BASIC RESEARCH

Abstract

Background: Oxidative stress is implicated in the pathogenesis of vitiligo. The function of DJ-1 in oxidative damage of melanocytes is still elusive. Aims: The aim of this study was to investigate the role of DJ-1 in oxidative damage of melanocytes. Material and Methods: The expression of DJ-1 in melanocytes was studied by reverse transcription-quantitative polymerase chain reaction and Western blot. Short-interfering RNAs (siRNA) were employed to downregulate DJ-1. The cells were pooled into three groups: mock group (cells with transfection reagent), negative control (NC) group (negative siRNA control), and siRNA group. After H₂O₂ treatment for 24 h, the morphological changes, cell viability, apoptosis, intracellular reactive oxygen species (ROS) levels, mitochondrial membrane potential (MMP), and mitochondrial respiration were measured in different groups. Results: DJ-1 was highly expressed in PIG1 melanocytes. DJ-1 knockdown rendered PIG1 melanocytes more susceptible to oxidative stress. Loss of DJ-1 led to apoptosis of PIG1 cells by impairing the function of mitochondria, including morphological abnormalities, ROS accumulation, depolarization of MMP, less adenosine-triphosphate (ATP) production, and less proton leak. Conclusions: DJ-1 plays a role in maintaining the antioxidative capacity in epidermal melanocytes.

Key Words: DJ-1, melanocyte, mitochondria, oxidative stress

Introduction

Vitiligo is an acquired depigmenting disorder characterized by the loss of melanocytes in lesional epidermis.[1] It is well documented that oxidative stress results in the damage of epidermal melanocytes in the pathogenesis of vitiligo.[2] Melanocytes are constantly exposed to environmental stressors, such as UV radiation and various chemicals, which further boost the production of reactive oxygen species (ROS). Excessive accumulation of ROS induces degeneration of melanocytes and finally results in white macules in the skin.[3-4] Mitochondria utilize oxygen to generate ATP and ROS was produced in this process. Moreover, the accumulation of ROS over threshold leads to mitochondria damage, which results in apoptosis.[7] Mitochondrial damage has been observed in vitiligo lesions and these disorders occurred in prostage of the disease, even before the lesion being noticed.[8] Therefore, the dysfunction of mitochondria is the initiator of melanocyte damage under oxidative stress in vitiligo.

The DJ-1 (PARK7) gene was first identified as an oncogene in 1997[9] and was found associated with familial Parkinson’s disease. It has a neuroprotective function against oxidative stress in dopaminergic neurons. The protective effect had been proved to be mediated through DJ-1 localizing to the mitochondria, but the mechanism had not been elucidated. In this study, we investigated the role of DJ-1 in oxidative stress in normal human melanocyte and related mechanisms.

Material and Methods

Cell culture, oxidative stress model, and short-interfering RNAs transfection

A normal human melanocyte cell line PIG1 was used in this study. It is immortalized by introducing a retroviral vector, E6 and E7 open reading frames of human papilloma virus 16 with unlimited growth potential and normal melanocytic properties[12] (a kind gift from Prof. Caroline Le Poole, Loyola University, Chicago, USA).

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The cells were cultured in Medium 254 (Gibco, USA) supplemented with 5% fetal bovine serum (Gibco, USA) and 1% human melanocyte growth supplement (Gibco, USA) at 37°C in a humidified incubator with 5% CO₂. PIG1 cells were seeded in 96-well plates (1 × 10⁵/ml, 100 µl per well) and cultured to 80% confluency. Cells were then treated with 0, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 mmol/l hydrogen peroxide (Sigma, USA). Treatment with 0.6 mmol/l of H₂O₂ for 24 h resulted in 50% cell death. We chose this concentration to induce the oxidative stress for subsequent experiments.

DJ-1-specific short-interfering RNAs (siRNAs) were designed and synthesized by GenePharma (Shanghai, China). The siRNA sequence was 5'-GGUUCCUACCAGGAGUAUTTAUCCUCCUGGUAGAACCTT-3'. PIG1 melanocytes were seeded in 6-well plates, with a density of 2 × 10⁵ cells per well. Next day, the cells were transfected with 30 nM siRNA (SiRNA group) in serum-free Dulbecco's modified eagle medium (DMEM) (Gibco, USA), using transfection reagent lipofectamine 2000 (Invitrogen, USA). About 30-nM negative siRNA (NC group) and non-siRNA treatment (mock group, cells only with transfection reagents) were used as control. DJ-1 knockdown efficiency was examined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot.

**Cell viability assay**

The medium in each well of the 96-well plate was aspirated and washed twice with PBS, and then, 100 µl of fresh medium supplemented with 10 µl of CCK-8 solution (Dojindo, Japan) was added to each well. After 3 h of incubation at 37°C, the absorbance of each well was measured at 450 nm using a microplate reader. Cell viability was calculated using the following formula: (A group – A blank)/(A control group – A blank) × 100%. Technical triplicates were performed for each group with three biological replicates.

**Reverse transcription-quantitative polymerase chain reaction**

Total RNA was isolated from PIG1 cells using Qiagen RNasy mini kit (Qiagen, German). The RNA (1 µg) was reverse transcribed into complementary DNA using ReverTra Ace qPCR RT kit (TOYOBO, Japan). The RT-qPCR was performed on Bio-Rad CFX96 Touch™ Real-Time PCR Detection System using SYBR green Real-time PCR Master Mix (TOYOBO, Japan). The primers for DJ-1 were forward 5'-AACCAGAAGGGCCCTGA-3' and reverse 5'-GCAAAGGGTTGTTGTAAC-3'. The cycling condition was 95°C for 2 min followed by 40 cycles of 95°C for 5 s, 55°C for 10 s, and 72°C for 15 s. A mean value was used for the determination of mRNA levels by the comparative Cq method with glyceraldehyde phosphate dehydrogenase (GAPDH) as reference gene and using the formula 2⁻ΔΔCT.

**Western blot analysis**

Cells were washed with PBS and lysed in RIPA buffer (Beyotime, China) containing phenylmethanesulfonyl fluoride (Protease inhibitor mix, 20:1, Sigma-Aldrich). Protein samples were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked with 5% nonfat dry milk for 30 min at room temperature, then washed and incubated with primary antibody overnight at 4°C (DJ-1 antibody at 1:2000 dilution, Abcam). The membrane was then washed and incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody (Proteintech, USA) for 1 h at room temperature. Bound secondary antibodies were detected using a chemiluminescence detection kit (KPL, Gaithersbury, MD). Band intensities were determined using the ImageJ software (National Institutes of Health, Bethesda, MD), and statistical analysis was performed to determine significant differences in protein expression.

**Transmission electron microscopy**

The ultrastructure of the cells was examined via transmission electron microscopy (TEM). Briefly, the cells were collected and washed in ice-cold PBS and then fixed in 1.5% glutaraldehyde for 24 h at 4°C. The cell pellets were then rinsed with Millonig's buffer and postfixed in 1.0% OsO₄. The cell pellets were cut into 90 nm sections, slide-mounted, and stained with 2.0% uranyl acetate, followed by ethanol dehydration, and subsequently embedded in epoxy resin. Ultrathin section analysis was visualized using a Tecnai G2 spirit (FEI, Hillsboro, OR).

**Apoptosis assays**

Apoptosis was detected by flow cytometry using an Annexin V FITC/PI Apoptosis Kit (Multisciences, USA) according to the manufacturer’s instructions. After treated with H₂O₂ for 24 h, the cells were washed and incubated with 500-µl binding buffer contained 5-µl Annexin V and 10-µl PI for 5 min at room temperature in the dark, thereafter measured by a BD FACSCalibur flow cytometry (BD Bioscience, USA). For each sample, the percentage of normal (Annexin V−, PI−), early apoptotic (Annexin V+, PI−), late apoptotic (Annexin V+, PI+), and necrotic cell (Annexin V−, PI+) population was calculated. Analysis was performed using the CellQuest software (BD FACS Calibur).

**Intracellular ROS detection**

Intracellular ROS was measured by staining cells with ROS detection reagent CM-H₂DCFDA (Invitrogen, USA). Cells were washed with PBS and incubated in PBS containing 1-mM CM-H₂DCFDA for 30 min at 37°C in the dark. Cells were subsequently washed twice in PBS, and fluorescence was detected using an inverted fluorescence microscope (Olympus IX70, Japan) within 1 h.
the cells were trypsinized and washed twice in PBS; the fluorescence was measured by flow cytometer (BD LSR II). The mean fluorescence intensity was quantified using the FACS Diva 6.0 software.

**Measurement of mitochondria membrane potential**

Cells in different groups were suspended in 1 ml warm phosphate-buffered saline at approximately 1 × 10^6 cells/ml. Then, the cells were incubated with 2 µmol/l JC-1 dye (Invitrogen, USA) at 37°C, 5% CO₂ for 20 min. After incubation, cells were pelleted by centrifugation and resuspended in 500-µl PBS and analyzed by flow cytometry (BD LSR II). The fluorescent emission of JC-1 shifted reversely from red (measured at 590 nm) to green (measured at 530 nm) with decreasing mitochondrial membrane potential (MMP) (ΔΨm) when excited at 488 nm, and the red/green emission ratio provided an estimate of ΔΨm. Flowjo 10 software was used for flow data analysis.

**Mitochondrial respiration assay**

Real-time mitochondrial respiration was assessed in PIG1 cells using a Seahorse XF96 Analyzer (Agilent, USA). Oxygen consumption rates (OCRs) were calculated as a measure of aerobic respiration. To do this, PIG1 cells were plated in Seahorse 96-well plates and treated with H₂O₂ for 24 h before the assay. On the day of the assay, the cells were washed with assay medium (pH 7.4) and then incubated at 37°C in a CO₂ free incubator for 60 min. The melanocyte-containing plates were then loaded into the instrument and three replicate baseline OCR measurements were collected. Three compounds (1 µM oligomycin, 0.5 µM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), and 1 µM retenone/antimycin A), which target the different components of the electron transport chain in mitochondrial respiration, were injected in succession. Three parameters of mitochondrial respiration were calculated according to the manufacturer's instruction. Results were normalized to protein concentration in each well.

**Statistical analysis**

Statistical analysis was performed using the SPSS 17.0 software (SPSS Inc., USA). Data are presented as mean ± standard deviation. ANOVA or a two-way Student's t-test was used to analyze the difference between groups. P < 0.05 was considered statistically significant.

**Results**

**DJ-1 was highly expressed in PIG1 cells and downregulated by DJ-1 siRNA**

DJ-1 mRNA expression was detected 48 h after DJ-1 siRNA delivery compared with the NC group (P < 0.01) [Figure 1a]. The corresponding DJ-1 protein reduction was observed at 72 h after transfection (P < 0.001) [Figure 1b and c].

**DJ-1 knockdown-induced morphological changes of PIG1 cells and abnormalities in mitochondria under oxidative stress**

To investigate the role of DJ-1 in cell characteristics, we observed the morphological changes in DJ-1 knockdown melanocytes. After exposure to H₂O₂ for 24 h, there were obvious morphological changes in PIG1 cells. The dendrites of cells in siRNA group were shorter or lacking; more cells became round and floated compared with the Mock and NC groups [Figure 1d-f]. Subsequent ultrastructural TEM analysis showed that cells in siRNA group displayed more cytoplasmic vesicles which had a typical single-membrane structure of autolysosomes, and the mitochondria were shrunked obviously [Figure 1g-i].

**DJ-1 knockdown impaired cell viability and induced apoptosis in PIG1 cells under oxidative stress**

Based on the morphological changes, the cell viability in siRNA group was dramatically decreased compared with the mock and NC group (P < 0.001) [Figure 2a]. The flow cytometry showed that the percentage of apoptotic cells was also significantly increased [Figure 2b and c] in siRNA group compared with the mock and NC group under oxidative stress induced by H₂O₂ (P < 0.01).

**DJ-1 knockdown affected the mitochondria respiration and decreased the ATP production and proton leak**

To study the mitochondrial respiration in different groups under oxidative stress, the OCR was measured by Seahorse XF96e analyzer in real time. The basal respiration that represented the energy demand of cells under baseline condition was decreased significantly in siRNA group (P < 0.01) [Figure 2d and e]. The ATP production and proton leak in siRNA group were also compromised significantly with the mock and NC group [Figure 2f and g].

**DJ-1 knockdown induced ROS accumulation and ΔΨm depolarization**

Mitochondria are the main source of ROS in cells; the depolarization of MMP is the hallmark of mitochondrial damage. As shown in Figure 3a, fluorescence intensity of CM-H₂DCFDA-stained cells was significantly stronger in siRNA group compared with the mock and NC group under fluorescence microscope. Flow cytometry demonstrated that the intracellular ROS was significantly increased in siRNA group [Figure 3b and c]. Then, we evaluated the MMP by flow cytometry, an apparent
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Discussion

It is well documented that DJ-1 is expressed in many tissues including the brain, pancreas, kidney, skeletal muscle, and the skin. DJ-1 performs a protective role against oxidative stress in dopaminergic neurons and some other cell types, but its role in oxidative damage of melanocytes has not been illustrated. We detected the DJ-1 expression in PIG1 melanocytes both at mRNA and protein levels. In subsequent studies, we found that downregulation of DJ-1 in PIG1 cells, prior to exposure to H₂O₂-induced oxidative stress, led to multiple morphological changes in melanocytes. Normal PIG1 melanocytes were polygonal with 2–3 dendrites, whereas DJ-1 knockdown PIG1 cells had shorter or even lacking dendrites. Additionally, there was a marked increase in round, floating, dead cells compared with the mock and NC groups. Cells in siRNA group also showed decreased cell viability and increased apoptosis, which strongly suggested that downregulation of DJ-1 in melanocytes made them more vulnerable to H₂O₂-induced oxidative stress (OS) in melanocytes.

Figure 1: DJ-1 was highly expressed in PIG1 cells and downregulated by DJ-1 siRNA. (a) A decrease of DJ-1 mRNA expression was detected 48 h after transfection; **P < 0.01. (b and c) DJ-1 protein reduction was observed at 72 h; ***P < 0.001. (d-i) DJ-1 knockdown induced morphological changes of cells and mitochondrial abnormality under oxidative stress. The siRNA group had shorter dendrites and more dead cells (arrow indicates dead cell). siRNA group displayed more autolysosomes and shrunken mitochondria (black arrow indicates autolysosomes, white arrows indicate shrunken mitochondria). Scale bars represented 1 µm.
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...oxidative stress. Consistent results were reported in other cell types and overexpression models. Inberg et al. reported that suppression of DJ-1 level using siRNA led to an accelerated cell death, whereas an increase in DJ-1 level attenuated cell death induced by H$_2$O$_2$ in pancreatic β-cell lines. Liu reported that downregulation of DJ-1 by siRNA decreased antioxidant gene expression and increased oxidative damage. Chang et al. found that knockdown of DJ-1 or overexpression of DJ-1 L166P mutation resulted in mitochondria damage and hypersensitivity to H$_2$O$_2$-induced cell apoptosis. Our results also suggested the antioxidative effect of DJ-1 in epidermal melanocytes, which was consistent with the aforementioned cell types.

Oxidative stress induces dysfunction of mitochondria and leads to apoptosis. In our experiment, the downregulation of DJ-1 in PIG1 cells caused morphological abnormalities of mitochondria, decrease of MMP, and the accumulation of ROS in melanocytes, indicating the dysfunction of mitochondria.

The ability of the mitochondria to make ATP and to consume oxygen in response to energy demands serves as another hallmark of its functional state. To investigate the mitochondrial respiration in DJ-1...
knockdown melanocytes, three key parameters were measured in real time by using the seahorse system. The basal respiration showed energetic demand of the cells under baseline condition. ATP production equaled to the decrease in OCR upon injection of the ATP synthase inhibitor oligomycin and showed ATP produced by the mitochondria. Proton leak equaled to remaining basal respiration not coupled to ATP production and could be a sign of mitochondrial damage. Our results revealed that DJ-1 knockdown decreased the basal respiration of melanocytes with less ATP production and less proton leak under oxidative stress.

**Conclusion**

DJ-1 protects melanocytes against oxidative stress caused by $\text{H}_2\text{O}_2$ through restoring mitochondrial homeostasis. Further studies are necessary to elucidate the mechanistic pathways for its protective role.

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**Conflicts of interest**

There are no conflicts of interest.
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