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# Protein phosphatase-1 is involved in the maintenance of normal homeostasis and in UVA irradiation-induced pathological alterations in HaCaT cells and in mouse skin

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ABSTRACT

The number of ultraviolet (UV) radiation-induced skin diseases such as melanomas is on the rise. The altered behavior of keratinocytes is often coupled with signaling events in which Ser/Thr specific protein kinases and phosphatases regulate various cellular functions. In the present study the role of protein phosphatase-1 (PP1) was investigated in the response of human keratinocyte (HaCaT) cells and mouse skin to UV radiation. PP1 catalytic subunit (PP1c) isoforms, PP1cα/γ and PP1cδ, are all localized to the cytoskeleton and cytosol of keratinocytes, but PP1cδ was found to be dominant over PP1α/γ in the nucleus. PP1c-silencing in HaCaT cells decreased the phosphatase activity and suppressed the viability of the cells. Exposure to a 10 J/cm$^2$ UVA dose induced HaCaT cell death and resulted in a 30% decrease of phosphatase activity. PP1c-silencing and UVA irradiation altered the gene expression profile of HaCaT cells and suggested that the expression of 19 genes was regulated by the combined treatments with many of these genes being involved in malignant transformation. Microarray analysis detected altered expression levels of genes coding for melanoma-associated proteins such as keratin 1/10, calcium binding protein S100A8 and histone 1b. Treatment of Balb/c mice with the PP1-specific inhibitor tautomycin (TM) exhibited increased levels of keratin 1/10 and S100A8, and a decreased level of histone 1b proteins following UVA irradiation. Moreover, TM treatment increased pigmentation of the skin which was even more apparent when TM was followed by UVA irradiation. Our data identify PP1 as a regulator of the normal homeostasis of keratinocytes and the UV-response.



## 1. Introduction

It is well established that many environmental and genetic factors contribute to the development of skin cancer, however, among these factors one of the most important causative factor is chronic exposure of the skin to solar UV radiation [1]. The solar UV spectrum includes UVA (320–400 nm), which is 90–95% of the total solar UV radiation and considered to be the "aging ray" causing benign tumor formation as well as malignant cancers [2]. UVB (280–320 nm) is thought to be responsible for a wide range of skin diseases such as non-melanoma and melanoma skin cancers. UVB acts as a tumor initiator and co-carcinogen by triggering DNA damage, immunosuppression and oxidative stress [3]. UVC radiation (200–280 nm) has no significant effect on biological systems since it is largely absorbed by stratospheric ozone [4].

UV irradiation initiates the transcription of numerous genes encoding multiple proteins, which play a role in skin cell apoptosis, malignant transformation, aging of the cells as well as collagen degradation [5]. Thus, UV light can be absorbed by several target chromophores in the skin such as nucleic acids, NADPH and heme-containing enzymes relevant for cellular signaling. DNA can absorb only UVB light, while it is affected by UVA light in an indirect manner whereby UVA-irradiated cells overproduce reactive oxygen species (ROS) resulting in ROS-induced DNA damage [6]. UV radiation can cause severe damage on many tissue components including proteins, membrane lipids and nucleic acids. It has also been recognized as a major regulator of cellular processes mediated by growth factors and cytokine-mediated signal transduction pathways. Its effects might include influence on phospholipids, protein kinases (MAP kinases, tyrosine kinases, Ser/Thr specific kinases) and protein phosphatases, leading to pathological (abnormal) gene expression [7]. The combined action of these pathways eventually

*Abbreviations:* PP1, protein phosphatase-1; PP1c, PP1 catalytic subunit; PP2A, protein phosphatase-2A; PP2Ac, PP2A catalytic subunit; TM, tautomycin; OA, okadaic acid; PBS, phosphate buffer saline; TBS, Tris-buffered saline solution; UV, ultraviolet; KRT, keratin; ROS, reactive oxygen species; NHEK, normal human epidermal keratinocyte; I-2, inhibitor-2; PARP, poly (ADP-ribose) polymerase

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determines the fate of UV-irradiated cells. Moreover, it is widely appreciated that the effects of UV light on certain signaling pathways are wavelength-specific [8,9] and are related mostly to the activation of MAPK, AKT/mTOR, AMPK and p53 signaling by protein phosphorylation [5]. The UV-induced changes in the phosphorylation of cellular proteins by protein kinases are relatively well established, while little is known about the regulatory role of the protein phosphatases which ensure the reversibility of these phosphorylation processes.

In the human genome almost 40 genes encode for phospho-Ser/Thr specific protein phosphatases (PP) [10] which are termed as PP1 to PP7 [11]. Among these types PP1 and PP2A are assumed to catalyze the dephosphorylation of over 90% of all eukaryotic phosphoproteins [12]. In fact, PP1 and PP2A are two of the most abundant enzymes and they are highly characterized in many cell types including skin cells [13]. Mammalian genomes contain three different genes that encode four distinct catalytic subunits termed PP1α, PP1β/δ and PP1γ1 [14], and a splice variant PP1γ2 [15] expressed ubiquitously except for the latter one. The substrate specificity and distinct subcellular localization of PP1 is regulated by various targeting subunits [16], and several of them (PNUTS, pRb, Repo-Man, GADD34, *etc.*) have recently been identified as regulators of UV-mediated pathways such as DNA damage, cell cycle, centrosome separation and apoptosis [17]. Hence, it is important to elucidate the mechanisms by which PP1 enzymes may regulate the UV radiation-induced pathologies and the underlying cellular events.

In this study, we report that PP1 enzymes play an important role in the maintenance of the homeostasis in human keratinocytes as well as in mouse skin. We also suggest that pathological processes could result from the selective regulation of PP1 enzyme activity by UVA irradiation.

## 2. Material and methods

### 2.1. Chemicals and antibodies

All chemicals were obtained from Sigma-Aldrich unless otherwise indicated. Antibodies were as follows: anti-KRT 1 (against middle region) and anti-KRT 10 (against N-terminal region) from Aviva System Biotechnology; affinity isolated anti-S100A8, horseradish-peroxidase (HRP) conjugated anti-rabbit IgG and anti-actin from Sigma-Aldrich; monoclonal anti-histone H1 (AE-4), anti-α-tubulin, anti-GAPDH and monoclonal anti-PP1 (E9) antibodies from Santa Cruz Biotechnology; affinity isolated anti-PP1cδ from Upstate Millipore; anti-PP2Ac antibody from BD Biosciences; anti-PARP, anti-cleaved PARP antibodies and HRP-linked anti-mouse IgG from Cell Signaling Technology; Alexa Fluor 488- and Alexa Fluor 546-conjugated anti-mouse and anti-rabbit IgG from Molecular Probes.

### 2.2. Cell culture, gene silencing and UV radiation

Human keratinocyte (HaCaT) cells (300493, CLS Cell Lines Service GmbH, Germany) were grown in high glucose DMEM supplemented with 10% heat-inactivated FBS and 2 mM L-glutamine in humidified 5% $CO_2$ atmosphere at 37 °C. All materials used for cell culture were purchased from PAA Laboratories, Austria. Normal human epidermal keratinocyte (NHEK) cultures were generously donated by Dr. Tamas Bíró (University of Debrecen, Department of Physiology) and maintained in Epilife medium complemented with Human Keratinocyte Growth Supplement (HKGS), 100 μg/ml streptomycin, 100 IU/ml penicillin and 50 ng/ml amphotericin B (all from Life Technologies) and were cultured on 1% collagen-coated plates. Double-stranded siRNA (100 nM) (Santa Cruz Biotechnology) was used to knock down the protein levels of endogenous PP1 isoforms (α, β/δ and γ) and a non-target sequence (Thermo Scientific Inc.) with the G–C content of the siPP1 sequence was applied in control experiments according to the manufacturer's protocol. 100 nM siRNAs were transfected into HaCaT cells using a Dharmafect 2 transfection reagent (Thermo Scientific Inc.) in serum-free medium. After 30 min, the medium was completed with 10% FBS and cells were incubated for 48 h. Then prior to UV irradiation, cells were kept in serum-free medium for 16 h in each case. UV irradiation was carried out using a Bio-Sun UV irradiation system (Vilbert Lourmat, France). After 24 hour incubation cells were either lysed for immunoblotting and microarray analysis and were fixed for immunofluorescent staining or utilized for MTT assay. 1 μM of tautomycin was applied for 1 h before UV irradiation and cells were treated as it was described before. In the case of protein phosphatase activity measurement cells were lysed and assayed right after TM treatment or UV irradiation.

### 2.3. Cell disruption and protein measurement

Treated cells were collected and washed by ice-cold Dulbecco's PBS without calcium, magnesium and phenol red (pH: 7.2; 2.7 mmol/l KCl, 1.5 mmol/l $KH_2PO_4$, 136.9 mmol/l NaCl, 8.9 mmol/l $Na_2HPO_4 \cdot 7H_2O$) and lysed in modified RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.1% (w/v) SDS, 1 mM DTT) containing protease inhibitor cocktail Complete Mini (Roche Diagnostics GmbH, Switzerland) followed by 3 × 15 s sonication on ice. After centrifugation (16,000 × *g* for 15 min at 4 °C), protein concentration of the supernatant was determined by the BCA method (BCA protein assay kit, Pierce Biotechnology, USA) using an ELISA reader (Labsystem Multiscan MS) at 540 nm with BSA as a standard as described before [18]. Alternatively subcellular factions of HaCaT cells were prepared as described before [19].

### 2.4. MTT assay

The assay was used to determine the viability of HaCaT cells. An MTT reagent was prepared by adding 5 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to 1 ml PBS. The cell culture medium was replaced and 20 μl of MTT was added to each well of the 96-well plate (200 μl). The plates were incubated for 1–3 h at 37 °C in 5% $CO_2$ atmosphere. The medium was removed and 100 μl DMSO was added to the wells for dissolving the formazan crystals in the cells and the absorbance at 540 nm was recorded.

### 2.5. Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were carried out as detailed before [19]. After blocking in 5% (w/v) non-fat dry milk, the membranes were washed with PBS containing 0.1% Tween 20 (PBST) and incubated with primary antibodies overnight at 4 °C, followed by application of the HRP-conjugated secondary antibodies. To determine the changes in the protein expression levels of histone 1b, keratin 1 and 10 as well as S100A8, specific antibodies were applied to detect changes. Immunoreactions were detected by enhanced chemiluminescence (ECL). Densitometry of the proteins of interest were performed by Image J. 1.46 and normalized to tubulin internal control (run on the same gel) and plotted as relative numbers (in %). To assess the extent of apoptosis of NHEK and HaCaT cells after TM treatment and UVA irradiation Western blot analysis was carried out on 30 μg of cell lysates using anti-PARP (1:500) and anti-cleaved PARP (1:500), antibodies.

### 2.6. Immunofluorescence microscopy

Paraformaldehyde (4%) was used for fixation (10 min) of HaCaT cells followed by three washes with ice-cold PBS. Cells were permeabilized with 0.02% (v/v) Triton X-100 dissolved in PBS for 10 min at room temperature and washed three times with PBS. After blocking in 1% (w/v) sterile BSA in PBS for 1 h, coverslips were incubated with anti-PP1c (E9), anti-PP1cδ, anti-PP2A and anti-KRT1 (all in 1:100 dilution) primary antibodies overnight at 4 °C. After extensive washing with PBS,