradiated group. These results indicated that low concentration of HA-R-PDT probably via cell cycle arrest to inhibit KFs proliferation.

Effects of HA-R-PDT on the migratory properties of KFs.

Human skin KFs represented bioenergetics that are similar to cancer cells, the increased migration ability of fibroblasts is a major feature in the formation of keloid disease[23]. In order to detect whether HA-R-PDT impact KFs cell action, we further monitored KFs migration ability after treatment. As shown in Fig. 2A, treatment with HA-R-PDT noticeably reduced the migration abilities of KFs, only about 30.6% of the scratched area was filled 20 hours after treatment ($p < 0.01$). While, the control group effectually migrated into the scratching area, and that the wound border was un-conspicuous after culture for 20 hours (Fig. 2A). Meanwhile, we made a trans-well assay verify the effect of HA-R-PDT on KFs migration. As observed in Fig. 2B, HA-R-PDT strikingly suppressed the invasive abilities of KFs at 20 hours after treatment. Compared with the control group, the number of cells migrated through the filter membrane to the bottom culture layer was greatly reduced to probably 7.7% ($p < 0.01$) (Fig. 2B). These results indicated that HA-R-PDT possesses a potential to reduce KFs' property of migration.

HA-R-PDT down-regulates collagen synthesis and ECM accumulation in KFs

There are several ECM-associated characteristic proteins overexpressed in KFs, such as α -SMA, collagen and MMP [16, 17]. Hence, we explored whether the expression of these genes was regulated by HA-R-PDT at mRNA level or not. The results demonstrated that exposure of KFs to HA-R-PDT for 20 hours significantly down-regulated extracellular matrix syntheses genes, for instance, collagen I, collagen III and α -SMA gene expression (Fig. 3A), compare with the control group. In addition, HA-R-PDT weaken the transcriptional level of fibronectin, which is a glycoprotein present in extracellular connective tissue matrices functions as a framework for the ECM network to promote cell migration and proliferation[24].

Furthermore, we monitored the expression level of matrix metalloproteinases (MMPs) and their inhibitors TIMPs, which are a family of zinc-dependent endopeptidases degrade essentially all ECM components[25]. As shown in Fig. 3B, HA-R-PDT reduced the ratio of MMP2/TIMP-1 and MMP9/TIMP-1, implying a breakdown of ECM by HA-R-PDT. In summary, all these results prompted that HA-R-PDT exhibits an anti-fibrosis effect in KFs.

HA-R-PDT induces KFs apoptosis

Based on our above results, we assumed that HA-R-PDT may accomplish its inhibitory effect on cell migration, invasion and proliferation via inducing apoptosis in KFs. With the purpose of elucidating the potential control mechanism, we first monitored the morphologic changes of KFs. After treating with HA-R-PDT, KFs were stained with Hoechst 33258. As shown in Fig. 4A, cells treated with HA-R-PDT exerted significant nuclear condensation and cell shrinkage, and observably increased apoptotic body forming. Whereas, the untreated control group represented weak fluorescence. And cells treated with HA alone or light irradiation alone showed no obvious nuclear condensation and apoptotic body formation. Meanwhile, we also assessed cell apoptosis by annexin V and propidium iodide (PI) double staining kit via flow cytometer. In the flow column, the cells in the lower left, lower right, upper right and upper left sector separately represent normal live cells, early apoptotic cells, late apoptotic cells and necrotic cells. As shown in Fig. 4B, KFs cells treated with HA-R-PDT caused the apoptotic cell percentage range from about 8.3% to 36.3% ($p < 0.05$), including both early apoptotic and late apoptotic cells. However, no remarkable manifestation of cell apoptosis was surveyed in HA alone or light irradiation alone treated cells. These data displayed that HA-R-PDT induced acute apoptosis in KFs; and HA and red light are cooperative promotion of apoptosis instead of simply merely supplement. Previous researches have reported that caspase pathway plays essential roles in cell apoptosis and caspase-3 is the final executioner of apoptotic process[26]. Otherwise, the Bcl-2 family formed by pro-apoptotic to anti-apoptotic proteins play vital role in the regulation of cell survival[27]. Therefore, we further detected the changes of caspase-3, BAX and Bcl-2, which are generally acknowledged to be involved in the mitochondrial-regulated apoptosis pathway. Through western blotting analysis, HA-R-PDT notably decreased the expression of Bcl-2, but the protein expression of caspase-3 and BAX were increased (Fig. 4C). HA alone and red light irradiation alone showed tinny impact on the expression of caspase-3, BAX and Bcl-2. Mitochondrial membrane potential (MMP) has been considered as a desirable biomarker for early detection of cell apoptosis[28]. In the meantime, we measured the variation of mitochondrial membrane potentials with TMRM staining in KFs. As shown in Fig. 4D, HA-R-PDT dramatically declined the mitochondrial membrane potential. These data were collected and indicated that HA-R-PDT induces apoptosis in KFs, which is mitochondrial-mediated and caspase-dependent.

HA-R-PDT inhibits autophagy in KFs

Autophagy is a conserved self-degradation system, which is critical for cell proliferation and cellular homeostasis under tension conditions. Autophagy can be neutral, tumor-inhibitive or tumor-promoting in different conditions. Abnormal autophagy relates to various diseases[19, 29]. For the sake of confirming

whether HA-R-PDT regulates autophagy in KFs, we assessed the punctual distribution of GFP-LC3 at the beginning to indicate the autophagosome level. The results of fluorescence microscopy showed that after treatment with HA-R-PDT, KFs displayed notably decreased accumulation of GFP-LC3 foci in compared with the negative controls (Fig. 5A). The reduction of autophagy was further verified by autophagy assay kit. Cells were monitored after treatment under fluorescence microscope, while the autophagy is revealed by the bright blue points stained autophagic vacuoles. As shown in Fig. 5B, KFs treated with HA-R-PDT exhibited less autophagic vacuoles compared with control groups ($p < 0.05$). Furthermore, we inspected the autophagosomes via acridine orange (AO) staining, which were diminished markedly after HA-R-PDT treatment (Fig. 5C). Otherwise, we detected the changes of expression of SQSTM1 and Beclin-1 and the conversion of LC3-I to LC3-II in KFs. Western blotting results revealed that, following treatment with HA-R-PDT, the expression of Beclin-1 and LC3-II/I were declined, however, the protein expression of SQSTM1 was enhanced (Fig. 5D). Later we further explored the impact of autophagy on the accumulation of ECM. We first exploited RNA interference to knockdown ATG5 (si-ATG5), a vital autophagy element, and found that the protein expression of Beclin-1 and the conversion of LC3-I to LC3-II were declined with the indication of autophagy inhibition (Fig. 5E and Supplementary Fig. 2). After that, we used RT-PCR method to test the expression of collagen I, collagen III and α -SMA gene. As shown in Fig. 5F, in si-ATG5 interfered cells, in relation to scramble group, autophagy blocked group represented reduced expression of extracellular matrix syntheses genes. The expression of MMP2, MMP9 and their inhibitors TIMPs was detected simultaneously. Shown in the results, both MMP2/TIMP-1 and MMP9/TIMP-1 were down-regulated by si-ATG5 transfection. In conclusion, all the results indicated that HA-R-PDT could suppress autophagy in KFs, and the blocking of autophagy potentially caused a failure in the migration procedure of KFs.

HA-R-PDT suppresses TGF- β /Smad pathway

Numerous researches have shown that TGF- β signaling plays an essential role in fibro pathogenesis[30]. TGF- β 1 is regarded as a vital regulatory factor in tissue fibrosis and scar tissue generation, mainly through the activation of the downstream Smad signaling pathway, and TGF- β /Smad signaling pathway was regarded to inhibit cancer proliferation[31, 32]. Accordingly, we firstly inspected the expression of TGF- β 1 by RT-PCR in KFs. As seen in Fig. 6A, the expression of TGF- β 1 was decreased evidently after the treatment with HA-R-PDT; while the expression of Smad7 was elevated as a negative regulator of TGF- β 1/Smad pathway ($p < 0.05$), which explained the reason why we further examined the extracellular secretion of TGF- β 1 by ELISA kit. The results showed that TGF- β 1 expression was declined after HA-R-PDT treatment (Fig. 6B). Studies have revealed that Smads were the central effectors of TGF- β signaling, and Smad2 and Smad3 were the two substantial downstream effectors[28]. To have a better understanding with the inhibitory effect of HA-R-PDT on TGF- β 1 pathway, we detected the expression of Smad2, p-Smad2, Smad3 and p-Smad3 by western blotting. Our results displayed that HA-R-PDT down-regulated the expression of p-Smad2 and p-Smad3, compared to the non-treated control group (Fig. 6C). Simultaneously, we detected that HA-R-PDT also declined the phosphorylation of Mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK), which was one potential effector

responsible for TGF- β 1 signaling pathway[33]. All these data suggested HA-R-PDT may block TGF- β /Smad pathway to suppress cell proliferation and down-regulate collagen synthesis and ECM accumulation in KFs.

HA-R-PDT exerts anti-keloid activity via regulating TGF- β /Smad/autophagy/apoptosis signaling pathway.

Studies have suggested TGF- β 1 may be an effective activator of autophagy[21, 34]. Nevertheless, the relevance between autophagy and TGF- β 1 in KFs remain less clear. To figure out the correlation between TGF- β 1 and autophagy, we inspected the expression of autophagy-related proteins in KFs treated with TGF- β R1 inhibitor LY-2157299 by western blotting. As shown in Fig. 7A, cells dealt with LY-2157299 reduced Beclin-1 expression and LC3II/LC3-I conversion. Since autophagy is mainly regulated via m-TOR dependent pathways [35], we investigated whether these pathways took effect in this procedure. Our results suggested that suppressing the AKT-PI3K-mTOR pathway by 3-MA, to a certain extent, could decline the autophagy level in KFs. Moreover, when cells treated with both HA-R-PDT and 3-MA, the apoptosis level was further enhanced (Fig. 7B) and we treated cells with ERK inhibitor U0126. Results suggested that this treatment decreased the expression of Beclin-1 and LC3II/LC3-I conversion (Fig. 7C), suggesting that ERK pathway might be one potential pathway involved in TGF- β 1 induced autophagy. Besides, when cells processed with both HA-R-PDT and U0126, the cell apoptosis was effectively increased. Accordingly, HA-R-PDT induced apoptosis may be acquired partly through suppressing autophagy. As our above results displayed, HA-R-PDT blocked TGF- β /Smad pathway, so that we detected whether the Smad pathway was involved in the regulation of autophagy. After transient transfection with si-Smad3, the expression of Beclin-1 and LC3II/LC3-I conversion were declined (Fig. 7D and Supplementary Fig. 3). Simultaneously, we assessed whether autophagy could conversely impact TGF- β 1 signaling. Our results showed that, when KFs treated with rapamycin, the ELISA assay showed that TGF- β 1 level was elevated, and the RT-PCR results suggested boosted autophagy by rapamycin down-regulated the expression of Smad7. Conversely, CQ down-regulated TGF- β 1 and enhanced the expression of Smad7 (Fig. 7E and 7F). As a consequence, all these results indicated that HA-R-PDT could inhibit autophagy in KFs through both Smad and ERK pathway, and suppress apoptosis partly through ERK pathway; while inhibiting autophagy could accommodate TGF- β 1 level by the negative feedback and further promote cell apoptosis. Therefore, HA-R-PDT might suppress cell hyper-proliferation, collagen synthesis and ECM accumulation in KFs via regulating TGF- β 1/Smad/autophagy/apoptosis signaling pathway.

Discussion

Keloid is a fibro-proliferative disorder characterized by overabundance deposition of ECM, which possesses some cancer-like features with high ability to surpass beyond the primary margins and invade surrounding normal tissue [36]. Keloid lesions usually arise from burn, surgery, trauma and acne procedure in genetically susceptible population, and with a high recurrence rate[3]. In our present research, we revealed that HA-R-PDT suppressed cell viability, multiplication and induced apoptosis in KFs (Figs. 1, 2 and 4), which suppressed TGF- β 1 pathway via inhibiting autophagy to down-regulate

collagen synthesis and ECM accumulation in KFs (Figs. 3, 5, 6 and 7). Accordingly, we convinced that HA-R-PDT may have wider practical effects than it is currently aware.

It has commonly known that excessive deposition of ECM components, particularly collagen I, collagen III, α -SMA and fibronectin, is one of the most prominent factors involved in keloid generation[1]. Collagen is the major components of the connective tissue, and its break down plays a vital role in generation, degradation, remodeling, and repair process[36]. α -SMA overexpression, a hallmark of fibrogenesis, that is considered to contribute essentially to the development to fibrosis[37]. Fibronectin is highly expressed in keloid tissues, and the expression of fibronectin receives more response to TGF- β 1 stimulation in KFs than in regular fibroblasts[38]. Matrix metalloproteinases (MMPs), a family of zinc-dependent endopeptidase, are the main proteases in charge of ECM decomposition and reconstruction. MMPs have been regarded as biological markers in many pathological circumstance, and conversion of given MMPs may impact tissue remodeling and cause various pathological disorders [39]. Endogenous tissue inhibitors of metalloproteinases (TIMPs) can mediate the level of MMPs, and the MMP/TIMP proportion decides the extend of ECM degradation and reshaping. Studies suggested that the pathological process of dermal fibrosis in KFs is related to the imbalance between MMPs and TIMP, especially MMP-2 and MMP-9 are favorable for the degradation of collagen I and collagen III in KFs[40]. Our results showed that HA-R-PDT not only down-regulated extracellular matrix syntheses genes (Fig. 3A), but also reduced the ratio of MMP2/TIMP-1 and MMP9/TIMP-1 to advance the degradation of ECM (Fig. 3B). Therefore, HA-R-PDT demonstrated an anti-fibrosis ability in KFs.

Fully understanding the mechanism of keloid pathogenesis and recurrence, and exploiting potential optional treatment options are essential and imperative. Several researches have shown that TGF- β 1, a pivotal factor in collagen synthesis and proliferation, is upregulated in KFs[41]. Disturbance of TGF- β 1/Smad pathway was a vital etiopathology in keloid generation, so TGF- β 1/Smad signaling has constantly been supposed to be a vital pharmacological target for healing keloids[31]. One study displaced that antagonizing TGF- β 1 pathway can restrain the deposition of ECM and reduce collagen synthesis in keloid[33]. Smad2/3 complexes, the critical regulatory elements of TGF- β 1 signaling pathway, are highly reactive and participated in keloid pathogenesis; while Smad7, as the negative regulator of TGF- β 1/Smad pathway, blocking TGF- β 1-mediated keloid generation. Besides, TGF- β 1 mediated the expression of MMP2 and MMP9 in keloid fibroblasts. Our present study manifested that HA-R-PDT significantly attenuated the expression of TGF- β 1 and up-regulated Smad7 (Fig. 6A and B); however, down-regulated p-Smad2 and p-Smad3, and declined the phosphorylation of MAPK/ERK (Fig. 6C). These data demonstrated that HA-R-PDT induced suppression of cell proliferation, migration, collagen accumulation and ECM deposition, to a certain extent, is on account of interfering with TGF- β 1/Smad signaling pathway.

TGF- β 1, as a multifunctional cytokine, is also involved in cell autophagy that is a cellular death form that participates in lysosomal degradation and recycling of damaged and redundant organelles[19]. A clinically related aspects of autophagy is its intimate function in tumorigenesis and its potentiality as a subsidiary target for cancer treatment [51]. Researches indicated that TGF- β 1 could induce autophagy to

assist cell survival and restrain apoptosis, and enhanced autophagy might relevant to the growth of keloid[42]. Our present results indicated that HA-R-PDT down-regulated Beclin-1 and LC3 II/I conversion in KFs, which consistent with the previous reports that keloid correlated to high autophagic activity. In addition, when KFs was treated with TGF- β R1 inhibitor LY-2157299, the expression of Beclin-1 and the conversion of LC3-I to LC3-II were declined (Fig. 7A). We found that treating KFs with ERK inhibitor U0126 also effectively decreased the expression of Beclin-1 and LC3II/LC3-I conversion (Fig. 7C). All of these results exhibited that HA-R-PDT suppressed autophagy in KFs and functioned as an efficient depressor of ERK and TGF- β 1 pathway. Moreover, transfected KFs fibroblast cells with si-Smad3 declined the expression level of Beclin-1 and LC3II/LC3-I conversion (Fig. 7D). When KFs fibroblast cells treated with autophagy activator rapamycin, the level of TGF- β 1 was elevated, and the expression of Smad7 was down-regulated. The autophagy inhibitor CQ suppressed TGF- β 1 and promoted the expression of Smad7 (Fig. 7E and 7F). In conclusion, all the present data revealed that autophagy is critical for the pathogenesis of keloids. TGF- β 1 could mediate autophagy via both Smad and ERK pathway in KFs, and accommodating autophagy could alter TGF- β 1 level by the negative feedback pathway. Consequently, HA-R-PDT could suppress cell hyper-proliferation and ECM accumulation in KFs via regulating TGF- β 1/Smad/autophagy signaling pathway.

Dysregulation of apoptosis is another significant pathophysiological characteristic in keloids. In the present study, Hoechst staining and annexin V and propidium iodide (PI) double staining showed that HA-R-PDT remarkably reduced cell viability and enhanced cell apoptosis (Fig. 4A and 4B). Caspase-3 plays a crucial role in intrinsic and extrinsic pathways of cell apoptosis[42]. The Bcl-2 protein plays a vital role in blocking of cell apoptosis, while BAX promoting apoptosis, and the balance of Bcl-2 to BAX is important for maintaining cell homeostasis [43]. At the same time, we further investigated the changes of caspase-3, Bcl-2 and BAX, which referred to the mitochondrial-mediated apoptosis pathway. In this study, we indicated that HA-R-PDT dramatically decreased the expression of Bcl-2, while significantly up-regulated the protein expression of caspase-3 and BAX (Fig. 4C). The results sustained the opinion that decreased ratio between Bcl-2/BAX initiates apoptosis. These findings demonstrated that HA-R-PDT induced apoptosis was regulated through mitochondrial-mediated and caspase-depended pathways. We also evaluated the alteration of mitochondrial membrane potentials in KFs, HA-R-PDT notably weakened the mitochondrial membrane potential (Fig. 4D). MAPK/ERK cascade activation is reported to play a vital role in cell proliferation and apoptosis, and MAPK/ERK is one potential effector that participated in TGF- β 1 signaling pathway [44]. Hence, the regulation of ERK was further examined. The present data showed that when cells treated with both HA-R-PDT and ERK inhibitor U0126, the cell apoptosis was effectively increased. It is well known that apoptosis and autophagy are two main form of cell death and intimately related to each other [45], so that we investigated the relationship between apoptosis and autophagy in KFs. As has been noted, treating KFs with HA-R-PDT united ERK inhibitor U0126 evidently reduced the expression of Beclin-1 and the conversion of LC3-I to LC3-II (Fig. 7C), which implying that the decreased autophagy may accelerate apoptosis and TGF- β 1 may block apoptosis by facilitating MAPK/ERK pathway. Consequently, we were convinced that HA-R-PDT regulated TGF- β 1-ERK-autophagy-apoptosis

pathway may be a pivotal mechanism to suppress cell proliferation and down-regulate collagen synthesis and ECM accumulation in KFs.

Overall, our present studies elucidated that HA-R-PDT was able to interdict TGF- β /Smad and MAPK/ERK signaling pathways to effectually suppress the proliferation, migration and ECM deposition of KFs (Fig. 8). HA-R-PDT could boost the apoptosis of KFs through the mitochondrial-mediated apoptotic pathway and inhibit autophagy via mediating TGF- β 1 pathway further promote apoptosis. Therefore, we believed that HA-R-PDT via regulating TGF- β 1-ERK-autophagy-apoptosis pathway is an attractive therapeutic approach for prevention and mitigation of keloid.

Declarations

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Author Contributions

T.N. and G.W. contributed to discuss and conceive experiments, T.N., Y.T. and G.G. executed all the tests and data analysis, T.N and G.W drafted the manuscript and Y.T. and Y.S. proofread the manuscript.

Declaration of Competing Interest

The authors have no conflict of interest to state.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure 1

Effect of HA-R-PDT on cell proliferation in KF. (A) The cell viability after treatment was tested by the CCK-8 assay kit. The result shows that HA-R-PDT remarkably reduced the cell viability by about 47.1 % compared with non-treated cells, while HA alone or red light irradiation alone treatment resulted in a small reduction of cell viability. (B) Cell proliferation ability detected by EdU incorporation assay. (i) After 20 h of treatments, KF cells were incubated with EdU (10 μM) for 4 h. The specimens were imaged under a fluorescent microscope. (Magnification: 100 $\times$). (ii) The proportion of EdU-positive cells to Hoechst-stained cells. (C) Flow cytometry analysis for inspecting the distribution of cell cycle after treating with HA alone and red light irradiation alone or HA-R-PDT. Data were presented by means $\pm$ SD of at least three replicates. Bars between control groups and treated groups were evidently different at $p < 0.05$ (*) or $p < 0.01$ (**) level.

Figure 2

Effects of HA-R-PDT on the migration ability of KF. (A) Wound healing assay. (i) KFs were developed to a confluent monolayer, then wounded by scratching with a sterile pipette tip and following further treatment with HA-R-PDT. The scratched areas that filled up with migrated KFs were surveyed under a fluorescent microscope in 20 h after scratching. (ii) The quantified ratio of migration. (B) Trans-well assay. (i) The migration of KFs was surveyed by imaging stained with crystal violet. The average number of invading in Ctrl, HA alone, red light alone and HA-R-PDT treated cells were calculated after 20 h of migration. (ii) The number of migrated cells calculated in four casual fields. (Magnification: 100 ×).

Figure 3

The down-regulation effect of HA-R-PDT on ECM generation and accumulation. (A) KFs detected by RT-PCR to determine the effects of HA-R-PDT on the extracellular matrix syntheses genes accumulation. RT-PCR analysis of col1, col3, α-SMA and Fibronectin expression in KFs. (B) The rate of MMPs/TIMPs. Data were performed in triplicate and presented by means ± SD of at least three replicates. $p < 0.05$ (*) or $p < 0.01$ (**).

Figure 4

Analysis of apoptosis upon the treatment of HA-R-PDT. (A) Hoechst 33258 staining to assess apoptotic morphology by fluorescence microscopy. The white arrow indicates the apoptotic body. (B) Cell apoptosis examined by AnnexinV/PI double staining with flow cytometry. In figure 2B, a, b, c and d represent normal cells, early apoptotic cells, late apoptotic cells and dead cells, respectively. The percentage of apoptotic cells is shown as means ± SD. (C) Western blotting was used for inspecting the expression of apoptosis related proteins. β-actin was probed as a loading control. (D) Mitochondrial Membrane Potential Assay Kit used to measure the mitochondrial membrane potential in KFs. After staining with TMRE, the mitochondria membrane potential of KFs was detected via a fluorescence microscope (Zeiss, Germany). Each data was repeated in three independent experiments and the results analyzed in three stochastically selected sites. The settings are as following: excitation at the wavelength of about 550 nm and emission at the wavelength of about 580 nm. (Magnification: 100 ×). Bars between control groups and treated groups are conspicuously different at $p < 0.05$ (*) or $p < 0.01$ (**) level.

Figure 5

Effects of HA-R-PDT on cell autophagy in KFs. (A) Inspection of punctual distribution of GFP-LC3 under fluorescence microscope. (i) Autophagosomes represented as cytosolic green dots in KFs. (ii) The proportion of cells with cytosolic GFP-LC3 dots. (B) Detection of autophagy using autophagy assay kit. (i) Autophagy represented as bright blue spots staining of autophagic vacuoles under fluorescent microscope. (ii) Data represented as a percentage of control. (C) Acridine orange staining of KFs. Cells were stained with acridine orange and imaged under fluorescence microscope. (D) Western blotting

analysis of the expression of autophagy related proteins and β -actin served as a loading control. (E) ATG5 knockdown processed in KFs using RNAi method. Western blotting was used to inspect the effect of ATG5 knockdown on the expression of Beclin-1 and LC3II/I. β -actin was probed as a loading control. (F) RT-PCR results of extracellular matrix syntheses genes after inhibiting autophagy with si-ATG5. Bars between control groups and treated groups are evidently different at $p < 0.05$ (*) or $p < 0.01$ (**) level.

Figure 6

Effects of HA-R-PDT on TGF- β /Smad pathway. (A) KFs detected by RT-PCR to determine the expression of TGF- β /Smad related genes. (B) ELISA results of TGF- β 1. ELISA kit was used to examine the expression of TGF- β 1. (C) Western blotting analysis of the expression of TGF- β /Smad pathway relevant genes. β -actin served as a loading control. Each assay was repeated in three independent experiments and data were performed in triplicate. $p < 0.05$ (*) or $p < 0.01$ (**).

Figure 7

The anti-fibrosis effect of HA-R-PDT on TGF- β 1 through inhibiting autophagy. (A) Western blotting analysis of the expression of autophagy related proteins after treated with TGF- β R1 inhibitor LY-2157299. (B) Western blotting analysis to suggest the expression of autophagy related proteins after treatment with autophagy inhibitor 3-MA. (C) Western blotting analysis to explore the expression of autophagy related proteins after treatment with ERK inhibitor U0126. (D) Western blotting analysis to indicate the effect of Smad pathway on the regulation of autophagy. β -actin was served as an equal loading control. (E) ELISA results of TGF- β 1. ELISA kit was used to examine the expression of TGF- β 1. (F) RT-PCR results of TGF- β /Smad related genes via regulating autophagy pathway. Data were performed in triplicate and presented by means $\pm$ SD of at least three replicates. $p < 0.05$ (*) or $p < 0.01$ (**).

Figure 8

Schematic diagram of the proposed model of the regulatory mechanism of HA united red light irradiation in keloid fibroblasts pathogenesis.

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