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Induction of differentiation in psoriatic keratinocytes by propylthiouracil and fructose☆

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ABSTRACT

Psoriasis is characterized by uncontrolled proliferation and poor differentiation. Sirtuin1 (SIRT1) a class III deacetylase, crucial for differentiation in normal keratinocytes, is reduced in psoriasis. Down regulated SIRT1 levels may contribute to poor differentiation in psoriasis. In addition, the levels of early differentiation factors Keratin1 (K1) and Keratin10 (K10) are depleted in psoriasis. We attempted to study a possible effect of fructose, a SIRT1 upregulator and Propylthiouracil (PTU) to augment differentiation in psoriatic keratinocytes. Keratinocytes were cultured from lesional biopsies obtained from psoriatic patients and control cells were obtained from patients undergoing abdominoplasty. Cells were treated with fructose and PTU individually. K1 and K10 transcript levels were measured to evaluate early differentiation; SIRT1 protein expression was also studied to decipher its role in the mechanism of differentiation. The K1, K10 transcript levels, SIRT1 protein and transcript levels in fructose treated psoriatic keratinocytes were improved. This suggests keratinocyte differentiation was induced by fructose through SIRT1 upregulation. Whereas PTU induced differentiation, as confirmed by improved K1, K10 transcript levels followed a non-SIRT1 mechanism. We conclude that the use of fructose and PTU may be an adjunct to the existing therapies for psoriasis.

1. Introduction

A hallmark of lesional psoriatic skin is premature keratinocyte differentiation and disturbed keratinization, altering the formation of cornified envelope in psoriasis. Human epidermal keratinocytes stratify into colonies and cells in stratum granulosum, stratum lucidum and stratum corneum gradually lose their mitotic potential to begin terminal differentiation. Differentiation is a highly organized process wherein the proteins K1, K10, profilaggrin, involucrin, loricrin, and other proteins of cornified envelope are sequentially expressed [1].

There had been a debate about the progress of differentiation pattern in psoriasis. Until 1992 it was thought that only the last step in differentiation was altered, however Bernerd et al. showed that alterations are found from the first stage itself [2]. The first step of differentiation involves heterodimerization of K1 and K10 to form cytoskeletal filaments. This keratin pair is the most abundant protein in differentiated keratinocytes [3].

Keratin K1/K10 regulates keratinocyte growth in the epidermis which is proved by *in vitro* experiments conducted by Paramio et al. (2001) [4]. They reported that K10 is directly involved in cell cycle control which onsets keratinocyte differentiation. Interestingly several other studies have shown that mutations either in K1/K10 or absence of K10 showed greater epidermal proliferation in the basal layer and hyperkeratosis [5,6]. Furthermore *in situ* hybridization of K10 transcripts proved a delayed keratin 10 synthesis in psoriatic epidermis [2]. These studies markedly prove the essential role of K1/K10 in differentiation and controlled proliferation. Many therapies in the past targeted differentiation, in the same lines we were on the lookout for a differentiation improving factor.

A study published by Blander et al. (2009) [7] exposed the potential role of SIRT1 in inducing keratinocyte differentiation and inhibiting keratinocyte proliferation. Elevated IFN gamma levels inhibits SIRT1 expression and sensitizes psoriatic keratinocytes to IL-22 mediated inflammatory response altering epidermal differentiation [8]. Pillai and colleague (2008) found that fructose augmented SIRT1 levels in heart [9]. Since SIRT1 mediated effects are tissue specific, we attempted to increase SIRT1 levels and improve differentiation in psoriatic keratinocytes, by

☆ Key message: A novel attempt to induce differentiation in poorly differentiated psoriatic keratinocytes.

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treating the cells with fructose. In the recent past our team has proved that PTU cleared lesions in psoriatic patients and improved differentiation as confirmed by modulated levels of involucrin [10,11]. In this study, we have focused on the role of fructose and PTU to improve differentiation in psoriatic keratinocytes and further explored the impact of these compounds on SIRT1 levels, a promoter of keratinocyte differentiation.

2. Materials and methods

2.1. Recruitment of patients and collection of samples

Patients (N = 7) with chronic plaque psoriasis, but otherwise in general good health who visited Saveetha Medical College Hospital, Chennai, India participated in this study. Psoriasis was confirmed by Psoriasis Area Severity Index (PASI) score by a dermatologist. None of the recruited patients had received any topical treatment for the last 2 weeks or any systemic treatment for the last 1 month. Skin from patients undergoing abdominoplasty served as control samples. Lesional biopsy specimens (5 mm) were obtained through punch biopsy from patients with chronic plaque psoriasis. The study was approved by Institutional Ethical Committee, Saveetha University (Chennai) and has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Informed consent was obtained from each participant included in the study.

2.2. Primary culture

The biopsy specimens were incubated overnight in 0.3% dispase (Sigma, USA) in Phosphate Buffered Saline (PBS) supplemented with 50 μg/ml Gentamicin (Life Technologies, USA) at 4 °C. After incubation the dermis layer was mechanically removed and discarded, the epidermal layer was treated with 0.25% trypsin (Life Technologies, USA) at 37 °C for 30 min. Cell dissociation was achieved by aspiration using Pasteur pipette followed by filtration (70 μm cell strainer). Keratinocytes Serum Free Medium (K-SFM) (Life Technologies, USA) was used to wash filtered keratinocytes. The viability of the cells was always >95% as determined by Trypan blue exclusion test. To generate keratinocyte cultures, suspension of primary epidermal cells (4×10^4 cells/cm^2) were plated in 100 mm petri dishes. The culture was supplemented with keratinocytes supplements such as Epidermal Growth Factor (EGF) 0.1 ng/ml, Bovine pituitary extract 25 μg/ml, and Gentamicin 50 μg/ml in K-SFM and the flasks were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. The medium was changed every 2 days and the third passage keratinocytes with 70–80% confluence was used for further experiments.

2.3. Cell proliferation assay

For analyzing cell proliferation via MTT assay, keratinocytes (1×10^4 cells/well) were incubated with different concentrations of PTU (2–10 mM) and fructose (1–20 mM) for 24 h, 48 h and 72 h in 96 well plates and were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Stock solutions of compounds were initially dissolved in DMSO and further diluted with fresh complete medium. MTT reagent (50 μl) (5 mg/ml in PBS), was added to each well and incubated at 37 °C for 4 h. At the end of the incubation period, the supernatant was removed completely without disturbing the cell layer, 150 μl of DMSO was added and read on a microplate reader at 570 nm.

2.4. RNA isolation and real-time polymerase chain reaction (RT-PCR)

TRIzol reagent (Life Technologies, USA) was used to extract total RNA to be analyzed by real-time PCR. The expression of SIRT1, K1, K10 and β-Actin mRNA were evaluated using SYBR Green PCR reagents following the manufacturer's protocol on an Applied Biosystem Thermocycler. The forward and reverse primers used in real-time PCR were as follows: For SIRT1, forward: 5′-TCAGTGTCATGGTTCCTTTGC-3′; reverse: 5′-AATCTG CTCCTTTGCCACTCT-3′, K1 forward 5′-ATTTCTGAGCTGAATCGTGTGATC-3′ reverse 5′-CTGATGGACTGCTGCAAGTT-3′ K10 forward 5′-ATGAGCTG ACCCTGACCAAG-3′ reverse 5′- TCACATCACCAGTGGACACA-3′ and for β-Actin forward: 5′-AGGCACCAGGGCGTGAT-3′; reverse 5′- GCCCACATAG GAATCCTTCTGAC-3′. The gene expression levels were determined by normalizing to β-Actin mRNA expression. The values obtained are presented as mean ± SD.

2.5. Western blot

Solubilized protein samples (40 μg; measured and equalized in each fraction using the Bio-Rad RC-DC protein assay; Bio-Rad) were separated by SDS-PAGE and transferred onto PVDF membrane (GE Healthcare, UK). Membranes were blocked with 3% (w/v) milk protein in Tris-buffered saline containing 0.1% Tween-20, and then incubated overnight with rabbit polyclonal anti-SIRT1 antibody (sc-15404, 1:1000 dilution, Santa Cruz Technologies, USA). Detection of bands was achieved by using the chemiluminescent substrate Super Signal West Pico (Pierce, Rockford, IL, USA). Blot is representative of three separate blots and densitometry was determined. Reference protein measurements were made with rabbit polyclonal anti-β-actin primary antibody in a 3% (w/v) Tris-buffered saline (1:1000 dilution, Santa Cruz Technologies, CA, USA).

2.6. Statistics

All values are represented as mean ± S.D. of the three measurements. A one-way analysis of variance test for post hoc multiple comparisons was used to determine significance. Probability values <0.05 were considered significant.

3. Results

MTT assay (Fig. 1A and B) shows percentage of live cells from control, untreated and treated psoriatic keratinocytes at 24 h, 48 h and 72 h. PTU treatment (2–10 mM) and fructose treatment (1–20 mM) decreased proliferation of psoriatic keratinocytes when compared to untreated cells. A dose of 4 mM of PTU and 5 mM of fructose was used for further experiments. Control keratinocytes obtained from abdominoplasty patients were treated with fructose and PTU independently and in combination. We measured the percentage of live cells. No significant changes were seen between the treated and untreated control cells.

3.1. Improved K1 and K10 transcript levels in cultured psoriatic keratinocytes after treatment with fructose and PTU

Poor differentiation in psoriatic keratinocytes was apparent from the decreased K1 & K10 levels compared to keratinocytes from control samples (0.56 and 0.60 fold change respectively, $p < 0.05$) (Fig. 2). Fructose treatment to psoriatic keratinocytes improved K1 and K10 levels by 1.66 and 2.5 fold respectively ($p < 0.001$). PTU treatment was also very effective in improving differentiation in psoriatic keratinocytes and the fold change for K1 and K10 were 2.04 and 2.79 respectively ($p < 0.001$). Control keratinocytes obtained from abdominoplasty patients were treated with fructose and PTU independently and in combination. We measured the mRNA expression of K1 and K10. No significant changes were seen between the treated and untreated control cells.

3.2. SIRT1 levels in cultured psoriatic keratinocytes was increased by fructose but not by PTU

The effect of PTU and fructose on SIRT1 mRNA transcript levels in psoriatic keratinocytes was studied by Real time PCR (Fig. 3). SIRT1 mRNA level was found to be 0.56 fold lesser in psoriatic keratinocytes