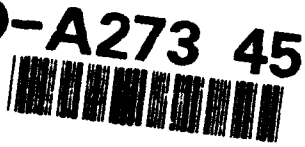

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This report presents results obtained during the first year of our study on the effects of sulfur mustard on free intracellular Ca^{2+} levels and synthesis of basement membrane zone proteins in human skin. So far, assays for determination of free intracellular Ca^{2+} levels, (ADP-ribosyl)ation and synthesis of basement membrane zone proteins have been almost established and some experiments with cultured human epidermal keratinocytes have been performed. The following preliminary results have been obtained. 1) Exposure of cells to 50 - 200 µM sulfur mustard caused little or no effect on free intracellular Ca^{2+} levels during the first 7 hr after treatment. 2) Following exposure of HEK cultured on coverslips to 50 or 250 µM sulfur mustard, no changes in the staining intensity of six different basement membrane proteins was observed at 24 hr. 3) The pattern of NAD^+ depletion induced by γ-irradiation is different from that induced by sulfur mustard.

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LIST OF ABBREVIATIONS

BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BMZ	basement membrane zone
BPA	bullous pemphigoid antigen
BSA	bovine serum albumin
$[Ca^{2+}]_i$	intracellular free Ca^{2+} levels
DHB-	dihydroxyboronyl-
DMF	dimethyl formamide
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
HEK	human epidermal keratinocytes
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KGM	keratinocyte growth medium
KSFM	keratinocyte serum free medium
LFMP	low fat milk powder
MADPRT	mono(ADP-ribosyl)transferase
MNNG	N-methyl-N'-nitro-N-nitroguanidine
MOPS	3-(N-morpholino)-propanesulfonic acid
NBT	4-nitro blue tetrazolium chloride
NC	nitrocellulose
PADPRT	poly(ADP-ribosyl)transferase
PBS	phosphate buffered saline (Dulbecco's; Ca^{2+} and Mg^{2+} free)
RT	room temperature
SSB	single strand breaks
TCA	trichloroacetic acid

INTRODUCTION

In human skin, subepidermal blisters develop upon contact with sulfur mustard. Characteristic for blister formation is loss of adherence between epidermis and dermis combined with the filling of the resulting cavity with fluid. Because vesication does not occur until 12 to 24 hr following exposure, there will be time to intervene in the processes that cause the weakening of the epidermal-dermal junction. This study will contribute to the development of therapeutic measures by investigating the relationship between sulfur mustard-induced changes in the basal epidermal cells and the development of blisters.

Well known biochemical effects observed in cells exposed to sulfur mustard are depletion of NAD^+ and ATP pools, inhibition of protein synthesis, and alkylation damage of DNA (Mol et al., 1989; Mol and de Vries-van de Ruit, 1992; Mol and van der Schans, 1993). Papirmeister et al. (1985) presented a hypothesis that linked the sulfur mustard-induced DNA damage to NAD^+ depletion and subsequent development of blisters. They postulated that DNA single strand breaks (SSB) due to spontaneous or enzymatically induced depurination of alkylated DNA bases activate the chromosomal enzyme poly(ADP-ribose)transferase (PADPRT). The latter would deplete cellular NAD^+ pools. In literature, this phenomenon has been reported to occur with several agents that cause different types of DNA damage, such as γ-irradiation, MNNG and hydrogen peroxide (Skidmore et al., 1979; Berger et al., 1979; Schraufstätter et al., 1986). In previous studies, we reported that the addition of nicotinamide, an inhibitor of PADPRT to the medium of sulfur mustard-treated keratinocytes, could only partially prevent the decrease of NAD^+ levels (Mol et al., 1989, 1991). It was concluded that following exposure of cells to sulfur mustard, activation of nuclear PADPRT is not likely to be the main factor responsible for NAD^+ depletion of cells. We postulate that NAD^+ depletion is also caused by the activation of another NAD^+-consuming enzyme, mono(ADP-ribose)transferase (MADPRT), which catalyzes mono-ADP-

ribosylation of proteins (Richter et al., 1985; Frei and Richter 1988; Richter and Kass, 1991). The mitochondrial-bound MADPRT might become activated due to enhancement of free intracellular Ca^{2+} levels ($[Ca^{2+}]_i$). So far, few data are known about early effects of sulfur mustard on $[Ca^{2+}]_i$ (Hua et al., 1993). Possibly, sustained elevation of the $[Ca^{2+}]_i$ will lead to impaired mitochondrial function followed by loss of NAD^+ and ATP.

Enhanced Ca^{2+} levels may also activate Ca^{2+}-dependent proteases such as calpain. This enzyme has been demonstrated in many cells and may disrupt cytoskeletal elements (Croall and Demartino, 1991; Melloni and Pontremoli, 1991). The intactness of the cytoskeleton may be important in the adhesion of the basal keratinocytes to the dermis. In fact, the cytoskeleton of the basal keratinocyte is connected with the dermal fibers via a comlex meshwork structure which is composed of various proteins, glycoproteins and proteoglycans (Uitto et al., 1989). Most of the components of this basement membrane zone (BMZ) are synthesized by dermal fibroblasts and by epidermal keratinocytes. In this respect, the observation that protein synthesis is inhibited in cells exposed to sulfur mustard leads to the proposal that the weakening of the epidermal-dermal junction might be caused by reduced synthesis of components of the basement membrane zone (Mol, 1992). As a result of damage to DNA, RNA or the endoplasmic reticulum, synthesis and/or secretion of basement membrane zone proteins may become reduced, leading to an easy rupture between basal epidermal keratinocytes and the dermis.

Summarizing, in this study the following effects of sulfur mustard in human epidermal cells will be examined: activation of poly- and mono-ADP-ribosyltransferase; enhancement of $[Ca^{2+}]_i$; activation of calpain and changes in cytoskeletal elements; reduced biosynthesis of basement membrane zone proteins. Cultured human epidermal keratinocytes (HEK) and, where possible, pieces of human skin in organ culture will be used in the following approaches:

- determine the effects of sulfur mustard on ADP-ribosyltransferase activity, thereby discriminating between the activities of poly-

and mono-ADPRT (task[1] 3).
- compare the relationship between DNA strand break formation, protein (ADP-ribosyl)ation and NAD^+ depletion following exposure of keratinocytes to sulfur mustard, γ-radiation, MNNG and hydrogen peroxide (task 3).
- measure the $[Ca^{2+}]_i$ levels as a function of sulfur mustard dose and time after exposure, subsequently, investigate whether the $[Ca^{2+}]_i$ levels of sulfur mustard-exposed cells can be manipulated by a Ca^{2+} entry blocker, an inhibitor of Ca^{2+} release from endogenous stores or by a Ca^{2+} chelator (task 2).
- assess the response of the Ca^{2+}-dependent neutral protease, calpain, to sulfur mustard exposure (task 5).
- determine the effects of sulfur mustard on cytoskeletal elements such as, microtubuli, microfilaments and intermediate filaments by qualitative immunohistochemistry (task 6).
- detect the biosynthesis of a number of basement membrane proteins in control and sulfur mustard-treated HEK by Western immunoblots and ELISA techniques. Proteins of choice are: bullous pemphigoid antigen (BPA), laminin, collagen type IV and type VII, and integrins α_6 and β_4. (task 10).
- if it appears to be possible to manipulate $[Ca^{2+}]_i$ in sulfur mustard-exposed cells, investigate the implications of that manipulation on ADPRT activity, NAD^+ levels, calpain and cytoskeleton (tasks 4,7,8 and 10).

This report describes the setup of assays for determination of $[Ca^{2+}]_i$, (ADP-ribosyl)ation and synthesis of BMZ proteins and several results obtained in experiments so far.

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[1] Tasks refer to the statement of work described for DAMD 17-92-V-2008.

EXPERIMENTAL METHODS

A) Cell culture

Primary cultures of HEK were grown with a feeder layer of irradiated 3T3 mouse fibroblasts as described before (Mol et al., 1989). After passage, HEK were cultured without feeder layer and were fed with Keratinocyte Serum Free Medium (KSFM) (GIBCO) or with Keratinocyte Growth Medium (KGM) (Clonetics), both supplemented with bovine pituitary extract. The advantages of these latter media were the absence from the medium of serum and a low calcium concentration of 0.09 - 0.15 mM which reduced differentiation of keratinocytes during culture period.

B) Protein ADP-ribosylation

- Preparation of Bio-Rex 70-aminophenyl boronate matrix (DHB-Biorex) and dihydroxyboryl Sepharose (DHB-Sepharose).

Methods for this assay were adopted from Jacobson and coworkers (Alvarez-Gonzales et al., 1983; Aboul-Ela et al., 1988).

DHB-Biorex. Stock solutions: 0.25 M NH_4OAc, pH 5.0; solution A: 0.1 M NaOAc, 1.0 M NaCl, pH 4.5; solution B: 0.1 M $NaHCO_3$, 1.0 M NaCl, pH 9.0; solution C: 6 M guanidine HCl, 50 mM MOPS, 10 mM EDTA, pH 6.0.

75 g of Bio-Rex 70 (200-400 mesh) (BioRad) was mixed with 300 ml of H_2O, pH 5.0 and washed with 3 L of deionized H_2O; the resin was suspended in 250 ml of deionized H_2O and adjusted to pH 5.0; after addition of 7.5 g carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Sigma)), pH was adjusted to 5.0 and the mixture was stirred for 15 min at room temperature (RT) while keeping pH at 5.0; 7.5 g m-aminophenyl boronic acid-hemisulfate (Sigma) was dissolved in 45 ml deionized H_2O and added to the resin mixture; pH was adjusted to 5.0 and the solution was kept in the dark while stirring for 18 hr; the resin was washed first with 3 L deionized H_2O, then with 3 L of solution A, 3 L of solution B, 1.5 L deionized H_2O and finally with 100 ml of solution C; the resin was suspended in solution C in a 1:1 ratio and stored at 4°C.

DHB-Sepharose. Stock solutions: buffer pH 8.1: mix 1 volume of 1 M Trisbuffer pH 8.1 with 9 volumes of 1 M NaCl; buffer pH 4.3: mix 8 volumes of 0.1 M HAc in 1 M NaCl with 5 volumes of 0.1 M NaAc in 1 M NaCl.

1.4 g 6-amino hexanoic acid-Sepharose 4B (Sigma) was swelled overnight in an excess of 0.5 M NaCl solution; then the resin was washed with 300 ml 0.5 M NaCl and 200 ml deionized H_2O; 30 mg m-aminophenyl boronic acid-hemisulfate was dissolved in 12 ml deionized H_2O and 2 volumes of this ligand solution were added to 1 volume of gel; pH was adjusted to between 4.5 and 6.0; the mixture was stirred at RT and carbodiimide powder was added to a final concentration of 0.1 M; pH was maintained at 4.5 - 6.0 for 1 hr; the reaction was allowed to proceed for 24 hr at RT; the resin was washed five times alternatively with buffer pH 8.1 and buffer pH 4.3 and finally washed with deionized H_2O; storage was in 250 mM ammonium formate, pH 4.5 at 4°C.

- Radiolabeling and cell extraction

Secondary confluent cultures of HEK grown in 35-mm wells of 6-well plates in KSFM were radiolabeled with 1 ml of medium containing 5 µM 2-^{3}H adenine (Amersham) per well. The incubation medium was KSFM, prepared without adenine. After 16 hr, medium was replaced with fresh medium for 1 hr. After experimental manipulation cultures were washed with phosphate buffered saline (PBS) and harvested by the addition of 1 ml of ice-cold 20% trichloroacetic acid (TCA). Following separation by centrifugation, the supernatant was saved for NAD^+ determination. The pellet was dissolved in 0.2 ml of ice-cold 98% formic acid and diluted by the addition of 10 volumes of ice-cold deionized H_2O. Bovine serum albumin (BSA) (1 mg) was added to facilitate reprecipitation. Samples were adjusted to a final concentration of 20% (w/v) TCA and the pellet was collected by centrifugation.

The NAD^+ content of the supernatant and the amount of protein-bound mono- and polymers of ADP-ribose of the final pellet have been analyzed by affinity chromatography.

- NAD^+ determination

To determine NAD^+, the TCA supernatant was diluted to 10 ml with 250 mM ammonium formate, pH 8.6, and adjusted to pH 8.6 ± 0.2 with concentrated ammonium hydroxide. The sample was applied to a 0.5 ml DHB-Sepharose column which had been prewashed with 15 ml of 250 mM ammonium formate pH 8.6. Following loading, the column was washed with 10 ml of 250 mM ammonium formate, pH 8.6, followed by 3 ml of deionized H_2O. NAD^+ was eluted with 8 ml of 250 mM ammonium formate, pH 4.5, and quantified by liquid scintillation counting.

- Determination of ADP-ribosyltransferase activity.

Total ADPRT activity has been measured as the total amount of ADP-ribose released from protein detected as radioactivity present in a precipitate of acid-insoluble proteins of the cells.

The acid-insoluble pellet was dissolved in 1 ml 6 M guanidinium chloride, 250 mM ammonium acetate, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 6.0 and 1 ml 100 mM MOPS, 10 mM EDTA, 2 mM ammonium chloride pH 7.0.

The sample was incubated at 37°C for 6 hr.

A 0.5-ml column of DHB-Biorex was prewashed with 5 ml deionized H_2O and 15 ml 1 M guanidinium chloride, 250 mM ammonium acetate, 10 mM EDTA, pH 9.0 (buffer A). Following application to the column of the sample, which was diluted 10 times with buffer A, the column was washed with 10 ml of buffer A and 20 ml of 1 M ammonium acetate, 10 mM EDTA. ADP-ribose was eluted with 1 ml 1 M HCl and 4 ml of deionized H_2O and quantified by liquid scintillation counting.

C) Enzymatic NAD^+ determination

NAD^+ was extracted from cultures with 0.5 M $HClO_4$ on ice during 20 min. The $HClO_4$ extract was removed by centrifugation, and the supernatants were assayed for NAD^+ using an enzymatic cycling assay (Jacobson and Jacobson, 1976).

D) Intracellular calcium determination

Intracellular free calcium ($[Ca^{2+}]_i$) was measured by means of the

fluorescent indicator Fura-2 (Molecular Probes), according to the procedure described by Tang and Ziboh, 1991. To load the fluorescent probe, secondary cultures of HEK grown on coverslips in KGM were incubated at RT during 60 min with 5 μM Fura-2 AM in a HEPES buffered saline solution (pH 7.3) containing 110 mM NaCl, 1 mM $MgSO_4$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.35 mM NaH_2PO_4, 5 mM glucose, 2 mM glutamine, 25 mM HEPES and 0.15 mM $CaCl_2$. To facilitate dye uptake 0.03% Pluronic F-127 (Calbiochem) was added. Unincorporated fluorescent dye was removed by washing the HEK with buffer. Subsequently, the cells were held for another 60 min in buffer at RT to allow intracellular deesterification.

Measurements were performed in a temperature-controlled cuvette (37°C), housed in a spectrofluorimeter (SLM-Aminco 500C). Changes in fluorescence were monitored at excitation wavelengths of 340 and 359 nm by manual adjustment of the wavelengths. The emission wavelength was 510 nm.

Coverslips were positioned diagonally in the cuvette which contained HEPES buffer. $[Ca^{2+}]_i$ was estimated from ratio measurements of Fura-2 fluorescence (340/359 nm excitation) using the following equation:

$$[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](S_{f2}/S_{b2})$$

K_d is the dissociation constant (220 nM at 37°C). R is the ratio of the sample, R_{min} and R_{max} are the ratios obtained with cells containing very low and very high Ca^{2+} levels, respectively. The parameter S_{f2}/S_{b2} is the ratio of fluorescence values measured at 359 nm before/after $CaCl_2$ addition.

To determine R_{min}, cells were loaded with Fura-2 in a Ca^{2+} free buffer containing 1 mM ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) and the ratio of fluorescence intensity at the excitation wavelength pair 340/359 nm was recorded. To provide R_{max}, $[Ca^{2+}]_i$ of the keratinocytes was raised by enhancing the Ca^{2+} concentration in the cuvette to 0.5 mM, together with the addition of ionomycin (Sigma) (final concentration 10 μM). The values

obtained for R_{min} and R_{max} were 0.75 and 1.32, respectively.
HEK were incubated for 30 min with various concentrations of sulfur mustard in KGM without bovine pituitary extract at RT. Cells were loaded with dye after exposure, except for the case that $[Ca^{2+}]_i$ was measured 1 hr after sulfur mustard treatment.

E) Detection of basement membrane zone proteins

- SDS-PAGE

Samples of HEK culture extracts in SDS-PAGE sample buffer were heated for 10 min at 95°C and loaded onto a 7.5% Phastgel (PhastSystem, Pharmacia). The SDS-PAGE protocol was run according to the manufacturer. Subsequently, proteins were visualized by staining with Coomassie brilliant blue according to the PhastSystem protocol or, alternatively, the gel was used for Western blotting.

- Western blotting

Proteins were blotted from a Phastgel onto a nitrocellulose (NC) filter of 4x4 cm (Schleicher & Schuell, 0.1 µm) using the electroblotting device of PhastSystem (Pharmacia). The NC filter was presoaked in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. Blotting was performed at 12.5 mA per gel during 15 min. The NC filter was then incubated with gentle shaking as follows (all steps at 20°C):

(1) 2 times 30 min in 20 ml 5% low fat milk powder (LFMP) in PBS.
(2) 90 min in 10 ml 0.5% LFMP in PBS with the specific antibody.
(3) 3 times 5 min in 20 ml PBS.
(4) 90 min in 10 ml 0.5% LFMP in PBS with the secondary antibody (normally Alkaline Phosphatase conjugated).
(5) 3 times 5 min in 20 ml PBS.
(6) 5 min in 20 ml of 100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$ (solution D).
(7) 1-3 min in 15 ml of solution D containing 100 µl 4-nitro blue tetrazolium chloride (NBT) (50 mg/ml in 70% dimethyl formamide (DMF)) and 50 µl 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (50 mg/ml in 100% DMF).

- Immunohistochemistry

Human keratinocytes were cultured as described earlier on coverslips in 12-well plates during 7-14 days. Medium was removed by rinsing with PBS (3 times). Cells were fixed in 2% paraformaldehyde in PBS (30 min 4°C) and rinsed with PBS (3 times). To improve intracellular detection, cells were then treated with 0.05% Nonidet P40 in PBS (10 min 20°C) and rinsed with PBS (3 times). The coverslips were then removed from the well plate and fixed onto glass slides using nail polish as glue. The fixed cells were subsequently incubated as follows (all steps in 50 µl at 20°C unless otherwise specified):

(1) 16 hr at 4°C in 0.1% BSA in PBS with the specific antibody.

(2) 3 times 5 min in PBS.

(3) 10 min in 0.02% H_2O_2 in PBS.

(4) 3 times 5 min in PBS.

(5) 2hr in 0.1% BSA + 1% serum in PBS with the secondary antibody (normally Peroxidase conjugated).

(6) 3 times 5 min in PBS. Coverslips were then incubated vertically during 7-10 min in 40 ml of 0.05 M Na-acetate pH 5.0 to which 3-amino-9-ethylcarbazole (16 mg in 1 ml DMF) and 200 µl 3% H_2O_2 were added.

RESULTS

A) Protein ADP-ribosylation

The fraction of radiolabeled 2-^{3}H adenine taken up by HEK varied in different experiments between 40 and 70%. Following precipitation with TCA 85-90% of the total amount of incorporated adenine remained in the supernatant and 10-15% was present in the pellet. Since non-protein-bound activity might be trapped in the pellet, the pellet was resolved with formic acid and reprecipitated with TCA. This final pellet contained proteins with covalently bound mono- or poly-ADP-ribose. The amount of radioactivity in the pellet was reduced to 2-5% of the total amount of incorporated 2-^{3}H adenine.

The maximum binding capacity of DHB-Biorex and DHB-Sepharose columns was determined by loading them with known quantities of NAD^+ and spectrophotometric measurement of NAD^+ in the eluent. The maximum binding capacity was defined as one-half the amount of NAD^+ loaded on a column that resulted in 50% binding. The DHB-Sepharose column had a capacity to bind 3.6 µmol of NAD^+ per ml packed resin, which is close to the 1.2 µmol reported by Alvarez-Gonzalez et al. (1983). The binding capacity of DHB-Biorex was 3.0 µmol NAD^+ per ml of resin, which is low compared to the 19.0 µmol reported by Alvarez-Gonzalez et al.

Figure 1 shows that NAD^+ could be effectively separated from other adenine-containing compounds in the TCA-soluble fraction on the DHB-Sepharose column. Label found in the NAD^+ eluate represented 13% of all incorporated ^{3}H adenine in the culture well. Mono- and poly-ADP-ribose residues from the TCA-insoluble pellet can be separated by application to a DHB-Biorex column (Figure 2).

Since it is known that treatment of cells with γ-irradiation leads to DNA SSB with subsequent activation of PADPRT and depletion of NAD^+, cultured HEK labeled with ^{3}H adenine were exposed to γ-irradiation (150 and 300 Gray). Determinations of NAD^+ and the total amount of ADP-ribosylated proteins were made immediately after irradiation and are shown in Table 1. A clear decrease of

cellular NAD^+ content and a small increase of the total amount of ADP-ribose residues were observed in γ-irradiated cultures.

B) NAD^+ depletion following γ-irradiation and sulfur mustard exposure

The cellular NAD^+ content of HEK cultured in KSFM was measured immediately, and at 4 hr and 24 hr after exposure to various doses of γ-irradiation. Results are shown in Figure 3. The NAD^+ content of HEK exposed to γ-irradiation of 70, 150 or 300 Gray was reduced in a dose-dependent manner immediately following treatment. The initial small drop in NAD^+ levels of cells exposed to 70 Gray of γ-irradiation decreased during the first 4 hr, and was completely restored at 24 hr after exposure. If HEK were exposed to 150 Gray, a considerable decrease of NAD^+ had developed at 4 hr following exposure, which was partly restored at 24 hr. Upon irradiation with 300 Gray, the NAD^+ content of HEK showed still a reduction of 40% at 24 hr after exposure.

Changes in the NAD^+ content of HEK immediately, 4 hr and 24 hr after exposure to various concentrations of sulfur mustard are shown in Figure 4. In contrast to the results obtained with γ-irradiation, no changes could be observed in the NAD^+ content of HEK cultures immediately after exposure to concentrations of sulfur mustard up to 150 μM. Treatment with concentrations up to 10 μM sulfur mustard had no effect on cellular NAD^+ levels for 24 hr following exposure. Upon treatment with concentrations of 25 and 50 μM sulfur mustard, a small decrease in NAD^+ was visible at 4 hr, which remained steady over 24 hr. If cells were exposed to 100 μM sulfur mustard or more, a considerable loss of NAD^+ was observed at 4 hr, which develops further over the next 20 hr.

C) Intracellular calcium determination

When HEK were exposed during 30 min to 50, 100 or 200 μM of sulfur mustard this resulted in little or no changes in $[Ca^{2+}]_i$ at 1, 4, or 7 hr after exposure compared to the $[Ca^{2+}]_i$ of untreated controls at these time points. Data are presented in Table 2. Two small changes

were observed. One is a slight decrease of [Ca]i at 1 hr after exposure to 100 μM sulfur mustard. The other is a little increase in cells that were exposed to 50 μM at 7 hr after exposure.

D) Biosynthesis of BMZ proteins

Antibodies against BMZ proteins that have been used to detect the production of these proteins in cultured HEK by Western blotting techniques are listed in Table 3. Four patient BPA sera reacted in a Western blot and recognized one or both forms of BPA, with molecular weights of 180 and 230 kD (see Figure 5). With all other antibodies, results were negative using conventional detection methods as well as dot-blots, immunoprecipitation or Western blots with sensitive chemiluminescent detection techniques.

To address the question of whether cultured HEK indeed produce the BMZ proteins under study, their presence in HEK cultured on coverslips was investigated with immunohistochemical techniques. Positive results were found for all antibodies available. Collagen types IV and VII, laminin and BPA were detected intracellularly, i.e., in the cytoplasm (presumably as precursors). Integrin subunits α_6 and β_4 were localized intracellularly, too, but antigen was also clearly present at the outer cell membrane. No changes in the staining intensity of each of the BMZ proteins were seen at 24 hr following exposure of HEK on coverslips to 50 or 250 μM sulfur mustard, although obvious effects on the morphology of the cells were observed. Results obtained for integrin α_6 are shown in Figure 6 as an example.

TABLE 1. Changes in the amounts of NAD^+ and total protein-bound ADP-ribose in HEK immediately following exposure to γ-irradiation. Results of one single experiment.

γ-Irradiation (Gray)	NAD (label present in specific fraction as % of total ^3H incorporation)	Total ADP-ribose
0	12.6	0.01
	12.5	
150	11.7	0.02
		0.03
300	8.4	0.03
	9.5	0.06
	10.4	
	10.3	

TABLE 2. $[Ca^{2+}]_i$ (nM) of HEK exposed to sulfur mustard (x ± s.d.; n=6 for each point).

Conc. HD (μM)	Time after exposure (hr)		
	1	4	7
0	148±10	101±7	128±6
50			154±13
100	110±8	110±8	138±10
200	138±7	101±4	

TABLE 3.

Available antibodies against BMZ proteins and their reaction in Western blots and immunohistochemistry.

Code	Antigen	Mono/Poly	Reaction in cultured cells	Reaction in Western blot
PS50	human coll IV	P	+	no
LH 7:2	human coll VII	M	+	prob.not
PS40	human laminin	P	+	prob.not
GoH3	human integrin α_6	M	+	no
J8H	human integrin α_6	M	+	no
3E1	human integrin β_4	M	+	no
439-9B	human integrin β_4	M	+	prob.yes
BP(8)	human BP antigen	P	+	yes
92F174	human BP antigen	P	+	yes
92F029	human BP antigen	P	+	yes
91F228	human BP antigen	P	+	yes
89F289	human BP antigen	P	+	yes

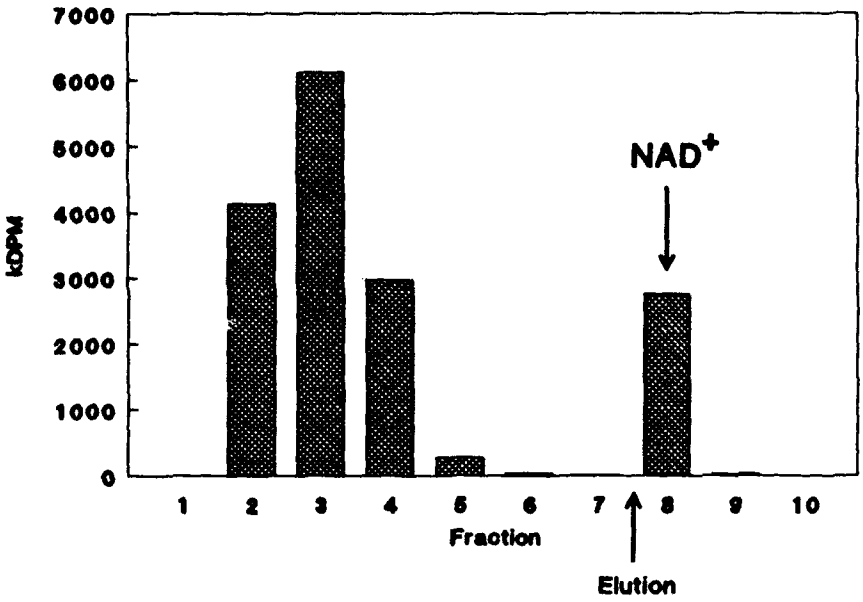

Figure 1. Boronate chromatography of the TCA-soluble pool of ^{3}H adenine-labeled control HEK. Typical elution profile of NAD^{+} over a DHB-Sepharose column.

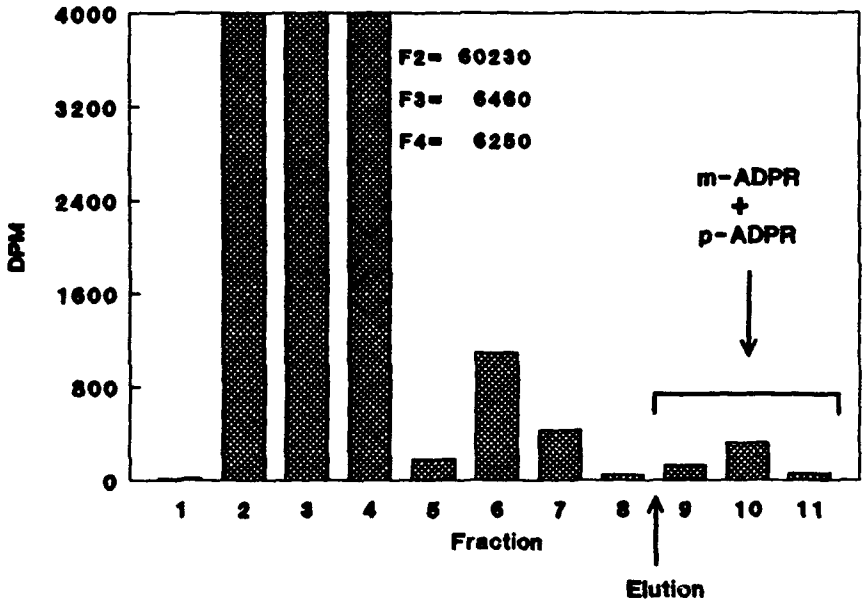

Figure 2. Boronate chromatography of the TCA-insoluble pool of ^{3}H adenine labeled control HEK. Typical elution profile over a DHB-Biorex column of mono- and poly-ADP-ribose released from protein.

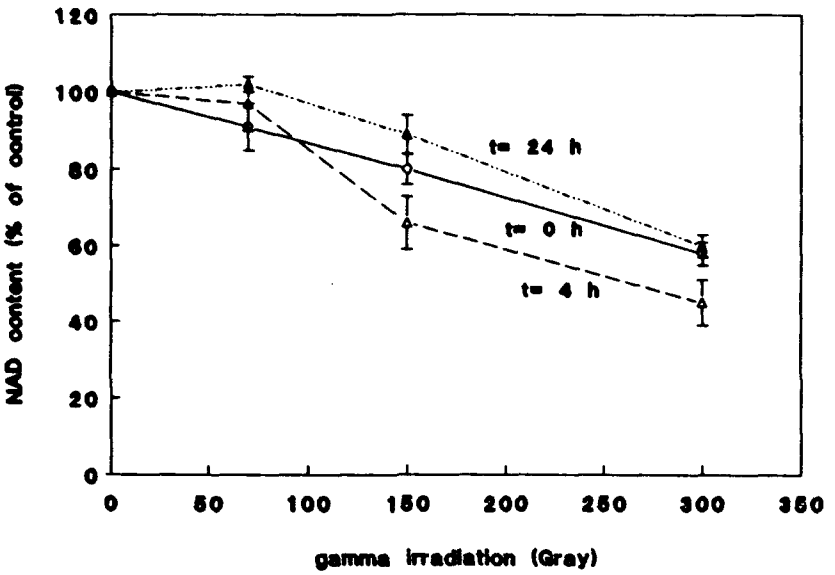

Figure 3. Decrease in NAD^+ levels of HEK cultures at 0, 4 and 24 hr following exposure to various doses of γ-irradiation. Each point represents the mean ± SE of at least 3 experiments with 3 cultures each.

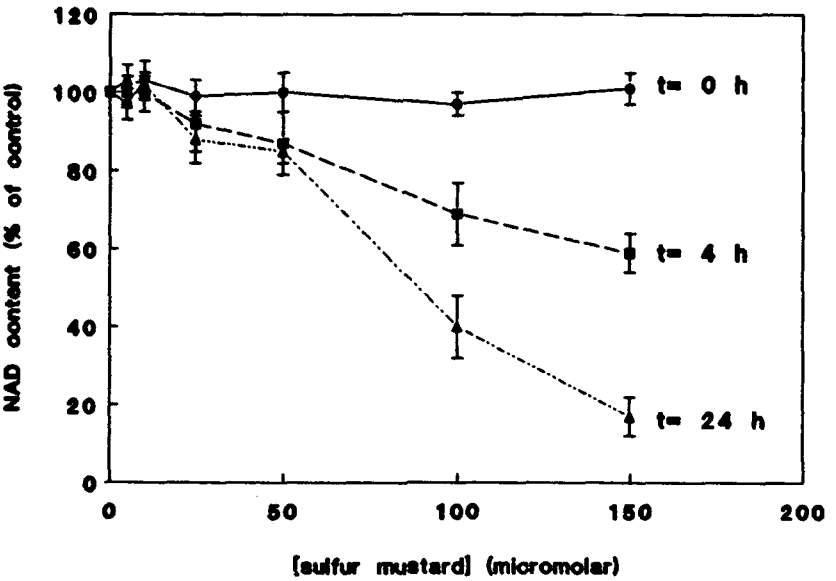

Figure 4. Decrease in NAD^+ levels of HEK cultures at 0, 4 and 24 hr following exposure during 30 min to various concentrations of sulfur mustard. Each point represents the mean ± SE of at least 3 experiments with 3 cultures each.

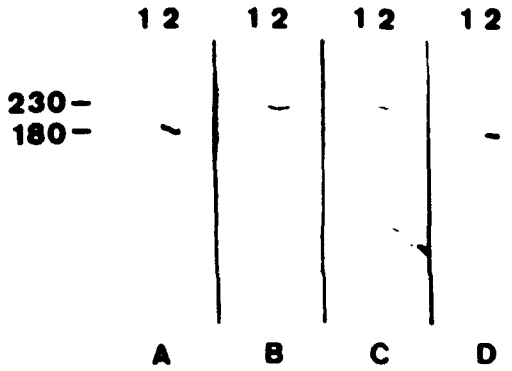

Figure 5. Immunoblot detection with four patient sera (A - D) of 180 kD and 230 kD BP antigen. Each serum was tested on an extract of a mammary tumor cell line (lanes 1) and on an extract of cultured HEK (lanes 2).

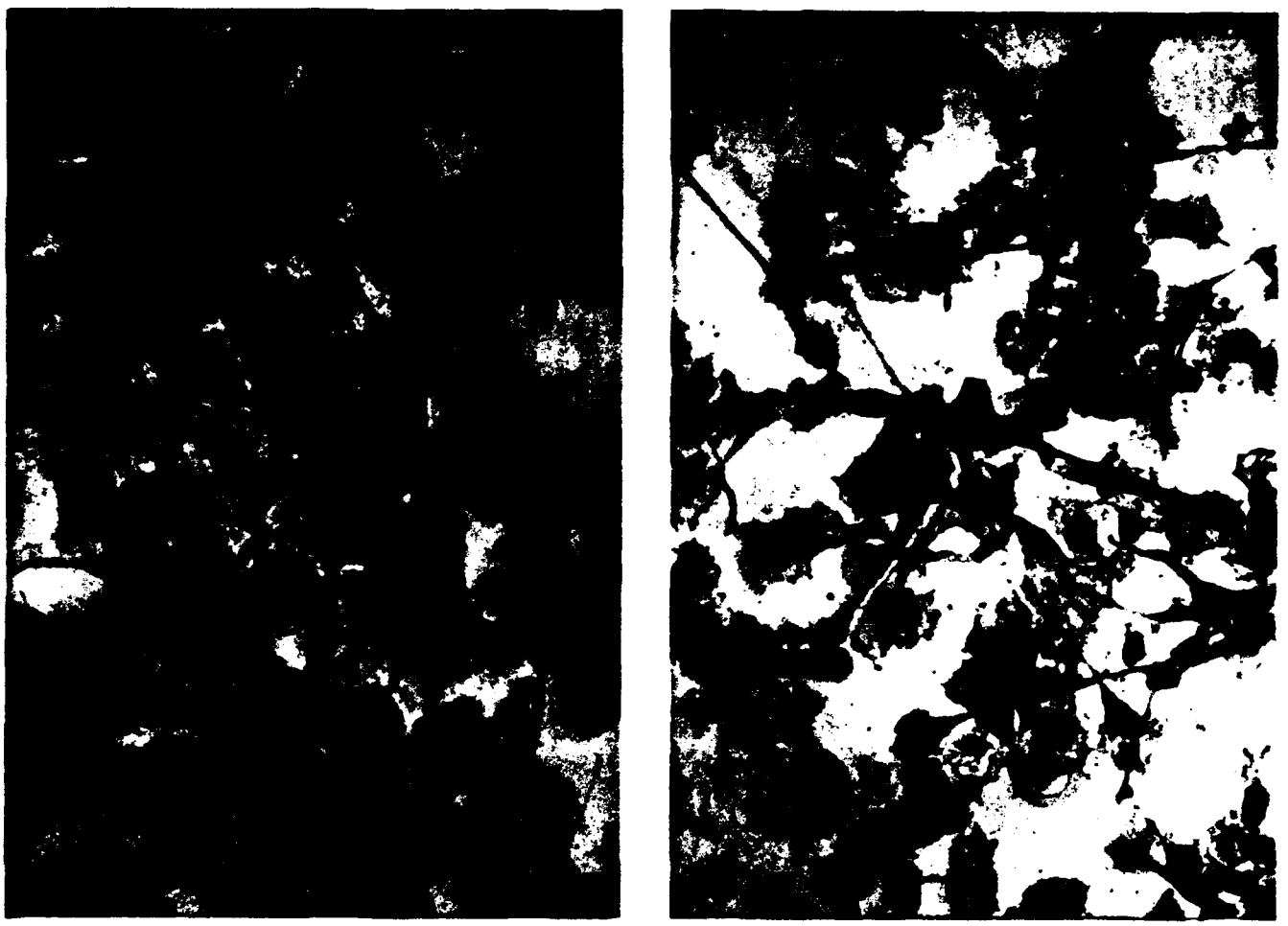

Figure 6. Immunohistochemical detection of integrin α_6 in HEK cultured on coverslips. The left panel shows control HEK, the right panel shows HEK, stained 24 hr after treatment with 250 µM sulfur mustard.

DISCUSSION

A) Protein ADP-ribosylation

DHB-Biorex and DHB-Sepharose resins have been synthesized and used for simultaneous quantification of NAD^+ as well as protein-bound polymers and monomers of ADP-ribose. It was decided that the relatively low binding capacity of 3.0 µmol on the DHB-Biorex resin was still sufficient for the ADP-ribose determinations because the amounts of ADP-ribose were expected to be in the nmol range. In a first experiment, HEK were treated with γ-irradiation which induces DNA SSB with, subsequently, activation of PADPRT and depletion of NAD^+. Quantification of NAD^+ levels by boronyl affinity chromatography showed that there was a dose-dependent depletion of NAD^+ (Table 1), although it was not as pronounced as was seen with the enzymatic NAD^+ assay (Figure 3). The total amount of ADP-ribose separated over DHB Biorex columns showed an increase with higher doses of irradiation. These results were encouraging and, with some technical improvements, this methodology will work well.

B) NAD^+ depletion following γ-irradiation and sulfur mustard exposure

By comparison of present results with those obtained with HEK cultured in DMEM/F12 medium (Mol et al. 1989), HEK cultured in KSFM appeared to be more vulnerable to sulfur mustard-induced NAD^+ depletion. This might be caused by the higher state of differentiation of HEK cultured in DMEM/F12 medium, which contains a high Ca^{2+} level. Differentiated cells contain more proteins which may act as scavengers for sulfur mustard.

The presence of DNA SSB caused by γ-irradiation will activate PADPRT to facilitate the DNA repair process. NAD^+ serves as a substrate for the synthesis of the nuclear ADP-ribose polymers. Figure 3 showed that irradiation with doses up to 70 Gy produced only a slight and transient decrease of the total cellular NAD^+ pool. Apparently, cells can buffer their NAD^+ pool to a considerable extent during the repair of an extensive amount of DNA

SSB like that induced by 70 Gy of radiation. If it is assumed that 1 Gy produces as much DNA SSB as 5 μM sulfur mustard immediately following exposure (Mol and van der Schans, 1993), the repair of SSB present immediately after treatment with 150 μM sulfur mustard will cause no loss of NAD^+ from cellular pools, as was observed (Figure 4). However, NAD^+ levels in sulfur mustard-treated cells decrease gradually over the next 24 hr to 20% of control levels. The reason for that drop is not known. Possibly, repair of DNA interstrand cross-links might cause SSB in DNA continuously, which might exhaust the cellular NAD^+ pool. A second possibility might be that other NAD^+-consuming processes are active, like mono-ADP-ribosylation of proteins or NAD^+ glycohydrolase activity.

C) Intracellular calcium determination

For a meaningful interpretation of the presented data, it should be kept in mind that they were based on a limited number of experiments and were obtained under suboptimal experimental conditions since the available spectrofluorometer was not quite fitted for $[Ca^{2+}]_i$ measurement with Fura-2. As a stirring device in the cuvette was lacking, cells in suspension could not be used. Therefore, cells were grown on coverslips which were placed diagonally in the cuvette. It appeared that after loading, unhydrolyzed Fura-2 AM remains attached to the cells. Probably, the ester is bound to cell membranes or trapped in organelles; both sites would be inaccessable to esterases. According to Malgaroli et al. (1987), who reported the same observations, the isosbestic point, 359 nm, was used as the second wavelength for ratio estimation. Although the fluorometer was not equipped for continuous dual excitation wavelength measurements, ratio estimation obtained by manual adjustment of the wavelengths was preferred over single wavelength measurements, since a number of variables that could perturb the measurements are eliminated by using a fluorescence ratio. Considering the thus obtained data, it has been concluded that, although some small changes in the $[Ca^{2+}]_i$ of HEK were observed, exposure of HEK to concentrations of sulfur

mustard up to 200 µM does not lead to an elevation of $[Ca^{2+}]_i$ levels within 7 hr after exposure. If exposure to sulfur mustard will cause any raise in $[Ca^{2+}]_i$, it will be so small that it could not be detected under the experimental conditions used. However, the presented data include neither the assessment of $[Ca^{2+}]_i$ at early time points after exposure nor time periods between 7 and 24 hr. Therefore, an early transient elevation of $[Ca^{2+}]_i$ or an irreversible increase starting later than 7 hr after exposure cannot be excluded.

D) Biosynthesis of BMZ proteins

Synthesis by cultured HEK of laminin, collagens type IV and type VII, integrins α_6 and β_4 and BPA could be demonstrated by immunohistochemistry. Of these BMZ proteins only BPA could be detected with Western immunoblot techniques. Either the cell extracts were not optimal or the antibodies available were not suited for antigen detection in Western blots.

No gross visible changes in the staining intensity of the six antibodies were observed over 24 hr in sulfur mustard-treated HEK. These results have to be confirmed quantitatively with ELISA.

CONCLUSIONS

During the past year, most of the time has been spent on the establishment of several detection methods. Except for Western immunoblots, assays seem to work fairly well. From preliminary experiments on cultured HEK we can conclude that:

- sulfur mustard induces at most a small increase in $[Ca^{2+}]_i$ during the first 7 hr following exposure.
- weakening of the epidermal-dermal junction is not caused by a considerable decrease in the amount of one of the investigated BMZ proteins within 24 hr following sulfur mustard exposure.
- comparison for differently acting DNA-damaging agents of the relationship between the formation of DNA SSB on one hand, and the metabolism of NAD^+ and ADP-ribose mono- and polymers on the other hand can be helpful to explain sulfur mustard-induced NAD^+ loss.

REFERENCES

ABOUL-ELA, N., JACOBSON, E.L., AND JACOBSON, M.K. (1988). Labeling methods for the study of poly- and mono(ADP-ribose) metabolism in cultured cells. Anal. Biochem. 174, 239 - 250.

ALVAREZ-GONZALEZ, R., JUAREZ-SALINAS, H., JACOBSON, E.L., AND JACOBSON, M.K. (1983). Evaluation of immobilized boronate for studies of adenine and pyridine nucleotide metabolism. Anal. Biochem. 135, 69 - 77.

BERGER, N.A., SIKORSKI, G.W., PETZOLD, S.J., AND KUROHARA, K.K. (1979). Association of poly (ADP-ribose) synthesis with DNA damage and repair in normal human lymphocytes. J. Clin. Invest. 63, 1164 - 1171.

CROALL, D.E., AND DEMARTINO, G.N. (1991). Calcium-activated neutral protease (calpain) system: structure, function and regulation. Physiol. Rev. 71, 813 -847.

FREI, B., AND RICHTER, C. (1988). Mono(ADP-ribosylation) in rat liver mitochondria. Biochemistry 27, 529 - 535.

HUA, A., DANIEL, R., JASSERON, M.P., AND THIRIOT, C. (1993). Early cytotoxic effects induced by bis-chloroethyl sulphide (sulphur mustard) - $[Ca^{2+}]_i$ rise and time-dependent inhibition of B77 fibroblast serum response. J. Appl. Toxicol. 13, 161 - 168.

JACOBSON, E.L., AND JACOBSON, M.K. (1976). Pyridine nucleotide levels as a function of growth in normal and transformed 3T3 cells. Arch. Biochem. Biophys. 175, 627 - 634.

MALGAROLI, A., MILANI, D., MELDOLESI, J., AND POZZAN, T. (1987). J. Cell Biol. 105, 2145 - 2155.

MELLONI, E., AND PONTREMOLI, S. (1991). The calpain-calpastatin system: structural and functional properties. J. Nutr. Biochem. 2, 467 - 476.

MOL, M.A.E. (1992). Molecular and cellular mechanisms of sulfur mustard-induced lesions of human skin (Thesis).

MOL, M.A.E., VAN DE RUIT, A.M.B.C., AND KLUIVERS, A.W. (1989). NAD+ levels and glucose uptake of cultured human epidermal cells exposed to sulfur mustard. Toxicol. Appl. Pharmacol. 98, 159-165.

MOL, M.A.E., AND VAN DER SCHANS, G.P. (1993). Quantification of sulfur mustard-induced DNA interstrand cross-links and single strand breaks in cultured human epidermal keratinocytes. Mut. Res. in press.

MOL, M.A.E., DE VRIES R., AND KLUIVERS, A.W. (1991). Effects of nicotinamide on biochemical changes and microblistering induced by sulfur mustard in human skin organ cultures. Toxicol. Appl. Pharmacol. 107, 439-449.

MOL, M.A.E., AND DE VRIES - VAN DE RUIT, A.M.B.C. (1992). Concentration- and time-related effects of sulphur mustard on human epidermal keratinocyte function. Toxicol. In Vitro 6, 245-251.

PAPIRMEISTER, B., GROSS, C.L., MEIER, H.L. PETRALI, J.P. AND JOHNSON, J.B. (1985). Molecular basis for mustard-induced vesication. Fund. Appl. Toxicol. 5, S134 - S149.

RICHTER, C., FREI, B., AND SCHLEGEL, J. (1985). Calcium transport and mono(ADP-ribosylation) in mitochondria. In: F.R. Althaus, H. Hilz, and S. Shall (Eds.), ADP-ribosylation of Proteins, Springer Verlag, Berlin/Heidelberg. pp 530 - 535.

RICHTER, C., AND KASS, G.E.N. (1991). Oxidative stress in mitochondria: its relationship to cellular Ca^{2+} homeostasis, cell death, proliferation, and differentiation. Chem.-Biol. Interactions 77, 1 - 23.

SCHRAUFSTÄTTER, I.U., HYSLOP, P.A., HINSHAW, D.B., SPRAGG, R.G., SKLAR, L.A., AND COCHRANE, C.G. (1986). Hydrogen peroxide - induced injury of cells and its prevention by inhibitors of poly (ADP - ribose) polymerase. Proc. Natl. Acad. Sci. U.S.A. 83, 4908 - 4912.

SKIDMORE, C.J., DAVIES, M.I., GOODWIN, P.M., HALLDORSSON, H., LEWIS, P.J., SHALL, S., AND ZIA'EE, A.A. (1979). The involvement of poly (ADP - ribose) polymerase in the degradation of NAD caused by γ - radiation and N - methyl -N - nitrosourea. Eur. J. Biochem.

101, 135 - 142.

TANG, W., AND ZIBOH, V.A. (1991). Agonist/inositol triphosphate-induced release of calcium from murine keratinocytes: a possible link with keratinocyte differentiation. J. Invest. Dermatol. 96, 134 - 138.

UITTO, J., OLSEN, D.R., AND FAZIO, M.J. (1989). Extracellular matrix of the skin: 50 years of progress. J. Invest. Dermatol. 92, 61 S - 77 S.