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Peptide reactivity assay using spectrophotometric method for high-throughput screening of skin sensitization potential of chemical haptens

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ABSTRACT

Haptens must react with cellular proteins to be recognized by antigen presenting cells. Therefore, monitoring reactivity of chemicals with peptide/protein has been considered an *in vitro* skin sensitization testing method. The reactivity of peptides with chemicals (peptide reactivity) has usually been monitored by chromatographic methods like HPLC or LC/MS, which are robust tools for monitoring common chemical reactions but are rather expensive and time consuming. Here, we examined the possibility of using spectrophotometric methods to monitor peptide reactivity. Two synthetic peptides, Ac-RWAACAA and Ac-RWAAKAA, were reacted with 48 chemicals (34 sensitizers and 14 non-sensitizers). Peptide reactivity was measured by monitoring unreacted peptides with UV–Vis spectrophotometer using 5,5′-dithiobis-2-nitrobenzoic acid as a detection reagent for the free thiol group of cysteine-containing peptide or fluorometer using fluorescamine™ as a detection reagent for the free amine group of lysine-containing peptide. Chemicals were categorized as sensitizers when they induced more than 10% depletion of cysteine-containing peptide or 20% depletion of lysine-containing peptide. The sensitivity, specificity, and accuracy of this method were 82.4%, 85.7%, and 83.3%, respectively. These results demonstrate that spectrophotometric methods can be easy, fast, and high-throughput screening tools for the prediction of the skin sensitization potential of chemical haptens.

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1. Introduction

Evaluation of the skin sensitization potential is an important part of the safety assessment of new ingredients in cosmetics and topical drugs. For many years, identification of the potential skin sensitization hazard and assessment of relative skin sensitizing potency of haptens have relied on *in vivo* methods such as the guinea pig maximization test (GPMT) (Magnusson and Kligman, 1969), the Buehler occluded patch test (BT) (Buehler, 1965) and the local lymph node assay (LLNA) (Kimber and Basketter, 1992). GPMT and BT have been considered the most appropriate skin sensitization test methods (Gerberick et al., 2000; Kimber and Dearman, 2002). Due to the restrictions of animal testing for cosmetics and its ingredients and the 3R guidance, which was developed to reduce animal abuse, several alternative test methods have been developed (EC, 2003; EEC, 1986).

The LLNA is an alternative method to traditional guinea pig methods and relies on measurement of lymphocyte proliferation in the draining lymph nodes (Kimber and Basketter, 1992). In comparison with guinea pig methods, LLNA achieves a similar degree of sensitivity and specificity in terms of hazard identification (Dean et al., 2001), but uses a reduced number of animals for a shorter test period and thus provides important animal welfare benefits. However, this method still requires animals for testing and cannot satisfy the demand for full replacement of animals. Major efforts are now focused on the development of alternative test methods at the cellular and molecular levels for full replacement of animals (Bauch et al., 2011; Martin et al., 2010).

Langerhans cells (LCs) are cutaneous, immature dendritic cells (DCs). They recognize, internalize and process hapten–protein conjugates and then migrate to the regional lymph nodes through afferent lymphatics to present the antigen to T-lymphocytes and to trigger allergen-specific T-cell responses. During migration, they differentiate into mature DCs and express various regulatory cytokines and cell-surface maturation biomarkers. Many research groups, including our own, have developed different *in vitro* skin sensitization test methods using human myeloid cell lines as source of DC-like cells and monitoring expression of the cytokines or the cell-surface maturation biomarkers such as CD86, CD54, CD40, MIP-1β, IL-8 or IL-1β [e.g., U937 (Sakaguchi et al., 2006), KG-1 (Hulette et al., 2002), THP-1 (Sakaguchi et al., 2006; Ashikaga et al., 2002, 2006; Yoshida et al., 2003; An et al., 2009; Lim et al., 2008), MUTZ-3 (Azam et al., 2006)].

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Chemical haptens/skin sensitizers are unable to directly induce skin sensitization. Protein haptenation, the formation of a stable adduct between the haptens and endogenous proteins and/or peptides in the skin, is a necessary step in the skin sensitization process [(Dupuis et al., 1980; Landsteiner and Jacobs, 1935; Patlewicz et al., 2001)]. In theory, almost any chemical that can make a stable hapten–protein conjugate can induce a skin sensitization. Therefore, the evaluation of the reactivity of chemicals with proteins or peptides can serve as an alternative *in vitro* test for skin sensitization (Divkovic et al., 2005; Gerberick et al., 2008). The reactivity of chemicals toward proteins and peptides has usually been measured by monitoring the depletion of a selected peptide in the presence of chemicals using liquid chromatography with ultraviolet (UV) detection (Gerberick et al., 2004, 2007) or by monitoring adduct formation between the peptide and the chemical using liquid chromatography–mass spectrometry (LC–MS) or nuclear magnetic resonance (NMR) (Ahlfors et al., 2005; Alvarez-Sanchez et al., 2004b; Nilsson et al., 2005; Aleksic et al., 2007, 2008). These chromatographic analysis-based methods are advantageous in that very small quantity of sample is needed for analysis (Alvarez-Sanchez et al., 2004a; Gerberick et al., 2007; Natsch et al., 2007). LC/MS- and NMR-based methods provide more detailed information than methods based on only chromatography, such as peptide depletion due to adduct formation between the peptide and the chemicals or dimerization or oxidation of the peptide. Although HPLC-, LC/MS-, and NMR-based analysis methods are robust tools for monitoring common chemical reactions and have the above-mentioned advantages, they are rather expensive and time-consuming. In contrast, spectrophotometric analysis is a conventional method to detect changes in colored samples, even with very small quantity. Previously a rapid and inexpensive spectrophotometric assay for determining the reactivity of chemicals to glutathione (GSH) was developed (Schultz et al., 2005). The free thiol group of GSH was monitored by derivatization of GSH with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB or Ellman's reagent) (Ellman, 1958) and subsequent spectrophotometric detection (Schultz et al., 2005). However, this spectrophotometric method for determining the reactivity of chemicals has not been widely used as an alternative test method, probably due to its limited applicability to only thiol reactive compounds.

In this study, we established spectrophotometric assay methods based on a 96-well plate platform to determine the reactivity of chemicals to the thiol group of a cysteine-containing peptide (cysteine peptide) and the amino group of a lysine-containing peptide (lysine peptide) and examined the possibility of using this method as an *in vitro* sensitization test. The combination of the two methods using each type of peptide achieved a high degree of sensitivity, specificity, and accuracy.

2. Materials and methods

2.1. Peptides

Peptides with generic peptide sequences AcRWAACAA and AcRWAAKAA were purchased from Peptron Co. (Daejeon, Korea) as >95% pure. These sequences were obtained by slight modification of the sequences reported by Gerberick et al. (2004).

2.2. Chemicals and materials

Thirty-four sensitizers and 14 non-sensitizers which are categorized based on their sensitization potential in human were used to react with two peptides and these materials are shown in Table 1. Most tested chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) except 2-aminophenol, 2-vinyl pyridine, octanoic acid, and 5-chloro-2-methyl-4-isothiazolin-3-one (MCI) (Fluka, Buchs, Switzerland), *n*-hexane (Daejung Reagent Chemical, Shiheung, Korea). Fluorescamine and methanol were purchased from Sigma–Aldrich (St. Louis, MO, USA) and DTNB was purchased from Interchim Inc. (San Pedro, CA, USA). Microtest™ 96-well ELISA plates and Clear and HTS MULTIWELL™ Storage 96-well plates (polypropylene) were purchased from BD Falcon™ (Franklin Lakes, NJ, USA), and µ CLEAR 96 well plates (black) were purchased from Greiner Bio-One™ (Frickenhausen, Germany).

3. Measurement of peptide depletion

3.1. Measurement of cysteine peptide depletion

Cysteine peptide stock solution (10 mM) was prepared by dissolving powdered peptide in dimethyl sulfoxide (DMSO) according to the manufacturer's protocol. Chemical stock solutions (20 mM) were prepared immediately before the experiment by solubilizing test chemicals in isopropanol, except nickel II sulfate heptahydrate and cobalt II sulfate in methanol, and potassium dichromate, ammonium persulfate, thimerosal, ammonium tetrachloroplatinate (II), zinc sulfate heptahydrate and sodium dodecyl sulfate (SDS) in 100 mM sodium phosphate buffer (pH 8.0, 1 mM EDTA).

Chemical reaction solutions were prepared by diluting chemical stock solutions to 2 mM with 100 mM sodium phosphate buffer (pH 8.0, 1 mM EDTA). Peptide reaction solution was prepared by diluting peptide stock solution to 400 µM with 100 mM sodium phosphate buffer (pH 8.0, 1 mM EDTA). Then, 90 µl of peptide reaction solution and 90 µl of chemical reaction solutions were allotted to the 96-well ELISA plate (Fig. 1a). This 96-well plate was incubated at room temperature for 24 h to allow any possible reaction between peptide and chemicals. After incubation, 20 µl of 10 mM DTNB solution solubilized in sodium phosphate buffer (pH 8.0) was added to each well of the plate and incubated for 3 min to achieve complete reaction between unreacted peptide and DTNB. Both before the addition of the DTNB solution and after the reaction between unreacted peptide and DTNB, the optical density (OD) was measured using UV–Vis spectrophotometer (SpectraMAX 190™, Molecular Devices, CA, USA, absorption wavelength: 412 nm) (Supplementary data 1). Three different controls were used in this experiment: chemicals only (Fig. 1b), peptide only (Fig. 1c), and solvents only (Fig. 1d). For the chemicals only control, 90 µl of chemical reaction solutions and 90 µl of peptide solvent [4% DMSO in 100 mM sodium phosphate buffer (pH 8.0, 1 mM EDTA)] were added to wells instead of peptide reaction solution (Fig. 1b). For peptide only control, 90 µl of peptide reaction solution and 90 µl of the chemical solvent [10% isopropanol or 10% methanol in 100 mM sodium phosphate buffer (pH 8.0, 1 mM EDTA) or just phosphate buffer, depending on the solvent of the original chemical stock solutions] were added to each well instead of chemical reaction solution (Fig. 1c). For the solvents only control, 90 µl of the solvent of peptide reaction solution and 90 µl of the solvent of chemical reaction solution were added to each well instead of peptide reaction solution and chemical reaction solution, respectively (Fig. 1d).

3.2. Measurement of lysine peptide depletion

On the basis of the manufacturer's protocol, lysine peptide stock solution (100 mM) was prepared in 100 mM sodium phosphate buffer (pH 10.0, 1 mM EDTA).

Chemical stock solutions were prepared as described above. Chemical reaction solutions were prepared by diluting chemical stock solutions to 2 mM with isopropanol. Peptide reaction solution was prepared by diluting peptide stock solution to 200 µM

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