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PRINCIPAL INVESTIGATOR: Marijke A.E. Mol, Ph.D.

CONTRACTING ORGANIZATION: Prins Maurits Laboratory TNO
2280 AA Rijswijk, The Netherlands

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LIST OF ABBREVIATIONS

CBB	coomassie brilliant blue
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
HD	sulfur mustard
HEK	human epidermal keratinocytes
HRP	horseradish peroxidase
KBM	keratinocyte basal medium
kDa	kilodalton
KGM	keratinocyte growth medium
MMP	matrix metalloproteinase
NP40	nonidet-p40
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
Ser(P)	phosphoserine
TNF α	tumor necrosis factor α

SUMMARY

Hypothetically, two possible causes underly the destruction of the epidermal-dermal junction of the skin after exposure to sulfur mustard (HD), i.e. improper functioning after HD-alkylation of proteins involved in the attachment structure, and breakdown of structural proteins by proteases. It will be of value to determine which of these processes occurs, in order to decide on the therapeutic strategy to be followed after exposure of skin to HD. In this report we describe the results so far of studies on protein alkylation and on proteolytic activity in skin or cultured keratinocytes after exposure to HD.

After exposure of cultured human keratinocytes to ^{14}C HD, circa 40% of label present in cell lysates is bound to proteins. Alkylation preference is observed for K14, an acidic type I keratin. Some other proteins, with molecular weights of 100 - 200 kD and 30 - 40 kDa are also preferentially alkylated. Almost 55% of the label in cell lysates is bound to components other than DNA, RNA and protein, or not bound at all.

The experiments on proteolytic activity demonstrate that there appears to be no involvement of matrix metalloproteinase (MMP)-9 in loosening the epidermal-dermal junction during HD-induced blistering. For MMP-2, it is doubtful whether its proteolytic activity is actually enhanced in HD-exposed skin.

1. INTRODUCTION

1.1 General introduction

Epidermal-dermal separation is a characteristic feature of sulfur mustard (HD)-induced pathology in the skin. Alleviation of skin damage can possibly be obtained by preventing tearing of the epidermal-dermal junction, an attachment layer composed of a complex of structural proteins. Hypothetically, two possible causes underly the destruction of this protein layer, i.e. improper functioning after HD-alkylation of proteins involved in the attachment structure, and breakdown of structural proteins by proteases. It will be of value to determine which of these processes occurs, in order to decide on the therapeutic strategy to be followed after exposure of skin to HD. If proteolysis proves to be a crucial step in the initiation of microblistering, application of protease inhibitors might provide an effective therapeutic treatment. However, if extensive protein alkylation is the dominant phenomenon, therapy will probably be constrained to generic cure.

1.2 Protein alkylation

So far, the role of protein alkylation in pathological effects of HD on the skin has been unclear. Probably, HD will bind to various proteins of the epidermis, dermis and the epidermal-dermal junction (Papirmeister et al., 1991; Zhang et al., 1995; Smith et al., 1997; Petralli and Oglesby-Megee, 1997). No data are available on the extent of formation of HD-protein adducts in keratinocytes, nor on preferential targets in these cells. Keratin, which is abundantly present in epidermal cells as part of the cytoskeleton, may be one of the targets for HD-alkylation. In basal epidermal keratinocytes, the cytoskeletal network regulates essential cell functioning and is, via the hemidesmosomes, involved in the anchorage system between the epidermis and the dermis. Alkylation damage to keratins or other structural proteins of the epidermal-dermal junction may disturb basic cell functions and cell-matrix attachment, leading to vesication. The present work is a first approach to study protein-alkylation by HD

in cultured human epidermal keratinocytes (HEK) using ^{14}C -labeled HD. A preliminary identification of cellular proteins bearing labeled adducts has been performed.

1.3 Proteolytic activity

Matrix metalloproteinases (MMPs) are a family of enzymes responsible for normal turnover of the extracellular matrix and capable of degrading all extracellular matrix proteins including those of the basement membrane zone (Kähäri and Saarialho-Kere, 1997). They are secreted by connective tissue cells, inflammatory cells and keratinocytes as latent proenzymes, requiring modification for the expression of enzyme activity. Although they are tightly regulated under normal conditions, their involvement has been suggested in the pathophysiology of several skin diseases in which the integrity of the basement membrane is destroyed, such as dermatitis herpetiformis, dystrophic epidermolysis bullosa and lichen planus (Kähäri and Saarialho-Kere, 1997).

The MMP family includes various subclasses having preferential substrates. MMP-1 is a representative of the interstitial collagenases and degrades many types of native and denatured collagen. Another subclass, i.e. the type IV collagenases or gelatinases, includes MMP-2 (72 kDa) and MMP-9 (92 kDa) which can specifically degrade type IV collagen, denatured collagens and laminin-5 (Gianelli et al., 1996). Stromelysin-1 (MMP-3) and matrilysin (MMP-7) are members of the subclass of stromelysins and are able to degrade type IV collagen, laminin-1, laminin-5 and integrin $\beta 4$ (Sires et al., 1993; von Bredow et al., 1997). In order to investigate the role of MMPs in the destruction of the cutaneous basement zone following HD exposure, their activity will be determined by proteolytic assays and immunohistochemistry. In this report period we measured by means of zymography the activity of the two type IV collagenases, MMP-2 and MMP-9, in the culture media of HD-exposed HEK or human skin pieces.

2. EXPERIMENTAL METHODS

2.1 Keratinocyte culture

Cultures of HEK were raised from basal keratinocytes, isolated from mammary skin obtained during cosmetic surgery. All patients gave their informed consent. In brief, primary epidermal cells were inoculated on a feeder layer of mitomycin C-treated 3T3 mouse fibroblasts in serum-containing medium as described earlier (Mol et al., 1989). When subconfluent, cells were trypsinized and cryopreserved as a stock. To do experiments, first passage HEK from cryovials were grown in serum-free keratinocyte growth medium (KGM; Clonetics/BioWhittaker, Walkersville, MD) at 37 °C in an atmosphere of 6% CO₂ in air.

Usually, confluent monolayer cultures were achieved at 7 days after plating.

2.2 Skin organ culture

Human mammary skin was obtained from cosmetic surgery with informed consent of the patient. Organ cultures of human skin were maintained as described by Varani et al. (1995). Skin pieces of 0.25 cm² were floated with the dermal side down on keratinocyte basal medium (KBM; Clonetics/BioWhittaker) supplemented with CaCl₂ to a final concentration of 1.4 mM (1ml medium/well of a 12 well cluster plate) and incubated at 37 °C in an atmosphere of 6% CO₂ in air for 24 or 48 hr.

2.3 Exposure of keratinocyte cultures to HD

HD was synthesized by the Chemical Toxicology Branch of TNO Prins Maurits Laboratory and had a purity of 97%. Stock solutions of HD were freshly prepared in dry acetone and diluted immediately before use in KBM to obtain the desired working concentrations. The final concentration of acetone in the incubation medium was 1%. One day after reaching confluence, HEK were exposed to various concentrations of HD (2.5 ml/well of a 6-well cluster plate) for 30 min at 25 °C. The cells were washed and processed either immediately or after an incubation period at 37 °C in KGM. Hydrocortisone was omitted from KGM in studies in which the media were analyzed for proteolytic activity.

2.4 Exposure of keratinocyte cultures to ^{14}C HD

^{14}C HD (specific activity 15 mCi/mmol) was synthesized by the Chemical Toxicology Branch of TNO Prins Maurits Laboratory (Fidder et al., 1998). The compound was 95% radiochemically pure as analyzed by thin layer chromatography. For exposure of HEK to ^{14}C HD, conditions were comparable with those used for cold HD. The desired HD concentrations in KBM were prepared by appropriate dilution of a freshly made ^{14}C HD stock solution in acetone based on the counts it contained. The concentrations of the ^{14}C HD solutions thus obtained, varied within a range of about 5% from the ones intended. One day after reaching confluence, HEK were exposed to various concentrations of ^{14}C HD (1.5 ml/well of a 6-well clusterplate) for 30 min at 25 °C. Then they were washed and processed either immediately or after an incubation period at 37 °C in KGM. For radioactivity determinations, samples containing ^{14}C activity were mixed with scintillation fluid (Hionic-Fluor; Packard, Groningen, The Netherlands) and counted in a Tricarb 2500 TR (Packard) scintillation counter. In all experimental devices using ^{14}C HD, recovery data for radioactive counts were calculated and found to be 95-97%.

2.5 Exposure of skin pieces to HD vapor

Human mammary skin was exposed to saturated HD vapor at 25 °C for 4 or 6 min using a vapor cup device as described earlier (Mol et al., 1991).

2.6 Isolation of DNA, RNA and protein from HEK

At various post-incubation times HEK were dissolved in TRIzol® reagent (Gibco BRL, Breda, The Netherlands). DNA, RNA and protein were isolated according to the instructions of the manufacturer. In brief, cells were lysed in TRIzol® (1 ml per 10 cm² cell surface area) and the reagent was transferred to sample tubes. Chloroform was added (0.2 ml/ml TRIzol®) and the closed tubes were shaken vigorously by hand and centrifuged. The mixture separated in a upper aqueous phase and a lower organic phase. RNA was precipitated from the aqueous

phase with isopropyl alcohol, washed with ethanol and dissolved in RNase-free water. After carefully removing the aqueous phase, DNA was isolated from the interphase and the organic phase by precipitation with ethanol. Following several washes, DNA was solubilized in 8 mM NaOH. Proteins were isolated from the phenol-ethanol supernatant by precipitation with isopropyl alcohol. Following several washes, proteins were dissolved in 1% SDS.

2.7 Total cell lysates

Total cell lysates were prepared by dissolving HEK in 0.1 M NaOH.

2.8 Quantitation of protein concentrations

Protein concentrations were determined in a microplate assay using BioRad Protein Assay (BioRad, Veenendaal, The Netherlands).

2.9 Extraction of keratins from HEK

The following method was used to obtain a keratin fraction (Breitkreutz et al., 1984). Cultures were extracted first with a low salt buffer (10 mM Tris-base, 150 mM NaCl, 3 mM EDTA and 0.1% nonidet-P40 (NP40), pH 7.4) and then with a high salt buffer (10 mM Tris-base, 150 mM NaCl, 1.5 M KCl, 3 mM EDTA, 0.1% NP40, pH 7.4). Both buffers were supplemented with 0.4 mM Pefabloc®, 0.5 µg/ml leupeptin and 0.5 µg/ml pepstatin (Boehringer Mannheim, Almere, The Netherlands). Then, insoluble residues were either dissolved in TRIzol® to isolate protein, in 0.1 M NaOH for protein quantitation, or in lysis buffer to perform SDS-PAGE.

2.10 SDS-PAGE and Western blotting

Samples were prepared by dissolving HEK or a keratin extract in lysis buffer consisting of 62.5 mM Tris pH 6.8, 1 mM EDTA, 2% SDS, 5 % β-mercaptoethanol and 0.05 % bromophenol blue. Protein content was determined in parallel samples that were dissolved in 0.1 M NaOH, using the

Bradford protein assay (BioRad). Samples containing equal amounts of protein were analyzed by electrophoresis on 10 % Tris-HCl Ready gels (BioRad) using the BioRad MiniProtean System.

Proteins on the gels were either stained with Coomassie Brilliant Blue (CBB) or transferred to nitrocellulose membranes (Costar, Badhoevedorp, The Netherlands) for immunodetection of phosphoserine (Ser(P)) groups with a mouse monoclonal antibody (Sigma, Zwijndrecht, The Netherlands). After incubation with a horseradish-peroxidase (HRP)-conjugated secondary antibody, blots were developed using the ECL Western blotting detection kit (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). Biotinylated molecular weight markers (BioRad) were included in each blot. Neutralite-avidin-HRP conjugate (Southern Biotechnologies Associates, Birmingham, AL) was added to the second antibody incubation for detection of the molecular weight markers with the ECL system.

2.11 Indirect autoradiography (fluorography)

¹⁴C labeled proteins on gels were visualized by fluorography. Gels were incubated with Amplify according to the instructions of the manufacturer (Amersham Pharmacia Biotech), then dried by air-heating (GelAir Dryer; BioRad) and exposed to radiographic film (Hyperfilm MP; Amersham Pharmacia Biotech) for up to four weeks. Films were developed in an automatic processor.

2.12 Zymography

Media of keratinocyte or skin organ cultures were analyzed for MMP activity by gelatin zymography using gelatin containing 10% Tris-HCl Ready Gels with 50 µl slots (BioRad). Briefly, fluids were concentrated (5-10 fold for organ culture media and 40-50 fold for cell culture media) by ultrafiltration using Centrex ultrafilters with molecular weight cut off of 30 kDa (Schleicher and Schuell, Den Bosch, The Netherlands), and subsequently prepared for electrophoresis without heating or reduction. After electrophoresis, the SDS was removed by washing the gels subsequently for 2 x 10 min in demi water containing 2.5% Triton X-100, in

50 mM Tris pH 7.5 containing 2.5% Triton X-100 and in 50 mM Tris pH 7.5. Next, the gels were incubated overnight at 37 °C in 50 mM Tris pH 7.5 containing 10 mM CaCl₂ and 20 mM ZnCl₂. The proenzyme forms of MMP-9 and MMP-2 can also be detected with this procedure, since they become activated during the process of renaturation after gel electrophoresis. The enzymes cleave the gelatin and leave a clear zone in the gel after staining with CBB and destaining. Purified proenzyme MMP-9 (Boehringer Mannheim) was included in each gel to determine the position of MMP-9.

2.13 Histology

Human skin pieces were fixed overnight at 4 °C in 2% paraformaldehyde in phosphate buffered saline. They were stored in 70% ethanol until embedding in paraffin. Sections were stained with hematoxylin/eosin and examined by light microscope.

3. RESULTS

3.1 ¹⁴C HD-adduct formation

HEK have been exposed to 1.5 ml of a solution of 100 μM ¹⁴C HD, corresponding to approximately 5. 10⁶ dpm. At various time periods after exposure radioactivity present in the total cell lysate as well as radioactivity bound to DNA, RNA and total precipitable protein was measured. The results are presented in Table 1. Immediately after exposure of HEK for 30 min to a solution of 100 μM ¹⁴C HD, the amount of dpm found in the cell lysate comprises approximately 0.8% of the total amount of radioactivity that was presented to the cells.

Assuming that the volume of the keratinocyte monolayer is approximately 0.5 μl (area of 1000 mm² and 0.5 μm thickness) and that equal distribution takes place between cells and the solution of ¹⁴C HD, it was expected that $0.5/1500 = 0.03\%$ of label would be found in the 0.5 μl cell volume. The amount of label present in the keratinocytes appears to be twenty five-fold higher, indicating considerable binding of ¹⁴C HD to cell components.

During the first hour of post-incubation, the radioactivity in the cell lysate has decreased, whereas the number of counts in the incubation medium has increased (not shown).

Apparently, part of the freely circulating labeled molecules diffused from the cells into the culture medium. From then until 5 h of post-incubation, the situation is rather stable and the amounts of label bound to DNA, RNA and protein contribute for 45% to the total amount of label present in the cells. This means that a considerable amount of label is either bound to other molecules in the cell or freely circulating, possibly as thioglycol. At 24 h of post-incubation, a further drop of the amount of label present in the cell lysate is observed, which is caused partly by a 30% decrease of label in the precipitable protein fraction. A small loss of label (13%) is observed for RNA, while no loss of label is seen for DNA.

In a second series of experiments, HEK have been exposed to 100, 200 and 300 μM ^{14}C HD. At 1 h after exposure the amounts of radioactivity present in the cells and bound to DNA, RNA, total precipitable protein and keratin have been estimated. The results as presented in Table 2, show that the amounts of label present in cell lysates and bound to the cellular macromolecules are approximately proportional to the concentration of HD used. It appears that the keratin fraction counts for about one-third of the label in the total protein fraction, except when HEK were exposed to 300 μM HD. In the latter case, keratins seem to have a disproportionately higher grade of alkylation. Since the protein contents of a total protein and a keratin extract from a confluent HEK culture amount to 750 - 800 μg and 200 - 250 μg , respectively, keratins seem to be not preferentially alkylated within the protein fraction, when concentrations up to 200 μM HD are used.

The purity of the keratin extract has been analyzed by SDS-PAGE and the keratins have been identified by western blotting and immunodetection with an antibody against phosphorylated serine residues, which are present in all keratins (Steinert et al., 1982). With regard to purity, Figure 1 shows that the keratin extract mainly contains proteins with molecular weights between 60 and 40 kDa (lane 3), which corresponds to the molecular weight range of keratins (Moll et al., 1982). Immunodetection of phosphorylated serine groups (lane 5) confirms that

the prominent five protein bands in the keratin extract are keratins. The molecular weights of the individual protein bands have been calculated. The keratins extracted from the keratinocyte cultures have molecular weights of 58, 56, 50, 48 and 46 kDa corresponding to keratins 5, 6, 14, 16 and 17. These are the keratins that are usually present in cultured HEK (Morley and Lane, 1994).

In order to investigate the formation of ^{14}C HD-protein adducts, total cell and keratin extracts were made at 1 h after exposure of HEK to 100, 200 or 300 μM ^{14}C HD. Proteins were separated by electrophoresis on 10% polyacrylamide gels. On one gel proteins were stained with CBB. On the other gel proteins containing radioactivity were visualized by fluorography (Figure 2). To obtain maximal signals on the fluorograms, the lanes of the gels had been overloaded with protein sample. The fluorograms show that, with equal amounts of protein applied to each lane (as CBB stained gels demonstrate), the amount of label increases with the applied concentration of ^{14}C HD. Radioactive label is prominently present in proteins with a molecular weight between 45 and 66 kDa, which is within the range of keratins. Furthermore, radioactive label is seen in proteins with molecular weights between 100 and 200 kDa, and in a number of low molecular weight proteins (30-40 kDa). This was confirmed by using 7.5 and 15% polyacrylamide gels on which, respectively, high and low molecular weight proteins are better resolved than on 10% gels (not shown). It is unknown which proteins are involved.

Comparison of the CBB stained gel and the fluorogram shows that within the group of keratins, the keratin with molecular weight of 50 kDa, i.e. K14, is preferentially alkylated. It is unclear from this fluorogram whether K16 (48 kDa) is also alkylated with preference.

Interestingly, SDS-PAGE analysis of a keratin extract made at 1 h after exposure of HEK to 850 μM HD showed the same pattern as a total cell extract (data not shown). Obviously, salt extraction did not remove efficiently the soluble cell proteins from the cell lysate. This is probably due to a decrease in solubility of proteins when they become alkylated.

3.2 Matrix metalloprotease activity

Culture media were collected at 6 and 24 h of post-incubation after exposure of HEK to 0, 75, 150, or 225 μM HD. After concentration by ultrafiltration they were analyzed by means of zymography on gelatin-containing gels. To determine the position of MMP-9 proenzyme on the gel, one lane was loaded with purified MMP-9 proenzyme. This commercially obtained preparation appeared to contain not only gelatinolytic activity at 92 kDa, but also at two sites with higher molecular weights. According to the information of the manufacturer proenzyme MMP-9 was isolated from human blood. We presume that polymorphonuclear leukocytes were used for isolation, which contain, in addition to proenzyme MMP-9 two other gelatinases with molecular weights of 135 and 225 kDa (Makowsky and Ramsby, 1996). The zymogram of the medium of control cells showed a narrow band at 92 kDa, the location of latent MMP-9 (Figure 3, lane 2). The second clear band there below probably represents the latent form of MMP-2 (72 kDa), which is also constitutively produced in low amounts by cultured human keratinocytes (Sarret et al., 1992). $\text{TNF}\alpha$ was added to untreated HEK for 6 or 24 h as a positive control, because secretion of latent MMP-9 by keratinocytes is enhanced by this cytokine (Salo et al., 1994). The media of $\text{TNF}\alpha$ -treated HEK showed an enhanced amount of proenzyme MMP-9 and no change in the level of MMP-2 (lane 6). At 6 h after exposure of HEK to the various concentrations of HD, the amounts of latent MMP-9 in the media were equal to (75 and 150 μM) or slightly lower (225 μM HD) than the amount found in the medium of control cells. The amounts of proenzyme MMP-2 appeared to be unchanged. At 24 h, there was less proenzyme MMP-9 secreted in medium of cells exposed to 75 μM HD than in that of control cells, whereas the proenzyme MMP-2 level was unchanged. Unfortunately, proteolytic activity could not be detected in media of cells exposed to 150 or 225 μM HD, since the presence of cellular proteins of lysed cells in the media obscured the clear zones of proteolytic activity on the zymogram.

In a similar way, the presence of proteolytic enzymes in tissue culture media of HD exposed human skin was investigated. Human skin was exposed for 4 or 6 min to saturated vapor of

HD and organ cultured for 24 or 48 h. Then, skin pieces were prepared for histology and media were collected for zymography. Histological examination showed that skin remained well preserved during the culture period and that HD exposure caused the well-known pathological signs of epidermal-dermal separation (not shown). In the medium of organ-cultured control skin the proenzymes MMP-9 and MMP-2 were observed, as well as a narrow band just below proenzyme MMP-2 (Figure 4, lanes 3 and 6). This band probably represents activated MMP-2 with a molecular weight of 62 kDa (Zeigler et al., 1996). In media of HD-exposed skin pieces collected at 24 and 48 h (lanes 4, 5, 7 and 8) the amounts of proenzyme MMP-9 were evidently decreased as compared to control medium. The amounts of proenzyme MMP-2 seemed to be slightly increased in these media, but the quantities of the active form of MMP-2 were not enhanced.

Table 1.

Radioactivity bound to cellular macromolecules at various time periods after a 30 min exposure of HEK to 100 μM ^{14}C HD, corresponding to approximately $5.000 \cdot 10^3$ dpm. Results are given in 10^3 dpm, and are expressed as mean ($x \pm \text{s.e.m.}$) of two or three experiments, each performed in duplicate.

Time after exposure (h)	Cell lysate (10^3 dpm)	DNA (10^3 dpm)	RNA (10^3 dpm)	Total protein (10^3 dpm)
0	40.4 ± 2.5	1.0 ± 0.0	0.9 ± 0.3	12.0 ± 0.8
1	28.8 ± 1.4	1.1 ± 0.2	0.8 ± 0.1	10.7 ± 0.1
3	28.8 ± 2.9	1.2 ± 0.1	0.8 ± 0.1	11.3 ± 0.7
5	28.6 ± 4.5	1.3 ± 0.0	0.8 ± 0.1	11.2 ± 0.5
24	18.4 ± 1.3	1.28 *	0.7 ± 0.1	8.0 ± 0.1

* n = 1

Table 2.

Radioactivity bound to cellular macromolecules at 1 h after a 30 min exposure of HEK to 100, 200 and 300 μM ^{14}C HD. Results are given in 10^3 dpm, and are expressed as mean ($x \pm \text{s.e.m.}$) of two or three experiments, each performed in duplicate.

Conc HD (μM)	Cell lysate (10^3 dpm)	DNA (10^3 dpm)	RNA (10^3 dpm)	Total protein (10^3 dpm)	Keratin (10^3 dpm)
100	28.4 ± 0.7	0.9 ± 0.0	0.8 ± 0.1	11.2 ± 0.8	3.5 ± 0.2
200	57.6 ± 2.1	1.7 ± 0.3	1.8 ± 0.1	26.1 ± 0.9	9.2 ± 0.5
300	83.8 ± 2.1	2.8 ± 0.2	2.7 ± 0.2	33.5 ± 2.3	15.3 ± 1.5

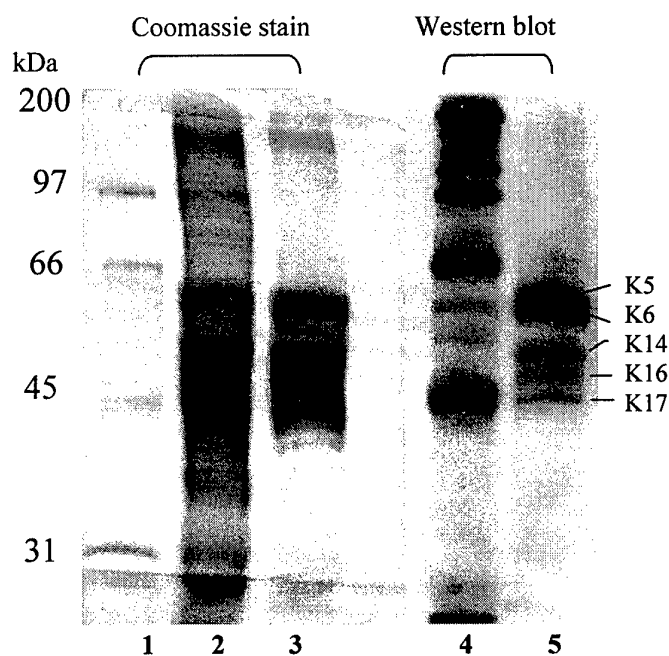


Figure 1.

SDS-PAGE and Western blot analysis of keratin extracts made from confluent control cultures of HEK (see Experimental Methods). Molecular weights of marker proteins are indicated on the left. Protein bands were either stained with Coomassie Brilliant Blue (lanes 1-3) or transferred to nitrocellulose filters for immunoblotting with monoclonal antibody against Ser(P) (lanes 4 and 5). Lanes 1 and 4: molecular weight markers. Lane 2: total protein extract from HEK. Lane 3: keratin extract from HEK. Lane 5: chemiluminescent detection of serine-phosphorylated proteins in keratin extract from HEK.

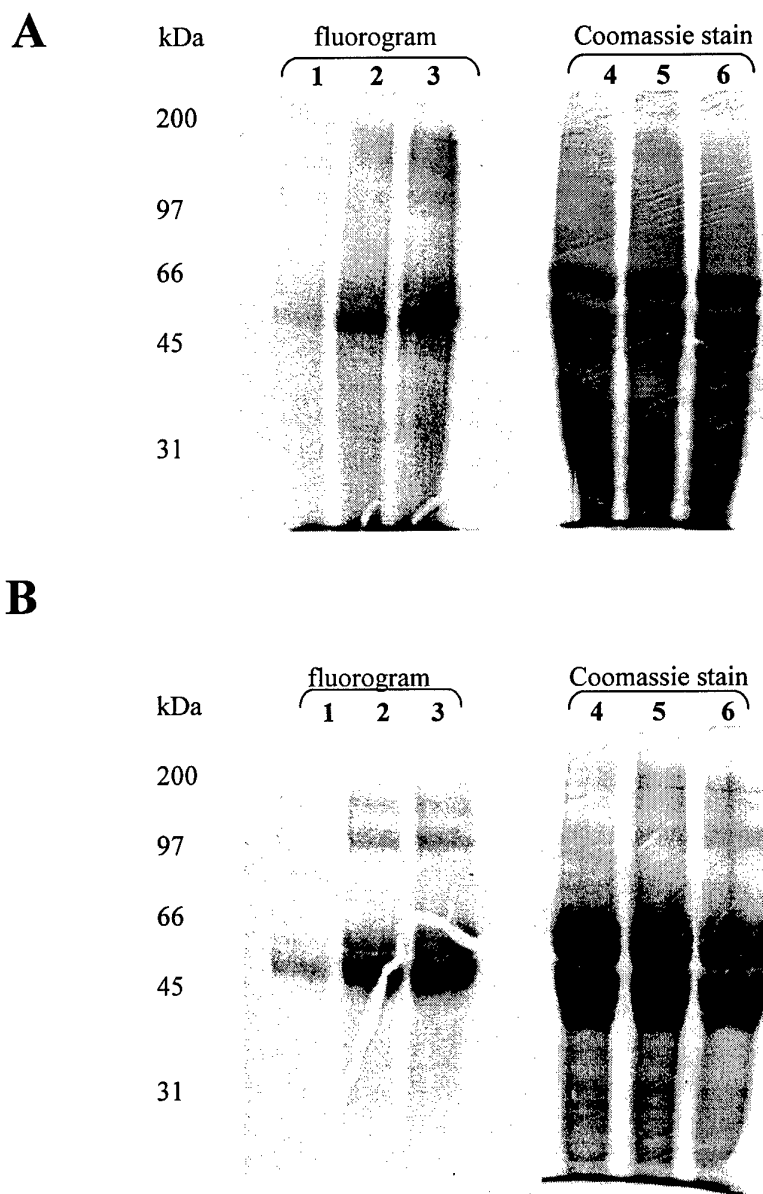


Figure 2.

Presence of ^{14}C HD-adducts in total protein extract (A) and keratin extract (B) of HEK cultures exposed to ^{14}C HD. Extracts were made after exposure of HEK to 100 μM (lanes 1 and 4), 200 μM (lanes 2 and 5) or 300 μM ^{14}C HD (lanes 3 and 6). Proteins in these extracts were separated on polyacrylamide gels and subsequently visualized by either fluorography (lanes 1-3) or CBB staining (lanes 4-6). For experimental details see Experimental Methods. Positions of molecular weight markers are indicated on the left.

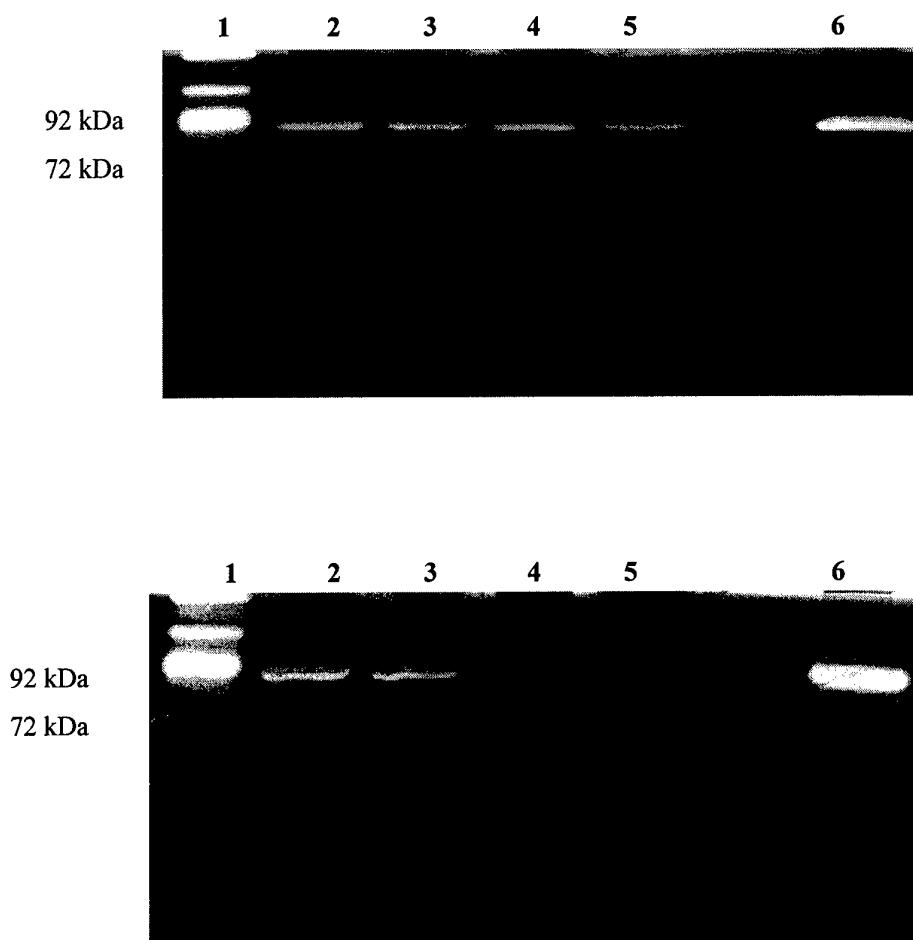


Figure 3.

Zymograms of post-incubation media from HEK exposed for 30 min to various concentrations of HD. Media were collected at 6 h (upper panel) or 24 h (lower panel) post exposure and prepared for zymography as described in Experimental Methods. Lane 1: purified proenzyme MMP-9; lane 2: medium from control HEK; lane 3: medium from HEK exposed to 75 μ M HD; lane 4: medium from HEK exposed to 150 μ M HD; lane 5: medium from HEK exposed to 225 μ M HD; lane 6: medium from control HEK incubated with 20 ng/ml TNF α during post-incubation time. The positions of the proenzymes MMP-9 (92 kDa) and MMP-2 (72 kDa) are indicated on the left.

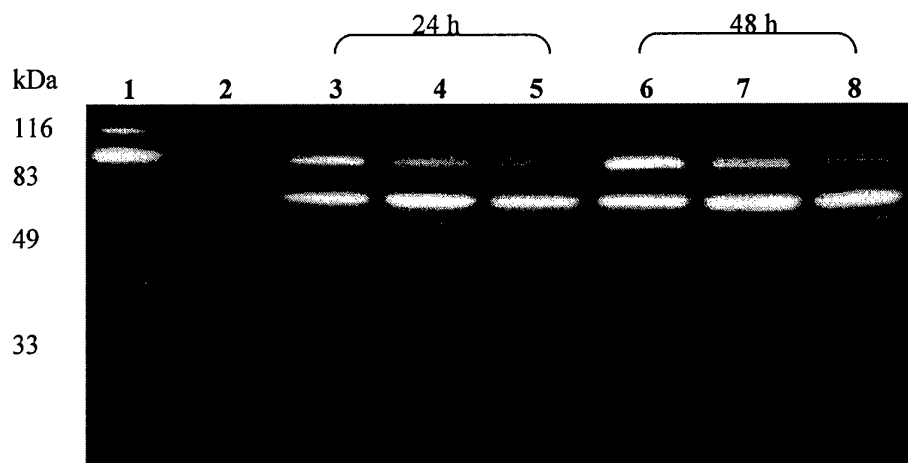


Figure 4.

Zymogram of post-incubation media from human skin pieces exposed for 4 or 6 min to saturated HD vapor. Media were collected at 24 h (lanes 3-5) and 48 h (lanes 6-8) and prepared for zymography as described in Experimental Methods. Molecular weights of marker proteins are indicated on the left. Lane 1: pure proenzyme MMP-9; lane 2: prestained molecular weight markers; lanes 3 and 6: media from control skin; lanes 4 and 7: media from skin exposed to saturated HD vapor for 4 min; lanes 5 and 8: media from skin exposed to saturated HD vapor for 6 min.

4. DISCUSSION

4.1 ^{14}C HD-adduct formation

As shown in Table 2, circa 40% of the total amount of label present in cell lysates of HEK is bound to proteins at 1 h after exposure to ^{14}C HD. This is tenfold more than the amount bound to DNA or RNA, but conform to the ratios of the quantities in which each group of macromolecules is present in the cells. Nevertheless, protein alkylation by HD appears to be substantial. Although one third of the label in the protein fraction is bound to keratins, they are not preferentially alkylated over other proteins. Interestingly, there is an alkylation preference within the keratin family for at least one keratin, K14 (50 kDa), and possibly also for K16 (48 kDa). The fluorogram (Figure 2) shows that these keratins are more sensitive to alkylation than keratins K5 and K6 (58 and 56 kDa, respectively). This might be explained from the acidic isoelectric point of the two keratins, which are both type I keratins containing a relatively high number of carboxyl groups (Steinert et al., 1985). These groups are active targets for HD alkylation (Papirmeister et al., 1991; Noort et al., 1997). The observation that keratin K14 is selectively alkylated might have consequences for the maintenance of tonofilament structures of the keratinocyte (Wilson et al., 1992). In addition, abnormalities of K14 are involved in the blistering disease of epidermolysis bullosa simplex (Fuchs, 1995). The fluorogram also shows that HD alkylates with preference some other proteins with molecular weights of 100 - 200 kDa and 30 - 40 kDa. The high molecular weight polypeptides may be subunits of laminin-5. This attachment protein of the epidermal-dermal junction has been described to become impaired after HD exposure (Smith et al., 1997), and is probably co-extracted with the keratin fraction.

The loss of label that occurs in the precipitable protein fraction at 24 h after exposure to 100 μM HD (Table 1) might be due to normal turnover of proteins, although induced degradation of damaged proteins cannot be ruled out. This large decrease in the amount of protein can not be ascribed to loss by floating of damaged cells since microscopic inspection showed a rather intact cell monolayer. Interestingly, no degradation is seen in DNA. It is probable that repair

enzymes become inactive by alkylation after exposure of cells to 100 μM HD. We noticed that in HEK that were exposed to 300 μM HD relatively more radioactive label was present in the keratin fraction than was expected (Table 2). This might be due to a decreased solubility of proteins after exposure to 300 μM HD, causing inefficient salt extraction of the soluble proteins, which leads to an impure keratin fraction. This phenomenon was clearly observed after exposure of HEK to the extremely high concentration of 875 μM HD.

The present results show that a considerable amount of label (55 %) is somewhere else in the cells than bound to DNA, RNA or protein. The label can be present as intact HD in a lipophilic environment, such as membranes and as the hydrolyzed metabolite of HD, i.e. thiodiglycol. It is also possible that the label is bound as HD adducts to other cell molecules. Based on the preference of HD for binding to sulfhydryl, phosphate or carboxy groups (Noort et al., 1997), HD might be bound to glutathion, ATP, cAMP or intermediates of the Krebs cycle.

Future experiments will learn more about alkylation of proteins in intact human skin after exposure to ^{14}C HD (Task 1). Special attention will be given to the alkylation of proteins of the epidermal-dermal junction (Task 6).

4.2 Matrix metalloprotease activity

Microblisters are formed in human skin pieces that are organ cultured for 24 or 48 h after a 4 or 6 min exposure to saturated HD vapor (present study, page 18; Mol et al., 1991). If MMPs were involved in this vesication process, this should be reflected by the presence of MMPs in the organ culture medium. However, the present experiments show that the secretion of proenzyme MMP-9 into the culture medium is reduced after exposure of cultured HEK or human skin pieces to HD. A slight increase is observed in the secretion of proenzyme MMP-2, but no increase was observed in the amount of active MMP-2 secreted into the medium. An increase should have been expected, if MMP-2 is actually involved in the degradation of extracellular matrix proteins. Altogether, it is doubtful whether proteolytic MMP-2 activity is

indeed involved in the pathophysiology of HD-exposed skin, whereas the reduction in the amount of secreted proenzyme MMP-9 suggests that there is no role for MMP-9 in inducing blister formation in HD-exposed skin.

The reduced secretion of proenzyme MMP-9 after HD exposure might be due to impaired mRNA transcription, protein synthesis, or protein kinase C activity in the epidermal keratinocytes. The production of proenzyme MMP-9 has been reported to be dependent on these processes (Mackay et al., 1992). While keratinocytes are mainly responsible for proenzyme MMP-9 secretion in skin, fibroblasts and endothelial cells of the dermis are the source of proenzyme MMP-2 (Zeigler et al., 1996). Secretion of proenzyme MMP-2 appeared to be not inhibited by HD. Possibly, the dose of HD that after penetration of the epidermis reaches the deeper layers of the dermis was not high enough to repress proenzyme MMP-2 production. Alternatively, proenzyme MMP-2 is kept in storage in intracellular pools and is released upon an HD-induced trigger. Although HD exposure of skin resulted in a slightly increased release of proenzyme MMP-2 into the culture medium, conversion of proenzyme MMP-2 into the active form did not occur. Lack of conversion into active MMP-2 could be due to inactivation by HD of the membrane-type MMP which is presumed to activate MMP-2 (Salo et al., 1994).

This study on the involvement of proteolysis in the epidermal-dermal separation (Task 2) will be continued by the assessment of the activity in culture media of two other members of the MMP family, i.e. MMP-1 and MMP-3. Because it is possible that activities of MMPs in the skin are not mirrored by their presence in culture medium, the occurrence of MMPs in the skin will be monitored immunohistochemically. Finally, assessment of the activity of other proteolytic enzymes in culture media will be performed by means of a fluorogenic assay.

5. CONCLUSIONS

From the studies conducted so far on alkylation of cellular macromolecules of HEK after exposure to ^{14}C HD, it can be concluded that

- almost 45% of the label present in cell lysates is bound to DNA, RNA and protein; the remainder is bound to other cell components or is not bound at all.
- circa 40% of the label present in cell lysates is bound to proteins.
- the group of keratin proteins seems to be not preferentially alkylated over other proteins.
- within the group of keratins, preferential alkylation is observed of at least one acidic type I keratin, i.e. K14.
- radioactive label is also prominently present on some proteins with molecular weights ranging from 100 to 200 kDa and from 30 to 40 kDa.

From the studies conducted so far on the role of proteolysis in the separation of epidermis and derms in skin that has been exposed to HD, it can be concluded that

- MMP-9 is not involved in HD-induced blister formation.
- it is doubtful whether proteolytic activity of MMP-2 is actually enhanced in HD-exposed skin.

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