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Direct binding of sulfur mustard and chloroethyl ethyl sulphide to human cell membrane-associated proteins; implications for sulfur mustard pathology☆,☆☆

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ABSTRACT

Sulfur mustard (SM) is a potent vesicating agent that produces debilitating blisters and ulcerating lesions on the skin which are characteristically slow to heal. There are currently no specific medical countermeasures to prevent SM-induced vesication and therefore SM remains a major military threat. To investigate the mechanism by which SM causes these injuries we aimed to identify the cellular proteins that are important in the vesicant response and pathology of SM. Membrane and membrane-associated proteins that are targets for direct binding by SM were compared to targets directly bound by CEES (chloroethylethylsulphide). As CEES is a less potent blistering agent compared to SM, it was hypothesised that differences in the binding of these two mustards could reveal key proteins directly involved in the mustard vesicant response. Human cellular membranes fractionated from HaCaT cells were exposed to ^{14}C-SM or ^{14}C-CEES and the membrane proteins to which SM or CEES bound were separated by 2D gel electrophoresis, located by fluorography and subsequently identified using mass spectrometry. A number of proteins were identified that were differentially labelled by SM and CEES. Actin, annexin A2 and keratin 9 were labelled with SM at a higher intensity than was seen with the same concentration of CEES. Therefore results from these studies suggest that SM binding to these proteins could contribute to the complex pathology seen following SM exposure.

1. Introduction

Sulfur mustard (SM) is a potent vesicant (blistering agent) and it has been used as a chemical weapon in a number of conflicts, including World War One [1] and the more recent Iran–Iraq war [2]. SM exposure can severely affect the eye, respiratory tract and skin and can even lead to death [3]. Although much is known about the clinical effects after SM exposure, the mechanism by which SM injures cells is still unclear and this has led to a paucity of therapies.

SM alkylates a variety of naturally occurring functional groups within biologically important molecules, such as amino and sulphide groups [4]. Therefore SM will modify the majority of proteins, and this alteration or cross-linking may affect their function by varying degrees. On exposure to SM the skin epidermal–dermal layer becomes separated beneath the hemidesmosomes within the upper portion of the lamina lucida [4,5]. This break down of the basement membrane is associated with the formation of micro blisters that can gradually coalesce to form large, pendulous blisters which take days/weeks to heal. Other features of SM exposure include the build up of necrotic and apoptotic cells and a recruitment of inflammatory cells including neutrophils. Delayed cell migration has also been observed *in vitro* [6].

Although the pathology of SM-induced injuries takes hours to days to appear there will be immediate effects on the cell after SM exposure, which are likely to be at the molecular level. If these immediate effects could be reduced or controlled it would provide greater protection against SM. To date a number of proteins have been shown to aggregate or breakdown on SM exposure, e.g. various keratins [7–9], and laminin [10,11]. There has been little research looking at the direct interaction of SM with cell membrane-associated proteins. These proteins are likely to be involved in cellular attachment to, and interactions with, the extracellular matrix and therefore could be involved in the pathology of SM. SM could also bind to cell surface receptors on the membrane and subsequently alter signal transduction. The purpose of this work was to investigate the direct interaction of SM with cell membrane proteins extracted from immortalized human keratinocytes (HaCaT cells) and to compare this interaction with CEES, an analogue of SM with a much lower vesicant potential (see Fig. 1 for structures).

Two-dimensional electrophoresis combined with fluorography techniques were used to identify cell membrane proteins directly bound by radio-labelled (^{14}C) SM or radio-labelled (^{14}C) CEES. The

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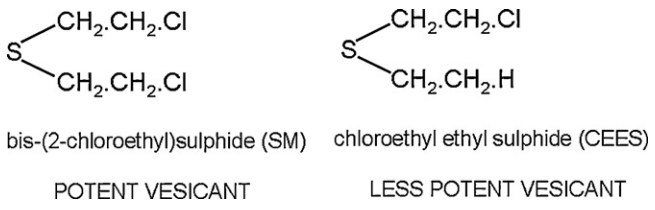

Fig. 1. Structure of sulfur mustard (bis-(2-chloroethyl)sulphide and chloroethyl ethyl sulphide (CEES), this study used radio-labelled molecules, each arm contained one C^{14} atom.

labelled proteins were then digested and analysed by liquid chromatography tandem mass spectrometry (LC–MS/MS).

The technique of two-dimensional electrophoresis has been used previously in the analysis of sulfur mustard adducts [9,12]. Dillman and Schlager used the technique to analyse changes in the cell proteome after SM exposure and saw increases in keratin aggregates and tubulins [12]. Recent work by Mol *et al.* labelled the whole cell proteome with ^{14}C-SM and identified a few proteins directly modified by SM including actin, stratifin (14-3-3σ) and galectin-7 [9]. Our work builds upon these findings by specifically labelling cell membrane/cell membrane-associated human keratinocyte proteins with either ^{14}C-SM or ^{14}C-CEES and comparing the binding profile to identify proteins that could be involved in SM pathogenesis.

We identified a number of proteins directly labelled by SM and also showed that the proteins bound by SM differed to the proteins bound by CEES. This has given us some insight into SM pathology and provided evidence that SM behaves differently to CEES.

2. Experimental

2.1. Chemicals and reagents

Antibodies against GAPDH, cadherin and DNA polymerase were purchased from Abcam, Cambridge, USA, and anti-IgG horse radish peroxidise (HRP) from Santa Cruz antibodies, Santa Cruz, USA. All other chemicals were purchased from Sigma–Aldrich Chemical Co. Ltd., Gillingham, UK unless otherwise stated. Proteinase inhibitors contained 104 mM AEBSF, 80 μM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A and 1.4 mM E-64. Radio-labelled SMs (^{14}C-SM or ^{14}C-CEES) were made by Dstl's chemistry group, specific activity 2.0 GBq/mmol (50% of the carbons were radio-labelled per molecule). HaCaT cells (immortalized human keratinocytes) [13] were provided by the Biomedical Research Centre, Dundee, UK.

2.2. Preparation of cell membrane proteins

Cell membrane samples were prepared as follows: HaCaT keratinocyte cells were grown to 80–90% confluency and then scraped off in PBS/proteinase inhibitors (15 μL/mL). The cells were homogenised and glucose added to a concentration of 0.32 M, this was centrifuged at 2500 × *g* to pellet the nuclear fraction (P1). The supernatant (S1) was diluted into PBS/proteinase inhibitors, centrifuged at 48,000 × *g* to give the supernatant (S2) and a cell membrane pellet. This pellet was resuspended in hypo-osmotic buffer (5 mM Tris, pH 7.5), freeze thawed to remove any cytoplasmic material trapped in sealed vesicles, centrifuged again at 48,000 × *g* and resuspended in PBS/proteinase inhibitors to give the cell membrane fraction (P2). The purity of the fractions was confirmed by Western blotting.

2.3. Labelling of cell membrane proteins with ^{14}C-SM or ^{14}C-CEES

Cell membrane proteins were labelled with varying concentrations of ^{14}C-SM or ^{14}C-CEES (0.3–4 mM) at 37 °C for 1 h, centrifuged at 48,000 × *g* to pellet the cell membranes and washed twice with PBS. Protein concentrations of 0.6 mg/mL protein labelled with 2–4 mM ^{14}C-SM gave the best incorporation (0.5–1% was incorporated). Proteins (450 μg) labelled with 4 mM ^{14}C-SM or ^{14}C-CEES were run on each 2D gel.

2.4. Gel electrophoresis and Western blotting

One-dimensional SDS PAGE and Western blotting were carried out as described in reference [14]. The cell fractions were diluted with Laemmli sample buffer (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) to the required protein concentration and then heated at 100 °C for 5 min. Electrophoresis of the cell preparations was carried out with the Bio-Rad Mini-PROTEAN® II electrophoresis system, and transblotting was performed with the Bio-Rad Mini Trans-Blot® electrophoretic transfer cell. Protein fractions were electrophoresed on 12% Tris–HCl Ready gels (Bio-Rad) and then transferred to nitrocellulose membrane. The nitrocellulose membranes were blocked with Tween buffer (0.1%, v/v Tween 20 in PBS) containing blotting grade non-fat dry milk powder (5%, w/v), washed and then incubated with primary antibodies (Abcam, Cambridge, UK) at the following dilutions: anti-GAPDH 1:5000, anti-Cadherin 1:1000 and anti-DNA polymerase 1:250, washed and then labelled with a goat anti-mouse HRP-conjugated IgG (Autogen Bioclear U.K. Ltd., Calne, UK) at a dilution of 1:500. The membranes were then washed and visualised by immersing in a stain comprised of 3,3′-diaminobenzidine tetrahydrochloride (0.8%, w/v), H_2O_2 (0.6%, v/v) in PBS for 8 min.

For 2D gels proteins were resuspended in 350 μL sample buffer (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3-10*, 40 mM Tris pH 7, 2 μM TBP* and 0.2% biolytes 3–10*(*Bio-Rad) and rehydrated with an IPG strip pH 3–10 (GE Healthcare, Chalfont St Giles, UK) overnight using the Multiphor II system. The strip was then equilibrated in buffer (8 M urea, 50 mM Tris pH 6.8, 30% glycerol, 2% SDS) plus 0.4% DTT for 30 min and then buffer plus 4.5% iodoacetamide for 30 min, rinsed and then run on a horizontal 12–14% ExcelGel (GE Healthcare) using a Multiphor II system. ^{14}C-labelled proteins were detected by fluorography (see below) and total proteins were detected by silver staining using SilverSNAP® stain (Perbio, Cramlington, UK). The methods described above are optimised conditions, for example, the reducing agent used in the sample buffer was changed to tributylphosphine (TBP) instead of dithiothreitol (DTT).

2.5. Detecting labelled proteins

The labelled proteins were separated on a 1D or 2D gel. The gels were then fixed (in 30% ethanol, 10% acetic acid aq.), incubated in Amplify™ fluorographic reagent (GE Healthcare) and placed in preserving solution (8.7% glycerol aq.) overnight. The following day the gels were incubated in Amplify™ fluorographic reagent again, dried in cellophane and then exposed to X-ray film at −80 °C for 1–3 weeks. The labelling was detected within a week on a 1D gel, whereas 2D gels were exposed for up to 5 weeks to provide an adequate signal.

Incubating the gels in Amplify™ fluorographic reagent before and after the preserving solution and then drying the gels in cellophane was found to greatly improve detection of the low levels of ^{14}C-SM-labelled or ^{14}C-CEES-labelled proteins. Using the same 2D gel for detection by both fluorography and silver staining allowed the X-ray film and silver stained gel to be overlaid exactly, using the markers on either side of the gel, ensuring the correct protein spots were excised for mass spectrometry analysis.

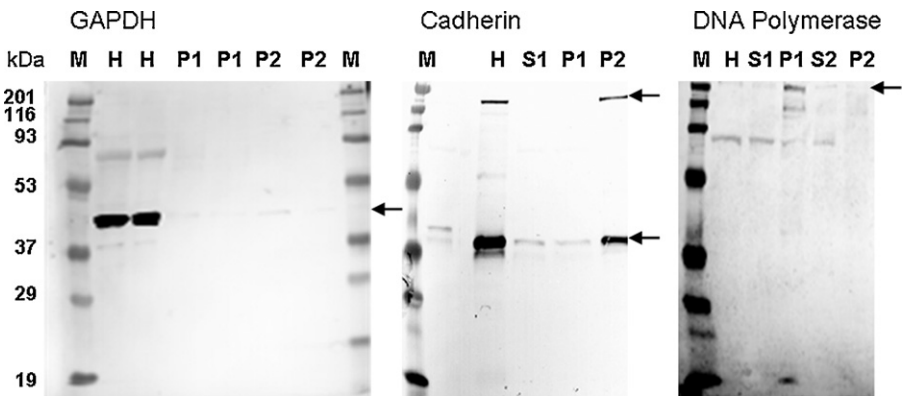

Fig. 2. Representative Western blots probing fractions from the cell membrane extraction. Molecular weight marker (M), with molecular weight in kDa shown on the left hand side, homogenate (H), supernatant from the first centrifugation (S1), nuclear fraction (P1), supernatant from the second centrifugation (S2) and cell membrane fraction (P2). Antibodies used were GAPDH to check for cytosolic contamination in other fractions, cadherin to check the membrane fraction and DNA polymerase epsilon to check the nuclear fraction, arrows indicate molecular weight of protein being probed. Proteins were loaded controlling for the volume of fraction. Antibodies were purchased from Abcam.

2.6. Mass spectrometry analysis

Excised protein spots were destained according to the SilverSNAP® protocol. Destained gel pieces were washed twice with acetonitrile (ACN), once with 0.1 M NH_4HCO_3 then with 0.1 M NH_4HCO_3:ACN (1:1), before drying in a vacuum concentrator. Protein spots were reduced for 45 min at 56 °C in 10 mM DTT in 0.1 M NH_4HCO_3. DTT solution was replaced by 55 mM iodoacetamide in 0.1 M NH_4HCO_3 and carboxamidomethylation allowed to proceed for 30 min at room temperature in the dark. Gel pieces were washed with 0.1 M NH_4HCO_3 followed by 0.1 M NH_4HCO_3:ACN (1:1) and dried in a vacuum concentrator. Dried gel pieces were rehydrated in 50 μL digestion buffer containing 12.5 ng/μL trypsin (Promega, Southampton, UK, sequencing grade), 0.1 mM $CaCl_2$ and 50 mM NH_4HCO_3. After 45 min on ice 50 μL 0.1 mM $CaCl_2$ in 50 mM NH_4HCO_3 was added and digestion allowed to proceed overnight at 37 °C. Peptides were extracted by addition of 25 mM NH_4HCO_3 followed by ACN, with vigorous shaking for 15 min after each addition. Two further extractions using 5% formic acid:ACN (1:1) were carried out. Extracts were pooled and reduced in volume using a vacuum concentrator.

Peptide samples were analysed by LC–MS/MS using an LCQ DECA ion-trap mass spectrometer (Thermo Scientific, Waltham, USA) coupled to an Agilent HP1100 HPLC system. Samples were injected onto a 150 × 1 mm Jupiter C18 column (Phenomenex, Macclesfield, UK). Mobile phase A consisted of 0.05% (v/v) trifluoroacetic acid (TFA) in water and mobile phase B 0.05% (v/v) TFA in ACN. Separation was performed at a flow rate of 50 μL/min with a hold at 5% B between 0 and 10 min, linear increase to 45% B at 80 min, linear increase to 65% B at 90 min and a final linear increase to 90% B at 95 min. Peptide analysis was performed using data dependent acquisition of one MS scan (550–2000 m/z) followed by an MS/MS scan of the most abundant ion in the MS scan.

MS/MS spectra were searched against the National Center for Biotechnology Information non-redundant (NCBI nr) database using Mascot (MatrixScience, London, UK) [15]. Search parameters were as follows: taxonomy all entries, maximum one missed cleavage, ±2 Da peptide mass tolerance, ±1 Da fragment mass tolerance, carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification. To incorporate all potential alkylation sites as search parameters would have required the use of an increased number of variable modifications leading to an impractical increase in search time. Positive identification of proteins required a minimum of two peptides, with at least one peptide having a significant ion score ($p < 0.05$).

2.7. Image analysis

Gels and film were scanned using Quantity One® (Bio-Rad). Gels were scanned using the gel/silver stain setting and films were scanned using the X-ray film/gray film setting.

Image analysis was carried out using PDQuest™ (Bio-Rad). Two sets of analysis were carried out: one on the fluorography images (showing radio-labelled protein spots present in the gel) and another on the silver stain images (showing all proteins present in the gel).

Quantitative analysis was then carried out to detect any spots that differed in labelling intensity by 2-fold or more between SM-labelled and CEES-labelled gels. The same analysis was carried out for both fluorography images and silver stain images. This enabled detection of changes in radio-labelled proteins between SM and CEES gels for the fluorography analysis and changes in all protein spots between SM and CEES gels for the silver stain analysis. Any proteins that varied in intensity by 2-fold or more between SM-labelled and CEES-labelled gels detected by silver stain were removed from the fluorography analysis set. This reduced the chance of identifying proteins that differed in labelling between SM and CEES due to a difference in the concentration of the protein in the sample, as opposed to a difference in reactivity of the mustard.

2.8. Actin polymerisation assay

This assay was carried out using the protocol and reagents supplied by Cytoskeleton Inc., Denver, UK, and adapted for use in black 96 well plates with a clear bottom. The plate was read on a fluorescence plate reader (SpectraMAX Gemini XS, Molecular Devices Corporation, Sunnyvale, USA) using the SoftMax Pro v 5.2 software at an excitation wavelength of 355 and an emission wavelength of 405 nm. Within the experiment all samples were assayed in duplicate, Fig. 5 shows data from five biological replicates. Buffer (200 μL of 5 mM Tris–HCl pH 8.0, 0.2 mM $CaCl_2$, 0.1 mM ATP) or actin stock (buffer containing 1 mg non-muscle actin and 0.1 mg muscle actin) was added to wells and the plate read for 3 min to give a baseline, 20 μL of each test agent (SM or ispropanol control or buffer) were added to their designated wells and the plate was read for 20 min. Actin polymerisation buffer (20 μL of 500 mM KCl, 20 mM $MgCl_2$, 10 mM ATP, 100 mM Tris–HCl pH 7.5) was then added to all wells and the plate read for 90 min. The data was then imported into Microsoft Excel where graphs were produced to determine the effect of SM on actin polymerisation. Data were also transferred to Prism, where graphs were plotted and one-way

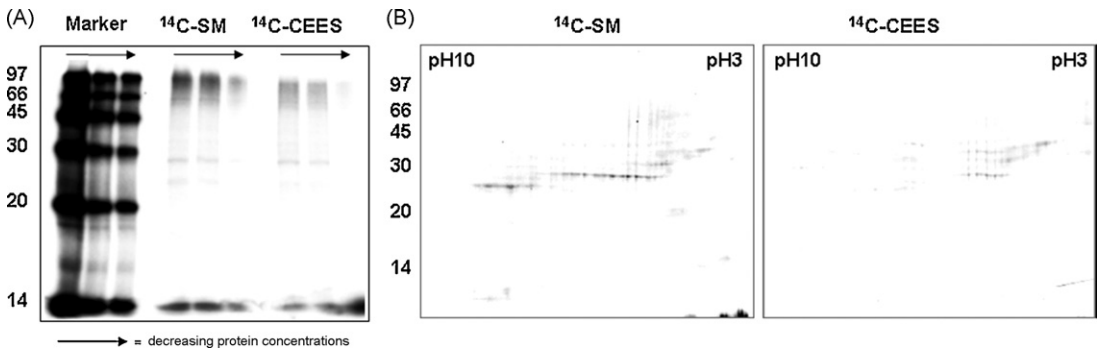

Fig. 3. 1D and 2D gels of cellular proteins labelled with ^{14}C-SM or ^{14}C-CEES. (A) 1D gel of membrane proteins (with protein loadings of 30, 15 and 7.5 μg, decreasing protein concentrations indicated by arrow left to right) labelled with either 3 mM ^{14}C-SM or ^{14}C-CEES and detected by fluorography (film exposed for 11 days). (B) ^{14}C-SM or ^{14}C-CEES-labelled membrane proteins (450 μg protein labelled with 4 mM ^{14}C-SM or ^{14}C-CEES) run on a 2D gel and detected by fluorography (films exposed for 5 weeks).

ANOVA with Bonferroni's multiple comparison test was carried out.

3. Results and discussion

3.1. Fractionation of cell membrane proteins

Human keratinocyte cells (HaCaT cells) were fractionated into cytosolic, nuclear and cell membrane fractions (see Section 2.2). The subcellular content of the fractions was confirmed by the distribution of specific marker proteins in Western blots (Fig. 2). As can be seen in Fig. 2 the cell membrane fraction (P2) contained the cell membrane marker cadherin, but not the cytosolic or nuclear markers (GAPDH and DNA polymerase, respectively). It is likely that the membrane fraction will contain mitochondria and other vesicular structures, such as the endoplasmic reticulum.

3.2. Labelling cell membrane proteins with ^{14}C-SM or ^{14}C-CEES

The cell membrane fraction was labelled with either ^{14}C-SM or ^{14}C-CEES. There was an increased proportion of labelled proteins at the higher molecular weights in the ^{14}C-SM labelled protein compared to ^{14}C-CEES-labelled protein when run on a 1D gel and visualised using fluorography (Fig. 3A).

Separation of the labelled proteins using 2D gels further demonstrated that the pattern of cell membrane labelling was different for these mustards (Fig. 3B), with an overall lower level of proteins labelled with ^{14}C-CEES. As both labelled mustards had the same specific activity the observed differences relate to their binding properties and may be due to the fact that CEES has a mono-functional alkylation ability and therefore is unable to cross-link proteins, unlike SM, which has a bi-functional alkylation ability and a higher vesicant potency.

3.3. Identification of proteins labelled with either ^{14}C-SM or ^{14}C-CEES

The intensity of binding of SM to membrane proteins was compared to that of CEES using PDQuest™. 2D gels showing SM-labelled proteins (group 1) and 2D gels showing CEES-labelled proteins (group 2) were loaded into the program. The protein spots on the gels were then matched within and between the two groups and those proteins that were labelled with a different proportion of the total incorporated adducts, i.e. different relative labelling, highlighted.

The labelled proteins were excised and identified using mass spectrometry. Not all of the proteins were of a high enough abundance to be identified using mass spectrometry, but those that were are shown in Fig. 4. Some proteins were not resolved in a single spot, but as a number of different spots, e.g. actin and annexin A2 (as shown in Fig. 4). This is due to a number of isoforms of the protein being present.

PDQuest™ creates a master gel image, which is representative of all the images loaded into the experiment. The master gel image for the fluorography data is shown in Fig. 4B, with the proteins differing in labelling intensity by 2-fold or more between SM and CEES gels numbered. Actin and annexin A2 had an increased intensity of labelling in the SM-labelled replicates; whereas pyruvate kinase (isoform M1 or M2) appeared to have an increased intensity of labelling in the CEES-labelled replicates. Annexin A2 was at a low level or was not present in the CEES-labelled sample. There was some actin labelled by CEES but generally this actin had a low p*I*. More actin was bound by SM including actin with a higher p*I* that did not appear to bind CEES. Another protein labelled by both SM and CEES was elongation factor 1 (EF-1), though this was not detected in all of the replicates. EF-1 is responsible for aminoacyl-tRNA transfer on the ribosome and has also been shown to interact with the cytoskeleton [16,17]; elongation factor 1 alpha has been shown to bundle actin filaments [16]. It could be envisaged that SM and CEES bind actin with a high intensity and as a result, may also bind proteins associated with actin.

3.4. Limitations to the methodology

Although a number of proteins bound by SM were identified there were several limitations to the methodology used in this study. First, the fluorography methods used were approaching the limits of detection determined by the specific activity of the ^{14}C radio-label. However, use of much higher specific activity levels was not possible as it would have resulted in molecule instability. Second, not all potential membrane proteins were investigated, as inevitably during the fractionating process some proteins were lost or found to be insoluble. Third, although the resolution of the 2D electrophoresis was improved, once labelled with SM or CEES some of the proteins became less soluble and were retained in the first dimension. Previous experiments were carried out labelling whole cells with ^{14}C-SM or ^{14}C-CEES and then extracting the cell membrane fraction to identify labelled proteins, but the level of incorporation was too low to make it a feasibly analytical method.

3.5. Differences between SM and CEES

Despite these limitations this study has shown differences in cellular membrane protein binding between SM and CEES, which may reflect SM's bi-functional alkylating property compared to the mono-functional CEES. A number of proteins appeared to preferentially bind SM over CEES and some such as pyruvate kinase appeared to exhibit higher intensity labelling with CEES. In all like-

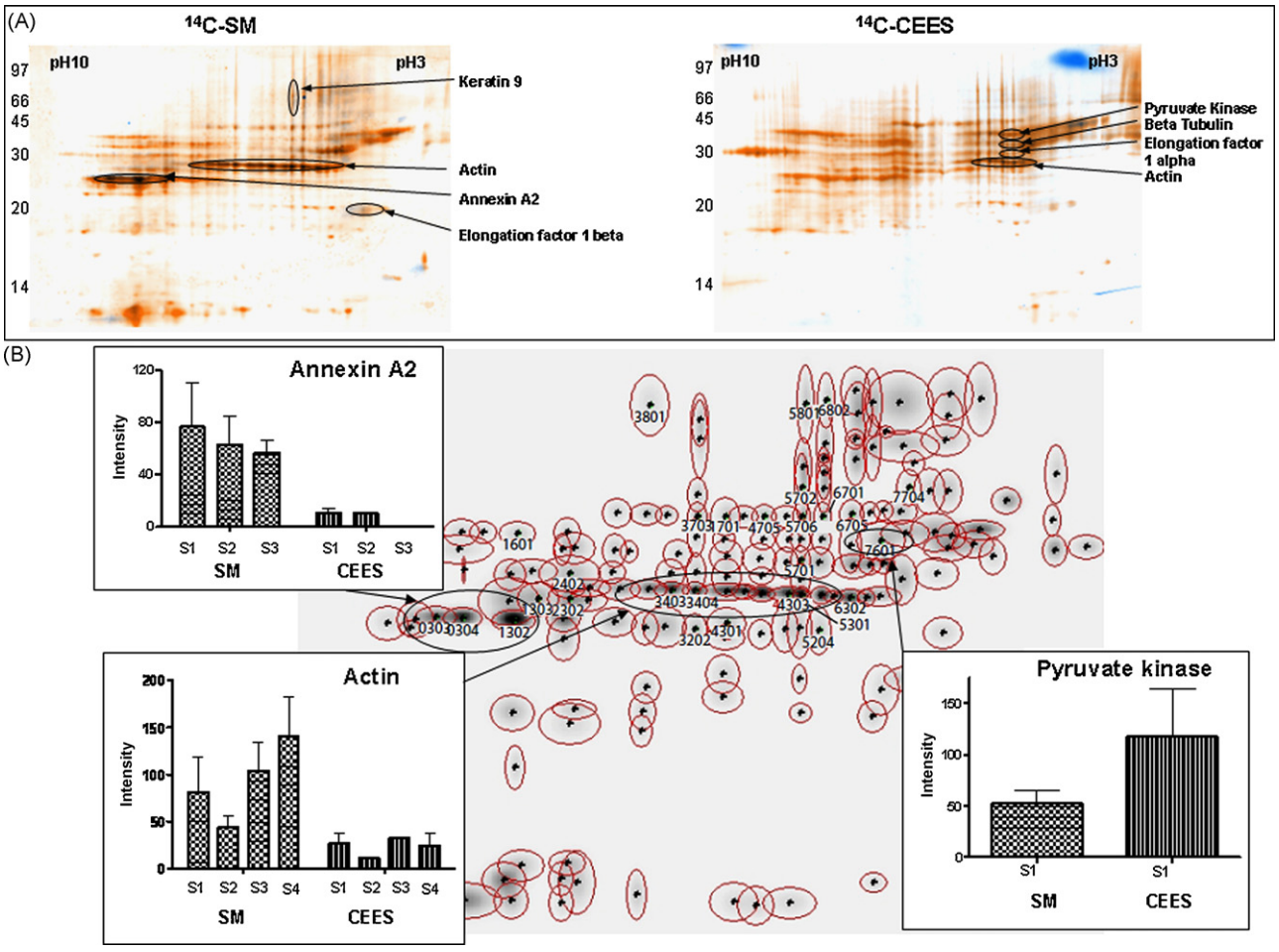

Fig. 4. Identification of proteins labelled with ^{14}C-SM or ^{14}C-CEES. (A) Overlay showing a 2D gel of ^{14}C-SM or ^{14}C-CEES-labelled membrane proteins (450 μg protein labelled with 4 mM ^{14}C-SM/^{14}C-CEES) detected by fluorography, and the same gel silver stained after detecting by fluorography to detect all proteins. Circled proteins are labelled with either ^{14}C-SM or ^{14}C-CEES (circled, annotated and brown in colour). (B) PDQuest™ analysis of 2D gels. Five biological replicates of 2D gels showing ^{14}C-SM labelled membrane proteins detected by fluorography (group 1) and four biological replicates of 2D gels showing ^{14}C-CEES-labelled membrane proteins detected by fluorography (group 2) were analysed. Images were cropped and loaded into the program and the protein spots matched between gels. A master gel image was created, representing a combination of all of the gels (image in the centre of B). Protein spots that differed between the two groups were then analysed. A number of protein spots were shown to differ in labelling intensity by 2-fold or more between SM and CEES images, and these are marked by numbers on the figure. The graphs show the intensity of the radio-labelled protein in the gel. Red bars represent the gels containing SM-labelled protein and blue represents the gels containing CEES-labelled protein. S = spot, representing different isoforms of protein. (For interpretation of the figure in colour, the reader is referred to the web version of the article.).

lihood there are many more proteins which exhibit this preferential binding but, as discussed above, the detection and identification of labelled proteins has been limited by various factors. This has, however, highlighted the issue that where possible CEES should not be used as a substitute for SM in experimental studies. Where CEES has been used, results should be interpreted with caution in the knowledge that this mustard potentially targets a different set of cellular proteins to SM.

3.6. SM binding proteins

From the SM binding proteins identified in this study the ones most likely to be involved in vesication associated with SM-induced injury are those labelled with greater intensity when compared to CEES, namely: actin, annexin A2, and keratin 9. It should be noted though that keratin 9 could only be detected in one out of five replicate samples, due to being at the limits of detection for the mass spectrometer. Keratins are flexible intracellular polymers that play a key role in providing structural resilience to cells. Previous reports have already shown that SM exposure induces the formation of keratin aggregates and have predicted that this has an effect on the structural integrity of the cell [18,19]. Although this disruption in structural integrity does not fully explain SM vesication, it may be integral to the loss in cell–cell adhesion seen on SM exposure.

3.6.1. Annexin A2

The present study has demonstrated the selective binding of SM (when compared to CEES) to annexin 2 (annexin A2 in humans) for the first time.

Annexin A2 is a Ca^{2+} and phospholipid binding protein with many roles within the cell, including the regulation of membrane–membrane and membrane–cytoskeletal interactions. Annexin A2 is found at membrane sites that function as assembly points for actin filaments and has Ca^{2+} dependent actin filament bundling activity, which is particularly pronounced when it is bound in a tetrameric complex with the protein S100A10 [20]. This regulation of cellular membrane interactions links two proteins shown to be directly bound by SM: annexin A2 and actin. It is tempting to postulate that the breakdown of the cytoskeleton on SM exposure involves modification of these proteins.

An inflammatory response is one of the first signs of SM-induced vesication and there are a number of links between annexin A2 and inflammatory pathways. Annexin A2 is a plasmin receptor on monocytes. Plasmin binds to the annexin A2 heterotetramer and

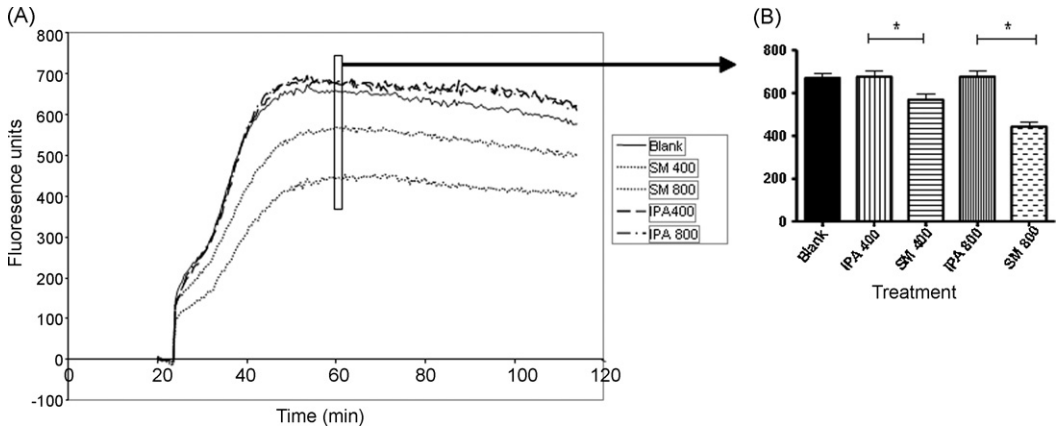

Fig. 5. Sulfur mustard's effect on actin polymerisation. The graph shows the effect of SM on actin polymerisation, data shown are from five biological replicates. (Blank = the actin control, SM 400 = 400 μM SM, SM 800 = 800 μM SM, IPA 400 = the isopropanol vehicle control for 400 μM SM, IPA 800 = the isopropanol vehicle control for 800 μM SM). The isopropanol controls have no effect on the level of actin polymerisation, however, as the concentration of SM increases the level of actin polymerisation decreases ($n = 5$). Graph B shows the effect of SM on actin polymerisation at the 60 min time point when the assay has reached an optimum plateau. One-way ANOVA with Bonferroni's multiple comparison test was carried out and demonstrated that 400 and 800 μM SM significantly lowered the amount of actin polymerisation compared to their respective vehicle controls (*p value <0.0001).

triggers various signalling pathways, including inflammatory cascades such as the p38 MAP Kinase signalling cascade [21]. p38 MAP Kinase has been shown to play a role in SM-induced cytokine production [22]. Therefore it could be postulated that SM-modified annexin A2 has an effect on the p38 MAP Kinase cascade and pro-inflammatory activation. Further investigation of this point was beyond the scope of this present study.

Annexin A2 is also involved in wound repair and wound repair is delayed in SM-induced injuries. Inhibition of annexin A2 has been shown to inhibit spreading and wound closure in epithelial cells [23]. Annexin A2 has been shown to be directly involved in MMP-1 production (a protease important in extracellular matrix breakdown and therefore involved in modulating the repair process) through a plasmin-dependent pathway [24]. Plasmin-dependant pathways have previously been implicated in SM pathogenesis by Lindsay *et al.* [25].

3.6.2. Actin

The finding that actin binds SM is important as actin is involved in many cellular processes such as exocytosis, cell adhesion and cell migration. Actin consists of monomers that polymerise into helical filaments and these can assemble into a wide variety of higher-order cellular structures, ranging from lamellipodia to microvilli [26]. Actin is essential for the correct functioning of all cells and therefore anything that prevents actin polymerisation will have a major effect on cell migration, adhesion and membrane trafficking. There has been little research into the effect of SM on actin in the cell. Werrlein *et al.* showed a collapse of the actin cytoskeleton in the cell on exposure to SM [27]. Dabrowska *et al.* showed that disruption of the long actin filament stress fibres and rounding of cells preceded other features of apoptosis, and noticed an increase in the amount of actin monomer compared to filaments [28] and recently Mol *et al.* showed that SM bound to actin in human keratinocyte cells [9]. Therefore, SM exposure evidently has an effect on actin structures.

To ascertain whether SM has an effect on the function of actin we investigated the effect that SM has on actin polymerisation (Fig. 5). As the concentration of SM was increased the level of actin polymerisation decreased. The amount of actin polymerisation was significantly lowered by 400 and 800 μM SM (one-way ANOVA with Bonferroni's multiple comparison test $p < 0.0001$). The isopropanol vehicle controls did not affect the level of actin polymerisation.

The concentrations of SM used in the polymerisation assay were at high micromolar levels and therefore there are likely to be other mechanisms involved in SM pathogenesis at lower concentrations of SM. SM reactivity may differ when interacting with actin filaments in an intact cell, therefore further experiments would be required to elucidate the effect that SM has on actin polymerisation within the cell. Despite this it can be envisaged how SM binding actin and reducing its polymerisation could contribute to the pathogenesis seen on SM exposure. SM-modification of actin could cause a disruption in actin polymerisation which in turn would have many consequences inter- and intra-cellularly.

Altering actin remodelling could disrupt cell adhesion via a number of mechanisms such as disruption of focal adhesions or adherens junctions. The migration of cells is reliant on the correct functioning of actin, so reduction of actin polymerisation would affect the formation of lamellipodium/lamellum and therefore cell migration. A reduction in cell migration would have an effect on wound healing and it has been shown that wounds are slower to heal in SM burns than in a comparable thermal burn [29]. It has also been recently shown that SM affects cell migration in human keratinocyte cells [6].

4. Conclusions

This study has identified a number of proteins that are directly bound by SM or CEES (an analogue with a much lower vesicant potential). It has also shown that the pattern of binding of SM compared to CEES is different. This differential binding has a number of implications. Since SM behaves differently to CEES, any experiments using CEES as a substitute for SM should be interpreted with care. Moreover, the proteins that bind SM with a higher intensity compared to CEES may have a role in the vesicant effects of SM. Two of these proteins, annexin A2 and actin, are of particular interest as their role in the cell can be linked to the pathology seen after SM exposure. The binding of SM to actin was associated with an effect on actin polymerisation. Our findings indicate that SM-modification of actin and annexin A2 could be integral to SM pathology and warrant further investigation.

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