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<b>13. ABSTRACT (Maximum 200 Words)</b> Sulfur mustard (SM) causes blisters in the skin through a series of cellular changes that we are beginning to identify. Previously, we found that SM induces markers of differentiation and apoptosis in human keratinocytes in response to SM. A number of studies in the past several years have shown that the central signaling proteins for many of the pathways that coordinate apoptosis are members of the caspase family of cysteine proteases (named for their preference for aspartate at their substrate cleavage site (Alnemri et al., 1996). Caspases cleave key proteins involved in the structure and integrity of the cell. Previously, we focused on caspase-3 in the SM response. To further understand the apoptotic response, we have devoted much of our effort to assay for the activation of other key caspases. These include the "upstream" caspases 8, 9, and 10, and the "executioner" caspases 3, 6, and 7. We have determined that caspase-8 is the first to be activated after SM treatment. Upregulation of different isoforms of both Fas and Fas ligand are observed following SM exposure. Caspase-6 is the first executioner caspase to be activated, and we have evidence that keratin K1, along with other structural proteins of the cell is cleaved by this caspase. Using retroviral constructs expressing antisense to calmodulin, we also obtained the results that SM-induced apoptosis and differentiation proceeds via a Ca <sup>2+</sup> -calmodulin-dependent pathway.			
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## 1. OVERVIEW

Sulfur Mustard (SM) is a highly reactive compound that induces the death and detachment of the basal cells of the epidermis from the basal lamina. This process may involve changes in intracellular  $\text{Ca}^{2+}$  and calmodulin (CaM; Meier et al., 1984; Gross et al., 1988; Petrali et al., 1990; Smith et al., 1990; Smulson, 1990; Papirmeister et al., 1991; Smith et al., 1991, Rosenthal et al., 1998). Keratinocytes respond to  $\text{Ca}^{2+}$ -signaling pathways in a fashion that makes them unique from other cells, which may explain the exquisite sensitivity of the skin to SM vesication. A number of laboratories, including our own, have shown that differentiation can be induced in both murine and human keratinocytes by the elevation of extracellular  $\text{Ca}^{2+}$  (Hennings et al., 1980; Stanley and Yuspa, 1983; Rosenthal et al., 1991). This in turn results in an increase in intracellular free  $\text{Ca}^{2+}$  ( $\text{Ca}_i$ ).  $\text{Ca}_i$  appears to be an important signal for terminal differentiation, since agents which chelate and buffer  $\text{Ca}_i$  can block markers of terminal differentiation (Li et al., 1995).  $\text{Ca}^{2+}$  has also been shown to play a role in apoptosis in a number of systems. Studies by Kaiser and Edelman (Kaiser and Edelman, 1977) first indicated that  $\text{Ca}_i$  may trigger apoptosis in glucocorticoid-stimulated thymocytes. Since then, a number of other studies have confirmed this role for  $\text{Ca}^{2+}$ . The observed  $\text{Ca}_i$  increase during apoptosis appears to occur by two different mechanisms. The first mechanism involves the activation of protein tyrosine kinases, leading to the activation of phospholipase C, the formation of IP<sub>3</sub>, and  $\text{Ca}^{2+}$  mobilization. The second pathway involves oxidative stress, which can occur in response to cytotoxic agents, such as SM, which generate reactive oxygen species. Oxygen radicals can damage  $\text{Ca}^{2+}$  transport systems localized in the endoplasmic reticulum, mitochondria, and plasma membrane, leading to a disruption in  $\text{Ca}^{2+}$  homeostasis and a sustained increase in  $\text{Ca}_i$  (Orrenius et al., 1989).

Published studies, including my own, utilizing specific inhibitors of CaM have demonstrated the importance of  $\text{Ca}^{2+}$ -CaM complexes in programmed cell death (Pan et al., 1996; Sasaki et al., 1996; Rosenthal, et al., 1998). Cyclosporin A-sensitivity of apoptosis in certain systems also suggests a role for  $\text{Ca}^{2+}$ -CaM complexes in programmed cell death. Cyclosporin binds to a family of cytosolic receptors (cyclophilins); the complex then binds to and suppresses the serine/threonine phosphatase calcineurin, which in turn is regulated by  $\text{Ca}^{2+}$ -CaM complexes in programmed cell death (Shi et al., 1989). Interestingly, in numerous recent studies, Bad, a pro-apoptotic member of the Bcl-2 family, has been implicated as a key player in programmed cell death

(Yang et al., 1995; Datta et al., 1997; del Peso et al., 1997; Hsu et al., 1997; Zha et al., 1997; Scheid and Duronio, 1998; Zundel and Giaccia, 1998). Calcineurin has been shown to interact with the Bcl-2 family members (Shibasaki et al., 1997), and to dephosphorylate Bad (Wang et al., 1997). This dephosphorylated form of Bad can interact with Bcl-2 or Bcl-X<sub>L</sub> and induce apoptosis (Zha et al., 1997). Ca<sup>2+</sup> also plays a role in the induction of an endonuclease responsible for internucleosomal DNA cleavage, yielding the characteristic apoptotic DNA ladders (Shiokawa et al., 1994), and recent studies have shown that the apoptotic endonuclease AP-24 is activated via CaM-dependent protein kinase II (Wright et al., 1997a).

Numerous studies now indicate that the targets for many of these signaling pathways that lead to apoptosis are a family of cysteine proteases, known as caspases (named for their preference for aspartate at their substrate cleavage site (Alnemri et al., 1996). Caspase-3 appears to be a converging point for different apoptotic pathways (Nicholson et al., 1995). In a number of apoptotic systems, caspase-3 cleaves key proteins involved in the structure and integrity of the cell, including PARP.

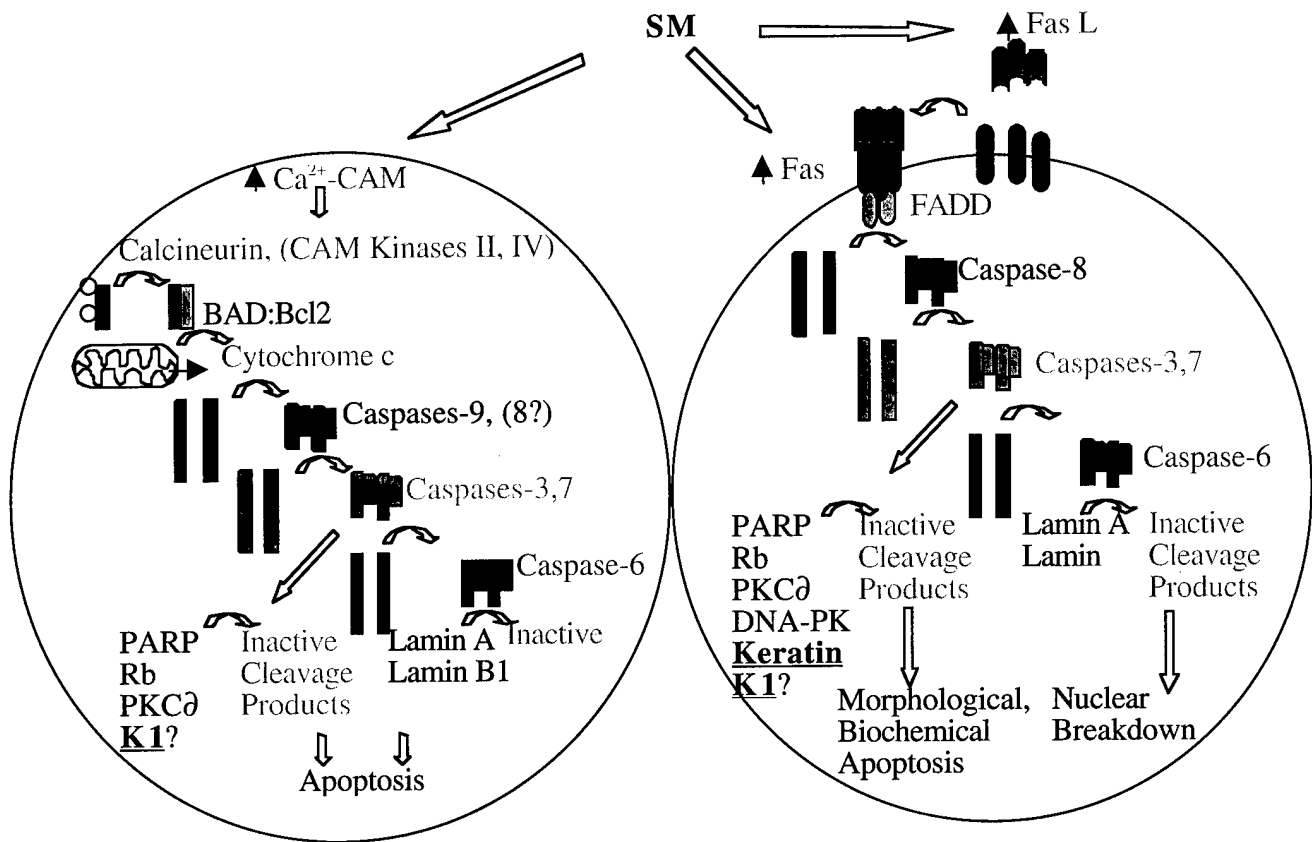
We have shown that SM induces both terminal differentiation and apoptosis in human keratinocytes. Further, we have demonstrated that these processes are Ca<sup>2+</sup> and CaM dependent, and involve the activation of caspase 3. These responses may, in part, explain the death and detachment of basal cells of the epidermis that occurs following exposure to SM.

## **2. CHARACTERIZATION OF THE SEQUENCE OF EVENTS DURING SM-INDUCED APOPTOSIS**

### **2.1 Introduction**

A number of studies for the past several years have shown that the central signaling proteins for many of the pathways that coordinate apoptosis are members of a family of cysteine proteases known as "caspases" (named for their preference for aspartate at their substrate cleavage site (Alnemri et al., 1996). Caspases cleave key proteins involved in the structure and integrity of the cell. Previously, we focused on caspase-3 in the apoptotic response. In order to further understand the apoptotic response, we have devoted much of our effort to assay for the activation of other key caspases, in particular the "upstream" caspases 8, 9, and 10, and the "executioner" caspases 3, 6, and 7. The sequence of their activation may give insight into the mechanism of

apoptosis. For example, caspase-8 is first activated following engagement of death receptors, while caspase-9 is activated via a mitochondrial pathway (Figure 1)



**FIGURE 13: Two related pathways for SM-induced apoptosis**

## 2.2 Materials and Methods

### (1) Culture of primary and immortalized human keratinocytes, and exposure to SM.

**Cells.** Normal human epidermal keratinocytes (NHEK) were either obtained as primary cultures from Clonetics (San Diego, CA), or prepared from human foreskin keratinocytes as described previously, and maintained in serum-free Keratinocyte Growth Medium (KGM). Cells are grown to 80% confluency and split 1:5. NHEKs in 75 cm<sup>2</sup> tissue culture flasks to 60-80% confluency, then exposed to HD diluted in KGM. Media is not changed for the duration of the experiments

**Chemicals.** SM (bis-(2-chloroethyl) sulfide; >98% purity) is obtained from the US Army Edgewood Research, Development and Engineering Center.

(2) *Measurement of proteolytic activation of caspase-2, -3, -6, -7, -8, -9, and -10.*

a. *Antibodies.* One of the first tasks was to determine the molecular ordering of events leading to SM-induced apoptosis. We have extensive experience utilizing Western analysis to detect the activation of several caspases. We first tested a number of antibodies from commercial and collaborative sources for their sensitivities and specificities using cells treated with known apoptosis-inducing agents, such as anti-Fas antibody, as controls. We developed an inventory of excellent antibodies specific for all relevant caspases (Table 1). In addition, we obtained antisera that detects the substrate cleavage products for caspase-3 and -7, several different PARP antisera, as well as for lamin B1, a substrate of caspase-6. Thus, by performing time-course experiments in Aim I, as well as inhibitor studies (to be performed), we have been able to begin to determine the sequence of events, as well as the regulatory molecules (such as Bcl-2), involved in SM-induced apoptosis.

b. *Immunoblot analysis.* SDS-polyacrylamide gel electrophoresis and protein transfer to nitrocellulose membranes were performed according to standard procedures. Membranes were stained with Ponceau S (0.1%) to confirm equal loading and transfer. After blocking of nonspecific sites, the blots were incubated with monoclonal or polyclonal antibodies (above) and then detected with appropriate peroxidase-labeled secondary antibodies (1:3000 dilution) and enhanced chemiluminescence (ECL, Amersham). Immunoblots were sequentially stripped 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 reprobbed with additional antibodies to accurately compare different proteins from the same filter. Typically, a filter could be reprobbed three times before there was detectable loss of protein from the membrane, which was monitored by Ponceau S staining after stripping.

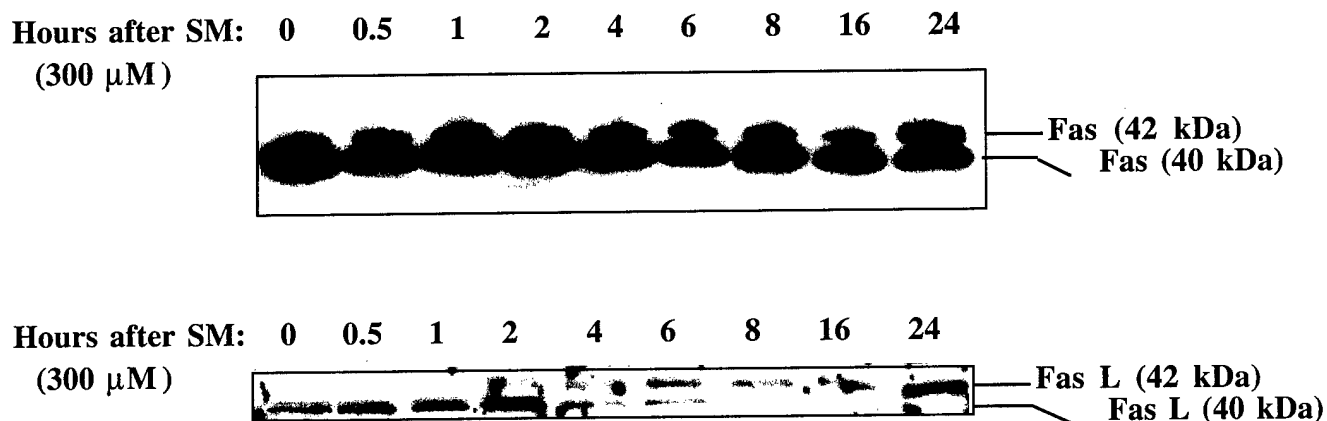
c. **Antibodies.** All antibodies indicated below were tested and used successfully in our laboratory.

Antibody (kDa)	type (clone)	Source	Dilution (conc.)
Calmodulin (17)	monoclonal (1F11 + 6D4)	Sigma	1:1000
K1 (67)	polyclonal	Babco	1:50
K1; K10 (67; 59)	monoclonal (8.60)	Sigma	1:100 (1 µg/ml)
K14 (50)	monoclonal	Sigma	1:200
Involucrin (68)	monoclonal (SY5)	Sigma	1:200
Fibronectin (220; 94)	polyclonal	Sigma	1:500
Fas (48)	monoclonal	Transduction Labs	1:250 (1 µg/ml)
Fas ligand	polyclonal	Santa Cruz	1:400 (0.5 µg/ml)
FADD (24)	monoclonal (1)	Transduction Labs	1:250
AU1	monoclonal (AU1)	Babco	1:1000 (1 µg/ml)
Caspase-3 (32; 17)	polyclonal	Dr. D. Nicholson	1:5000
Caspase-3 (propeptide)	polyclonal	Transduction Labs	1:500
Caspase-6 (34; 11)	monoclonal (B93-4)	PharMingen	1:250 (2 µg/ml)
Caspase-7 (35;17)	monoclonal	PhaMingen	1:500 (1 µg/ml)
Caspase-7 (17)	polyclonal	Dr. E. Gelmann	1:1000
Caspase-8 (20)	polyclonal	Dr. E. Gelmann	1:1000
Caspase-8 (55)	monoclonal	PharMingen	1:100 (1 µg/ml)
Caspase-9	monoclonal	PharMingen	1:400
Caspase 10 (55)	polyclonal	PharMingen	1:500 (1 µg/ml)
PARP (116, 89)	monoclonal (c210)	BioMol	1:5000
PARP DBD (24)	polyclonal	Dr. I. Hussein	1:400
PAR	polyclonal	Dr. M. Smulson;D. Rosenthal	1:500
Lamin B1	monoclonal	Calbiochem	1:100 (1 µg/ml)
Rb (110)	monoclonal	Calbiochem	1:100 (1 µg/ml)
DFF45 (45; 30)	polyclonal	PharMingen	1:1000
p53 (53)	monoclonal (pAb421)	Calbiochem	1:200 (0.5 µg/ml)
Bcl-2 (25)	monoclonal (4D-7)	Biomol	1:200 (1 µg/ml)
Bcl-X <sub>L</sub>	polyclonal	Calbiochem	1:40 (2.5 µg/ml)
Bax (21)	polyclonal	Calbiochem	1:50 (2 µg/ml)
Apoptotic endonuclease	polyclonal	Dr. Yoshihara	1:1000

**Table 1**

### 2.3 Results

*Changes in endogenous levels of apoptosis-mediating receptors Fas and Fas-ligand (Fas-L), as well as caspases and "death domain" signaling intermediates (FADD) during SM-induced apoptosis.*



**Fig. 2. Time course for expression of Fas and Fas ligand**

Some DNA damaging agents, including chemotherapeutic agents, have been shown to increase the expression levels of the Fas receptor or its ligand. In addition, overexpression of either Fas or Fas ligand can lead to apoptosis. We performed a time course, in which NHEKs were exposed to a vesicating dose (300  $\mu$ M) of SM and extracts were subjected to immunoblot analysis. As shown in **Fig. 2**, there is a change in the expression levels of both Fas receptor as well as Fas ligand. A time-dependent increase in the levels of both proteins is seen. In addition, an increase in the relative abundance of the higher molecular forms of both the Fas receptor and Fas ligand (both 42 kDa) is observed.

The molecular ordering of caspase activation was next determined. Cell extracts of NHEK exposed to 300  $\mu$ M SM were subjected to immunoblot analysis utilizing antibodies specific to caspases-3, 6, -8, -9, or -10. **Fig. 3** shows that the upstream caspases -8 and -9 are both activated in a time-dependent fashion, although caspase-8 appears to be cleaved prior to caspase-9 (0.5 h vs. 2 h), and little cleavage of caspase-10 is observed. Activation of caspase-8 is consistent with a Fas-mediated pathway of apoptosis, while activation of caspase-9 is consistent with a mitochondrial pathway of apoptosis. These results are consistent with the activation of both

death receptor and mitochondrial pathways by SM (Fig. 1). Caspase-3 and -7 are both proteolytically activated after

### Proteolytic Activation of Caspases

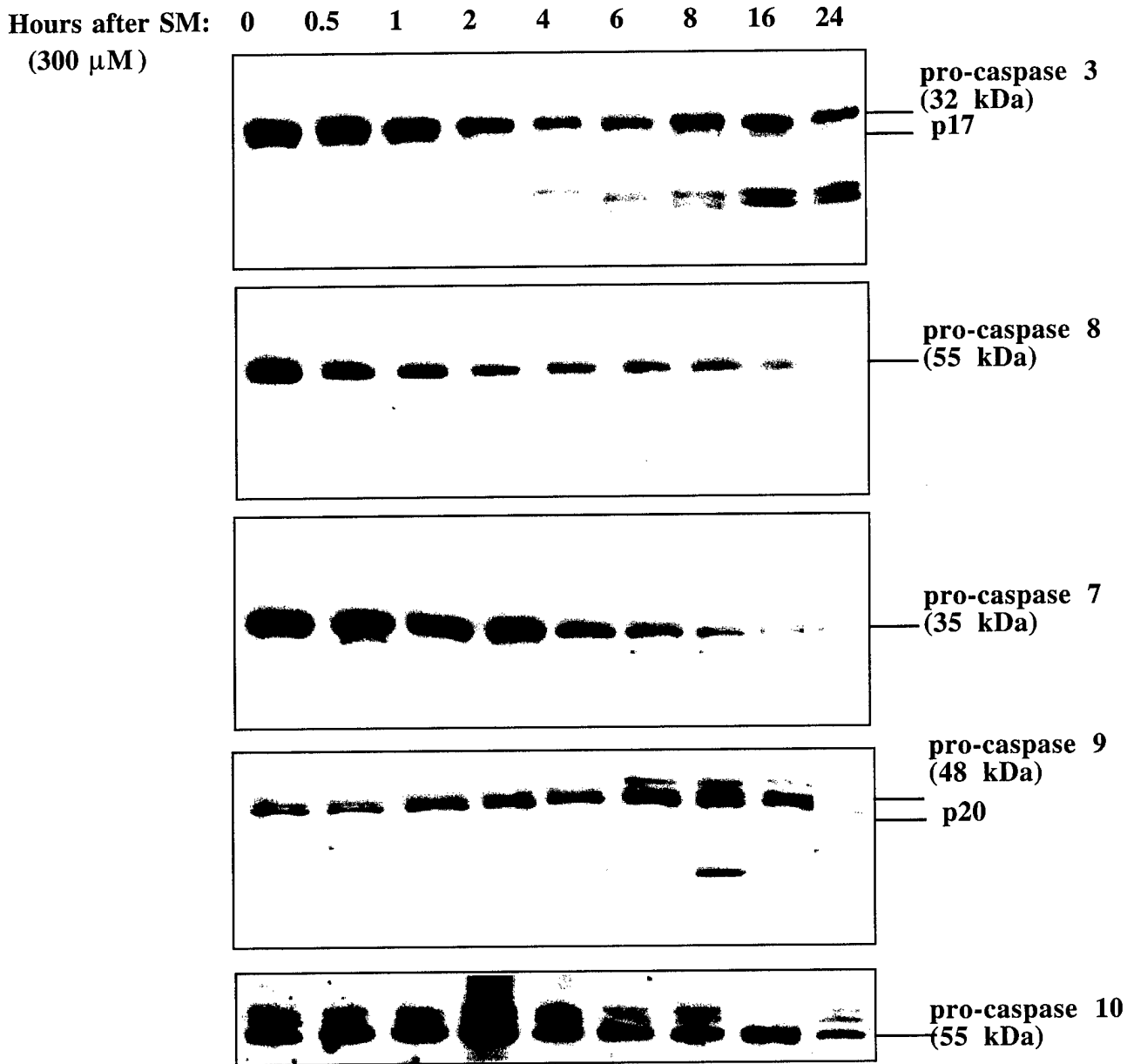


Fig. 3. Time course for proteolytic activation of caspases

## Caspase-Mediated Cleavage of Substrates PARP, DFF-45, Lamin B1, and HK-1

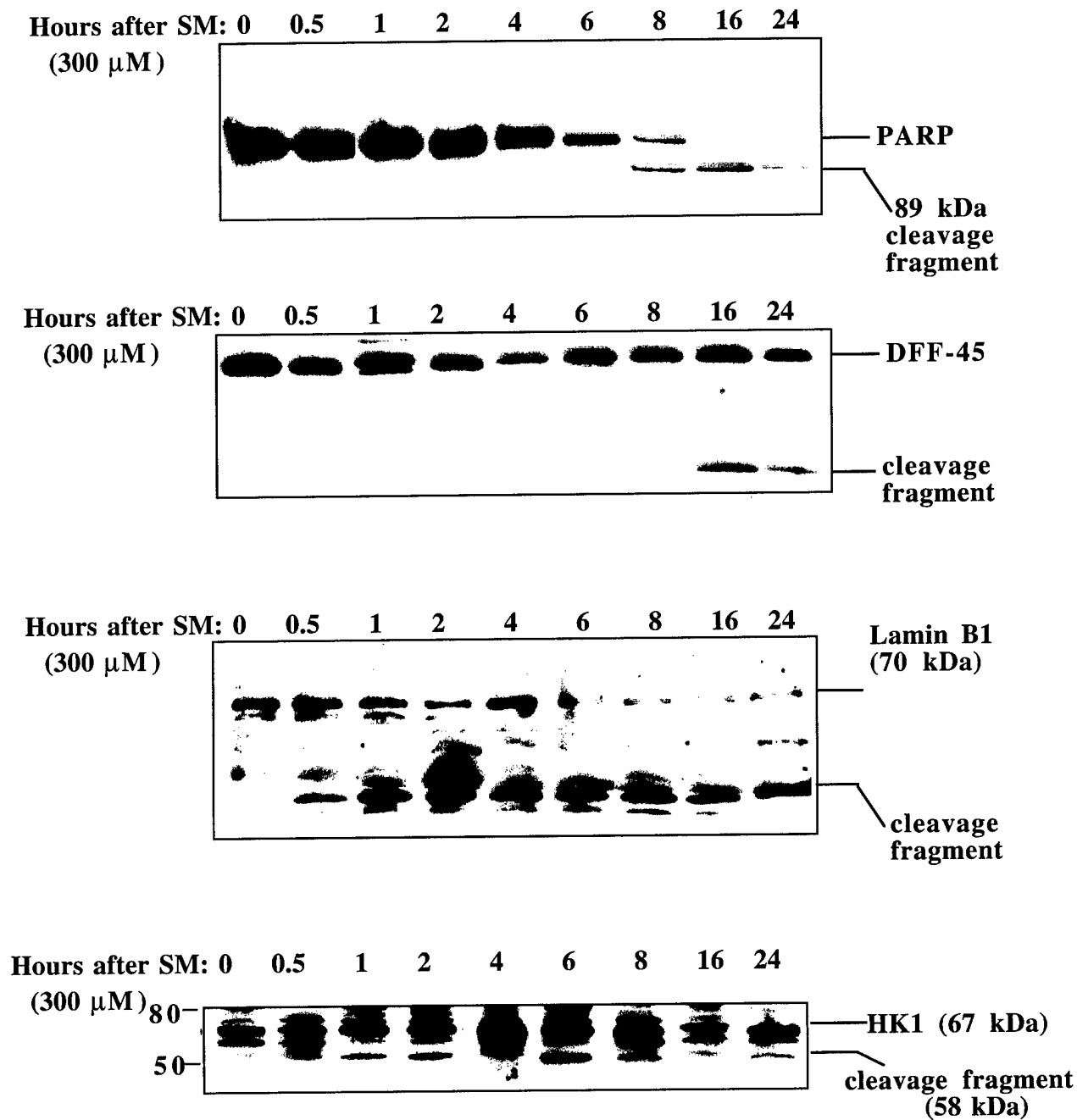
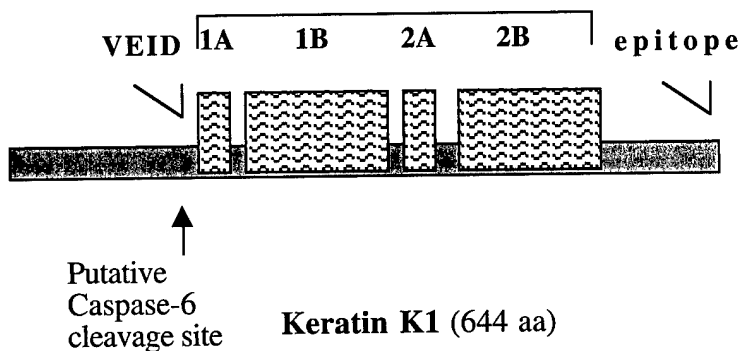


Fig. 4. Time course for proteolytic cleavage of caspase substrates

exposure to SM, with caspase-3 activation detectable at 1 h after SM exposure, and caspase-7 cleavage detected 2 h after exposure. To detect caspase-6 activity, we utilized antisera specific for lamin B1, which is specifically cleaved *in vivo* by active caspase-6 at the peptide sequence VEID<sup>A</sup>. **Figure 4** shows that the caspase-6 cleavage product was in fact formed following SM treatment. Surprisingly, this substrate was one of the first to be cleaved (within 30 min), when compared to poly (ADP-ribose) polymerase (PARP; 6 h), or the apoptotic DNA fragmentation factor (DFF 45; 16 h). PARP has been shown to be a substrate of caspase-3 and -7, while 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.

*Immunoblot and pulse labeling/immunoprecipitation analysis of newly discovered caspase-mediated cleavage of epidermal keratins during SM-induced differentiation and apoptosis.*

In order to study the differentiation response to SM, we originally focused on the suprabasal-specific keratins, K1 and K10, which are tightly regulated at the level of transcription in keratinocytes both *in vitro* and *in vivo*. **However, many changes may occur at the post-translational level, including a putative caspase-mediated breakdown of keratin K1 that occurs during apoptosis.** Thus, we continued to employ Western analysis, as well as immunofluorescent analysis to examine the changes in these gene products in the first year.

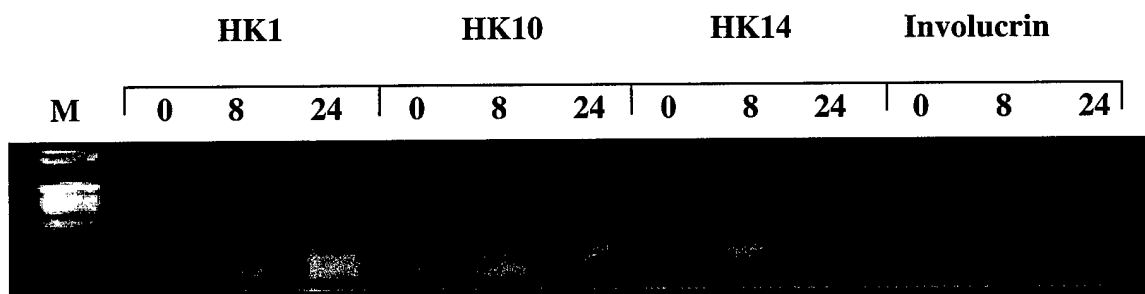


**Fig. 5**

To determine if **SM** alters keratin expression, we performed a time course, followed by Western analysis using a monoclonal antibody specific for the suprabasal keratins K1 and K10, which are the major proteins expressed in keratinocytes in response to differentiating agents. K1 was strongly induced in the presence of 100  $\mu$ M **SM**, between 8 and 24 h following treatment (not shown). When we recently used a different polyclonal antibody directed against the C-terminus of K1, and treated cells with higher concentrations of **SM**, we discovered an apparent cleavage product of K1 (**Fig. 4**, bottom panel). The size of this product maps near a perfect consensus sequence for a site of cleavage by caspase-6 (**Fig. 5**). Moreover, point mutations near this region of K1 give rise to a genetic **blistering disorder**, epidermolytic hyperkeratosis (McLean et al., 1994). Thus, it is of interest to determine if K1 can be cleaved by caspase-6 following treatment with **SM**. We are therefore performing the same experiments in the presence of the caspase-6 inhibitor, VEID-CHO. Inhibition of the cleavage product in the presence of the inhibitor would strongly suggest that K1 is in fact the substrate for caspase-6, and that K1 is a target during keratinocyte apoptosis. In order to determine if the smaller molecular weight fragment arises from K1 cleavage, a pulse-labeling experiment will be performed:

*Expression of markers of differentiation at the RNA level*

We next examined whether alterations in the expression of differentiation-specific markers could be observed following exposure to **SM**. NHEK were exposed to **SM**, RNA was isolated, and RT-PCR was performed using primers specific for either keratin 1, keratin 10, keratin 14, or involucrin. **Fig. 6** shows that all markers are induced at the RNA level, with the exception of involucrin.



**Fig. 6**

Interestingly, in contrast to normal keratinocyte differentiation, K10 is induced prior to K1, indicating that this represents an aberrant or uncoordinated form of differentiation.

## 2.4 Discussion

### Future course for K1 cleavage

**Pulse-labeling.** Transfected cells will be cultured in cysteine- and methionine-free DMEM for 12 h and then switched to the same medium containing 0.2 mCi/ml  $^{35}\text{S}$ -methionine plus  $^{35}\text{S}$ -cysteine ("EXPRE $^{35}\text{S}^{35}\text{S}$ " system, New England Nuclear) for 1 h as described previously (Rosenthal et al., 1994). Cells will then be switched to normal medium with cold methionine (15 mg/ml) and cysteine (pulse-chase) for 12 h, and then treated with SM for different amounts of time.

**Immunoprecipitation** will be performed with the polyclonal antibody to K1 (AF87; Babco), according to procedures I have published previously (Rosenthal et al., 1994). Briefly, equal amounts of cell extracts (10  $\mu\text{g}$ ) are precleared overnight at 4°C with 200  $\mu\text{l}$  of EBC buffer [50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% NP-40, and aprotinin (0.1 TIU/ml)] and 10  $\mu\text{l}$  of protein A-Sepharose beads (Pharmacia). After centrifugation, the supernatants were then incubated for 1 h with 0.5 ml of NET-N buffer [20 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40] containing the anti-K1 pAb (2  $\mu\text{g}/\text{ml}$ ). The samples were then incubated for an additional 20 min with 20  $\mu\text{l}$  of a 1:1 suspension of protein A-Sepharose beads in Tris-buffered saline containing 10% bovine serum albumin. The beads are washed five times with NET-N buffer, and the proteins bound to the beads were then separated by SDS-PAGE. gels, dried, and subject to fluorography. If label appears initially in the 67 kDa K1 band, and then appears in the smaller molecular weight band over time, this indicates that the cleavage product arises from proteolysis of K1. Inhibition by VEID-CHO will indicate that this occurs at the VEID caspase-6 recognition site.

### 3. ROLE OF CALMODULIN

#### 3.1 Introduction

During the first year, I have focused on the roles of  $\text{Ca}^{2+}$  /CaM in the modulation of differentiation and apoptosis in epidermal cells, and potentially involved in vesication. I have utilized much of the same technology that I have successfully employed previously to answer an essential question- How does  $\text{Ca}^{2+}$  /CaM alter the apoptotic and differentiation responses in keratinocytes, and can these pathways be modulated to alter SM vesication in animal models (and ultimately, in humans)?

In the first year, we showed that SM induces both terminal differentiation and apoptosis in human keratinocytes. Further, we have demonstrated that these processes are  $\text{Ca}^{2+}$  and CaM dependent, and involve the activation of a number of caspases. These responses may, in part, explain the death and detachment of basal cells of the epidermis that occurs following exposure to SM.

#### 3.2 Materials and Methods

##### (1) *Culture of primary and immortalized human keratinocytes, and exposure to SM.*

**Cells.** Normal human epidermal keratinocytes (NHEK) are prepared from human foreskin keratinocytes as described previously, and maintained in serum-free Keratinocyte Growth Medium (KGM). Cells are grown to 80% confluency and split 1:5. NHEKs in 75 cm<sup>2</sup> tissue culture flasks to 60-80% confluency, then exposed to HD diluted in KGM. Media is not changed for the duration of the experiments

(2) **RT-PCR Reverse transcription-polymerase chain reaction (RT-PCR).** Unique oligonucleotide primer pairs for human keratin K1, K10, K14 and involucrin, mRNA were designed and prepared. Total RNA, purified from cell pellets with Trizol Reagent (Gibco BRL), was subjected to RT-PCR with a Perkin Elmer Gene Amp EZ tTh RNA PCR kit. The reaction mix (50  $\mu\text{l}$ ) contained 300  $\mu\text{M}$  each of dGTP, dATP, dTTP, and dCTP, 0.45  $\mu\text{M}$  of each primer, 1  $\mu\text{g}$  of total RNA, and rTth DNA polymerase (5 U). RNA was transcribed at 65°C for 40 min, and DNA was amplified by an initial incubation at 95 °C for 2 min, followed by 30 cycles of 95° C for 1 min, 60°C for 1.5 min, and 65 °C for 0.5 min, and a final extension at 70 °C for 22 min. The PCR products were then separated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

### 3.3 Results

#### Determining the effects of modulation of CaM levels by chemical inhibitors or antisense on progression of SM-induced apoptosis and differentiation.

In humans, 3 genes encode CaM. In keratinocytes, the predominant form is CaM 1 as determined by RTPCR analysis (Fig. 7). Furthermore, we found that during a time course following exposure to SM, the levels of CaM protein increased and subsequently decreased (Fig. 8). This, along with our previous results, indicated that CaM may play a role in the response of NHEK to SM.

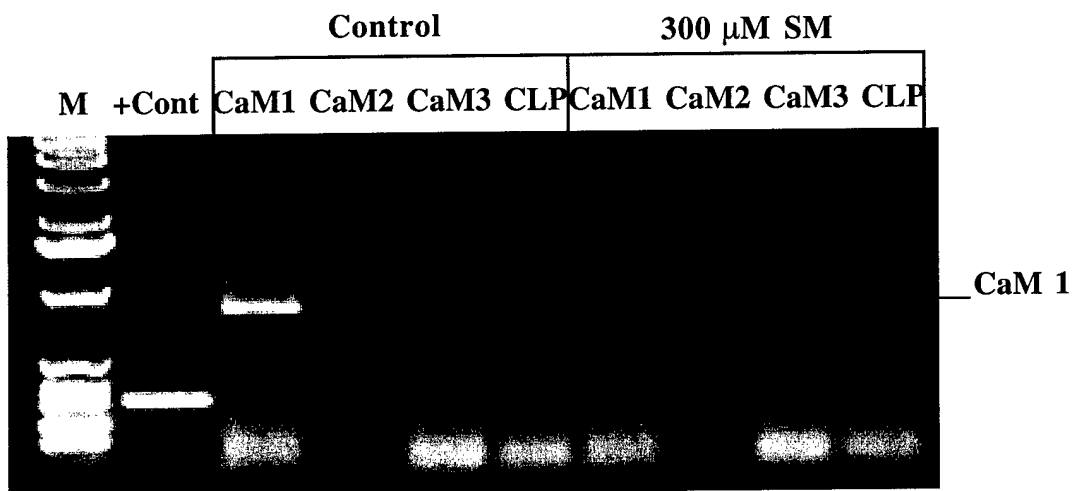


Fig. 7

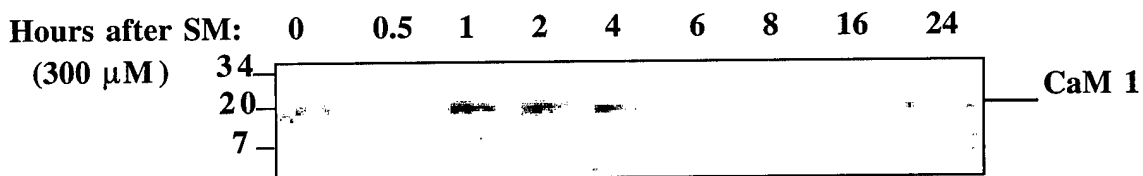
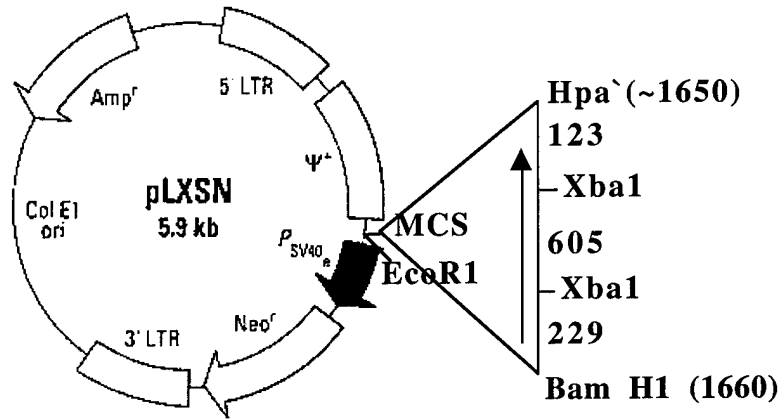
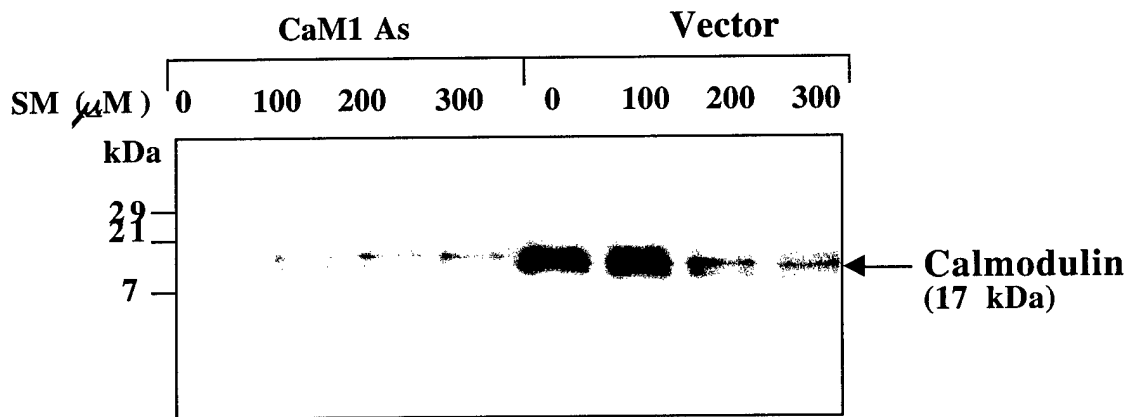


Fig. 8

We therefore constructed a retroviral vector expressing CaM RNA in the antisense orientation (**Fig. 9**). Following packaging and infection, NHEK were then analyzed for the endogenous levels of CaM protein by immunoblot analysis (**Fig. 10**).



**Fig. 9**



**Fig. 10**

**Fig. 10** shows that NHEK expressing CaM antisense RNA had lower levels of CaM protein. These cells were then exposed to different levels of SM and the expression of apoptotic markers was analyzed by Western analysis. **Fig. 11** shows that processing of caspases-3, 6, 7, and 8, as well as the cleavage of PARP were inhibited by the expression of CaM antisense.

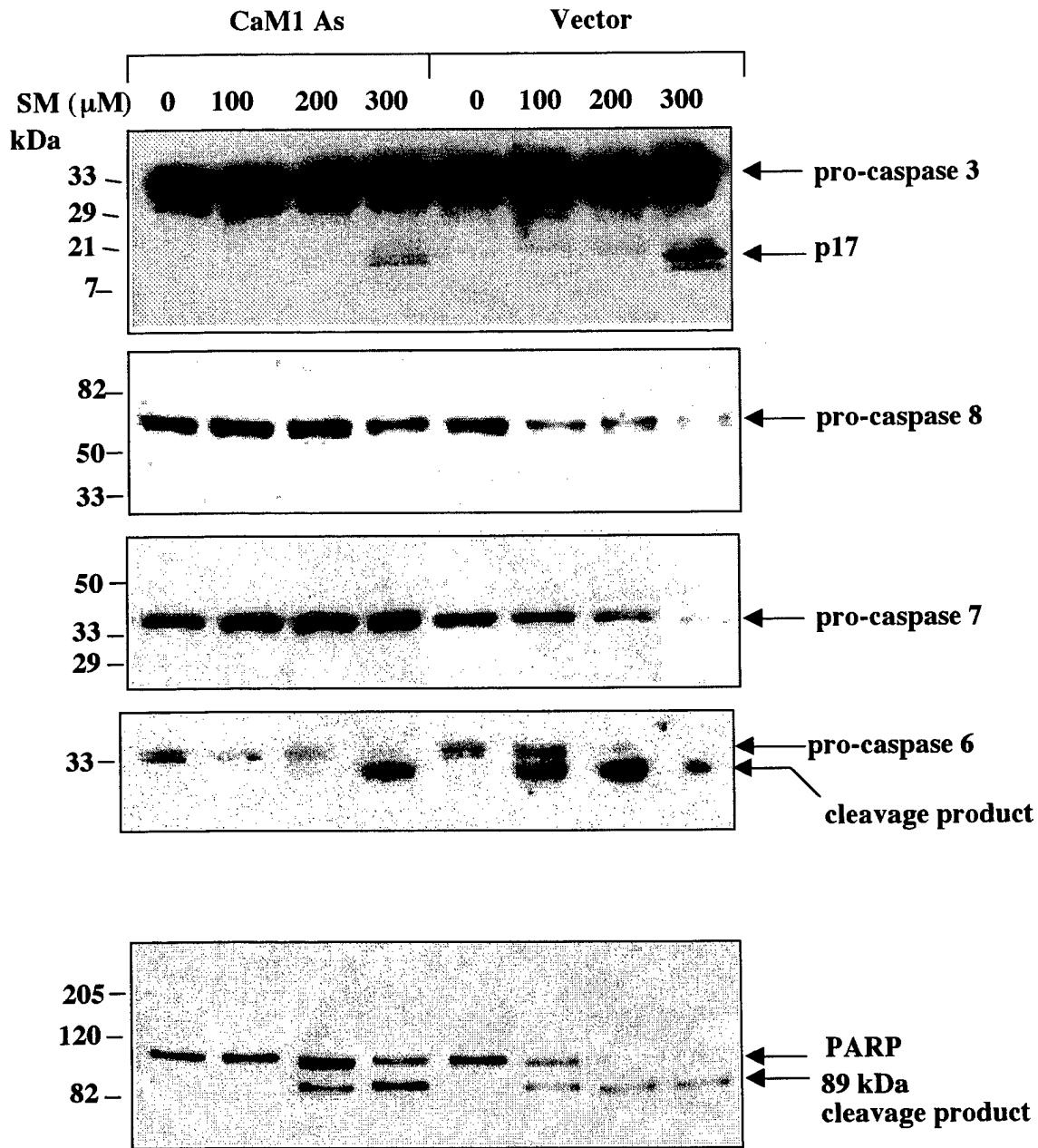
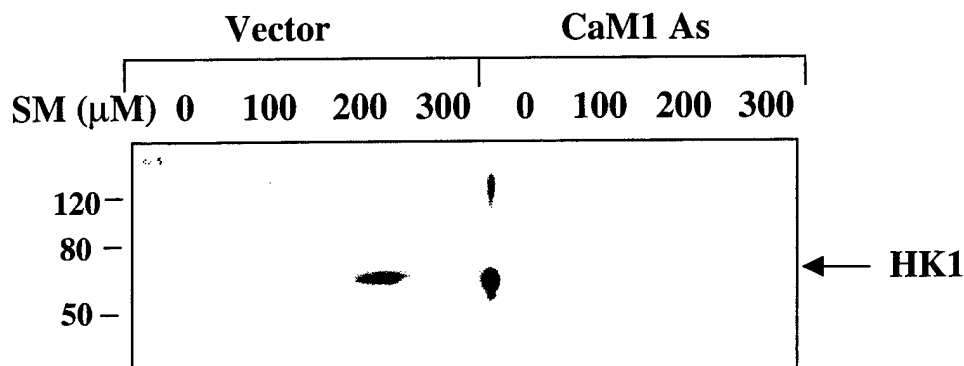


Fig. 11



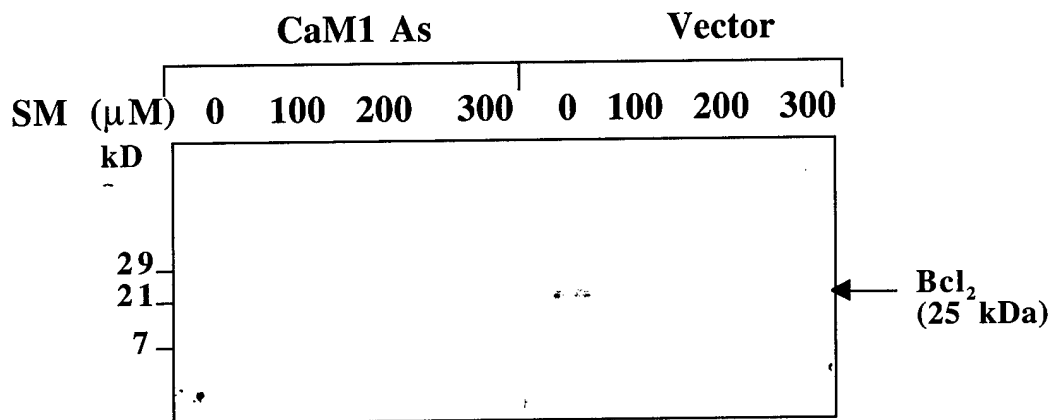
**Fig. 12**

Furthermore, the induction of the differentiation-specific marker K1 by SM was inhibited by CaM antisense RNA expression (Fig. 12).

*Examination of changes in members of the anti-apoptotic Bcl-2 family to examine their role in  $Ca^{2+}$ /CaM mediated pathways.*

Bcl-2 is an anti-apoptotic protein located on the nuclear membrane, ER, and outer mitochondrial membrane. Several Bcl-2-related proteins have been described, including Bcl-X<sub>L</sub> and Bcl-w, both of which are anti-apoptotic. In addition, some Bcl-2-related proteins are anti-apoptotic, including Bax, Bak, and Bad. In numerous recent studies, Bad has been implicated as a key player in programmed cell death (Yanget al., 1995; Dattaet al., 1997; del Pesoet al., 1997; Hsuet al., 1997; Zhaet al., 1997; Scheid and Duronio, 1998; Zundel and Giaccia, 1998) and the  $Ca^{2+}$  /CaM-regulated protein, calcineurin, has been shown to interact with the Bcl-2 family members (Shibasakiet al., 1997), and to dephosphorylate Bad (Wanget al., 1997). This dephosphorylated form of Bad can interact with Bcl-2 or Bcl-X<sub>L</sub> and induce apoptosis (Zhaet al., 1997).

NHEKs were treated with SM for 24 h, and then subjected to Western analysis, using a Bcl-2-specific antibody. Figure 13 shows the significant decrease in the expression of Bcl-2 protein levels following SM treatment. p53 has been shown to antagonize this activity of Bcl-2, perhaps via the induction of Bax, since p53 has been shown to induce *bax* gene transcription via p53 response elements within the *bax* promoter (Miyashita et al., 1995). Moreover, p53 levels rapidly rise following SM treatment.



**Fig. 12**

We are therefore examining the expression and phosphorylation of Bcl-2, Bcl-XL, Bad and Bax following SM treatment, initially by Western analysis. Any alterations in these proteins (as seen in the Bcl-2 protein) will also be further examined at the transcriptional level by PCR to see if they are a primary event in the repression/derepression of apoptosis. Since these proteins regulate mitochondrial function and stability, **they may be important with regards to both apoptotic and necrotic responses to SM.**

## **2.4 Discussion**

The initial results presented above will be repeated and verified utilizing W-7 as well as other inhibitors of CaM, including TR and TFP, since these latter two compounds have been used in clinical settings for the treatment of thermal burns and frostbite (Beitner et al., 1989a; Beitner et al., 1989b; Beitner et al., 1991). In addition these experiments will be repeated and the levels of K1 mRNA analyzed by RT-PCR in order to determine if the inhibition of K1 occurs at the level of transcription. In addition, we will perform immunofluorescent analysis to determine if there is any alterations in the keratin filament structure following K1 induction by SM, and whether these structural alterations are blocked by CaM inhibitors.

**Examination of the role of CaM in altering other proteins that control terminal differentiation and apoptosis.** Calmodulin plays pleiotropic roles in cell signaling. However, several recent studies suggest likely targets for CaM in the modulation of apoptosis. The first is calcineurin, a

serine/threonine phosphatase is regulated by  $\text{Ca}^{2+}$ -CaM complexes in programmed cell death (Shiet al., 1989). Calcineurin plays a role in the dephosphorylation of Bcl-2 family members involved in apoptosis. The phosphorylation state of these proteins regulates their degradation or sequestration.  $\text{Ca}^{2+}$  also plays a role in the induction of an endonuclease responsible for internucleosomal DNA cleavage, yielding the characteristic apoptotic DNA ladders (Shiokawa et al., 1994), and recent studies have shown that the apoptotic endonuclease AP-24 is activated via CaM-dependent protein kinase II (Wright et al., 1997a). If we observe changes in the phosphorylation patterns of the Bcl-2-related proteins, then we will directly assay for changes in calcineurin expression and activity, utilizing specific inhibitors of this enzyme, Cyclosporin A and FK506 (Moia et al., 1994). In addition, we will test the expression and role of the serine protease AP-24 utilizing specific inhibitors as described (Wright et al., 1997b), and if it is positive we will investigate the potential role of CaM kinase II utilizing specific inhibitors KN62 and KN93 (Marley and Thomson, 1996) (Wright et al., 1997a). If there are any alterations in the expression of differentiation or apoptotic markers following treatment with these inhibitors then the enzyme can be further analyzed for activity (autophosphorylation in the case of calcineurin) and expression by techniques described above.

#### 4. CONCLUSIONS

- **SM** induces markers of terminal differentiation as well as apoptosis in NHEK.
- When NHEK are exposed **SM** for 24 h, markers of terminal differentiation are strongly induced **at the protein level** (as determined by immunoblot analysis) including keratins K1, K10, and the cross-linked forms of involucrin. In addition, fibronectin synthesis is drastically reduced.
- When NHEK are exposed to markers of terminal differentiation are strongly induced at the mRNA level, including keratins K1 (within 24 h), K10 (within 8 h), and K14 (within 8 h).
- Fas and Fas L mediate many apoptotic responses. Following **SM** treatment, there is a significant increase in the total levels of both Fas and FasL. Additionally, the appearance of new isoforms of both Fas and FasL are induced.

- Time-course experiments show that caspase-8 is the first to be activated, consistent with a Fas-mediated pathway of apoptosis.
- Caspase-9 is also activated within 2 h showing that a cytotoxic/mitochondrial pathway of apoptosis is also stimulated.
- Caspase-6 is the first of the executioner caspases to be activated, followed by caspases-3 and -7. Lamin B1 is cleaved early by caspase-6, as is keratin K1. Cleavage of the latter protein has not been described in the literature.
- Retroviral constructs expressing antisense to CaM were made. Expression of CaM antisense RNA lowered endogenous levels of CaM, and differentiation of keratinocytes exposed to **SM** as measured by the expression of keratin K1.
- Keratinocytes with reduced levels of CaM were also more resistant to **SM**-induced apoptosis as measured by PARP cleavage and processing of caspases-3, 6, 7, and -8 to their active forms.

#### **Plans/Milestones for the Next Quarter**

- Further analysis of caspase-6 activation and K1 cleavage.
- Beginning of grafting experiments using NHEK expressing CaM antisense RNA.

## 5. ACCOMPLISHMENT OF TASKS

**C.2 Specific Aim I:** Ca<sup>2+</sup>-, CaM-, and Fas/TNF receptor-mediated changes in differentiation and apoptosis induced by SM in human epidermal keratinocytes will be further characterized in order to establish a molecular sequence of events following SM exposure.

**Task 1:** The sequence of events during SM-induced apoptosis were extensively characterized, as monitored by proteolytic cleavage of caspases and their substrates (**Task 1.1, 1.2**). We are currently performing time course analysis for nuclear morphology (**Task 1.3**) DNA laddering, (**Task 1.4**), annexin V binding, (**Task 1.5**) and LDH release (**Task 1.6**).

**Task 2:** Time-course and dose-response experiments were performed to look for alterations in differentiation proteins, including K1, K10, K14 and involucrin as marker for SM-induced skin damage (**Task 2.1**). Characterization of newly discovered caspase-mediated cleavage of epidermal keratin K1 has begun (**Task 2.2**).

**Task 3:** Time- and dose-specific onset of changes in specific pro- apoptotic and anti- apoptotic proteins (Bcl-2 family) during SM-induced apoptosis is being further characterized, to determine their importance as potential modulators of SM toxicity. Levels of CaM protein and mRNA during SM-induced apoptosis were measured, as monitored by immunoblot analysis and RT-PCR (**Task 3.1**). Changes in endogenous levels and interactions of apoptosis-mediating receptors Fas and Fas-ligand (Fas-L) were be examined (**Task 3.2**). Changes pro- and anti-apoptotic Bcl-2 family members will have been examined in the presence and absence of CaM to determine their role in Ca<sup>2+</sup> /CaM mediated pathways (**Task 3.3**).

**C.3 Specific Aim 2:** Calmodulin and the Fas/TNF receptor pathway will be modulated in SM-exposed cultured epidermal keratinocytes by chemical inhibitors, antisense technology, neutralizing antibodies, and dominant-negative strategies

### C.3.1

**Task 1:** The effects of modulation of CaM levels were determined by using antisense technology. It was determined that antisense constructs to CaM can alter the response of cultured epidermal keratinocytes to SM with respect to differentiation and apoptosis.

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