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HSP27 as a biomarker for predicting skin irritation in human skin and reconstructed organotypic skin model

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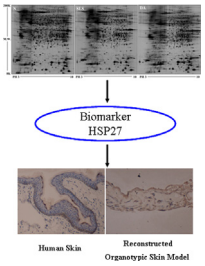
HIGHLIGHTS

- Proteomics was used to analyze the altered protein in human skin exposed to SLS..
- New skin-equivalent was constructed and was suitable for the skin irritation test.
- HSP27 was up-regulated significantly after acid and basic chemicals exposure.

GRAPHICAL ABSTRACT



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ABSTRACT

In vitro alternative tests aiming at replacing the traditional animal test for predicting the irritant potential of chemicals have been developed, but the assessing parameters or endpoints are still not sufficient. To discover novel endpoints for skin irritation responses, 2DE-based proteomics was used to analyze the protein expression in human skin exposed to sodium lauryl sulfate (SLS) following the test protocol of the European Centre for the Validation of Alternative Methods (ECVAM) in the present study. HSP27 was up-regulated most significantly among the eight identified proteins, consistent with our previous reports. Acid and basic chemicals were applied on human skin for further validation and results showed that the up-regulated expression of HSP27 was induced in 24 h after the exposure. Skin-equivalent constructed with fibroblasts, basement membrane and keratinocytes was used to investigate the potential of HSP27 as a biomarker or additional endpoint for the hazard assessment of skin irritation. Our skin-equivalent (Reconstructed Organotypic Skin Model, ROSM) had excellent epidermal differentiation and was suitable for the skin irritation test. HSP27 also displayed an up-regulated expression in the ROSM in 24 h after the irritants exposure for 15 min. All these results suggest that HSP27 may represent a potential marker or additional endpoint for the hazard assessment of skin irritation caused by chemical products.

1. Introduction

Dermal irritation is defined as the production of "reversible damage of the skin following the application of a test substance" (OECD, 2002). Determining the potential of chemicals to cause acute skin irritation is important for establishing procedures for the safe handling, packaging and transport of chemicals, as well as for the general safety assessment purposes. For reasons of safety assessment, new chemicals are often evaluated for irritant potential

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by application to animals and the investigation of visible changes such as erythema and oedema (Draize et al., 1944). For ethical reasons the European Council decided to pursue the policy of reduction, refinement and replacement (3R) of animal testing within the Cosmetic Directive 2009/1223/EC (2009). In order to replace animal testing and improve the prediction of irritants, a few reconstructed human epidermal models were developed and used in alternative test in vitro. Keratinocytes make up 95% of the cells in the epidermis and play an integral role in initiating, modulating and regulating skin irritation (Beissert et al., 2006). Therefore, normal human epidermal keratinocytes were cultured to form a multilayer, differentiated model and seeded on matrices of either dermal components or non-biological origins (de Brugerolle de et al., 1999; Demetrulias et al., 1998; Hoffmann et al., 2008; Jirova et al., 2013). The most prominent skin equivalents are EpiDerm (MatTek, Ashland, MA, USA), Episkin (Episkin, Chaponost, France), Apligraf (Organogenesis Inc., MA, USA) and models engineered by Skinethic (Skinethic, Nice, France) (Welss et al., 2004).

Following irritation, changes in cell viability and morphous, differential expression of genes or proteins, differential synthesized biochemical molecules are revealed, which could be used as biomarkers or endpoints to assess irritant responses. Among these changes, the most commonly used parameters are measurement of cell viability by MTT assay (Osborne and Perkins, 1994); but measuring cytotoxicity alone does not always reveal the right prediction between irritants and non-irritants (Fentem et al., 2001). Immunological processes play an important role in irritant contact dermatitis, and keratinocytes are the major source of cytokines, including interleukin family, Tumor Necrosis Factor-α (TNF-α), TNF-β, monocyte chemoattractant protein-1 (MCP-1) and interferon induced protein-10 (IP-10) (Corsini and Galli, 2000). Single and repeated irritation induced differential and concerted expression of various inflammatory mediators and markers. Despite the variation, interleukin-1α (IL-1α) clearly increased after single exposure and interleukin-1 receptor antagonist (IL-1RA) increased after repeated exposure (de Jongh et al., 2007). A validation study of an in vitro skin irritation testing method using EpiSkin has been conducted by the European Centre for the Validation of Alternative Methods (ECVAM), and the protocol standard has been documented based on the validated test methods (ECVAM SIVS, 2007). In the recent studies, test substances were also applied onto Epiderm (Casas et al., 2013; Kidd et al., 2007) and LabCyte EPI-MODEL (Aichi, Japan) (Katoh et al., 2009) according to the protocol of ECVAM, and the measurement of IL-1α and MTT assay were used to distinguish skin irritant from non-irritant agents. IL-1α is only released from leaky cells following cell injury or membrane perturbation (Dinarello, 1998). Irritants without membrane-damaging characteristics probably do not initiate the inflammatory response. Different irritants are often characterized by different cytokine patterns (Fulzele et al., 2007). IL-1α may not be sensitive and effective enough for skin irritation assessment.

Skin irritation is a complex response, and the currently used biomarkers are potential options for the discrimination between irritants and non-irritants of some classes of substances (Welss et al., 2004). Searching for new parameters or endpoints is necessary and urgent in vitro testing systems for predicting cytotoxic effects and assessing skin irritation. Toxicogenomic technologies were used to identify novel mechanistic endpoints for skin irritation responses, and it was found that differentially expressed genes were involved in cell signaling, stress response, cell cycle, protein metabolism and cell structure after exposure to irritant and non-irritant chemicals (Borlon et al., 2007). Proteomics assessment was also applied in searching for new protein markers: Comparing the expression profile of sodium lauryl sulfate (SLS) treated human skin by 2D-PAGE, small Heat Shock Protein 27 (HSP27) was prominently up-regulated (Boxman et al., 2002b). In our previous reports, the expression of HSP27 also displayed a significant alteration in keratinocytes exposed to SLS, potassium hydroxide (PH), 10-undecenoic acid (UA) (Zhang et al., 2011) and heavy metals Chromium and Neodymium (Zhang et al., 2010). Partial breakdown of type I cytokeratins K14, K16 and K17 as well as the emergence of new charge variants of the proteins heat shock protein 27 and ribosomal protein P0 were observed in keratinocytes exposed to sulfur mustard (Mol et al., 2008).

In the present study, following the irritation test protocol of ECVAM in vitro, altered proteins were identified by proteomics technology in human skin exposed to SLS. Acid and basic chemicals were used to investigate the potential of HSP27 as a biomarker or additional endpoint for the hazard assessment of skin irritation >in human skin and reconstructed organotypic skin model (ROSM).

2. Materials and methods

2.1. Excised skin and treatment

Foreskin specimens from children who underwent circumcision (provided by Guangzhou Children's Hospital) were used into 8 h after surgery. The tissues were cut into small pieces (8 mm × 8 mm) and then transferred to 0.9 ml fresh DMEM medium (Gibco, U.S.) supplemented with 10% FBS. The specimens were placed epidermal side up and exposed to SLS (Sigma Aldrich, U.S.), UA (Alfa, Spain), PH (Alfa, Spain) and 3,3′-dithiodipropionic acid (DA, Sigma Aldrich, U.S.). Liquids (25 μl) were applied with a micropipette, and a nylon mesh (8-mm diameter) was placed over the surface of the tissue. Solids (25 mg) were applied with a small spoon and wetted with 25 μl sterile water. After 15 min of exposure, each tissue was carefully rinsed with Ca^{2+} and Mg^{2+}-free PBS in a soft stream for 10 times. Subsequently, the tissue was completely submerged and shaken three times in 150 ml PBS, and finally, again rinsed once with a stream of PBS. The samples were cultured in air–liquid interface for another 24 h at 37 °C in a 5% CO_2 incubator and collected in 0, 2, 6, 12 and 24 h for the next detection (time point after exposure for 15 min was set as 0 h).

2.2. Two-dimensional gel electrophoresis, image analysis and mass spectrometry peptide sequencing

Total proteins of the skin tissues after exposure to SLS for another 24 h were extracted and the concentration was detected by BCA method. Two-dimensional gel electrophoresis, image analysis and mass spectrometry peptide sequencing >were performed by following the procedures described in the report of Ge et al. (2009).

2.3. Western blot analysis

All the protein samples were electrophoresed in a 12% SLS-polyacrylamide gel and electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked for 1–2 h at room temperature and then incubated at 4 °C overnight with goat anti-human HSP27 antibody (Santa Cruz, CA) in 1:2000 and rabbit anti-human GAPDH antibody (Santa Cruz, CA) in 1:1000. After incubation with the corresponding horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich, U.S.) for 2 h at room temperature, the membranes were detected with SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, U.S.).

2.4. Construction of the reconstructed organotypic skin model (ROSM)

Normal keratinocytes and fibroblasts were prepared from foreskin specimens of children who underwent circumcision (provided by Guangzhou Children's Hospital). Each skin graft was treated overnight with the solution of 0.25% Dispase II (Neutral protease, grade II, Sigma-Aldrich, U.S.) at 4 °C to separate epidermis from dermis. For keratinocytes: the epidermis was digested in the solution of 0.25% trypsin-0.02% EDTA for 10 min; the cells suspension was filtered through a 200 mesh stainless steel mesh filter and cells were cultured in Keratinocyte-SFM (Serum-Free Keratinocyte Medium for Culture of Human Keratinocytes, Gibco, U.S.). For fibroblasts: the dermis was dissected into small pieces and the monolayers were obtained following the method as described by Matzner et al. (1986).

Human amniotic membrane (AM) was obtained from placenta at cesarean section. Under sterile conditions, amnion was separated from chorion, and washed with PBS supplemented with a variety of antibiotics (100 U/ml penicillin, 100 U/ml streptomycin and 100 μg/ml gentamicin) for several times. After cut into pieces (5 cm × 5 cm), AM was put into 1% Triton X-100 solution and shook for 24 h at normal temperature. The epithelial cells were removed from the AM by incubation in the solution of 0.25% trypsin-0.02% EDTA at 37 °C for 2 h and then AM was gentle scraped with a cell scraper under a microscope. Before use, the AM was thawed, washed three times with PBS and stored in −80 °C. The complete removal of the epithelial cells was confirmed by hematoxylin and eosin (HE) staining.