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Expression of cytosolic NADP$^+$-dependent isocitrate dehydrogenase in melanocytes and its role as an antioxidant

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ABSTRACT

Background: Cytosolic NADP$^+$-dependent ICDH (IDPc) has an antioxidant effect as a supplier of NADPH to the cytosol, which is needed for the production of glutathione.
Objective: To evaluate the expression of IDPc in melanocytes and to elucidate its role as an antioxidant.
Methods: The knock-down of IDPc expression in immortalized mouse melanocyte cell lines (melan-a) was performed using the short interfering RNA (siRNA)-targeted gene silencing method. After confirming the silencing of IDPc expression with mRNA and protein levels, viability, apoptosis and necrosis, as well as ROS production in IDPc-silenced melanocytes were monitored under conditions of oxidative stress and non-stress. Also, the ratio of oxidized glutathione to total glutathione was examined, and whether the addition of glutathione recovered cell viability, decreased by oxidant stress, was checked.
Results: The expression of IDPc in both primary human melanocytes and melan-a cells was confirmed by Western blot and RT-PCR. The silencing of IDPc expression by transfecting IDPc siRNA in melan-a cells was observed by Western blotting and real-time RT-PCR. IDPc knock-down cells showed significantly decreased cell viability and an increased number of cells under apoptosis and necrosis. IDPc siRNA-treated melanocytes demonstrated a higher intensity of DCFDA after the addition of H_2O_2 compared with scrambled siRNA-treated melanocytes, and a lower ratio of reduced glutathione to oxidized glutathione were observed in IDPc siRNA transfected melanocytes. In addition, the addition of glutathione recovered cell viability, which was previously decreased after incubation with H_2O_2.
Conclusions: This study suggests that decreased IDPc expression renders melanocytes more vulnerable to oxidative stress, and IDPc plays an important antioxidant function in melanocytes.

1. Introduction

The skin is directly exposed to harmful conditions such as ultraviolet light, pollution and other stresses that contain or generate free radicals [1]. Appropriate levels of reactive oxygen species (ROS) modify regulatory signaling pathways, while high concentrations of ROS can induce oxidative damage to various kinds of cells, even leading to cell death [2]. An integrated antioxidant defense mechanism exists in the skin to protect against damage from ROS. Enzymatic and nonenzymatic antioxidants interact to provide protection from oxidant stress. The enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione reductase (GR) detoxify superoxide, hydrogen peroxide, and hydroperoxide [3]. Glucose-6-phosphate dehydrogenase (G6PD) and isocitrate dehydrogenase (ICDH), which produce reduced nicotinamide adenine dinucleotide phosphate (NADPH) [4–6], an essential reducing agent for the regeneration of glutathione, also act as enzymatic antioxidants in the skin [1].

The pathogenesis of vitiligo, an acquired depigmenting disorder, still remains unclear, but autoimmunity and oxidative stress are thought to be the most probable mechanisms in the loss of functional melanocytes [7–9]. The association of oxidant stress with vitiligo has been demonstrated in various clinical and experimental studies. An increased production of H_2O_2 has been

Abbreviations: ICDH, isocitrate dehydrogenase; IDPc, cytosolic NADP$^+$-dependent isocitrate dehydrogenase; G6PD, glucose-6-phosphate dehydrogenase; SOD, superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; GSSG, glutathione disulfide; Nrf2, nuclear factor E2-related factor 2; NADPH, nicotinamide adenine dinucleotide phosphate; DTNB, dithio-2-nitrobenzoic acid; DCFDA, dichlorofluorescein diacetate.

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found in the epidermises of patients with active vitiligo and may be associated with lower expression and activity of the antioxidant enzyme catalase [10,11]. Additionally, several studies have shown dysregulation of redox balance in various kinds of cells and cell components of vitiligo [12–14].

In one of our previous studies, via proteomics and 2-dimensional Western blotting, we detected 10 candidate melanocyte antigens that reacted with IgM antibodies in the sera of vitiligo patients [15]. Among these 10 antigens, cytosolic $NADP^+$-dependent ICDH (IDPc) has an antioxidant effect during oxidative stress as a supplier of NADPH to the cytosol, which is needed for the production of glutathione [16]. Therefore, we postulated that the loss of IDPc activity from autoantibodies in vitiligo sera might cause a loss in the repair capacity of melanocytes. The expression and function of IDPc in melanocytes have not been examined, although the anti-oxidative function of IDPc has been studied, albeit limited to a few types of cells including cells of the kidney, liver and fibroblasts [11–13].

Thus, in this study, to establish the role of IDPc in protecting against oxidative stress through efficient glutathione recycling, the function of IDPc in melanocytes was investigated by silencing IDPc expression therein with transfecting IDPc short interfering RNA (siRNA).

2. Materials and methods

2.1. Cell culture

Melan-a, a copiously pigmented non-tumorigenic mouse melanocyte cell line, was gratefully obtained as a gift from Prof. Dorothy C. Bennett of St George's Hospital Medical School in London, UK. Culture procedures were carried out as previously described [17] with only minor modification. The cells were maintained in RPMI 1640 growth medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 200 nM 2-*O*-tetradecanoylphorbol-13-acetate (TPA) at 37 °C in a humidified atmosphere of 5% CO_2. Normal human melanocytes (NHM) were purchased from ATCC (American Type Culture Collection, USA) and were maintained in Dermal Cell Basal Medium with a Melanocyte Growth Kit (ATCC). HeLa cells were grown in EMEM supplemented with 10% FBS and 1% penicillin–streptomycin. NHM and HeLa cells were used as positive controls for IDPc expression.

2.2. Short interfering RNA-targeted gene silencing

To suppress IDPc expression, melan-a cells were transfected with 20 nM of siRNA (Bioneer, Daejeon, South Korea) using INTERFERin™, a transfection reagent (Polyplus Transfection, NY, USA), according to the manufacturer's protocol. One day before transfection, melan-a cells were seeded at a density of 1×10^5 cells onto 6-well plates in growth medium. At 30–50% confluence, cells were transfected with IDPc siRNA, scrambled siRNA, or growth medium alone.

2.3. Cell viability assay

Melan-a cells transfected with scrambled siRNA or IDPc siRNA were seeded at 1×10^4 cells in a 96-well plate. Cell proliferation was estimated with a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) staining. Viability was assessed by measuring absorbance at 570 nm with a molecular device spectra Max 340 microplate reader. Experiments were repeated three times, and data are presented as mean values ± standard deviations (SDs). In addition, on days 1 and 2 after transfection, cells were incubated with 100 μM of H_2O_2 and 3 mM of glutathione for 2 h. To confirm whether glutathione recovered the cell viability decreased by H_2O_2 treatment, cell viability was compared between groups with and without the addition of glutathione after H_2O_2 treatment.

2.4. Western blot

Proteins isolated from melan-a cells on days 1 and 2 after treatment with IDPc siRNA or scrambled siRNA were separated in a 10% SDS-PAGE gel. These proteins were then transferred to an NC membrane according to standard procedures. The membrane was blocked with 5% (v/v) skim milk in a TBS-T buffer (TBS with 0.01%, w/v Tween-20) and reacted with the anti-IDPc antibody (Santa Cruz Biotechnology, CA, USA) overnight at 4 °C. The membrane was then washed three times for 15 min each time with TBS-T buffer and incubated for 1 h with 1% skim milk in the TBS-T buffer containing horseradish peroxidase-conjugated anti-rabbit, anti-mouse or anti-goat antibody (diluted to 1:2000). The proteins were visualized using an ECL system (Thermo Scientific, Bremen, Germany) using horseradish peroxidase-conjugated anti-rabbit secondary antibody. GAPDH (Cell Signaling, Beverly, MA, USA) was used as a control for protein loading.

2.5. Real-time RT-PCR

Total RNA was isolated from melan-a cells transfected with IDPc siRNA and scrambled siRNA on days 1 and 2 using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following the manufacturer's recommendations. One microgram of the total RNA was used for cDNA synthesis with Maxime™ RT PreMix (Oligo(dT)15 Primer (iNtRON). For quantitative real-time RT-PCR, amplification was performed in a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and was visualized with a Power SYBR GREEN MASTER MIX (Applied Biosystems, Foster City, CA, USA). The cycling conditions were 2 min at 50 °C followed by 10 min at 95 °C and 50 cycles of 15 s at 95 °C and 1 min at 60 °C. The primer sequences were as follows: sense primer for IDPc, 5′-ATGCAAGGAGATGAAATCACACG-3′ and anti-sense primer, 5′-GCATCACGATTCTCTATGCCTAA-3′; sense primer for ACTIN, 5′-GGCTGTATTCCCCTCCATCG-3′ and anti-sense primer, 5′-CCAGTTGGTAACAATGCCATGT-3′. Relative quantification was calculated using the ΔΔCt (threshold cycle) method, where ΔCt equaled (target Ct − β-actin Ct), and ΔΔCt equaled (ΔCt sample − ΔCt untreated control). The relative quantity of the sample to control mRNA was $2^{-\Delta\Delta Ct}$. The expression of mRNA was indicated with a Ct value and was corrected according to the expression of β-actin.

2.6. Annexin V-FITC/PI double staining

Annexin V-FITC/PI double staining was employed to quantify the apoptosis of melan-a cells treated with IDPc siRNA or scrambled siRNA. Briefly, cells were seeded in a 6-well plate (1×10^5 cell/well) and exposed to scrambled siRNA or IDPc siRNA for 24 h and 48 h. The cells were then stained using an Annexin V-FITC/PI double-fluorescence apoptosis detection kit (BD Pharmingen, San Diego, CA, USA) following the manufacturer's instruction. Flow cytometry was performed using LSRII (BD Biosciences), then compensated and analyzed with WINMDI software (Scripps Research Institute, San Diego, CA, USA).

2.7. Measurement of the ratio of reduced glutathione to oxidized glutathione

The levels of total glutathione and reduced form glutathione were determined by the rate of formation of dithio-2-nitrobenzoic acid (DTNB) at 412 nm using a Glutathione Detection Kit (BioVision, CA, USA) following the manufacturer's instructions,