Toxicology in Vitro 27 (2013) 1127–1134

Contents lists available at SciVerse ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

NCTC 2544 and IL-18 production: A tool for the identification of contact allergens

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ARTICLE INFO

Article history:
Available online 9 October 2012

Keywords:
Allergic contact dermatitis
Keratinocytes
Cytokines
Immunotoxicology
In vitro toxicology
Alternative method

ABSTRACT

Progress in understanding the mechanisms of skin sensitization, provides us with the opportunity to develop in vitro tests as an alternative to in vivo sensitization testing.

Keratinocytes play a key role in all phases of skin sensitization. We have recently identified interleukin-18 (IL-18) production in keratinocyte as a potentially useful endpoint for determination of contact sensitization potential of low molecular weight chemicals. The aim of the present article is to further exploit the performance of the NCTC 2544 assay.

NCTC 2544 is a commercially available skin epithelial-like cell line originating from normal human skin, which posses a good expression of cytochrome P450-dependent enzymatic activities. Cells were exposed to contact allergens (2-bromo-2-bromomethyl glutaronitrile, cinnamaldehyde, citral, diethylmaleate, dinitrochlorobenzene, glyoxal, 2-mercaptobenzothiazole, nickel sulfate, 4-nitrobenzylbromide, oxazolone, penicillin G, resorcinol, tetramethylthiuram disulfide), to pre- pro-haptens (cinnamyl alcohol, eugenol, isoeugenol, *p*-phenylediamine), to respiratory allergens (ammonium hexachloroplatinate, diphenylmethane diisocyanate, glutaraldehyde, hexamethylenediisocyanate, maleic anhydride, trimellitic anhydride) and to irritants (benzaldehyde, cholorobenzene, diethylphtalate, hydrobenzoic acid, lactic acid, octanoic acid, phenol, salicylic acid, sodium lauryl sulphate, sulfamic acid). Cell associated IL-18 was evaluated 24 later by ELISA. At not-cytotoxic concentrations (cell viability higher of 80%, as assessed by MTT reduction assay), all contact sensitizers, including pre-pro-haptens, induced a dose-related increase in IL-18, whereas both irritants, with the exception of sulfamic acid, and respiratory allergens failed. A total of 33 chemicals were tested, with an overall accuracy of 97%.

Overall, results obtained indicated that cell-associated IL-18 might provide an in vitro tool for identification and discrimination of contact vs. respiratory allergens and/or irritants.

1. Introduction

At present, the identification of potential sensitization of chemicals relies on animal models, such as the guinea pig maximization test (Magnusson and Kligman, 1969) and the local lymph node assay (Kimber et al., 1995). However, in the screening of new chemicals, it should be very important both from ethical, safety and economic points of view to have biological markers to discriminate allergy and irritation events without the use of animals. Furthermore, due to the new European policy on chemicals (REACH) and the EU cosmetic directive, in vitro methods are likely to play a major role in the near future. Over the last decade, several in vitro methods have been proposed to identify the potential of chemicals to induce skin sensitization (reviewed by Corsini and Roggen, 2009; Galbiati et al., 2010), but no accepted in vitro method for the identification of sensitizing chemicals are yet available.

The NCTC 2544 assay, based on the selective induction of IL-18 in human keratinocytes by contact allergens, was developed within the SENS-IT-IV project sponsored by the European Union. The assay proved to be useful in the identification and discrimination of contact allergens from respiratory sensitizers and irritants (Corsini et al., 2009; Galbiati et al., 2011).

Skin sensitization is an immune mediated reaction to low molecular weight exogenous chemicals. Both skin resident cells, such as keratinocytes (KC), Langerhans cells (LC) and mast cells, and immigrating leucocytes, including T lymphocytes and natural killer cells, actively participate in contact allergy (Cavani et al., 2007).

KC were chosen as they play a role in all phases of allergic contact dermatitis, from the early initiation phase with the elaboration of inflammatory cytokines, that plays a role in LC migration, and T-cell trafficking, through the height of the inflammatory phase

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http://dx.doi.org/10.1016/j.tiv.2012.05.018

with direct interactions with epidermotrophic T-cells, through the resolution phase of allergic contact dermatitis with the production of anti-inflammatory cytokines and tolerogenic antigen presentation to effector T-cells (Gober and Gaspari, 2008). KC sense haptens, and in turn initiate a program of enhances or de novo expression of inflammatory molecules representing the starting point of primary inflammation.

IL-18, formerly known as IFN-γ-inducing factor (IGIF), which belongs to the IL-1 cytokine family, is a potent inducer of IFN-γ by activated T cells (Okamura et al., 1995). We had focused our attention on IL-18 since this cytokine has been shown to play a key proximal role in the induction of allergic contact sensitization and to favor Th-1 type immune response by enhancing the secretion of pro-inflammatory mediators such as TNF-α, IL-8 and IFN-γ (Okamura et al., 1995; Cumberbatch et al., 2001; Antonopoulos et al., 2008). IL-18 has no apparent role in irritant contact dermatitis (Antonopoulos et al., 2008), indicating that the role of IL-18 in contact hypersensitivity is not simply part of a general requirement for IL-18 in skin inflammation. Furthermore, Hartwig et al. (2008) demonstrated using IL-18-deficient mice that the absence of IL-18 does not affect any of the asthma-specific parameters, including allergen-specific IgE, histological changes of the lungs, infiltrating leukocytes, serum cytokine levels and airway hyperresponsiveness. Human keratinocytes constitutively express IL-18 mRNA and protein (Naik et al., 1999), and works published by Naik et al. (1999) and Van Och et al. (2005) showed the induction of IL-18 following exposure to contact sensitizers.

In the context of the sensitization process, the NCTC 2544 assay was designed to cover the initial phase 'local trauma – proinflammatory cytokine production (danger signals)', necessary for maturation and migration of dendritic cells, and T cells activation. Haptens, acting as danger signals, stress the cells, stimulate the assembly of the inflammasome and NF-κB activation, resulting in IL-18 neosynthesis and release.

The aim of the present article was to further exploit the performance of the NCTC 2544 assay. Overall, our data suggests that IL-18 represents a promising marker for the screening of potential contact allergens. Its technical simplicity and the good predictivity may make this assay a candidate for rapid validation studies.

2. Materials and methods

2.1. Chemicals

As respiratory allergens the following chemicals were used diphenylmethane diisocyanate (MDI), trimellitic anhydride (TMA), ammonium hexachloroplatinate (HClPt), glutaraldehyde, hexamethylenediisocyanate (HMD), maleic anhydride. As skin sensitizers 2-bromo-2-bromomethyl glutaronitrile, cinnamaldehyde, citral, diethylmaleate, dinitrochlorobenzene (DNCB), glyoxal, 2-mercaptobenzothiazole, nickel sulfate, 4-nitrobenzylbromide, oxazolone, penicillin G, resorcinol, tetramethylthiuram disulfide (TMTD), as pre- prohaptens cinnamyl alcohol, eugenol, isoeugenol, *p*-phenylediamine (PPD) and, as irritants benzaldehyde, cholorobenzene, diethylphtalate, hydrobenzoic acid, lactic acid, octanoic acid, phenol, salicylic acid (Sal. Ac.), sodium lauryl sulphate (SLS), sulfamic acid were tested. Glycerol was used as negative compound. The SENS-IT-IV program mainly dictated the choice of chemicals. Chemicals have been selected by a dedicated workpackage as relevant and representative of the 'universe' of irritants, respiratory and contact allergens. All reagents were purchased from Sigma (St. Louis, MO, USA) at the highest purity available. All chemicals with the exception of HClPt, glyoxal, nickel sulfate, glycerol, and SLS dissolved in PBS, were dissolved in DMSO (0.1% final concentration). Cell culture media and all supplements were from Sigma. Solutions were freshly prepared for each experiment. The maximum concentration tested was 1000 μg/ml.

2.2. Cell culture

For experiments using NCTC 2544 cells (Institute Zooprofilattico di Brescia, Brescia, Italy), cells were cultured in 24-well plate at a cell density of $1.5–2.5 \times 10^5$/ml (0.5 ml/well). Cells were treated in 0.5 ml of RPMI 1640 containing 2 mM L-glutamine, 0.1 mg/mL streptomycin, 100 IU/mL penicillin, supplemented with 10% heated-inactivated foetal calf serum (media) and cultured at 37 °C in 5% CO_2 for 24 h.

2.3. Cell viability

Prior to study the effects of the selected chemicals on IL-18 production, their cytotoxicity was assessed by the MTT test (Gerlier and Thomasset, 1986). One hundred microliter of cells ($1.5–2.5 \times 10^5$/ml) were seeded in a 96-well plate and treated with increasing concentrations of the test chemical or DMSO as vehicle control (0.2% final concentration). After an incubation period of 24 h, medium was discarded and 100 μl/well of MTT solution 0.75 mg/ml in culture medium was added. Cells were incubated for 3 h at 37 °C, medium was discarded and cells lysed in 100 μl/well of a mixture of HCl 1N:isopropanol (1:24). The absorbance of the resulting solutions was read at a wavelength of 595 nm in a microplate reader (Molecular Devices). Eighty percent cell viability (CV80) was calculated for each chemical by linear regression analysis of data.

2.4. Cytokine production

For IL-18 production cells were incubated with increasing concentrations of the selected chemicals, or DMSO as vehicle control (0.2% final concentration). CV80 was the highest concentration tested. PPD 60 μg/ml was used as positive control. After incubation, culture medium was discarded, monolayers gently washed once with 1 ml of PBS and cells lysed in 0.25 ml of 0.5% Triton X-100 in PBS. Plates were stored at −80 °C until measurement. Intracellular IL-18 content was assessed by specific sandwich ELISA commercially available (MBL, Nagoya, Japan). Results are expressed in pg/mg of total intracellular protein content. The protein content of the cell lysate was determined by the BCA method.

The following PREDICTION MODEL was used: if the fold increases in intracellular IL-18 is $\geqslant 1.2$ and the increase in IL-18 is statistically significant from vehicle treated cells (Dunnett multiple comparisons test) the chemical is classified as contact sensitizer (R43, risk 43: may cause sensitisation by skin contact, as defined in Annex III of European Union Directive 67/548/EEC). If the fold increase in intracellular IL-18 is <1.2 and there is no statistical significance the chemical is classified as non-contact sensitizer. The positive control is included in each plate. The positive control meets the acceptance if the fold increase in intracellular IL-18 is >1.4 compared to vehicle treated cells (if the tested chemical is dissolved in PBS or water, the relative control cells for PPD will be the same control cells used for the chemical even if PPD stock solution is dissolved in DMSO. The intracellular IL-18 content of naïve cells or treated with DMSO is very similar). For a given chemical, the same classification must be obtained in at least two out of three independent experiments. The 1.2-fold increase is meant for at least one of the concentrations tested. In Table 1 is reported the effective concentration resulting in 1.2-fold induction in intracellular IL-18 (EC1.2), as assessed by linear regression analysis of data for the allergens tested.