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Review

Lipid deregulation in UV irradiated skin cells: Role of 25-hydroxycholesterol in keratinocyte differentiation during photoaging

Elodie Olivier[a,b], Mélody Dutot[a,c], Anne Regazzetti[a], Delphine Dargère[a], Nicolas Auzeil[a], Olivier Laprévote[a], Patrice Rat[a,*]

[a] UMR CNRS 8638-Chimie Toxicologie Analytique et Cellulaire, Université Paris Descartes, Sorbonne Paris Cité, Faculté de Pharmacie de Paris, 4 Avenue de l'Observatoire, 75006 Paris, France
[b] Soliance-Givaudan, Route de Bazancourt, 51110 Pomacle, France
[c] Recherche et Développement, Laboratoire d'Evaluation Physiologique, Yslab, 2 rue Félix le Dantec, 29000 Quimper, France

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ABSTRACT

Skin photoaging due to UV irradiation is a degenerative process that appears more and more as a growing concern. Lipids, including oxysterols, are involved in degenerative processes; as skin cells contain various lipids, the aim of our study was to evaluate first, changes in keratinocyte lipid levels induced by UV exposure and second, cellular effects of oxysterols in cell morphology and several hallmarks of keratinocyte differentiation. Our mass spectrometry results demonstrated that UV irradiation induces changes in lipid profile of cultured keratinocytes; in particular, ceramides and oxysterols, specifically 25-hydroxycholesterol (25-OH), were increased. Using holography and confocal microscopy analyses, we highlighted cell thickening and cytoskeletal disruption after incubation of keratinocytes with 25-OH. These alterations were associated with keratinocyte differentiation patterns: autophagy stimulation and intracellular calcium increase as measured by cytofluorometry, and increased involucrin level detected by immunocytochemistry. To conclude, oxysterol deregulation could be considered as a common marker of degenerative disorders. During photoaging, 25-OH seems to play a key role inducing morphological changes and keratinocyte differentiation.

1. Introduction

Population aging constitutes one of the most significant trends of the 21st century; indeed, nowadays one in eight people in the world are aged 60 or over and this proportion tends to increase dramatically [1]. As a result, skin aging appears as a growing concern. Skin aging is characterized in particular by wrinkles, sagging skin and elasticity loss. Physiologically, the most abundant cells of the skin are keratinocytes, which are located in the epidermis, the outermost layer of the skin. Epidermis continuously regenerates throughout the life: keratinocytes migrate into the upper layer of the epidermis during a process called differentiation. During this process, keratinocytes are gradually modified to become flat cells named corneocytes, which have lost their nucleus and cytoplasmic organelles [2]. Changes occurring through keratinocyte differentiation are characterized, amongst others, by remodeling of actin cytoskeleton [3], increased levels of involucrin and keratin [4], increased calcium level [5] and autophagy [6]. Keratinocyte differentiation can be abnormal or accelerated under certain stresses such as UV irradiation after sun exposure [7]. In this particular case, the abnormal and accelerated keratinocyte differentiation is part of photoaging.

Abbreviations: 27-OH, 27-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; 24-OH, 24-hydroxycholesterol; 7-βOH, 7-βhydroxycholesterol; 7-KC, 7-ketocholesterol; BSA, bovine serum albumin; Cer, ceramide; CHO, cholesterol; DMEM, Dulbecco's modified Eagle's medium; DR, down-regulated; ESI, electrospray ionization; GlcCer, glucosylceramide; HRMS, high resolution mass spectrometry; LacCer, lactosylceramide; MDC, monodansylcadaverine; OPLS-DA, orthogonal partial least squares discriminant analysis; PBS, phosphate buffered saline; PC, phosphatidylcholine; PCA, principal component analysis; PC-P, phosphatidylcholine plasmalogen; PE, phosphatidylethanolamine; PE-P, phosphatidylethanolamine-plasmalogen; SM, sphingomyelin; UPLC, ultra-performance liquid chromatography; UR, up-regulated.

* Corresponding author.
E-mail address: patrice.rat@parisdescartes.fr (P. Rat).

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At a molecular level, skin cells contain various lipids such as sphingolipids, phospholipids, cholesterol and triglycerides. Lipid deregulation, particularly in ceramides and oxidized derivatives of cholesterol called oxysterols, has been associated with Alzheimer disease and age-related macular degeneration (AMD) [8–15]. As photoaging is considered as a multisystem degenerative process [16], we hypothesize that some key lipids could play an important role in photoaging.

In this *in vitro* study, our aim was to evaluate first, the modifications of keratinocyte lipid levels induced by UV irradiation, and second, the cellular effects of oxysterols on cell morphology and hallmarks of keratinocyte differentiation.

2. Materials and methods

2.1. Cell culture

HaCaT cells, spontaneously transformed human keratinocytes, were obtained from Cell lines service (Cell lines service-CLS-Germany). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Eurobio, Courtaboeuf, France) supplemented with 10% fetal calf serum, 2 mM of glutamine, 50 IU/ml of penicillin and 50 IU/ml of streptomycin (Eurobio). Cell cultures were maintained in controlled atmospheric conditions: CO_2 5%, humidity 95% and temperature 37 °C. When the cells reached confluency, they were dispersed using trypsin and counted. Depending on cellular concentration, the cellular suspension was diluted and seeded in flasks or microplates.

2.2. UV irradiation experiments

2.2.1. UV irradiation

UV irradiation was performed with a solar light simulator Suntest CPS+ (Atlas, Mount Prospect, IL, USA). This simulator equipped with a xenon arc lamp and special glass filters restricting transmission of light below 290 nm, provides irradiance that approximates sunlight. HaCaT cells were seeded in flasks and irradiated at a dose of 1 or 2.5 J/cm^2. The cells were subsequently rinsed and incubated for 24 h in culture medium. Non-irradiated cells were used as control.

2.2.2. Lipidomic analysis

Lipid composition of keratinocytes was analyzed by ultra-performance liquid chromatography coupled to high resolution mass spectrometry (UPLC-HRMS). After cell dispersion using trypsin, cell pellets were dissolved in 600 μL double-distilled water, vortexed for 30 s and sonicated for 5 min. Total lipids were extracted by the method of Bligh and Dyer [17]. Lipids extracts were resuspended in a 35:35:20:10 v/v/v/v acetonitrile/isopropanol/chloroform/water solution and analyzed using UPLC-HRMS on a Synapt™ G2 HDMS™ mass spectrometer (Waters MS Technologies, Manchester, UK). Data were analyzed using unsupervised principal component analysis (PCA) and supervised partial least squares discriminant analysis (PLS-DA). Moreover, an orthogonal partial least squares discriminant analysis model (OPLS-DA) was built on the PLS model using SIMCA-P+ software version 13.0.3 (Umetrics, Umeå, Sweden). Annotation of lipid species was performed using LIPID MAPS and METLIN online databases with a tolerance window for the mass accuracy of 5 ppm. Expected and actual retention times were compared for each lipid to confirm the previous annotation, a relative difference between these two retention times below 15% was accepted [18].

2.2.3. Sterols dosage

Quantitation of sterols in human keratinocytes was performed according to the method developed by Ayciriex et al. [19] using ultra-performance liquid chromatography–high resolution mass spectrometry analysis (UPLC-HRMS). After cell dispersion using trypsin, cell pellets were dissolved in 600 μL double-distilled water, vortexed for 30 s and sonicated for 5 min. Sterols were extracted with a hexane/methanol mixture (7:1, v/v) under agitation for 40 min and dried under reduce pressure. Sterols were derivatized into carbamate using a solution of 4-(dimethylamino)phenyl isocyanate in dichloromethane. Dichloromethane was evaporated under reduced pressure, derivatized sterols were resuspended in an acetonitrile/isopropanol mixture (1:1, v/v) and analyzed using UPLC-ESI-HRMS on a Synapt™ G2 HDMS™ mass spectrometer (Waters MS Technologies, Manchester, UK). Oxysterol levels were normalized to protein content measured by BCA method.

2.3. Oxysterol incubation experiments

2.3.1. Keratinocytes incubation with 25-hydroxycholesterol

HaCaT cells were incubated with 25-hydroxycholesterol (25-OH, Sigma-Aldrich, Saint Louis, MO, USA). 25-OH was dissolved in absolute ethanol to obtain a 40 mM stock solution. Solutions were sonicated to solubilize oxysterol. 25-OH was diluted in culture medium to obtain targeted concentrations ranging from 5 to 40 μM.

2.3.2. Cell viability evaluation

Cell viability was evaluated through membrane integrity using the neutral red assay. HaCaT cells, cultured in 96-well microplates, were incubated with 25-OH for 48 h. After this incubation time, the cells were washed with PBS and incubated with a 50 μg/mL neutral red solution for 3 h at 37 °C according to Borenfreund and Puerner validated protocol [20]. Then, the cells were rinsed with PBS and lysed with a solution of acetic acid-ethanol (ethanol 50.6%, water 48.4% and acetic acid 1%). After homogenization, the fluorescence signal was scanned (λexc = 540 nm, λem = 600 nm) using a cytofluorometre (Safire, Tecan, Männedorf, Switzerland).

2.3.3. Necrosis evaluation

Cell necrosis was assessed using the lactate dehydrogenase (LDH) release assay. In case of membrane damage, LDH, a cytoplasmic enzyme, is released in the extracellular compartment. The extracellular rate of lactate dehydrogenase is therefore correlated with cell death [21]. LDH mixture was prepared according to manufacturer's instructions (Sigma-Aldrich). 50 μL of cell supernatant were added to 50 μL of LDH mixture, and the microplate was agitated for 30 min at room temperature. Reaction was stopped with 10 μL HCl 1 N and absorbance was read at 490 nm (λref = 690 nm) using a cytometer (Safire, Tecan).

2.3.4. Cell morphology assessment

Cell morphology was studied on living cells with the label-free technique digital holographic microscopy using a HoloMonitor M3 (Phase Holographic Imaging PHI AB, Lund, Sweden). HaCaT cells, cultured in 6-well microplates, were incubated with 25-OH for 48 h. Cell morphological changes (thickness, roughness, volume, area) were analyzed by the software provided with the HoloMonitor (HoloStudio).

2.3.5. Cell cytoskeleton actin analysis

Cytoskeletal changes in actin stained with phalloidin were observed by confocal microscopy. HaCaT cells were cultured in