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Retinoic acid and hydroquinone induce inverse expression patterns on cornified envelope-associated proteins: Implication in skin irritation

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ABSTRACT

Background: Hydroquinone (HQ) with or without retinoic acid (RA) is routinely used for the treatment of hyperpigmented conditions. Skin irritation is a major problem with popular depigmenting agents, resulting in postinflammatory hyperpigmentation.
Objective: To examine the molecular mechanism associated with skin irritation by RA or HQ.
Methods: A genome-wide transcriptional profiling analysis was performed using monolayer cultures of human keratinocytes treated with or without irritant doses of RA, HQ, or sodium lauryl sulfate (SLS), a representative irritant. Differentially expressed genes (DEGs) were mapped on human chromosomes using a Manhattan plot. For the validation of candidate DEGs, the chemicals with different concentrations of varying irritation intensities were applied *in vitro* and *in vivo* and analyzed using real time-PCR and Western blotting.
Results: DEGs mapped to the 1q21 locus, which is composed of a cluster of genes encoding the cornified envelope precursors, showed an inverse expression pattern in response to HQ and RA. Concentrations of RA and HQ that induced a broad range of irritant responses in cultured cells or mice skin also induced inverse effects on the expression of cornified envelope-associated proteins.
Conclusions: Genetic modulation on cornified envelope-associated proteins by RA-induced irritation, which may be involved in physiological skin barrier disturbance, could be inverse to that by HQ- or SLS-induced irritation.

1. Introduction

Topical phenolic agents, including hydroquinone (HQ) and non-phenolic agents, including retinoids are used for hyperpigmentation disorders. Topical HQ is the most popular treatment modality and is frequently used in combination therapies, such as a triple combination with topical all-trans retinoic acid and corticosteroids [1–4]. Although HQ is used in over-the-counter formulations for lightening skin pigmentation, it can cause skin irritation [5]. The most common adverse event associated with topical retinoic acid (RA), is also an irritant reaction of variable intensity, which presents with dryness, scaling, erythema, burning, and/or stinging [6–8]. Because skin irritation could lead to post-inflammatory hyperpigmentation, efforts have focused on the reduction of the irritant potential without loss of efficacy. Skin irritation can be induced by different mechanisms, such as skin barrier disruption, induction of a cytokine cascade, and involvement of the oxidative stress network [9,10]. Different classes of substances could potentially exert skin irritation through different mechanisms.

A dose–response relationship is seen for adverse events, as well as effectiveness [11–13]. Irritant reaction from the same chemical shows variable intensities *in vivo*, if their concentrations are different. Gene expression may also differ at different intensities of irritation. Although concentrations that inhibit cell viability by 50% have been used as the threshold concentration of irritation on human skin [14], the exact proportion of cell survival or cytotoxicity that is indicative of irritation *in vivo* is not defined. Therefore, validation using sequentially increasing concentrations

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including concentration of 50% cell survival may be helpful in the identification of molecular mechanism of irritation.

Skin irritation is one of the most common adverse reactions to chemicals. Every substance is an irritant to some degree. To reduce irritant reactions, the molecular mechanisms require to be identified. This study was performed to elucidate the molecular mechanisms associated with skin irritation by RA and HQ. Toward this aim, a genome-wide transcriptional profiling analysis and a Manhattan plot were performed after treatment of cultured normal human keratinocytes, a main constituent of skin, with subcytotoxic doses of HQ or RA. The data were validated by *in vitro* mRNA studies using increasing concentrations of each chemical (subcytotoxic, 50%, and 20–30% cell survival). The expression of corresponding proteins was examined *in vivo* by using increasing concentrations which induced mild erythema, definite erythema, and severe erythema with focal erosion, to corroborate the *in vitro* result.

2. Materials and methods

2.1. Cell culture

Adult skin specimens obtained from Cesarean sections and circumcisions, were used to establish cells in culture. For keratinocyte culture, individual epidermal cells were suspended in EpiLife Medium (Invitrogen, Carlsbad, CA) supplemented with bovine pituitary extract, bovine insulin, hydrocortisone, human epidermal growth factor, and bovine transferrin (HKGS; Invitrogen). The harvested cells were resuspended at 7.5×10^4 cells/mL in each culture medium and seeded at 1.5×10^5 cells/well in 6-well plates. One day after seeding, appropriate concentrations of each chemical were added. After 2 days, the cultured cells were harvested and used in cell viability assay, cytotoxicity assay, microarray, real time PCR, and Western blot. All experiments were repeated thrice.

2.2. Preparation of chemicals

For *in vitro* studies, RA (Sigma Aldrich, St Louis, MO, USA) and HQ (Sigma Aldrich) were prepared at the concentration of 1 mM and 10 mM stock solution, respectively, using dimethylsulfoxide (DMSO; Sigma Aldrich). Sodium lauryl sulfate (SLS; Sigma Aldrich) was solved in distilled water to make 10 mM stock solution. For *in vivo* studies, RA and HQ were prepared as 0.1–1.0% and 10–40% ointment, respectively.

2.3. Cell viability test

Cell viability was evaluated by the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) reduction method. The cells were stained with MTT for 4 h. The precipitated formazan was dissolved in DMSO and optical density was measured using a spectrophotometer, at 570 nm with background subtraction at 630 nm. Cell viability was calculated as the ratio of cell growth in response to RA, HQ, or SLS to that with the appropriate solvent.

2.4. Cytotoxicity test

Cytotoxicity was evaluated by the lactate dehydrogenase (LDH) release method. LDH activity was measured in the culture medium using the cytotoxicity detection kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. LDH release in the supernatant of the experimental cultures was determined by measuring optical density at 490 nm and then subtracting the optical density at 620 nm. Effects of chemicals on cytotoxicity were calculated from the percent of LDH release with each chemical relative to the appropriate solvent.

2.5. Oligonucleotide microarray analysis and differentially expressed genes (DEG) mapping by a Manhattan plot

Total RNA from cells cultured under each condition was isolated using QuickGene RNA cultured cell kit S (Life Science, Tokyo, Japan), according to the manufacturer's instructions. Total RNA (100 ng) was used for labeling. GeneChip microarrays (Affymetrix, Santa Clara, CA) were prepared, hybridized, and scanned by the local authorized Affymetrix service provider (DNA Link, Seoul, South Korea). RNA was converted to cDNA and transcribed into cRNA in the presence of biotinylated ribonucleotides, according to standard Affymetrix protocols ('Expression Analysis Technical Manual', #701021 Rev. 5). Hybridization was carried out using Affymetrix GeneChip Human U133 Plus 2.0 oligonucleotide arrays. The GeneChip procedure of the biotin-labeled cRNA by *in vitro* transcription, hybridization to the array, washing, and scanning was performed according to the manufacturer's protocol. The hybridized probe array was stained and washed with GeneChip hybridization, stain and wash kit using the Fluidics Station 450 (Affymetrix). The stained GeneChip probe array was scanned with a GeneChip Scanner 3000+7G (Affymetrix). The signal intensity corresponding to the gene expression level was calculated by the Expression Console Software, Version 1.1 (Affymetrix) based on the MAS 5.0 algorithm. The individual probe expression levels for each chemical treatment and corresponding solvent control were compared. We selected DEGs by using the following steps: (i) selection of Affymetrix probe sets called as "present" for HQ, RA, or SLS treated samples; (ii) selection of Affymetrix probe sets with comparative signal sample/control ratios >2 as "up-regulated", <0.5 as "down-regulated", and (iii) selection of Affymetrix probe sets with simultaneously significant p-values (threshold, 0.05) in the Wilcoxon rank test when compared with vehicle treated control samples. In order to analyze the DEG distribution in the chromosome, a Manhattan plot was conducted using R (http://www.R-project.org).

2.6. Real-time PCR

cDNA was synthesized from the total RNA using the First Strand cDNA Synthesis Kit for RT-PCR (AMV; Boehringer Mannheim, Germany). The amount of target mRNAs was quantified by real-time PCR using Light Cycler real-time PCR machine (Roche, Penzberg, Germany). The relative amount of mRNAs was calculated as the ratio of each target relative to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences for real-time PCR were as follows: loricrin 5′-GCTCTGTCTGCGGCTACTCT-3′ (forward) and 5′-AGTGACCTGCTGCGAGGA-3′ (reverse); filaggrin 5′-GTTACAATTCCAATCCTGTTGTTTTC-3′ (forward) and 5′-CGTTGCATAATACCTTGGATGATC-3′ (reverse); involucrin 5′-CTCTCCCCTGCCCTCAGT-3′ (forward) and 5′-TCATTTGCTCCTGATGGGTATT-3′ (reverse); late cornified envelope (LCE) 3D 5′-CCTCCTCTGCACCTGGAC-3′ (forward) and 5′-CGTTGCATAATACCTTGGATGATC-3′ (reverse); small proline rich protein (SPRR) 1A 5′-CATTCTGCTCCGTATACCAGAAA-3′ (forward) and 5′-GCAAGGTTGTTTCACCTGCT-3′ (reverse); SPRR1B 5′-GACCATACAGAGTATTCCTCTCTTCAC-3′ (Forward) and 5′-TGCTGCTGCTGAAGCTGA-3′ (reverse); GAPDH 5′-TCCACTGGCGTCTTCACC-3′ (forward) and 5′-GGCAGAGATGATGACCCTTT-3′ (reverse).

2.7. Western blot analysis

Equal amounts of extracted proteins were resolved and transferred to nitrocellulose membranes. The membranes were