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Determination of Protein Haptenation by Chemical Sensitizers Within the Complexity of the Human Skin Proteome

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ABSTRACT

Skin sensitization associated with the development of allergic contact dermatitis occurs via a number of specific key events at the cellular level. The molecular initiating event (MIE), the first in the sequence of these events, occurs after exposure of the skin to an electrophilic chemical, causing the irreversible haptenation of proteins within skin. Characterization of this MIE is a key step in elucidating the skin sensitization adverse outcome pathway and is essential to providing parameters for mathematical models to predict the capacity of a chemical to cause sensitization. As a first step to addressing this challenge, we have exposed complex protein lysates from a keratinocyte cell line and human skin tissue with a range of well characterized sensitizers, including dinitrochlorobenzene, 5-chloro-2-methylisothiazol-3-one, cinnamaldehyde, and the non (or weak) sensitizer 6-methyl coumarin. Using a novel stable isotope labeling approach combined with ion mobility-assisted data independent mass spectrometry (HDMS[E]), we have characterized the haptenome for these sensitizers. Although a significant proportion of highly abundant proteins were haptenated, we also observed the haptenation of low abundant proteins by all 3 of the chemical sensitizers tested, indicating that within a complex protein background, protein abundance is not the sole determinant driving haptenation, highlighting a relationship to tertiary protein structure and the amino acid specificity of these chemical sensitizers and sensitizer potency.

Key words: cinnamaldehyde; DNCB; keratinocytes; MCI; DIA; HDMS[E]; proteomics; sensitization; skin.

Skin sensitization, which leads to the development of allergic contact dermatitis (ACD), is the most common manifestation of immunotoxicity found in humans. Approximately 15%–20% of people living in North America and Western Europe become sensitized to at least 1 contact allergen (Thyssen *et al.*, 2007) in an occupational or a domestic setting. Contact allergy occurs in 2 stages: first, the sensitization phase, whereby a chemical penetrates through the stratum corneum and reaches the viable epidermis, covalently modifying (haptenating) skin proteins, inducing the generation of allergen-specific T cells; second, the

elicitation phase in which re-exposure to the same (or cross-reactive) chemical allergen leads to a cascade of biochemical and cellular processes, effectively recalling the allergen-specific T cells to the exposure site, resulting in a clinical manifestation of ACD (Basketter *et al.*, 1995; Landsteiner and Jacobs, 1935; Lepoittevin, 2006). Our current understanding of the sequence of events involved in the development of sensitization and ACD is reflected in the more recent literature (Karlberg *et al.*, 2008; Koppes *et al.*, 2017; Martin, 2015) aspects of which are used in defining the adverse outcome pathway (AOP) for skin

sensitization (OECD, 2012). This framework links our existing knowledge of the direct molecular initiating event (MIE), the haptenation of proteins within skin, to the adverse outcome, ACD, via a number of specific key events at the cellular level (Ezendam et al., 2016; Vinken, 2013). Although a simplified view, the use of AOPs provides the basis for a mechanistic understanding of the effect of a chemical at the molecular and subcellular level. Through use of mathematical modeling, AOPs underpin the development and improvement of strategies for chemical and drug safety assessment (Burden et al., 2015; Maxwell and Mackay, 2008; Strickland et al., 2016). Characterization of the MIE, ie, skin protein haptenation, is a key step in understanding the skin sensitization AOP leading to more reliable mathematical models and their use in risk assessment (Jaworska et al., 2013; MacKay et al., 2013).

Most sensitizers are electrophilic in nature, or can easily be converted to an electrophile. As such, they are likely to react with nucleophilic side chains of protein amino acid residues, mainly lysine and cysteine, and to a lesser extent tyrosine, histidine and arginine (Ahlfors et al., 2003). The modification of proteins by chemical sensitizers is generally regarded as an irreversible reaction and, given the importance of this step to skin sensitization, has been studied extensively. To this end, a number of experimental approaches have been utilized to determine the reactivity of sensitizing chemicals and the reaction rates of chemicals with model nucleophiles. Previously, researchers studied chemical reactivity using nucleophilic chemicals analogous to side chains of nucleophilic amino acids (Alvarez-Sanchez et al., 2003; Chipinda et al., 2011; Sanderson et al., 2016) whereas others used simple short peptides with single or multiple nucleophilic amino acids as biological target surrogates (Aeby et al., 2010; Aleksic et al., 2009; Gerberick et al., 2004, 2007; Natsch and Gfeller, 2008; Roberts and Natsch, 2009). Further understanding was obtained using a variety of model proteins (Aleksic et al., 2007; Alvarez-Sanchez et al., 2004b; Parkinson et al., 2014). These studies define 3 main factors that determine the binding of sensitizers to nucleophiles: electrophilicity of the sensitizer, nucleophilicity of the target and steric constraints. However, these simple experimental systems used to determine protein haptenation differ from the complex milieu of skin in a number of ways, such as: competition for binding between the proteins present, differences in protein expression levels, differences in local pH, micro-bioavailability, and steric hindrance.

The identity and location of haptenated skin proteins is currently unknown, e.g. whether intra- or extracellular space or location within the membrane of a specific cell type may provide optimum conditions for haptenation. However, the epidermis and dermis are generally regarded as the skin sites where these modifications become available to the immune system (Kimber et al., 2011; Kimber and Dearman, 2003; Pickard et al., 2009).

The limited number of detailed investigations of protein haptenation in complex protein mixtures, including cell lines and tissues such as human skin, have so far focused on the use of antibodies to specific sensitizer adduct(s) (Elahi et al., 2004), biotin-tagged electrophiles (Codreanu et al., 2009; Hong et al., 2005), click chemistry (Jacobs and Marnett, 2010), derivatization of protein bound carbonyls and aldehydes with biotin hydrazides (Conrad et al., 2001; Mello et al., 2007; Shearn et al., 2016; Spiess et al., 2011) or dependent upon intrinsic features of certain sensitizers (such as fluorescent adducts of monobromobimane) to pinpoint the amino acid site of haptenation (Simonsson et al., 2011).

Identifying sites of protein modification in complex mixtures clearly represents a considerable analytical challenge and, to date, there is no globally applicable methodology. We have previously demonstrated an increased sensitivity in detecting haptenated peptides within the model protein human serum albumin (HSA) by combining a stable isotope labeling approach with data-independent acquisition mass spectrometry (MS) (Parkinson et al., 2014). This approach revealed more about the modification of HSA by a range of sensitizing chemicals than had previously been known. To further advance our understanding of the qualitative and quantitative aspects of skin protein haptenation, an assessment of protein modification by sensitizers within the complex skin proteome is required. To address this challenge, we have exposed protein lysates from a keratinocyte cell line and human skin tissue to the well characterized sensitizers, dinitrochlorobenzene (DNCB), 5-chloro-2-methylisothiazol-3-one (MCI), cinnamaldehyde (CA), and 6-methyl coumarin (6-MC), which has been classed as a nonsensitizer in the murine local lymph node assay (Ashby et al., 1995).

The results presented here demonstrate that in a complex protein mixture, protein abundance is not the sole determinant of protein haptenation. We observe a degree of specificity of some chemicals towards binding certain amino acid side chains and a possible effect of the protein tertiary structure on the likelihood of haptenation. Additionally, we observe a relationship between the extent of haptenation and sensitizer potency; however we refrain from making firm conclusions due to a low number of chemicals tested. Based upon these experimental data, we have highlighted useful parameters for advancing the development of *in silico* mathematical models of skin sensitization (Maxwell et al., 2014).

MATERIALS AND METHODS

Chemical Sensitizers
DNCB (99% purity; MW 202.55 Da) was obtained from Sigma-Aldrich (Poole, UK), and DNCB-D$_3$ (99% purity; MW 205.57 Da) was obtained from QMX Laboratories (Dunmow, UK).

Trans-CA (99% purity; MW 132.16 Da), Diphenylcyclopropenone (DPCP) (98% purity; MW 206.24 Da) and 6-MC (99% purity; MW 160.17 Da) were obtained from Sigma-Aldrich and trans-CA-D$_5$ (98% purity; MW 137.12 Da), DPCP-D$_{10}$ (97.1% purity; MW 211.27 Da) and 6-MC-^{3}D (99% purity; MW 163.15 Da) were custom synthesized by Quotient Amersham Radiochemicals (Irvine, California).

MCI (MW 149.60 Da) and ^{13}C labeled MCI (MW 150.8 Da) were synthesized and kindly donated by Prof Jean-Pierre Lepoittevin and Dr Elena Gimenez Arnau, Labaratoire de Dermatochimie, Strasbourg. Isotopically modified atoms for each chemical are shown in Table 1.
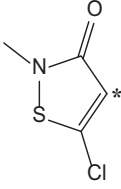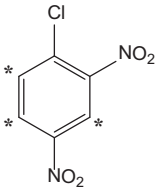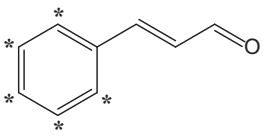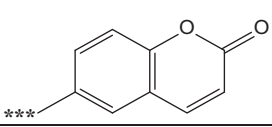
Collection of Human Skin Samples
Full-thickness human skin samples were obtained from mastectomy surgery at Southampton General Hospital with the patients' signed consent, under the guidelines stated in ethics protocol 07Q170459, snap frozen and stored at −80 °C.

Culturing of Keratinocyte Cells
The adherent keratinocyte cell line (HaCaT) was cultured in Dulbecco's Modified Eagle Medium, high glucose, (supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml Penicillin and

Table 1. Structures, Position of Stable Isotope, Potency Category (Including EC3 Value, as Derived From the Local Lymph Node Assay), Δ Mass (Da) Expected Following Haptenation, Potential Reactivity Domain, and Possible Amino Acid Residue for Modification, Based on Data Shown in Parkinson et al. (2014)

Chemical	Structure and Position of Stable Isotope Labels (*)	Potency Category (% EC3)	Δ Mass (Da) Expected for Unlabeled and (Labeled) Adduct	Potential Reactivity Domains	Residue
5-chloro-2-methyl-4-isothiazol-3-one (MCI)		Extreme[a] (0.0009)	+99.032[b] (+100.035)	Amide adduct[b]	Cys, Lys, His, Tyr[a]
			+112.9935[b] (+113.9965)	Addition-Elimination[b]	Cys, Lys, Tyr[a]
			+115.0092[b] (+116.0122)	Thioamide adduct[b]	Cys, Lys, His, Tyr[a]
1-chloro-2, 4-dinitrobenzene (DNCB)		Extreme[c] (0.05)	+166.0015[d] (+169.0195)	S_NAr[d]	Cys, His, Lys, Tyr[e]
Cinnamaldehyde (CA)		Moderate[f] (3.0)	+114.047 (+119.078)	Schiff base[g]	Arg, Lys[e]
			+132.0575 (+137.0885)	Michael adduct; acylation	Arg, Cys, His, Lys[e]
6-methylcoumarin (6-MC)		Nonsensitizer[e]	+158.0368 (+161.0548)	Michael adduct	Cys, Lys[e]
			+160.0525 (+163.0705)	Acylation	

[a]Parkinson et al. (2014).
[b]Alvarez-Sanchez et al. (2004a).
[c]Loveless et al. (1996).
[d]Aleksic et al. (2007).
[e]Aleksic et al. (2009).
[f]Basketter et al. (2001).
[g]Majeti and Suskind (1977).

100 µg/ml Streptomycin) (Gibco, ThermoFisher, Paisley, UK), at 37 °C and 5% CO_2. Once the cells had reached 70% confluency the media was removed and the cells washed twice with phosphate buffered saline (PBS). Cells were scraped into 10 ml of PBS and centrifuged for 5 min at 300 × g and the cell pellets stored at −80 °C until required.

Processing of Skin Tissue and Cell Line Pellets
Full-thickness skin tissue samples were thawed on ice, washed in Hanks' Buffered Saline Solution (HBSS, Gibco) and cut into pieces approximately 0.5 × 0.5 cm and placed into reinforced 1.5 ml tubes containing ceramic beads (Matrix D – QBioGene, Cambridge, UK) and 500 µl of lysis buffer (0.1% SDS in 0.1 M TEAB).

Cell line pellets were thawed on ice and transferred in a small volume of HBSS into tubes containing Matrix D and lysis buffer to a final concentration of 0.1% SDS in 0.1 M TEAB as described earlier.

The skin samples and cell pellets were then processed in the same way using a FastPrep macerator (MP Biomedical, Fisher Scientific, Loughborough, UK) for 5 cycles of 45 s; speed setting 6, chilled on ice for 1 min between cycles. Insoluble material was pelleted by centrifugation at 9000 × g for 5 min and discarded and the supernatant stored at −80 °C until required.

Estimation of Protein Concentration
The protein concentrations of the lysates generated were determined using the bicinchoninic acid method (Smith et al., 1985; Wiechelman et al., 1988) using a kit from Sigma-Aldrich.

Protein Modification With Sensitizers
To investigate the differences in protein haptenation with a range of chemicals, stock solutions of sensitizers were prepared in 100% DMSO (for DNCB) or 100% ethanol (for DNCB, CA, MCI and 6-methyl-coumarin) containing 50%, by molar concentration, of unlabeled sensitizer and 50% stable isotope labeled sensitizer (see Table 1). Lysates of keratinocyte cells or ex vivo skin were diluted to a concentration of 1 mg/ml in 0.1 M TEAB (pH 8.0) + 0.1% SDS prior to treatment with a 1:100 molar excess of sensitizer to protein and incubated at 37 °C for 4 weeks. The molarities of the protein lysates were approximated based upon the average molecular weight (66 kDa) of proteins within the

samples. Control samples were prepared at the same concentration in 0.1 M TEAB with the addition of 0.2% of the relevant solvent.

Sample Cleanup and Digestion

Proteins were precipitated using an adapted Bligh Dyer method (Bligh and Dyer, 1959). To 100 μg of modified protein lysate, 4 volumes of methanol were added and the sample vortexed. One volume of chloroform was added to the sample/methanol solution and vortexed before finally adding 3 volumes of water followed by vortexing. The sample was centrifuged at 20 000 × g for 1 min, focusing the proteins between the organic and inorganic phases. The aqueous phase was removed and 4 volumes of ethanol were added, followed by a short vortex. The precipitate was pelleted by centrifugation at 20 000 × g for 2 min, the ethanol removed, and the pellet air-dried.

The pellet was resolubilized in buffer containing 6 M urea, 2 M thiourea and 10 mM HEPES, pH 7.5. Proteins were reduced with dithiothreitol for 1 h at 60 °C, alkylated with 5.5 mM iodoacetamide for 45 min in the dark at room temperature, and then digested for 4 h with the protease Lys-C (Thermo Pierce, Loughborough, UK) (1/50 w/w). Peptides were then diluted 4 times with 20 mM ammonium bicarbonate and further digested using sequencing grade modified trypsin (1/50 w/w, Promega, Southampton, UK) overnight at 37 °C.

Fractionation of Peptides

To increase proteome coverage of the haptenated peptide digests, the samples were separated into 12 fractions based on their isoelectric points. This was performed using the Agilent 3100 OFFGEL Fractionator in combination with 13 cm Immobiline IPG strips, pH 3-10. 100 μg peptide samples were made up to a final volume of 1.4 ml with a 1:50 solution of IPG buffer, pH 3-10 (GE Life Sciences, Buckinghamshire, UK) diluted in 5% Glycerol. Peptides were focused for 20 kVh at a maximum current of 50 μA and a maximum power of 200 mW.

Each fraction was collected and acidified by adding 10 μl of solvent containing 10% TFA. Each acidified fraction was loaded onto a conditioned C_{18} reverse-phase Empore Plates (3 M, Maplewood, Minnesota, USA), and washed with 20 μl of 0.5% acetic acid. Peptides were eluted from the tip using 40 μl of 80% acetonitrile + 0.5% acetic acid. Samples were lyophilized using a vacuum concentrator to 6 μl and mixed with 6 μl of 2% acetonitrile + 1% TFA.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Analysis

In duplicate, 10 μl of the fractionated sample was loaded onto a reverse phase trap column (Symmetry C18, 5 μm, 180 μm × 20 mm, Waters Corporation, Milford, Massachusetts, USA), at a trapping rate of 5 μl/min and washed for 10 min with buffer A prior to the analytical nanoscale LC separation using a C18 reversed phase column (HSS T3, 1.8 μm, 200 mm × 75 μm, Waters, Wilmslow, UK). The eluted peptides were fractionated over a 90 min linear gradient from 1% acetonitrile + 0.1% formic acid to 60% acetonitrile + 0.1% formic acid, at a flow rate of 300 nl/min. Eluted samples were sprayed directly into a Synapt G2-S MS (Waters Corporation, Wilmslow, UK) operating in the data independent High Definition MS (HDMS^E) mode. Data were acquired from 50 to 2000 m/z using alternate low and high collision energy (CE) scans. Low CE was 5 V and elevated CE was ramped from 15 to 40 V. Ion mobility was implemented prior to fragmentation using a wave height of 650 m/s and wave velocity of 40 V. The lockmass Glu[1]-Fibrinopeptide B ((M + 2 H)$^{+2}$, m/z =

785.8426) was infused at a concentration of 100 fmol/μl at a flow rate of 250 nl/min and acquired every 60 s.

Database Searches

The raw mass spectra were processed using ProteinLynx Global Server (PLGS) 3.0 (Waters Corporation, Wilmslow, UK) to generate reduced charge state and deisotoped precursor and associated product ion peak lists. These peak lists were searched against the UniProt *Homo sapiens* sequence database (obtained from UniProt 03/2010). A maximum of 2 missed cleavages was allowed for tryptic digestion and the variable modification was set to contain oxidation of methionine, carboxyamidomethylation of cysteine and sensitizer-specific haptenation(s) as detailed in Table 1.

Precursor ion and product ion mass tolerances were calculated automatically during data processing and the allowed protein false discovery rate was set at 4%.

Data Filtering

Following database searching the data was filtered to eliminate falsely identified sensitizer modified peptides. Precursor ion peak pairs were extracted from the monoisotopic deconvoluted spectrum files generated from processing raw data using the data processing software based on the following criteria; ion pairs with a fixed mass difference, (corresponding to the number of stable isotopes incorporated into the labeled sensitizer), with similar ion intensity and according to retention time (with a retention time window of 1 min). Extracted peptide pairs were correlated with modified peptide masses identified after database searching using m/z and retention time. Extracted ion chromatograms of the filtered modified peptides were compared with those from the control samples to filter any remaining false positives. Fragmentation spectra were subsequently manually inspected and the amino acid site of modification determined, where possible.

Calculating Protein Abundance

Protein abundance was calculated based upon the method (Silva et al., 2006) where the sum of the intensity of the 3 most abundant peptides of an enolase digest standard (Waters Corporation, Wilmslow, UK) at a known concentration was used as a response factor to estimate the concentration of each protein in the samples based on the sum of the intensity of their 3 most intense peptide signals.

Calculating Nucleophile Content

The final nucleophile concentration for each protein was calculated as follows:

1. Protein amount in ng: Estimated protein abundance in ng was calculated as described above, normalized to total protein loaded in each MS run, and then averaged across all MS runs for either HaCaT or skin lysates.
2. Protein concentration in fmol: (ng protein/MW)*1000; where MW is protein molecular weight in kDa.
3. Nucleophile concentration: Sum of nucleophilic residues (excluding cysteine residues that are known to form disulfide bridges) * protein concentration (fmol).

RESULTS

Using a previously published dual labeling approach (Parkinson et al., 2014), HaCaT cell and human skin protein lysates were haptenated by DNCB, MCI, cinnamic aldehyde, and 6-MC, with a

100-fold molar excess of chemical to protein, for 4 weeks. By using an artificially high (nonclinical) concentration ratio as well as an extended exposure we hoped to ensure that all possible haptenation reactions would occur at levels that were detectable. This would firstly enable us to gain confidence in detecting these reactions in complex mixtures (i.e. inability to detect any haptenated sites at this level would render the method unsuitable for more clinically relevant experiments with lower concentrations and shorter exposures), and secondly help us to gain baseline data for future experiments where we would want to quantify the levels of haptenation. Haptenated samples were precipitated using chloroform/methanol to remove excess sensitizer prior to digestion with LysC and trypsin. The resulting peptides mixtures were fractionated via OFFGel Fractionation and each fraction was analyzed by MS. Due to the stochastic nature of peptide haptenation, modified peptides are present in low abundance (Figure 1). A ion-mobility assisted data-independent mode of acquisition (HDMS^E) in combination with a dual isotope labeling method was used to confidently identify haptenated peptides within these complex lysates.

Raw MS data were processed and searched against the UniProt *H. sapiens* sequence database using PLGS. Modified peptides were confirmed where a peptide signature consisting of 2 peptide isotope clusters of fixed Δ m/z were observed in an MS spectrum. The product ion spectrum of the modified precursor ion was subsequently manually inspected to determine the site of haptenation.
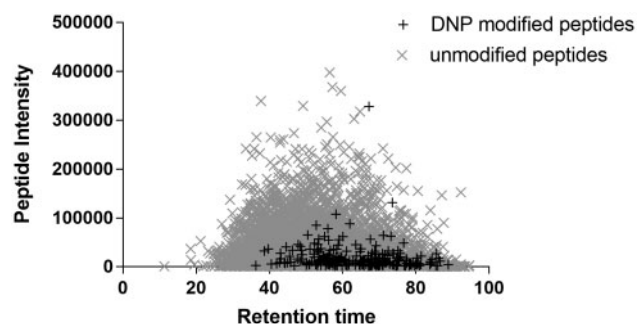

Figure 1. Distribution of ion intensity between unmodified and sensitizer modified peptides. Modified peptides are observed at lower ion intensity compared with unmodified peptides within the same sample.

A total of 7208 proteins (≥2 peptides) were identified across the 2 datasets, 6396 proteins in the keratinocyte lysates and 2423 in lysates from the skin tissue. From the total 7208 proteins identified, 400 proteins (5.5%) were modified by at least 1 chemical sensitizer. Analysis of gene ontology terms associated with the proteins identified exhibited a high level of similarity across both datasets with 70% of the proteins assigned in skin also identified in the keratinocyte cell lysates (Supplementary Figure 1). The proportion of modified proteins assigned within each dataset were equally similar demonstrating the utility of the keratinocyte cell line as a useful model for assessing global protein haptenation in skin tissue. In the keratinocyte cell lysates, 213 peptides related to 162 proteins (2.5% of keratinocyte cell proteome) were modified by DNCB; 204 peptides related to 159 proteins (2.5%) by MCI; and 85 peptides related to 71 proteins (1.1%) by CA. In the skin lysates, 66 peptides related to 43 proteins (1.8% of the skin proteome) were modified by DNCB; 41 peptides related to 30 proteins (1.2%) by MCI; and 50 peptides related to 41 proteins (1.7%) by CA. Only a single peptide was modified by 6-MC in the keratinocyte cell lysates and no 6-MC modifications were observed in the skin lysates (see Supplementary Table 1). Despite the high concentration of reactive chemicals used, prolonged incubation time and cell lysis/tissue maceration, the level of haptenation observed was relatively low.

Quantified proteins were ranked in order of abundance from the highest to least abundant to test the hypothesis that high abundant proteins were more likely to be modified because of a greater number of available modifiable sites (Figure 2). Although a large proportion of the haptenated proteins were from proteins present at high abundance, we observed the haptenation of proteins across the abundance range by DNCB, MCI, and CA, demonstrating that protein abundance is not the only single factor driving protein haptenation.

The total theoretical nucleophile concentrations within the HaCaT cell line and skin lysates were similar (19.0% of the keratinocyte and 18.6% of the skin proteome containing modifiable nucleophilic residues Lys, Arg, Cys, Tyr, and His). The percentage nucleophile content within each protein identified in both keratinocytes and skin samples was calculated and the proteins ranked according to their nucleophile concentration (high to low). Mapping of sensitizer modified proteins to this ranked list showed a correlation between protein modification and
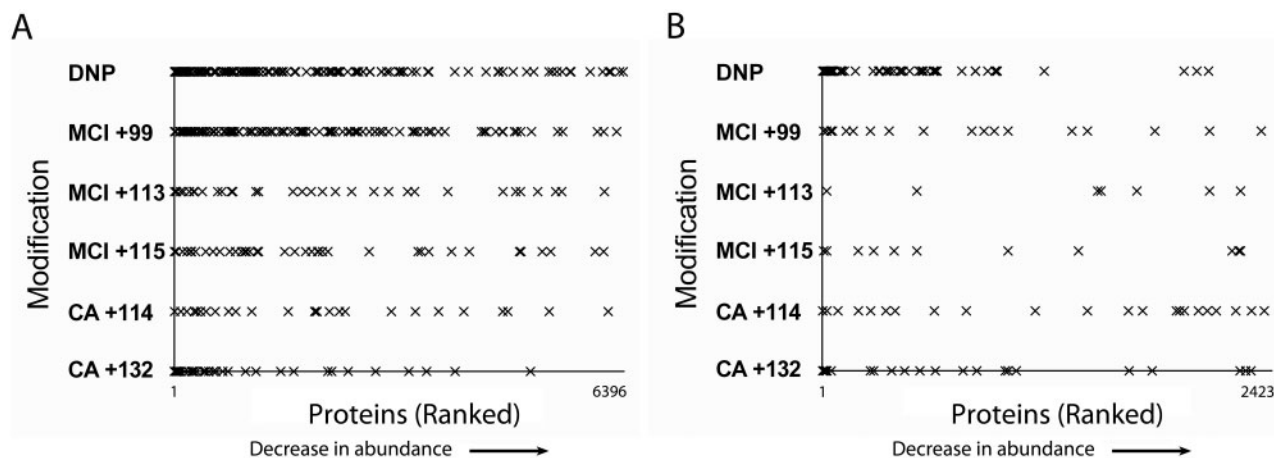

Figure 2. Graphical representation of all the modified proteins identified with the keratinocyte cell (A) and *ex vivo* skin (B) lysates, ranked by their abundance which was based on their estimated protein concentration (fmol). The x-axis represents all of the proteins identified in each lysate from most abundant to least; the y-axis shows which of those proteins were modified by each of the 3 chemical sensitizers tested in this study.
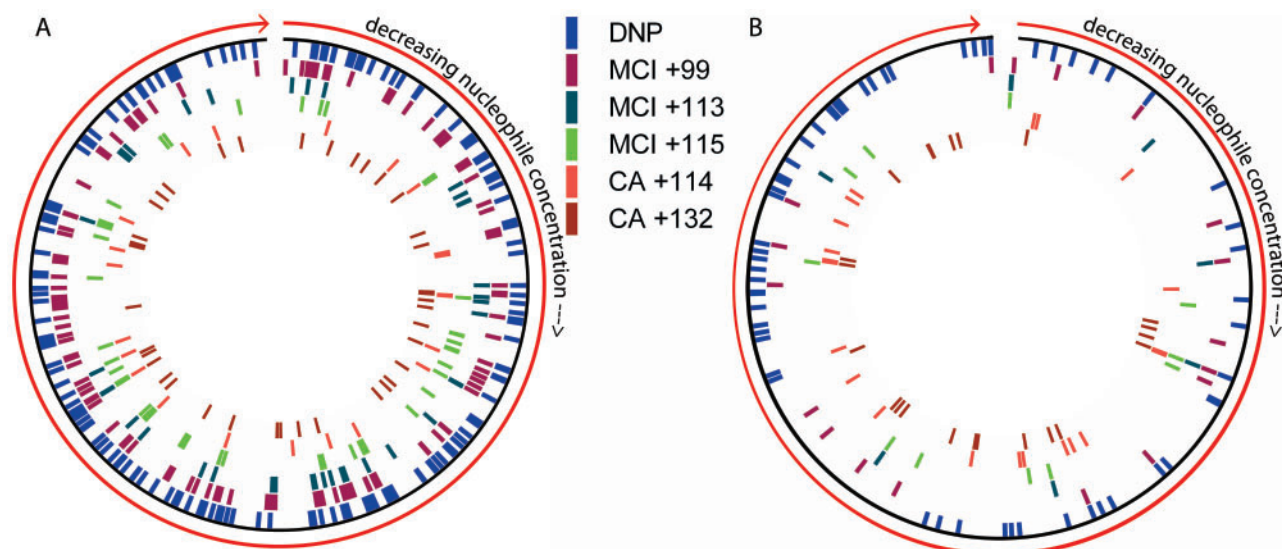
Figure 3. Circular representation of the nucleophilic concentration of each protein identified within the keratinocyte (A) and *ex vivo* skin (B) lysates, from highest concentration moving clockwise to the lowest concentration of nucleophiles. Proteins that were modified by each of the adducts observed in this study are highlighted within these circular representations, to show the distribution of nucleophile concentration amongst the haptenated proteins.
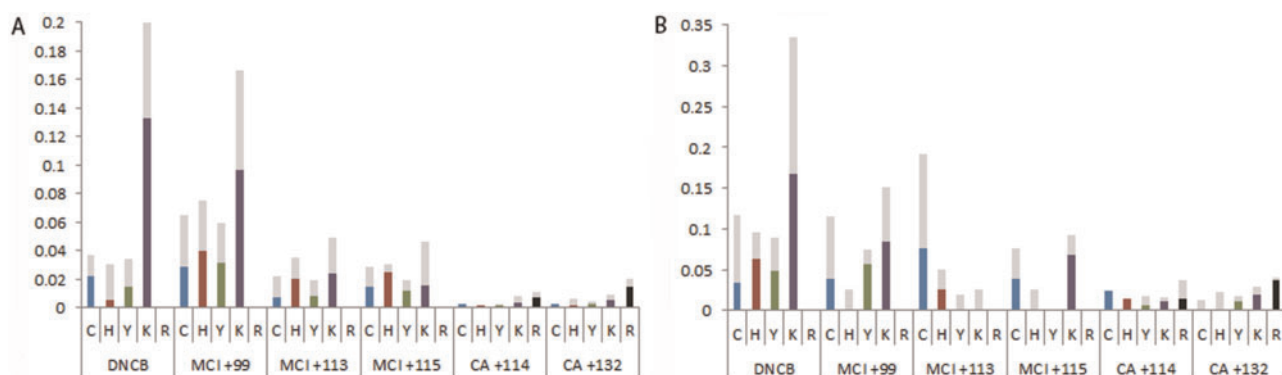

Figure 4. Percentage of available nucleophilic residues haptenated by adducts of DNCB, MCI, and CA in the keratinocyte (A) and *ex vivo* skin (B) lysates. Light Gray bars indicate where the exact site of sensitizer modification could not be confirmed from the fragmentation spectra.

nucleophile concentration within the HaCat cell lysates, but was not observed for the *ex vivo* skin lysate data (Figure 3).

Across the haptenated peptides identified (haptenome), a total of 252 amino acid residues in HaCaT cell lysates were haptenated by DNCB, 210 by MCI, 98 by CA and 1 by the non-sensitizing chemical 6-MC. In human skin lysates, 102 amino acid residues were haptenated by DNCB, 43 by MCI and 57 by CA. No confirmed haptenated amino acids were found for 6-MC. The percentage of haptenated residues observed for each sensitizer adduct as a proportion of the total number of each amino acid residue observed within each proteome were calculated (Figure 4). DNCB and the +99 adduct of MCI both modified the largest number of residues with a preference for lysine. The +113 and +115 adducts of MCI do not show any nucleophile preference, whilst CA appears to modify a higher percentage of arginine and lysine residues. The CA preference for formation of Schiff bases (in particular with arginine) was previously observed when using the model protein HSA (Parkinson *et al.*, 2014). This is particularly interesting since CA reactivity has mainly been studied from the basis that Michael addition reaction with protein thiols is dominant (Roberts *et al.*, 2007). For all chemicals tested, we detected a number of potential further

sites of haptenation where the exact site of haptenation could not be confirmed from the fragmentation spectra. These modifications are shown as gray within Figure 4.

Many proteins were found to be modified at more than 1 amino acid residue as well as by more than 1 sensitizer. The 20 proteins found to have the highest number of observed modifications are summarized in Supplementary Table 1, these included Complement C3 with 8 residues modified by DNCB alone; Prelamin-A/C (P02545) with 8 residues modified by DNCB, MCI, and CA; Heat shock cognate 71 (P11142) with 8 residues modified by DNCB and MCI; Pyruvate kinase (P14618) with 10 residues modified with DNCB, MCI and CA; and Serum Albumin (P02768) with 29 residues modified by all 3 chemicals. A summary of all the modified proteins can be found in the Supplementary Table 2.

DISCUSSION

Despite the prevalence of skin allergy, our knowledge about the process of protein haptenation, a key MIE, is limited. Current mechanistic knowledge of haptenation is derived from studies utilizing model peptides or isolated single proteins (Ahlfors

et al., 2003; Aleksic et al., 2007, 2008, 2009; Gerberick et al., 2007; Parkinson et al., 2014). Haptenation of a single protein (HSA) has also been shown to stimulate hapten-specific T-cell responses in a number of studies, such as the production of a stable HSA-penicillin G complex (Brander et al., 1995), the occurrence of DNP adducts after the modification with the extreme sensitizer 2, 4-dinitrobenzesulfoinc acid (Dietz et al., 2010) and via p-phenylenediamine modification of cysteine 34 on HSA (Jenkinson et al., 2010). Although useful in understanding the reactivity of a variety of chemicals, these studies do not provide any insights on haptenation within the milieu of the skin proteome. As a first important step towards understanding the complexity of haptenation in a complex protein mixture we have sought to identify sensitizer haptenated peptides in protein lysates of the HaCaT cells and human skin tissue. This was achieved using a novel approach combining isotopic labeling with the data independent MS acquisition method (HDMS[E]), which successfully pinpointed and identified low abundance haptenated proteins, initially in a single model protein (Parkinson et al, 2014) and now in these complex mixtures.

In total, 7208 proteins were identified in this study, 6396 proteins in keratinocyte cell lysates, and 2423 in lysates from skin tissue. The difference in the numbers of proteins identified likely reflects differences in the efficiency of protein extraction from a monolayer of cells versus whole skin tissue, albeit comparison of the gene ontology terms associated with the identified proteins across the datasets of both sample types are similar (Supplementary Figure 1). Moreover, percentages of proteins that were modified by a sensitizer within each sample type were comparable, and many proteins were found to be modified in both HaCat and ex vivo skin lysates (eg, Keratin's, Annexin A2, HSP90, Glutathione S transferase, alpha actinin). Although modified samples were analyzed in duplicate, the majority of modifications identified were mainly observed within a single replicate. Detected modified peptides were present at very low abundance with MS measurements close to the lower limits of detection resulting in increased missingness, challenging the repeatability of observed modified peptides across samples.

The underlying concept for the induction of sensitization is that a chemical must be able to covalently react with proteins, either directly or indirectly in skin. Based upon the data obtained in this study, there are clear indications that the previous assumption that only highly abundant proteins are likely to be modified preferentially (Hopkins et al, 2005) may not necessarily be correct. Although the majority of haptenated proteins identified within the keratinocyte cell lysates were highly abundant, the data indicates that low abundant proteins are also haptenated by all 3 of the chemical sensitizers tested. Equally, in skin lysates, where fewer haptenated proteins were identified overall, we found that both high- and low-abundant proteins were modified, indicating specificity in protein haptenation.

To further understand this specificity, and to provide useful parameters for building in silico mathematical models of sensitization, we investigated the relationship between protein modification and proteome nucleophile concentration, ie, whether the numbers of theoretically available reactive sites correlate with their likelihood of modification by a chemical sensitizer. For HaCat cell lysates, our data showed a correlation between the total protein nucleophile concentration and the number of proteins haptenated, whereas for ex vivo skin lysates, this was not evident. This difference is likely to be attributable to the greater proteome coverage obtained for HaCat cell lysates,

compared with ex vivo skin. Although we would expect to see more modifications at higher nucleophile concentrations, we also observed modifications of proteins at lower nucleophile concentration, which may reflect differences in the accessibility of nucleophilic residues to modification. Our data demonstrated a specificity for modification of certain nucleophilic residues over others between each of the different sensitizers tested. A similar finding was observed in the direct peptide reactivity assay, although the amino acid specificity in this study differs slightly (Aleksic et al., 2009). For example, after correcting for overall abundance of each residue within each dataset, our data show that DNCB binds predominantly to lysine residues and, to a much lesser extent, available cysteine residues, with very few tyrosine and histidine modifications observed. This is in contrast to the direct peptide reactivity assay, which showed almost 100% depletion of cysteine, lysine and tyrosine containing peptides. Although we believe the observed differences are more likely to be attributed to the effect of secondary and tertiary structure on protein modification as well as an overall high abundance of amines (Parkinson et al, 2014), it is possible that this is the result of an experimental artifact (decreased number of free thiols as a consequence of cysteine oxidation).

The complex reactivity of MCI obtained in this study was in agreement with previously published studies (Alvarez-Sanchez et al., 2003, 2004a,b; Parkinson et al., 2014) in terms of the nucleophile specificity.

Very strong bias for reaction with amines was also observed for CA. As already indicated, experimental Cys oxidation and high abundance of amines may be responsible, but there are additional indications from the literature that may explain this bias in case of CA. Although there is no direct evidence from these experiments, it is plausible that initial Michael addition of CA to thiols may be reversed. This would result first in a thiazolidine type product (making a cross-link between Cys and eg, Lys) followed ultimately by Schiff base adduct formation and release of the thiol originally conjugated to CA. These reactions were observed for similar compounds (α-β unsaturated aldehydes) by several authors (eg, Cai et al., 2009; Esterbauer et al., 1975; Jackson et al., 2016; Randall et al., 2013; Wlodek, 1988). These events are worthy of investigation, however, in light of the complexity of cell/tissue lysates, it would be technically challenging using the current experiments. We have also observed unusual adduct types of CA (+114) with Cys, His, and Tyr, however, we believe that these are an experimental artifact (hemiacetal type products, which lose water in the interaction with the ionized peptide backbone in the electrospray source of the mass spectrometer).

We identified 162 proteins that were haptenated by DNCB, 159 by MCI and 71 for CA. Nonetheless, these account for only approximately 2.5%, 2.5%, and 1.1% of the total protein content in HaCaT cell lysates, respectively. This low proportion of protein haptenation was unexpected, especially for extreme sensitizers such as DNCB and MCI and only further emphasizes the specificity in this initial event. Although the number of modifications was lower than expected, a relationship, albeit weak, between increased levels of protein haptenation and sensitizer potency (as indicated by published in vivo data) was observed. The total number of proteins modified by each of the sensitizers decreased with decreasing sensitizing potency (MCI > DNCB > CA > 6MC). This positive correlation between protein reactivity and the intensity of sensitization reactions is consistent with previous studies (Basketter et al., 1997; Godfrey and Baer, 1971; Roberts and Aptula, 2008; Roberts and Natsch, 2009).

It is important to highlight that these experiments are not directly representative of the protein haptenation that may occur in human skin following topical exposure to a reactive chemical. The data presented here are based upon experiments where the bioavailability aspect was not taken into consideration and is part of generating a baseline haptenome, ie, the skin relevant cells and human skin tissue were lysed prior to contact with study chemicals. However, the protein lysate samples were prepared in buffers containing 0.1% SDS, conditions where proteins are likely to be in their native state, (eg, where trypsin still maintains proteolytic activity), and may sterically influence the availability of nucleophiles for their modification (Gudiksen *et al.*, 2006). It is most likely that the realistic and physiologically relevant haptenation will be a subset of the modifications determined in this baseline study and is an important bridge between previous studies using a single model protein in isolation and the goal of physiologically relevant data.

We have demonstrated the applicability of this approach to provide a robust assessment of global protein haptenation within complex mixtures for a wide range of sensitizers and thereby bringing mechanistic insights into sensitizer reactivity. It is still unclear however whether protein reactivity, selectivity of binding for certain nucleophilic residues, the rate of the protein-binding reaction, or most likely a combination of all 3 provides the best correlate for sensitizer potency (Enoch and Roberts, 2013; Jaworska et al., 2013; Natsch and Gfeller, 2008; Patlewicz et al., 2007).

Having addressed the challenge of method sensitivity, we have the opportunity to investigate these research questions and generate new insights into the types and levels of haptenation closely relevant to human exposure. Further studies bringing a quantitative assessment of protein haptenation to a model cellular system, more relevant to *in vivo* skin, will be required. This could be achieved by using clinically relevant exposure scenarios in either a hapten-treated 3D cell model or by direct exposure of *ex vivo* skin tissue. In silico studies that explore and compare the microenvironment of the identified haptenated residues would also be of considerable value, in addition to understanding how the skin proteome responds at the cellular level after sensitizer exposure, particularly in individuals susceptible to skin allergy. However, the findings of this study clearly indicate that haptenation is more than just a statistical process (where all nucleophiles are equally likely to be modified) and that elements such as specificity may play a more important role.

Advancing our knowledge of the skin proteome, the protein targets of modification and the immunogenicity of these covalent protein modifications will ultimately enable us to better interpret reactivity data obtained from studies using model peptides enhancing our understanding of the skin sensitization AOP and its use in quantitative risk assessment. The methodology used within these studies is applicable beyond the investigation of skin sensitization, and has general utility for studying global protein haptenation events across a range of biological research areas, such as identifying drug-haptens in drug allergy.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

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