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IGF-1 resist oxidative damage to HaCaT and depigmentation in mice treated with H_2O_2

Cui-ping Guan [a, *], Qing-tian Li [b], Hongyan Jiang [a], Qing-wei Geng [c], Wen Xu [a], Liu-yu Li [a], A.-E. Xu [a]

[a] Department of Dermatology, The Third People's Hospital of Hangzhou, 310009, Hangzhou, Zhejiang, PR China
[b] Department of Medicine, Baylor College of Medicine, Houston, TX, 77030, USA
[c] Department of Dermatology, Anhui Medical University Affiliated Hangzhou Clinical College, 310009, Hangzhou, Zhejiang, PR China

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ABSTRACT

Vitiligo, an acquired pigmentary disorder of the skin, is characterized by a chronic and progressive loss of melanocyte from the epidermis and follicular reservoir. Growth factor of surrounding cells impacted on melanocytes survival. In this study, lower level of IGF-1 in the lesion was found than that in the donor area of vitiligo patients. IGF-1 improved activation of Nrf2, and inhibited ROS generation and endoplasmic reticulum dilation in HaCaT. C57BL/6 mice were treated with 5% H_2O_2, and combined with 50 μg/kg of IGF-1 pre-treatment or not once every day for 50 consecutive days. After 50 days, IGF-1 obviously ameliorated depigmentation of mice skin and reduced hair follicle length, skin thickness and Tyrosinase induced by H_2O_2. Moreover, IGF-1 significantly suppressed $CD8^+$ T cells infiltration in mice skin, inhibited the production of IL-2 and IFN-γ, and decreased the expression of CXCL10 and CXCR3. Thus, the results indicated that IGF-1 could resist oxidative damage to HaCaT, suppress $CD8^+$ T cells infiltration and pro-inflammatory cytokines secretion, and suppresses the thinning of epidermal layer *in vivo*. It suggests that IGF-1 inhibits oxidative damage to HaCaT and immunosuppressive effects on $CD8^+$ T cells proliferation and activation to resist depigmentation induced by H_2O_2. This disclosed its multiple roles in the vitiligo, and shed a light on developing the application potential for IGF-1 in vitiligo.

1. Introduction

Vitiligo, an acquired pigmentary disorder of the skin, is characterized by a chronic and progressive loss of melanocyte from the epidermis and follicular reservoir. Growth factor of surrounding cells impacted on melanocytes survival. In the skin, insulin-like growth factor-1 (IGF-1)is expressed exclusively by mesenchymal cells of the dermis and the DP, and its receptor, IGF-1R, is synthesized in both mesenchymal and epithelial cells [1]. As a paracrine/autocrine growth factor, IGF-1 promotes hair growth by regulating cellular proliferation and migration during the development of hair follicles [2,3].

IGF system plays a crucial role in regulating cell proliferation, differentiation, and apoptosis [4]. It is being investigated as a potential therapeutic agent for the treatment of growth hormone-resistant and insulin-resistant disorders because of its acute insulin-like metabolic effects and long-term anabolic actions [5]. Related to vitiligo, IGF-1 plays a critical role in keratinocyte maintenance [6] and had the important effects on the melanogenesis in melanocyte [7]. IGF-1 endues monocytes with immune suppressive ability to inhibit inflammation in the intestine [8]. IGF-I treatment reduced immune cell extravasation to improve blood-brain barrier function of Ischemic Middle-Aged Female Rats [9]. Whether IGF-1 can regulate the immune process of vitiligo has not been investigated. Higher level of H_2O_2 was demonstrated in vitiligo epidermis than that in healthy controls [10], and much $CD8^+$ T cells infiltration in the dermal skin [11]. Increasing evidence supported interplay between H_2O_2 and $CD8^+$ T cells, such as increased ROS are thought to be involved in onset of vitiligo, and the infiltration of melanocyte-specific cytotoxic $CD8^+$ T cells into the perilesional margin directly result in melanocyte loss [12,13]. More evidence linking oxidative stress and immune system support a convergent terminal pathway of oxidative stress–autoimmunity mediated melanocyte loss [14]. Oxidative stress leads to chemokines production, causes $CD8^+$ T cell skin trafficking and melanocyte

* Corresponding author.
E-mail address: imgcp@hotmail.com (C.-p. Guan).

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destruction in vitiligo. Blockade of oxidative stress can ameliorate melanocyte apoptosis through anti-inflammatory and anti-apoptotic processes [15]. The goal of this study was to address the effect of IGF-1 on human keratinocyte and the role of IGF-1 in the depigmentation of mice induced by H_2O_2.

2. Materials and methods

2.1. Study subjects

This study was approved by the ethics committee of the third people's hospital of Hangzhou. Ten vitiligo patients (female 5, 33.2 ± 9.6; male 5, 30.6 ± 4.9) treated with epidermal transplantation were collected randomly from outpatient department of dermatology of the hospital, and each signed an informed consent form of the surgery. Suction blisters (1 cm in diameter) were induced on the skin by using the Negative Pressure Instrument Model NP-4 (Electronic Diversities, Finksburg, MD), and the protocol was carried out according to [11]. After blister formation, the blister fluid was collected for analysis by enzyme-linked immunosorbent assays (ELISA).

2.2. qPCR analysis

Total RNA extracts from cells were extracted using Trizol reagent (Thermo, Rockford, IL, USA) according to the manufacture's instructions. Two micrograms of total RNA were reversely transcribed using the first strand cDNA synthesis kit (Roche, Mannheim, Germany), and the relative amount of target mRNA was determined using the comparative threshold (Ct) method by normalizing target mRNA Ct values to those for β-actin (ΔCt). The primer sequences were as follows, Nrf2 forward primer: 5′-TCAGCGACGGAAAGAGTATGA-3′, Nrf2 reverse primer: 5′-CCACTGGTTTCTGACTGGATGT-3′; β-actin forward primer: 5′-ATCAAGATCATTGCTCCTCCTGAG-3′,β-actin reverse primer 5′-CTGCTTGCTGATCCACATCTG-3′.

2.3. Flow cytometry for ROS level

ROS level were determined by measuring the oxidative conversion of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to fluorescent compound dichlorofluorescin (DCF) according to reactive oxygen species assay kit (Cell Biolabs, San Diego, CA, USA). The protocol was briefly described as follows, the cells were washed twice and stained in FACS tubes with DCF-DA in PBS or media for 15 min at 37 °C in the dark. Then the cells were washed once with 1 × PBS and analyze immediate by FACSCanto II flow cytometer (BD Biosciences, San Diego, CA, USA). The experiments were repeated independently for 3 times.

2.4. Electron microscopy observation

HaCaT were seeded in a 6-well plate, and then the medium was replaced with serum-free medium for 24 h starvation. Cells were pretreated with optimal concentration of 6-OHDA for 24 h or 48 h at 37 °C. Method of observation of endoplasmic reticulum (ER) configuration with electron microscopy was described by us previously [16].

2.5. Animals and treatment

Four-week-old female pathogen-free C57BL/6 mice (weighting 18–20 g) were purchased from Changzhou Cavens Experimental Animal Co., Ltd (Changzhou, Jiangsu, P. R. China) and fed in the laboratory animal research center of Zhejiang Chinese medical university. Mice were housed in groups under specific pathogen-free conditions (22 ± 2 °C, RH 50–60%, and a 12-h light/dark cycle). Each mouse was individually weighed and randomly assigned to an experimental group. The mice were housed in polycarbonate cages and fed a standard animal diet with water. All mice were treated in strict accordance with the Zhejiang Chinese Medical University Animal Care and Use committee's guidelines for the care and use of laboratory animals. Before treatment, the back skin of all mice was shaved (area: 2 × 2 cm) and a depilatory cream (Veet, London, UK) was applied to areas. This is aimed to promote hair follicles transferred from telogen stage to anagen stage. Mice were grouped into three: One group of mice were smeared with 1 ml of PBS as control. One group of mice was smeared with 1 ml of 5% H_2O_2 in the experimental skin area for 3 min at 3 p.m. The third group of mice were administered with IGF-1 at 50 μg/kg of per day by gastric administration at 9 a.m. and H_2O_2 was smeared at 3 p.m. The mice were treated once a day for continuous 50 days and shaved daily. Three mice were used in one group. The experiments were repeated independently for 3 times.

2.6. Measurement of hair growth, skin thickness and pigmentation observation

Hair follicle (HF) length was measured as the distance from the dermal papilla to the epidermis. The width of the surface of the epidermis to the muscle in the photomicrograph was measured as skin thickness. Irregular shape was simulated the shaved area, and pigmentation percentage was estimated. All data were normalized to the controls, and analyzed statistically.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Human IGF-1, mouse IL-2 and IFN-γ ELISA kits were used to analyze human or mice serum refer to the manufacturer's instructions (R&D Systems, Minneapolis, USA). The absorbance at 450 nm was measured with a microplate reader (SpectraMAX 190, MD, USA). The experiments were repeated independently for 3 times.

2.8. Immunohistochemistry/immunofluorescence

Immunohistochemistry and immunofluorescence of the mice skin were performed. Mice sections were blocked with endogenous peroxidase (3% hydrogen peroxide solution) for 5 min at room temperature and then blocked in 10% goat serum for another 1 h at room temperature. The sections were incubated with one of the following antibodies: anti-CXCL10 mouse monoclonal antibody (ab8098, Abcam, USA) at a dilution 1:300, anti-CXCR3 rabbit polyclonal antibody (ab71864, Abcam, USA) at a dilution 1:500, anti-Tyrosinase mouse monoclonal antibody (ab54447, Abcam, USA) at a dilution 1:1000, anti-Nrf2 mouse monoclonal antibody (ab89443, Abcam, USA), and anti-CD8 monoclonal antibody (ab22378, Abcam, USA). In immunohistochemistry, sections were incubated with biotinylated anti-rabbit IgG before being incubated with the avidin–biotin–peroxidase complex about 30 min at room temperature, and finally visualized using aminoethyl carbazole (AEC) as a peroxidase substrate. Images were captured under an Olympus BX51 microscope installed with ImageJ software. In immunofluorescence, sections were incubated for 30 min at room temperature with goat anti-rabbit IgG H&L (Alexa Fluor® 594) (1:1000 dilution) (ab150080, Abcam, USA), and/or with DAPI for 10 min, and then observed with confocal laser scanning microscope (TCS SP2, Leica, Germany).

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