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13. ABSTRACT ***(Maximum 200 Words)***

In this report the results are described of studies on protein alkylation and on proteolytic activity in human skin *ex vivo* or in cultured human keratinocytes after exposure to sulfur mustard (HD). Hypothetically, these two events can lead to the destruction of the epidermal-dermal junction of the skin after exposure to HD.
On microgram basis, proteins in the skin become alkylated by HD to the same extent as DNA or RNA and in proportion to the amount of HD used. Keratins, that are the most abundantly present proteins in epidermis, bind half of the available HD. In addition, two-dimensional gel electrophoresis shows that HD binds with preference to a selection of the proteins of the non-keratin protein pool. The observed alkylation of keratin and selected proteins may have consequences for the functioning of basal cells.
Matrix metalloproteases (MMPs) play a role in the weakening of cutaneous basement membrane zone following HD exposure. In the presence of specific inhibitors of MMPs, BB94 or BB3103, no epidermal-dermal separation occurred in human skin *ex vivo*. However, neither upregulated expression of MMPs, nor reduced expression of tissue inhibitors of metalloproteinases (TIMPs) are responsible for HD-induced epidermal-dermal separation. The results suggest that HD causes a disturbance of the basement membrane protein homeostasis as a result of diminished availability or binding capacity of the adhesion molecules rather than of enhanced degradation. Therapeutic treatment of HD-exposed skin with MMP inhibitors will keep epidermis and dermis together, thereby eliminating one of the causal factors of vesication

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Contents

2 Introduction

Vesication is a characteristic feature of sulfur mustard (HD)-induced pathology in the human skin. Alleviation of this phenomenon can possibly be obtained by preventing tearing of the basement membrane zone (BMZ), the attachment layer between epidermis and dermis consisting of a complex of structural proteins. Two possible causes that underly the destruction of the BMZ are, firstly, improper functioning of proteins involved in the attachment structure after HD-alkylation, and, secondly, breakdown of the structural proteins by proteases. It will be of value to determine to which extent these processes occur, 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 blistering, application of protease inhibitors might provide an effective therapeutic treatment. However, if extensive protein alkylation is a dominant phenomenon, therapy will probably be constrained to generic cure. In this report the results of research efforts to elucidate these items are described in conformity with the Tasks outlined in the Statement of Work.

During the 1940's the effects of HD on many proteins were investigated, generally by exposing purified proteins to extremely high concentrations of HD (for review see Papirmeister et al., 1991). It was observed that HD reacts with a wide variety of proteins, and that at neutral pH adducts of HD are formed predominantly with the carboxylic groups. The studies showed that most enzymes are resistant to inactivation by HD, only phosphokinases and some proteinases are relatively sensitive to HD. Indeed, the preference of HD for carboxylic groups in proteins has been confirmed recently with mass spectrometric techniques for HD-adduct formation in hemoglobin (Noort et al., 1997). In addition, these authors found that sulfhydryl and histidine moieties in proteins are also prone to alkylation. Despite the occurrence of protein alkylation by HD, most investigators believed for a long time that there is no direct relationship with the pathological effects of HD. However, the role of protein alkylation in the onset of skin vesication has become subject of discussion again. Recent studies have focused on the alkylation of the structural proteins of the BMZ (Zhang et al., 1995, 1998; Smith et al., 1997). The investigators found indications for alkylation by HD of laminin-1, fibronectin, uncein, and laminin-5 and they suggested that the attachment properties of the BMZ proteins could be reduced due to this alkylation, thereby leading to the loss of function of the BMZ. In direct line with that, HD-alkylation of keratins may be another determinant for the onset of vesication. In basal epidermal keratinocytes, keratins are part of the cytoskeleton, which controls essential cell functioning and, via the hemidesmosomes, regulates the anchorage between the epidermis and the dermis. From this point of view, alkylation damage to keratins may disturb basic cell functions and cell-matrix attachment, leading to vesication. Interestingly, certain keratins will be prone to alkylation by HD as they contain a relatively high amount of carboxylic groups (Steinert et al., 1985).

To date, scarce data are available on the extent of HD-protein adduct formation in keratinocytes and on the preferential protein targets in these cells. This study presents data on protein alkylation by HD in cultured human epidermal keratinocytes (HEK) and human skin *ex vivo* using ^{14}C-labeled HD. Special attention has been given to the alkylation of keratins. In addition, a prelimi-

nary study was performed by using two-dimensional gel electrophoresis, in order to reveal the distribution of labeled adducts over the total cellular protein pool.

The second option to explain dysfunctioning of the BMZ during HD-induced vesication is the breakdown of structural proteins by proteases. It was assumed that serine proteases play an important role in the onset of epidermal-dermal separation (Papirmeister et al., 1991). However, the matrix metalloproteinases (MMPs) are more likely to be involved in the degradation of the BMZ. Normal turnover of the extracellular matrix is regulated by MMPs, a family of enzymes capable of and responsible for degrading all extracellular matrix proteins including those of the BMZ (for reviews see Nagase, 1994, 1997; Kähäri and Saaralhio-Kere, 1997; Nagase and Woessner, 1999). The enzymes are secreted as latent proenzymes by fibroblasts, inflammatory cells and keratinocytes and require modification for the expression of enzyme activity. Although they are tightly regulated under normal conditions, their involvement has been shown in various pathological processes, including inflammation, tumor invasion and chronic degenerative diseases. In addition, a role is 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 Saaralhio-Kere, 1997). MMPs are also involved in the separation of basal epidermal cells from their basement membrane in equine laminitis (Pollitt et al., 1998).

The MMP family includes three subclasses, each having preferential substrates, but acting synergistically in the degradation of all major components of the extracellular matrix. 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 and MMP-9 which can specifically degrade type IV collagen, denatured collagens and laminin-5 (Gianelli et al., 1997). 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 β4 (Sires et al., 1993; von Bredow et al., 1997). Two naturally occurring inhibitors, i.e., tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2, can reduce the proteolytic activities of MMPs and keep control of protein degradation. A high expression of MMPs together with a low expression of TIMPs is thought to be indicative of basement membrane degradation.

In order to investigate the role of MMPs and TIMPs in the destruction of the BMZ following HD exposure, their expression has been determined by substrate zymography, ELISA and immunohistochemistry. An indirect proof of the involvement of MMPs in vesication of human skin *ex vivo* following HD exposure has been obtained by testing the efficacy of selected MMP inhibitors in the attenuation of the effects of HD on skin. Since laminin-5 is very susceptible to digestion by MMPs, immunohistochemical staining of this protein was used to visualize possible changes evoked by HD. Finally, the contribution of serine proteases to epidermal-dermal separation in HD-exposed skin was investigated.

3 Body of work

3.1 Experimental methods

3.1.1 Keratinocyte culture

Cultures of HEK were raised from basal keratinocytes, isolated from mammary skin obtained during cosmetic surgery. 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. For experiments, first passage HEK from cryovials were grown in serum-free keratinocyte growth medium (KGM; Clonetics/BioWhittaker[1]) at 37 °C in an atmosphere of 6% CO_2 in air. Usually, confluent monolayer cultures were achieved at 7 days after plating.

3.1.2 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 cm^2 were floated with the dermal side down in keratinocyte basal medium (KBM; Clonetics/BioWhittaker[1]) supplemented with $CaCl_2$ to a final concentration of 1.4 mM (KBMCa; 1 ml medium/well of a 12 well cluster plate) and incubated at 37 °C in an atmosphere of 6% CO_2 in air for desired time periods.

3.1.3 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 solutions with 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.

3.1.4 Exposure of keratinocyte cultures to ^{14}C-labeled HD

^{14}C HD was synthesized by the Chemical Toxicology Branch of TNO Prins Maurits Laboratory. Two batches were used during this research period. Experiments described in section 3.2.1.1 were performed with a batch of ^{14}C HD having a specific activity of 15 mCi/mmol. This compound was 95% radiochemically pure as analyzed by thin layer chromatography. Experiments described in sections 3.2.1.2 and 3.2.1.3 were performed with a second batch of ^{14}C HD having a specific activity of 56 mCi/mmol (Fidder et al., 1999). The chemical purity of this batch was 99%, determined by gas chromatography.

[1] see List of Suppliers

For exposure of cultured 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 solutions with various concentrations of ^{14}C HD (1.5 ml/well of a 6-well clusterplate) for 30 min at 25 °C. Next, 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[1]) and counted in a Tricarb 2500 TR scintillation counter (Packard[1]). In all experiments using ^{14}C HD, recovery data for radioactive counts were calculated and found to be 95-97%.

3.1.5 Exposure of skin pieces to vapor of HD or ^{14}C-labeled HD

Human mammary skin was exposed to saturated HD vapor at 25 °C for indicated time periods using a vapor cup device as described earlier (Mol et al., 1991).
To generate saturated vapor of ^{14}C HD, the filter paper in the vapor cup was impregnated with 3 µl of ^{14}C HD with a specific activity of 45 µCi/µl HD (=58 µCi/mg HD), obtained by mixing the stock of ^{14}C HD (specific activity 56 mCi/mmol) with cold HD in a ratio of 1:5.

3.1.6 Epidermal-dermal separation of human skin

Human skin was cut into pieces of 0.25 cm^2 and submerged into distilled water of 60 °C for 2 min. Next, the skin pieces were cooled in an ice-water bath for 5 min. The epidermis was then torn from the dermis using a forceps. If necessary, the epidermis and dermis were stored frozen at –70 °C until use.

3.1.7 Isolation of DNA, RNA and soluble protein from cultured HEK

Cultured HEK were dissolved in Trizol® reagent (Gibco BRL[1]). DNA, RNA and protein were isolated according to the instructions of the manufacturer. In brief, cells were lysed in Trizol® (1 ml per 10 cm^2 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 an 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 dissolved 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.
The quality of isolated DNA and RNA was checked by measuring the A_{260}/A_{280} ratio. Typical ratios for DNA and RNA were between 1.65 and 1.80, and between 1.85 and 2.00, respectively. Typical yields for DNA as well as RNA from confluent HEK cultures on a 10 cm^2 culture dish were between 35 and 50 µg. A typical yield for soluble protein was approx. 600 µg (see section 3.1.10).

[1] see List of Suppliers

To prevent RNase contamination during this isolation procedure, gloves were worn and sterile bottles and plasticware were used.

3.1.8 Isolation of DNA, RNA and soluble protein from epidermis

For isolation of RNA and soluble protein, 0.5 cm^2 of heat-separated epidermis was minced and homogenized on ice in 0.8 ml of Trizol® with 5 or 6 strokes of a glass/glass Potter homogenizer. The homogenate was left on ice for 10 min and then the homogenization was repeated. If necessary, this procedure was repeated. The homogenate was allowed to reach room temperature and was subsequently treated as described in section 3.1.7 to isolate RNA and soluble protein. Prior to precipitation of the RNA with isopropanol, 10 µl of a 1 mg/ml glycogen solution (Roche Diagnostics[1]) was added to the aqueous phase as an RNA carrier. The quality of the isolated RNA was investigated by measuring the A_{260}/A_{280} ratio. A typical RNA content of epidermis was 20 to 30 µg per cm^2, the amount of soluble protein was approx. 2.0 mg per cm^2 of epidermis. To prevent RNase contamination during this isolation procedure gloves were worn and sterile bottles and plasticware were used.
The Trizol® extraction was not suitable to isolate DNA from an epidermal homogenate. Therefore, an alternative method for isolation of DNA from epidermal samples was used, based on the Puregene® DNA isolation Kit (Biozym Nederland[1]). Heat-separated epidermal tissue (0.5 cm^2) was minced and lysed overnight at 37 °C under gentle agitation in 1 ml of 0.01 M Tris-HCl buffer (pH 7.8) containing 1 mM EDTA, 1% SDS and 0.1 mg/ml proteinase K. Thereafter, 10 µl of an RNAse solution of 2.5 mg/ml was added and the lysate was further incubated for 1 h at 37 °C. After cooling down the sample to room temperature, 400 µl of Puregene® Protein Precipitation Solution was added and the solution was vortexed at high speed for 20 sec. The tube was stored for 5 min on ice and then centrifuged for 10 min at 14,000 g. DNA was precipitated from the supernatant with 100% isopropanol and collected as a pellet after centrifugation for 5 min at 14,000 g. After washing the pellet with 70% ethanol, the pellet was dried on air for 15 min. The DNA pellet was solved in 8 mM NaOH and the quality of the isolated DNA was investigated by measuring the A_{260}/A_{280} ratio. A typical yield of DNA was approx. 65 µg per cm^2 of epidermis.

3.1.9 Total cell lysates

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

3.1.10 Quantitation of protein concentrations

Protein concentrations in samples not containing detergents were determined in a microplate assay using BioRad Protein Assay (BioRad[1]). Samples containing detergents were assessed with BioRad DC Protein Assay. Bovine serum albumin has been used as a standard.

3.1.11 Extraction of keratins from cultured HEK or from epidermis

The following method was used to obtain a keratin fraction from cultured HEK (Breitkreutz et al., 1984). Cultures were extracted for 10 min at 4 °C with an ice-cold low salt buffer (10 mM Tris-base, 150 mM NaCl, 3 mM EDTA and 0.1% nonidet-P40 (NP40), pH 7.4) and then for 10 min at 4 °C with an ice-cold high salt buffer (10 mM Tris-base, 150 mM NaCl, 1.5 M KCl, 3 mM EDTA, 0.1% NP40, pH 7.4). Next, insoluble residues were washed twice in ice-cold wash

[1] see List of Suppliers

buffer (10 mM Tris-base, 150 mM NaCl, 3 mM EDTA, pH 7.4) and dissolved in lysis buffer (0.02M Tris, pH 7.4, 1 mM EDTA, 2% SDS, 1 mM dithiothreitol (DTT)). Alternatively, the residues can be dissolved in Trizol® or in 0.1 M NaOH. All buffers contained 0.4 mM Pefabloc®, 0.5 µg/ml leupeptin and 0.5 µg/ml pepstatin (Roche Diagnostics[1]).
Epidermal pieces were minced and essentially the same procedure for keratin extraction was used as described for cultured HEK. Extractions from the epidermal pieces took place under gentle agitation. Incubation times with extraction buffers were extended to 1 h and the final extraction with lysis buffer occurred overnight at room temperature.

3.1.12 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[1]). Samples containing equal amounts of protein were analyzed by electrophoresis on 10 % Tris-HCl Ready gels (BioRad[1]) using the BioRad MiniProtean System.
Proteins on the gels were either stained with Coomassie Brilliant Blue (CBB) or transferred to nitrocellulose membranes (Costar[1]) for immunodetection of phosphoserine (Ser(P)) groups with a mouse monoclonal antibody (Sigma[1]). After incubation with a horseradish-peroxidase (HRP)-conjugated secondary antibody, blots were developed using the ECL Western blotting detection kit (Amersham Pharmacia Biotech[1]). Biotinylated molecular weight markers (BioRad[1]) were included in each blot. Neutralite-avidin-HRP conjugate (Southern Biotechnologies Associates[1]) was added to the second antibody incubation for detection of the molecular weight markers with the ECL system.

3.1.13 Indirect autoradiography (fluorography)

^{14}C labeled proteins on gels were visualized by fluorography. Gels were incubated with Amplify according to the instructions of the manufacturer (Amersham Pharmacia Biotech[1]), then dried by air-heating (GelAir Dryer; BioRad[1]) and exposed to radiographic film (Hyperfilm MP; Amersham Pharmacia Biotech[1]) for up to four weeks. Films were developed in an automatic processor.

3.1.14 Zymography

Media of keratinocyte or skin organ cultures were analyzed for the presence of MMP-2 and MMP-9 activity by gelatin zymography using gelatin containing 10% Tris-HCl Ready Gels with 50 µl slots (BioRad[1]). 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[1]), and subsequently prepared for electrophoresis without heating or reduction. After electrophoresis, the SDS was removed by washing the gels successively 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 $CaCl_2$ and 20 mM $ZnCl_2$. Gels were fixed and stained with CBB and subsequently destained. Proteolytic activity was identified as clear bands. The proenzyme forms of MMP-9 and MMP-2 can also be detected with this proce-

[1] see List of Suppliers

dure, 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 (Roche Diagnostics[1]) was included in each gel to determine the position of MMP-9.
Casein containing gels were used to detect MMP-3, which has weak gelatinolytic activity.

3.1.15 Histology

Human skin pieces were fixed overnight at 4 °C in 2% paraformaldehyde in phosphate buffered saline (PBS). They were stored in 70% ethanol until embedding in paraffin. Sections were stained with hematoxylin/eosin and examined by light microscope.
Alternatively, skin specimens were embedded in TissueTek (Sakura Finetek[1]), frozen in liquid nitrogen and stored at –70 °C until use.

3.1.16 Immunohistochemistry

Paraffin-embedded sections were dewaxed in xylene and rehydrated through graded ethanol concentrations. Cryostat sections (5 µm) were placed on gelatin-coated slides and air dried. Endogenous peroxidase activities of both paraffin-embedded and frozen sections were quenched in 0.3% hydrogen peroxide. The immunoperoxidase staining was performed using a streptavidin-biotin system (DAKO[1]) in which 3-amino-9-ethyl-carbazole (AEC) is applied as a chromogenic substrate. Monoclonal antibodies against MMP-1 (clone 41-1E5), MMP-2 (clone 42-5D11), MMP-3 (55-2A4), MMP-9 (56-2A4), TIMP-1 (clone 7-6C1) and TIMP-2 (clone T2-101) were obtained from Calbiochem[1]. Monoclonal antibodies against laminin-5 (clone GB3) was obtained from Harlan Sera-Lab[1]. For immunostaining of MMPs, TIMPs and laminin-5 frozen sections were used. After blocking non-specific antigens with 10% fetal calf serum in PBS, slides were incubated with primary antibodies for 90 min at room temperature in a humidified chamber. Biotinylated goat anti-mouse IgG antibody was applied to the sections for 10 min. The slides were then incubated with HRP-labeled streptavidin solution for 10 min and finally, AEC was converted into its coloured product. Slides were finally counterstained with hematoxylin. Negative controls were performed using 1% fetal calf serum in PBS instead of the primary antibody.

3.1.17 ELISA for MMP-1 and MMP-3

Media of HEK or skin organ cultures were analyzed for the presence of MMP-1 or MMP-3 by using Biotrak ELISA kits (Amersham Pharmacia Biotech[1]). The assays recognize total MMP-1 and MMP-3 present as proenzyme, active enzyme or (pro)enzyme complexed with its inhibitor TIMP. The assays were performed according to the instructions of the manufacturer.

3.1.18 Serine protease assay

Media of HEK or skin organ cultures were analyzed for serine protease activity by using the EnzChek protease assay from Molecular Probes[1]. with BODIPY TR-X casein as substrate. The assay was performed according to the instructions of the manufacturer with slight modifications. Trypsin was used as a positive control to create a standard curve. Samples were incubated for 18

[1] see List of Suppliers

h at 37°C. The fluorescence was measured in a CytoFluor 2350 (PerSeptive Biosystems[1]) using a 530 nm excitation filter and a 645 nm emission filter. The detection limit was defined as the amount of enzyme required to cause a 10% change in fluorescence relative to the fluorescence of the appropriate control sample.

3.1.19 Isotopic labeling of cells

Confluent cultures of HEK were incubated during 90 min with methionine/cysteine deficient KGM (Clonetics/BioWhittaker[1]) at 37 °C. Next, cells were labeled with 75 µCi/ml of ^{35}S methionine/cysteine in methionine/cysteine deficient medium (Redivue™PRO-MIX™ Cell labeling mix, specific activity ≥ 37 TBq/mmol, Amersham Pharmacia Biotech[1]) during 15 min at 37 °C. Following labeling, cultures were washed and incubated for 2 h with KGM at 37 °C before preparation for two-dimensional SDS-polyacrylamide gel electrophoresis (2-D SDS-PAGE).

3.1.20 Two-dimensional SDS-polyacrylamide gel electrophoresis (2-D SDS-PAGE)

Two-dimensional gel electrophoresis was performed at the Laboratory of Pediatrics of the Erasmus University of Rotterdam. Cultured HEK that were either isotopically labeled with ^{35}S methionine/cysteine or exposed to 400 µM ^{14}C HD and post-incubated for 4 h were lysed in buffer consisting of 9M urea, 2% (w/v) Triton-X-100, 130 mM DTT, 2%(v/v) Pharmalyte 3-10 (Amersham Pharmacia Biotech[1]) and 0.02% (w/v) Pefabloc®. Isoelectric focusing was performed with Immobiline dry strips of pH range 4 to 7 (Amersham Pharmacia Biotech[1]). Isoelectric focusing was performed for 3 h at 300 V, followed by overnight focusing at 2,200 V. Thereafter, the strips were mounted onto SDS 12 to 20% polyacrylamide gradient gels containing 3% bisacrylamide. After 2D SDS-PAGE gels were exposed to radiographic films and the films were developed.

3.1.21 Preparation of a dermal protein extract containing basement membrane proteins

The epidermis was removed from the dermis by heat separation (see section 3.1.6). Dermal extracts were prepared according to the method described by Gao and Byrstyn (1994). Tiny pieces of human dermis were incubated for 48 h at 4 °C into extraction buffer containing 50 mM Tris-HCl pH 6.8, 4% SDS, 2 M urea, 2 mM EDTA and 1 mM Pefabloc®. The extract was then dialyzed against PBS, that was supplemented with 1 mM Pefabloc®. To remove small proteins the dialyzed extract was passed over a Sephacryl S100 HR column (Amersham Pharmacia Biotech[1]) equilibrated in buffer containing 50 mM Tris-HCl pH 6.8 and 0.15 M NaCl. Fractions with high molecular weight proteins were concentrated using polyethylene glycol 40,000 and a Slide-a-Lyzer dialysis cassette (Pierce[1]). Samples were analyzed under non-reducing conditions by electrophoresis on 7.5 % Tris-HCl Ready gels (BioRad[1]), see section 3.1.12. Proteins were visualized by CBB staining. Their molecular weights were estimated with the help of molecular weight standards.

[1] see List of Suppliers

3.2 Results

3.2.1 Determination of dose- and time-dependency of HD-protein adduct formation

3.2.1.1 HD-protein adduct formation in HEK

In a first series of experiments the time-dependency of HD-adduct formation to proteins as well as DNA and RNA has been investigated. HEK have been exposed to 1.5 ml of a solution of 100 µM ^{14}C HD, corresponding to approximately 5. 10^6 dpm. Radioactivity present in the total cell lysate as well as radioactivity bound to DNA, RNA and Trizol®-soluble protein was measured at various time periods after exposure. The results are presented in Table 1. Immediately after exposure of HEK for 30 min to a solution of 100 µM ^{14}C HD, 4. 10^4 dpm was found in the cell lysate which 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 mm^2 and 0.5 µm thickness) and that equal distribution takes place between cells and the solution of ^{14}C 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 ^{14}C 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 Trizol®-soluble protein contribute for 45% to the total amount of label present in the cells. Evidently, a considerable amount of label is either bound to other molecules in the cell or freely circulating, possibly as thiodiglycol. 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 Trizol®-soluble 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, dose-dependency of HD-protein adduct formation has been studied. HEK have been exposed to 100, 200 and 300 µM ^{14}C HD. The amounts of radioactivity present in the cells and bound to DNA, RNA, Trizol®-soluble protein and keratin have been estimated at 1 h after exposure . The results as presented in Table 2 show that the amounts of label present in cell lysates and bound to DNA, RNA and Trizol®-soluble protein are approximately proportional to the concentration of HD used. For keratins a slightly higher grade of alkylation is observed with increasing concentrations of HD. However, the ratio for alkylation of the various macromolecules at a given concentration of HD is in good agreement with that for the quantities of the macromolecules in the cell culture on a microgram basis. Typical yields for DNA as well as RNA from confluent HEK cultures on a 10 cm^2 culture dish were between 35 and 50 µg. A typical yield for Trizol®-soluble protein was approx. 600 µg and for keratin approx. 200 µg. Thus, in a culture of HEK DNA, RNA, Trizol®-soluble protein and keratin are present in a ratio of approximately 1 : 1 : 15 : 5. Comparable ratios are found for bound radioactivity at the various concentrations of HD that were 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). In addition, 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 K5, K6, K14, K16 and K17. 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, with equal amounts of protein applied to each lane (as CBB stained gels demonstrate), show that 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 high and low molecular weight proteins, respectively, 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 875 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.

3.2.1.2 HD-protein adduct formation in human skin *ex vivo*

The formation of HD-adducts to protein, DNA and RNA in skin pieces after a vapor exposure of varying duration has been investigated. Pieces of human skin have been exposed to saturated vapor of ^{14}C HD for 1, 2 and 4 min at 25 °C. The filter paper reservoir in the vapor cup contained 24 µmol ^{14}C HD corresponding to 300.10^6 dpm. Square skin pieces with an area of 0.25 cm^2 were excised immediately after exposure. Two pieces of 0.25 cm^2 were used to determine the amount of label in complete skin and the distribution of label between epidermis and dermis. The results are presented in Table 3. The amounts of ^{14}C label present in the complete skin pieces increased with the duration of exposure. At 4 min after exposure about 60 % of the total

amount of label in the skin is present in the dermis and about 40 % in the epidermis. Since there is a rather linear increase in adducts in skin with the time of exposure, the amount of label found in the skin pieces after 1, 2 and 4 min of exposure to saturated vapor of ^{14}C HD was used to calculate the penetration rate of HD in this particular skin model. Based on the observation that 65,200 dpm is present in 0.5 cm^2 of skin after exposure for 4 min it was calculated that the penetration rate of HD vapor at 25°C is 0.25 µg/ cm^2.min in human skin *ex vivo*. For comparison, Renshaw et al. (1946) reported a penetration rate of 1 - 4 µg/cm^2.min at 21°C in human skin.

In addition, the amount of label was estimated that was bound to DNA, RNA, Trizol®-soluble protein and keratin in the epidermis. Table 4 shows the distribution of label over these macromolecules per 0.5 cm^2 of epidermis, measured at 4 min after exposure to ^{14}C HD at 25°C. Of the total number of counts in epidermis 0.9 % is bound to DNA, 1.4% to RNA, 26 % is associated with proteins that are soluble in Trizol®, and 47 % with the keratin fraction. The remaining 23 to 48% of labeled HD (see section 3.4.1) is bound to other cell constituents, hydrolysed into thiodiglycol or is still intact.

In order to relate the amounts of radioactive label to the occurrence of the various macromolecules in the epidermis, the amounts of DNA, RNA, soluble protein and keratin in epidermis have been assessed. Per 0.5 cm^2 of epidermis, 31 ± 2.2 µg DNA, 11 ± 1.8 µg RNA, 992 ± 147 µg Trizol®-soluble protein, and 1547 ± 57 µg keratin are present. On microgram basis, the amounts of DNA, RNA, Trizol®-soluble protein and keratin are in the ratio of 1 : 0.3 : 32 : 50., The amounts of radioactive label bound to these macromolecules at 4 min after exposure to ^{14}C HD are in the ratio of 1 : 1.5 : 27 : 57. Unless the RNA content of epidermis is underestimated, there seems to be a preference for RNA alkylation, whereas the other macromolecules have become alkylated in proprotion to their occurrence.

3.2.1.3 Survey of proteins bearing ^{14}C HD-adducts

2-D SDS-PAGE was used to reveal the distribution of labeled adducts over the total cellular protein pool. Figure 3a shows a 2-D gel map of proteins from confluent cultures of HEK, obtained by isotopic labeling of cultures with ^{35}S methionine/cysteine. The proteins have been separated in the first dimension between pH 4 and 7, covering the proteins with an acidic to neutral isoelectric point. The map shows the proteins within this range that are potentially available in HEK for adduct formation by HD. Figure 3b shows a 2-D gel pattern of proteins in the same range that contain ^{14}C HD-adducts following exposure of HEK to 1 mM ^{14}C HD. Comparison of both gels shows that a limited number of proteins from the total protein pool of HEK bears ^{14}C HD-adducts. It is expected that one of the two prominent spots with a MW of appoximately 50 kDa is keratin 14 indicated with an arrow in Figure 3b. Keratin 5 with an isoelectric point of 7.4 is beyond the pH range of this gel. This result indicates that adducts are formed in a selective instead of a non-discriminative way. So far, it has not been investigated which proteins had become alkylated by HD. However, identification will be possible in future experiments by extracting the protein spots from the gel and subjecting them to mass spectrometric analysis. For now, it can be concluded from these photographs that keratins, which are easily recognized by their location on the gel, belong to the group of proteins that are preferably alkylated by HD.

3.2.2 Determination of the onset of proteolysis induced by HD

3.2.2.1 Serine protease activity

Cultured HEK were exposed for 30 min to various concentrations of HD and after a post-incubation period of 24 h culture media were collected and analysed for proteolytic activity with the EnzCheck assay. Similarly, proteolytic activity was determined in organ culture media collected at 24 and 48 h after exposure of human skin pieces to saturated vapor of HD at 25°C. A standard curve was created using trypsin dissolved in KGM or KBMCa, depending on the origin of the samples. It appeared that the activity of trypsin is reduced when KGM or KBMCa were used as assay buffers instead of Tris-HCl buffer as recommended by the manufacturer. Using KGM or KBMCa as assay buffer, the detection limit of this assay was 100 ng trypsin/ml which is two orders of magnitude lower than with the use of Tris-HCl buffer. The results are shown in Figure 4. Very low serine protease activity was found in media of control HEK or control human skin pieces. A small increase (<10%) in serine protease activity is only seen in media of HEK that were exposed to the highest HD concentration used, i.e., 150 µM (Figure 4a). No significant increase in serine protease activity is seen in organ culture media of skin pieces that were exposed to saturated vapor of HD (Figure 4b).

3.2.2.2 Zymographic analysis of MMP-2 and MMP-9 present in culture media

Culture media were collected at 6 and 24 h of post-incubation after exposure of HEK for 30 min 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 (92 kDa) 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 places 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 two other gelatinases with molecular weights of 135 and 225 kDa, in addition to proenzyme MMP-9 (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 5, lane 2). The second clear band represents the latent form of MMP-2 (72 kDa), which is constitutively produced in low amounts by cultured human keratinocytes (Sarret et al., 1992). TNFα 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 TNFα-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 0, 4 or 6 min to saturated vapor of HD at 25 °C 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 6, 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. However, the amounts of proenzyme MMP-2 are apparently increased in these media compared to medium of control skin, while the quantities of the active form of MMP-2 remained unchanged.

3.2.2.3 Immunochemical analysis of MMP-1 and MMP-3 present in culture media

As casein zymography in our hands appeared to be not successful for detection of MMP-3, ELISA kits were used to measure the presence of MMP-1 and MMP-3 in culture media of HEK or skin pieces that had been exposed to HD. These assays detect the combined amounts of proenzyme, activated enzyme and enzyme-TIMP complex, but not the activity of the enzyme. At 24 h after exposure of cultured HEK to HD, the amounts of MMP-1 and MMP-3 were elevated with increasing concentrations of HD, with a maximum effect of 2- to 4- fold of the control values in media from HEK exposed to 100 µM HD (Figures 7a and 7b). When cells were exposed to higher concentrations of HD, toxic effects are probably dominating, leading to a decrease in the secretion of the two enzymes. Control pieces of human skin secrete MMP–1 and –3 in the medium constitutively over 24 or 48 h. When the human skin pieces had been exposed to saturated vapor of HD for 4 or 6 min the amount of MMPs secreted into the medium during 24 or 48 h is significantly inhibited (Figures 8a and 8b).

3.2.2.4 Immunohistochemical analysis of MMP-1, -2, -3, -9, TIMP-1 and TIMP-2

Skin pieces that had been exposed for 5 min to saturated HD vapor at 25 °C were organ cultured for 6, 16, 24 or 48 h and processed for immunohistochemistry. Antibodies to MMP–1, -2, -3, and -9 have been used to study spatial and temporal localization of the MMPs in the skin after exposure to HD. The antibodies recognize the latent as well as the active forms of the enzymes. Photographs of control and HD-exposed skin after post-incubation times of 6, 24 or 48 h are shown in Figures 9 through 12. The staining of skin collected at 16 h after exposure to HD was not different from that observed in skin at 24 h after exposure.

In fresh control skin, staining of MMP-2, -3 and –9 is absent or very faint (not shown).For MMP-1, a staining of fibroblasts and basal epidermal cells is observed, which resembles closely that of the control section at 6 h post-incubation (Figure 9a). The staining intensity of MMP-1 in control as well as in HD-treated skin increases with culture time and no differences in expression of MMP-1 are observed between control skin and HD-exposed skin at the various time points. Interestingly, the expression of MMP-1 in control skin and HD-exposed skin are compa-

rably intense after 48 h of organ culture, while in epidermal-dermal separation occurs only in the HD-exposed skin.

Also the expression of MMP-2 increases slightly with time in control skin that is maintained in organ culture (Figure 10). Staining of MMP-2 shows a maximal intensity in the epidermis at 24 h and is slightly decreased at 48 h. In HD-exposed skin a faint staining of MMP-2 is observed at 6, 24 and 48 h of organ culture. The intensity of this staining is comparable or even lower than that observed in control skin.

Expression of MMP-3 appears in small amounts in the epidermis and the upper dermis of control skin during organ culture (Figure 11). The highest level of expression is seen at 48 h. When skin has been exposed to saturated vapor of HD the expression of MMP-3 seems to be suppressed.

The expression of MMP-9 in the epidermis of control skin increases somewhat with culture time and reaches its highest level at 24 h of organ culture (Figure 12). The staining intensity returns to a lower level at 48 h. Exposure of skin to saturated vapor of HD causes a moderate peak in the staining intensity of MMP-9 at 6 h of post-incubation. At later time points this staining had diminished to a lower intensity.

Since not only a high expression of MMPs, but also a low expression of the TIMPs might be indicative for proteolytic activity, it was investigated whether the expressions of TIMP-1 and –2 varied between control skin and HD-exposed skin. Staining of TIMP-1 and TIMP-2 was negative in fresh control skin (not shown). During the organ culture period a faint staining of TIMP-1 is observed in control skin (Figure 13). In the epidermis of HD-treated skin, staining for TIMP-1 shows a slight increase at 6 and 24 h after exposure compared to control skin. At 48 h this staining is reduced. The expression of TIMP-2 in control skin is faint and was only slightly increased at 48 h of organ culture (Figure 14). However, when skin has been exposed to vapor of HD the expression of TIMP-2 at 6 h of organ culture is enhanced compared to control skin. The staining is less intense at 24 and 48 h of organ culture and is seen under the basal cells, along the BMZ. In a microblister TIMP-2 is present at the blister roof.

3.2.3 Determination of protease inhibitor effects on proteolysis induced by HD

We have not performed experiments with serine protease inhibitors on cultured HEK since no serine protease activity was found in the culture media. The effects of MMP-inhibitors on epidermal-dermal separation were studied in human skin pieces. These experiments have been described in section 3.2.4.

3.2.4 Determination of the efficacy of selected protease inhibitors in the attenuation of the effects of HD on skin tissue

An indirect proof of the involvement of MMPs in vesication of human skin *ex vivo* following HD exposure was obtained by testing the efficacy of several MMP inhibitors in the attenuation of the effects of HD. Two synthetic inhibitors of MMPs were kindly provided by British Biotech, Oxford UK, i.e., BB94, or batimastat, and BB3103. Another inhibitor of MMPs that has been tested is *trans* retinoic acid (tRA), that is reported to inhibit MMP-1, -3 an –9 (Fisher et al.,

1996). These three MMP inhibitors have been added to the culture medium of HD-exposed skin pieces. BB94 and tRA were dissolved in DMSO and added with a maximum concentration of 1% vehicle.

Without any additive, exposure of human skin pieces to saturated vapor of HD at 25 °C for 5 min results in clear epidermal damage with pyknotic nuclei and in microvesication after a culture period of 48 h in KBMCa (Figure 15b). When the BB94 (0.5 µg/ml) or BB3103 (0.5 mg/ml) were added to the culture medium, the epidermal-dermal separation was completely prevented (Figures 15c and 15d). Addition of tRA (30 µg/ml) to the culture medium caused partial protection against epidermal-dermal separation (Figure 15e). The MMP inhibitors did not reduce the HD-induced cellular necrosis within the overlying epidermis. The presence of the vehicle DMSO (1%) had no attenuating effect on the lesion (Figure 15f).

3.2.5 Characterisation of hemidesmosomal damage sites with regard to HD-induced changes and protection by protease inhibitors

The proteins of the BMZ in human skin are targets for the proteolytic degradation by MMPs that is induced following HD exposure. Since laminin-5 is very susceptible to digestion by MMPs (Gianelli et al., 1997), this protein was immunohistochemically stained to assess changes due to HD-induced MMP-activities. Antibody staining was performed on sections of human skin obtained at 48 h after exposure for 5 min to saturated vapor of HD at 25 °C. Under these conditions, epidermal-dermal separation occurs at the basement membrane as observed by histological examination (Figure 15 b). As shown in Figure 16, laminin-5 is present as a continuous line along the BMZ of control skin. Laminin-5 staining is present exclusively at the dermis of the microblister that developed in HD-exposed skin, which indicates that HD-induced cleavage in the BMZ occurs in the upper lamina lucida, close to the basal cells The observed staining in HD-exposed skin is at least as intense as in control skin, but some interruptions of the red staining occur along the blister floor. When HD-exposed skin has been cultured in medium containing 0.5 µg/ml BB94, the expression of laminin-5 is continuous and comparable to that in control skin.

3.2.6 Examination of specific hemidesmosomal and extracellular matrix molecules for evidence of HD alkylation

To examine the extent of alkylation by HD of BMZ proteins in human skin, it was envisaged to purify selected BMZ proteins from a dermal protein extract by immunoprecipitation. Next, the presence of ^{14}C HD-adducts could be detected on each of the proteins purified from human skin that had been exposed to saturated vapor of ^{14}C HD. Only a first step of this plan, i.e., the preparation of a protein fraction with high molecular weight BMZ proteins from a dermal extract, has been performed and is described here.

Heat separation was used to separate the epidermis from the dermis of normal human skin. With this treatment the BMZ proteins of the lower lamina lucida, i.e., laminin-1, laminin-5 and collagen type IV, are present on top of the dermis (Allen et al., 1997). The dermis is used to prepare a protein extract. Next, a fraction with high molecular weight proteins was obtained by gel filtration and separated on a 7.5 % SDS-PAGE gel under nonreducing conditions. Figure 17 shows that several high molecular weight proteins are present in the gel, while one or more protein

complexes with very high molecular weights can not enter the gel. Sharp bands of at least six proteins are visible in the gel. Two protein bands with a molecular weight of about 120 and 150 kDa and two doublets of about 240 and 300 kDa are observed. Time was lacking to identify the proteins by immunochemistry, but it is supposed that the high molecular weight protein fraction of the dermal extract contains glycoproteins like nidogen (150 kDa), the 120 kDa proteolytic fragment of BP180, laminin-1 (900 kDa) and laminin-5 (400 kDa). The extracts will be used in future experiments to purify individual BMZ proteins by immunoprecipitation and to determine the extent of ^{14}C HD-protein adduct formation in skin that had been exposed to ^{14}C HD.

In a preliminary study, a protein extract was prepared from dermis of skin that had been exposed for 5 min to saturated vapor of ^{14}C HD at 25 °C. After gel filtration, the amount of radioactivity present in the fraction containing high molecular weight proteins was determined. It was calculated that in this protein extract about 1 molecule of HD was present on 100 molecules of high molecular weight protein with an assumed molecular weight 140 kDa.

3.2.7 Study of the changes in hemidesmosome formation and functioning in cells that are able to assemble hemidesmosomes, e.g. in cell line 804G

The studies on protein alkylation and protein degradation in skin exposed to HD yielded so much interesting information, that the task on the formation and functioning of hemidesmosomes has received low priority.

3.3 Tables and figures

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 approx. 5.10^6 dpm. Results are given in 10^3 dpm, and are expressed as mean (x ± 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)	Trizol®-soluble 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 ± s.e.m.) of two or three experiments, each performed in duplicate. Percentage of total counts in cell lysate is given between brackets.

Conc HD (μM)	Cell lysate (10^3dpm)	DNA (10^3dpm)	RNA (10^3dpm)	Trizol®-soluble protein (10^3dpm)	Keratin (10^3dpm)
100	28.4 ± 0.7	0.9 ± 0.0	0.8 ± 0.1	11.2 ± 0.8	3.5 ± 0.2
	(100%)	(3 %)	(3 %)	(39%)	(12%)
200	57.6 ± 2.1	1.7 ± 0.3	1.8 ± 0.1	26.1 ± 0.9	9.2 ± 0.5
	(100%)	(3%)	(3%)	(45%)	(16%)
300	83.8 ± 2.1	2.8 ± 0.2	2.7 ± 0.2	33.5 ± 2.3	15.3 ± 1.5
	(100%)	(3%)	(3%)	(40%)	(18%)

Table 3

Amounts of radioactive label present in 0.5 cm^2 of total human skin, epidermis or dermis immediately following exposure to saturated vapor of ^{14}C HD at 25 °C. In each determination two skin pieces of 0.25 cm^2 were used. Results are presented as the mean (x ± s.e.m.) of two or three experiments.

Duration of exposure (min)	Radioactive label in 0.5 cm^2 of total skin (10^3 dpm)	Radioactive label in 0.5 cm^2 of epidermis (10^3 dpm)	Radioactive label in 0.5 cm^2 of dermis (10^3 dpm)
1	18.3 ± 3.7	8.2 ± 1.8	8.1 ± 1.2
2	38.5 ± 3.2	10.4 ± 0.9	16.8 ± 1.2
4	65.2 ± 4.3	23.3 ± 1.1	40.2 ± 4.8

Table 4

Amounts of radioactive label bound to cellular macromolecules present in 0.5 cm^2 of human epidermis immediately following exposure for 4 min to saturated vapor of ^{14}C HD at 25 °C. In each determination two skin pieces of 0.25 cm^2 were used. Results are presented as the mean (x ± s.e.m.) of two or three experiments. The mean of total counts present in 0.5 cm^2 of epidermis is 24,300 ± 900 dpm.

Cellular macromolecules	Counts bound to specific macromolecule in 0.5 cm^2 of epidermis			Amount of specific macromolecule present in 0.5 cm^2 epidermis	
	(10^3 dpm)	(% of total counts)	ratio	µg	ratio
DNA	0.22 ± 0.01	0.9	1.0	31 ± 2.2	1.0
RNA	0.33 ± 0.04	1.4	1.5	11 ± 2.8	0.3
Trizol ®-soluble protein	6.0 ± 0.3	24.7	27.4	992 ± 147	32.0
Keratin	12.6 ± 0.8	51.9	57.6	1547 ± 57	49.9

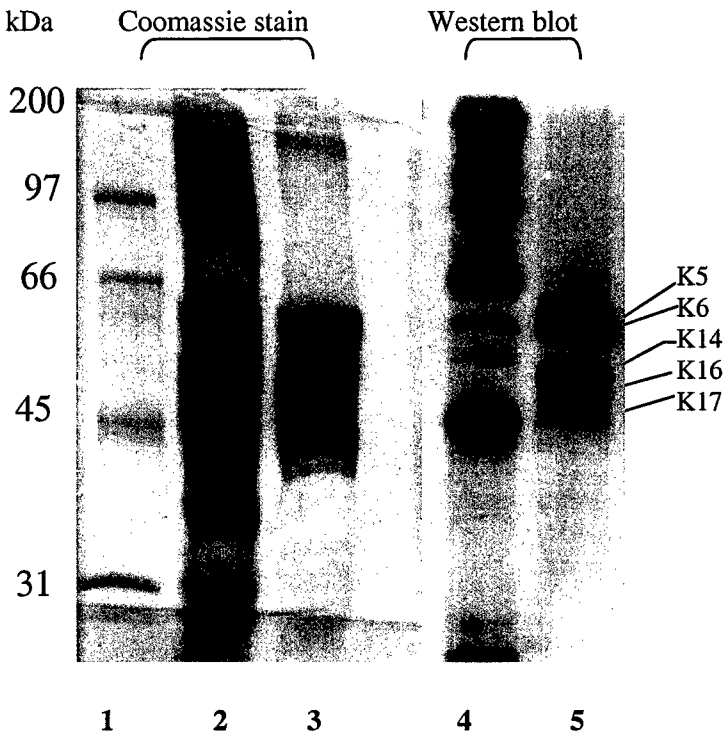


Figure 1

SDS-PAGE and Western blot analysis of keratin extracts made from confluent control cultures of HEK. 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 (lanes 4 and 5). Lanes 1 and 4: molecular weight markers. Lane 2: total protein extract prepared from HEK. Lane 3: keratin extract prepared from HEK. Lane 5: chemiluminescent detection of serine-phosphorylated proteins with monoclonal antibody against Ser(P) in a keratin extract prepared from HEK.

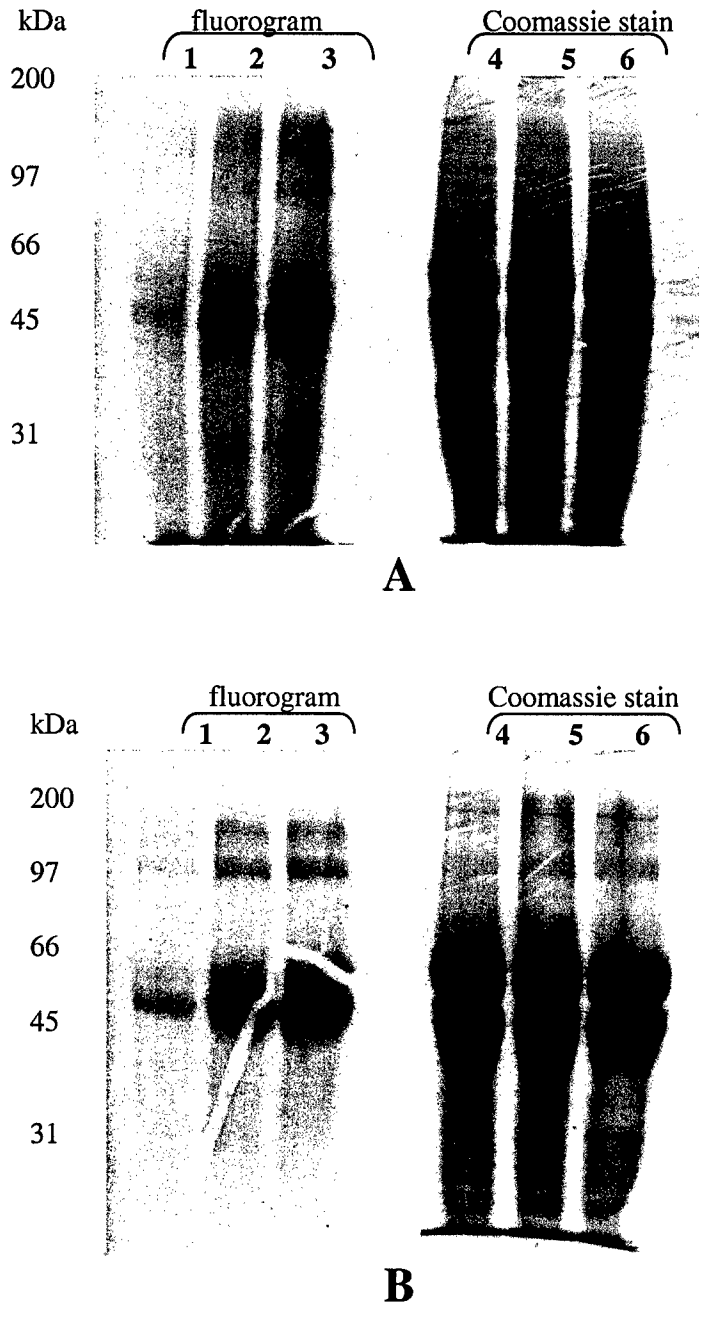


Figure 2

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 1 h after a 30 min 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). Positions of molecular weight markers are indicated on the left.

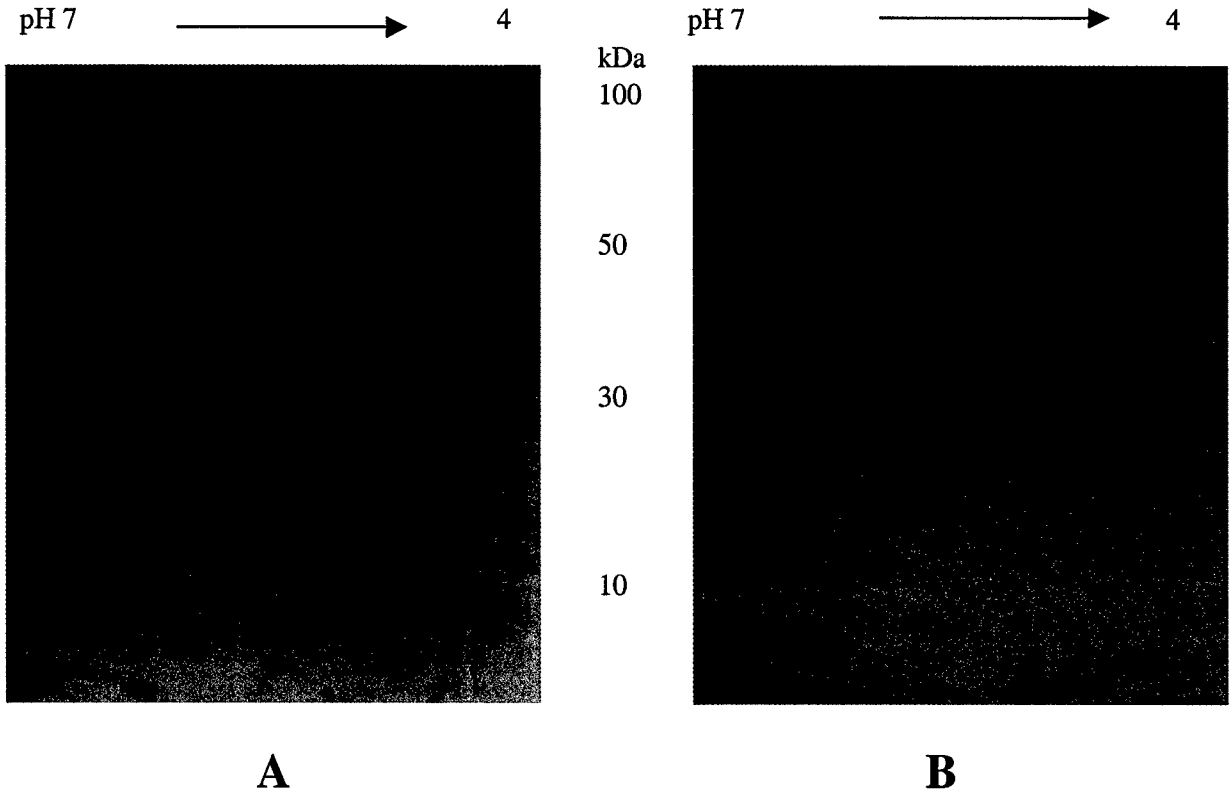


Figure 3

2-D SDS-PAGE patterns of (A) ^{35}S-methionine/cysteine labeled proteins from total cell lysates of cultured control HEK and of (B) ^{14}C-HD-adducted proteins from total cell lysates of cultured HEK that were exposed to 1 mM ^{14}C-HD. A limited number of proteins in the HEK has bound adducts of HD, indicating a selective instead of a non-discriminative alkylation of proteins by HD. The spot indicated with an arrow is thought to be keratin 14.

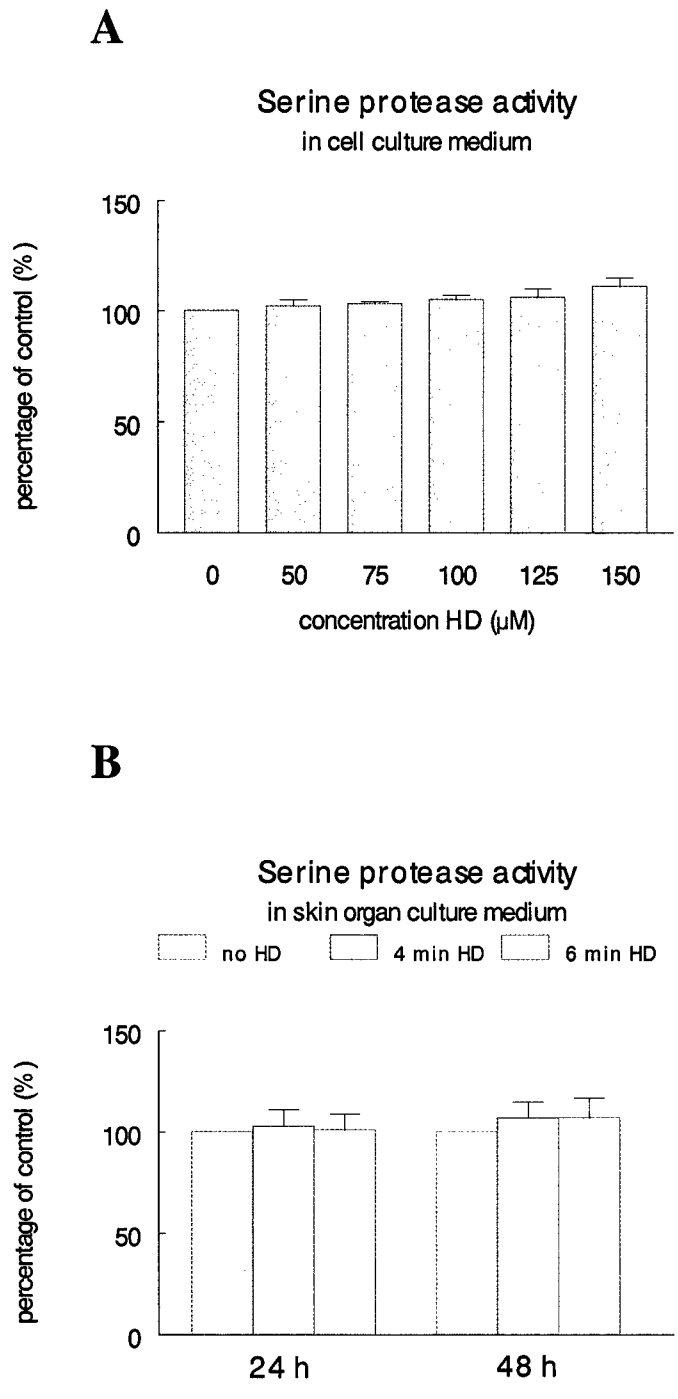


Figure 4

Serine protease activity in (A) culture media of HEK or (B) human skin pieces after exposure to HD. Cultured HEK were exposed for 30 min to 0 – 150 µM HD and media were collected after a post-incubation period of 24 h. Human skin pieces were exposed for 0, 4 or 6 min to saturated HD vapor at 25 °C and media were collected after 24 and 48 h of post-incubation. Data are presented as mean (x ± s.e.m) of four determinations.

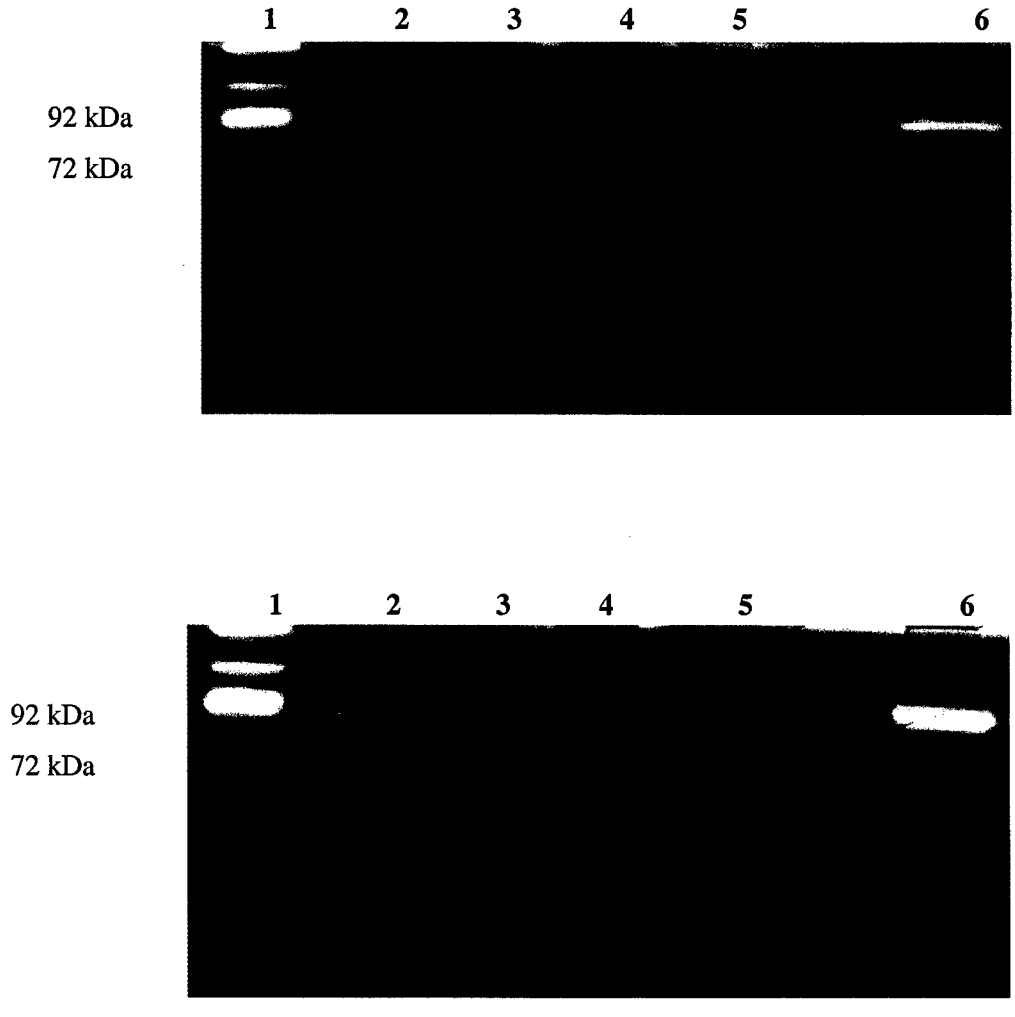


Figure 5

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. Lane 1: purified proenzyme MMP-9; lane 2: medium from control HEK; lane 3: medium from HEK exposed to 75 µM HD; lane 4: medium from HEK exposed to 150 µM HD; lane 5: medium from HEK exposed to 225 µM HD; lane 6: medium from control HEK incubated with 20 ng/ml TNFα during post-incubation time. The positions of the proenzymes MMP-9 (92 kDa) and MMP-2 (72 kDa) are indicated on the left.

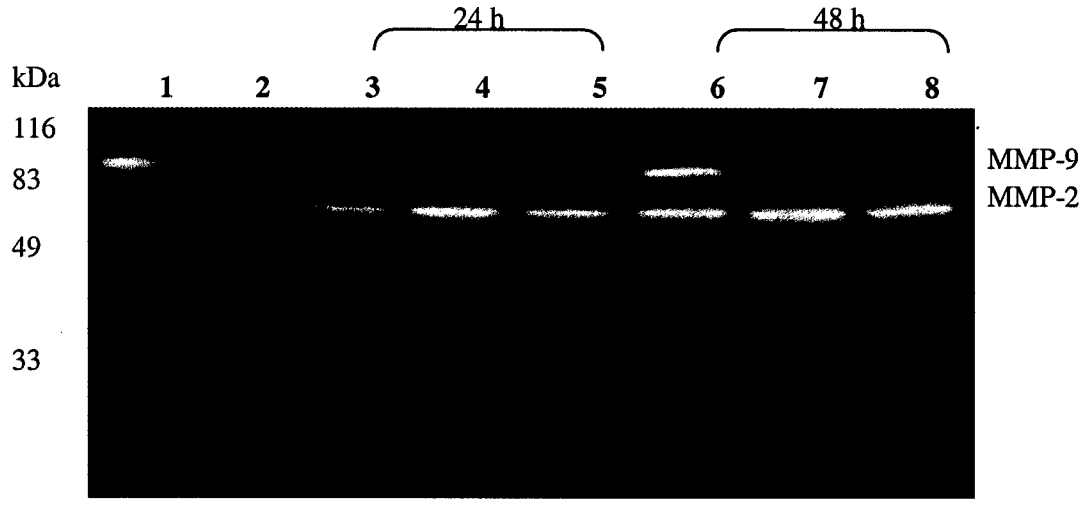


Figure 6

Zymogram of post-incubation media from human skin pieces exposed for 0, 4 or 6 min to saturated HD vapor at 25 °C. Media were collected at 24 h (lanes 3-5) and 48 h (lanes 6-8) and prepared for zymography. Molecular weights of marker proteins are indicated on the left. Lane 1: proenzyme MMP-9; lane 2: 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.

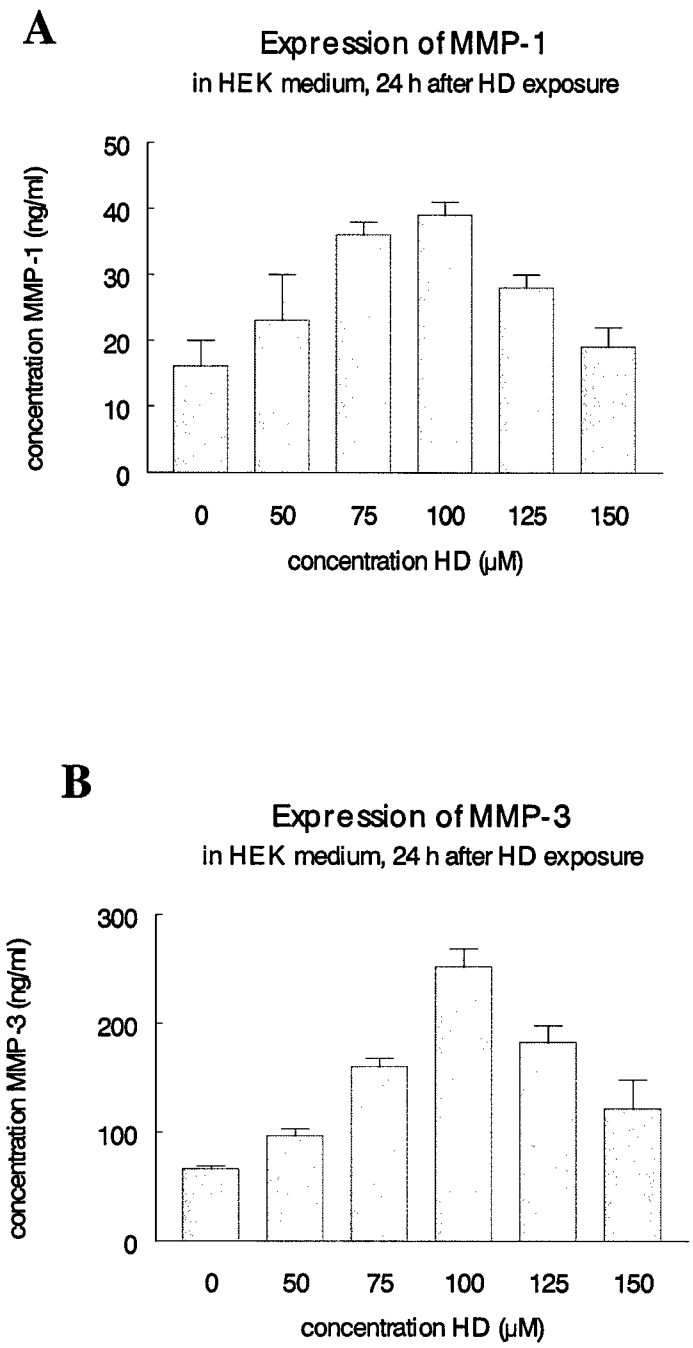


Figure 7

Release of (A) MMP-1 and (B) MMP-3 in culture media at 24 h after exposure for 30 min of HEK to 0 – 150 µM HD. Values represent the mean (x ± s.e.m.) of eight determinations.

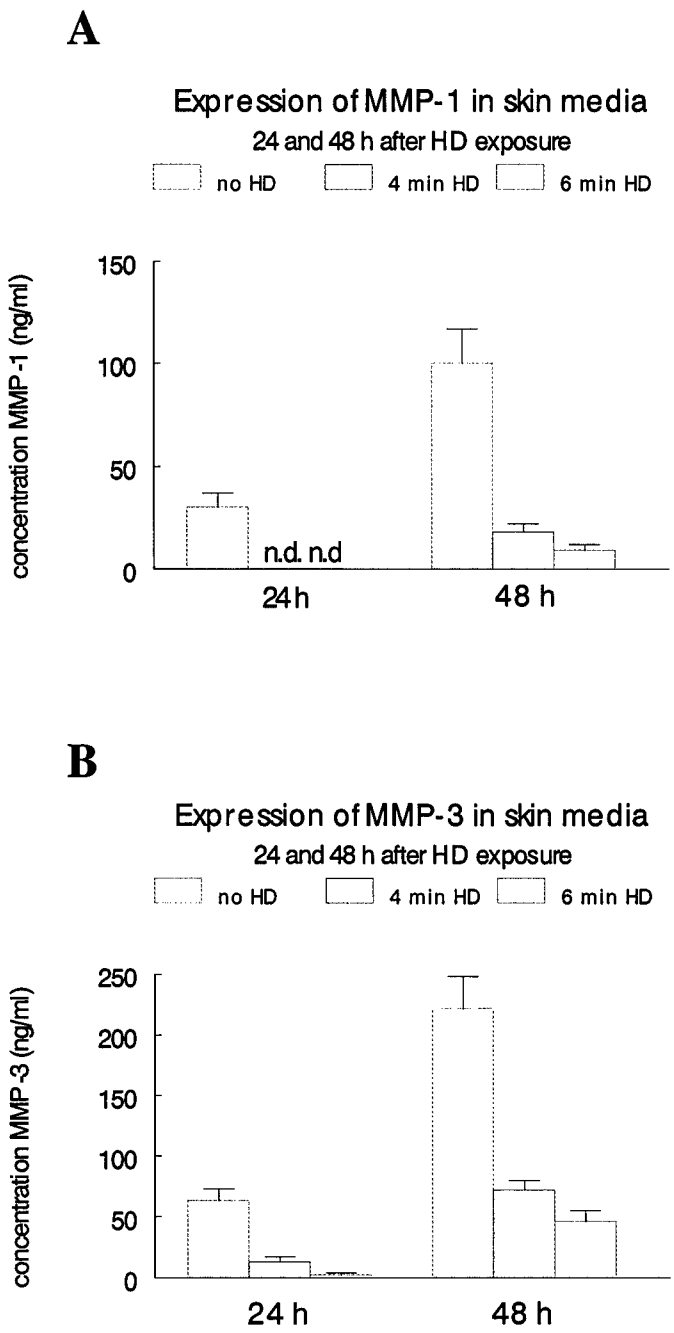
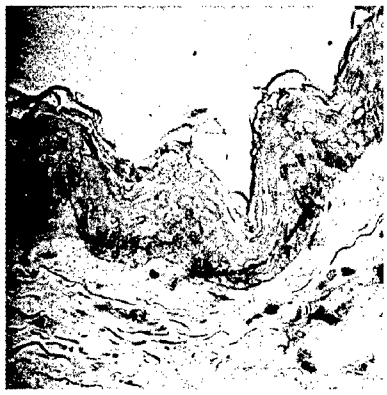


Figure 8

Release of (A) MMP-1 and (B) MMP-3 in culture media at 24 h and 48 h after exposure of human skin pieces to saturated HD vapor for 0, 4 or 6 min at 25 °C. Values represent the mean (x ± s.e.m.) of five to nine determinations; n.d. is below detection limit.

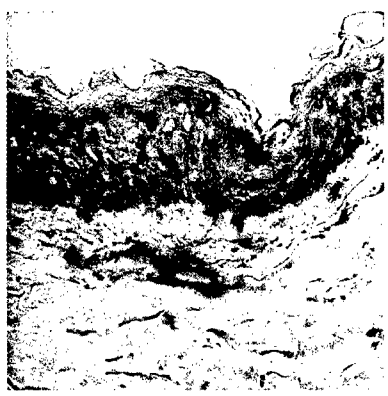


A. Control, 6 h post-incubation time

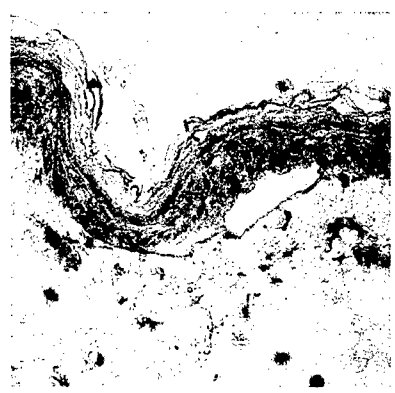
B. HD, 6 h post-incubation time

C. Control, 24 h post-incubation time

D. HD, 24 h post-incubation time



E. Control, 48 h post-incubation time

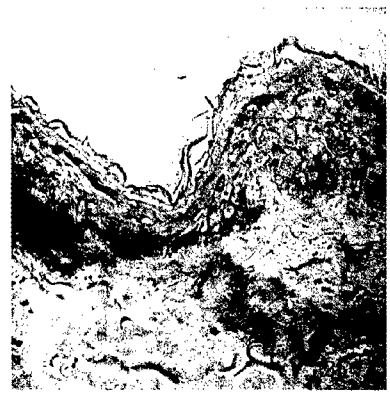


F. HD, 48 h post-incubation time

Figure 9

Immunohistochemical staining of MMP-1 in human control skin (A, C, E) and in HD-exposed human skin (B, D, F) at 6, 24 and 48 h of organ culture after exposure for 5 min to saturated vapor of HD at 25 °C. The staining intensity of MMP-1 in control as well as in HD-treated skin increases with culture time and no differences in expression of MMP-1 are observed between control skin and HD-exposed skin at the various time points.

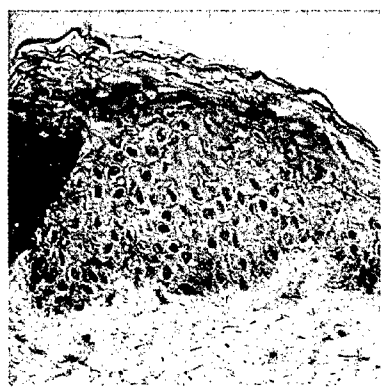
A. Control, 6 h post-incubation time



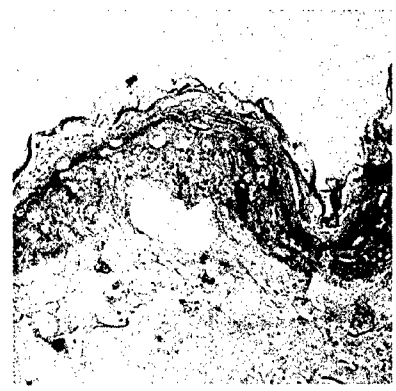
B. HD, 6 h post-incubation time

C. Control, 24 h post-incubation time

D. HD, 24 h post-incubation time



E. Control, 48 h post-incubation time

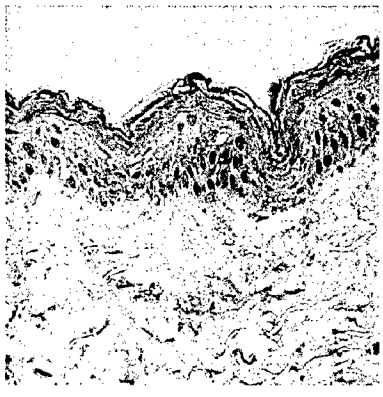


F. HD, 48 h post-incubation time

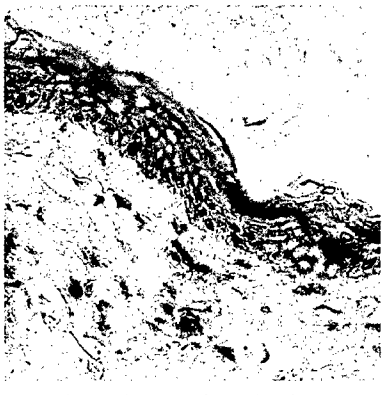
Figure 10

Immunohistochemical staining of MMP-2 in human control skin (A, C, E) and in HD-exposed skin (B, D, F) at 6, 24 and 48 h of organ culture after exposure for 5 min to saturated HD vapor at 25 °C. In control skin staining of MMP-2 shows a maximal intensity in the epidermis at 24 h and is slightly decreased at 48 h. In HD-exposed skin a faint staining of MMP-2 is observed at 6, 24 and 48 h of organ culture.

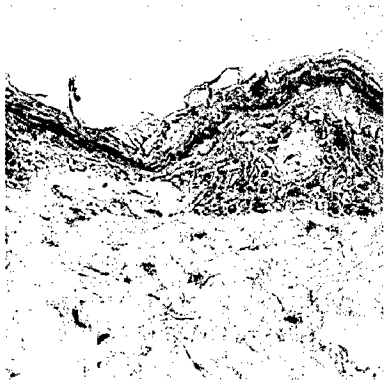
A. Control, 6 h post-incubation time



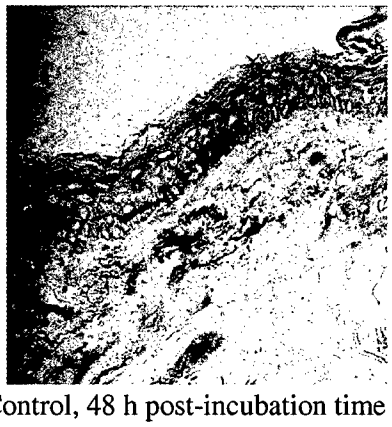
B. HD, 6 h post-incubation time



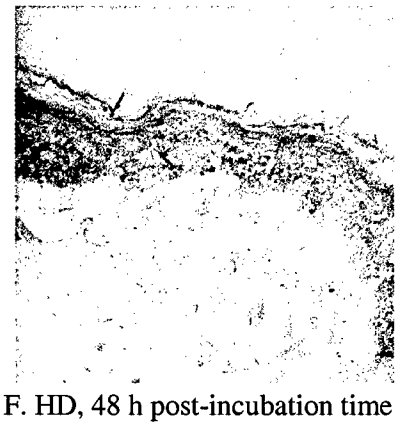
C. Control, 24 h post-incubation time



D. HD, 24 h post-incubation time



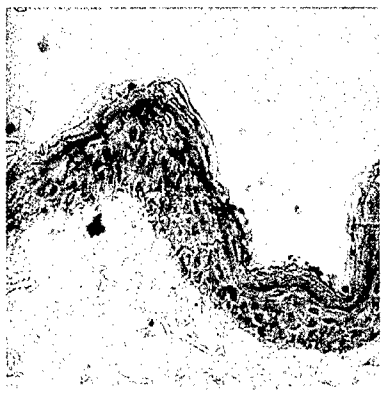
E. Control, 48 h post-incubation time



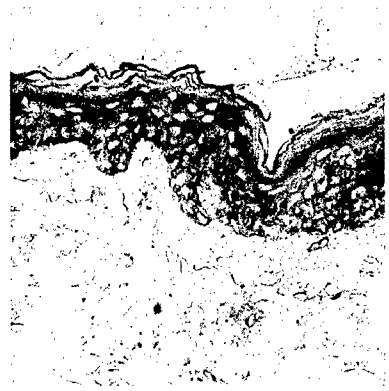
F. HD, 48 h post-incubation time

Figure 11

Immunohistochemical staining of MMP-3 in human control skin (A, C, E) and in HD-exposed skin (B, D, F) at 6, 24 and 48 h of organ culture after exposure for 5 min to saturated vapor of HD at 25 °C. Expression of MMP-3 appears in small amounts in the epidermis and the upper dermis of control skin during organ culture. The highest level of expression is seen at 48 h. When skin has been exposed to saturated vapor of HD the expression of MMP-3 seems to be suppressed.

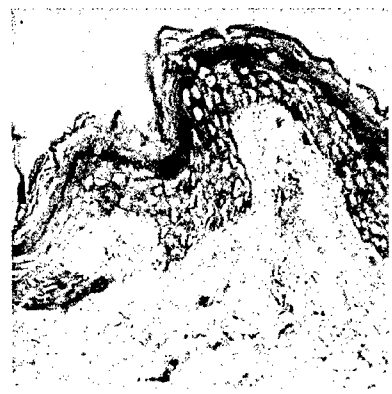


A. Control, 6 h post-incubation time

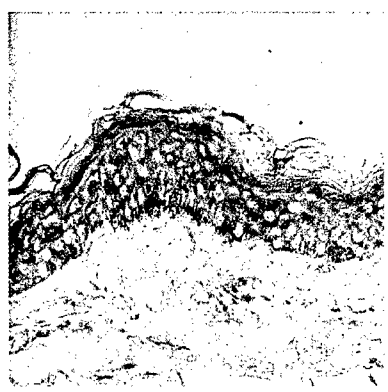


B. HD, 6 h post-incubation time

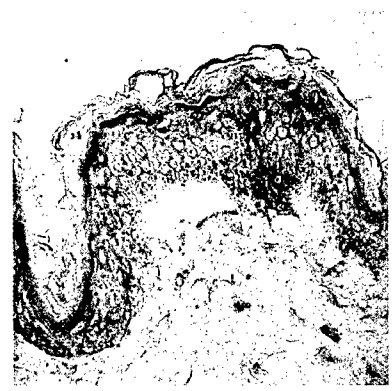
C. Control, 24 h post-incubation time



D. HD, 24 h post-incubation time



E. Control, 48 h post-incubation time

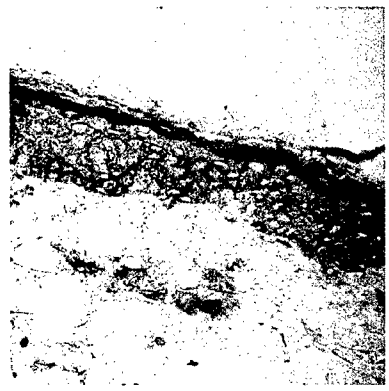


F. HD, 48 h post-incubation time

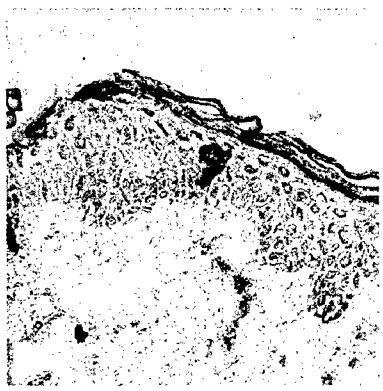
Figure 12

Immunohistochemical staining of MMP-9 in human control skin (A, C, E) and in HD-exposed human skin (B, D, F) at 6, 24 and 48 h of organ culture after exposure for 5 min to saturated vapor of HD at 25 °C. The expression of MMP-9 in the epidermis of control skin increases with culture time and reaches its highest level at 24 h of organ culture. The staining intensity returns to a lower level at 48 h. After exposure of skin to saturated vapor of HD the highest staining intensity of MMP-9 is observed at 6 h of post-incubation. At later time points this staining had diminished to a lower intensity.

A. Control, 6 h post-incubation time

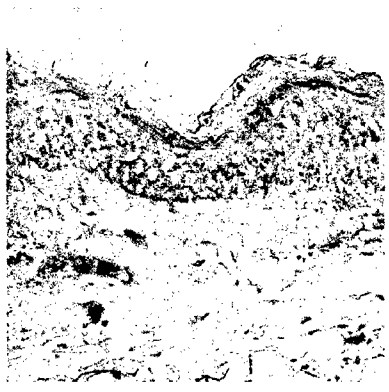


B. HD, 6 h post-incubation time

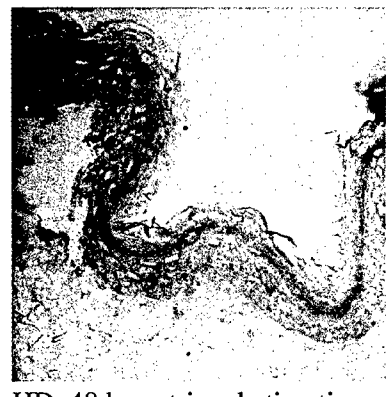


C. Control, 24 h post-incubation time

D. HD, 24 h post-incubation time



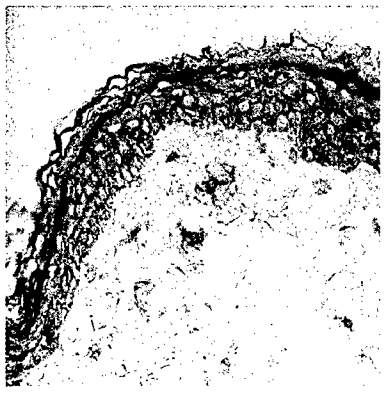
E. Control, 48 h post-incubation time



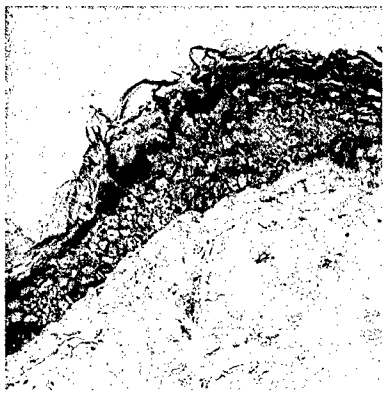
F. HD, 48 h post-incubation time

Figure 13

Immunohistochemical staining of TIMP-1 in control human skin (A, C, E) and in HD-exposed human skin (B, D, F) at 6, 24 and 48 h of organ culture after exposure for 5 min to saturated vapor of HD at 25 °C. During the organ culture period a faint staining of TIMP-1 is observed in control skin. In the epidermis of HD-treated skin, staining for TIMP-1 shows a slight increase at 6 and 24 h after exposure compared to control skin. At 48 h this staining is strongly reduced.



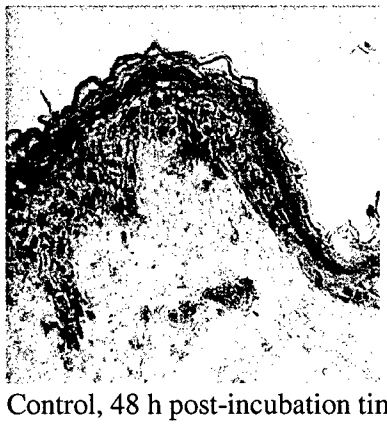
A. Control, 6 h post-incubation time



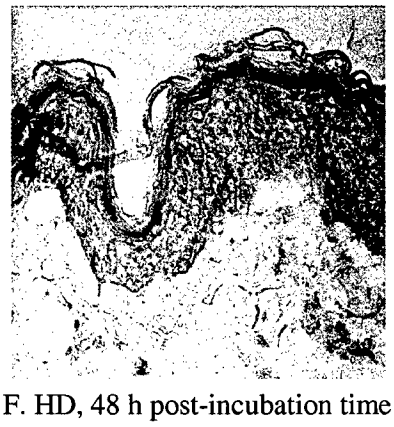
B. HD, 6 h post-incubation time

C. Control, 24 h post-incubation time

D. HD, 24 h post-incubation time



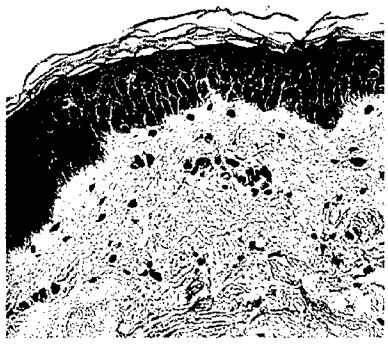
E. Control, 48 h post-incubation time



F. HD, 48 h post-incubation time

Figure 14

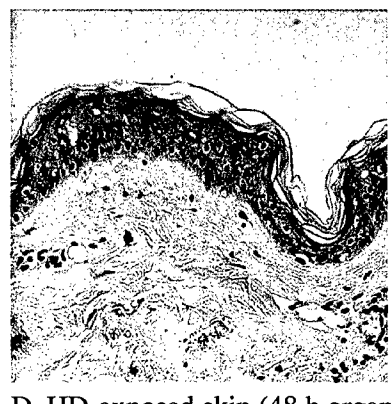
Immunohistochemical staining of TIMP-2 in control human skin (A, C, E) and in HD-exposed human skin (B, D, F) at 6, 24 and 48 h of organ culture after exposure for 5 min to saturated vapor of HD at 25°C. The expression of TIMP-2 in control skin is only slightly increased at 48 h of organ culture. When skin has been exposed to vapor of HD the expression of TIMP-2 at 6 h of organ culture is enhanced compared to control skin. The staining is less intense at 24 and 48 h of organ culture and is seen under the basal cells, along the BMZ. In a microblister TIMP-2 is present at the blister roof.



A. Control skin (48 h organ culture)

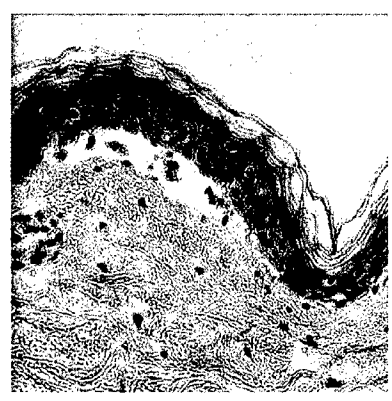
B. HD-exposed skin (48 h organ culture)

C. HD-exposed skin (48 h organ culture with BB94 0.5 μg/ml)



D. HD-exposed skin (48 h organ culture with BB3103 0.5 mg/ml)

E. HD-exposed skin (48 h organ culture with tRA 30 μM)

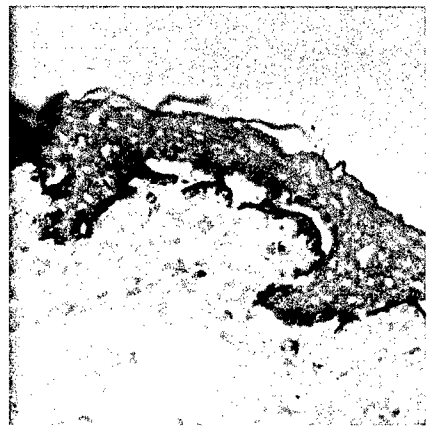


F. HD-exposed skin (48 h organ culture with with DMSO 1%)

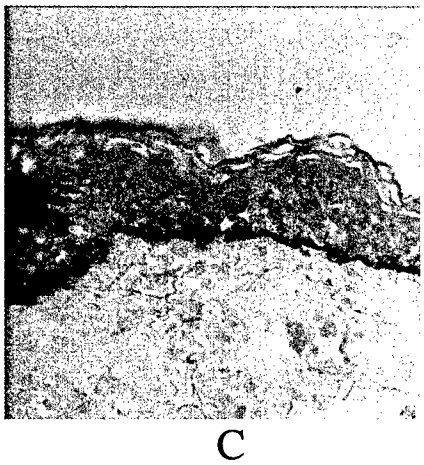
Figure 15

Effects of protease inhibitors on epidermal-dermal separation in human skin that has been exposed to saturated vapor of HD for 5 min. After exposure skin is organ cultured in KBMCa for 48 h in the presence of no inhibitor (B), BB94 (C), BB3103 (D), tRA (E) or DMSO (F). Epidermal-dermal separation is completely inhibited by BB94 (C) and by BB3103 (D) and partially by tRA (E).

A



B



C

Figure 16

Immunohistochemical staining of laminin-5 in the basement membrane. (A) In control human skin the BMZ is continously stained. (B) In human skin exposed for 5 min to saturated vapor of HD at 25 °C and cultured in KBMCa for 48 h the staining is on the floor of the blister and appears to be interrupted locally. (C) In human skin exposed for 5 min to saturated vapor of HD at 25 °C and cultured in KBMCa supplemented with 0.5 μg/ml BB94 for 48 h the BMZ is stained continuously as in control skin.

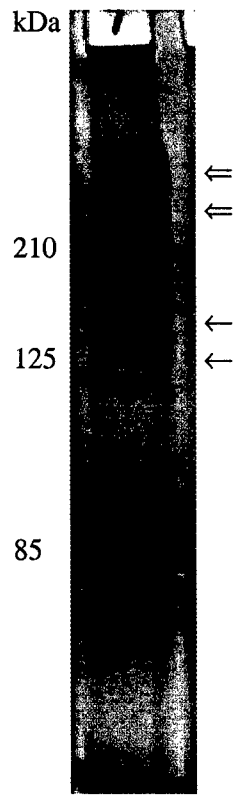


Figure 17

Non-reduced electrophoretic separation of high molecular weight proteins from a dermal extract on a 7.5% SDS-PAGE gel. Sharp bands of proteins are present at approx. 120 and 150 kDa (arrows) and doublets are seen at approx. 240 kDa and 300 kDa (double arrows). In addition, at the top protein complexes are visible that can not enter the gel.

3.4 Discussion

3.4.1 Alkylation of proteins by HD

From the experiments that have been described in this report it is concluded that alkylation by HD of proteins in cultured HEK or in human skin pieces *ex vivo* is substantial. As shown in Table 2, circa 40% of the total amount of label present in cell lysates of HEK is bound to Trizol®-soluble proteins at 1 h after exposure to ^{14}C HD. In addition, 18% of the total amount of label is bound to keratins. Precise assessment of the amount of radioactive label that is bound to the total protein pool in cultured HEK is difficult. It is not known to which extent keratins of cultured HEK are co-extracted with the other cellular proteins in Trizol® and form part of the Trizol®-soluble protein pool. On the other hand, the keratin extract contains all keratins, i.e., the Trizol®-soluble as well as the Trizol®-insoluble ones. Thus, the sum of the radioactive label present in the Trizol® extract and of that in the keratin extract might be an overestimation of the amount of label bound to the total protein pool. Similarly, in epidermis from intact skin it is unpredictable how much keratin is extracted with Trizol®. The keratins that reside in the uppermost layers, i.e. the stratum granulosum and the stratum corneum, certainly do not dissolve into moderate solvents like Trizol®. Therefore, it is assumed that between 40 and 58% of the total amount of label is bound to cellular proteins in cell lysates of ^{14}C HD-treated HEK (Table 2) and that between 52 and 77 % of the total amount of radioactive label is bound to proteins in human epidermis after exposure to saturated HD vapor (Table 4).

The alkylation of Trizol®-soluble proteins in cultured HEK is found to increase linearly with the dose of HD (Table 2). We noticed that in HEK that were exposed to 300 µM HD slightly more radioactive label was present in the keratin fraction than was expected. 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 phenemenon was clearly observed after exposure of HEK to the extremely high concentration of 875 µM HD. The experiments investigating the time-effect relationship in cultured HEK showed that at each concentration maximal alkylation already has been reached within the 30 min exposure period (Table 1).

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

Although in absolute numbers the amount of HD-adducts bound to proteins is many times higher than the amounts of radioactive label associated with DNA or RNA, from a relative point

of view the extent of alkylation is corresponding with the ratios of the quantities in which each group of macromolecules is present in the cultured cells or in the intact epidermis. The observation that in intact epidermis RNA is alkylated with a higher preference than the other macromolecules might be an artefact. Extraction of RNA from epidermis might have been hampered by an excess of protein in the sample. In addition, comparison between the ratios for the occurrence of macromolecules in microgram quantities in cultured HEK or in intact epidermis and the ratios for radioactivity bound to these macromolecules leads to the conclusion that keratins as a group are not preferentially alkylated over other proteins.

In intact skin which contains a large amount of keratins many HD molecules are trapped by these proteins (52 % of all present label in the epidermis). Keratins may therefore be considered to act like scavengers and a thick epidermis or callus will be protective against HD-induced skin injury. This feature of keratins to easily bind HD molecules may be used for detection and verification of the use of HD by demonstration of HD-adducts in skin scales or in hair.

Whereas alkylation of keratins in the dead upper epidermis is not harmful, alkylation of keratins in the basal cells could be, since the keratin filaments contribute to the maintenance of cell integrity. Cultured HEK serve as a model for the basal layer of epidermis. In HEK it is found that 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 given in 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 by the relatively high content of aspartic and glutamic acid in the rod domains of K14 and K16 compared to K5 and K6 (Steinert et al., 1985). Both are type I keratins with an acidic isoelectric point of 5.3 and 5.1, respectively, whereas K5 and K6 are type II keratins with neutral to basic isoelectric points of 7.4 and 7.8, respectively (Moll et al., 1982). In addition, the carboxyl-terminal tail of K14 (see reference SWISSPROT P02533) contains 4 acidic amino acids out of 49 amino acids, whereas the carboxyl-terminal tail of K5 (see reference SWISSPROT P13647) has only one acidic amino acid out of 113 amino acids. As the tails of keratin subunits are thought to be at the periphery of the assembled keratin filaments, the carboxyl groups could well be targets for HD alkylation (Papirmeister et al., 1991; Noort et al., 1997). Although the significance of keratin alkylation remains to be determined, it might be envisaged that when alkylation sites on the keratin end domain are present, the (de)phosphorylation of serine residues is hampered, leading to impaired functioning of the intermediate filaments and hence to a disturbed cellular integrity and anchorage to the dermis.

The fluorogram in Figure 2 also shows that HD alkylates a small number of proteins other than keratins. This selective alkylation of proteins by HD is better illustrated on a two-dimensional protein map of proteins with an acidic to neutral isoelectric point (Figure 3). Although the cells were exposed to an extremely high concentration of 1 mM ^{14}C HD, causing extensive alkylation of proteins, only a small number of the cellular proteins is bearing a ^{14}C HD-adduct. In addition, there is a rather large difference in labeling intensity between the spots, which might not only be due to the different quantities of protein. The results obtained with this preliminary experiment are encouraging to apply protein separation by 2-D SDS-PAGE combined with peptide mass fingerprinting analysis using mass spectrometric techniques as a manner to identify the proteins

that become preferentially alkylated by HD. The elucidation of their identity may help to analyse the role of protein alkylation in the mechanism of action of HD.

The question whether proteins of the BMZ belong to the target proteins of HD has been answered marginally in this study. In our opinion, alkylation by HD could easily be overestimated when purified proteins are exposed to HD (Zhang et al., 1995, 1998). Therefore, we initiated an experiment by exposing intact skin to saturated vapor of HD so that the proteins of the BMZ are in their natural conformation with only selective targets accessible to HD. However, with this approach the BMZ proteins like laminin-1, nidogen or laminin-5 have to be isolated from intact skin, in order to study the effect of HD alkylation. Isolation of BMZ proteins from intact skin is not an easy task. In literature, BMZ proteins are often purified from a tumor or from cultured cells. Only one study was found that described the extraction of laminin-1 from pig skin (Lindsay and Rice, 1996). However, protein detection with a laminin antibody on Western blots in that study showed a major protein band of 160 kDa and a minor protein band of 240 kDa, instead of the usual 200, 220 and 400 kDa subunits of laminin-1. This finding questioned the reliability of the results. So far, we have made a dermal extract containing several high molecular weight proteins. The extract has been used to obtain an impression of the presence of HD adducts bound to these proteins as a group. It was calculated that about 1 molecule of HD is present on 100 molecules of high molecular weight protein. This finding suggests that with our approach it will be difficult to detect HD adducts on the particulate proteins. The alkylation of BMZ proteins is probably limited and might be of minor importance in the mechanism of HD-induced vesication.

The present results show that in cultured HEK 36 to 54 % of label in the cells is not bound to DNA, RNA or protein. In intact epidermis this percentage is between 23 and 48 %. 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 to other cell molecules. Based on the preference of HD for binding to sulfhydryl, phosphate or carboxyl groups (Noort et al., 1997), HD might be bound to glutathione, ATP, cAMP or intermediates of the Krebs cycle, all targets of which alkylation will have impact on cell functioning.

3.4.2 Proteolytic activity induced by HD

Our results show that HD does not induce enhanced proteolytic activity of serine proteases in cultured HEK, which is in contrast with the reported stimulation of serine protease activity in the culture medium of cells exposed to HD (Cowan et al., 1993, 1994; Ray and Ali, 1998). An explanation for this discrepancy might be that we have measured substrate conversion in cell-free media and not in the presence of cells, so that we might have missed cell bound activity. Indeed, a membrane-bound 80 kDa protein with trypsin-like activity has recently been purified from HEK that were exposed to HD (Chakrabarti et al., 1998). Equally, our findings that trypsin-like activity in media of HD-exposed human skin explants is not enhanced are in contrast with other reports using human skin (Lindsay and Rice, 1996), hairless guinea pig skin (Cowan et al., 1993) and rabbit skin (Higuchi et al., 1988). This discrepancy of results may be ascribed to the EnzCheck proteolytic assay itself, as it seems to be suitable for only a limited group of

serine proteases. According to the supplier, the EnzCheck assay detects activities of serine proteases such as trypsin, chymotrypsin and elastase. Thus, it is thinkable that proteolytic activity of, for example, plasmin remains undetected with the EnzCheck assay. Nevertheless, it remains doubtful whether serine proteases act on structural proteins of the BMZ, since serine protease inhibitors did not prevent epidermal-dermal separation in an *ex vivo* human skin system (Lindsay et al., 1996).

We supposed that MMPs are more likely to be involved in blister formation induced by HD. The MMPs have a central function in maintaining the integrity of the basement membranes and other extracellular matrix structures that undergo constant remodeling. HD may disturb the fine balance between synthesis and degradation of the BMZ proteins. This presumption has been tested using three approaches: 1) measurement of the release of MMP-1, -2, -3 and –9 in culture media from human skin *ex vivo* and cultured HEK after exposure to HD, 2) localization by means of immunohistochemistry of MMPs and TIMPs in human skin *ex vivo* after exposure to HD, and 3) the assessment of the effects of specific MMP-inhibitors on HD-induced epidermal-dermal separation in human skin *ex vivo*.

After exposure of human skin to saturated vapor of HD at Ct values that cause epidermal-dermal separation, only the release of the latent form of MMP-2 is higher than the release from control skin, whereas the release of MMP-1, -3 and –9 is lower than that from control skin (Figures 6 and 8). Exposure of cultured HEK to HD causes an increased release of MMP-1 and –3 compared to control cells (Figure 7). However, the rise shows a maximum at 100 µM, which concentration is considered to be non-vesicating when extrapolated to the *ex vivo* situation. At higher concentrations of HD the release of MMP-1 and –3 into the medium drops towards the level of unexposed cells. This phenomenon might be indicative for reduction of protein synthesis or impairment of the protein secretion pathway caused by HD. In earlier studies, a similar maximal release into the culture medium upon exposure of HEK to 100 - 125 µM HD was observed for urokinase-type plasminogen activator (unpublished) and for interleukin-6 and -8 (Mol, 1997). The quantity of MMP-9 in the culture media of HEK that were exposed to HD remains equal to that found in control medium, except for a reduction after exposure to 225 µM HD (Figure 5). Control cultures of HEK secrete only a very low amount of MMP-2 into the medium which is not altered by exposure to HD. The results obtained with this release study suggest that of the MMPs that have been investigated, only MMP-2 is probably involved in HD-induced epidermal-dermal separation.

However, since pericellular proteolysis by MMPs is a local event in the skin, measurement of released MMPs may not be representative for the presence and the activity of the enzymes in the skin. It has been expected that immunohistochemical staining of skin sections will give a better impression of presence of MMPs after exposure to HD. Antibodies have been used that recognize active as well as latent forms of the MMPs. At various time points after exposure to HD, the expressions of four different MMPs in HD-exposed skin sections have been compared to those in control skin. Although quantitative interpretation of the immunohistochemical stainings is rather indeterminate, it is concluded that of none of the MMPs tested the expression in HD-exposed skin is greater than in control skin. In fact, the staining intensity in HD-exposed skin is equal to or even lower than in control skin. Only the rather early expression of MMP-9 in skin at

6 h after exposure to HD, may be an effect that is related to HD. However, detection of MMPs by immunostaining does not necessarily mean that the enzymes are active. Their activity is determined by the balance of MMPs to TIMPs and by the conversion from the proenzyme into the active enzyme. Although the immunostaining of TIMP-1 and –2 is faint, the results suggest that in HD-exposed skin even more inhibitor protein is present than in control skin. Thus, no imbalance of MMPs over TIMPs occurs in HD-exposed skin. Measurements of the activity of MMPs in skin sections by means of in situ zymography have not been performed in this study. Altogether, the results of the immunohistochemical approach do not indicate an involvement of MMP-1, -2, or -3 in HD-induced blister formation, while a role for MMP-9 is inconclusive.

However, proof of the participation of MMPs in HD-induced epidermal-dermal separation in human skin *ex vivo* has been obtained indirectly by the use of specific inhibitors of MMPs. The complete inhibition of microvesication in HD-exposed skin by the inclusion of the hydroxamate inhibitors BB94 or BB3103 in the organ culture medium strongly suggests that MMPs actually contribute to HD-induced epidermal-dermal separation. BB94 inhibits in particular MMP-2 and -9 (Wojtowicz-Praga et al., 1997). When tRA was included in the culture medium, the epidermal-dermal separation was somewhat diminished but not abolished as was seen with both BB inhibitors. tRA is an effective inhibitor of MMPs that are transcriptionally regulated via the induction of cJun which stimulates the MMP promotor via activator protein-1 (Fisher et al., 1996). MMP-1, -3 and –9 are regulated in this manner. MMP-2 has a different regulatory system and is insensitive to tRA inhibition (Corcoran et al., 1996). Therefore, from this inhibitor study it is concluded that MMP-2 is probably involved in HD-induced epidermal-dermal separation, which is in agreement with our conclusion from the MMP release studies. Participation of other MMPs than those investigated, in particular membrane type 1-MMP (MT-1 MMP), cannot be excluded. Recently it was reported that BB94 has also an inhibitory effect on MT-1 MMP (Koshikawa et al., 2000). The MMP inhibitors had only an effect on the epidermal-dermal junction and did not reduce the HD-induced cellular necrosis within the overlying epidermis.

Our observation that laminin-5 is on the floor of the microblister in HD-exposed skin agrees with the study of Monteiro-Riviere and Inman (1995). This finding suggests that HD-induced cleavage in the BMZ occurs in the upper lamina lucida, between laminin-5 and the basal cells. Laminin-5 plays a very important role in the connection of basal cells to the structural proteins of the BMZ. As a ligand for various integrins, laminin-5 is involved in stable attachment via hemidesmosomes (Baker et al., 1996) as well as in the migration of the cells over the matrix (Gianelli et al., 1997). Movement of cells, which occurs normally in the epidermis when cells withdraw from the basal layer to enter differentiation, requires breakage of existing attachments and formation of new ones. This is regulated by proteolytic processing of laminin-5 by MMP-2 or MT-1 MMP. The observed separation in the BMZ of HD-exposed skin suggests that after cleavage of the existing receptor/ligand complexes, the formation of new attachments between laminin-5 and integrins is hampered. The observed focal defects in the immunostaining of laminin-5 might be an artefact, since MMPs only remove a part of the laminin-5 molecule and do not fully degrade it. It is expected that the epitope of the GB3 antibody remains available.

Diminished availability or reduced binding capacity of the attachment molecules of the BMZ could be caused by several factors. A down-regulation of adhesion molecules at the BMZ might occur as a consequence of the terminal differentiation pathway that cells enter when their mitotic cell cycle is ceased by HD-induced DNA damage. Low availability of adhesion proteins might also be caused by a defect in the intracellular transport of newly synthesized proteins that are destined for delivery at the cell surface. If HD interferes with the protein trafficking of the endoplasmic reticulum (ER) and Golgi apparatus (GA), delivery of membrane bound proteins will be hampered. A third cause might be a disruption of hemidesmosomes due to a disturbance of their phosphorylation status. Disruption of hemidesmosomes might occur when the phosphorylation of integrin subunit β4 on tyrosine or serine residues is enhanced (Mainiero et al., 1996; Rabinovitz et al., 1999). Since phosphate groups may become preferentially alkylated by HD (Papirmeister et al., 1991), a disturbance of the phosphorylation status of proteins after exposure to HD is possible.

In conclusion, based on the results of the present study it is hypothesized that the destruction of the BMZ of human skin by HD does not only depend on an increased degradation of the proteins that are involved in attachment but also on a diminished availability or reduced binding capacity of proteins to reattach to properly. The application of MMP inhibitors on HD-exposed skin will suppress all proteolytic activity in the BMZ thereby preserving the epidermal-dermal attachment. The elimination of epidermal-dermal separation by the use of MMP inhibitors will contribute to the prevention of blister formation as it is supposed that no blister can develop when the epidermis remains attached to the dermis. Interestingly, several MMP inhibitors are in clinical trials now to treat patients and new ones are under development. The MMP inhibitors are considered to be promising therapeutics in various pathological situations, such as cancer and arthritis.

4 Key Research Accomplishments

- In cell lysates of HEK that have been exposed to ^{14}C HD, among 40 to 58% of the total amount of ^{14}C label present is bound to proteins. In human epidermis that was exposed to saturated vapor of ^{14}C HD, between 52 and 77 % of the total amount of radioactive label is bound to proteins.

- The amounts of label bound to DNA, RNA and protein of cultured HEK are approximately proportional to the concentration of HD used.

- The extent of alkylation of macromolecules in cultured HEK or in intact human epidermis corresponds to the quantities in which each group of macromolecules is present.

- In lysates of cultured HEK that have been exposed to ^{14}C HD, 36 to 54 % of ^{14}C label is bound to other molecules than DNA, RNA and protein or is freely circulating as intact HD in a lipophilic environment such as membranes, or as thiodiglycol, the hydrolyzed metabolite of HD. In intact human epidermis this percentage is between 21 and 46 %.

- The amounts of ^{14}C label present in the intact human skin pieces increases with the duration of exposure to ^{14}C HD vapor (Ct). After exposure, about 60 % of the total amount of label in the skin is present in the dermis and about 40 % in the epidermis.

- Maximal alkylation levels are reached within the 30 min exposure period, when cultured HEK have been exposed to 100, 200 or 300 µM HD.

- Immediately after exposure of cultured HEK for 30 min to a solution of 100 µM ^{14}C HD, the amount of label present in the keratinocytes appears to be twenty five-fold higher than may be expected from the volume of the cultured cells. This indicates a considerable binding of ^{14}C HD to cell components.

- Keratins as a group are not preferentially alkylated over other proteins of cultured HEK or human epidermis.

- In epidermis of human skin that has been exposed to saturated vapor of ^{14}C HD, 52 % of all present ^{14}C label is trapped by keratins.

- Keratin 14 (50 kDa) is preferentially alkylated by HD. This might indicate an alkylation preference for keratins with an acidic isoelectric point.

- In the *ex vivo* model for exposure of human skin to saturated vapor of HD, the penetration rate of HD is estimated to be 0.25 $\mu g/cm^2.min$ at 25°C. For comparison, Renshaw et al. (1946) reported a penetration rate of 1 - 4 $\mu g/cm^2.min$ at 21°C in human skin.

- Comparison of the protein pattern of the total cellular protein pool and pattern of the proteins bearing ^{14}C HD-adducts shows that a limited number of proteins from the total cellular protein pool of HEK are alkylated by ^{14}C HD. This points to a selective instead of a non-discriminative way of alkylation by HD.

- HD does not induce enhanced proteolytic activity of serine proteases in cultured HEK. A small increase (<10%) in serine protease activity is only seen in media of HEK that have been exposed to the highest concentration of HD used, i.e., 150 μM. No significant increase in serine protease activity is seen in organ culture media of human skin pieces that have been exposed to saturated vapor of HD.

- Only the release of latent MMP-2 was higher than the release from control skin, after exposure of human skin to saturated vapor of HD at Ct values that cause epidermal-dermal separation. The release of MMP-1, -3 and –9 from HD-exposed skin was lower than that from control skin.

- Exposure of cultured HEK to 0 - 150 μM HD causes an increased release of MMP-1 and –3 compared to control cells. However, the rise shows a maximum at 100 μM and drops with higher concentrations of HD. The quantity of MMP-9 in the culture media of HEK that were exposed to HD remains equal to that found in control medium, except for a reduction following exposure to 225 μM HD. Control cultures of HEK secrete only a very low amount of MMP-2 into the medium and exposure to HD does not change that amount.

- Up to 48 h after exposure of human skin for 5 min to saturated vapor of HD at 25 °C the expression of MMP-1, 2-, -3 or –9 does not exceed that in control skin. Only the rather early expression of MMP-9 in skin at 6 h after exposure to HD may be an effect that is related to HD. Furthermore, examination of the staining patterns of TIMP-1 and –2 revealed that no imbalance of MMPs over TIMPs occurs in HD-exposed skin.

- The inclusion of the hydroxamate inhibitors BB94 or BB3103 in the organ culture medium of human skin pieces that have been exposed to saturated vapor of HD for 5 min completely inhibited microvesication. Addition of tRA to the culture medium caused partial protection against epidermal-dermal separation. The MMP inhibitors did not reduce the HD-induced cellular necrosis within the overlying epidermis.

- Immunohistochemical staining shows that laminin-5 is present exclusively at the dermis of the microblister that developed after 48 h in human skin *ex vivo* exposed to saturated vapor of HD at 25 °C. Some focal defects occur in the linear staining of laminin-5 after exposure to HD vapor.

- Some evidence is obtained that MMP-2 is involved in HD-induced epidermal-dermal separation in human skin.

- A high molecular weight protein fraction was prepared from a dermal extract made from skin that was exposed to ^{14}C HD. From the radioactivity present in this extract it was calculated that 1 molecule HD was present on 100 molecules of high molecular weight protein.

5 Reportable Outcomes

Manuscripts, abstracts, presentations:
M.A.E. Mol, S.W. Alblas and H.P.Benschop (1998). Alkylation of epidermal cell proteins by sulfur mustard. Proceedings of the 1998 Bioscience Review, Hunt Valley MD.

M.A.E. Mol (1999). The involvement of matrix metalloproteinases and serine proteases in the onset of sulphur mustard-induced blister formation. Abstract Book of NATO TG 004 Meeting, Brussels

M.A.E. Mol, S.W. Alblas, A. Hammer and H.P. Benschop (2000). Synthetic inhibitors of matrix metalloproteinases prevent sulfur mustard-induced epidermal-dermal separation in human skin pieces. To be presented at the 2000 Bioscience Review, Hunt Valley MD

Manuscripts in preparation:
M.A.E. Mol, S.W. Alblas and H.P. Benschop (2000). Alkylation of epidermal cell proteins by sulfur mustard. To be submitted to J. Appl. Toxicol.

M.A.E. Mol, S.W. Alblas, A. Hammer and H.P. Benschop (2000). Synthetic inhibitors of matrix metalloproteinases prevent sulfur mustard-induced epidermal-dermal separation in human skin pieces. To be submitted to J. Invest. Dermatol.

Funding applied for based on work supported by this award:
M.A.E. Mol (1999). New approaches towards the elucidation of epidermal-dermal separation in sulfur mustard-exposed human skin and directions for therapy. Submitted to U.S. Army Medical Research Acquisition Activity, ERMS# 00352002

6 Conclusions

Vesication is a characteristic feature of HD-induced pathology in the human skin. Alleviation of this phenomenon can possibly be obtained by preventing tearing of the BMZ, the attachment layer between epidermis and dermis consisting of a complex of structural proteins. In this report the results have been described of studies on two possible causes that underly the destruction of the BMZ. Firstly, we have investigated the extent of protein alkylation in skin or cells that had been exposed to HD and, secondly, we have examined the role of proteolytic enzymes in damaging the structural proteins of the BMZ in HD-exposed skin. Cultured HEK and human skin *ex vivo* have been used in these studies as models for human skin.

There is substantial alkylation of proteins in cultured HEK and in human epidermis that have been exposed to HD. On a microgram basis, proteins in the skin become alkylated by HD to the same extent as DNA or RNA and in proportion to the amount of HD used. In human epidermis that was exposed for 5 min to saturated vapor of HD at 25 °C, between 52 and 77 % of the total amount of HD present is bound to proteins. Keratins, that are the most abundantly present proteins in epidermis, bind half of the available HD. Alkylation of keratins in the dead upper epidermis can be regarded as a beneficial scavenger action. On the contrary, alkylation of keratin filaments in the vital cells of the basal layer might have harmful consequences for cell functioning. In these cells also a selection of the proteins of the non-keratin protein pool bears HD-adducts. To understand the consequences of selected protein alkylation for cell attachment to the basement membrane, the alkylated proteins should be identified by means of 2-D PAGE and MALDI-MS. Information on the susceptibility of proteins in the BMZ to alkylation by HD is still minimal. In a preliminary experiment, a low degree of alkylation by HD was found in a partially purified extract of the dermis, containing several high molecular weight proteins of the BMZ.

The results of the present research further show that, besides considerable binding of HD to proteins, a large amount of HD, between 36 to 54 %, is somewhere else in the cells. Part of this HD fraction may be hydrolyzed into the non-toxic thiodiglycol. But a considerable amount of HD may be bound to cell metabolites such as glutathione, ATP, cAMP or intermediates of the Krebs cycle. These are all targets of which alkylation might have impact on cell functioning.

The present studies demonstrate that following exposure to HD there is considerable HD-protein adduct formation in keratinocytes and that certain protein targets become preferentially alkylated by HD. These findings suggest that protein alkylation forms part of the toxicological implications of HD exposure on skin cells. It is conceivable that the observed alkylation of keratins in basal cells might cause a collapse of the cytoskeleton, which will have implications for the attachment of basal cells to the BMZ.

The second option to explain dysfunctioning of the BMZ during HD-induced vesication is the breakdown of structural proteins by proteases. The results of the present study do not show an involvement of serine proteases in the onset of epidermal-dermal separation. However, evidence is obtained that MMPs play a role in weakening of the BMZ following HD exposure. In the

presence of the specific inhibitors of MMPs, BB94 or BB3103, no epidermal-dermal separation occurs in human skin *ex vivo* that had been exposed to saturated HD vapor. However, neither upregulation of the expression of MMPs nor reduction of TIMPs seem to be responsible for HD-induced epidermal-dermal separation. These results suggest that the destruction of the BMZ of human skin by HD does not only depend on an increased degradation of the proteins that are involved in attachment but also on a diminished availability or reduced binding capacity of proteins to reattach to properly. Diminished availability or reduced binding capacity of the attachment molecules of the BMZ could be caused by several factors such as 1) functional damage of the ER/GA, 2) a shift of basal cells from a proliferative status towards terminal differentiation, or 3) an increased phosphorylation of hemidesmosomal proteins. In future research it will be investigated which of these possible causes contributes to the epidermal-dermal separation.

Based on the results of the present study it is hypothesized that application of MMP inhibitors on HD-exposed skin will suppress all proteolytic activity in the BMZ thereby preserving the epidermal-dermal attachment. The elimination of epidermal-dermal separation by the use of MMP inhibitors will contribute to the prevention of blister formation as it is supposed that no blister can develop when the epidermis remains attached to the dermis.

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8 Appendices

8.1 List of abbreviations

2-D SDS-PAGE	two-dimensional SDS-polyacrylamide gel electrophoresis
AEC	3-amino-9-ethyl-carbazole
BMZ	basement membrane zone
CBB	coomassie brilliant blue
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EDJ	epidermal-dermal junction
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzym linked immunosorbent assay
ER	endoplasmic reticulum
GA	Golgi apparatus
HD	sulfur mustard
HEK	human epidermal keratinocytes
HRP	horseradish peroxidase
KBM	keratinocyte basal medium
KBMCa	keratinocyte basal medium containing 1.4 mM Ca^{2+}
kDa	kilodalton
KGM	keratinocyte growth medium
MMP	matrix metalloproteinase
MT-1 MMP	membrane type-1 matrix metalloproteinase

NP40	nonidet-P40
PAGE	polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulphate
Ser(P)	phosphoserine
TIMP	tissue inhibitor of metalloproteinase
TNFα	tumor necrosis factor α
tRA	*trans* retinoic acid
Tris	Tris[hydroxymethyl]aminomethane

8.2 List of suppliers

Amersham Pharmacia Biotech, Roosendaal, The Netherlands

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