Expression of Dominant-negative Fas-associated Death Domain Blocks Human Keratinocyte Apoptosis and Vesication Induced by Sulfur Mustard*

Received for publication, September 17, 2002, and in revised form, December 2, 2002
Published, JBC Papers in Press, December 12, 2002, DOI 10.1074/jbc.M209549200

Dean S. Rosenthal‡§, Alfredo Velena‡, Feng-Pai Chou‡, Richard Schlegel‡, Radharaman Ray¶, Betty Benton§, Dana Anderson‡, William J. Smith¶, and Cynthia M. Simbulan-Rosenthal‡

From the Departments of ‡Biochemistry and Molecular Biology and ¶Pathology, Georgetown University School of Medicine, Washington, D. C. 20007 and the §United States Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, Maryland 21010

Sulfur mustard (bis-(2-chloroethyl) sulfide; SM),1 the vesicant agent used as recently as 1988/1989 in the Iraq/Iran conflict and implied to have been used in the Gulf War, induces vesication in human skin by its ability to cause cytotoxic, genotoxic, or a combination of both effects in the skin. SM is a highly reactive compound that induces the death and detachment of the basal cells of the epidermis from the basal lamina (1–6). SM causes blisters in the skin via poorly understood mechanisms. In an effort to help develop medical countermeasures for potential exposure of military personnel and civilians, we have been attempting to define the molecular series of events leading to SM toxicity in cell culture, in transgenic animal models, and in grafted human epidermis.

Whereas human dermal fibroblasts may contribute to the vesication response by releasing degradative cytosolic components extracellularly after a poly(ADP-ribose) polymerase (PARP)-dependent SM-induced necrosis (7), keratinocytes display markers of an apoptotic death, as well as those of terminal differentiation (8). SM-induced apoptosis in keratinocytes appears to be controlled by both death receptor and mitochondrial pathways (9). The targets of these apoptotic pathways are a family of aspartate-specific cysteine proteases or caspases (10). Caspase-3 appears to be a converging point for different apoptotic pathways (11). In most apoptotic systems, caspase-3 is proteolytically activated, and in turn cleaves key proteins involved in the structure and integrity of the cell, including PARP (11–14).

In the present study, we demonstrate that SM induces both Fas and its ligand (FasL) in primary human epidermal keratinocytes. We also observed the activation of markers of apoptosis that are consistent with a Fas-FasL-receptor interaction, including cleavage of caspase-8, caspase-3, and PARP. Utilizing a combination of techniques including the stable expression of a dominant-negative inhibitor of Fas-associated death domain protein (FADD), we demonstrate a role for the Fas/TNF receptor family in mediating the response of human keratinocytes to SM. Stable expression of FADD-DN blocks SM-induced markers of keratinocyte apoptosis, such as caspase-3 activity and proteolytic processing of procaspases-3, -7, and -8, internucleosomal DNA cleavage, and caspase-6-mediated nuclear lamin cleavage.

† This work was supported by United States Army Medical Research and Materiel Command contract DAMD17-00-C-0026 (to D. S. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Georgetown University School of Medicine, 3900 Reservoir Rd. NW, Washington, D. C. 20007; Tel: 202-887-1056; Fax: 202-887-7186; E-mail: rosenthld@georgetown.edu.

1 The abbreviations used are: SM, sulfur mustard; NHEK, normal human epidermal keratinocytes; FADD, Fas-associated death domain; DN, dominant-negative; PARP, poly(ADP-ribose) polymerase; FasL, Fas ligand; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; SFM, serum-free medium; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; AMC, aminomethylcoumarin; DFF, DNA fragmentation factor; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered saline.
We have shown earlier that NHEK as well as an immortalized line, Nco, could be used to establish a histologically and immunocytochemically normal epidermis when grafted onto nude mice (8, 9, 15). The present study demonstrates that markers of apoptosis are induced in basal cells of SM-exposed grafts, particularly in regions where microvesicles are formed. We have now also utilized the graft system to genetically engineer human keratinocytes prior to grafting to ectopically express a dominant-negative FADD and generate a human epidermis containing FADD-DN keratinocytes. These human grafts were exposed to SM, and showed a reduced vesication response compared with control keratinocyte. Topical SM exposure of Fas-deficient mice in the current study also indicates the viability of this strategy to suppress vesication by using inhibitors of the death receptor pathway.

An understanding of the mechanisms for SM vesication will hopefully lead to therapeutic strategies for prevention or treatment of SM toxicity. Importantly, our experiments indicate that the Fas/FADD pathway is required for caspase-3 processing, because inhibitors of this pathway block SM-induced apoptosis. Because the FADD pathway can be manipulated at the level of a cell surface (Fas), receptor, Fas/FADD as well as the caspases represent attractive targets for the modulation of the effects of SM. Inhibition of the Fas/FADD pathway by specific pharmacological inhibitors such as neutralizing antibodies to Fas or peptide inhibitors of caspases may therefore be of therapeutic value in the treatment of or prophylaxis against SM injury in humans.

MATERIALS AND METHODS

Cells, Plasmids, and Transfection—Primary human keratinocytes were derived from neonatal foreskins and grown in keratinocyte serum-free medium (SFM) supplemented with human recombinant epidermal growth factor and bovine pituitary extract (Invitrogen). Primary keratinocytes were immortalized by transduction with the HPV16 E6/E7 genes (16) to generate the Nco cell line as described previously (17). The FADD-DN plasmid construct in pcDNA 3.1 (Invitrogen), a generous gift from Dr. V. Dixit, expresses a truncated FADD protein, which lacks the N-terminal domain that is responsible for recruiting and activating caspase-8 at the death receptor complex (Fig. 5A). Nco cells were transfected with empty vector or with FADD-DN using LipofectAMINE (Invitrogen), and stable clones were selected in G418 and maintained in SFM. Cells were grown to 60–80% confluency, and then exposed to SM diluted in SFM to final concentrations of 100, 200, or 300 μM, with or without pretreatment with Fas- (clone ZB-4; Upstate Biotech, Wal- tham, MA) or TNFR1- (clone H398; Bender MedSystems, Vienna, Austria) neutralizing antibodies. Media was not changed for the duration of the experiments. At different time points after SM exposure, cells were harvested for further analyses.

Chemicals—SM (bis-(2-chloroethyl) sulfide; >98% purity) was ob- tained from the United States Army Edgewood Research, Development and Engineering Center.

Fluorometric Assay of Caspase-3 Activity—Cells were resuspended in lysis buffer containing 50 mm Tris-HCl (pH 7.5), 150 mm NaCl, 1 mm EGTA, 0.25% sodium deoxycholate, 0.5% Nonidet P-40, 10 μg/ml apro- tinin, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mm phenylmeth- ylsulfonfluoride. Cells were incubated for 10 min on ice, and freeze-thawed 3 times. The cell lysate was centrifuged at 14,000 × g for 5 min, and the protein concentration of the cytosolic extract was determined by the Bio-Rad DC protein assay kit. For the fluorometric caspase-3 activity assay, 25 μg of cytosolic extract was initially diluted to a volume of 50 μl with Nonidet P-40 lysis buffer, to which 50 μl of caspase assay buffer (100 mm Tris-Cl, pH 7.4, 2 mm EDTA, 0.1% Triton X-100, 20% glycerol) was added. The aliquots were then mixed with equal amounts (100 μl) of 40 μM fluorescent tetrapeptide substrate specific for caspase-3 (Ac-DEVD-AMC; BACHEM) in caspase assay buffer and transferred to 96-well plates. Free aminomethylcoumarin (AMC), generated as a result of cleavage of the aspartate-AMC bond, was monitored continu- ously by fluorescence (excitation 380 nm, emission 460 nm) in an Enzyme Systems, Framingham, MA) at excitation and emission wavelengths of 360 and 460 nm, respectively. The emission from each well was plotted against time, and linear regression analysis of the initial velocity (slope) for each curve yielded the activity.

Immunoblot Analysis—SDS-PAGE and transfer of separated pro- teins to nitrocellulose membranes were performed according to stand- ard procedures. Proteins were measured (DC protein assay; Bio-Rad) and normalized prior to gel loading, and all filters were stained with Ponceau S, to reduce the possibility of loading artifacts. They were then incubated with antibodies to the p17 subunit of caspase-3 (1:200; Santa Cruz Biotechnology), caspase-7 (1:1000; BD Pharmingen), caspase-8 (1:1000; Trevigen), or caspase-10 (1:1000; Trevigen), lamin A (1:100; Santa Cruz Biotechnology), DNA fragmentation factor (DDF) (15:400; BD Pharmingen), or PARP (1: 1000; BD Pharmingen). Immune complexes were detected by subse- quent incubation with appropriate horseradish peroxidase-conjugated antibodies to mouse or rabbit IgG (1:3000) and enhanced chemilumi- nescence reagents. Slides were sequentially exposed to x-ray films by incubation for 30 min at 50 °C with a solution containing 100 mm 2-mercaptoethanol, 2% SDS, and 62.5 mm Tris-HCl (pH 6.7), blocked again, and reprobed with additional antibodies to accurately compare different proteins from the same filter. Typically, a filter could be reprobed three times before there was detectable loss of protein from the membrane, which was monitored by Ponceau S staining after striping.

Analysis of DNA Fragmentation—Cells were harvested and lysed in 0.5 ml of 7 M guanidine hydrochloride, and total genomic DNA was extracted and purified using a Wizard Miniprep DNA Purification Resin (Promega). After RNase A treatment (20 μg/ml) of the DNA samples for 30 min, apoptotic DNA fragmentation was detected by gel electrophoresis on a 1.5% agarose gel at 4 V/cm. DNA ladders were visualized by staining with ethidium bromide (0.5 μg/ml) and images were captured with the Kodak EDAS 120 (Kodak) gel documentation system.

Annexin V and Propidium Iodide Staining, and FACS Analysis—Cells were plated in culture plates and exposed to various concentra- tions of SM. 16 h after induction of apoptosis, the cells were trypsinized, washed with ice-cold phosphate-buffered saline (PBS), and subse- quently resuspended in and incubated in the dark with 100 μl of annexin V incubation reagent that includes fluorescein isothiocyanate-conjugated annexin V (Trevigen, Gaithersburg, MD) and propidium iodide (Molecular Probes). Flow cytometric analysis was conducted on a BD Biosciences FACStar Plus cytometer using a 100- milliwatt air-cooled argon laser at 488 nm.

Grafting Protocols and Exposure of Human Skin Grafts to SM—A 1-cm diameter piece of skin was removed from the dorsal surface of athymic mice, and a pellet of cells containing 5 × 106 fibroblasts + 5 × 106 keratinocytes (NHEK or Nco) was pipetted on top of the muscular layer within a silicon dome to protect the cells during epithelization (Fig. 10A). The dome was removed after a week and the graft was allowed to develop for 6–8 weeks. SM exposure was performed by placing a small amount of SM liquid into an absorbent filter at the bottom of a vapor cup, which was then inverted onto the dorsal surface of the athymic mouse, to expose the graft site to the SM vapor. Frozen and fixed sections were derived from punch biopsies taken from the graft site, and analyzed for the expression of FADD-DN using the AU1 antibody, which recognizes the specific AU1 epitope tag on the FADD-DN protein. Histological analysis of the SM-exposed human skin grafts trans- planted onto nude mice was also performed utilizing an end point of micro- or macroblisters or SM-induced microvesication.

Assays for in Vivo Markers of Apoptosis on Human Skin Grafts—Paraffin-embedded sections derived from SM-exposed human skin grafts were subjected to analysis for markers of in vivo apoptosis, including indirect immunofluorescence microscopy with antibodies to the active form of caspase-3 (Cell Signaling Technology, Beverly, MA). Antibodies were incubated overnight in a humid chamber at room temperature with antibodies to active caspase-3 (1:250 dilution) in PBS containing 12% bovine serum albumin. After a PBS wash, slides were incubated for 1 h with biotinylated anti-mouse IgG (1:400 dilution in PBS/bovine serum albumin), washed, and incubated for 30 min with streptavidin-conjugated Texas Red (1:900 dilution in PBS/bovine serum albumin). Cells were finally mounted with PBS containing 90% glycerol and observed with a Zeiss fluorescence microscope.

DNA breaks characteristic of the late stage of apoptosis were de- tected in situ using a Klenow fragment-based assay system (Derma- TACS; Trevigen). For fixation, slides were equilibrated to room temper- ature and rehydrated for 2 h on a slide warmer at 45 °C, rehydrated in 100, 90, 70, and 50% ethanol, washed twice in PBS, fixed in 3% glutaral- dehyde for 10 min at room temperature, and washed in PBS. Slides were then incubated with 50 μl of Cytoxin for 30 min at room temperature, washed twice in deionized water, and immersed in quenching solution containing 90% methanol and 3% H2O2 for 5 min at room temperature.
After a PBS wash, slides were incubated in terminal deoxynucleotidyltransferase labeling buffer for 5 min at room temperature, and visualized under a bright field microscope.

RESULTS

Characterization of the Sequence of Events during SM-induced Apoptosis—We determined the sequence of events involved in SM-induced apoptosis by performing dose-response and time course experiments. Fas, a cell-surface receptor found in most cell types including keratinocytes, mediates some forms of apoptosis. Upon activation by its specific ligand (FasL), or by agonist antibody, Fas forms a homotrimeric complex, which in turn recruits the FADD to the membrane-bound complex. In turn, one or more of the upstream caspases (caspase-8 or -10) localize to the Fas-FADD complex, and become autocatalytically activated. We first determined whether SM induces expression of the Fas receptor or its ligand because enhanced expression of Fas or FasL has been shown to occur in cells exposed to DNA damaging agents, leading to activation of upstream caspase-8 and downstream apoptotic events such as caspase-3-mediated PARP cleavage (19, 20). Immunoblot analysis of extracts derived from keratinocytes exposed to different doses of SM revealed a dose-dependent increase in the levels of both Fas receptor and FasL in response to SM (Fig. 1A).

By immunoblot analysis using antibodies that recognize both the full-length (116 kDa) and 89-kDa cleavage products of PARP, we also demonstrated that SM-induced apoptosis is accompanied by complete cleavage of PARP into 89- and 24-kDa fragments that contain the active site and the DNA-binding domain of the enzyme, similar to the caspase-3-mediated cleavage of PARP induced by exposure to anti-Fas (Fig. 1C).

The central signaling proteins for many of the pathways that coordinate apoptosis are the caspases, cysteine proteases named for their preference for aspartate at their substrate cleavage site (10), which cleave key proteins involved in the structure and integrity of the cell. We previously focused on the upstream caspases-3 activation in the SM apoptotic response (8, 9), because caspase-3 has been shown to be a converging point for different apoptotic pathways (11). In a number of apoptotic systems, caspase-3 cleaves key proteins involved in the structure and integrity of the cell. To further understand the apoptotic response of keratinocytes following SM exposure, we assayed for the activation of other key caspases, in particular the upstream caspases-8, -9, and -10, and the executioner caspases-6, -8, and -7. When the blot in Fig. 1A was stripped of antibodies and reprobed with anti-caspase-8, SM-induced proteolytic processing of caspase-8 was noted in cells exposed to vesicating doses of SM (200 and 300 μM; Fig. 1B).

The sequence of caspase activation provides insight into the mechanism of apoptosis because caspase-8 is first activated following engagement of death receptors, whereas caspase-9 is activated via a mitochondrial pathway. We therefore investigated the molecular ordering of caspase activation in response to SM. NHEK were exposed to 300 μM SM for various times, and cell extracts were derived and subjected to immunoblot analysis utilizing antibodies specific to caspases-3, -7, -8, -9, or -10. Upstream caspases-8 and -9 were both activated in a time-dependent fashion, with caspase-8 cleaved prior to caspase-9 (1 versus 4 h) (Fig. 2). Because activation of caspase-8 correlates with a Fas-mediated pathway of apoptosis and activation of caspase-9 is consistent with a mitochondrial pathway, these results are in agreement with the activation of both death receptor and mitochondrial pathways by SM. In contrast, no cleavage of caspase-10 was observed (Fig. 6C).

The executioner caspases-3 and -7 were both proteolytically activated after SM exposure, with caspase-3 activation detectable 3 h after SM exposure, and caspase-7 cleavage noted 4 h after exposure. To detect caspase-6 activity, we utilized antisera specific for lamin A, which is cleaved in vivo by active caspase-6 at the peptide sequence VEID. Caspase-6 activity is essential for lamina A cleavage, which is necessary for chromatin condensation during apoptotic execution (21). Fig. 3 shows the time course of caspase-6-mediated lamin A cleavage in NHEK following SM exposure. Surprisingly, this substrate was one of the first to be cleaved (within 1 h), relative to cleavage of PARP (6 h), or the apoptotic DFF/ inhibitor of caspase-activated DNase (16 h; Fig. 3). PARP has been shown to be a substrate of caspase-3 and -7, whereas DFF 45 is primarily cleaved by caspase-3. Taken together, these data suggest that caspase-6 may be the first of the executioner caspases to be activated following exposure of NHEK to SM, followed by caspase-3 and -7.

Caspase-6-mediated Cleavage of Epidermal Keratin K1 following SM Exposure—We previously found that the suprabasal-specific keratins, K1 and K10, are induced upon exposure of NHEK to 100 μM SM, using monoclonal antibodies (8). In the current study, we utilized a polyclonal antibody directed against the C terminus of K1, and found that exposure of cells to higher concentrations of SM resulted in proteolytic cleavage of keratin K1 (Fig. 4A). The size of the K1 cleavage product maps near a perfect consensus sequence for a site of cleavage
by caspase-6 (Fig. 4B). Moreover, point mutations near this region of K1 give rise to a genetic blistering disorder, epidermolytic hyperkeratosis, very similar to SM-induced vesication (22). K1 may therefore be a substrate for caspase-6 and a target during SM-induced keratinocyte apoptosis.

Expression of FADD Dominant-negative in Human Keratinocytes Inhibits SM-induced Activation and Processing of Caspases-3 and -8—Up-regulation of the Fas ligand or receptor (23) causes recruitment of FADD (24), FLASH (25), and caspase-8 (26), to the death-inducing signaling complex (27), and induces the activation of caspase-8 (26), followed by the activation of the executioner caspases-3,-6, and -7. SM induces a dose-dependent increase in the levels of both Fas receptor as well as FasL (Fig. 1), and caspase-8 is activated within 2 h after exposure of NHEK to SM (Fig. 2). To further analyze the importance of the death receptor pathway for SM toxicity, we utilized a dominant negative inhibitor of FADD (FADD-DN), which expresses a truncated FADD protein containing an AU1 epitope tag and lacking the N-terminal domain necessary for recruitment and activation of caspase-8 at the death receptor complex (Fig. 5A). Thus, the recruitment of FADD to the death receptor complex is inhibited in cells expressing FADD-DN.

Nco cells were transfected with empty vector or with FADD-DN; stable clones were selected in G418 and maintained in SFM. Immunoblot analysis of extracts derived from different FADD-DN clones with antibodies to FADD confirmed the presence of both FADD and FADD-DN in positive clones, whereas parental Nco cells expressed only full-length FADD protein (Fig. 5B, left panel). Expression of the AU1 tag in one clone (DN3), which was chosen for high levels of FADD-DN and used in subsequent experiments, was further confirmed by immunoblot analysis with anti-AU1 (Fig. 5B, right panel).

We first tested whether expression of the FADD-DN construct could in fact suppress the death receptor pathway of apoptosis. Control Nco (transfected with vector alone) or Nco stably expressing FADD-DN were incubated with a Fas agonist antibody (clone CH11) to induce apoptosis. We measured caspase-3 activity as a marker of apoptosis, by quantitative fluorometric analysis with DEVD-AMC as a substrate. Cytosolic extracts were derived 16 h after SM exposure and analyzed for caspase-3 activity. Fig. 6A (right panel) shows that, following incubation with agonist antibodies to Fas, caspase-3 activity is suppressed in cells expressing the FADD-DN protein. Control Nco and Nco-FADD-DN keratinocytes were then treated with increasing doses of SM for 16 h, and extracts were analyzed for caspase-3 activity. Similar to Fas-mediated apoptosis, SM-induced caspase-3 activity was markedly inhibited by expression of FADD-DN (Fig. 6A, left panel). At all SM doses, Nco keratinocytes displayed substantially more caspase-3 activity than cells expressing FADD-DN.

We next analyzed whether expression of FADD-DN in kera-
A hallmark of apoptosis is the fragmentation of nuclei, which occurs partly because of the caspase-6-mediated cleavage of nuclear lamin A at a specific sequence (21). We therefore analyzed the cleavage of lamin A following exposure to SM. Whereas control Nco keratinocytes displayed a dose-dependent increase in the caspase-6-mediated cleavage of lamin A in response to SM (Fig. 7B), this cleavage was almost completely inhibited in keratinocytes that stably expressed FADD-DN. Thus, blocking the death receptor complex by expression of FADD-DN inhibits SM-induced internucleosomal DNA cleavage, as well as caspase-6-mediated nuclear lamin cleavage.

Expression of FADD-DN in Keratinocytes Suppresses SM-induced Cleavage of PARP and Caspase-7, an Effect That Is Dependent on Caspase-3—To verify whether cleavage of downstream targets of caspase-3 is also blocked by expression of FADD-DN, immunoblot analysis was performed on extracts from control and SM-exposed cells with antibodies to PARP and caspase-7. Whereas both caspase-3-mediated cleavage of PARP and caspase-7 were observed following exposure of control Neo keratinocytes to 300 μM SM, these apoptotic markers were completely abolished by expression of FADD-DN (Fig. 8). To examine whether caspase-3 was in fact responsible for SM cytotoxicity in human keratinocytes, we next determined whether pretreatment of keratinocytes with the peptide inhibitor of caspase-3 (Ac-DEVD-CHO; Biomol) could block SM-induced cleavage of PARP and caspase 7. A 30-min pretreatment of cells with 50 μM Ac-DEVD-CHO prior to SM exposure suppressed PARP cleavage. This effect was dose dependent, with inhibition of PARP cleavage over 80% when cells were pretreated with 50 μM Ac-DEVD-CHO for 30 min before SM exposure (Fig. 9A). In agreement with these findings, caspase-3 activity in extracts of FADD-DN keratinocytes pretreated with 50 μM Ac-DEVD-CHO for 30 min before SM exposure was much lower than that in extracts of FADD-DN keratinocytes preincubated with 0.01% DMSO only (Fig. 9B). Lowering caspase-3 activity by pretreatment of FADD-DN keratinocytes with the peptide inhibitor was also associated with a decrease in the number of apoptotic cells, as determined by an Annexin V assay (Fig. 9C). These results suggest that caspase-3 plays a key role in SM-induced apoptosis and vesication of human keratinocytes.
suppressed activation of caspase-7 and PARP cleavage, which are both cleaved by caspase-3 (Fig. 8).

Inhibition of the Fas, but Not TNFR1, Pathway with Blocking Antibodies Inhibits Markers of SM-induced Apoptosis—Elevation of both Fas and TNFR1 suggested that activation of Fas is responsible for SM toxicity. To directly test the role of Fas and TNFR1 in SM-induced apoptosis, we utilized neutralizing antibodies specific for each receptor. Because phosphatidylserine is exposed on the surface of apoptotic cells, and the presence of these residues can be detected by their ability to bind to annexin V, we analyzed the cells for annexin V binding by FACS analysis 16 h after SM exposure. Fig. 9A shows that untreated NHEK are more sensitive to SM-induced apoptosis at the doses tested than those pretreated with Fas-blocking antibody (ZB4). A plot of the survival rates (propidium iodide negative, annexin V-negative) also confirms that control cells are more sensitive to SM-mediated killing (Fig. 9B). Fig. 9, C and D, further show that pretreatment of NHEK to ZB4 attenuates caspase-3 activity and proteolytic processing. In contrast, TNFR1-blocking antibody had no effect on SM-induced apoptotic markers and cell survival. Thus, SM exerts its effects primarily through a Fas-mediated pathway.

FADD-DN Expression in Human Keratinocytes Partially Blocks the Vesication Response in Grafted Human Keratinocytes—Human skin grafts transplanted onto nude mice have been used successfully to examine SM-induced biochemical alterations, utilizing an end point of micro- or macroblisters (1–6, 28). We also previously determined that NHEK as well as Nco cells could be used to establish a histologically and immunocytochemically normal epidermis when grafted onto nude mice that exhibits SM-induced vesication (8, 9, 15). Utilizing human keratin-specific antibodies, we additionally demonstrated the correct expression of human keratins K1, K10, and K14 within the grafted epidermis previously (15). In an attempt to test the effects of inhibitors of the death receptor pathway on apoptosis and vesication in intact human epidermis, we utilized this system to genetically engineer human keratinocytes prior to grafting to ectopically express FADD-DN. Nco and Nco-FADD-DN human grafts were subsequently exposed to SM by the vapor cup method 6–8 weeks after grafting. Frozen and fixed sections derived from graft sites of these animals were first analyzed for the expression of FADD-DN using the AU1 antibody, which recognizes the specific AU1 epitope on the FADD-DN protein. Immunofluorescence analysis of these sections with antibodies to FADD or AU1 verified that Nco keratinocytes stably expressing FADD-DN attached with an AU1 epitope tag could be grafted, and that the AU1 epitope could be detected within the grafted human skin (Fig. 10).

Significantly, histological analysis of SM-exposed animals grafted with Nco (control), and those grafted with the FADD-DN clone of Nco revealed that SM microvesication is reduced by FADD-DN. Table I shows that while there was no difference in the response of the athymic nude mouse host epidermis to SM (bottom half of Table I), there was a decrease in the amount of microvesication in the FADD-DN grafts (Table I, sixth column, boldface).

SM Induces Markers of Apoptosis in Basal Cells in Human Skin Grafts, Particularly in Regions of Microvesication, an Effect That Is Inhibited by FADD-DN Expression—Because the epidermis comprises less than half of the weight of the grafted skin, it is difficult to measure epidermal-specific markers of apoptosis by immunoblot analysis. We thus performed cytochemical and immunofluorescent analysis to examine the expression of markers within individual cells. In addition to increased sensitivity, cytochemical staining and immunofluorescent labeling of individual cells allowed us to localize and identify the cell type within the epidermis undergoing apoptosis (i.e. basal, spinous, granular, or cornified). This information coupled with the vesication data ultimately permits correlation between the apoptotic pathways and blistering.

DNA breaks can be detected in situ using a Klenow fragment-based assay system (DermaTACS; Trevigen). We tested the relationship between apoptotic DNA breaks, vesication, and the Fas/TNF pathway by two different approaches. In the first approach, we grafted control Nco keratinocytes, or FADD-DN-expressing Nco, followed by exposure to SM. 24 h after exposure, animals were sacrificed and skin biopsies were obtained, fixed, and sectioned. DNA breaks were then detected by the DermaTACS method as described under "Materials and
Methods. Fig. 11A shows that SM induces apoptosis in basal cells of grafts derived from Nco. In addition, apoptotic cells were concentrated in the areas of microvesication. In contrast, Nco-FADD-DN skin grafts did not display the same degree of apoptosis or microvesication.

The second approach involved exposing control and Fas knockout (lpr) newborn pups to SM by the vapor cup method. SM strongly induced apoptosis primarily in the basal cells of control animals in the areas of microvesication, but DNA breaks were markedly diminished in skin derived from genetically matched mice with a disrupted Fas gene (Fig 11B). Taken together, the data suggest that SM activates a Fas/TNF apoptotic pathway resulting in the activation of caspase-3 and apoptosis of basal cells, contributing to the vesication response.

To observe caspase-3 activation in skin sections, we performed immunofluorescent staining utilizing antibodies that recognize the cleavage products of caspase-3 but not the full-length protein to localize active caspase-3 in individual cells following exposure of human skin grafts to SM. Immunostaining of mouse epidermis exposed to SM by the vapor cup method using anti-active caspase-3 reveals that caspase-3 is activated in basal epidermal cells of control mouse skin treated with SM (Fig. 12). On the other hand, caspase-3 activation in basal cells was markedly diminished in skin derived from genetically matched mice with a disrupted Fas gene (knockout).2

2 D. S. Rosenthal, A. Velena, F-P. Chou, R. Schlegel, R. Ray, B. Benton, D. Anderson, W. J. Smith, and C. M. Simbulan-Rosenthal, unpublished data.
FADD-DN Blocks SM-induced Apoptosis and Vesication

**DISCUSSION**

SM vesication involves both cytotoxicity and detachment of the epidermal basal cell layer in vivo. Using a cell culture model in the present study, we have described a potential mechanism for SM-induced keratinocyte basal cell death and detachment: apoptosis in keratinocytes via a Fas/TNF death receptor pathway. Keratinocyte basal cell death is primarily because of apoptosis at the doses tested (100–300 μM SM), contributing to SM vesication (8). We have further observed the activation of markers of apoptosis that are consistent with a Fas ligand-receptor interaction, including caspase-3, caspase-8, and PARP cleavage (7–9). Several investigators have also examined the mode of cell death induced by SM in other cell types. SM induces an apoptotic response in HeLa cells (10–100 μM) (29), peripheral blood lymphocytes (6–300 μM) (30), keratinocytes (50–300 μM) (8, 17), and endothelial cells (<250 μM) (31). However, a time-dependent shift to necrosis was observed in SM-treated lymphocytes (30), whereas markers of necrosis were observed at higher levels of SM in endothelial cells (>500 μM) (31) and HeLa (1 mM) (29).

SM is a strong bifunctional alkylating agent with a high affinity for DNA, and has been shown to induce DNA strand breaks in keratinocytes (8, 32), which is confirmed by our results showing the presence of DNA breaks in SM-exposed human skin grafts. It is therefore likely that DNA strand breaks play a role in the SM-induced apoptosis in human skin grafts. These results suggest that the Fas/TNF pathway of apoptosis is activated in individual basal cells by SM, particularly in regions of microvesication. We also obtained similar results in which basal cells of SM-treated human skin grafts derived from Nco keratinocytes displayed immunostaining for active caspase-3 in areas of microvesication in the skin grafts. In contrast, preliminary results indicate that grafts derived from FADD-DN keratinocytes exhibit less active caspase-3 in the basal cells, consistent with the results of immunoblot analysis.  

**TABLE I**

| Animal       | Site        | Exposure time (min) | Pustular epidermitis | Epidermal necrosis | Microvesicle (cleft) | Follicular involvement |
|--------------|-------------|---------------------|----------------------|--------------------|----------------------|------------------------|
| FADD-DN 1    | Graft       | 6                   | 0                    | 0                  | 0                    | 0                      |
| FADD-DN 2    | Graft       | 6                   | 2                    | 0                  | 1                    | 0                      |
| FADD-DN 3    | Graft       | 8                   | 0                    | 0                  | 0                    | 0                      |
| FADD-DN 4    | Graft       | 8                   | 0                    | 1                  | 0                    | 0                      |
| Control 1    | Graft       | 6                   | 0                    | 0                  | 0                    | 0                      |
| Control 2    | Graft       | 6                   | 2                    | 0                  | 2                    | 0                      |
| Control 3    | Graft       | 8                   | 0                    | 0                  | 2                    | 0                      |
| Control 4    | Graft       | 8                   | 2                    | 3                  | 3                    | 0                      |
| FADD-DN 1    | Host        | 6                   | 2                    | 4                  | 2                    | 3                      |
| FADD-DN 2    | Host        | 6                   | 2                    | 3                  | 1                    | 3                      |
| FADD-DN 3    | Host        | 8                   | 1                    | 2                  | 0                    | 2                      |
| FADD-DN 4    | Host        | 8                   | 1                    | 3                  | 3                    | 3                      |
| Control 1    | Host        | 6                   | 2                    | 4                  | 2                    | 4                      |
| Control 2    | Host        | 6                   | 0                    | 1                  | 0                    | 1                      |
| Control 3    | Host        | 8                   | 4                    | 1                  | 2                    | 3                      |
| Control 4    | Host        | 8                   | 2                    | 4                  | 3                    | 2                      |

**FIG. 11.** SM induces markers of apoptosis in basal cells in human skin grafts, particularly in regions of microvesication, an effect that is inhibited by Fas-knockout or FADD-DN expression. A, control human keratinocytes (Nco), or FADD-DN-expressing Nco were grafted onto nude mice, which were then exposed to SM by vapor cup. The SM-exposed human skin grafts were obtained, fixed, sectioned, and subjected to DNA break detection by DermaTACS. Slides were then observed by bright field microscopy. The positions of the basal cells, the dermis, and areas of vesication are indicated. B, control and Fas knockout newborn pups were exposed to SM by the vapor cup method. 24 h after exposure, animals were sacrificed, and skin biopsies were obtained, fixed, and sectioned. DNA breaks were then detected by the DermaTACS method as described under “Materials and Methods.”

**FIG. 12.** Caspase-3 is activated in basal epidermal cells of mouse skin treated with SM by vapor cup, particularly in regions of microvesication. Newborn mice were exposed to SM by the vapor cup method, and paraflin-embedded sections were derived from the sites of SM-exposed mouse skin. Sections were deparaffinized, incubated with antibodies to active caspase-3 with biotinylated antimouse IgG, and with streptavidin-conjugated Texas Red, and then observed with a Zeiss fluorescence microscope as described under “Materials and Methods.” Immunostaining of mouse epidermis treated with SM by vapor cup exposure using anti-active caspase-3 (left) or phase-contrast (right) are shown. The positions of the basal cells, cells with active caspases-3, as well as areas of microvesication are indicated.
 keratinocytes. In an attempt to define the molecular series of
events leading to SM vesication, we elucidated important path-
ways by which SM induces cell death in cultured keratinocytes,
as well as in intact mouse and grafted human skin. Members of
the Fas/TNFFR family and their ligands may be induced at the
level of transcription following stimulation by apoptosis-induc-
ing agents, such as doxorubicin (19, 20), and p53 has been
shown to play a role in the up-regulation of Fas (33). Consis-
tently, we have shown that p53 is also rapidly up-regulated in
keratinocytes following SM treatment, and that p53 may play a
role in SM-induced apoptosis (9, 17). Similarly, ectopic over-
expression of either Fas or FasL directly leads to apoptosis. In
the present paper, we observed activation of a death receptor
pathway for apoptosis, in which Fas receptor and FasL play a
role. Following SM exposure, keratinocytes significantly up-
regulate levels of both Fas receptor and FasL, followed by the
rapid activation of the upstream caspase-8, mediated by re-
cruitment of the adaptor protein FADD, and the consequent
activation of the executioner caspases-3, -6, and -7.

To better understand the contribution of FADD-regulated
pathways in the cutaneous response to SM, we blocked the
death receptor pathway utilizing keratinocytes stably express-
ing a truncated FADD adaptor protein (FADD-DN); this pro-
tein lacks the N-terminal domain responsible for recruitment
and activation of caspase-8 at the death receptor complex.
Keratinocytes expressing FADD-DN exhibited reduced levels
of FADD signaling and were found to be more resistant to
SM-induced PARP cleavage and processing of caspases-3, -6,
-7, and -8 into their active forms. In most apoptotic systems,
caspase-3, the primary executioner caspase, is proteolytically
activated, and in turn cleaves key proteins involved in the
structure and integrity of the cell, including PARP, DFF 45,
fodrin, gelsolin, receptor-interacting protein, X-linked inhibitor
of apoptosis protein, topoisomerase I, vimentin, Rb, and lamin
B (11–14, 34). Caspase-3 is also essential for apoptosis-associated
chromatin margination, DNA fragmentation, and nuclear
collapse (34).

Utilizing the stable expression of a dominant-negative inhib-
itor of FADD, we also demonstrated a role for the Fas/TNFFR
receptor family in mediating the response of grafted human
keratinocytes to SM. Significantly, we noted that blocking the
Fas/FADD death receptor pathway in human skin grafted onto
nude mice reduces vesication and tissue injury in response to
SM, thus indicating that this pathway is an excellent target for
therapeutic intervention to reduce SM injury. Fas-blocking
antibody experiments in cultured keratinocytes also show that
SM partially exerts its apoptotic effect via a Fas-FasL interac-
tion (Fig. 9). In addition, our recent studies with Fas-deficient
mice indicate the viability of this strategy to prevent vesication
by using inhibitors of the death receptor pathway.

Both SM and UV, another agent that induces apoptosis in
keratinocytes, have been shown to up-regulate the levels of
another member of the Fas/TNF family, TNFα, and partial
protection of keratinocytes from UV can be obtained by incu-
bating keratinocytes with antibody that neutralizes TNF (35,
36). Targeted gene disruption (knockout) studies have shown
that the majority of pathophysiological responses to TNFα are
mediated by the p55 TNF receptor (TNFR1) (37, 38). TNFα was
also shown to be elevated in SM-treated epidermal cells (39),
and TNFα-blocking treatments have demonstrated a clinical
usefulness for a wide variety of lesions, including systemic
lupus erythematosus (40), rheumatoid arthritis (41), psoriasis
(42–45), and cutaneous necrosis. However, in the current
study, TNFR1-neutralizing antibody was unable to block SM-
induced apoptosis.

An understanding of the mechanisms for SM-induced cell
death in keratinocytes will hopefully lead to strategies for
prevention or treatment of SM vesication. The present study
suggests that inhibition of FADD (upstream) or caspase-3
(downstream) may alter the response of the epidermis to SM.
With an understanding of the biochemical pathways for SM
vesication and having attenuated SM-induced toxicity in vivo
using a genetic approach, we are currently further testing the
effects of specific pharmaceutical inhibitors of Fas/caspase-
death receptor pathway of apoptosis to block this pathway, and
alter the cytotoxic response of keratinocytes to SM in cell cul-
ture, as well as the vesication response in vivo. To assay
whether the SM-induced apoptotic response is altered upon
 treatment with inhibitors of the Fas/caspase pathway, we are
examining the biochemical, morphological, and structural
changes that we have previously established as characteristic
markers of apoptosis (7, 8, 17). Our present study shows that
we can detect activation of caspase-3 in single cells, thus,
whether other caspases of the Fas/TNF receptor pathway are
coactivated by SM in vivo, and whether this activation can be
prevented by using inhibitors of this pathway, also remain to
be clarified.

Toxic epidermal necrolysis, a blistering lesion similar to that
resulting from SM exposure, has been successfully treated with
intravenous immunoglobulins, containing naturally occurring
neutralizing antibodies specific for human-Fas (46). FasL
blocking antibodies, 5 mg/kg, injected into the tail vein, have
also shown to be effective in blocking ethanol-induced liver
apoptosis in mice (47). Using antibodies that have been
clinically used for other lesions, such as toxic epidermal
necrolysis, systemic lupus erythematosus, rheumatoid arthri-
tis, and psoriasis (40–46), we are currently testing the effects
of inhibiting Fas/TNF binding to their ligands with neutralizing
antibodies to Fas/TNFFR in grafted human epidermis.

The effects of suppressing the function of the upstream
caspases-3 and -9 as well as the downstream central execution-
caspase-3 with cell-permeable peptide inhibitors are also cur-
rently being investigated. An inhibitor that blocks the activity
of all caspases, N-benzoyloxycarbonyl-Val-Ala-Asp-(O-methyl)fluoromethyl ketone (zVAD-fmk) has been used in a number of
cell culture studies and in mouse in vivo studies. For example,
two intraperitoneal injections of 0.25 mg/mouse on days 0, 5,
and 10 were recently found to be sufficient to prevent silicon
(48). For in vivo inhibition of Fas/TNFFR, systemically admin-
istered neutralizing antibodies against Fas/TNF, as well as
systemic and topical peptide inhibitors of caspases are pres-
ently being evaluated. The use of pharmacological Fas/TNF/
caspase inhibitors to study SM pathology, in the context of the
whole animal grafted with human skin offers a better under-
standing of the mechanism of this damage for human personnel.

Acknowledgments—We are grateful to Wen Fang Liu and Ruibai Luo
for technical assistance.

REFERENCES
1. Papirmeister, B., Feister, A. J., Robinson, S. L., and Ford, R. D. (1991) Medical
Defense Against Mustard Gas: Toxic Mechanisms and Pharmacological
Implications, 1st Ed., CRC Press, Boca Raton, FL.
2. Meier, H. L., Gross, C. L., Papirmeister, B., and Daszkiewicz, J. E. (1984) in
Proceedings of the Fourth Annual Chemical Defense Bioscience Review,
Aberdeen Proving Ground, MD, May 30–June 1, 1984, U. S. Army Medical
Research Institute of Chemical Defense, Aberdeen Proving Ground, MD
3. Gross, C. L., Innace, J. K., Smith, W. J., Krebs, R. C., and Meier, H. L. (1988)
in Proceedings of the Meeting of NATO Research Study Group, Panel VI/ RSF-3,
Washington, D. C., September 25–29, 1988, NATO, Brussels, Belgium
4. Petrali, J. P., Oglesby, S. B., and Mills, K. R. (1990) J. Toxicol. Cutaneous Ocuc.
Toxicol. 9, 193–214
5. Smith, W. J., Gross, C. L., Chan, P., and Meier, H. L. (1990) Cell Biol. Toxicol.
6, 285–291
6. Smith, W. J., and Dunn, M. A. (1991) Arch. Dermatol. 127, 1207–1213
7. Rosenthal, D. S., Simbalan-Rosenthal, C. M., Liu, W. F., Velena, A., Anderson,
D., Benton, B., Wang, Z. Q., Smith, W., Ray, R., and Smulson, M. E. (2001)
FADD-DN Blocks SM-induced Apoptosis and Vesication

8. Rosenthal, D. S., Simbulan-Rosenthal, C. M., Iyer, S., Spoonde, A., Smith, W., Ray, B., and Smulson, M. E. (1998) J. Invest. Dermatol. 111, 64–71
9. Rosenthal, D. S., Simbulan-Rosenthal, C. M., Iyer, S., Smith, W. J., Ray, R., and Smulson, M. E. (2000) J. Appl. Toxicol. 20, 543–549
10. Alnemri, E., Livingston, D., Nicholson, D., Salvesen, G., Thornberry, N., Wong, W., and Yuan, J. (1996) Cell 87, 17–34
11. Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., Munday, N. A., Raju, S. M., Smulson, M. E., Yamin, T. T., Yu, V. L., and Miller, D. K. (1995) Nature 376, 37–43
12. Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Porier, G. Q., Salvesen, G. S., and Dixit, V. M. (1995) Cell 81, 801–809
13. Song, Q., Lees-Miller, S., Kumar, S., Zhang, Z., Chan, D., Smith, G., Jackson, S., Ahnemri, E., Litwaek, G., Khanna, K., and Lavin, M. (1996) EMBO J. 15, 3238–3246
14. Casciola-Rosen, L., Nicholson, D., Chong, T., Rowan, K., Thornberry, N., Miller, D., and Rosen, A. (1996) J. Exp. Med. 183, 1957–1964
15. Rosenthal, D. S., Shima, T. B., Celii, G., De Luca, L. M., and Smulson, M. E. (1995) J. Invest. Dermatol. 105, 38–44
16. Sherman, L., and Schlegel, R. (1996) J. Virol. 70, 3269–3279
17. Stoppeler, H., Stoppeler, M. C., Johnson, E., Simbulan-Rosenthal, C. M., Smulson, M. E., Iyer, S., Rosenthal, D. S., and Schlegel, R. (1998) Oncogene 17, 1207–1214
18. Choi, K. B., Wong, F., Harlan, J. M., Chauftthary, P. M., Hood, L., and Karsan, A. (1998) J. Biol. Chem. 273, 20185–20188
19. Herr, I., Wilhelm, D., Bohler, T., Angel, P., and Debatin, K. M. (1997) EMBO J. 16, 6200–6208
20. Friesen, C., Herr, I., Krammer, P. H., and Debatin, K. M. (1996) Nat. Med. 2, 574–577
21. Ruchaud, S. N. K., Villa, P., Ketke, T., Dingwall, C. S. K., and Earnshaw, W. (2002) EMBO J. 21, 1967–1977
22. McLean, W. H., Eady, R. A., Dopping-Henepenat, P. J., McMillan, J. R., Leigh, I. M., Navaux, H. A., Higgins, C., Harper, J. I., Paige, D. G., Morley, S. M., and Lane, E. B. (1994) J. Invest. Dermatol. 102, 24–30
23. Takahashi, H., Kobayashi, H., Hashimoto, Y., Matsuo, S., and Iizuka, H. (1995) J. Invest. Dermatol. 105, 810–815
24. Chinnaia, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
25. Imai, Y., Kimura, T., Murakami, A., Yajima, N., Sakamaki, K., and Yonehara, S. (1999) Nature 398, 777–785
26. Medema, J. P., Scaffidi, C., Kischkel, F. C., Shchervenko, A., Mann, M., Krammer, P. H., and Peter, M. E. (1997) EMBO J. 16, 2794–2804
27. Kischkel, F. C., Hellhardt, S., Behrmann, I., Gerner, M., Pawlika, M., Krammer, P. H., and Peter, M. E. (1995) EMBO J. 14, 5579–5588
28. van Genderen, J., Mol, M. A., and Wolthuis, O. L. (1985) Fundam. Appl. Toxicol. 5, 998–1111
29. Sun, J., Wang, Y., and Sun, M. J. (1999) Chang Kuo Yao Li Hsueh Pao 20, 445–448
30. Meier, H. L., and Millard, C. B. (1998) Biochim. Biophys. Acta 1404, 367–376
31. Dabrowowska, M., Iii, J. L., Lelli, J. L., Jr., Levee, M. G., and Minshw, D. B. (1996) Toxicol. Appl. Pharmacol. 141, 568–583
32. Hinsnaw, D. B., Lodhi, I. J., Hurley, L. L., Atkins, K. B., and Dabrowwksa, M. E. (1999) Toxicol. Appl. Pharmacol. 156, 1–17
33. Owen-Schaub, L. B., Chung, K., Cusack, J. C., Angelo, L. S., Santee, S. M., Fujisawa, T., Roth, J. A., Deisseroth, A. R., Zhang, W. W., Kruzel, E., and Radinsky, R. (1995) Mol. Cell. Biol. 15, 3032–3040
34. Snee, E., Adrain, C., and Martin, S. (2001) J. Biol. Chem. 276, 7320–7326
35. Schwarz, A., Bhardwaj, R., Aragane, Y., Mahnke, K., Riemann, H., Metze, D., Luger, T. A., and Schwarz, T. (1995) J. Invest. Dermatol. 104, 922–927
36. Schwarz, A., Mahnke, K., Luger, T. A., and Schwarz, T. (1997) Exp. Dermatol. 6, 1–5
37. Zhuang, L., Wang, B., Sander, G. A., Shiavi, G. M., Mak, T. W., and Sauder, D. N. (1999) J. Immunol. 162, 1440–1447
38. Zhuang, L., Wang, B., and Sauder, D. N. (2000) J. Interferon Cytokine Res. 20, 445–454
39. Arroyo, C. M., Schafer, R. J., Kurt, E. M., Broomfield, C. A., and Carmichael, A. J. (2000) J. Appl. Toxicol. 20, Suppl. 1, S61–S72
40. Aringer, M., Feierl, E., Steiner, G., Stummvoll, G. H., Hofler, E., Steiner, C. W., Rudda, I., Smole, J. S., and Graninger, W. B. (2002) Lupus 10, 102–108
41. Butler, D. M., Manni, R. N., Fieldman, M., and Brennan, F. M. (1995) Eur. Cytokine Netw. 6, 225–230
42. Mang, R., Stege, H., Ruzwicka, T., and Krtumann, J. (2002) Dermatology 204, 156–157
43. Schof, R. E., Aust, H., and Knap, J. (2002) J. Am. Acad. Dermatol. 46, 891–896
44. O'Quinn, R. P., and Miller, J. L. (2002) Arch. Dermatol. 138, 644–648
45. Scallon, B., Cai, A., Solowosi, N., Rosenberg, A., Song, X. Y., Shealy, D., and Wagner, C. (2002) J. Pharmacol. Exp. Ther. 301, 415–426
46. Viard, I., Wehrli, P., Bollani, R., Schneider, P., Holler, N., Salomen, D., Hunziker, T., Saurat, J. H., Tschopp, J., and French, L. E. (1998) Science 282, 490–493
47. Zhou, Z., Sun, X., and Kang, Y. J. (2001) Am. J. Pathol. 159, 329–338
48. Borges, V. M., Lopes, M. F., Falcao, H., Leite-Juiner, J. H., Rocco, P. R., Davidson, W. F., Linden, R., Zin, W. A., and DesReis, G. A. (2002) Am. J. Respir. Cell Mol. Biol. 27, 78–84
Expression of Dominant-negative Fas-associated Death Domain Blocks Human Keratinocyte Apoptosis and Vesication Induced by Sulfur Mustard
Dean S. Rosenthal, Alfredo Velena, Feng-Pai Chou, Richard Schlegel, Radharaman Ray, Betty Benton, Dana Anderson, William J. Smith and Cynthia M. Simbulan-Rosenthal

J. Biol. Chem. 2003, 278:8531-8540.
doi: 10.1074/jbc.M209549200 originally published online December 12, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209549200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 11 of which can be accessed free at
http://www.jbc.org/content/278/10/8531.full.html#ref-list-1