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TITLE: The Molecular Biological Basis for the Response of
Poly(ADP-RIB) Polymerase and NAD Metabolism to DNA Damage
Caused by Mustard Alkylating Agents

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13. ABSTRACT (Maximum 200 Words) Poly(ADP-ribose) polymerase (PARP), modifies a variety of nuclear proteins utilizing NAD. DNA is required for the catalytic activity of the enzyme and the activity is dependent upon the presence of strand breaks in this DNA. It has been hypothesized that human skin exposed to mustards may develop blisters due to a generalized lowering of NAD/ATD in exposed skin cells. We have now utilized primary dermal fibroblasts, immortalized fibroblasts, and keratinocytes derived from PARP -/- and their wild type littermates (PARP +/+) and mice to determine the contribution of PARP to SM toxicity. Primary skin fibroblasts from PARP-deficient mice demonstrated increased caspase-3 activity, compared to those from PARP +/+ animals. Propidium Iodide staining and PARP cleavage patterns revealed a SM dose-dependent increase in necrosis in PARP +/+, but not PARP -/- cells. Using immortalized PARP -/- keratinocytes stably transfected with the human PARP cDNA or control cells, the presence of PARP and its activity, inhibited markers of apoptosis in these cells as well. Primary keratinocytes were derived from newborn PARP+/+ and PARP -/- mice, and immortalized with the E6 and E7 genes of human papilloma virus. In contrast to fibroblasts, keratinocytes from both PARP -/- and PARP +/+ mice expressed markers of apoptosis in response to SM exposure. However, deletion of the PARP gene resulted in the enhanced expression of apoptotic markers in the keratinocytes derived from PARP -/- animals. The effects of PARP on the mode of cell death in <u>different skin cell types</u> may determine the severity of vesication, and thus have implications for the design of PARP inhibitors to reduce SM pathology. Studies in which newborn wild-type and PARP-deficient mice have been exposed to SM by vapor cup indicate that PARP modulates the response of intact animals as well.				
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Appendices

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Appended Publication B..... Rosenthal, D. S., Simbulan-Rosenthal, C. M., Smith, W., Benton, B., Ray, R., and **Smulson, M. E.** Poly(ADP-ribose) polymerase is an active participant in programmed cell death and maintenance of genomic stability. In Cell Death: The Role of PARP (Szabo, C. (ed.), 227-250 (2000).

Appended Publication C..... Rosenthal, D. S., Simbulan-Rosenthal, C. M., Anderson, D., Benton, B., Smith, W., Ray, R. & **Smulson, M.E.** PARP inhibits sulfur mustard-induced apoptosis in skin fibroblasts and keratinocytes. *J Invest Dermatol* Submitted (2001).

Appended Publication D.....Stoppler, H., Stoppler, C., Johnson, E., Simbulan-Rosenthal, C., Iyer, S., Rosenthal, D. S., Smulson, M. E., Schlegel. The E7 protein of human papillomavirus type 16 sensitizes primary human keratinocytes to apoptosis. *Oncogene Sep 10;17(10):1207-14* (1998)

Appended Publication E..... Bhat, K. R., Benton, B. J., Rosenthal, D. S., , S., *Smith, W.J.*, Ray, R. & **Smulson, M.E.** Role of Poly(ADP-ribose) Polymerase (PARP) in DNA Repair in Sulfur-mustard-exposed Normal Human Epidermal Keratinocytes (NHEK). *J Appl Toxic* 20, 00-00- (2000)

Appended Publication F..... Rosenthal, D. S., Simbulan-Rosenthal, C.M., Iyer, S., Smith, W.J., Ray, R. & **Smulson, M.E.** Calmodulin, Poly(ADP-Ribose) Polymerase and p53 are Targets for Modulating the Effects of Sulfur Mustard. *J Appl Toxic* 21, 00-00 (2001)

INTRODUCTION

Sulfur mustard (**SM**) and other chemical warfare agents induce strand breaks in the DNA of exposed cells [1]. The nuclear enzyme poly(ADP-ribose) polymerase (PARP) binds to such DNA strand breaks and is thereby activated [2]. With nicotinamide adenine dinucleotide (NAD) as its substrate, PARP catalyzes the covalent attachment of chains of poly(ADP-ribose) (PAR) to specific proteins in the vicinity of DNA strand breaks and thereby modulates their functional activity and contributes to repair of the damaged DNA [2]. Agents that induce substantial DNA strand breakage and consequent PARP activation—including **SM**, chemical carcinogens, and ionizing radiation—therefore also trigger a marked decrease in the intracellular concentration of NAD as well as in that of its biosynthetic precursor, adenosine triphosphate (ATP) [1, 3-7]. Such a depletion of intracellular NAD and ATP in keratinocytes, together with other effects—such as Ca^{2+} accumulation, oxidative stress, and the activation and release of proteases—has been proposed to contribute to the development of subepidermal blisters and cell death in response to **SM** [1].

The localization of PARP to cells in the lower layers of the epidermis, including the basal or proliferating cells [8], which are more susceptible to the effects of DNA-damaging agents than are more superficial cells [8], is consistent with an important role for this enzyme in the repair of DNA in this tissue. In addition to its role in DNA repair, PARP is thought to contribute to both cellular proliferation and differentiation [9, 10]. The role of PARP in epidermal differentiation is of interest in the context of **SM** action in that perturbations in this process can result in a variety of skin lesions, including squamous cell and basal cell carcinoma.

Elucidation of the molecular mechanism of **SM** action will likely facilitate both the development of treatments to reduce its pathological effects on skin as well as the identification of molecular markers for assessment of exposure to subsymptomatic doses of this and similar agents. The overall goal of the studies funded by the current contract was to investigate, with the use of various molecular biological techniques, the role of PARP and poly(ADP-ribosyl)ation in **SM**-induced skin damage with special reference to markers of apoptosis, especially caspase-3 and its activation to its active form from pro-caspase. We further exploited the mouse PARP knockout system, both in whole animals and in derived fibroblasts and keratinocytes. The studies below outline our major observations obtained over the last period, showing that **SM** exposure causes different routes of death, depending upon skin cell type. In certain cells excessive NAD/ATP lowering due to poly (ADP-ribosyl)ation activation early in the apoptosis pathway leads

ultimately to necrosis, while in other cells, the full apoptotic cascade progresses toward death via scheduled cell death.

BODY

(Research from April 1, 1998 – March 31, 2001)

1.1 Statement of Work: Aim 1

To test directly whether SM induces apoptosis in skin cells, keratinocytes, and tissues exposed to SM by *in-vitro* assessment of the PARP cleavage enzyme (apopain/caspase-3).

1.2 Description of Work Accomplished:

The overall experimental strategy here proposed to assess the testable hypothesis that an early marker for scheduled cell death caused by SM is the induction of activity of the PARP cleavage enzyme, caspase-3. Secondly, to test whether, in conjunction with other assays, caspase-3 would provide a biological indicator in cells or tissues for damage due to vesicating agents.

We had already identified from the five known human members of the ICE protease family, apopain (i.e. caspase-3) is responsible for the cleavage of PARP and necessary for apoptosis. The human cDNA for PARP, which our laboratory first isolated, in part with funding from this contract, served as a template for transcription/translation of a [³⁵S] labeled substrate PARP to allow a direct assay for PARP cleavage activity in cell extracts. This has provided a new, very sensitive **marker** for initiation of early steps in apoptosis, in which many potentially genotoxic agents such as SM most probably participate in.

The cleavage site within PARP (DEVD216-G217) had been identified by our group and others and represents the separation of the two zinc-finger DNA-binding motifs in the amino terminus of PARP, from the automodification and catalytic domain of the enzyme located at the carboxyl terminus of the polypeptide. Since PARP must bind to DNA strand breaks to be catalytically active, we have found that this simple cleavage by caspase-3 renders this enzyme totally inactive in the presence of massive DNA strand breaks which occur during late apoptosis. (Specifically related to experiments performed in these latter 3 years of the contract, we recently published work [Boulares, A.H., Yakovlev, A., Ivanova, V., Stoica, B. A., Wang, G., Iyer, S., Smulson, M.E. Role of PARP cleavage in apoptosis: caspase 3 resistant PARP mutant increases rates of apoptosis in transfected osteosarcoma cells. *J. Biol. Chem.*, 274: 22932-2294 (1999)], which has formed the basis for a better understanding of why PARP must be cleaved

early in apoptosis. We believe this is a mechanism to allow cells to maintain their NAD/ATP levels, under severe DNA strand breaking conditions, and these experiments have been exploited for new experimental approaches contained in a new contract proposal, which was submitted in late November, 2000, to continue this work)

Thus, a very convenient *in vitro* assay for apoptosis had been established which could be potentially be adapted for use with respect to cell toxicity induced by **SM**, and that was in part, the rationale for the studies performed during the last 3 years in this particular area. It was therefore of interest to determine whether **SM**, in fact does, as part of its genotoxic effects, set off the cascade of proteases involved in the apoptotic cascade; these experiments are discussed in more detail in the Final Report Accomplishments, of AIM II outlined below.

SM suppresses Bcl-2 and induces p53. The expression of p53 has been postulated to play important roles in both differentiation and apoptotic responses. The data obtained on this topic in this section of the report is predominantly found in *appended publication A* (attached) and the methodological details, references and figures for these research accomplishments performed during the last 3 years, are provided in this attached publication. Accordingly, immunoblot analysis showed a significant increase in the protein level of p53 after exposure to 100 μ M **SM**, and this increase in p53 level occurred within 2 h (**Fig 3A**), *appended publication A*). The legend of this figure and all figures in the appended publications to this report, as noted, provide precise details of how these experiments were performed. We also examined the levels of the *bcl-2* gene product, which inhibits keratinocyte apoptosis. Following **SM** treatment a dependent decrease in *bcl-2* protein levels in NHEK, as determined by immunoblot analysis was observed. (**Fig 3B**). (*appended publication A*)

SM induces apoptosis via caspase-3. The striking decrease in Bcl-2 levels and the increase in p53 levels suggested that **SM** may induce apoptosis via a p53-related signal. Thus, we assayed for markers of apoptosis following **SM** treatment. A hallmark of apoptosis in several cell types is the appearance of nucleosome-sized ladders due to the presence of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease that is induced in apoptotic cells. DNA isolated from NHEK treated with 0 or 100 μ M **SM** was intact; however at 300 μ M **SM**, NHEK showed nucleosome-sized ladders analyzed by agarose gel electrophoresis, although some nonspecific fragmentation was also apparent (shown in the data of *appended publication A Fig 4*, p. 67). Accordingly, trypan blue exclusion (i.e. viability) at 24 h was 98% in control cells, 90% following 100 μ M **SM** treatment, and 60% after 300 μ M **SM**.

We have recently determined that the activation of PARP plays a role in the etiology of apoptosis in osteosarcoma cells induced to undergo programmed cell death, in which poly (ADP-ribosyl)

ation corresponds with the early reversible stages of apoptosis. We therefore determined whether we could detect a similar increased level of poly (ADP-ribose) ation following exposure of NHEK to SM. Because PARP is the main acceptor protein for poly(ADP-ribose)ation, via intermolecular “automodification,” the presence of a 116 kDa antipoly(ADP-ribose)-cross reactive band is a sensitive indicator of poly(ADP-ribose)ation within the nucleus. Anti-sera specific for poly (ADP-ribose) **polymer** did in fact detect a strong band at 116 kDa in extracts of primary keratinocytes treated with all concentrations of SM tested, whereas no such band was present in extracts of control keratinocytes, indicating that SM induces DNA strand breaks and PARP is activated. The data in **Fig 5(A)** (*appended publication A p.68*) shows that, at 100 μ M SM, activation of PARP occurs and automodification is apparent as early as 2h. The molecular weight of automodified PARP was similar to that of unmodified PARP(116 kDa), indicating that the average polymer chain length is relatively short at this time point. After this time, the level of poly(ADP-ribose) was observed to decrease precipitously, similar to our previous observations using osteosarcoma cells induced to undergo apoptosis, in work performed under the earlier Contract. The abundance of both poly(ADP-ribose) and PARP, after its early burst at day 3, decreased markedly, corresponding to the appearance of the proteolytic cleavage product containing the DNA-binding domain of PARP; no poly(ADP-ribose)polymer was observed during this time, in spite of the fact that there was massive DNA internucleosomal degradation.

In **Section 2** of the **Final Report** which concerns Specific Aim II of the Contract Proposal, further data on the use of immuno-fluorescence to detect this activation of poly(ADP-ribose)ation in intact cells will be discussed. Other evidence will be reported indicating a requirement for this “burst” of PARP during apoptosis (in certain cell systems) as well as recent work using a caspase-3-resistant PARP mutant expressions vector which was transfected into PARP knockout cells to clarify these effects further.

We previously determined that this characteristic rise and decline in poly(ADP-ribose) levels during apoptosis could be attributed not only to poly(ADP-ribose) glycohydrolase activity but also to the caspase-3 proteolytic cleavage of PARP into the characteristic 89 kDa and 24kDa fragments, the latter of which contains the Zn^{2+} finger region and DNA-binding domain. We therefore performed western analysis to monitor the **SM**-induced cleavage of PARP, using an antibody that recognizes both the full-length 116kDa protein as well as the 89kDa fragment of PARP (**Fig 5 B** of the above appended publication); this data demonstrates a significant conversion of full-length PARP to 89kDa fragment following 300 μ M SM.

We have also established a sensitive technique to verify that SM induces apoptosis by determining proteolytic processing of caspase-3 from its precursor (pro-caspase-3; CPP32) via

the use of *in vitro* translated PARP. We therefore used a combination transcription/translation system to radiolabel full-length PARP (see *appended publication paper A*) that was subsequently incubated with extracts derived from keratinocytes treated with SM. The data in *amended publication A*, **Figure 6 (A)**; shows that PARP cleavage activity was clearly noted in NHEK in 300 μ M SM (but not 100 μ M SM) after 24 h, as evidenced by the strong appearance of the 24 kDa and 89 kDa cleavage products. **Figure 6(B)** from the same paper shows, by quantitative phosphorimage analysis, the relative PARP cleavage activities that resulted from the treatment of NHEK with SM for 24h. The high level of PARP-cleavage activity observed in 300 μ M SM is indicative that this vesicant is also a strong inducer of apoptosis in primary keratinocytes and that apoptosis is occurring via a caspase-3 like pathway.

This, accordingly, summarizes the relevant results concerning work performed on caspase-3 and SM as proposed in Aim I. In the next section of the **Final Report** concerning Aim II, data will be provided indicating immuno fluorescent studies using antibody to poly(ADP-ribose) polymer with cells after exposure to SM as well as briefly describing the unique effects observed on cells during excessive poly(ADP-ribosyl)ation, after SM treatment or other DNA strand breaking agent treatment. This will be followed by a discussion of the accomplishments in Aim III, using PARP knockout animals and skin cells derived from these animals, which has introduced a new paradigm in PARP's participation in SM induced cell death in dermal cells versus keratinocytes. This appears to be directly related to the extent of this extreme activation of poly(ADP-ribosyl)ation by SM and its concurrent lowering of both NAD and ATP levels.

1.3 List of Publications and Presentations Relevant to Aim 1 (see *Appended Publications and REPORTABLE OUTCOMES*)

1.4 Figures and Graphs (see *appended publications*)

1.5 Recommended Future Work (see **CONCLUSIONS)**

2.1 Statement of Work Aim 2

Analysis of SM induced skin pathology via initiation of apoptosis by utilizing immunofluorescence methods to assess degradation of PARP, the generation and loss poly(ADP-ribose) (PAR), the activation of ICE proteases, and alterations general nuclear morphology.

2.2 Description of Work Accomplished

It was proposed in this Aim to establish and verify that SM induces apoptosis by determining whether the observed caspase-3 activity in vitro (**Aim 1.1**) could be associated with the SM processing of pro-caspase-3/CPP 32 into its active protease form. During apoptosis, pro-caspase-3/CPP 32 is processed into 17 kDa and 12kDa peptides, with the removal of a pro-peptide sequence from the N-terminus. The 17kDa and 12kDa fragments then form the active proteolytic heterodimer. We used an antibody that recognizes both the active (p17) and inactive CPP32 forms a caspase-3, a slightly smaller form of CPP32. Formation of a CPP32 degradation product was observed 24 hours after the cells were exposed to 100 μ M SM, of equivalent size to CPP32 minus the pro-peptide sequence, suggesting that processing of the N-terminus of the precursor protein was occurring. This is shown in the data of *appended publication A Figure 7A*, top and the explanation for the experimental protocol is described in the legend to this appended publication.

To confirm the activation of pro-caspase by SM treatment, the same extracts used above were examined utilizing an antibody that is specific for the pro-sequence that is removed from caspase-3, as processed into its active form. The disappearance of the slightly smaller MW band previously observed at 24 h in 100 μ M SM (Fig 7A bottom; above publication), indicated that this band is missing the pro-peptide sequence, and is thus the result of CPP32 N-terminal processing. Following treatment with 300 μ M SM, NHEK showed complete processing of a portion of CPP32 into the active P17 form (Figure 7B, *same appended publication*). A small amount of the partially processed P20, which represents P17 and the terminal pro-sequence, is also observed. Thus with both 100 μ M and 300 μ SM, markers of apoptosis are induced by SM, although complete activation of caspase-3, PARP cleavage, and DNA fragmentation are only observed at higher concentrations of SM at least under these in vitro conditions.

We also observed the induction by SM, under specific conditions of the expected transient, early “burst” of poly(ADP-ribosyl)ation of nuclear protein by both immunofluorescence as well as electrophoretic analysis of modified proteins using anti-PAR antibody apoptotic inducers, such as anti-Fas, and conditions. This early transient activation of poly(ADP-ribosyl)ation was essential for apoptosis to proceed in most instances. It should be noted that recent experiments to be, described below, indicate that specific skin cell severe and transient lowering of NAD, due

to early poly(ADP-ribosyl)ation, and die due to necrosis rather than apoptosis. The necrotic death would contribute greatly to an inflammatory and blistering greater than apoptotic death.

Human osteosarcoma cells incubated with 300 μ M **SM** were subjected to immunofluorescent analysis with antibodies that recognize the DNA binding domain of PARP (DBD) but not intact PARP. As with the other markers, samples were analyzed each day throughout the total 10-day period; samples from immediate (day 1), early (day 3), mid- (day 6), and late (10) stages of apoptosis are shown in the (*Fig. 11.1 A*) (*Appended publication B, p. 229*). A more detailed explanation of the experiment is provided in the legend to this figure. Immunofluorescence analysis detected the PARP DBD in human osteosarcoma cells only after 6 to 7 days in culture, a time at which the abundance of both PARP and PAR is decreasing, PARP-cleavage activity is increasing, and internucleosomal DNA cleavage is present. The pattern of staining for the DBD of PARP differed markedly from that of full-length PARP.

We initially wished to demonstrate that the transient poly(ADP-ribosyl)ation could be observed in cells treated with **SM** and caused apoptosis. We measured PARP activity as well as the total amount of cellular PAR at different times of apoptosis induced by sulfur mustard. PAR (i.e. poly(ADP-ribose) polymer) was observed to be strongly induced by **SM** in the early stages of apoptosis (within 30min), but not at later stages (*Figure 11.1B*); (*appended publication B*) Importantly, the appearance of PAR precedes the cleavage of DNA fragmentation factor (DFF45; see below), and the appearance of DNA ladders at 24 h (*Fig. 11.1C*; *appended publication B*). Although PARP is completely cleaved, extracts derived from 24-h apoptotic, cells as induced by **SM**, retained approximately 20% of their *in vitro* polymerizing activity (*Fig. 11.1 D*; *appended publication B*), reflecting the low-level DNA-independent activity of the catalytic domain. However, this activity is apparently insufficient to synthesize or sustain detectable steady-state levels of PAR *in vivo* by **SM** treatment in the presence of PAR-degrading enzymes. Furthermore, addition of the PARP inhibitor benzamide to the incubations with **SM** significantly delayed the onset of apoptosis as induced by apoptosis as illustrated by the lack of DNA fragmentation in keratinocytes experienced to sulfur mustard for 24 h (*Fig 11.1 C*; *appended publication B, p. 229*).

Although reported in an earlier **Mid-Term Report** for the first earlier 3 years of this contract, the generality of an early burst of poly(ADP-ribosyl)ation was confirmed with human HL-60 cells, mouse 3T3-L1, and immortalized fibroblasts derived from mice. As noted above in certain systems (including apoptosis as induced by **SM**) this early burst of poly(ADP-ribosyl)ation severely effects progression of apoptosis using the depletion of PARP protein, their by antisense RNA expression or by gene disruption and examining the various morphological and

biochemical markers of apoptosis. (This is illustrated in figure 11.2; *appended publication B, p. 231*).

For example, relevant to later studies using SM, in 3T3 L1 cells, stably transfected with a construct expressing dexamethasone (Dex)-inducible PARP antisense RNA, Dex induced a time-dependent depletion of PARP, with only ~5% of the protein remaining after 72 h. A combination of anti-Fas and cycloheximide induced a marked increase in caspase-3-like activity in control 3T3-L1 cells that had been preincubated in the absence or presence of Dex. This effect was maximal 24 h each after induction of apoptosis, as indicated by the generation of the 89- and 24-kDa cleavage fragments of PARP in an *in vitro* assay (Fig. 11.2A, top; *appended publication B*). Similar results were observed in this publication on the activation of pro-caspase-3 to caspase-3. Furthermore using DNA fragmentation analysis as an assay for apoptotic cells, several cell lines, depleted of PARP by antisense did not exhibit marked DNA fragmentation (DNA ladders) (Figure 11.2C), or apoptosis. Although the use of the PARP knockout system will be more extensively summarized in **Aim III**, below, it is of relevance to our later experience with SM that PARP^{+/+} cells showed substantial nuclear fragmentation and chromatin condensation 24 h after induction of Fas-mediated apoptosis; ~97% of nuclei exhibited apoptotic morphology by this time. It is additionally of importance to later experiments on this current contract, as well as on a contract, under review at this time, in contrast, no substantial changes in nuclear morphology characteristic of apoptosis were apparent in the PARP^{-/-} fibroblasts even after exposure to anti-Fas and cycloheximide for 24 h or 48 h.

PARP^{-/-} fibroblasts were stably transfected with a plasmid expressing wild-type PARP. Individual as well as pooled clones expressed PARP protein at levels similar to those of PARP^{+/+} cells. These cells were to undergo apoptosis by exposure to anti-Fas and cycloheximide for up to 48 h. in contrast to the cells derived from knockout animals which had not been re-introduced with PARP. (Figure 11.3 A-D; *appended manuscript B, p. 233*) and assessed by all the various assays described above for markers of apoptosis. Thus, depletion of PARP by antisense in 3T3L1, or by knockout of PARP attenuates Fas plus cycloheximide-mediated apoptosis. In addition, the reintroduction of PARP in independent clones of PARP^{-/-} cells re-establishes the response. *New data obtained during the study describes a new paradigm in SM pathology between different types of skin cells which has been clarified by the use of the PARP knockout system described above.*

The mechanisms for the generation of blisters in the skin caused by SM is still not completely understood, although the news observations discussed in detail below in Aim 3 from our laboratory on necrosis and apoptosis effects, have recently made important molecular contributions towards a better understanding of this phenomenon. We have previously shown

that SM induces markers of terminal differentiation and apoptosis in normal human epidermal keratinocytes (NHEK), (i.e. discussed in the results of **Specific Aim I**, including the early activation and late cleavage of PARP). In addition to the direct effect of SM on keratinocytes, dermal fibroblasts are also considered important for the vesication response. Human dermal fibroblasts may contribute to the vesication response by releasing degradative cytosolic components extracellularly after SM exposure. Lactate dehydrogenase (LDH), a cytosolic marker of necrotic cell death, was shown to be released in a time-dependent fashion after exposure of a dermal preparation to 2 μ M SM, suggesting a steady leakage of cytosolic constituents after the initial SM exposure. Elastase levels also increased to over 740% of those in control culture medium 24 h after exposure and also there is a dose and time dependent cytotoxicity of a dermal equivalent, and a decreased synthesis of fibronectin by dermal fibroblasts due to SM.

PARP knockout mice have now been independently generated by the interruption of exon 2, and by exon 4, and most recently exon 1 has been interrupted on the gene for PARP-1, located on chromosome 1. PARP knockout mice with a disrupted PARP gene neither express intact PARP nor exhibit significant poly(ADP-ribosyl)ation. The potential role for PARP in cell death via NAD and ATP depletion has been supported by recent studies in which both exon 1 and exon 2 of PARP^{-/-} animals have been shown to be resistant to streptozotocin-induced pancreatic islet cell death, associated with NAD depletion in PARP^{+/+} animals. We have also collaborated in a study that demonstrated that exon 2 PARP^{-/-} animals are resistant to the neurotoxin MPTP-induced Parkinsonism. Exon 2 PARP^{-/-} animals are also more resistant to ischemic injury. (See "Reportable Outcomes" below).

It has recently been shown in our laboratory that expression of caspase-3 resistant PARP in exon 2 PARP^{-/-} cell as well as expression of exogenous wild-type PARP in osteosarcoma cells results in an earlier onset of both the apoptotic and necrotic responses, a finding that is consistent with an active role for PARP and poly(ADP-ribosyl)ation early in apoptosis as well as necrosis. Further evidence for role of PARP in necrosis was determined in studies in which exon 2 PARP^{-/-} fibroblasts were found to be more resistant to necrosis and ATP depletion induced by the alkylating agent MNNG. Induction of PARP can drastically deplete levels of cellular NAD and ATP which could contribute to either apoptotic or necrotic cell death. The ATP-depleted cells display a significant upregulation of Fas, Fas-ligand, and FADD, resulting in induction of caspase-8 and caspase-3 activity. Further depletion of ATP below a threshold level might be expected to inhibit the later events of apoptosis, depending on the cell type and inducing agent.

Thus, the cellular response to DNA damage may depend upon the level and type of damage, as well as the cell type and the concomitant lowering of NAD and ATP levels via activation of PARP activity resulting ultimately in cell death, the form of which (apoptosis vs. necrosis) may depend upon a number of factors, including the time of onset of caspase activation and proteolytic cleavage of PARP.

In recent experiments performed and submitted for publication as part of **Specific Aim III** of the current contract, we have found that PARP plays a role in cell death induced by SM in primary dermal and immortalized fibroblasts by **shifting the mode of cell death from apoptosis to necrosis**. (*Appended publication C*). Keratinocytes, on the other hand, express markers of apoptosis in the presence or absence of a functional PARP-1 gene, although apoptotic markers are upregulated in the absence of PARP. These results thus indicate that:

- dermal fibroblasts and keratinocytes, which both contribute to SM vesication, undergo different mechanisms of cells death in response to SM.
- PARP shifts the mode of cell death from apoptosis to necrosis in dermal fibroblasts. Therefore, inhibition of PARP **may be of therapeutic value in the treatment of or prophylaxis against SM injury, since apoptotic cell death may prevent the release of inflammatory or degradative enzymes contributing to vesication or inhibition of healing of SM-induced wounds.**

2.3 List of Publications and Presentations Relevant to Aim II (*see REPORTABLE OUTCOMES*)

2.4 Figures and Graphs (see appended publications)

2.5 Recommended Future Work (see CONCLUSIONS)

3.1 Statement of Work: Aim 3

The use of PARP K/O animals and derived immortalized keratinocytes to evaluate SM skin pathology and activation of apoptosis.

3.2 Description of Work Accomplished

As noted above, Sulfur mustard exerts differing cytotoxic effects of dermal fibroblasts and epidermal keratinocytes. Recent studies performed at the end of the current Contract Period; submitted for publication *J. Invest. Dermatol.* demonstrated a new paradigm in PARP's participation in SM induced cell death in dermal versus keratinocytes which we feel supports a significant new aspect of the potential control of SM pathology. We compared primary dermal fibroblasts, immortalized fibroblasts, and keratinocytes derived from PARP^{-/-} and PARP^{+/+} mice to determine the contribution of PARP to SM toxicity. The precise details in methodology of experimental protocols and their literature citations the figures and legends of these current experiments is provided in *appended publication C* and thus will only be highlighted and summarized here. As detailed in results of specific Aim I above, SM induces caspase-3 activity, an established marker of apoptosis in various skin cells, especially keratinocytes. We were therefore interested in determining whether cleavage of endogenous PARP and the apoptotic cascade would also be observed in SM exposed primary skin fibroblasts. Immunoblot analysis was performed using antibodies that recognize both the full length as well as the caspase-3 induced products of PARP. Although PARP is degraded at the higher doses of SM, no apoptotic cleavage fragments were observed (Figure 1A; *amended manuscript C*), suggesting that the mode of cell death due to SM in these cells might be necrotic.

As expected, no immunodetectable PARP was observed in the PARP^{-/-} fibroblasts, confirming the PARP^{-/-} genotype. While PARP^{+/+} primary fibroblasts did not exhibit SM-induced caspase activity at any of the doses tested, PARP^{-/-} fibroblasts exhibited marked caspase-3 activity in response to SM at all doses tested (Figure 1B; *appended manuscript C*). The greatest caspase-3 activity was observed following treatment of PARP^{-/-} fibroblasts with 300 μ M SM. Thus, PARP activity appeared to inhibit apoptosis in *primary dermal fibroblasts*. It must be noted that caspase-3, besides cleaving PARP during apoptosis, also cleaves a large number of other key cellular components, such as Rb gene product, nuclear lamins, etc. required for apoptosis.

An early marker of apoptosis is the exposure of phosphatidylserine residues in the outer plasma membrane leaflet. The presence of these residues can be detected by their ability to bind to annexin V. To further examine the level of apoptosis in these primary dermal fibroblasts, we therefore exposed cells to increasing doses of SM and then analyzed the cells for annexin V binding by FACS analysis 24 h after SM exposure. While only a small percentage of PARP^{+/+}

primary fibroblasts were apoptotic at all doses of SM tested (< 20%), a dose-dependent increase in the number of PARP^{-/-} apoptotic fibroblasts was observed, up to a maximum of 65% (*Fig. 2A; appended publication C*). These results suggested that the absence of PARP shifted the mode of cell death by SM from necrosis to apoptosis. To verify if this was the case, we examined the percentage of cells that were positive for Propidium Iodine (PI) staining, and were also annexin V negative, indicating a primarily necrotic mode of cell death. PARP^{+/+} primary fibroblasts exhibited a SM dose-dependent increase in the level of necrosis, while PARP^{-/-} fibroblasts underwent little necrosis at all doses tested (Figure 2B). In a separate set of experiments, we confirmed the mode of cell death induced by SM in wild-type and PARP^{-/-} dermal fibroblasts, using annexin V plus Propidium Iodide (PI) staining. Figure 3A (*appended publication C*) clearly shows that the primary mode of cell death in general, in PARP^{+/+} cells is necrotic, while PARP^{-/-} cells undergo apoptosis.

Since the absence of PARP-induced caspase-3 activity and apoptosis in SM-exposed PARP^{-/-} fibroblasts, we examined whether caspase-3 was in fact responsible for SM cytotoxicity in PARP^{-/-} cells. We therefore preincubated PARP^{-/-} cells with either an inhibitor of caspase-3 (DEVD-CHO), or caspase-1 (YVAD-CHO) for 30 min prior to and during SM exposure. PARP^{-/-} fibroblasts that were either not pretreated or pretreated with YVAD-CHO underwent apoptosis following SM exposure (Figure 3B). However, pretreatment with DEVD-CHO abolished the apoptotic response. Thus the deletion of PARP by gene knockout leads to an apoptotic mode of death that is still dependent on caspase-3.

To more firmly establish the role of PARP in the mode of cell death in fibroblasts, we utilized immortalized PARP^{-/-} fibroblasts that were stably transfected with human PARP-1 cDNA or with empty vector alone. Western analysis shows that PARP is expressed in immortalized wild-type fibroblasts (PARP^{+/+}), as well as PARP^{-/-} fibroblasts stably transfected with the human PARP cDNA [PARP^{-/-} (+PARP)], but not in immortalized PARP^{-/-} fibroblasts (Figure 4A, *appended publication C*). PARP^{-/-} or PARP^{-/-} (+PARP) fibroblasts were then exposed to similar concentrations of SM, and then analyzed for caspase-3 activity by performing an *in vitro* PARP cleavage assay. PARP cleavage activity was induced in PARP^{-/-} fibroblasts at SM doses greater than 100 μ M, with >60% cleavage observed at the highest dose of SM tested (500 μ M; Figure 4B *appended publication C*). In contrast, the reintroduction of PARP suppressed the *in vitro* PARP cleavage activity, *suggesting that expression of PARP rendered these cells more susceptible to necrosis*. To further analyze caspase-3 processing, cells were exposed to SM and analyzed for the correct processing of procaspase-3 (32 kDa) into its active form (p17). Caspase-3 processing was clearly observed at 300 μ M and 500 μ M SM in the PARP^{-/-} cells but not in the cells stably retransfected with PARP cDNA (Figure 4C).

To analyze the level of apoptosis after reintroduction of PARP, annexin V plus PI staining was again utilized. Both PARP^{+/+} and PARP^{-/-}(+PARP) immortalized fibroblasts showed only low levels of annexin V staining at all doses of SM tested (<6%; *appended publication C*; Figure 5A). On the other hand, immortalized PARP^{-/-} fibroblasts demonstrated a dose-dependent increase in annexin V-positive cells, up to a maximum of 22%, corresponding to exposure to 500 μ M SM. Conversely, dose-dependent increases in PI-positive cells were observed in both PARP^{+/+} and PARP^{-/-} (+PARP) cells, but not in PARP^{-/-} cells, indicating that the expression of PARP increases the level of necrosis in immortalized fibroblasts.

3.3 List of Publications and Presentations Relevant to Aim III (*see REPORTABLE OUTCOMES*)

3.4 Figures and Graphs (*see appended publications*)

3.5 Recommended Future Work (*see CONCLUSIONS*)

SM induces apoptosis in keratinocytes that is enhanced in the absence of PARP.

We next assessed the role of PARP in the induction of apoptosis in keratinocytes. Primary mouse keratinocytes derived from PARP^{-/-} animals grow poorly under culture conditions that we normally use. In order to obtain a sufficient supply of keratinocytes, we derived keratinocyte clones from PARP^{-/-} and PARP^{+/+} animals following immortalization of cells with a retrovirus that expresses both the E6 and E7 genes of human papillomavirus 16 (HPV 16). A number of clones were found to be both keratin-positive and vimentin-negative by Western analysis, indicating that these cells were in fact keratinocytes, and not fibroblasts (Fig. 6A; *appended publication C*) middle and bottom panels). As with all other cells utilized in the study, these clones were also confirmed to be either PARP^{-/-} or PARP^{+/+} by immunoblot analysis (Fig. 6A, top panel). Two different clones of each type were then exposed to SM and caspase-3-like activity was determined by a fluorometric assay as described above. All clones demonstrated a very strong apoptotic response to SM (Figure 6B). At the lower dose (100 μ M), there was no significant difference observed in the level of caspase-3 activity induced by SM, while at the higher dose (300 μ M), extracts from the PARP^{-/-} keratinocytes showed a slightly higher activity (30%). Thus, SM induces apoptosis in keratinocytes that is enhanced in the absence of PARP.

Direct Examination of Apoptosis, Necrosis and Other Parameters in Skin from Animals Exposed to SM.

During the last three years we initiated a number of collaborative experiments (and are still) active with the laboratory of Dana Anderson (USAMRICD), which used animals directly exposed to SM. We have examined the type of cell death occurring *in situ* in SM-exposed skin of wild type and PARP^(-/-) mice, followed by histopathological analysis. Based on the encouraging results summarized below, it would be appropriate in the future to follow through by using immunofluorescence microscopy of skin sections with a number of PARP and apoptotic-related antibodies mentioned already. This will test whether apoptotic or necrotic chemical markers (i.e. caspase-3 activation, etc.) can be demonstrated in the SM-exposed skin.

Thus far Dana Anderson has performed the SM-vapor cup exposures and Dr. Larry Micheltree has performed the pathology and analysis of morphology.

Sulfur mustard exposure was performed by placing a small amount of sulfur mustard liquid into an absorbent filter at the bottom of a vapor cup, which was then inverted onto the dorsal surface of the animal, which was exposed to the SM vapor for the indicated times and conditions. Additional anesthetic was administered if corneal or toe pinch reflexes were returning, or changes in respiration noted. Neonatal PARP K/O mice were placed on a frozen isothermal pad until their respirations were shallow and their bright pink color had faded. Once immobilized,

the mice were placed alternatively between the frozen pad and non-chilled gauze to maintain sedation while being exposed. Once exposure is complete the mice were aroused with manual stimulation and placement on a warm isothermal pad.

The results of several interesting experiments are provided below, both in detailed pathological analysis (Tables 1), and by summary bar graphs (Fig. 1). In general, the PARP K/O phenotype had a profound effect on differential killing by SM in different skin layers. Clearly more work needs to be done in the future Contract.

Table 1. Exposure of Animals to SM, Pathology Analysis

Accession#	Animal#	Pustular Epidermitis	Epidermal Necrosis	Vesicle	Follicular damage	Dermal necrosis	Comments	Time of Exposure
99-1246	26	0	0	0	0	0	KO/C	0
99-1225	5	0	0	0	0	0	wt/C	0
99-1231	11	0	0	0	0	0	wt/C	0
99-1243	23	0	1	0	1	2	KO/HD	10 min vapor
99-1244	24	0	1	0	0	1	KO/HD	10 min vapor
99-1223	3	0	0	0	0	0	wt/HD	10 min vapor
99-1224	4	0	0	0	0	0	wt/HD	10 min vapor
99-1229	9	0	0	0	1	1	wt/HD	10 min vapor
99-1232	12	0	1	0	0	1	wt/HD	10 min vapor
99-1235	15	0	0	0	0	0	wt/HD	10 min vapor
99-1239	19	0	1	0	1	1	wt/HD	10 min vapor
99-1242	22	0	0	0	1	2	KO/HD	15 min vapor
99-1245	25	1	2	0	1	2	KO/HD	15 min vapor
99-1222	2	0	1	0	1	2	wt/HD	15 min vapor
99-1227	7	0	1	0	1	2	wt/HD	15 min vapor
99-1228	8	0	1	0	1	1	wt/HD	15 min vapor
99-1234	14	0	1	0	1	2	wt/HD	15 min vapor
99-1236	16	0	1	0	1	1	wt/HD	15 min vapor
99-1238	18	0	0	0	1	2	wt/HD	15 min vapor

99-1240	20	0	0	0	1	2	KO/HD	20 min vapor
99-1241	21	0	0	0	1	2	KO/HD	20 min vapor
99-1221	1	1	2	2	2	3	wt/HD	20 min vapor
99-1226	6	1	1	0	1	1	wt/HD	20 min vapor
99-1230	10	0	1	1	2	2	wt/HD	20 min vapor
99-1233	13	1	1	1	1	2	wt/HD	20 min vapor
99-1237	17	0	1	0	0	2	wt/HD	20 min vapor
99-1246	26	0	0	0	0	0	KO/C	0
99-1225	5	0	0	0	0	0	wt/C	0
99-1231	11	0	0	0	0	0	wt/C	0
99-1243	23	0	1	0	1	2	KO/HD	10 min vapor
99-1244	24	0	1	0	0	1	KO/HD	10 min vapor
99-1223	3	0	0	0	0	0	wt/HD	10 min vapor
99-1224	4	0	0	0	0	0	wt/HD	10 min vapor
99-1229	9	0	0	0	1	1	wt/HD	10 min vapor
99-1232	12	0	1	0	0	1	wt/HD	10 min vapor
99-1235	15	0	0	0	0	0	wt/HD	10 min vapor
99-1239	19	0	1	0	1	1	wt/HD	10 min vapor
99-1242	22	0	0	0	1	2	KO/HD	15 min vapor
99-1245	25	1	2	0	1	2	KO/HD	15 min vapor
99-1222	2	0	1	0	1	2	wt/HD	15 min vapor
99-1227	7	0	1	0	1	2	wt/HD	15 min vapor
99-1228	8	0	1	0	1	1	wt/HD	15 min vapor
99-1234	14	0	1	0	1	2	wt/HD	15 min

99-1236	16	0	1	0	1	1	wt/HD	vapor 15 min
99-1238	18	0	0	0	1	2	wt/HD	vapor 15 min vapor
99-1240	20	0	0	0	1	2	KO/HD	20 min
99-1241	21	0	0	0	1	2	KO/HD	vapor 20 min
99-1221	1	1	2	2	2	3	wt/HD	vapor 20 min
99-1226	6	1	1	0	1	1	wt/HD	vapor 20 min
99-1230	10	0	1	1	2	2	wt/HD	vapor 20 min
99-1233	13	1	1	1	1	2	wt/HD	vapor 20 min
99-1237	17	0	1	0	0	2	wt/HD	vapor 20 min vapor

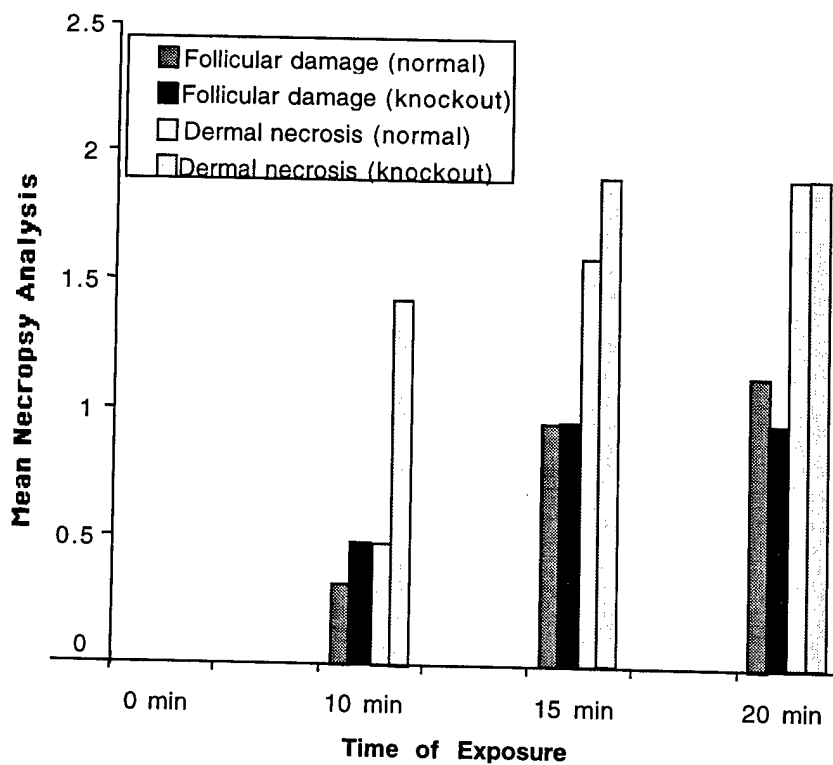
Averages

Time	Type	Pustular Epideritis	Epider mal Necros is	Vesicle	Follicular damage	Dermal necrosis	Time
10 min vapor	KO/HD	0.00	1.00	0.00	0.50	1.50	10 min vapor
	wt/HD	0.00	0.33	0.00	0.33	0.50	
15 min vapor	KO/HD	0.50	1.00	0.00	1.00	2.00	15 min vapor
	wt/HD	0.00	0.83	0.00	1.00	1.67	

20 min vapor	KO/HD	0.00	0.00	0.00	1.00	2.00	20 min vapor
	wt/HD	0.60	1.20	0.80	1.20	2.00	

Figure 1.

24 h Pathology Following Vapor Cup Exposure



3.3 List of Publications and Presentations Relevant to Aim III (*see Appended Publications and REPORTABLE OUTCOMES*)

3.4 Figures and Graphs (*see appended publications*)

3.5 Recommended Future Work (*see CONCLUSIONS*)

Key Research Accomplishments

Human keratinocytes were treated with SM and examined for various properties of apoptosis. For example, caspase-3 activity was shown to increase.

- Our initial studies report indicated that immortalized PARP^{-/-} cells are more sensitive to **SM** killing than PARP K/O cells retransfected with a vector expressing PARP cDNAs. Consistent with these findings, primary fibroblasts derived from PARP^{-/-} mice were also more sensitive to **SM** induced cytotoxicity than those derived from PARP^{+/+} animals.
- During the last contract period we exposed newborn PARP^{-/-} and ^{+/+} pups to **SM** Vapor. The pathological analysis of stained skin slices showed that they are more sensitive to SM induced vesication.
- To further dissect the role of PARP in apoptosis, certain nuclear DNA binding proteins were found to become significantly more poly(ADP-ribosyl)ated during the early stages of apoptosis. These proteins include the tumor suppressor, p53, a protein that **is elevated early in SM induced apoptosis.**
- The elevation of p53 by the viral HPV E7 protein rendered cells more sensitive to **SM** apoptosis, while E6 protein binds p53 and reversed this effect. (*appended publication D*).
- PARP appears to play a role in the stabilization and accumulation of p53 given that PARP^{-/-} mice already have markedly reduced levels of p53 protein compared to PARP^{+/+} animals. The mechanism was investigated by utilizing purified p53 and PARP, and determining that p53 is poly(ADP-ribosyl)ated *in vitro* and this may play a role in the stabilization and accumulation of p53 in **SM** induced apoptosis.
- We have showed that SM induces markers of terminal differentiation and apoptosis in primary human epidermal keratinocytes (NHEK), including the early activation and late cleavage of (PARP) by caspase-3. Primary skin fibroblasts from PARP^{-/-} and PARP^{+/+} animals were derived and exposed them to three different doses of SM. Although PARP is degraded at the higher doses of SM, no apoptotic cleavage fragments were observed, suggesting that the mode of cell death is necrotic.
- The PARP^{+/+} primary fibroblasts did not exhibit caspase activity at any of the SM doses tested. The PARP^{-/-} fibroblasts induced caspase-3 activity in response to SM at all doses,

although most strongly at 300 μ M. Thus, high PARP activity (i.e. drastic NAD/ATP depletion) appears to inhibit apoptosis in primary dermal fibroblasts.

- Cells were exposed to increasing doses of SM and then analyzed for Annexin V positivity by FACS analysis. While only a small percentage of PARP ^{+/+} primary fibroblasts were apoptotic at all doses of SM tested (< 20%), a dose-dependent increase of apoptotic ^{-/-} fibroblasts were observed, up to a maximum of 65%. These results suggested that the absence of PARP shifted the mode of cell death from necrosis to apoptosis.
- To verify if this is the case, we examined the percentage of cells that were positive for propidium iodide (PI) staining, and were also Annexin V negative, indicating a primarily necrotic mode of cell death. The data indicated that PARP ^{-/-} primary fibroblasts underwent a dose-dependent increase in the level of necrosis, while the PARP ^{+/+} fibroblasts underwent little necrosis at all doses tested.
- The new data, obtained in the past period clearly shows that the primary mode of cell death in PARP ^{+/+} cells is necrotic, while PARP ^{+/+} cells undergo apoptosis.
- The PARP ^{+/+} primary fibroblasts did not exhibit caspase activity at any of the SM doses tested. The PARP ^{-/-} induced caspase-3 activity in response to SM at all doses, although most strongly at 300 μ M. Thus, high PARP activity appears to inhibit apoptosis in primary dermal fibroblasts.

REPORTABLE OUTCOMES
(APRIL 1, 1998 – MARCH 31, 2001)
CHAPTERS, ABSTRACTS, PRESENTATIONS, AND MANUSCRIPTS
DIRECTLY RELEVANT TO SPECIFIC AIM 1

Rosenthal, D. S., Simbulan-Rosenthal, C., Liu, W.F., and **Smulson, M.E.** Sulfur mustard induces terminal differentiation and apoptosis in keratinocytes via calmodulin and FADD-dependent pathways. American Association for Cancer Research 91st Annual Meeting, San Francisco, CA (2000).

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Supporting Publication with USAMRICID Acknowledgments

Rosenthal, D. S., Simbulan-Rosenthal, C. M., Liu, W.F., Stoica, B., and **Smulson, M.E.** Mechanisms of JP8 jet fuel toxicity II: induction of necrosis in skin fibroblasts and keratinocytes and modulation of levels of Bcl-2 family members. *Toxicol. Appl. Pharmacol.* 171:107-116 (2001).

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REPORTABLE OUTCOMES
(APRIL 1, 1998 – MARCH 31, 2001)
CHAPTERS, ABSTRACTS, PRESENTATIONS, AND MANUSCRIPTS
DIRECTLY RELEVANT TO SPECIFIC AIM 2

Rosenthal, D. S., Simbulan-Rosenthal, C., Liu, W.F., and **Smulson, M.E.** Sulfur mustard induces terminal differentiation and apoptosis in keratinocytes via calmodulin and FADD-dependent pathways. *New Biosciences*, In Press (2001).

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Supporting Publication with USAMRICD Acknowledgments

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REPORTABLE OUTCOMES
(APRIL 1, 1998 – MARCH 31, 2001)
CHAPTERS, ABSTRACTS, PRESENTATIONS, AND MANUSCRIPTS
DIRECTLY RELEVANT TO SPECIFIC AIM 3

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CONCLUSIONS

The work performed during the last 2.5 years of the current contract has clearly established that anti-Fas induces apoptosis in a variety of skin cells including immortalized human keratinocytes as well keratinocytes (PARP^{+/+}) derived from the PARP knockout mice system. However, a DNA damaging and crosslinking agents such as **SM** appears to provide complex patterns as far as mechanisms for a cell killing and skin pathology; for example, causing necrosis in fibroblasts while, in contrast inducing apoptosis in keratinocytes. Past data using PARP K/O animals, also indicated that SM induces necrosis in PARP^(+/+) primary fibroblasts and also PARP^(-/-), fibroblasts re-transfected with a PARP vector. Other findings, established a requirement for PARP in **SM** induced apoptosis in keratinocytes derived from PARP^(-/-) mice (only when PARP activity was restored). We also exposed newborn PARP^(+/+) and PARP^(-/-) pups to **SM** vapor. All the indications suggest that the dermis from PARP^(+/+) animals are more sensitive to **SM**-induced vesication, probably due to PARP-induced necrosis, but these experiments must be expanded during the next contract period. DNA damaging agents (**SM**) apparently cause necrosis in fibroblasts containing PARP (i.e. due to excessive ATP depletion?).

SIGNIFICANCE OF CONCLUSIONS AND RECOMMENDED FUTURE WORK

Necrosis is undesirable since it may contribute to the inflammatory pathology caused by **SM**. Alternatively, inhibiting PARP by the use of the PARP K/O system or by antisense tranfection causes **SM** to induce apoptosis mainly in keratinocytes. This new data emphasizes how a detailed knowledge of PARP activity in skin may allow development of new modulation strategies for controlling pathology caused by **SM** exposure.

This new paradigm indicates the importance of completing future work (proposed in a new contract proposal, submitted November, 2000) and discussed below with respect to recommended changes on the future work to better address this problem. Accordingly, Specific **Aim I** p.15 of the newly submitted Contract) focuses on follow-through on recent specific data concerning **SM** induction of cell death by apoptosis in keratinocytes and by necrosis of skin fibroblasts includes the following suggested recommended future work).

1. We have proposed to determine the role of PARP in **SM**-induced necrosis and apoptosis and why this response is skin cell-type specific. Here we feel it will be important to further explore the use of the PARP knockout fibroblasts and keratinocytes, both in immortalized as well as primary culture as a major strategy to establish this difference in biochemical susceptibility of skin cells to **SM**.
2. We have earlier established that **SM** at 300 μ M induces apoptosis with a wild-type PARP^(-/-) keratinocytes and apoptosis and PARP^(+/+), nevertheless, we feel is important to determine the "upstream" targets for **SM** in the apoptotic cascade. For example, are caspase-8, 9, 10 also activated by **SM**? It will also be important to establish a role for Fas receptor with the Fas activated death domain (FADD) already initiated in research performed during the current contract period. (i.e. *appended publications B and C*).
3. Given that the mitochondria is a potential target of **SM** toxicity (possibly resulting in the early release of cytochrome-c from the mitochondria into the cytosol) and the subsequent activation of the downstream caspase cascade, it will be important to investigate the biochemical and molecular changes that occur in the mitochondria following exposure to **SM**.
4. Differences in the abundance of the pro- and anti-apoptotic members of the BCL-2 family gene fibroblasts keratinocytes should be examined to ascertain whether they effect responses to **SM**.
5. The reintroduction of PARP cDNA to immortalized PARP^(-/-) fibroblasts causes a shift in the cell death pathway in response to **SM** exposure from apoptosis towards necrosis. It will be

important to determine whether this effect is attributable to PARP activity, PARP protein interactions, specific poly(ADP-ribosyl)ation modification of relevant DNA binding proteins, such as p53 or due to the depletion of NAD/ATP levels, or a combination of all of these.

6. Similar to the burst of poly(ADP-ribosyl)ation at the early stages of Fas-mediated apoptosis noted in our earlier studies, there is also a burst of PARP activity at the early stages of **SM**-induced apoptosis in keratinocytes or during necrotic death in wild type fibroblasts. Given that PARP has been shown to modify specific proteins during apoptosis such as histones, we feel it important to examine how this PAR burst contributes to alter the chromatin and nuclear structure/ultrastructure during early apoptosis, in a new contract funded, [to collaborate as before, in part on electron microscopy of chromatin coupled with PAR antibody staining, with Dr. John Petrali (**USAMRICD**)].

Finally, with regard to future directions, it will be necessary to understand SM pathology in general a major priority of the new contract submitted in November to the **USAMRICD** will involve the use of the PARP knockout K/O system, coupled with Comparative Genomic Hybridization (CGH), and Spectral Karyotyping (SKY) to test the hypothesis that the well-established mutagenesis effects of **SM** on cells, especially those of the skin, are due to deletions and additions of specific chromosomal regions. We propose to test the hypothesis that specific genes within these regions are altered or amplified even further (than those noted already by the PARP K/O genotype due to exposure to **SM**). The addition of SM exposure to the PARP K/O genotype will add a new database which may be useful towards modulating the pathology of SM and we feel that this toxogenetic approach should yield rather important genotoxic and pathological effects after modulation of SM exposure.

Additionally, in the new contract we propose to utilize DNA Array analysis of various skin cells, including those involved in the PARP K/O system, with the general hypothesis that significant additional changes in gene expression will be noted by the additional stress imposed by exposure of cells and animals to vesicating doses of **SM**. Thus based on the recent data obtained by my laboratory with the PARP K/O system and DNA microarray as published in PNAS and listed above under **REPORTABLE OUTCOME**. It is thus anticipated that a detailed molecular knowledge of the changes in protein expression in **SM** exposed tissues and cells will lead to novel methods for preventing the pathology associated with **SM** not only in skin but also perhaps in other organs, such as the lungs. However, the major stress of the future work is mainly focused providing a better understanding of the novel effects on skin cell death elicited by **SM**, as revealed in the last 3 years and summarized in this **Final Report**.

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(Most of relevant references are included in the Appended Publications A-C)

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CURRICULUM VITAE

NAME	POSITION TITLE
MARK E. SMULSON	Professor of Biochemistry and Molecular Biology and Radiation Biology (Joint Appointment), Co-Director Radiation Biology Program, Lombardi Cancer Center

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Washington & Lee University, Lexington, VA	A.B.	1958	Chemistry
Cornell University, Ithaca, NY	M.S.	1961	Nutrition
Cornell University, NY	Ph.D.	1964	Biochemistry
Einstein Medical Center, Philadelphia, PA	Post-doc	1965-65	Nucleoside Analogs

PROFESSIONAL EXPERIENCE

1967-1972; 1972-1978 Assistant Professor; Associate Professor of Biochemistry, Georgetown University Medical School, Professor of Biochemistry

1978 - present Professor of Biochemistry and Molecular Biology, Georgetown University

1989 - present Lombardi Cancer Center, Co-Director, Program of Radiation Biology

SPECIAL RECOGNITION

- Awarded First Annual Dean's Prize for Biomedical Research, 1988
- Meeting Organizer International Meeting on Poly(ADP-Rib), 6/73 at NIH.
- Organizer Princess Takamatsu Cancer Meeting, Tokyo, Japan (9/82).
- Organizer, session of Winter Gordon Conference on DNA repair, February 1991.

AUTHORSHIP OF BOOKS AND MONOGRAPHS

Smulson, M.E. and Sugimura, T., eds, *Novel ADP-Ribosylations of Regulatory Enzymes and Proteins, Developments in Cell Biology*, Vol. 6, Elsevier/North Holland, 1980.

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Simbulan-Rosenthal, C., Rosenthal, D. S., Iyer, S., Boulares, H., and **Smulson, M.E.** Requirement for a transient poly(ADP-ribosyl)ation of p53 and other nuclear proteins at the early stages of apoptosis. Proc. American Association for Cancer Research 40: 222, 90th Annual Meeting, Philadelphia, PA (1999).

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Appended Publication A

(Rosenthal D. S., Simbulan-Rosenthal C. M., Iyer S., Spoonde A, Smith W, Ray R, Smulson M. E. Sulfur mustard induces markers of terminal differentiation and apoptosis in keratinocytes via a Ca²⁺-calmodulin and caspase-dependent pathway. *J Invest Dermatol.* Jul;111(1):64-71. (1998).

Sulfur Mustard Induces Markers of Terminal Differentiation and Apoptosis in Keratinocytes Via a Ca^{2+} -Calmodulin and Caspase-Dependent Pathway

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Sulfur mustard (SM) induces vesication via poorly understood pathways. The blisters that are formed result primarily from the detachment of the epidermis from the dermis at the level of the basement membrane. In addition, there is toxicity to the basal cells, although no careful study has been performed to determine the precise mode of cell death biochemically. We describe here two potential mechanisms by which SM causes basal cell death and detachment: namely, induction of terminal differentiation and apoptosis. In the presence of 100 μM SM, terminal differentiation was rapidly induced in primary human keratinocytes that included the expression of the differentiation-specific markers K1 and K10 and the cross-linking of the cornified envelope precursor protein involucrin. The expression of the attachment protein, fibronectin, was also reduced in a time- and dose-dependent fashion. Features common to both differentiation and apoptosis were also induced in 100 μM SM, including the

rapid induction of p53 and the reduction of Bcl-2. At higher concentrations of SM (i.e., 300 μM), formation of the characteristic nucleosome-sized DNA ladders, TUNEL-positive staining of cells, activation of the cysteine protease caspase-3/apopain, and cleavage of the death substrate poly(ADP-ribose) polymerase, were observed both *in vivo* and *in vitro*. Both the differentiation and the apoptotic processes appeared to be calmodulin dependent, because the calmodulin inhibitor W-7 blocked the expression of the differentiation-specific markers, as well as the apoptotic response, in a concentration-dependent fashion. In addition, the intracellular Ca^{2+} chelator, BAPTA-AM, blocked the differentiation response and attenuated the apoptotic response. These results suggest a strategy for designing inhibitors of SM vesication via the Ca^{2+} -calmodulin or caspase-3/PARP pathway. **Key words:** BAPTA/caspase-3/poly(ADP-ribose) polymerase/W-7. *J Invest Dermatol* 111:64-71, 1998

Sulfur mustard [bis-(2-chloroethyl) sulfide; SM] causes blisters in the skin via poorly understood mechanisms. Because SM is a strong alkylating agent, its ability to induce DNA damage via apurinic sites and endonucleolytic activation has been advanced as one possible pathway leading to vesication (Papirmeister *et al*, 1985). Similar to other agents that induce DNA strand breakage, SM activates the nuclear protein poly(ADP-ribose) polymerase (PARP), which drastically depletes levels of cellular nicotinamide adenine dinucleotide and adenosinetriphosphate (Wielckens *et al*, 1982; Alvarez *et al*, 1986), a mechanism proposed to induce cell death (Berger *et al*, 1983). We have recently examined this mechanism using a human skin graft derived from human keratinocytes stably transfected with a PARP anti-sense inducible vector (Rosenthal *et al*, 1995). Recent studies have further implicated PARP as an important player in apoptosis. Proteolytic cleavage of PARP was first demonstrated in

chemotherapy-induced apoptosis (Kaufmann *et al*, 1993) and the specific proteolysis of PARP is now closely associated with apoptosis in different systems (Neamati *et al*, 1995; Nicholson *et al*, 1995; Tewari *et al*, 1995). We recently showed that the reversible stage of apoptosis is characterized by the transient activation of PARP, and poly(ADP-ribosylation of nuclear proteins followed by the breakdown of poly(ADP-ribose) and PARP (Rosenthal *et al*, 1997b).

Ca^{2+} also plays an important role in apoptosis, as well as in the maintenance and homeostasis of the skin, and SM has been shown to elevate intracellular levels of Ca^{2+} in keratinocytes (Ray *et al*, 1995; Mol and Smith, 1996). Several laboratories, including our own, have shown that terminal differentiation can be induced in both murine and human keratinocytes by the elevation of extracellular Ca^{2+} (Hennings *et al*, 1980; Stanley and Yuspa, 1983; Rosenthal *et al*, 1991b). This in turn results in an increase in intracellular free Ca^{2+} (Ca_i). Ca_i appears to be an important signal for terminal differentiation, because agents that chelate and buffer Ca_i can block markers of terminal differentiation (Li *et al*, 1995). Ca^{2+} has also been shown to play a role in apoptosis in a number of systems. Studies by Kaiser and Edelman (1977) showed the first evidence that Ca_i may trigger apoptosis in glucocorticoid-stimulated thymocytes. Since then, several other studies have confirmed this role for Ca^{2+} . The observed 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 IP₃, and Ca^{2+}

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Abbreviations: Ca_i , intracellular free calcium; NHEK, normal human epidermal keratinocytes; PARP, poly(ADP-ribose) polymerase, SM, sulfur mustard.

mobilization (Takata *et al*, 1995; Silvennoinen *et al*, 1996). The second pathway involves oxidative stress, which can occur in response to cytotoxic agents, such as SM, that generate reactive oxygen species. Oxygen radicals can damage Ca^{2+} transport systems localized in the endoplasmic reticulum (ER), mitochondria, and plasma membrane, leading to a disruption in Ca^{2+} homeostasis and a sustained increase in Ca^{2+} (Orrenius *et al*, 1989).

Ca^{2+} -buffering experiments have supported the role of Ca^{2+} in the etiology of SM-induced cytotoxicity (Ray *et al*, 1996). Experiments utilizing specific inhibitors of calmodulin have also demonstrated the importance of Ca^{2+} -calmodulin complexes in programmed cell death. Cyclosporine A-sensitivity of apoptosis in certain systems also suggests a role for Ca^{2+} -calmodulin complexes in programmed cell death. Cyclosporine 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 Ca^{2+} -calmodulin complexes in programmed cell death (Shi *et al*, 1989). Ca^{2+} also plays a role in the induction of the endonuclease responsible for the internucleosomal DNA cleavage, yielding the characteristic apoptotic DNA ladders (Shiokawa *et al*, 1994).

Numerous recent studies have suggested that the ultimate 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). In collaboration with others, our laboratory has been particularly active in studying the characterization of caspase-3 (also known as "apopain"), which appears to be a converging point for different apoptotic pathways (Nicholson *et al*, 1995). In several apoptotic systems, caspase-3 cleaves key proteins involved in the structure and integrity of the cell, including PARP (Nicholson *et al*, 1995; Tewari *et al*, 1995; Casciola-Rosen *et al*, 1996; Song *et al*, 1996).

In this study, we show that SM induces both terminal differentiation and apoptosis in human keratinocytes. Further, we demonstrate that these processes are Ca^{2+} and/or calmodulin dependent, and involve the activation of caspase-3 as well as the activation and specific cleavage of PARP. These responses may, in part, explain the death and detachment of basal cells of the epidermis that occurs following exposure to SM.

MATERIALS AND METHODS

Cells Normal human epidermal keratinocytes (NHEK) were obtained as primary cultures from Clonetics (San Diego, CA) and maintained in serum-free keratinocyte growth medium. NHEK were grown in 75 cm^2 tissue culture flasks to 60–80% confluency, then exposed to SM diluted in keratinocyte growth medium to final concentrations of 100 μM or 300 μM . Media was not changed for the duration of the experiments. Cell viability was measured by the ability of cells to exclude trypan blue. For all studies, similar results were obtained from three independent experiments utilizing human primary adult keratinocytes and from three independent experiments utilizing human primary neonatal keratinocytes.

Chemicals SM (>98% purity) was obtained from the US Army Edgewood Research, Development and Engineering Center. Glycine, N,N'-[1,2-ethane-diylbis(oxy-2,1-phenylene)]bis[N-[2-(acetoxy)methoxy]-2-oxoethyl]-bis[acetoxy)methyl]ester (BAPTA-AM) was purchased from Molecular Probes (Eugene, OR), as was Pluronic F-127, used to facilitate loading of BAPTA-AM into cells. N-(6-Aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7) was obtained from Sigma (St. Louis, MO). The tetrapeptide aldehyde inhibitor of caspase-3 (AcDEVD-CHO) was obtained from Biomol (Plymouth Meeting, PA).

Antibodies

Immunoblotting The following antibodies were obtained from Sigma: (i) mouse monoclonal antibody (clone 8.60) that recognizes both K1 and K10 keratins; (ii) monoclonal antibody (clone SY5) against the 68 kDa cornified envelope precursor, involucrin; (iii) a mixture of three monoclonal antibodies (clones 2D1, 1F11, and 6D4), recognizing a 17 kDa band corresponding to calmodulin; and (iv) affinity purified rabbit anti-serum against the attachment protein, fibronectin, recognizing both a 220 kDa and a 94 kDa form of the protein. Rabbit affinity-purified anti-peptide anti-sera specific for human K1 (AF87) was from Babco (Richmond, CA). Mouse monoclonal antibody (clone DO-1) recognizing human p53 is from Calbiochem (Cambridge, MA). Monoclonal

anti-human Bcl-2 antibody (clone 4D7) is from Biomol. Rabbit anti-peptide anti-sera specific for the N-terminal propeptide sequence of pro-caspase-3/apopain (CPP32) was obtained from Transduction Labs (Lexington, KY). Rabbit anti-sera against the p17 subunit of CPP32 was obtained from Donald Nicholson (Merck Frosst Center for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada). Rabbit anti-sera recognizing both full-length PARP, as well as the 89 kDa apoptotic cleavage product of PARP, was a kind gift from Eric Ackerman (Pacific NW National Laboratories, Richland, WA). Guinea pig anti-sera specific for poly(ADP-ribose) has been described (Rosenthal *et al*, 1995).

Fluorescence-activated cell sorter analysis FITC-conjugated anti-human cytokeratin ("CK") antibody (MNFI16), recognizing keratins 10, 17, and 18, was from DAKO (Carpinteria, CA).

Immunoblot analysis For immunoblot analysis, sodium dodecyl sulfate-polyacrylamide gel electrophoresis-separated proteins were transferred to nitro-cellulose filters. Proteins were measured (DCA protein assay; BioRad), and normalized prior to gel-loading, and all filters were stained with Ponceau-S, in order to reduce the possibility of loading artifacts. The details for using rabbit anti-serum to human PARP (Ding *et al*, 1992) and guinea pig anti-serum to poly(ADP-ribose) (Rosenthal *et al*, 1995) for immunoblot analysis have been described previously in detail. Immune complexes were visualized by electrochemiluminescence (Amersham Life Sciences, Arlington Heights, IL).

Fluorescence-activated cell sorter analysis At the designated time points, monolayer culture medium was decanted, trypsin-ethylenediamine tetraacetic acid was added for 5 min, and the cells were removed from the flasks by scraping. The cell suspension was mixed with trypsin neutralizing solution, washed in keratinocyte growth medium, and fixed with 1% formaldehyde for 15 min followed by 70% ethanol. Fixed cells were stored at -200°C until stained for cytometry. Flow cytometric analyses were conducted on a Becton-Dickinson (Franklin Lakes, NJ) FACStar Plus cytometer using a 100 mW air-cooled argon laser at 488 nm.

PARP cleavage assay The full-length cDNA clone for PARP (pcD-12) (Alkhatib *et al*, 1987) was excised and ligated into the XhoI site of pBluescript-II SK+ (Stratagene, La Jolla, CA), and then used to drive the synthesis of PARP labeled with [^{35}S]methionine (Dupont-NEN, Wilmington, DE) by coupled T7 transcription/translation in a reticulocyte lysate system (Promega, Madison, WI). [^{35}S]PARP was separated from the other constituents by gel filtration chromatography on a Superdex-75 FPLC column (Pharmacia, Piscataway, NJ; 1×30 cm) in 10 mM HEPES-KOH (pH 7.4), 2 mM ethylenediamine tetraacetic acid, 0.1% (wt/vol) CHAPS, and 5 mM dithiothreitol.

Cytosolic extracts were prepared from NHEK by scraping phosphate-buffered saline-washed monolayers in 10 mM HEPES/KOH (pH 7.4), 2 mM ethylenediamine tetraacetic acid, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 10 μg pepstatin A per ml, 20 μg leupeptin per ml, and 10 μg aprotinin per ml (at 1×10^8 cells per ml). The post-100,000 $\times g$ supernatant was recovered after centrifugation.

PARP cleavage activity was measured in mixtures containing 5 μg protein from the cytosol fractions of keratinocytes. Assay mixtures also contained purified [^{35}S]PARP ($\approx 5 \times 10^4$ cpm), 50 mM PIPES-KOH, 2 mM ethylenediamine tetraacetic acid, 0.1% (wt/vol) CHAPS, and 5 mM dithiothreitol in a total volume of 25 μl . Incubations were performed at 37°C for 1 h, and terminated by the addition of 25 μl of 2 $\times$ sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer containing 4% sodium dodecyl sulfate, 4% β -mercaptoethanol, 10% glycerol, 0.125 M Tris-HCl (pH 6.8), and 0.02% bromophenol blue. Samples were resolved by 10% sodium dodecyl sulfate-polyacrylamide gels.

PARP cleavage products were visualized either by fluorography, or else the 89 kDa cleavage product of [^{35}S]PARP was quantitated relative to the full-length PARP using a Storm 840 PhosphorImage analyzer (Molecular Dynamics, Sunnyvale, CA). Quantitation included a correction for background, as well as for the difference in methionine residues present in the 89 kDa fragment (18 met residues) versus full-length PARP (25 met residues).

DNA isolation Cells were lysed for 2 h in 10 mM Tris-Cl pH 7.5, 10 mM ethylenediamine tetraacetic acid, 0.5% sodium dodecyl sulfate, containing 144 μg proteinase K per ml, and 500 μg RNase A per ml (Boehringer Mannheim, Indianapolis, IN). Lysates were extracted twice with phenol:chloroform (1:1), and precipitated by the addition of ethanol to 70%. Precipitates were resuspended in distilled water. Glycerol was added to 10% and DNA was resolved on 1% agarose gels and visualized by ethidium bromide staining.

[^{35}S]Methionine labeling To measure total protein synthesis, cells were pulse-labeled with [^{35}S]methionine (10 μCi per ml; Dupont-NEN) for 1 h.

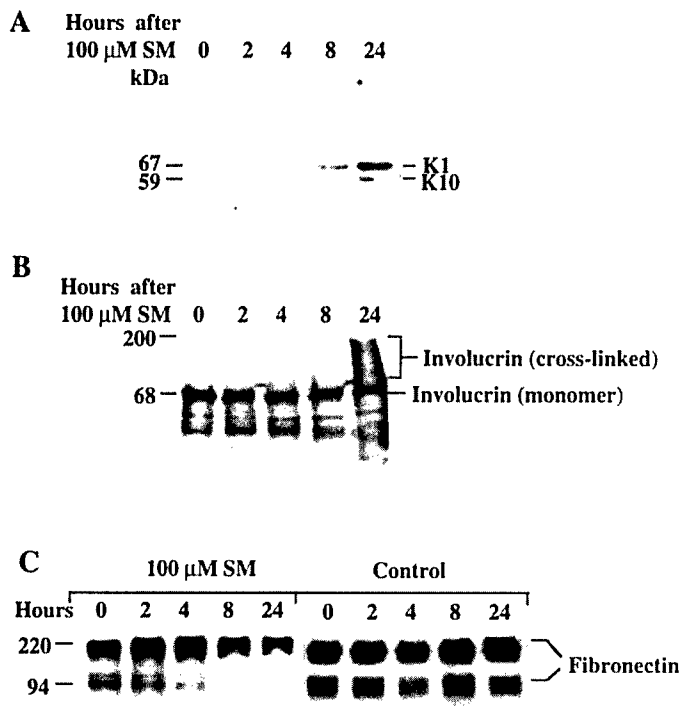


Figure 1. Modulation of differentiation markers and attachment proteins by SM. NHEK were treated with 100 μ M SM for the indicated times, harvested, and total cell extracts were immunoblotted using antibodies specific for K1 + K10 (A), involucrin (B), or fibronectin (C). For all studies, similar results were obtained from three independent experiments utilizing human primary adult keratinocytes and three independent experiments utilizing human primary neonatal keratinocytes.

Cells were then washed twice with phosphate-buffered saline, and harvested. Cells were lysed in 10% trichloroacetic acid, and the precipitated protein was collected on glass filters. Filters were washed successively with 10% trichloroacetic acid, 70% ethanol, and absolute ethanol, and dried. Protein-incorporated [^{35} S]methionine on filters was measured by scintillation spectroscopy.

RESULTS

SM induces markers of terminal differentiation in both primary and immortalized keratinocytes To determine if SM altered keratin expression, NHEK were exposed to 100 μ M SM, fixed after 24 h, and then subjected to fluorescence-activated cell sorter analysis, using the broad-range-reactive cytokeratin (CK) antibody as a tag. Following SM exposure, the number of CK+ cells increased significantly (data not shown). Because the CK antibody recognizes the suprabasal keratin K10, we were curious to determine if SM altered the expression of any differentiation-specific proteins. Immunoblot analysis with specific anti-sera revealed that both K1 and K10 were induced in the presence of 100 μ M SM (Fig 1A). The viability of NHEK throughout the time course was greater than 90% as measured by dye exclusion. We also examined the expression of involucrin, a precursor protein that becomes cross-linked in the fully differentiated cornified envelope (Yaffe *et al*, 1993; Robinson *et al*, 1996; Steinert and Marekov, 1997). In extracts derived from untreated cells, involucrin migrated as a 68 kDa monomer form. Following 24 h exposure to SM, the staining pattern shifted to higher molecular weight forms (Fig 1B), suggesting that the protein is cross-linked in response to SM.

We next examined the levels of fibronectin expressed in NHEK following SM treatment, for two reasons. First, fibronectin is expressed in basal cells, but is suppressed in suprabasal cells *in vivo*, and in response to differentiating agents *in vitro* (Adams and Watt, 1990; Nicholson and Watt, 1991). In turn, contact with fibronectin also inhibits keratinocyte differentiation (Staiano-Coico and Higgins, 1992; Drozdoff and Pledger, 1993; Watt *et al*, 1993). Second, fibronectin is

a major component of the basal lamina, and forms an attachment site for the α 5 β 1 integrin of the basal cells (Adams and Watt, 1990). Thus, suppression of this protein by SM could in part explain the detachment of basal cells from the basal lamina during vesication *in vivo*. Fibronectin is produced in keratinocytes (as well as fibroblasts), in two isoforms. Both the 220 kDa and the 94 kDa forms of fibronectin were reduced with time after SM exposure. In contrast, untreated NHEK showed no decrease in the levels of fibronectin (Fig 1C).

Because previous studies have shown that SM can induce an increase in Ca_i (Ray *et al*, 1995), and because we have observed that a rise in Ca_i is associated with the normal terminal differentiation response of keratinocytes (Yuspa *et al*, 1989; Rosenthal *et al*, 1991), the expression of these markers suggested a role for Ca^{2+} in the SM response that was observed. Furthermore, we have shown that the K1 gene contains specific Ca^{2+} -inducible enhancer sequences located 3' to the gene (Huff *et al*, 1993) and expression of K1 (and other differentiation-specific genes) can be blocked by the Ca_i chelator BAPTA (Li *et al*, 1995). BAPTA also enhances the survival of keratinocytes in the presence of SM (Ray *et al*, 1996). We therefore preincubated keratinocytes with 20 μ M BAPTA-AM (+ Pluronic F-127; see *Materials and Methods*) for 30 min prior to SM treatment. When NHEK were subsequently treated for 24 h with 100 μ M SM, keratin K1 was suppressed (Fig 2A). Although BAPTA treatment suppressed total protein synthesis by 50% after 24 h (not shown), this effect was not enough to account for the complete suppression of K1. Ca^{2+} may induce differentiation via its role in the activation of protein kinase C (Dlugosz and Yuspa, 1993). In addition, Ca^{2+} -calmodulin complexes are also generated that modulate this protein kinase C response (Chakravarthy *et al*, 1995). We therefore determined whether the calmodulin inhibitor W-7 could alter the differentiation response to SM. Figure 2(A) shows that a 30 min pretreatment with W-7 prior to exposure to 100 μ M SM inhibited the expression of K1. Protein calibration prior to gel-loading, followed by Ponceau-S-staining of the immunoblot (Fig 2B), eliminated the possibility of loading artifact, indicating that SM induces the expression of this differentiation-specific marker via a Ca^{2+} -calmodulin-dependent pathway. Interestingly, calmodulin itself was downregulated by SM (Fig 2C).

SM suppresses Bcl-2 and induces p53 To examine possible mechanisms by which SM altered the differentiation response, we initially examined the expression of p53, which has been postulated to play important roles in both the differentiation and the apoptotic responses. Immunoblot analysis showed a significant increase in the protein levels of p53 after exposure to 100 μ M SM, and that this increase in p53 levels occurs within 2 h (Fig 3A).

We also examined the levels of the *bcl-2* gene product, which inhibits both keratinocyte differentiation and apoptosis. Bcl-2 levels are high in basal keratinocytes and are reduced in the differentiating layers of the epidermis (Hockenberry *et al*, 1991). Furthermore, expression of *bcl-2* anti-sense RNA can lower endogenous levels of Bcl-2 and induce markers of terminal differentiation in mouse keratinocytes (Marthinuss *et al*, 1995). Following SM treatment, there is a time-dependent decrease in Bcl-2 protein levels in NHEK as determined by immunoblot analysis (Fig 3B).

SM induces apoptosis via caspase-3 The striking decrease in Bcl-2 levels and the increase in p53 levels suggested that in addition to modulating differentiation, SM may induce apoptosis as well. Thus, we assayed for markers of apoptosis following SM treatment. A hallmark of apoptosis in several cell types is the appearance of nucleosome-sized ladders due to the presence of a $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonuclease that is induced in apoptotic cells. DNA isolated from NHEK treated with 0 or 100 μ M SM was intact; however, at 300 μ M SM, NHEK showed nucleosome-sized ladders analyzed by agarose gel electrophoresis, although some nonspecific fragmentation was also apparent (Fig 4). Accordingly, trypan blue exclusion at 24 h was 98% in control cells, 90% following 100 μ M SM treatment, and 60% after 300 μ M SM (not shown). W-7 suppressed DNA fragmentation, whereas BAPTA partially inhibited DNA cleavage (see below).

We have recently determined that the activation of PARP plays a role in the etiology of apoptosis in osteosarcoma cells induced to

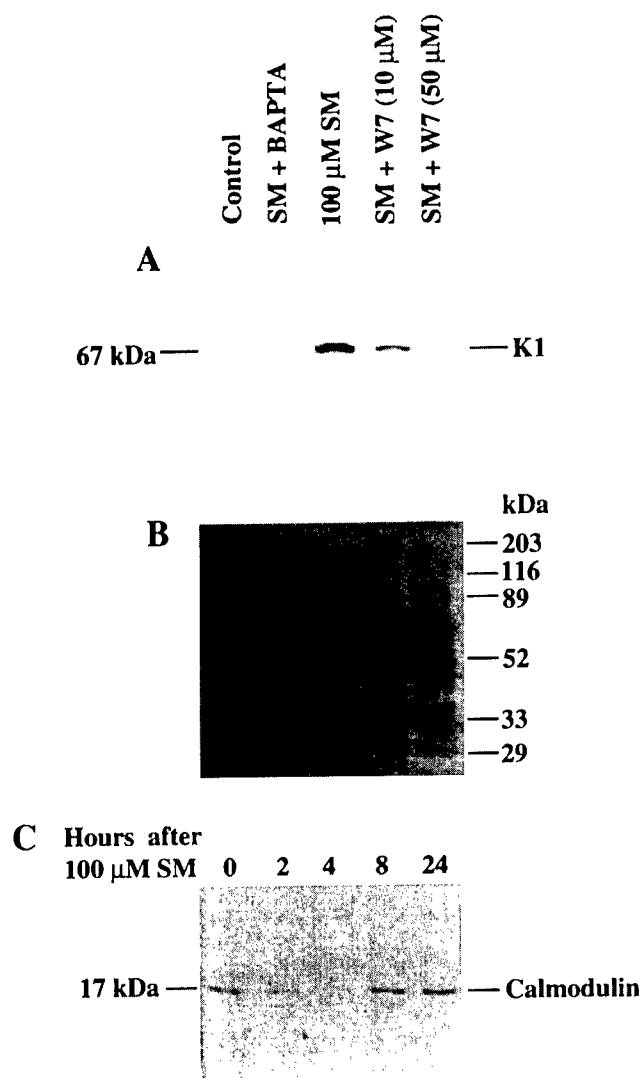


Figure 2. Ca^{2+} and calmodulin are required for the induction of markers of terminal differentiation by SM. NHEK were treated with SM as above, either with or without pretreatment with 20 μ M BAPTA-AM or W-7 (A). Cell extracts were immunoblotted using antibodies specific for K1 (A), or calmodulin (C). (B) Ponceau-S stain of total cell protein on nitrocellulose prior to immunostaining shown in (A).

undergo programmed cell death, in which poly(ADP-ribosylation) corresponds with the early reversible stages of apoptosis (Rosenthal *et al.*, 1997b). We therefore determined whether we could detect increased levels of poly(ADP-ribosylation) following exposure of NHEK to SM. Because PARP itself is the main acceptor protein for poly(ADP-ribosylation), via intermolecular "automodification" (Mendoza-Alvarez and Alvarez-Gonzalez, 1993), the presence of a 116 kDa antipoly(ADP-ribose)-crossreactive band is a sensitive indicator of poly(ADP-ribosylation) within the nucleus. Anti-sera specific for poly(ADP-ribose) did in fact detect a strong band at 116 kDa in extracts of primary keratinocytes treated with all concentrations of SM tested, whereas no such band was present in extracts of control keratinocytes, indicating that SM induces DNA strand breaks and PARP is activated. **Figure 5(A)** shows that, at 100 μ M SM, activation of PARP occurs and automodification is apparent by 2 h. The molecular weight of automodified PARP was similar to that of unmodified PARP (116 kDa), indicating that the average polymer length is relatively short at this time point. After this time, the level of poly(ADP-ribose) decreases precipitously, similar to our previous observations using osteosarcoma cells induced to undergo apoptosis (Rosenthal *et al.*, 1997b).

We previously determined that this characteristic rise and rapid

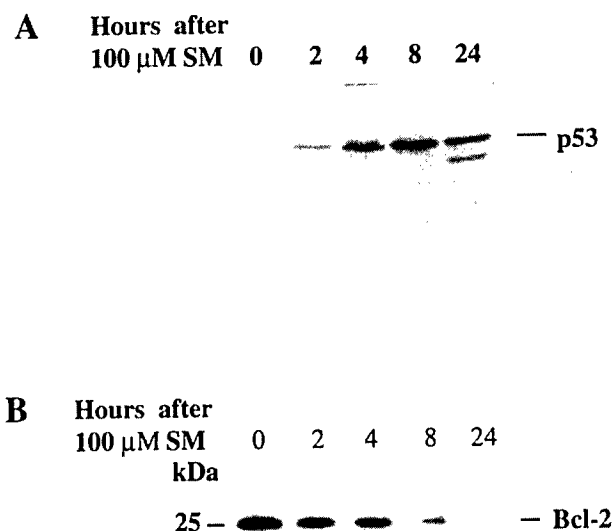


Figure 3. SM induces an increase in p53 levels, and a decrease in Bcl-2. NHEK were treated with 100 μ M SM for the indicated times. Total cell extracts were derived, resolved by polyacrylamide gel electrophoresis, and subjected to immunoblot analysis using anti-sera specific for p53 (A) or Bcl-2 (B).

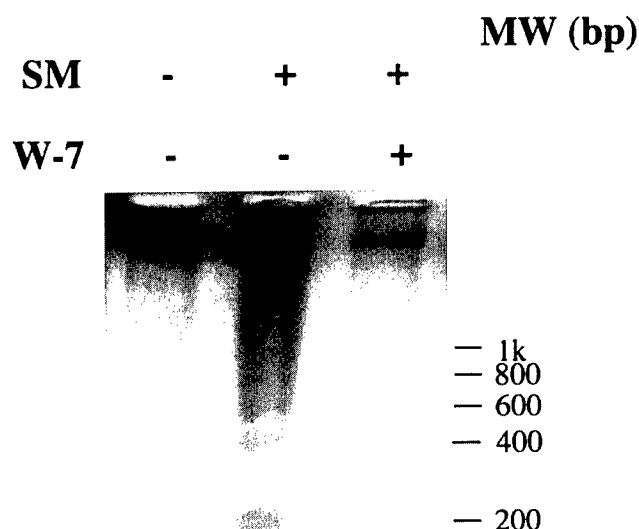


Figure 4. SM treatment induces DNA fragmentation in keratinocytes. NHEK were treated with 100 μ M or 300 μ M SM, with or without W-7 or BAPTA. Total genomic DNA was isolated, resolved by agarose gel electrophoresis, and stained with ethidium bromide.

decline in poly(ADP-ribose) levels could be attributed not only to poly(ADP-ribose) glycohydrolase activity (Wielckens *et al.*, 1983), but also to the proteolytic cleavage of PARP into the characteristic 89 kDa and 24 kDa fragments, the latter of which contains the Zn^{2+} finger region and DNA-binding domain. We therefore performed western analysis to monitor the cleavage of PARP using an antibody that recognizes both the full-length 116 kDa protein as well as the 89 kDa fragment of PARP. **Figure 5(B)** shows a significant conversion of full-length PARP to the 89 kDa fragment following 300 μ M SM treatment.

We have previously described an ICE-like protease similar to the Ced-3 protein of *C. elegans* and closely associated with apoptosis (Nicholson *et al.*, 1995), now known as "caspase-3" (Alnemri *et al.*, 1996). Accordingly, a sensitive technique to verify that SM induces apoptosis is to determine the activation of caspase-3 from its precursor (pro-caspase-3; CPP32) via the use of *in vitro* translated PARP. We therefore used a combination transcription/translation system to radiolabel full-length PARP (see *Materials and Methods*) that was subsequently incubated with extracts derived from keratinocytes treated

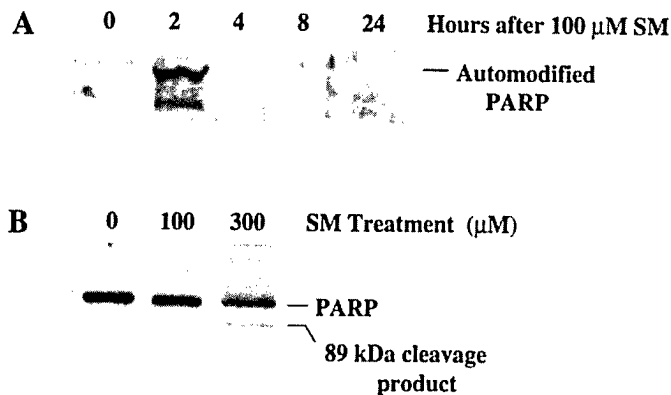


Figure 5. SM treatment induces *in vivo* PARP activation and cleavage in keratinocytes. NHEK were treated with 100 μ M (A, B) or 300 μ M (B) SM. Total cell extracts were derived, resolved by polyacrylamide gel electrophoresis, and subjected to immunoblot analysis using anti-sera specific for poly(ADP-ribose) (A), or for both the full-length and the 89 kDa cleavage product of PARP (B).

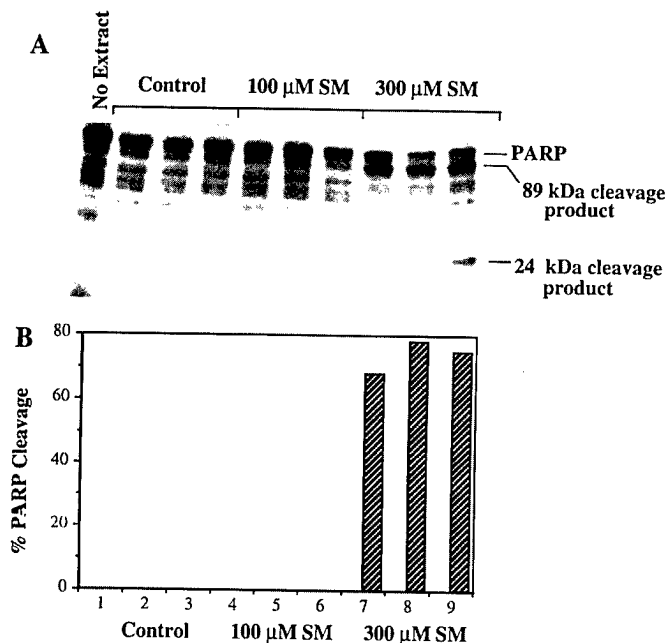


Figure 6. Extracts of keratinocytes treated with SM show *in vitro* PARP-cleavage activity. Triplicate cultures of NHEK were treated with 100 μ M or 300 μ M SM. Cytoplasmic extracts were then derived after 24 h and assayed for caspase-3 activity, using [^{35} S]PARP as a substrate (Materials and Methods). (B) Quantitation of PARP-cleavage activity by phosphorimage analysis.

with SM. **Figure 6(A)** shows that PARP cleavage activity is clearly seen in NHEK in 300 μ M SM (but not 100 μ M SM) after 24 h, as evidenced by the strong appearance of the 24 kDa and 89 kDa cleavage products. **Figure 6(B)** shows, by quantitative phosphorimage analysis, the relative PARP cleavage activities that result from the treatment of NHEK with SM for 24 h. The high level of PARP-cleavage activity observed in 300 μ M SM is indicative that this vesicant is also a strong inducer of apoptosis in primary keratinocytes, and that apoptosis is occurring via a caspase-3-like pathway.

We next further verified that SM induces apoptosis by determining whether the observed caspase-3 activity *in vitro* could be associated with the processing of pro-caspase-3/CPP32 into its active protease form. During apoptosis, procaspase-3/CPP32 is processed into 17 kDa and 12 kDa peptides, with the removal of a pro-peptide sequence from the N-terminus. The 17 kDa and 12 kDa fragments then form the active proteolytic heterodimer. Using an antibody that recognizes both the active (p17) and the inactive (CPP32) forms of caspase-3, a

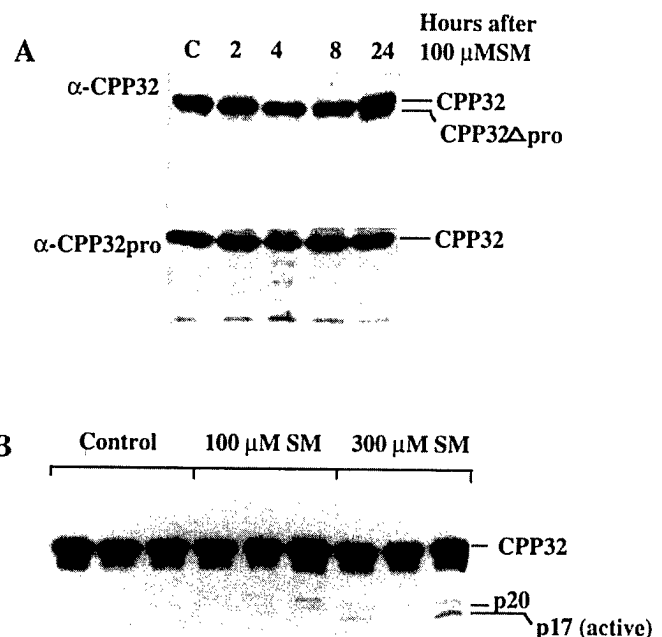


Figure 7. SM induces processing of procaspase-3/CPP32 to its active form. Triplicate cultures of NHEK were treated with 100 μ M or 300 μ M SM for the indicated times (A), or for 24 h (B). Total cell extracts were derived, resolved by polyacrylamide gel electrophoresis, and subjected to immunoblot analysis using anti-sera specific for the p17 subunit (A, top; B), or the N-terminal pro-peptide sequence (A, bottom) of caspase-3.

slightly smaller form of CPP32 was observed 24 h after the cells were exposed to 100 μ M SM, equivalent in size to CPP32 minus the pro-peptide sequence, suggesting that processing of the N-terminus of the precursor protein was occurring (**Fig 7A, top**). To confirm this, the same extracts were analyzed utilizing an antibody that is specific for the pro-sequence that is removed when caspase-3 is processed into its active form. The disappearance of the slightly smaller MW band previously observed at 24 h in 100 μ M SM (**Fig 7A, bottom**), indicates that this band is missing the pro-peptide sequence, and is thus the result of CPP32 N-terminal processing. Following treatment with 300 μ M SM, NHEK showed complete processing of a portion of CPP32 into the active p17 form (**Fig 7B**). A small amount of the partially processed p20, which represents p17 and the N-terminal pro-sequence, is also observed. Thus, in both 100 μ M and 300 μ M SM, markers of apoptosis are induced, although complete activation of caspase-3, PARP cleavage, and DNA fragmentation are only observed at the higher concentration of SM.

A Ca^{2+} calmodulin-dependent pathway for SM-induced apoptosis To determine if SM-induced apoptosis was proceeding via Ca^{2+} -calmodulin dependent pathways, BAPTA and W-7 were utilized as pretreatment agents. BAPTA had a small effect on *in vitro* PARP-cleavage activity, whereas greater suppression was observed following W-7 pretreatment (**Fig 8A**). W-7 also suppressed the level of DNA fragmentation (**Fig 4**).

We next determined whether this W-7-sensitive repression was related to the processing of CPP32 into its active form. In control NHEK, one of the two subunits of the active form of apopain, p17, can clearly be detected 24 h following treatment with 300 μ M SM; however, p17 is completely suppressed by 50 μ M W-7 (**Fig 8B**). Thus, SM induces apoptosis via a calmodulin-dependent pathway that involves the activation of caspase-3.

DISCUSSION

SM vesication clearly involves both cytotoxicity and detachment of the epidermal basal layer *in vivo*. Using a cell culture model in this

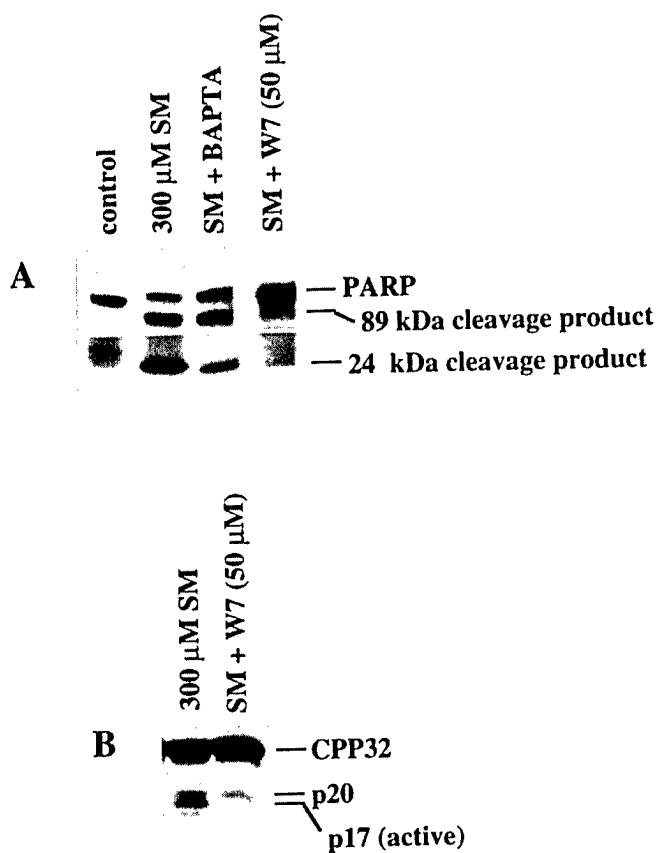


Figure 8. Caspase-3 activity and processing induced by SM is suppressed by inhibitors of Ca_i and calmodulin. Cells were treated with 300 μM SM for 24 h with or without a 30 min pretreatment with BAPTA-AM or W-7. Cytoplasmic extracts were then derived and assayed for caspase-3 activity, using [^{35}S]PARP as a substrate. (B) Extracts of cells treated with 300 μM SM, plus a 30 min W-7 pretreatment, were resolved by polyacrylamide gel electrophoresis, and subjected to immunoblot analysis using anti-sera specific for p17.

study, we have described two potential mechanisms for SM-induced keratinocyte basal cell death and detachment: induction of terminal differentiation and apoptosis. SM induced the differentiation-specific markers K1 and K10, cross-linking of the cornified envelope precursor protein involucrin, and suppressed fibronectin. SM also induced markers of apoptosis in NHEK, including the transient elevation of poly(ADP-ribose), the processing of CPP32 into caspase-3, and the specific cleavage of PARP both *in vivo* and in cell-free PARP-cleavage assays. Differentiation markers were suppressed by the intracellular Ca^{2+} -chelator BAPTA-AM, and the calmodulin inhibitor W-7, whereas markers of apoptosis were suppressed by W-7. Thus, both the differentiation and the apoptotic responses appear to be Ca^{2+} -calmodulin dependent.

Because SM induces an increase in Ca_i (Ray *et al.*, 1995; Mol and Smith, 1996), it seems likely that this elevation in Ca_i accounts for the induction of the markers of terminal differentiation observed in this study, because terminal differentiation of keratinocytes is closely associated with an elevation in Ca^{2+} (Hennings *et al.*, 1980; Stanley and Yuspa, 1983; Kruszewski *et al.*, 1991a, b; Rosenthal *et al.*, 1991; Li *et al.*, 1995). The suppression of these markers by BAPTA also lends support to the idea that the rise in Ca_i is an important event in SM-induced expression of differentiation markers (Fig 2). How SM induces a rise in Ca_i is unknown, but may stem from its ability to alkylate several molecules within the cell, including glutathione (Gross *et al.*, 1993) or other molecules involved in cellular homeostasis, ultimately resulting in disruptions of plasma, ER, and mitochondrial membranes. Disruption of these membranes could easily lead to perturbations in Ca_i within the cell.

The role of SM in the induction of apoptosis may also involve Ca_i and/or Ca^{2+} -calmodulin complexes, because these molecules have been shown to be involved in apoptosis in other systems (Kaiser and Edelman, 1977; Shi *et al.*, 1989). The suppression of apoptotic markers by the Ca_i buffer BAPTA and the calmodulin inhibitor W-7 in this study supports this idea. It is also likely that DNA strand breaks and the activation of PARP play a role in the SM-induced expression of apoptotic markers in keratinocytes. SM is a strong alkylating agent with a high affinity for DNA, and has been shown to induce DNA strand breaks and consequently activate PARP. PARP inhibitors can extend the lifespan of lymphocytes treated with SM (Meier and Johnson, 1992; Meier, 1996), and inhibitors of PARP have previously been reported to significantly affect the extent of apoptosis in response to other agents (Rice *et al.*, 1992; Ghibelli *et al.*, 1994; Monti *et al.*, 1994; Kuo *et al.*, 1996). PARP may thus be an important signaling molecule for cell death either via the lowering of NAD and/or ATP levels (Wielckens *et al.*, 1982; Berger *et al.*, 1983; Alvarez *et al.*, 1986), or by poly(ADP-ribosylation) of other key cellular proteins involved in apoptosis, such as p53 (Whitacre *et al.*, 1995) and the $\text{Ca}^{2+}/\text{Mg}^{2+}$ dependent nuclease involved in the apoptotic cleavage of DNA (Rice *et al.*, 1992).

The early and short-lived poly(ADP-ribosylation), followed by caspase-3 activation and PARP cleavage detected in this study (Figs 5–7), is consistent with our previous findings during spontaneous apoptosis in the osteosarcoma system (Rosenthal *et al.*, 1997a,b). The synthesis of poly(ADP-ribose) from nicotinamide adenine dinucleotide increased early after initiation of apoptosis. The abundance of both poly(ADP-ribose) and PARP then decreased markedly, corresponding to the appearance of the proteolytic cleavage product containing the DNA-binding domain of PARP; no poly(ADP-ribose) was observed during this time, in spite of the fact that there was massive DNA internucleosomal degradation. This coincided with earlier data indicating that caspase-3 activity maximized in osteosarcoma cells at this time. These data suggested that there appears to be not only a requirement for destruction of PARP during apoptosis, but also a stage in early apoptosis that appeared to require the presence of PARP protein and presumably poly(ADP-ribosylation) of certain proteins. Recently, we utilized antisense RNA expression to deplete endogenous PARP in order to examine the requirement of this protein early in apoptosis, and found that the apoptotic response was modulated (Simbulan-Rosenthal *et al.*, 1998). Likewise, in recent PARP-knockout animals, apoptosis was found to be altered (de Murcia *et al.*, 1997).

The involvement of such varied molecules as Ca^{2+} , calmodulin, p53, Bcl-2, and caspase-3 suggests a complex network involved in SM-induced apoptosis; however, it seems that the activation of caspase-3 (or the closely related caspase-7) may be a final converging point for apoptosis, because (i) procaspase-3/CPP32 knockout mice show altered programmed cell death in the nervous system (Kuida *et al.*, 1996), (ii) caspase-3 (and -7) is a target for granzyme B-mediated apoptosis, as well as apoptosis mediated by other caspases (Darmon *et al.*, 1995, 1996; Chinnaiyan *et al.*, 1996; Gu *et al.*, 1996; Quan *et al.*, 1996), and (iii) a tetrapeptide aldehyde (Ac-DEVD-CHO) that inhibits caspase-3 activity blocks apoptotic events in isolated nuclei (Nicholson *et al.*, 1995). In this study, the convergence of these signals at the level of caspase-3 activation is supported by the facts that: (i) p53 and Bcl-2, which act upstream of caspase-3, are strongly and rapidly modulated by SM; (ii) inhibitors of Ca^{2+} and calmodulin, which have been found in previous studies to prolong the lifespan of keratinocytes exposed to SM (Ray *et al.*, 1996), and to block apoptosis in this study, prevent the processing and activation of caspase-3; and (iii) in preliminary experiments, NHEK pretreated with AcDEVD-CHO showed both inhibition of *in vitro* PARP-cleavage activity and a reduced apoptotic index in response to SM, as determined by TUNEL labeling (not shown).

An understanding of the mechanisms for SM vesication will hopefully lead to strategies for prevention or treatment of SM toxicity. This study suggests that inhibition of calmodulin (upstream) or caspase-3 (downstream) may protect the epidermis from SM-induced apoptosis. Although the mechanism for their protection has not been described, calmodulin inhibitors have already been successfully employed in the

treatment of both thermal burns and frostbite (Beitner *et al*, 1989a, b), and may prove effective for SM as well, either alone or in combination with caspase-3 inhibitors.

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11 Poly(ADP-Ribose) Polymerase is an Active Participant in Programmed Cell Death and Maintenance of Genomic Stability

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11.1 INTRODUCTION

Poly(ADP-ribose) polymerase (PARP) is a major nuclear protein associated with chromatin that contains zinc fingers and binds to either double- or single-strand DNA breaks. PARP is activated upon binding to DNA and forms covalent homopolymers of poly(ADP-ribose) (PAR) attached to a number of nuclear proteins, including itself and proteins involved in DNA replication, DNA repair, and apoptosis. Nuclear NAD, which comprises 95% of the total cellular NAD, is the substrate for polymer formation. PARP has been implicated in numerous biological functions involved with the breaking and rejoining of DNA.¹⁻⁶ In addition, other functions have been

ascribed for PARP in which the role of DNA strand breaks is not so clear. For example, PARP has been demonstrated to play a role as a coactivator of gene transcription.^{7,8} In addition, the binding of PARP to specific nuclear proteins has been shown to alter their activity, in the absence of DNA breaks or NAD.⁹

11.2 PARP AND CELL DEATH

PARP knockout mice have now been independently generated from the interruption of exon 2,¹⁰ exon 4,¹¹ and most recently, exon 1¹² of the PARP gene on chromosome 1. PARP knockout mice with a disrupted PARP gene neither express intact PARP nor exhibit significant poly(ADP-ribosyl)ation.¹⁰⁻¹² Because poly(ADP-ribosyl)ation is stimulated by DNA fragmentation, the potential role for PARP in cell death via NAD and ATP depletion had been proposed previously.^{13,14} This idea has been supported by recent studies in which both exon 1¹² and exon 2^{15,16} PARP^{-/-} animals have been shown to be resistant to streptozotocin-induced pancreatic islet cell death, associated with NAD depletion in PARP^{+/+} animals. We have also collaborated in a study that demonstrated that exon 2 PARP^{-/-} animals are resistant to the neurotoxin MPTP-induced parkinsonism.¹⁷ Exon 2 PARP^{-/-} animals are also more resistant to ischemic injury.¹⁸⁻²¹

To investigate whether PARP might play an active role in programmed cell death, we first used a human osteosarcoma cell line that undergoes a "slow," spontaneous apoptotic death.²² On reaching confluency, approximately 6 days under our culture conditions, these cells undergo the morphological and biochemical changes characteristic of apoptosis. Internucleosomal DNA cleavage was apparent at day 7 and increased until day 10, at which time virtually all of the cells have undergone apoptosis. Cells from duplicate cultures were incubated for up to 10 days and fixed at daily intervals for examination of nuclear poly(ADP-ribosyl)ation with antiserum to PAR. After 3 days, the nuclei of all attached cells stained intensely for the PAR. The *in vivo* synthesis of PAR was markedly reduced afterward (Figure 11.1A). Our results support the idea that nuclear disruption involving strand breaks may be present in the earliest stages of apoptosis, before morphological changes and the appearance of the characteristic nucleosome ladder. The substantial extent of nuclear poly(ADP-ribosyl)ation apparent early during apoptosis is consistent with the appearance of large (1-Mb) chromatin fragments at this reversible stage,²³ given that the activity of PARP is absolutely dependent on DNA strand breaks. A marked decrease in NAD concentration, indicative of increased PAR synthesis, and a subsequent recovery in NAD levels prior to the appearance of internucleosomal DNA cleavage have also been previously observed.²⁴

Kaufmann et al.²⁵ first demonstrated that PARP undergoes proteolytic cleavage during chemotherapy-induced apoptosis. By immunoblot analysis with epitope-specific antibodies, it was demonstrated that programmed cell death was accompanied by early cleavage of PARP into 85- and 24-kDa fragments that contain the active site and the DNA-binding domain (DBD) of the enzyme, respectively. This latter domain is required for full PARP activity. The purification and characterization of caspase-3, responsible for the cleavage of PARP during apoptosis was performed by Nicholson et al.²⁶ This enzyme is composed of two subunits of 17 and 12 kDa

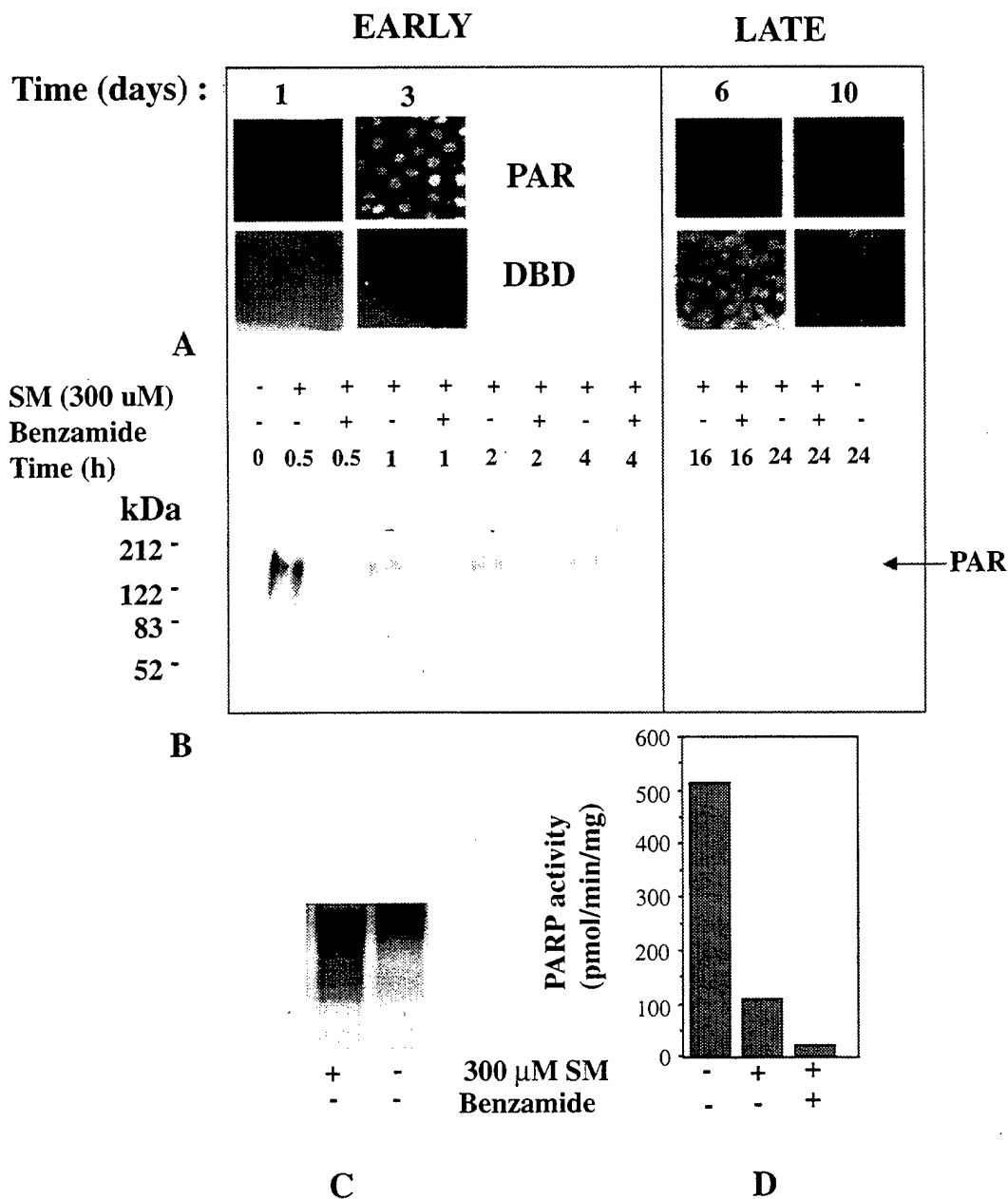


FIGURE 11.1 PARP activity and poly(ADP-ribosylation) are characteristic of early stages of apoptosis, while PARP cleavage and inactivation are associated with DNA laddering. (A) Osteosarcoma cells undergoing 10-day spontaneous apoptosis were fixed at the indicated times and subjected to immunofluorescent analysis utilizing antibodies specific for PAR (top), or the caspase-3-cleavage product of PARP (DBD; bottom). (B) Primary human keratinocytes were treated with 300 μ M sulfur mustard for the indicated times. Cell extracts were derived and subjected to immunoblot analysis using monoclonal antibody specific for PAR. (C) Keratinocytes were treated with sulfur mustard for 24 h. DNA was isolated, separated by electrophoresis in 1.5% agarose gels, and detected with ethidium bromide. (D) Cell extracts were derived from keratinocytes treated as in (C) and assayed for PARP activity using [^{32} P]NAD as a substrate.

that are derived from a common proenzyme, which is related to interleukin-1 β -converting enzyme and to CED-3, the product of a gene required for programmed cell death in *Caenorhabditis elegans*.²⁷ The identity of this protease was also demonstrated by Tewari et al.²⁸ To measure PARP cleavage in intact cells, we subjected human osteosarcoma cells to immunofluorescence analysis with antibodies that recognize the DBD but not intact PARP.^{22,29} As with the other markers, samples were analyzed each day throughout the total 10-day period; samples from immediate (day 1), early (day 3), mid (day 6), and late (day 10) stages of apoptosis are shown in Figure 11.1A. Immunofluorescence analysis detected the PARP DBD in human osteosarcoma cells only after 6 to 7 days in culture, a time at which the abundance of both PARP and PAR is decreasing, PARP-cleavage activity is increasing, and internucleosomal DNA cleavage is present.²⁶ The pattern of staining for the DBD differed markedly from that of full-length PARP. Whereas PARP staining was present throughout the nucleus, the DBD showed a more localized punctate pattern in the region of the nucleolus and throughout the nucleus-disrupted cytoplasm.

Therefore, catalytic activation of PARP occurs early in osteosarcoma cell growth, while the cleavage of PARP and the accumulation of a large number of DNA strand breaks occur later in the apoptotic process. The concomitant loss of poly(ADP-ribosyl)ation of target proteins appears to be characteristic of later stages of apoptosis during which cells become irreversibly committed to death. This may conserve NAD and ATP during the later stages of apoptosis. Recently, the requirement for PARP cleavage to prevent necrosis associated with depletion of NAD has been confirmed using PARP^{-/-} cells that express a caspase-resistant mutant of PARP.³⁰

These results are in contrast to those of Negri et al.,³¹ who reported the presence of PAR in cells at the late stages of apoptosis, although, as the authors point out, it is difficult to reconcile the quantitative cleavage of PARP with its activation late in apoptosis (see below). To determine if a minor fraction of uncleaved PARP could be responsible for PAR formation that has been reported late in apoptosis, as well as to determine if transient poly(ADP-ribosyl)ation could be observed in another system, we recently measured PARP activity as well as the total amount of cellular PAR at different stages of apoptosis induced by the alkylating agent sulfur mustard. PAR is strongly induced in the early stages of apoptosis (within 30 min), but not at later stages (Figure 11.1B). Importantly, the appearance of PAR *precedes* the cleavage of DNA fragmentation factor (DFF45; see below), and the appearance of DNA ladders at 24 h (Figure 11.1C). Although PARP is completely cleaved, extracts derived from 24-h apoptotic cells retain approximately 20% of their *in vitro* polymerizing activity (Figure 11.1D), reflecting the low-level DNA-independent activity of the catalytic domain.³² However, this activity is apparently insufficient to synthesize or sustain detectable steady-state levels of PAR *in vivo* in the presence of PAR-degrading enzymes including poly(ADP-ribose) glycohydrolase. Furthermore, addition of the PARP inhibitor benzamide in this system significantly delays the onset of apoptosis.³³ Clearly, the activation of PARP is associated with DNA breaks, although chromosomal degradation to large fragments precedes the formation of DNA ladders in response to apoptotic signals generated from DNA-damaging agents as well as from stimulation of the Fas receptor (see below).

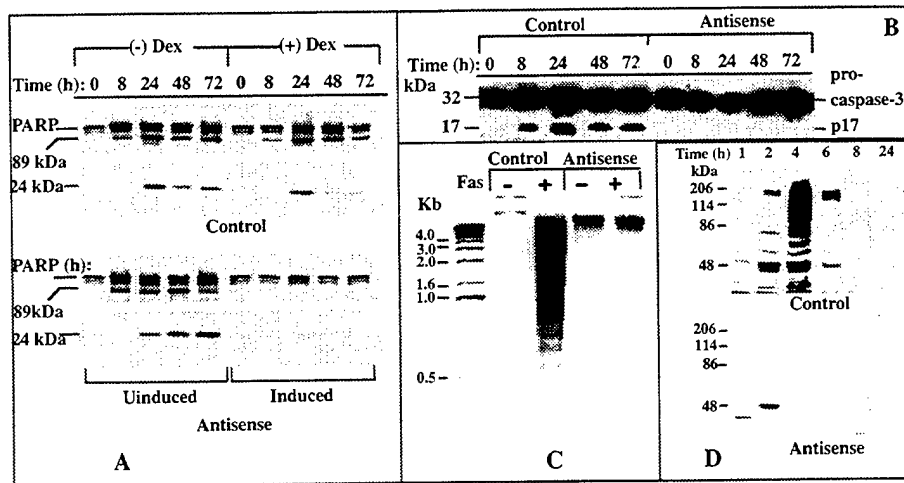


FIGURE 11.2 Effects of PARP depletion by antisense RNA expression on the increase in caspase-3-like activity (A), proteolytic processing of procaspase-3 (B), internucleosomal DNA fragmentation (C), and synthesis of PAR (D) during Fas-mediated apoptosis in 3T3-L1 cells. Mock-transfected (A, top) and PARP-antisense (A, bottom) 3T3-L1 cells were preincubated in the absence or presence of 1 μ M Dex for 72 h and then incubated with anti-Fas (100 ng/ml) and cycloheximide (10 μ g/ml) for the indicated times. Cytosolic extracts were prepared and assayed for *in vitro* PARP-cleavage activity with [^{35} S]PARP as substrate. (B) 3T3-L1 control and antisense cells were preincubated with Dex for 72 h and then exposed to anti-Fas and cycloheximide for the indicated times as in (A). Cell extracts were subjected to immunoblot analysis with a monoclonal antibody to the p17 subunit of caspase-3 (B) or to PAR (D). The positions of procaspase-3 and p17 are indicated. (C) Total genomic DNA was extracted and internucleosomal DNA ladders characteristic of apoptosis was detected by agarose gel electrophoresis and ethidium bromide staining.

The generality of an early burst of poly(ADP-ribosyl)ation was confirmed with human HL-60 cells, mouse 3T3-L1, and immortalized fibroblasts derived from wild-type mice.³⁴ The effects of eliminating this early transient modification of nuclear proteins by depletion of PARP protein either by antisense RNA expression or by gene disruption on various morphological and biochemical markers of apoptosis were then examined.

In 3T3-L1 cells stably transfected with a construct expressing dexamethasone (Dex)-inducible PARP antisense RNA, Dex induced a time-dependent depletion of PARP, with only ~5% of the protein remaining after 72 h. A combination of anti-Fas and cycloheximide induced a marked increase in caspase-3-like activity in control 3T3-L1 cells that had been preincubated in the absence or presence of Dex. This effect was maximal 24 h after induction of apoptosis, as indicated by the generation of the 89- and 24-kDa cleavage fragments of PARP in an *in vitro* assay (Figure 11.2A, top). No caspase-3 activity was apparent in PARP-antisense cells that had been depleted of PARP by preincubation with Dex before exposure to anti-Fas and cycloheximide (Figure 11.2A, bottom).

To confirm that procaspase-3 is proteolytically processed to an active p17 subunit during apoptosis in control 3T3-L1 cells, and to determine whether the transient

early poly(ADP-ribosyl)ation is necessary for this activation, control and antisense cells were preincubated with Dex, exposed to anti-Fas and cycloheximide for indicated times, and cell extracts were subjected to immunoblot analysis with antibodies to the p17 subunit of caspase-3. Whereas procaspase-3 was proteolytically processed to p17 by 24 h, coinciding with the peak of *in vitro* caspase-3-like PARP-cleavage activity, in control cells, proteolytic processing of procaspase-3 was not apparent in the PARP-depleted antisense cells (Figure 11.2B). Furthermore, using DNA fragmentation analysis as another assay for apoptosis, control 3T3-L1 cells exposed to anti-Fas and cycloheximide for 24 h exhibited marked internucleosomal DNA fragmentation (DNA ladders), but not the PARP-depleted antisense cells exposed to these inducers for the same time (Figure 11.2C). Similar to our previous studies, we noted that the earliest stages of apoptosis were associated with a burst of PAR synthesis. This early synthesis of PAR was eliminated by the expression of PARP antisense RNA (Figure 11.2D).

Cells derived from animals depleted of PARP were also unable to undergo Fas-mediated apoptosis. Anti-Fas and cycloheximide induced a rapid synthesis of PAR in PARP^{+/+} cells, which was not observed in PARP^{-/-} cells (Figure 11.3A). To determine if the activation of PARP and synthesis of PAR was the result of cleavage of the inhibitor of caspase-activated DNase (ICAD/DFF45)³⁵ and the concomitant oligonucleosomal cleavage of DNA, a time course was first performed for PAR; the same filter was then reprobed with ICAD-specific antibody. Figure 11.3B shows that PAR synthesis occurred at 1 to 2 h, while ICAD cleavage did not occur until 4 to 6 h (Figure 11.3C). In contrast to PARP^{+/+} cells, no processing of ICAD was evident in PARP^{-/-} cells after exposure to anti-Fas and cycloheximide for up to 24 h (not shown).

PARP^{+/+} cells showed substantial nuclear fragmentation and chromatin condensation 24 h after induction of Fas-mediated apoptosis; ~97% of nuclei exhibited apoptotic morphology by this time. In contrast, no substantial changes in nuclear morphology were apparent in the PARP^{-/-} fibroblasts even after exposure to anti-Fas and cycloheximide for 24 or 48 h³⁴.

PARP^{-/-} fibroblasts were stably transfected with a plasmid expressing wild-type PARP.³⁶ Individual as well as pooled clones expressed PARP protein at levels similar to those of PARP^{+/+} cells. These cells were induced to undergo apoptosis by exposure to anti-Fas and cycloheximide for up to 48 h. PARP^{+/+} cells, as well as PARP^{-/-} transfected with PARP, exhibited significant caspase-3-like activity after 48 h. As expected, PARP was not expressed in the PARP^{-/-} fibroblasts nor in PARP^{-/-} cells transfected with vector alone. Consistently, whereas exposure to anti-Fas and cycloheximide induced marked internucleosomal DNA fragmentation in PARP^{+/+} fibroblasts and in PARP^{-/-} cells stably transfected with PARP, no apoptotic DNA ladders were evident in the PARP^{-/-} cells when similarly treated (Figure 11.3D). Furthermore, exposure to anti-Fas plus cycloheximide for 48 h induced apoptotic nuclear morphology in PARP^{-/-} cells transfected with PARP almost to the same extent as the PARP^{+/+} cells.³⁴

Thus, depletion of PARP by antisense in 3T3-L1, or by knockout of PARP attenuates Fas plus cycloheximide-mediated apoptosis. In addition, the reintroduction of PARP in independent clones of PARP^{-/-} cells reestablished the response. We interpret these results to indicate that PARP plays an active role early in apoptosis

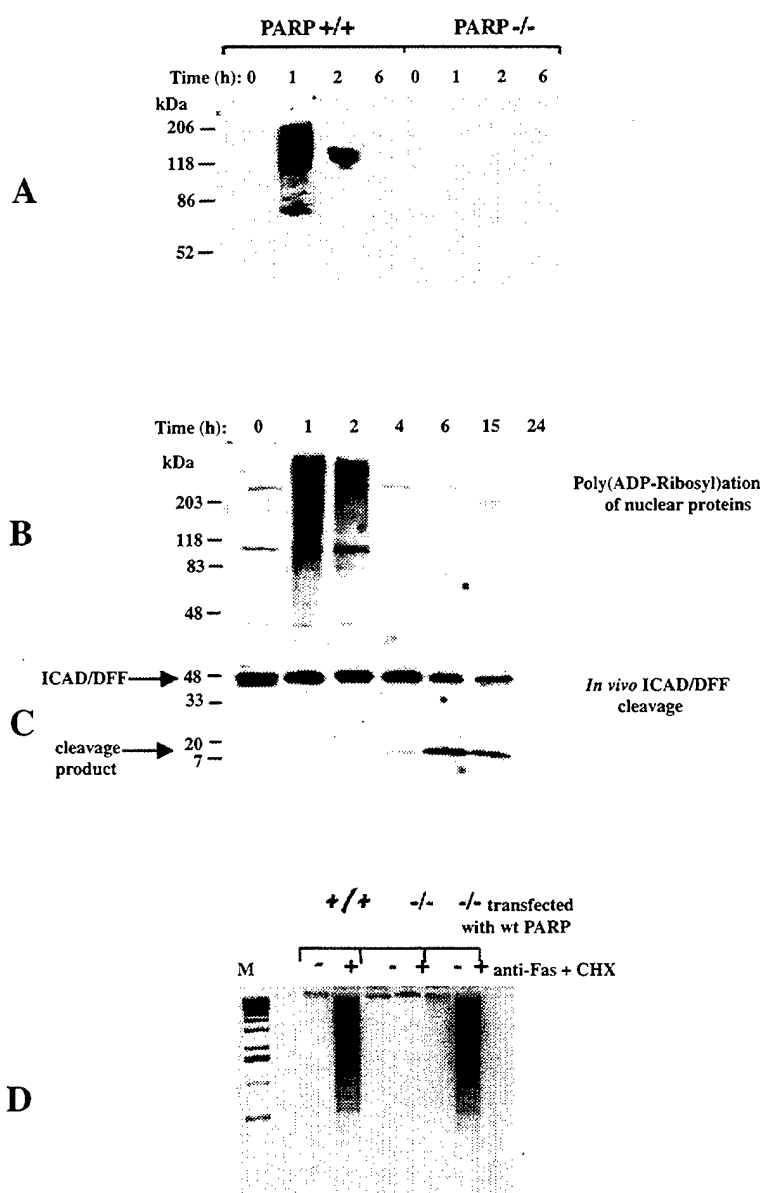


FIGURE 11.3 Effects of anti-Fas and cycloheximide on poly(ADP-ribosyl)ation, ICAD cleavage, and internucleosomal DNA fragmentation in immortalized fibroblasts from wild-type and PARP knockout mice. (A) PARP^{+/+} and PARP^{-/-} fibroblasts were exposed to anti-Fas (100 ng/ml) and cycloheximide (10 μ g/ml) for the indicated times, after which extracts were subjected to immunoblot analysis with antibodies to PAR. (B) PARP^{+/+} fibroblasts were treated as in (A) in an independent experiment, after which extracts were subjected to immunoblot analysis with antibodies to PAR. (C) The identical filter used in (B) was stripped of antibodies 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), and reprobed using an antibody specific for ICAD. (D) PARP^{+/+} and PARP^{-/-} fibroblasts as well as PARP^{-/-} cells stably transfected with wild-type PARP were exposed to anti-Fas and cycloheximide for 48 h. Apoptosis was monitored by extraction of total genomic DNA and detection of characteristic apoptotic internucleosomal DNA ladders by agarose gel electrophoresis and ethidium bromide staining.

either by depletion of NAD and ATP or via the modification of nuclear proteins involved in apoptosis. Furthermore, these studies are consistent with earlier results using chemical inhibitors (which require a degree of caution in their interpretation), indicating that the activation of PARP is required for apoptosis to occur in some systems.^{24,37-39} It should be pointed out that another study indicated that primary PARP^{-/-} fibroblasts underwent similar apoptosis compared to PARP^{+/+} cells.⁴⁰ Whether these differing observations are due to the use of different cells or reagents remains to be determined. However, it has been shown recently that expression of caspase-3-resistant PARP in exon 2 PARP^{-/-} cells,^{30,41} as well as expression of exogenous wild-type PARP in osteosarcoma cells, results in an earlier onset of the apoptotic response, a finding that is consistent with an active role for PARP and poly(ADP-ribosyl)ation early in apoptosis. Both of these findings are in contrast to a study by Oliver et al.⁴² who found a presumptive decrease in apoptotic response, as determined by morphological changes, upon expression of uncleavable PARP in cells derived from different PARP knockout animals. However, as pointed out previously,^{30,43} these animals are derived from an interruption in exon 4,¹¹ and could potentially express a truncated protein encompassing the first zinc finger of the DNA-binding region of PARP. This zinc finger is sufficient to bind both single- and double-stranded DNA breaks,⁴⁴ and may therefore act as a dominant-negative mutant.^{45,46}

How could PARP play a role in the pathway leading from the Fas/TNF receptor to apoptosis? There is clearly cross talk between the mitochondrial and death receptor-mediated pathways for apoptosis, and in fact "type II" cells do not form a significant amount of death-inducing signaling complex (DISC), comprising Fas, Fas-associating protein with death domain (FADD), and procaspase-8.⁴⁷ The characteristics of type II cells include the relatively slow activation of caspase-3, followed by the activation of caspase-8, and direct processing of caspase-3 into a p17 active form without the appearance of the intermediate p20. The immortalized exon 2 PARP^{-/-} fibroblasts, as well as 3T3-L1 cells expressing PARP antisense RNA, utilized in our study, fit both of these criteria. In addition, 3T3 cells demonstrate Bcl-2-inhibitable Fas killing,⁴⁸ another hallmark of type II cells.⁴⁷

In type II cells, caspase-3 activation is secondary to the release of proapoptotic factors from the mitochondria, including cytochrome c, procaspase-2, procaspase-9,⁴⁹ and apoptosis-inducing factor (AIF).⁵⁰ AIF is a noncaspase inducer of apoptosis that can translocate from the mitochondrial intermembrane space to the nucleus and induce DNA cleavage into 50-kb fragments.⁵¹ Cytosols from anti-Fas-treated human lymphoma cells have been shown to accumulate AIF activity.⁵²

In this system, the cleavage of ICAD/DFF45 by caspase-3, which allows the translocation of CAD/DFF40 to the nucleus and internucleosomal cleavage, is a relatively late event. However, chromosomal DNA is not directly cleaved into nucleosome ladders during apoptosis. Studies with isolated nuclei⁵³ and intact cells⁵⁴ demonstrate that DNA is first fragmented into high-molecular-weight fragments of 1 to 2 Mb, followed by the appearance of 200- to 800-kb fragments, which may reflect the higher-order chromatin structure of nuclei. Afterward, DNA is further degraded into 50-kb fragments, and then finally into the characteristic nucleosome-sized ladders visible by conventional agarose gel electrophoresis, although certain cell types, such as MCF-7, do not demonstrate this final stage of chromatin degra-

dation. Although elegant studies have demonstrated that ICAD/DFF45 and CAD/DFF40 play important roles in apoptotic DNA fragmentation,⁵⁵⁻⁵⁷ these studies have not excluded the roles of other nucleases. Hughes et al.⁵⁸ have identified a 260-kDa factor that is responsible only for the cleavage of DNA into 30- to 50-kb fragments, while a 25-kDa factor generated both 30- to 50-kb fragments, as well as smaller fragments.⁵⁸ Other nucleases that have been shown to play a role in DNA degradation in apoptosis include DNase II,⁵⁹ DNase I,^{60,61} DNase I-related protein,⁶² and cyclophins A, B, and C.⁶³ Similar to the endonuclease activity induced by AIF, cyclophilin C only cleaves DNA into 50-kb fragments.

Thus, a model may be proposed whereby stimulation of the Fas receptor on immortalized fibroblasts induces the gradual release of mitochondrial factors, such as AIF, that may translocate to the nucleus and induce low levels of caspase-independent cleavage of chromatin into large fragments. These DNA breaks would then stimulate PARP activity, amplifying apoptotic events by either poly(ADP-ribosyl)ating p53 or other factors involved in the upregulation or altered intracellular trafficking of Fas, Bax, or IGFBP3 (see below). Additionally, PARP activation rapidly depletes NAD and ATP, which could contribute to both receptor and mitochondrial pathways of apoptosis. Feldenberg et al.⁶⁴ have shown that partial depletion of ATP (approximately 10 to 65% of control) can induce apoptosis of cultured renal epithelial cells including internucleosomal DNA cleavage, morphological changes, and plasma membrane alterations. The ATP-depleted cells display a significant upregulation of Fas, Fas ligand, and FADD, resulting in induction of caspase-8 and caspase-3 activity.⁶⁴

Further depletion of ATP below a threshold level might be expected to inhibit the later events in apoptosis. Eguchi et al.⁶⁵ have shown that Fas-induced apoptosis is completely blocked by reducing the intracellular ATP level in both type I and type II cells. In type I cells, ATP-dependent step(s) of Fas-mediated apoptotic signal transduction are only located downstream of caspase-3 activation. However, in type II cells, activation of caspase-3, -8, and -9, as well as cleavage of ICAD/DFF45, was blocked by reduction of intracellular ATP, whereas release of cytochrome c was not affected. This may reflect the requirement for dATP/ATP in the activation of caspase-9.⁶⁶⁻⁶⁸ Cleavage of PARP at later stages of apoptosis would prevent ATP from falling below this critical level.

11.3 POLY(ADP-RIBOSYL)ATION OF p53

In addition to undergoing automodification, PARP catalyzes the poly(ADP-ribosyl)ation of such nuclear proteins as histones, topoisomerases I and II,^{69,70} SV40 large T antigen,⁷¹ DNA polymerase α , proliferating cell nuclear antigen (PCNA), and approximately 15 protein components of the DNA synthesome.⁷⁰ The modification of nucleosomal proteins also alters the nucleosomal structure of the DNA containing strand breaks and promotes access of various replicative and repair enzymes to these sites.^{72,73} We have obtained some potentially relevant targets for poly(ADP-ribosyl)ation during the burst of PAR synthesis at the early stages of apoptosis, including p53. p53, a tumor suppressor nuclear phosphoprotein, reduces the occurrence of mutations by mediating cell cycle arrest in G₁ or G₂/M or inducing

apoptosis in cells that have accumulated substantial DNA damage, thus preventing progression of cells through S phase before DNA repair is complete.⁷⁴⁻⁷⁶ p53 is induced by a variety of apoptotic stimuli and is required for apoptosis in many cell systems.⁷⁷ Overexpression of p53 is sufficient to induce apoptosis in various cell types.⁷⁸ Interestingly, p53 can utilize transcription activation of target genes and/or direct protein-protein interaction to initiate p53-dependent apoptosis.

Both PARP activity and p53 accumulation are induced by DNA damage, and both proteins have been implicated in the normal cellular responses to such damage. Whereas PAR synthesis increases within seconds after induction of DNA strand breaks,⁷⁹ the amount of wild-type p53, which is usually low because of the short half-life (20 min) of the protein, increases several hours after DNA damage as a result of reduced degradation.^{80,81} A functional association of PARP and p53 has recently been suggested by coimmunoprecipitation of each protein *in vitro* by antibodies to the other.^{82,83} It was recently shown that p53 is poly(ADP-ribosyl)ated *in vitro* by purified PARP, and that binding of p53 to a specific p53 consensus sequence prevents its covalent modification.⁸⁴ We recently showed that modification of p53 by poly(ADP-ribosyl)ation also occurs *in vivo*, and that it represents one of the early acceptors of poly(ADP-ribosyl)ation during apoptosis in human osteosarcoma cells.⁸⁵ Given that the *in vivo* half-life of PAR chains on an acceptor has been estimated to be about 1 to 2 min, we additionally explored how this post-translational modification of p53 is altered at the onset of caspase-3-mediated cleavage and inactivation of PARP during the later stages of the death program.

Human osteosarcoma cells were plated under conditions that result in spontaneous apoptosis over a 10-day period.^{22,26} Biochemical markers of apoptosis were initially observed at day 5 and maximized around days 7 to 9, including caspase-3-mediated *in vitro* PARP-cleavage activity (Figure 11.4A, top), proteolytic processing of the caspase-3 proenzyme (CPP32) to its active form (p17; Figure 11.4A, middle), and internucleosomal DNA fragmentation.

Consistent with previous studies showing p53 accumulation during early apoptosis in different cell lines, immunoblot analysis with anti-p53 mAbs of extracts of osteosarcoma cells at various stages of spontaneous apoptosis revealed that endogenous levels of p53 protein were significantly increased as early as days 2 to 3, maximized at day 4, and declined thereafter. Immunoblot analysis with antibodies to PARP to monitor *in vivo* PARP cleavage during the same time frame showed that ~50% of endogenous PARP was cleaved to its 89-kDa fragment by day 7 and complete cleavage of PARP was noted by day 9.⁸⁵

When the same extracts were subjected to immunoblot analysis with antibodies to PAR, low levels of polymer were observed at day 2 of apoptosis (Figure 11.4A, bottom), indicating the absence of DNA strand breaks, PARP activity, or both. However, poly(ADP-ribosyl)ation of nuclear proteins was markedly increased at day 3 and was maximal at day 4, a stage at which all the cells were still viable and could be replated, prior to any evidence of internucleosomal DNA fragmentation. Subsequently, a marked decline in poly(ADP-ribosyl)ation of nuclear proteins was observed at later time points (days 7 to 9), concomitant with the onset of substantial DNA fragmentation, proteolytic activation of caspase-3, and caspase-3-mediated *in vitro* and *in vivo* cleavage of PARP.

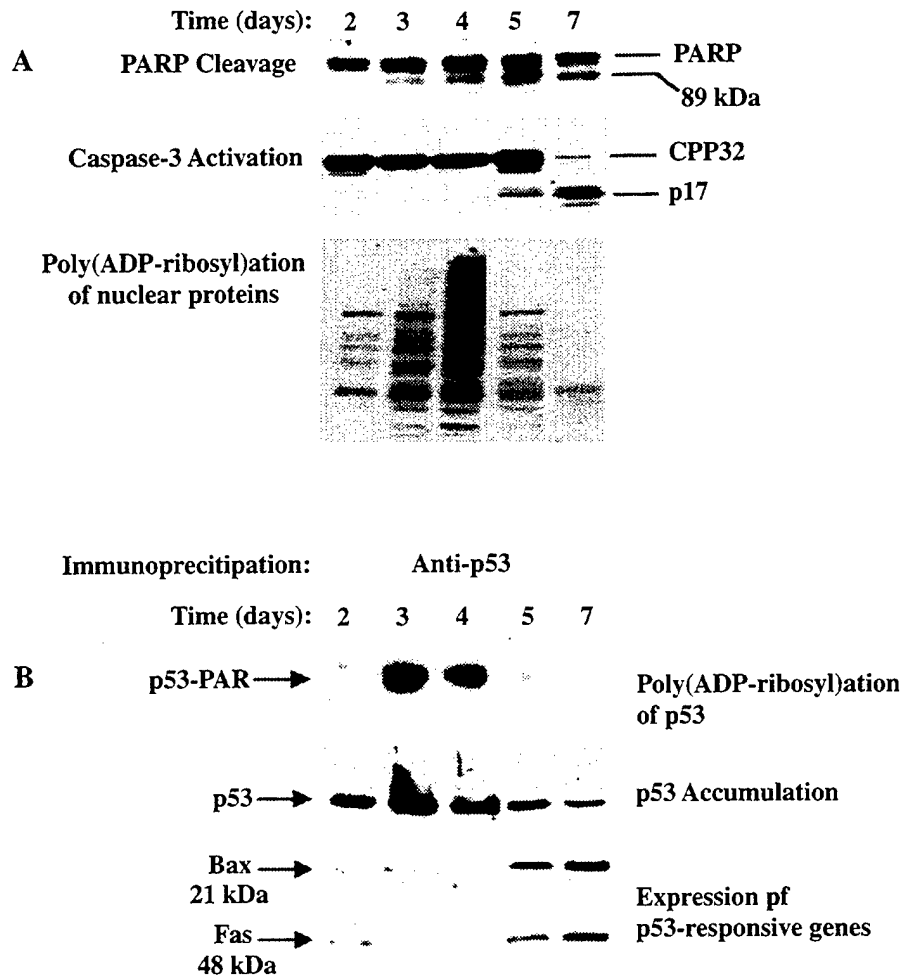


FIGURE 11.4 Time courses of *in vitro* PARP cleavage, activation of caspase-3, and poly(ADP-ribosyl)ation of nuclear proteins (A) vs. poly(ADP-ribosyl)ation of p53 and expression of Bax and Fas (B) during spontaneous apoptosis in human osteosarcoma cells. (A) At the indicated times of confluence-associated spontaneous apoptosis, cell extracts were prepared and caspase-3-like PARP-cleavage activity in cytosolic extracts was assayed with [^{35} S]PARP as substrate (top panel). Extracts were also subjected to immunoblot analysis with mAb to the p17 subunit of caspase-3 (middle panel), or to PAR (bottom panel). (B) At the indicated times during spontaneous apoptosis, cell extracts were prepared and equal amounts of total protein (100 μ g) were subjected to immunoprecipitation with an mAb to p53. The immunoprecipitated proteins were then subjected to immunoblot analysis with an mAb to PAR (top panel). The immunoblot shown in A was stripped of antibodies 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), and reprobed sequentially with antibodies to p53, Bax, and Fas.

To confirm if p53 undergoes poly(ADP-ribosyl)ation *in vivo* during apoptosis in human osteosarcoma cells, cell extracts were derived at various times during spontaneous apoptosis and subjected to immunoprecipitation with an anti-p53 mAb. The immunoprecipitated proteins were then subjected to immunoblot analysis with mAb to PAR. This approach revealed marked poly(ADP-ribosyl)ation of p53 at the

early stages of apoptosis (days 3 to 4; Figure 11.4B, top), coincident with the burst of PAR synthesis during this stage. The extent of poly(ADP-ribosyl)ation of p53 declined concomitant with the onset of both *in vitro* and *in vivo* caspase-3-mediated PARP cleavage. Reprobing of the blot with polyclonal antibodies to p53 confirmed that the modified protein was in fact p53 (Figure 11.4B, second panel). The observation that p53 is specifically poly(ADP-ribosyl)ated during the early stages of spontaneous apoptosis in human osteosarcoma cells suggests that this post-translational modification may play a role in regulating its function during the early phases of the cell death cascade.

PARP can modulate the catalytic activity of a number of DNA-binding nuclear enzymes by catalyzing their poly(ADP-ribosyl)ation. In most instances, poly(ADP-ribosyl)ation inhibits the activity of the modified protein, presumably because of a marked decrease in DNA-binding affinity caused by electrostatic repulsion between DNA and PAR. Thus, post-translational modification of p53 may also alter DNA binding to specific DNA sequences in the promoters of target genes associated with the induction of p53-mediated apoptosis, such as those encoding Bax, IGF-BP3,⁸⁶ or Fas.⁸⁷ The time course of accumulation and poly(ADP-ribosyl)ation of p53 during the early stages of apoptosis was thus correlated with the induction of expression of the p53-responsive genes Bax and Fas. Immunoblot analysis of extracts of cells at various stages of apoptosis in osteosarcoma cells with antibodies to either Bax or Fas revealed that expression of both Bax and Fas (Figure 11.4B, bottom) were negligible before and at the peak of p53 accumulation and poly(ADP-ribosyl)ation (days 3 and 4). Although p53 accumulation was already significantly elevated by day 2, expression of Bax and Fas was markedly induced only at day 5, concomitant with a decline in PAR attached to p53 and the onset of caspase-3-mediated PARP cleavage and inactivation. The coincident decrease in PAR covalently bound to p53 and induction of Bax and Fas expression suggests that poly(ADP-ribosyl)ation may regulate p53 function early in apoptosis; caspase-3-mediated cleavage of PARP may release p53 from poly(ADP-ribosyl)ation-induced inhibition at the later stages of the apoptotic cascade.

Accordingly, p53 may represent a potentially relevant target for poly(ADP-ribosyl)ation during the burst of PAR synthesis at the early periods of apoptosis. Colocalization of PARP and p53 in the vicinity of large DNA breaks and their physical association^{82,83} suggest that poly(ADP-ribosyl)ation may regulate the DNA-binding ability and, consequently, the function of p53. The accumulation of p53 may be due to induced expression of the protein by the apoptotic stimuli or stabilization by inhibition of p53 degradation via modification of the protein. These results suggest a negative regulatory role for PARP and/or PAR early in apoptosis, since subsequent degradation of PAR attached to p53 coincided with the increase in caspase-3 (PARP-cleavage) activity as well as the induction of expression of the p53-responsive genes Bax and Fas at a stage when cells are irreversibly committed to death. Although the mechanism(s) of action of Bax/Bcl-2 family of gene products during apoptosis remain to be clarified, induction of Bax expression may influence the decision to commit to apoptosis since homodimerization of Bax promotes cell death and heterodimerization of Bax with Bcl-2 inhibits the antiapoptotic function of Bcl-2.⁸⁶ Wild-type p53, but not mutant p53, also upregulates Fas expression during

chemotherapy-induced apoptosis, and p53-responsive elements were recently identified within the first intron and the promoter of the Fas gene.⁸⁷ Binding of Fas to Fas ligand recruits the adapter molecule FADD via shared protein motifs (death domains), resulting in subsequent activation or amplification of the caspase cascade leading to apoptosis.

Electrophoretic mobility-shift analysis has shown that PAR attached to p53 *in vitro* can block its sequence-specific binding to the palindromic p53 consensus sequence, suggesting that poly(ADP-ribosyl)ation of p53 may regulate p53-mediated transcriptional activation of genes important in the cell cycle and apoptosis.⁸⁸ PARP cycles on and off DNA ends in the presence of NAD, and its automodification during DNA repair *in vitro* presumably allows access to DNA-repair enzymes.⁴⁻⁶ Our results with *in vivo* poly(ADP-ribosyl)ation of p53 suggest that p53 may, similarly, cycle on and off its DNA consensus sequence depending on its level of negative charge based on its poly(ADP-ribosyl)ation state. This may represent a mechanism for regulating transcriptional activation of Bax and Fas by p53 during apoptosis. Alternatively, a polymer-binding site in p53 has been localized near a proteolytic cleavage site,⁸⁸ indicating that PAR binding could protect this sequence from proteolysis; similar protection has been noted after binding of monoclonal antibodies adjacent to this region.⁸⁹ The significant poly(ADP-ribosyl)ation of p53 early in apoptosis, therefore, suggests that this post-translational modification could also play a role in p53 upregulation by protecting the protein from proteolytic degradation.

11.4 PARP AND GENOMIC STABILITY

Similar to p53, the active role of PARP in cell death may serve to eliminate cells that have accumulated excessive levels of DNA damage, and may therefore function in the maintenance of genomic stability. A number of studies have employed chemical inhibitors,⁹⁰⁻⁹² dominant negative mutants,^{45,46} and PARP antisense RNA^{93,94} to examine the function of PARP. These studies have demonstrated that PARP plays a role in reducing the frequency of DNA strand breaks, recombination, gene amplification, micronuclei formation, and sister chromatid exchanges (SCE), all of which are markers of genomic instability, in cells exposed to DNA-damaging agents. PARP-deficient cell lines are hypersensitive to carcinogenic agents and also display increased SCE, implicating PARP as a guardian of the genome that facilitates DNA repair and protects against DNA recombination.⁹⁵

Primary fibroblasts derived from exon 2 PARP knockout mice show an elevated frequency of spontaneous SCE and micronuclei formation in response to treatment with genotoxic agents,^{10,40} providing further support for a role of PARP in the maintenance of genomic integrity. Exon 4 PARP knockout mice exhibit extreme sensitivity to γ irradiation and methylnitrosourea and also show increased genomic instability as revealed by a high level of SCE.¹¹ Immortalized cells derived from these animals are characterized by retarded cell growth, G₂/M block, and chromosomal instability on exposure to DNA-alkylating agents, presumably because of a severe defect in DNA repair.⁹⁶

We recently utilized immortalized fibroblasts derived from exon 2 PARP knockout mice (PARP^{-/-}), as well as from control animals of the same strain (PARP^{+/+}),

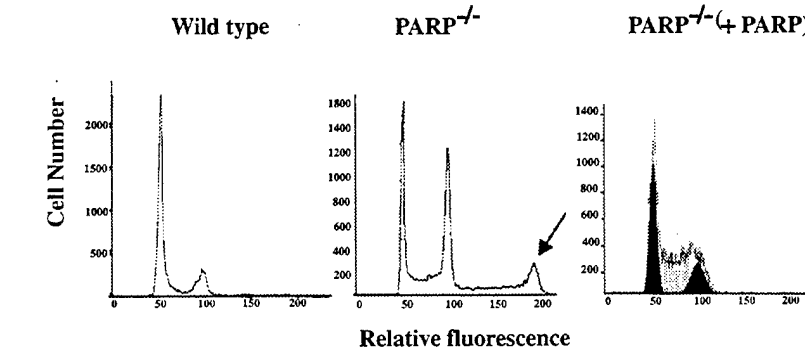
to study the role of PARP in genomic stability. FACS analysis initially revealed that these cells exhibit mixed ploidy, including a tetraploid cell population, indicative of genomic instability.⁹⁷ The tetraploid population was not observed in PARP^{+/+} cells. Further, this tetraploid cell population was no longer apparent in PARP^{-/-} cells retransfected with PARP cDNA, suggesting that the reintroduction of PARP into PARP^{-/-} cells may have stabilized the genome and resulted in selection against this genomically unstable population (Figure 11.5A).

We characterized the genetic alterations associated with PARP depletion by comparative genomic hybridization (CGH) analysis, a cytogenetic technique that detects chromosomal gains and losses in the test DNA as a measure of genetic instability.^{98,99} Although CGH is now commonly used for mapping DNA copy number changes in human tumor genomes, few studies to date have utilized this technique to evaluate genetic instability in transgenic mouse models.^{100,101} CGH analysis revealed that PARP^{-/-} mice or immortalized PARP^{-/-} cells exhibited gains in regions of chromosomes 4, 5, and 14, as well as a deletion in chromosome 14 (Figure 11.5B). We further investigated the effect of stable transfection of immortalized PARP^{-/-} fibroblasts with PARP cDNA on the genetic instability of these cells. Reintroduction of PARP cDNA into PARP^{-/-} cells appeared to confer stability because these chromosomal gains were no longer detected in these cells, further supporting an essential role for PARP in the maintenance of genomic stability (Figure 11.5B).

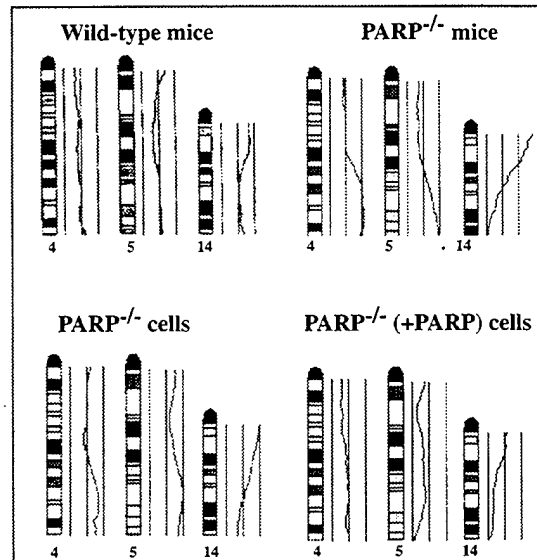
In our study, we noted the absence of immunoreactive p53 from these cells as revealed by immunoblot analysis. A previous report indicated that primary fibroblasts from exon 2 PARP^{-/-} mice¹⁰² also show reduced basal levels of p53 as well as a defective induction of p53 in response to DNA damage, indicating that PARP-dependent signaling may influence this response. Cells that are unable to synthesize PAR because of unavailability of NAD¹⁰³ also show a reduced p53 response. Thus, PARP may regulate genomic stability, at least in part, via p53. Given that the loss of p53 from diploid cells promotes the survival of cells with severe DNA damage and the development of tetraploidy,¹⁰⁴⁻¹⁰⁶ the presence of a tetraploid population among the immortalized PARP^{-/-} cells was consistent with the apparent absence of p53. p53 is involved in the maintenance of diploidy as a component of the spindle checkpoint¹⁰⁶ and by regulating centrosome duplication.¹⁰⁷ A functional association of PARP and p53 has been suggested by immunoprecipitation experiments (see above). Thus, both the increased sensitivity of PARP^{-/-} mice and cells to DNA-damaging agents^{10,11,96} and their genetic instability are consistent with their deficiencies in PARP and p53.

11.5 CONCLUSIONS

PARP has been shown to play active roles in the response to diverse forms of cellular damage resulting from normal metabolic processes, as well as environmental factors, leading to genetic instability. The response may depend upon the level and type of damage, as well as the cell type. In mildly damaged cells, PARP may signal a repair response. In severely damaged cells, PARP activation induces poly(ADP-ribosyl)ation of key nuclear proteins, including p53, and a concomitant lowering of NAD



A



B

FIGURE 11.5 Flow cytometric analysis of immortalized wild-type, $\text{PARP}^{-/-}$, and $\text{PARP}^{-/-}$ (+PARP) fibroblasts (A), and comparison of the CGH profiles of chromosomes 4, 5, and 14 (B). (A) Cells were harvested 18 h after release from serum deprivation. Nuclei were then prepared and stained with propidium iodide for flow cytometric analysis. In addition to the two major peaks of nuclei at G_0/G_1 and G_2/M apparent in the DNA histograms of wild-type and $\text{PARP}^{-/-}$ (+PARP) cells, the DNA histograms of $\text{PARP}^{-/-}$ cells exhibit a third peak corresponding to the G_2/M peak of an unstable tetraploid cell population (arrow). (B) Average ratio profiles were computed for all chromosomes and used for the mapping of changes in copy number, with only the results for chromosomes 4, 5, and 14 shown. The three vertical lines to the right of the chromosome ideograms represent values of 0.75, 1, and 1.25 (left to right, respectively) for the fluorescence ratio between the test DNA and the normal control DNA. The ratio profile (curve) was computed as a mean value of at least eight metaphase spreads. A ratio of ≥ 1.25 was regarded as a gain and a ratio of ≤ 0.75 as a loss. $\text{PARP}^{-/-}$ (+PARP) fibroblasts did not show the gains at 4C5-ter, 5F-ter, or 14A1-C2 that were apparent in both $\text{PARP}^{-/-}$ mice and immortalized $\text{PARP}^{-/-}$ cells, although they retained the partial loss at 14D3-ter.

and ATP levels, resulting ultimately in cell death, the form of which (apoptosis vs. necrosis) may depend upon the time of onset of caspase-mediated PARP cleavage.

ABBREVIATIONS

The abbreviations used are AIF, apoptosis-inducing factor; CAD, caspase-activated DNase; CGH, comparative genomic hybridization; DBD, DNA-binding domain; Dex, dexamethasone; DFF, DNA fragmentation factor; DISC, death-inducing signaling complex; FADD, Fas-associating protein with death domain; ICAD, inhibitor of caspase-activated DNase; PAR, poly(ADP-ribose); PARP, poly(ADP-ribose) polymerase; PCNA, proliferating cell nuclear antigen; SCE, sister chromatid exchange.

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Appended Publication C

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PARP inhibits sulfur mustard-induced apoptosis in skin fibroblasts and keratinocytes

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Abbreviations used: NHEK, normal human epidermal keratinocytes; PARP, poly(ADP-ribose) polymerase, SM, sulfur mustard.

ABSTRACT

Sulfur mustard (SM) is cytotoxic to dermal fibroblasts as well as epidermal keratinocytes. We recently demonstrated that poly(ADP-ribose) polymerase (PARP) modulates Fas-mediated apoptosis, and other groups and we have shown that PARP plays a role in the modulation of other types of apoptotic and necrotic cell death. We have now utilized primary dermal fibroblasts, immortalized fibroblasts, and keratinocytes derived from PARP^{-/-} and PARP^{+/+} mice to determine the contribution of PARP to SM toxicity. Following SM exposure, primary skin fibroblasts from PARP-deficient mice demonstrated increased caspase-3 activity by quantitative fluorometric analysis, and annexin V binding by FACS analysis, compared to those derived from PARP^{+/+} animals. Conversely, PI staining and PARP cleavage patterns revealed a dose-dependent increase in necrosis in PARP^{+/+}, but not PARP^{-/-} cells. Using immortalized PARP^{-/-} fibroblasts stably transfected with PARP cDNA or with empty vector alone, we show that PARP inhibits markers of apoptosis in these cells as well. Finally, primary keratinocytes were derived from newborn PARP^{+/+} and PARP^{-/-} mice, and immortalized with the E6 and E7 genes of HPV. In contrast to fibroblasts, keratinocytes from both PARP^{-/-} and PARP^{+/+} mice express markers of apoptosis in response to SM exposure. However, deletion of the PARP gene results in the enhanced expression of apoptotic markers in the keratinocytes derived from PARP^{-/-} animals. The effects of PARP on the mode of cell death in different skin cell types may determine the severity of vesication *in vivo*, and thus have implications for the use of PARP inhibitors to reduce SM pathology.

INTRODUCTION

Sulfur mustard (bis-(2-chloroethyl) sulfide; SM) causes blisters in the skin via poorly understood mechanisms. We have previously shown that SM induces markers of terminal differentiation and apoptosis in normal human

epidermal keratinocytes (NHEK), including the early activation and late cleavage of poly(ADP-ribose) polymerase (PARP) (Rosenthal et al., 1998). In addition to the direct effect SM on keratinocytes, dermal fibroblasts are important for the vesication response. Human dermal fibroblasts may contribute to the vesication response by releasing degradative cytosolic components extracellularly after SM exposure. Lactate dehydrogenase (LDH), a cytosolic marker of necrotic cell death, was shown to be released in a time-dependent fashion after exposure of a dermal equivalent to 2 mM SM, suggesting a steady leakage of cytosolic constituents after the initial exposure. Elastase levels also increased to over 740% of those in control culture medium 24 h after exposure (Lindsay and Upshall, 1995). In another study, SM was shown to produce both dose- and time-dependent cytotoxicity of a dermal equivalent, and a decreased synthesis of fibronectin by dermal fibroblasts. These SM-induced dermal alterations were correlated with secondary modifications of epithelial adhesion and maturation of unexposed normal human keratinocytes (Gentilhomme et al., 1998). We have also shown decreased levels of fibronectin in NHEK exposed to SM (Rosenthal et al., 1998). In 3T3 fibroblasts, total protein synthesis was reduced to < 20% of controls 24 h after SM exposure (Detheux et al., 1997). Cell viability was not strongly affected, although the SM was used at a concentration of only 100 μ M, corresponding to the lowest dose we used in the present study.

Since SM is a strong alkylating agent, its ability to induce DNA damage via apurinic sites and endonucleolytic cleavage has been advanced as one possible pathway leading to vesication (Papirmeister et al., 1985). Similar to other agents that induce DNA strand breakage, SM induces PARP activity and the poly(ADP-ribosyl)ation of a number of nuclear proteins using NAD as a substrate. We recently examined this process using a human skin graft derived from human keratinocytes stably transfected with a PARP antisense inducible vector (Rosenthal et al., 1995).

PARP has been implicated as an important regulator of both apoptosis and necrosis. Proteolytic cleavage of PARP was first demonstrated in chemotherapy-induced apoptosis (Kaufmann et al., 1993) and the specific proteolysis of PARP is now closely associated with apoptosis in different systems (Neamati et al., 1995; Nicholson et al., 1995; Tewari et al., 1995). We recently showed that the reversible stage of apoptosis is characterized by the transient activation of PARP, and poly(ADP-ribosyl)ation of nuclear proteins followed by the breakdown of poly(ADP-ribose) and PARP, in a human osteosarcoma cell line (Rosenthal et al., 1997). The concomitant loss of poly(ADP-ribosyl)ation of target proteins appears to be characteristic of later stages of apoptosis during which cells become irreversibly committed to death. This may conserve NAD and ATP during the later stages of apoptosis.

PARP knockout mice have now been independently generated from the interruption of exon 2 (Wang et al., 1995), exon 4 (de Murcia et al., 1997), and most recently, exon 1 (Masutani et al., 1999) of the PARP gene on chromosome 1 (PARP-1). PARP knockout mice with a disrupted PARP gene neither express intact PARP nor exhibit significant poly(ADP-ribosyl)ation (Wan et al., 1995; de Murcia et al., 1997; Masutani et al., 1999). The potential role for PARP in cell death via NAD and ATP depletion (Berger et al., 1983; Berger, 1985) has been supported by recent studies in which both exon 1 (Masutani et al., 1999) and exon 2 (Burkart et al., 1999; Pieper et al., 1999) PARP^{-/-} animals have been shown to be resistant to streptozotocin-induced pancreatic islet cell death, associated with NAD depletion in PARP^{+/+} animals. We have also collaborated in study that demonstrated that exon 2 PARP^{-/-} animals are resistant to the neurotoxin MPTP-induced parkinsonism (Mandir et al., 1999). Exon 2 PARP^{-/-} animals are also more resistant to ischemic injury (Eliasson et al., 1997; Endres et al., 1997; Szabo et al., 1997; Zingarelli et al., 1999).

We found that depletion of PARP by antisense in 3T3L1, or by knockout of PARP attenuates Fas plus cycloheximide-mediated apoptosis, and that exon 2 PARP^{-/-} fibroblasts stably transfected with a plasmid expressing wild-type PARP were induced to undergo apoptosis by exposure to anti-Fas and cycloheximide (Simbulan-Rosenthal et al., 1998). It has recently been shown that expression of caspase-3-resistant PARP in exon 2 PARP^{-/-} cells (Boulares et al., 1999; Herceg and Wang, 1999), as well as expression of exogenous wild-type PARP (Boulares et al., 1999) in osteosarcoma cells results in an earlier onset of both the apoptotic (Boulares et al., 1999; Herceg and Wang, 1999) and necrotic responses (Herceg and Wang, 1999), a finding that is consistent with an active role for PARP and poly(ADP-ribosyl)ation early in apoptosis as well as necrosis. Further evidence for a role of PARP in necrosis was determined in studies in which exon 2 PARP^{-/-} fibroblasts were found to be more resistant to necrosis and ATP depletion induced by the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Ha and Snyder, 1999).

Induction of PARP can drastically deplete levels of cellular NAD and ATP (Wielckens et al., 1982; Alvarez et al., 1986), which could contribute to either apoptotic or necrotic cell death. Feldenberg *et al.* (1999) have shown that partial depletion of ATP (approximately 10-65% of control) can induce apoptosis of cultured renal epithelial cells including internucleosomal DNA cleavage, morphological changes, and plasma membrane alterations. The

ATP-depleted cells display a significant upregulation of Fas, Fas ligand, and FADD, resulting in induction of caspase-8 and caspase-3 activity.

Further depletion of ATP below a threshold level might be expected to inhibit the later events in apoptosis, depending on the cell type and inducing agent. Eguchi *et al.* (1999) have shown that Fas-induced apoptosis is completely blocked by reducing the intracellular ATP level in both type I and type II cells. In type I cells, ATP-dependent step(s) of Fas-mediated apoptotic signal transduction are only located downstream of caspase 3 activation. However, in type II cells, activation of caspase-3, -8, and -9, as well as cleavage of ICAD/DFF45, was blocked by reduction of intracellular ATP, whereas release of cytochrome c was not affected. This may reflect the requirement for dATP/ATP in the activation of caspase-9 (Hu *et al.*, 1999; Saleh *et al.*, 1999; Zou *et al.*, 1999). Cleavage of PARP at later stages of apoptosis would prevent ATP from falling below this critical level.

Thus, the cellular response to DNA damage may depend upon the level and type of damage, as well as the cell type. In less severely damaged cells, PARP may signal a repair response and protect against deleterious DNA recombination (Chatterjee *et al.*, 1999). In more severely damaged cells, PARP activation induces poly(ADP-ribosyl)ation of key nuclear proteins, including p53 (Whitacre *et al.*, 1995; Simbulan-Rosenthal *et al.*, 1999), and a concomitant lowering of NAD and ATP levels, resulting ultimately in cell death, the form of which (apoptosis vs. necrosis) may depend upon a number of factors, including the time of onset of caspase activation and proteolytic cleavage of PARP.

In the present study, we have found that PARP plays a role in cell death induced by SM in primary and immortalized fibroblasts by shifting the mode of cell death from apoptosis to necrosis. Keratinocytes, on the other hand, express markers of apoptosis in the presence or absence of a functional PARP-1 gene, although apoptotic markers are upregulated in the absence of PARP. These results indicate that 1) dermal fibroblasts and keratinocytes, which both contribute to SM vesication, undergo different mechanisms of cells death in response to SM, and 2) PARP shifts the mode of cell death from apoptosis to necrosis in dermal fibroblasts. Therefore, inhibition of PARP may be of therapeutic value in the treatment of or prophylaxis against SM injury, since apoptotic cell death may prevent the release of inflammatory or degradative enzymes contributing to vesication or inhibition of healing of SM-induced wounds.

MATERIALS AND METHODS

Cells. NHEK were prepared from human foreskins as described (Stöppler *et al.*, 1998) and maintained in serum-free medium (SFM; Life Technologies). NHEK were grown in 75 cm² tissue culture flasks to 60-80% confluency, then exposed to SM diluted in SFM to final concentrations of 100 μ M or 300 μ M. Media was not changed for the duration of the experiments. Primary fibroblasts were derived from newborn dermis as described previously (Yuspa *et al.*, 1981). Newborn mouse keratinocytes were derived as described previously (Yuspa *et al.*, 1981; Rosenthal *et al.*, 1991), and immortalized utilizing the E6/E7 genes of HPV 16 (Sherman and Schlegel, 1996) in the LXSIN retroviral vector (Clontech, Palo Alto, CA) as described previously (Stöppler *et al.*, 1998). Retrovirus-infected cells were selected in G418, and resistant clones were passaged and analyzed for the expression of keratins and the absence of vimentin utilizing the anti-pan cytokeratin (Sigma-Aldrich, St. Louis, MO) or anti-vimentin (Sigma-Aldrich) by both Western blot and immunofluorescent analysis.

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

Antibodies. Rabbit antisera against the p17 subunit of caspase-3 was obtained from Donald Nicholson (Merck Frosst Centre for Therapeutic Research, Montreal, Quebec, Canada). Rabbit antisera recognizing both full-length PARP, as well as the 89-kDa apoptotic cleavage product of PARP was a kind gift from Eric Ackerman (Pacific Northwest National Laboratory, Richland, WA). Guinea pig antisera specific for poly(ADP-ribose) have been described (Rosenthal *et al.*, 1995). Anti pan-keratin and anti-vimentin are from Sigma-Aldrich.

Immunoblot analysis. For immunoblot analysis, SDS-PAGE-separated proteins were transferred to nitrocellulose filters. Proteins were measured (DCA protein assay; BioRad, Hercules, CA), and normalized prior to gel loading, and all filters were stained with Ponceau-S, in order to reduce the possibility of loading artifacts. The details for using rabbit antiserum to human PARP (Ding *et al.*, 1992) and guinea pig antiserum to poly(ADP-ribose) (Rosenthal *et al.*, 1995) for immunoblot analysis have been described previously in detail. Immune complexes were visualized by electrochemiluminescence (Pierce, Rockford, IL).

Annexin V and Propidium Iodide (PI) Staining, and FACS analysis. Cells were plated in culture plates and exposed to SM. At the indicated times after induction of apoptosis, the cells were trypsinized, washed with ice cold PBS, and subsequently resuspended in and incubated in the dark with 100 μ l of annexin V incubation reagent which includes FITC-conjugated annexin V (Trevigen, Gaithersburg, MD) and PI for 15 min at RT. Flow Cytometric analyses were conducted on a Becton-Dickinson FACStar Plus cytometer using a 100 mW air-cooled argon laser at 488 nm.

PARP cleavage assay. The full-length cDNA clone for PARP (pcD-12) (Alkhatib et al., 1987) was excised and ligated into the Xho I site of pBluescript-II SK+ (Stratagene, La Jolla, CA) then used to drive the synthesis of PARP labeled with [^{35} S]methionine (Dupont-NEN, Boston, MA) by coupled T7 transcription/translation in a reticulocyte lysate system (Promega, Madison, WI). [^{35} S]PARP was separated from the other constituents by gel filtration chromatography on a Superdex-75 FPLC column (Pharmacia, Peapack, NJ; 1X30 cm) in 10 mM HEPES-KOH (pH 7.4), 2 mM EDTA; 0.1% (w/v) CHAPS, and 5 mM dithiothreitol.

Cytosolic extracts were prepared from NHEK by scraping PBS-washed monolayers in 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulphonylfluoride, 10 μ g/ml pepstatin A, 20 μ g/ml leupeptin, 10 μ g/ml aprotinin (at 1×10^8 cells/ml). The post-100,000 g supernatant was recovered after centrifugation.

PARP cleavage activity was measured in mixtures containing 5 μ g protein from the cytosol fractions of keratinocytes. Assay mixtures also contained purified [^{35} S]PARP (5×10^4 cpm), 50 mM PIPES-KOH, 2 mM EDTA, 0.1% (w/v) CHAPS, and 5 mM dithiothreitol in a total volume of 25 μ l. Incubations were performed at 37°C for 1 h, and terminated by the addition of 25 μ l of 2x SDS-PAGE sample buffer containing 4% SDS, 4% -mercaptoethanol, 10% glycerol, 0.125 M Tris-HCl (pH 6.8), and 0.02% bromophenol blue. Samples were resolved by 10% SDS-polyacrylamide gels.

PARP cleavage products were visualized either by fluorography, or else the 89 kDa cleavage product of [^{35} S]PARP was quantified relative to the full-length PARP using a Storm 840 PhosphorImage analyzer (Molecular Dynamics, Sunnyvale, CA). Quantification included a correction for background, as well as for the difference in methionine residues present in the 89 kDa fragment (18 met residues) vs. full-length PARP (25 met residues).

Assay of caspase-3 activity. Briefly after harvesting the cells were resuspended in Triton X-100 lysis buffer (60 mM Tris HCl (pH 7.5), 5mM EDTA, 10% glycerol, 2mM sodium orthovanadate, 25 mM sodium fluoride, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM AEBSF, 75 mM NaCl, 1 μ g/ml pepstatin A, 1% Triton X-100) and incubated for 30 min at 4°C. The cell lysate was centrifuged at 14,000 x g for 15 min and the supernatant containing the postnuclear extract was recovered. The protein concentration in the extract was determined using the Micro BCATM protein assay kit (Pierce) and then the extract was stored until use at -80°C. For the caspase-3 assay, 25 μ g of extract was initially resuspended up to a volume of 50 μ l with Triton X-100 lysis buffer, to which 50 μ l of caspase assay buffer (10 mM HEPES (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM AEBSF, 1 μ g/ml Pepstatin A) was added. The aliquots were preincubated at 37 for 30 min in a microtiter plate, then mixed with equal amount (100 μ l) of 40 μ M fluorescent tetrapeptide substrate specific for caspase-3 (Ac-DEVD-AMC, Biomol, Plymouth Meeting, PA) in caspase assay buffer. The plate was read immediately. Free aminomethylcoumarin (AMC), generated as a result of cleavage of the aspartate-AMC bond, was monitored continuously over 10 min with a Cytofluor 4000 fluorometer (PerSeptive Biosystems, 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.

RESULTS

SM induces PARP-dependent necrosis in primary skin fibroblasts.

We have previously shown that SM induces markers of terminal differentiation and apoptosis in normal human epidermal keratinocytes (NHEK), including the early activation and late cleavage of poly(ADP-ribose) polymerase (PARP) (Rosenthal et al., 1998). In addition to the direct effect SM on keratinocytes, fibroblasts are important for the vesication response (Lindsay and Upshall, 1995; Detheux et al., 1997; Gentilhomme et al., 1998). We therefore first derived primary skin fibroblasts from PARP^{-/-} and PARP^{+/+} animals. These fibroblasts were then exposed to three different doses of SM.

PARP undergoes proteolytic cleavage during chemotherapy-induced apoptosis (Kaufmann et al., 1993). By immunoblot analysis with epitope-specific antibodies, it was demonstrated that programmed cell death was accompanied by early cleavage of PARP into 89- and 24- kDa fragments that contain the active site and the DNA-binding domain (DBD) of the enzyme, respectively. We therefore determined whether cleavage of endogenous PARP could be observed in primary skin fibroblasts. Immunoblot analysis was performed using antibodies that recognize both the full length (113 kDa) and 89 kDa cleavage products of PARP. Although PARP is degraded at the higher doses of SM, no apoptotic cleavage fragments were observed (Figure 1A), suggesting that the mode of cell death is necrotic. As expected, no immunodetectable PARP was observed in the PARP^{-/-} fibroblasts, confirming the PARP^{-/-} genotype.

The purification and characterization of caspase-3, responsible for the cleavage of PARP during apoptosis was performed by Nicholson *et al.* (1995). This enzyme is composed of two subunits of 17 and 12 kDa that are derived from a common proenzyme, which is related to interleukin-1-converting enzyme and to CED-3, the product of a gene required for programmed cell death in *Caenorhabditis elegans* (Yuan et al., 1993).

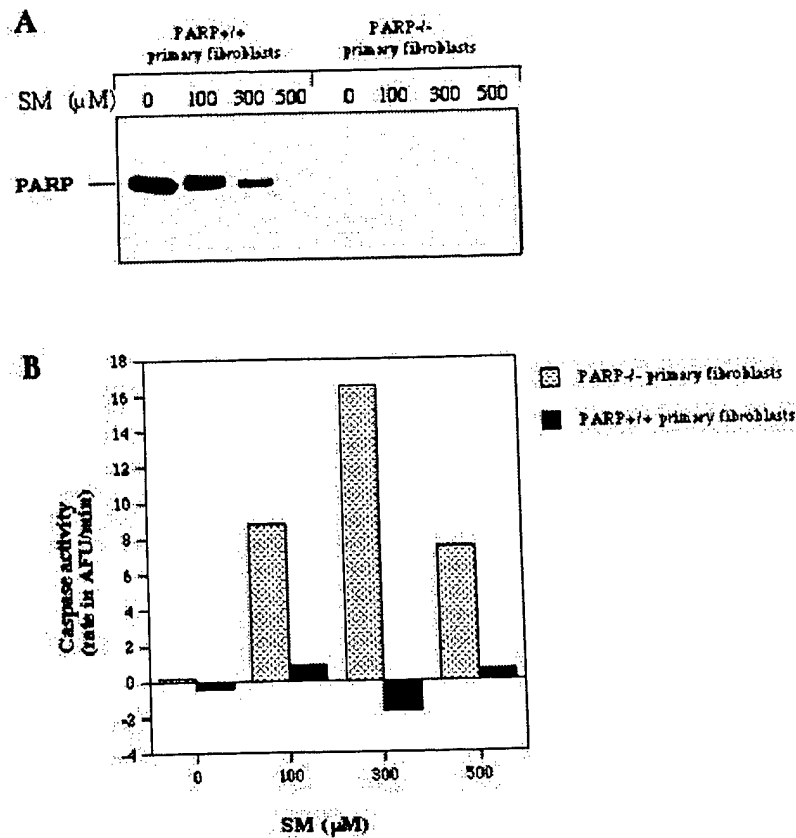


Figure 1

Legend Figure 1

Exposure of primary dermal fibroblast cells derived from PARP^{-/-}, but not PARP^{+/+} mice results in

caspase-3 activation. Primary dermal fibroblasts were derived from newborn mice as described in Materials and Methods. Cells were incubated for 24 h with the indicated concentrations of SM in KGM, after which whole cell extracts were prepared and assayed for the presence of PARP by immunoblot analysis (A), or caspase-3 activity with the specific substrate DEVD-AMC (B).

The identity of this protease was also demonstrated by Tewari *et al.* (1995). 24 h after exposure, extracts were derived and analyzed for caspase-3 activity using a fluorometric assay (Materials and Methods). While PARP^{+/+} primary fibroblasts did not exhibit SM-induced caspase activity at any of the doses tested, PARP^{-/-} fibroblasts exhibited marked caspase-3 activity in response to SM at all doses tested (Figure 1B). The greatest caspase-3 activity was observed following treatment of PARP^{-/-} fibroblasts with 300 μ M SM. Thus, PARP appears to inhibit apoptosis in primary dermal fibroblasts.

An early marker of apoptosis is the exposure of phosphatidylserine residues in the outer plasma membrane leaflet (Fadok *et al.*, 1992). The presence of these residues can be detected by their ability to bind to annexin V (Koopman *et al.*, 1994). To further examine the level of apoptosis in these primary dermal fibroblasts, we therefore exposed cells to increasing doses of SM and then analyzed the cells for annexin V binding by FACS analysis 24 h after SM exposure. While only a small percentage of PARP^{+/+} primary fibroblasts were apoptotic at all doses of SM tested (< 20%), a dose-dependent increase in the number of PARP^{-/-} apoptotic fibroblasts was observed, up to a maximum of 65% (Figure 2A).

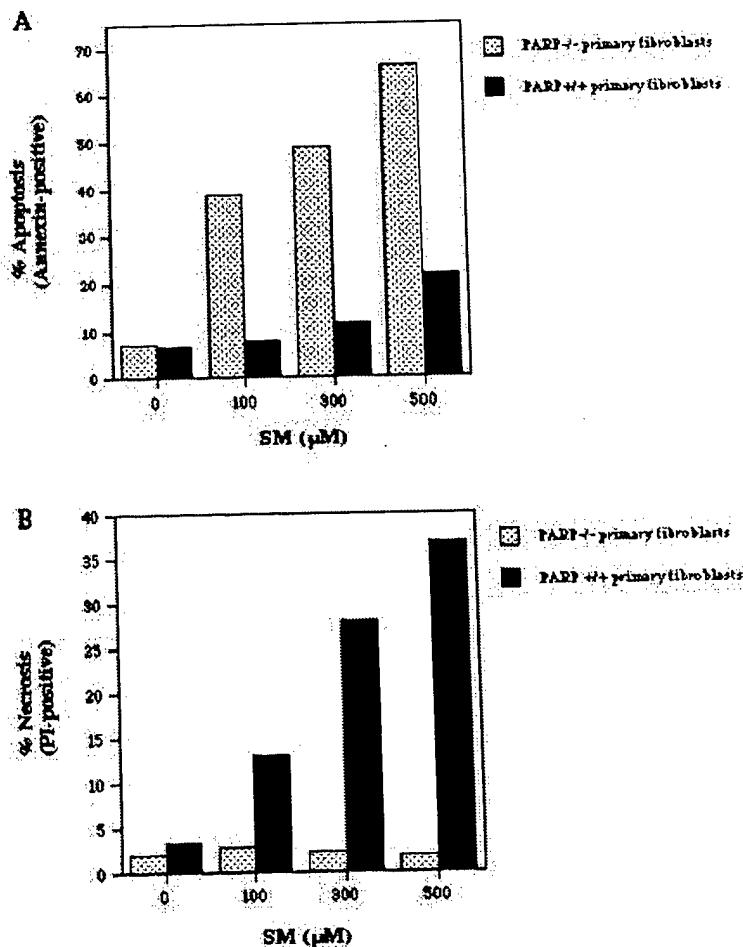


Figure 2

Legend Figure 2

Exposure of primary dermal fibroblast cells derived from PARP^{-/-}, but not PARP^{+/+} mice results in a dose-dependent increase in annexin V-positive cells. Primary dermal fibroblasts were derived from newborn mice as described in Materials and Methods. Cells were incubated for 24 h with the indicated concentrations of SM in KGM, after which cells were prepared and assayed for annexin V binding (A) or PI staining (B) by FACS analysis.

These results suggested that the absence of PARP shifted the mode of cell death from necrosis to apoptosis. To verify if this is the case, we examined the percentage of cells that were positive for PI staining, and were also annexin V negative, indicating a primarily necrotic mode of cell death. PARP^{+/+} primary fibroblasts exhibited a dose-dependent increase in the level of necrosis, while PARP^{-/-} fibroblasts underwent little necrosis at all doses tested (Figure 2B). In a separate set of experiments, we confirmed the mode of cell death induced by SM in wild-type and PARP^{-/-} dermal fibroblasts, using annexin V plus PI staining. Figure 3A clearly shows that the primary mode of cell death in PARP^{+/+} cells is necrotic, while PARP^{+/+} cells undergo apoptosis.

Since the absence of PARP-induced caspase-3 activity and apoptosis in SM-exposed PARP^{-/-} fibroblasts, we examined whether caspase-3 was in fact responsible for SM cytotoxicity in PARP^{-/-} cells. We therefore preincubated PARP^{-/-} cells with either an inhibitor of caspase-3 (DEVD-CHO), or caspase-1 (YVAD-CHO) for 30 min prior to and during SM exposure. PARP^{-/-} fibroblasts that were either not pretreated or pretreated with YVAD-CHO underwent apoptosis following SM exposure (Figure 3B). However, pretreatment with DEVD-CHO abolished the apoptotic response. Thus the deletion of PARP leads to an apoptotic mode of death that is dependent on caspase-3.

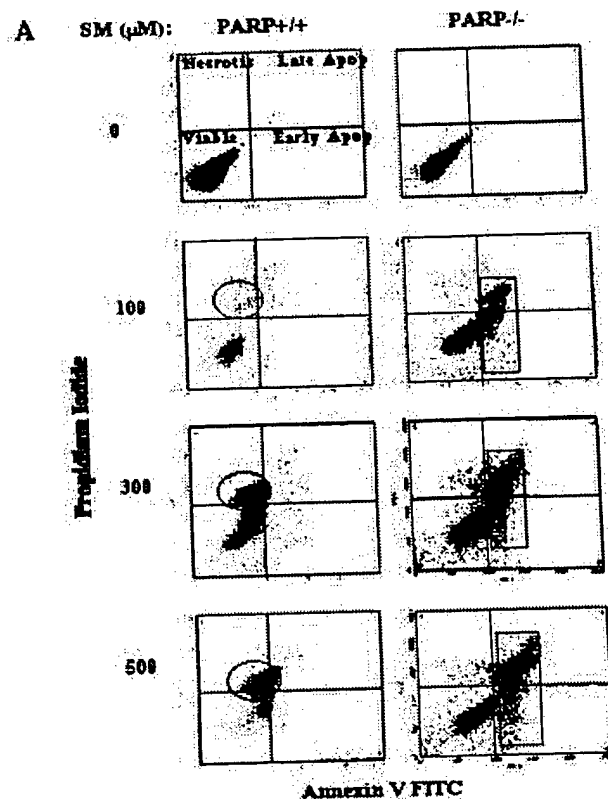
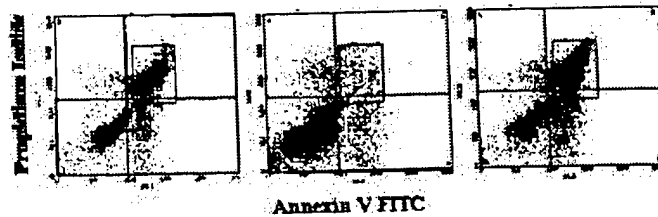


Figure 3





Legend Figure 3

Exposure of primary dermal fibroblast cells derived from PARP^{-/-} mice results in a dose-dependent increase in annexin V-positive cells dependent upon caspase-3 activity. Primary dermal fibroblasts were derived from newborn mice as described in Materials and Methods. Cells were incubated for 24 h with the indicated concentrations of SM in KGM, after which cells were prepared and assayed for annexin V binding plus PI staining at the doses indicated (A), or at 500 μ M SM with or without the indicated caspase inhibitors (B) by FACS analysis. Dot plots of the results show viable (lower left quadrant), early apoptotic (Annexin V-FITC-positive; lower right quadrant), late apoptotic (upper right quadrant), and necrotic (PI-positive; upper left quadrant).

To more firmly establish the role of PARP in the mode of cell death in fibroblasts, we utilized immortalized PARP^{-/-} fibroblasts that were stably transfected with human PARP-1 cDNA or with empty vector alone. Western analysis shows that PARP is expressed in immortalized wild-type fibroblasts (PARP^{+/+}), as well as PARP^{-/-} fibroblasts stably transfected with the human PARP cDNA [PARP^{-/-} (+PARP)], but not in immortalized PARP^{-/-} fibroblasts (Figure 4A). PARP^{-/-} or PARP^{-/-} (+PARP) fibroblasts were then exposed to similar concentrations of SM, and then analyzed for caspase-3 activity by performing an *in vitro* PARP cleavage assay (Materials and Methods). PARP cleavage activity was induced in PARP^{-/-} fibroblasts at SM doses greater than 100 μ M, with >60% cleavage observed at the highest dose of SM tested (500 μ M; Figure 4B). In contrast, the reintroduction of PARP suppressed the *in vitro* PARP cleavage activity, suggesting that expression of PARP rendered these cells more susceptible to necrosis. To further analyze caspase-3 processing, cells were exposed to SM and analyzed for the correct processing of procaspase-3 (32 kDa) into its active form (p17). Caspase-3 processing can clearly be observed at 300 μ M and 500 μ M SM in the PARP^{-/-} cells but not in the cells stably retransfected with PARP cDNA (Figure 4C).

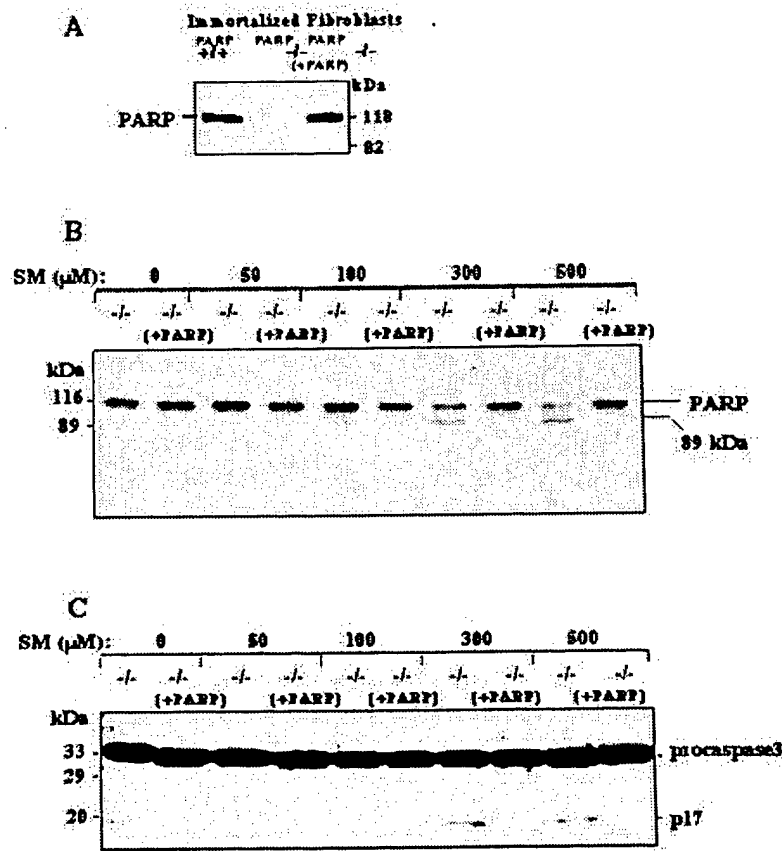


Figure 4

Legend Figure 4

Stable Expression of PARP in immortalized PARP^{-/-} fibroblasts eliminates SM-induced *in vitro* PARP-cleavage activity and inhibits processing of procaspase-3 to its active form. Cultures of fibroblasts were analyzed for expression of PARP by immunoblot analysis (A), and treated with the indicated concentrations of SM (B and C). Extracts were then derived after 24 h and assayed for caspase-3 activity, using [³⁵S] PARP as a substrate (B), or analyzed for caspase-3 processing by immunoblot analysis (C).

To analyze the level of apoptosis after reintroduction of PARP, annexin V plus PI staining was again utilized. Both PARP^{+/+} and PARP^{-/-} (+PARP) immortalized fibroblasts showed only low levels of annexin V staining at all doses of SM tested (<6%; Figure 5A). On the other hand, immortalized PARP^{-/-} fibroblasts demonstrated a dose-dependent increase in annexin V-positive cells, up to a maximum of 22%, corresponding to exposure to 500 μM SM. Conversely, dose-dependent increases in PI-positive cells were observed in both PARP^{+/+} and PARP^{-/-} (+PARP) cells, but not in PARP^{-/-} cells, indicating that the expression of PARP increases the level of necrosis in immortalized fibroblasts.

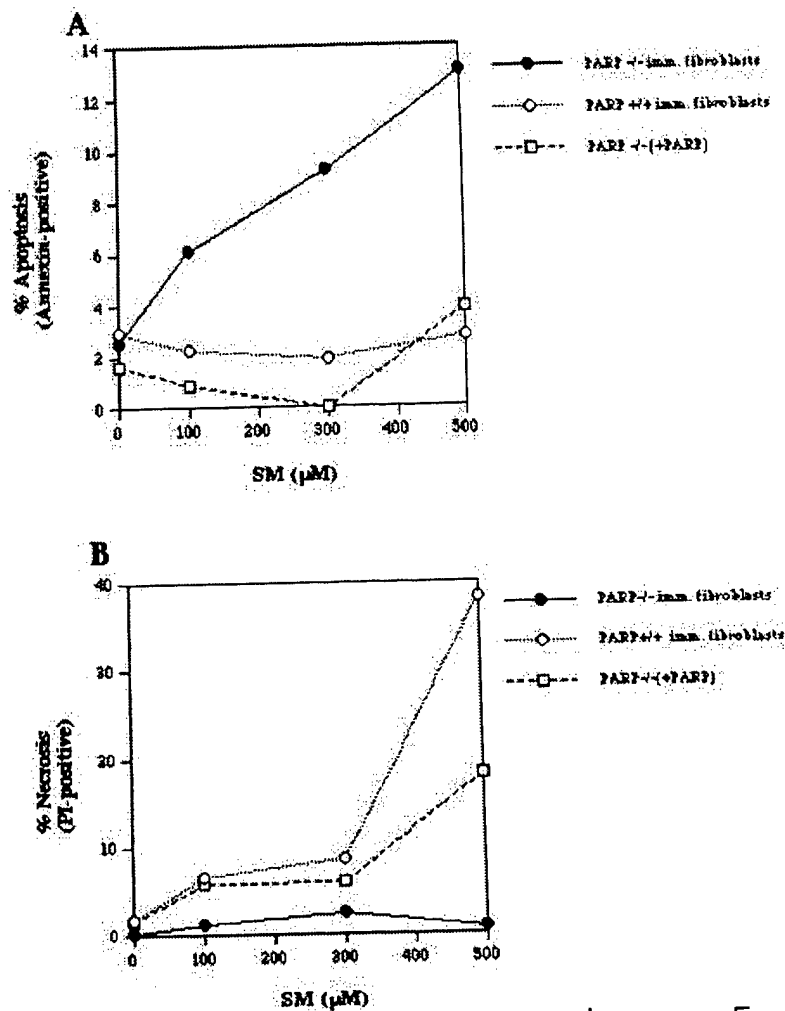


Figure 5

Legend Figure 5

Stable expression of PARP in immortalized PARP^{-/-} fibroblasts results in the elimination of a dose-dependent increase in annexin V-positive cells. Immortalized PARP^{+/+}, PARP^{-/-}, or PARP^{-/-} (+PARP) fibroblasts were incubated for 24 h with the indicated concentrations of SM in KGM, after which cells were prepared and assayed for annexin V binding (A) or PI staining (B) by FACS analysis.

SM induces apoptosis in keratinocytes that is enhanced in the absence of PARP.

We next assessed the role of PARP in the induction of apoptosis in keratinocytes. Primary mouse keratinocytes derived from PARP^{-/-} animals grow poorly under culture conditions that we normally use. In order to obtain a sufficient supply of keratinocytes, we derived keratinocyte clones from PARP^{-/-} and PARP^{+/+} animals following immortalization of cells with a retrovirus that expresses both the E6 and E7 genes of human papillomavirus 16 (HPV 16). A number of clones were found to be both keratin-positive and vimentin-negative by Western analysis, indicating that these cells were in fact keratinocytes, and not fibroblasts (Fig. 6A, middle and bottom panels). As with all other cells utilized in the study, these clones were also confirmed to be either PARP^{-/-} or PARP^{+/+} by immunoblot analysis (Fig. 6A, top panel). Two different clones of each type were then exposed to SM and caspase-3-like activity was determined by a fluorometric assay as described above. All clones demonstrated a very

strong apoptotic response to SM (Figure 6B). At the lower dose (100 μ M), there was no significant difference observed in the level of caspase-3 activity induced by SM, while at the higher dose (300 μ M), extracts from the PARP^{-/-} keratinocytes showed a slightly higher activity (30%). Thus, SM induces apoptosis in keratinocytes that is enhanced in the absence of PARP.

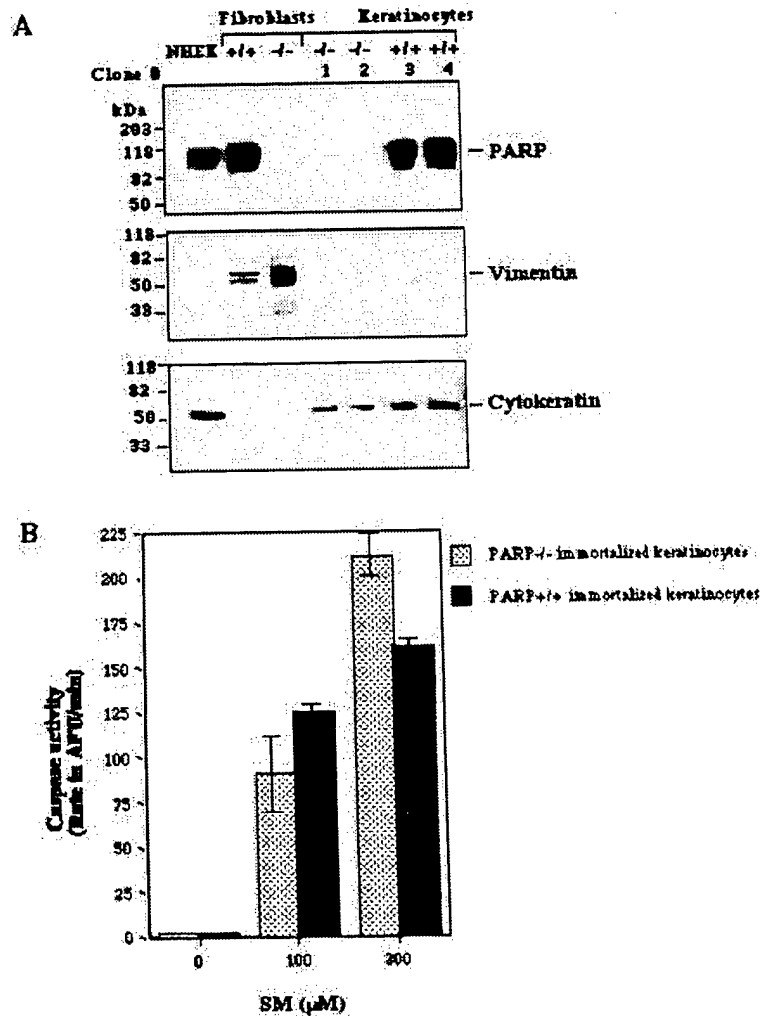


Figure 6

Legend Figure 6

SM induces caspase-3 activity in different clones of both PARP^{-/-} and PARP^{+/+} cells. Primary keratinocytes were derived from PARP^{+/+} or PARP^{-/-} newborn mice and immortalized with the E6 and E7 genes of HPV 16 as described in Materials and Methods. Cell extracts were subjected to immunoblot analysis using antibodies specific for PARP (A, top), vimentin (A, middle), or cytokeratin (A, bottom). Two clones of PARP^{-/-} keratinocytes, and two clones of PARP^{+/+} keratinocytes were then or incubated for 24 h with the indicated concentrations of SM in KGM, after which whole cell extracts were prepared and assayed for caspase-3 activity with the specific substrate DEVD-AMC. Bars = range of average activities for two different clones.

DISCUSSION

SM vesication clearly involves both cytotoxicity and detachment of the epidermal basal layer *in vivo*. Using a cell culture model in the present study, we have described two potential mechanisms for SM-induced keratinocyte basal cell death and detachment: induction of necrosis in dermal fibroblasts and apoptosis in keratinocytes. Since the primary target of SM is the skin, we derived primary skin fibroblasts from PARP^{+/+} or PARP^{-/-} newborn animals to determine the contribution of PARP to SM toxicity. Using quantitative fluorometric assays, primary skin fibroblasts from PARP deficient-mice showed increased caspase-3 activity compared to those derived from PARP^{+/+} animals. Furthermore, FACS analysis with annexin V revealed no binding to PARP^{+/+} cells compared to cells derived from PARP^{-/-} animals. PI staining revealed that PARP shifted the mode of cell death from apoptosis to necrosis. We also utilized immortalized PARP^{-/-} fibroblasts transfected with vector alone or with PARP cDNA. *In vitro* PARP cleavage assays as well as immunoblot analysis revealed that only the PARP^{-/-} cells displayed markers of apoptosis in response to SM exposure. Primary keratinocytes were also derived from newborn PARP^{+/+} and PARP^{-/-} mice, and immortalized with the E6 and E7 genes of human papilloma virus (HPV). In contrast to fibroblasts, keratinocytes from both types of mice express markers of apoptosis in response to SM exposure, consistent with our earlier studies with NHEK (Rosenthal et al., 1998). However, deletion of the PARP gene results in the stronger expression of apoptotic markers in response to 300 μ M SM in the keratinocytes derived from PARP^{-/-} animals.

SM is a strong bifunctional alkylating agent with a high affinity for DNA, and has been shown to induce DNA strand breaks and consequently activate PARP. It is therefore likely that DNA strand breaks and the activation of PARP play a role in the SM-induced cell death in dermal fibroblasts and keratinocytes. SM induces both epidermal cell death and detachment from the basal lamina *in vivo*. The modes of cell death of keratinocytes and fibroblasts both contribute to SM vesication since detachment may be the result of the release of basal lamina-degrading cytosolic components during dermal fibroblast necrosis (Lindsay and Upshall, 1995; Gentilhomme et al., 1998), while keratinocyte basal cell death is primarily due to apoptosis (Rosenthal et al., 1998). Consistent with the results of the present study, our preliminary studies in which newborn wild-type and PARP-deficient mice have been exposed to SM by vapor cup indicate that PARP modulates the response of intact animals as well (Rosenthal et al., manuscript in preparation). The mechanisms that underlie the differences in the modes of cell death in these two cell types remain to be elucidated, but may be either directly related to the levels or activity of PARP in these cells, or may be the result of other events that regulate cellular NAD and ATP levels.

PARP inhibitors can extend the lifespan of lymphocytes treated with SM (Meier and Johnson, 1992; Meier, 1996), and inhibitors of PARP have previously been reported to significantly affect the extent of apoptosis in response to other agents (Rice et al., 1992; Ghibelli et al., 1994; Monti et al., 1994; Kuo et al., 1996). PARP may thus be an important signaling molecule for cell death either via the lowering of NAD and/or ATP levels (Wielckens et al., 1982; Berger et al., 1983; Alvarez et al., 1986), or by poly(ADP-ribosyl)ation of other key cellular proteins, such as p53 (Whitacre et al., 1995; Simbulan-Rosenthal et al., 1999) and the Ca²⁺/Mg²⁺ dependent nuclease involved in the apoptotic cleavage of DNA (Rice et al., 1992). Depletion of cellular NAD and ATP levels via PARP activation can contribute to either apoptotic or necrotic cell death, since partial depletion of ATP induces apoptosis (Feldenberg et al., 1999), while depletion of ATP below a threshold level blocks the later events in apoptosis (Eguchi et al., 1999), thereby shunting the mode of cell death to necrosis.

Since no mutants of PARP have yet been found in the human population, we attempted to regulate the apoptotic response of NHEK to SM by inhibiting endogenous PARP with benzamide. NHEK were exposed to 100 or 300 μ M SM followed by FACS or immunoblot analysis. Similar to our previous results (Rosenthal et al., 1998), and similar to the result for mouse keratinocytes, NHEK showed a strong apoptotic response to SM. We then performed a time course to examine the burst of PARP activity that we had shown to be necessary for Fas-mediated apoptosis in fibroblasts (Simbulan-Rosenthal et al., 1998). Addition of benzamide either decreased or enhanced the apoptotic response depending upon when it was administered relative to SM exposure (Rosenthal et al., manuscript in preparation).

An understanding of the mechanisms for SM-induced cell death in keratinocytes and dermal fibroblasts will hopefully lead to strategies for prevention or treatment of SM vesication. The present study suggests that inhibition of PARP (upstream), or caspase-3 (downstream) may alter the response of the epidermis to SM. PARP inhibitors have already been successfully employed in the prevention of tissue damage in other models, including diabetes (Masiello et al., 1985), ischemia (Endres et al., 1997; Takahashi et al., 1999), and arthritis (Szabo et al., 1998), and may prove effective for SM as well, either alone, or in combination with caspase-3 inhibitors.

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Appended Publication D

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The E7 protein of human papillomavirus type 16 sensitizes primary human keratinocytes to apoptosis

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The 'high risk' human papillomaviruses are associated with the development of anogenital carcinomas and their E6 and E7 genes possess immortalizing and transforming functions in several *in vitro* culture systems. Recently the E6 gene has also been shown to enhance the apoptosis of human mammary epithelial cells. To determine the apoptotic activity of these oncogenes in the natural host cell, we infected genital keratinocytes with retroviruses expressing either HPV-16 E6, E7, or both the E6 and E7 (E6/7) genes. Apoptosis was quantitated under normal growth conditions or when induced by tumor necrosis factor α /cycloheximide or sulfur mustard. In contrast to previous findings with mammary epithelial cells, the E6 gene did not significantly augment either spontaneous or induced apoptosis. E6 also did not suppress apoptosis in normal keratinocytes (despite dramatically reducing their p53 levels), suggesting that p53-independent events mediated this effect. In contrast, E7 increased both spontaneous and induced apoptosis as well as the cellular levels of p53 and p21 protein. Interestingly, co-expression of E6 abrogated E7-facilitated apoptosis by tumor necrosis factor α nearly completely, but had only a minor protective effect on sulfur mustard induced apoptosis in these cells, demonstrating at least in part the p53-dependence and -independence of these two apoptotic pathways. Finally, our results indicate that the apoptosis of normal and E7-expressing keratinocytes is differentially affected by E6 expression and that E7, when unaccompanied by E6, sensitizes keratinocytes to apoptosis.

Keywords: HPV E6 and E7 oncogenes; apoptosis; p53; primary keratinocytes; extended life span

Introduction

Human papillomaviruses (HPV) infect and replicate in stratified squamous epithelia at specific anatomic sites and induce a concomitant hyperplasia of the infected tissues (de Villiers, 1989). A subgroup of the HPV's infecting the genital mucosa are the 'high-risk' human papillomaviruses (e.g. HPV-16 and -18) which are strongly associated with the malignant conversions of

anogenital tract lesions (zur Hausen, 1991). The E6 and E7 genes of the 'high-risk' HPV's are responsible for the transforming/immortalizing activity of the viral genome (Mansur and Androphy, 1993; Stöppler *et al.*, 1994). For example, the co-expression of the E6 or E7 gene with an activated oncogene (e.g. ras) leads to immortalization of primary rodent cells (Chesters and McCance, 1989; Crook *et al.*, 1991a; Liu *et al.*, 1994; Peacock *et al.*, 1990; Phelps *et al.*, 1988; Storey and Banks, 1993; Storey *et al.*, 1988). The expression of either the E6 or E7 genes is also sufficient to transform immortalized rodent cells (Bedell *et al.*, 1989; Kanda *et al.*, 1988; Sedman *et al.*, 1991; Tanaka *et al.*, 1989; Vousden *et al.*, 1988). Finally, the combined expression of E6 and E7 efficiently immortalizes primary human keratinocytes (Barbosa and Schlegel, 1989; Hawley-Nelson *et al.*, 1989; Hudson *et al.*, 1990; Mürner *et al.*, 1989; Sedman *et al.*, 1991), although it does not induce directly the tumorigenic phenotype. Additional cellular genetic changes appear requisite for malignant progression.

The oncogenic properties of E6 and E7 viral proteins correlate with their ability to interfere respectively with the functions of two cellular tumor suppressor proteins, p53 (Huibregtse *et al.*, 1991, 1993; Scheffner *et al.*, 1990, 1992) and Rb (or Rb-related proteins) (Davies *et al.*, 1993; Dyson *et al.*, 1989, 1992). Thus, the oncogenic activity of 'high risk' E7 proteins is at least partly due to its interference with Rb/E2F interactions and the consequent loss of cell cycle control functions of the Rb protein (Goodrich and Lee, 1993). Similarly, the transforming activity of E6 appears partly due to its ability to target p53 protein for ubiquitination and consequent degradation (Crook *et al.*, 1991b; Goodrich and Lee, 1993; Hubbert *et al.*, 1992; Huibregtse *et al.*, 1991, 1993; Li and Coffino, 1996; Scheffner *et al.*, 1990, 1992; Werness *et al.*, 1990). However, since E6 proteins which are unable to target p53 for degradation have a weak immortalizing activity (Band *et al.*, 1993), it is possible that there is an additional cellular target(s) for the E6 protein. Recently two additional E6-associated proteins, E6-BP (Chen *et al.*, 1995) and paxillin (Tong and Howley, 1997), have been discovered which may play a role in cellular transformation. Furthermore, the expression of high risk E6 proteins in primary keratinocytes increases telomerase activity prior to cell immortalization. Normally, telomerase activity is lost in keratinocytes prior to cell senescence (Klingelutz *et al.*, 1996; Stöppler *et al.*, 1997).

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The p53 degradation functions of the E6 proteins are necessary for the efficient immortalization of mammary epithelial cells (MEC) (Band *et al.*, 1990, 1991; Dalal *et al.*, 1996; Wazer *et al.*, 1995) and human kidney cells (Nakagawa *et al.*, 1995). However, despite the loss of p53 protein in E6-immortalized mammary epithelial cells, there is an increased sensitivity to the induction of apoptosis (Xu *et al.*, 1995). In direct contrast, the expression of E6 in transformed cells increases the resistance to the induction of apoptosis (Xu *et al.*, 1995; Thomas *et al.*, 1996; Hickman *et al.*, 1997). The influence of an E6 or E7 expression on apoptosis has been further analysed in the lens morphogenesis of transgenic mice expressing E6, E7 or E6 and E7 (Pan and Griep, 1994, 1995). E6 expression inhibits apoptotic events necessary for the normal development of the eye, whereas E7 transgenic mice demonstrate spatially inappropriate cell proliferation and apoptosis during lens development.

To determine the effects of a HPV-16 E6 or E7 expression on the apoptosis of the natural host cell, the genital keratinocyte, we used retroviruses expressing the E6, E7, or E6 plus E7 (E6/7) genes. Foreskin keratinocytes transduced with any of these recombinant retroviruses exhibit an 'extended life span', compared to keratinocytes infected with control retrovirus (Klingelutz *et al.*, 1994, 1996; Stöppler *et al.*, 1997). 'Extended life span' cells have not yet undergone an immortalization (M2) crisis, which allows the analysis of effects of viral gene expression independently from the immortalization event. When HPV oncogene transduced cells were evaluated for apoptosis induced by either TNF α or sulfur mustard, E6-transduced cells showed a slight increase in comparison to control cells whereas E7-transduced cells showed a 5–10-fold increase in apoptotic signaling. Cells transduced with E6/7 showed either a slight increase in apoptotic response in comparison to control cells or an intermediate apoptotic response between that of control cells and E7 transduced cells, depending upon the agent used for inducing apoptosis. E7 was also able to enhance spontaneous apoptosis in keratinocytes, although the effect was less pronounced than that observed during induced apoptosis.

Results

Induction of an 'extended life span' in primary human keratinocytes

Primary human foreskin keratinocytes, which possess a limited life span *in vitro* and can be passaged only 10–12 times (equivalent to 50–60 population doublings) prior to senescence, were infected with recombinant retroviruses encoding E6, E7, or E6/7 genes as well as the neomycin resistance gene. The cells were then selected in G418 and analysed for their sensitivity to apoptosis. The cells could be cultured for at least twenty passages, indicating that they had an 'extended life span' and had bypassed M1 crisis (the point at which control-transduced cells ceased cell division) (Klingelutz *et al.*, 1994; Stöppler *et al.*, 1997). These cells, however, had not bypassed M2 crisis (cell immortalization) (Shay *et al.*, 1991; Wright *et al.*, 1989) and could not routinely be established

into cell lines. All evaluations of apoptosis were performed on these non-immortalized, post M1 crisis cells.

E7-transduced keratinocytes exhibit the highest levels of spontaneous apoptosis

The percentage of apoptotic cells in post M1 crisis cultures of primary keratinocytes was evaluated by both morphological and biochemical methods. Figure 1 demonstrates an *in situ* DNA-labeling of fragmented DNA of confluent, primary epithelial cells infected with the control (LXSN), E6, E7, or E6/7 retroviruses. This staining technique visualizes cells with nicked chromosomal DNA, a hallmark of apoptotic cells. E7-expressing cells showed the highest level of nuclear staining, indicating a greater degree of DNA breakage. Control transduced cells showed the lowest levels of staining.

The *in situ* DNA labeling results were confirmed by staining these same keratinocyte strains with bisbenzamide (Hoechst 33258) to detect chromatin condensation and fragmentation in the nuclei of apoptotic cells. Figure 2 summarizes the results of three independent experiments in which the percentage of apoptotic cells was quantitated. Approximately 1.5–2.0% of the E7-expressing cells were spontaneously apoptotic, in contrast to 0.5% of control cells. An intermediate apoptosis rate (1%) was observed in E6 or E6/7 cells. The overlap in experimental values between E6 and E6/7 expressing cells precluded making definitive conclusions regarding the differences in the activities of these constructs.

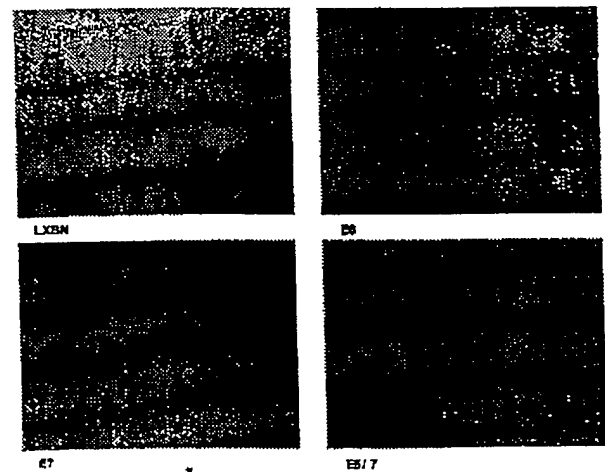


Figure 1 *In situ* labeling of fragmented DNA is highest in the nuclei of E7-transduced keratinocytes. Keratinocytes were transduced with retroviruses expressing either the empty vector, LXSN, or the HPV-16 E6, E7, or E6/7 genes. Spontaneous keratinocyte apoptosis during growth in culture medium was monitored by *in situ* DNA labeling of fragmented DNA (see Materials and methods) in which staining of the nucleus is an indicator of DNA breakage. The highest number of *in situ* DNA-labeled nuclei was observed in E7-expressing cells whereas the nuclei of vector- and E6-transduced were nearly completely negative. Cells transduced with E6/7 showed an intermediate number of positive nuclei. The small spots in E6/7-expressing cells are artifacts of the staining procedure. Microscopic magnification 100 $\times$

TNF α /cycloheximide treatment augments the apoptotic-inducing activity of E7

In an effort to amplify the apoptotic signal in the transduced keratinocytes and to better differentiate between control and transduced cells, we utilized a well-characterized inducer of apoptosis, TNF α . Primary keratinocytes were treated with 10 ng/ml TNF α and 30 μ g/ml cycloheximide and then assayed for apoptotic cells by staining with bisbenzamide as described in the legend of Figure 3a. Qualitatively, E7-transduced cells showed the highest percentage of apoptotic nuclei. When the staining was quantitated (Figure 3b), approximately 1.0% of the TNF α -treated control cells were apoptotic, representing a twofold increase compared to the spontaneous rate (0.5%). Primary keratinocytes were relatively resistant to this induction procedure. In contrast, TNF α induced 12% of the E7-expressing keratinocytes to undergo apoptosis, indicating that E7 had preferentially sensitized these cells. There was now a 12-fold difference in apoptosis between control and E7-transduced cells. E6- and E6/7-transduced cells showed only minimal induction of apoptosis by TNF α (2–3-fold).

A cell death ELISA confirms the sensitization of E7-transduced cells to apoptosis

To validate the morphologic increase of apoptosis in E7-transduced keratinocytes, we performed an ELISA technique (see Materials and methods) to quantitate the amount of histone/DNA fragment complexes which are present in the cytoplasm of apoptotic cells (Figure 4). This independent assay confirmed that E7-transduced keratinocytes were more sensitive (in this case, eightfold) to TNF α -induced apoptosis than control cells. As previously shown in Figure 3, the co-expression of E6 reduced E7-induced apoptotic sensitivity in E6/7-transduced cells close to the levels of E6 cells. The values for apoptosis in non-induced

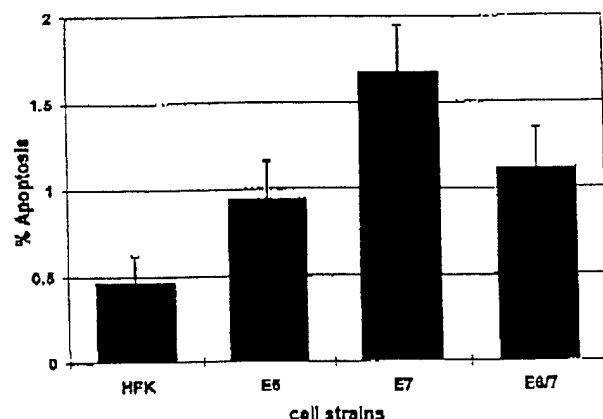


Figure 2 E7-transduced keratinocytes display increased nuclear fragmentation. The spontaneous rate of apoptosis in subconfluent, transduced keratinocytes was analysed in three separate experiments utilizing bisbenzamide (Hoechst 33258) staining (see Materials and methods) to detect the nuclear fragmentation characteristic of apoptosis. In each experiment 300 cells were counted and evaluated for nuclear changes (e.g. Figure 3). Bars indicate the standard error of the mean

control cells was near the limit of experimental detection and, although no reliable conclusion can be made with respect to quantitative differences, cells expressing the E7 and E6/7 genes showed a slight increase in apoptosis in comparison to E6 or control cells in this assay.

Keratinocytes sensitized to apoptosis by E7 contain increased amounts of p53 and p21 protein

To determine whether there was any correlation between the apoptotic sensitivity of the transduced keratinocytes and their expression of p53 protein, a regulator of cellular apoptosis, we screened the above cell strains by Western blotting analysis using a p53-specific monoclonal antibody (Figure 5). The expression of E6 or E6/7 induced a dramatic decrease in p53 levels, a finding which is consistent with the known

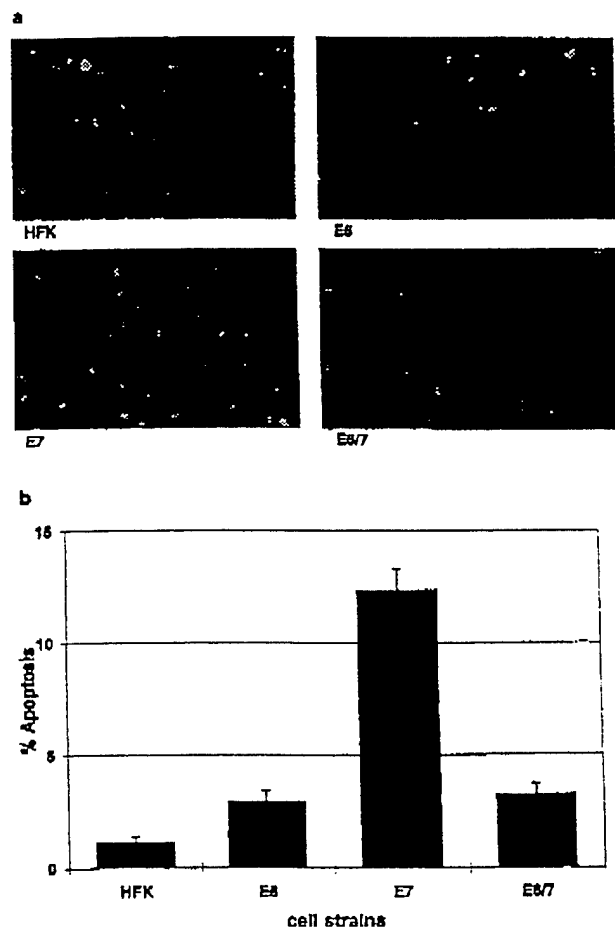


Figure 3 TNF α /cycloheximide treatment augments E7-facilitated apoptosis. (a) Keratinocytes transduced with vector (HFK), E6, E7, and E6/7 were treated for 6 h with TNF α /cycloheximide (see Materials and methods) and then fixed with methanol and stained with bisbenzamide (Hoechst 33258). Characteristic apoptotic changes of chromatin condensation and nuclear fragmentation were observed in a portion of the cell nuclei. E7-expressing cells demonstrated the most prominent degree of nuclear fragmentation. Microscopic magnification 400 $\times$. (b) For quantitation, 300 cells were counted and evaluated for nuclear morphological evidence of apoptosis. The percentage of apoptotic nuclei for each of the keratinocyte strains is indicated and the bar indicates the standard error of the mean for three independent experiments

ability of E6 to target p53 for ubiquitination and degradation. In marked contrast, E7-transduced cells with an 'extended life span' showed increased amounts of p53 protein, which is in agreement with the previous finding of elevated steady state p53 levels in cells immortalized by E7 (Demers *et al.*, 1994).

To determine whether the increase in p53 had functional consequences, we evaluated the expression

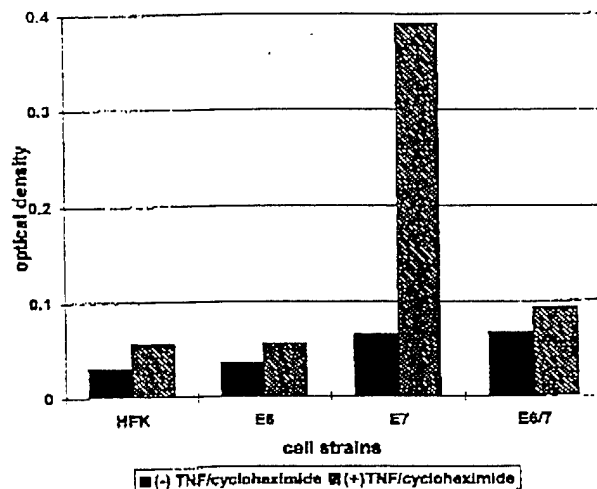


Figure 4 A cell death ELISA confirms that TNF α /cycloheximide treatment enhances the apoptosis of E7-transduced keratinocytes. Cytoplasmic extracts of untreated and TNF α /cycloheximide-treated keratinocytes were prepared as described in Materials and methods. An ELISA technique was used to quantitate the amount of histone/DNA fragment complexes present in the cytoplasm, an independent indicator of apoptotic change. Similar to the morphological findings in Figure 3, the ELISA demonstrated that TNF α -treated, E7-transduced keratinocytes displayed the highest level of apoptosis

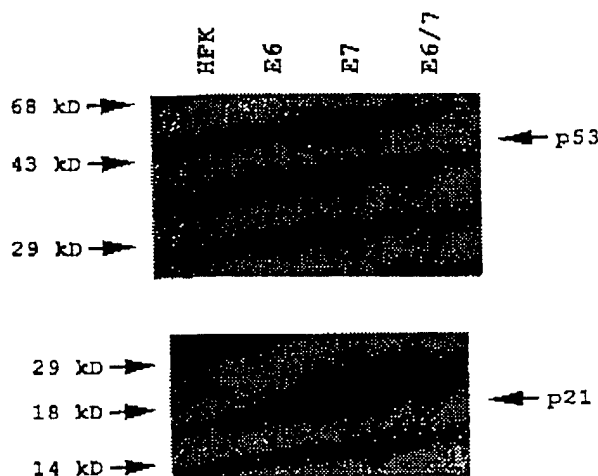


Figure 5 E7-transduced keratinocytes have elevated levels of p53 and p21 protein. The expression of p53 and p21 in control primary keratinocytes and HPV-16 E6, E7 and E6/7 transduced primary keratinocytes was analysed by Western blot using 50 μ g of whole protein cell extract (see Materials and methods). The steady state level of p53 was elevated in E7-expressing cells in comparison to vector-transduced keratinocytes. Little or no p53 protein was detected in the E6 or E6/7 transduced cells. The increased steady state level of p53 in E7-expressing cells correlated with an increased steady state level of p21 in these cells

of a cell regulatory protein, p21, which is normally up-regulated at the transcriptional level by p53 (El-Deiry *et al.*, 1993, 1994). p21 protein was increased in E7-transduced cells (high p53) and decreased in E6- or E6/7-expressing cells (low p53), although the decrease of p21 in E6-expressing cells was not as profound as the loss of cellular p53 (Figure 5). Regardless, it is apparent that the increase in cellular p53 in E7-transduced cells was biologically functional.

Sulfur mustard further increases keratinocyte apoptosis, permitting the detection of poly(ADP-ribose)polymerase (PARP) breakdown

Treatment of keratinocytes by TNF α cycloheximide induced an apoptosis rate of approximately 12%. However, these levels were insufficient to detect the apoptotic-specific breakdown of poly(ADP-ribose) polymerase (PARP) through the ICE like protease, caspase (also known as 'apopain'). Caspase appears to be a converging point for distinct apoptotic pathways (Nicholson *et al.*, 1995) and, in a number of analysed systems, caspase-3 cleaves key proteins involved in the structure and integrity of the cell, including PARP. We treated transduced keratinocytes with sulfur mustard in order to induce a higher rate of apoptosis in these cells (Dabrowska *et al.*, 1996; Rosenthal *et al.*, 1998) and thereby allow us to determine whether the caspase pathway was activated during E7-augmented apoptosis. Preliminary studies showed that the treatment of the cells with 50 μ M sulfur mustard was sufficient to induce apoptosis (by bisbenzimid staining) in 40% of the E7-transduced cells (data not shown). Protein extracts of sulfur mustard treated cells were evaluated for their ability to cleave the 116 kD PARP protein to an 89 kD product (Figure 6a). Control cells and E6-transduced keratinocytes demonstrated the weakest response; in each case approximately 3% of PARP (measured with a Phosphorimager; see Materials and methods) was cleaved following treatment with 50 μ M sulfur mustard (Figure 6b). E7-transduced cells, however, cleaved approximately 24% of PARP and E6/7-expressing cells showed a somewhat lower conversion (20%). Treatment of the cells with 100 μ M sulfur mustard increased the apoptotic breakdown of PARP in primary keratinocytes and E6-expressing cells to approximately 12%, while E7- and E6/7-expressing cells demonstrated no further increase in PARP breakdown.

Discussion

In the current study, we analysed the effects of expressing the E6 and E7 oncogenes (alone or together) on the rate of spontaneous and induced apoptosis in the natural host cell for HPV-16, the genital keratinocyte. In contrast to previous studies with mammary epithelial cells (Xu *et al.*, 1995), the E6 gene had only a minor effect on cellular apoptosis. Rather, the E7 gene was found to strongly sensitize keratinocytes to apoptosis, despite the observation that this gene can independently, but infrequently, immortalize keratinocytes (Halbert *et al.*, 1991). There are parallels for E7 biological activity at the molecular

level; the interaction of E7 with Rb appears not only responsible for the transforming and immortalizing activity of E7, but it might also be responsible for cell sensitization to apoptosis. E7 is known to uncouple E2F function from Rb regulation, and a deregulated E2F activity has been shown to induce apoptosis (Qin *et al.*, 1994; Shan and Lee, 1994; Wu and Levine, 1994) in 32D.3 cells, apparently by a p53-independent mechanism (Hiebert *et al.*, 1995).

The observed sensitization to apoptosis in the E7-transduced cells is in agreement with the effect of E7 expression on the lens development in transgenic mice (Pan and Griep, 1994, 1995). E7 expression in the murine lens, in which apoptosis is necessary for an orderly development of the eye during embryogenesis,

leads to spatially inappropriate cell proliferation and apoptosis. The cell proliferation and apoptotic effects of E7 on murine lens development correlate with its known extension of life span (Klingelutz *et al.*, 1994; Stöppler *et al.*, 1997) as well as its apoptosis sensitizing activity (current study).

The E6 and E6/7-dependent induction of the 'extended life span' in primary keratinocytes was also accompanied by a slight increase in spontaneous apoptosis in comparison to the non HPV gene expressing control cells. Interestingly, even the expression of E6 alone in these cells, which led to a drastic reduction in cellular p53 levels, was unable to protect the keratinocytes from a higher rate (about twofold) of spontaneous apoptosis in comparison to the control cells. A similar effect was observed in E6-transduced cells following the induction of apoptosis by TNF α . The increased rate of apoptosis in E6 expressing cells together with the drastic decrease of p53 levels in these cells suggests that keratinocytes are able to undergo, at least in part, a p53 independent apoptosis. The inability of E6 to inhibit, or at least reduce, the apoptotic rate of primary control keratinocytes is in contrast with the observed ability of E6 to inhibit p53 dependent and independent apoptosis events in the transgenic mouse lens (Pan and Griep, 1994, 1995).

When expressed alone, E7 induced p53 expression in post M1 crisis ('extended life span') cells in comparison to control cells. The increase in p53 levels subsequent to E7 expression has been described previously in epithelial cells (Demers *et al.*, 1994), and in our study the elevated levels of p53 were accompanied by an increase in p21 protein, a downstream target of p53. It is probable that the overexpression of p53 plays a role in E7-induced apoptosis as it does in other systems (Gottlieb and Oren, 1996) and that the reduction of apoptosis in E6/7-transduced cells (either spontaneous or TNF α -induced) is a consequence of degradation of cellular p53 protein by E6 as observed in other cell types (Thomas *et al.*, 1996; Yu *et al.*, 1997). Nevertheless the observation that E6/7-transduced primary human keratinocytes demonstrated slightly higher levels of spontaneous and TNF α -induced apoptosis than control cells, suggested that the E6 expression in E6/7 transduced cells could not fully counteract the E7 induced apoptotic effects. This was most obvious when using a stronger inducer of apoptosis, sulfur mustard. Sulfur mustard-induced E6/7 cells clearly demonstrated a significantly higher apoptotic rate than E6 expressing cells, which was intermediate between the apoptotic rates of E6 and E7 cells. The simplest explanation for these opposite phenotypes induced by TNF α and sulfur mustard is the hypothesis that the apoptotic pathways used are at least in part p53-dependent and -independent mechanisms, respectively.

It is possible that the apoptotic-tempering effects of E6 on E7 may explain the synergistic interactions between these two genes for mediating cell immortalization. By reducing E7-induced apoptosis, E6 would facilitate the progression of a greater number of cells from the M1 'extended life span' phase of cell growth to the M2 phase of cell immortalization crisis. The frequency of cell immortalization would thereby be increased.

In contrast to our current studies with pre-immortalized keratinocytes, it appears that immorta-

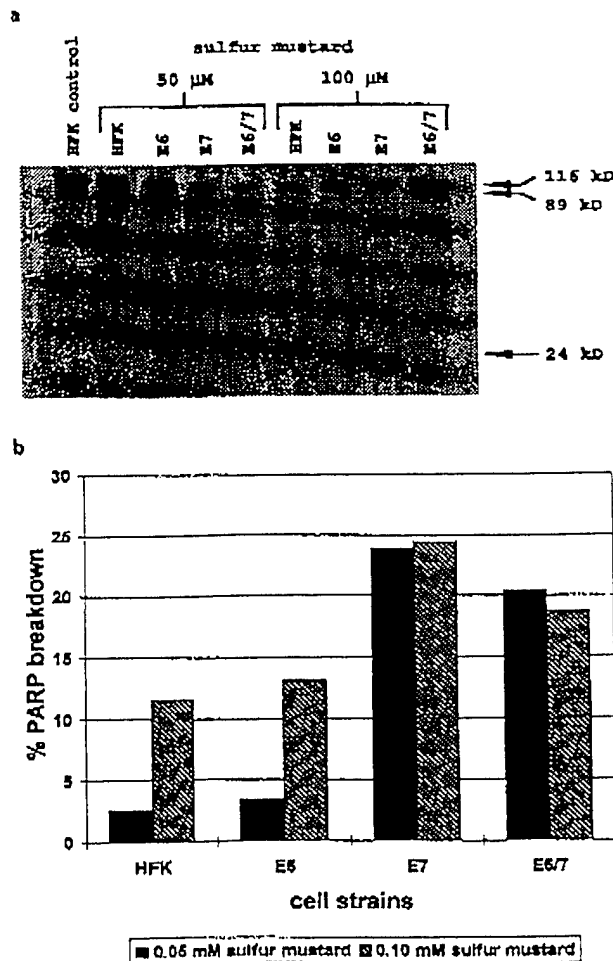


Figure 6 Sulfur mustard-induced apoptosis is enhanced by both E7 and E6/7 and is accompanied by PARP cleavage. (a) The keratinocyte strains described in the previous figures were treated with 50 or 100 μ M sulfur mustard for 24 h. Cytoplasmic extracts were then prepared and assayed for apopain activity using [^{35} S]PARP as a substrate (see Materials and methods). Full-length PARP (119 kD) can be visualized in all lanes. The 89 kD PARP cleavage product is readily detected in cells expressing E7 following treatment with 50 μ M sulfur mustard. (b) Quantitative autoradiography of the above experiment was performed with a phosphorimage analyser and evaluated for the conversion of the 119 to the 89 kD form of PARP. Both the E7- and E6/7-transduced keratinocytes displayed increased apopain cleavage activity indicative of an enhanced apoptotic response

lized keratinocytes (expressing the E6/E7 genes) are resistant to some effects of TNF α (Villa *et al.*, 1992). For example, while primary keratinocytes are sensitive to TNF-mediated growth inhibition, HPV-16- or HPV-18-immortalized keratinocytes are resistant, with HPV-18 cells being the most resistant. Evidently there are additional genetic or epigenetic alterations which occur during immortalization which render keratinocytes less sensitive to the effects of TNF. However, as shown in the current study, pre-immortal cells expressing E6/E7 still retain their sensitivity to TNF-induced apoptosis.

Materials and methods

Cell culture

Primary human keratinocytes were derived from neonatal foreskins as described and grown in KSF medium (Life Technologies Inc.) supplemented with gentamycin. The primary cells were infected with derivatives of the amphotropic LXS_N retrovirus expressing the various HPV-16 open reading frames (E6, E7, or E6 plus E7). The retroviruses were generated as described (Miller and Rosman, 1989) using existing recombinant vectors (Sherman and Schlegel, 1996). Retrovirus-infected cells were selected in G418 (100 μ g/ml medium) for 10 days. G418-resistant colonies were pooled from each transduction and passed every 3–4 days (ratio of 1:5).

TNF α /cycloheximide treatment

The various cells strains were trypsinized 24 h before the induction of apoptosis and passaged at equal cell densities. Twenty-four hours after passaging, cells were treated for 6 h with medium containing 10 ng/ml TNF α (human recombinant expressed in yeast, Sigma) and 30 μ g/ml cycloheximide (Sigma) in tissue culture medium.

Sulfur mustard treatment

Sulfur mustard (bis-(2-chloroethyl)sulfide; >98% purity) was obtained from the US Army Edgewood Research, Development and Engineering Center. Cells were grown in 75 cm² tissue culture flasks to 60–80% confluency, then exposed to sulfur mustard in KFS medium to final concentration of 50 or 100 μ M. Media was not changed for the duration of the experiments (24 h).

Bisbenzimidazole (Hoechst 33258) staining

Following TNF α treatment, the cells were fixed for 5–10 min over methanol fumes at room temperature and then submerged for an additional 5–10 min in methanol at room temperature. Fixed cells were then allowed to air dry and stained for 10 min with bisbenzimidazole (Hoechst 33258 dye (Sigma) 0.5 μ g/ml in PBS). The cells were washed three times with PBS followed by mounting (Fluoromount-G, Southern Biotechnology Associates, Inc.) the cells with a cover slip. The morphology of nuclei was evaluated under a Zeiss Axioskop microscope at a magnification of 400 $\times$.

Detection of DNA fragmentation / in situ DNA-labeling

DNA breaks were detected *in situ* using a Klenow fragment-based assay. Cells were fixed and labeled with biotinylated dUTP (200 μ M) for 30 min at room temperature in reaction buffer containing 200 μ M dA,G,CTP, 50 U/ml Klenow, 50 mM Tris-Cl (pH 7.5), 5 mM MgCl₂, 10 mM β -mercaptoethanol, and 50 μ g/ml BSA. Biotinylated dUTP was

detected using streptavidin-conjugated horseradish peroxidase and VIP substrate (Vector).

PARP cleavage assay

The full-length cDNA clone for PARP (pcD-12 (Alkhatib *et al.*, 1987)) was excised and ligated into the *Xho*I site of pBluescript-II SK⁺ (Stratagene) then used to drive the synthesis of PARP labeled with ³⁵S-methionine (Dupont-NEN) by coupled T7 transcription/translation in a reticulocyte lysate system (Promega). [³⁵S]PARP was separated from the other constituents by gel filtration chromatography on a Superdex-75 FPLC column (Pharmacia; 1 $\times$ 30 cm) in 10 mM HEPES-KOH (pH 7.4), 2 mM EDTA, 0.1% (w/v) CHAPS and 5 mM dithiothreitol.

Cytosolic extracts were prepared from the cells by scraping PBS-washed monolayers in 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 10 μ g/ml pepstatin A, 20 μ g/ml leupeptin, 10 μ g/ml aprotinin (at 1 $\times$ 10⁶ cell/ml). The post-100 000 g supernatant was recovered after centrifugation.

PARP cleavage activity was measured in mixtures containing 5 μ g protein from the cytosol fractions of keratinocytes. Assay mixtures also contained purified [³⁵S]PARP (~5 $\times$ 10⁴ c.p.m.), 50 mM PIPES-KOH, 2 mM EDTA, 0.1% (w/v) CHAPS, and 5 mM dithiothreitol in a total volume of 25 μ l. Incubations were performed at 37°C for 1 h, and terminated by addition of 25 μ l of 2 $\times$ SDS-PAGE sample buffer containing 4% SDS, 4% β -mercaptoethanol, 10% glycerol, 0.125 M Tris-Cl (pH 6.8) and 0.02% bromophenol blue. Samples were resolved by 10% SDS-polyacrylamide gels.

PARP cleavage products were visualized by fluorography and the 89 kDa cleavage product of [³⁵S]PARP was quantified relative to full-length PARP using a Storm 840 PhosphorImage analyzer (Molecular Dynamics). Quantification included a correction for background, as well as for the difference in methionine residues present in the 89 kDa fragment (18 methionine residues) vs full-length PARP (25 methionine residues).

Cell death ELISA

A cell death ELISA kit from Boehringer Mannheim (Indianapolis, IN) was used for the ELISA assay. The cells were prepared as follows for the ELISA. The cells of the various cell strains were trypsinized and then suspended in DMEM medium containing 10% fetal calf serum. 7.5 $\times$ 10⁴ cells of each cell strain were aliquoted into plastic tubes and pelleted by centrifugation (4°C, 900 g for 10 min). After removing the supernatant, 500 μ l of lysis buffer was added. The pellets were carefully resuspended and kept for 30 min on ice for complete cell lysis. The ELISA conditions were in accordance with the manufacturer's instructions.

Western blot

Whole cell extracts were either obtained by repeated freezing and thawing (three times) of harvested cell pellets or by extracting the cells in protein sample buffer containing 2% SDS. The extracts were centrifuged at 10 000 g for 10 min at 4°C the supernatant was separated from undissolved debris, and the protein concentrations of the supernatants were determined using a DC Protein Assay kit (Bio-Rad). Fifty μ g of protein extract were separated on 10% SDS-polyacrylamide gels and blotted onto PVDF membranes (Millipore). p53 was detected using a monoclonal anti p53 antibody (anti p53 Ab2, Oncogene Science) and p21 with a monoclonal anti p21 antibody (clone 6B6, Pharmingen) in conjunction with a chemiluminescence kit (Western-Light, Tropix).

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Appended Publication E

Bhat, K. R., Benton, B. J., Rosenthal, D. S., , S., *Smith, W.J.*, Ray, R. & *Smulson, M.E.* Role of Poly(ADP-ribose) Polymerase (PARP) in DNA Repair in Sulfur-mustard-exposed Normal Human Epidermal Keratinocytes (NHEK). *J Appl Toxic* 20, 00-00- (2000)

Role of Poly(ADP-ribose) Polymerase (PARP) in DNA Repair in Sulfur-mustard-exposed Normal Human Epidermal Keratinocytes (NHEK)[†]

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Key words: sulfur mustard; DNA repair; poly(ADP-ribose) polymerase; keratinocytes; DNA ligase

We previously reported that, in normal human epidermal keratinocytes (NHEK) cultures exposed to the alkylating compound sulfur mustard (bis-(2-chloroethyl) sulfide, HD, 0.3–1 mM), there is a rapid (≤ 1 h) activation (100% above unexposed control) of the DNA repair enzyme DNA ligase I (130 kD) followed by a first-order decay (1–5 h). The DNA ligase activation is accompanied by a time-dependent (0.5–4 h) and significant DNA repair. Inhibition of another putative DNA repair enzyme, poly(ADP-ribose) polymerase (PARP), by using 3-amino benzamide does not affect DNA ligase activation following HD exposure, but increases the half-life of the activated enzyme threefold. To examine the role of PARP in HD-induced DNA ligase activation and subsequent DNA repair, we conducted studies using cultured keratinocytes in which the level of PARP had been selectively lowered ($\geq 85\%$) by the use of induced expression of antisense RNA. In these cells, there was no stimulation of DNA ligase up to 3 h, and a small stimulation (ca. 30% above unexposed control) at 5–6 h after HD exposure. A time-course (0.5–6 h) study of DNA repair in HD-exposed PARP-deficient keratinocytes revealed a much slower rate of repair compared with HD-exposed NHEK. The results suggest an active role of PARP in DNA ligase activation and DNA repair in mammalian cells, and also indicate that modulation of PARP-mediated mechanisms may provide a useful approach in preventing HD toxicity. Copyright © 2000 John Wiley & Sons, Ltd.

INTRODUCTION

When cellular DNA is damaged, poly(ADP-ribose) polymerase (PARP), a DNA-break-dependent enzyme, is activated. The specific role of PARP activation in DNA repair is yet to be delineated. A study of base excision repair of 5-hydroxymethyl-2'-deoxythymidine in Chinese hamster cells suggests that normal base excision repair stimulates PARP.¹ Concurrent with PARP activation, DNA ligase activation in mammalian, including human, cells has been reported.² On inhibition of PARP by 3-amino benzamide (3-AB), DNA ligase appears to be inhibited, and this observation has led to the conclusion that PARP participates in DNA repair at the ligation step.^{3–6} Sulfur mustard (bis-(2-chloroethyl) sulfide, HD), a toxic vesicant, causes activation of PARP⁷ and DNA ligase in normal human epidermal keratinocytes (NHEK).^{2,8–10} Exposure of NHEK to HD results in alkylation of cellular DNA and single- and double-strand breaks. The major product of

alkylation is N-7 alkyl guanine and minor alkyl adducts of other bases.¹¹ Activation of these two enzymes strongly suggests that the cells' DNA repair pathway is activated in response to HD insult. We have reported previously that the activation of DNA ligase does not involve ADP ribosylation of the enzyme.⁹ However, in the presence of 3-AB, the activated state of DNA ligase was prolonged, suggesting a linked role of the two enzymes in responding to HD insult. The 3-AB is a non-specific inhibitor and, as such, the results are less precise to define the role of PARP in DNA repair. With the availability of a human keratinocyte cell line in which PARP can be inhibited specifically by induction of PARP antisense expression, the role of PARP in the repair of HD damage can be defined more precisely. In addition, we presented a hypothetical mechanism where both PARP and DNA ligase simultaneously participate in DNA repair,¹² and this cell line is a useful model to study how PARP affects HD damage repair by its presence or absence. In this report, we present results suggesting that in PARP-deficient cells DNA repair is slower, and the HD-induced DNA ligase activation usually seen in NHEK is very low and delayed. These data suggest a specific role of PARP in mammalian DNA repair.

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[†] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Army or the Department of Defense.

EXPERIMENTAL

Materials

Sulfur mustard (HD) was obtained from the US Army Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, MD, and subsequently analyzed (98% pure) in house before use. The NHEK and keratinocyte growth medium (KGM) were purchased from the Clonetics Corp (San Diego, CA). Oligo-dT cellulose and poly-dA were purchased from Pharmacia LKB (Piscataway, NJ). [^3H]-Thymidine triphosphate (activity, 2.5 mCi ml^{-1}) was obtained from New England Nuclear Corp. (Boston, MA). The 3-AB, nucleotides, Klenow enzyme and spin column-

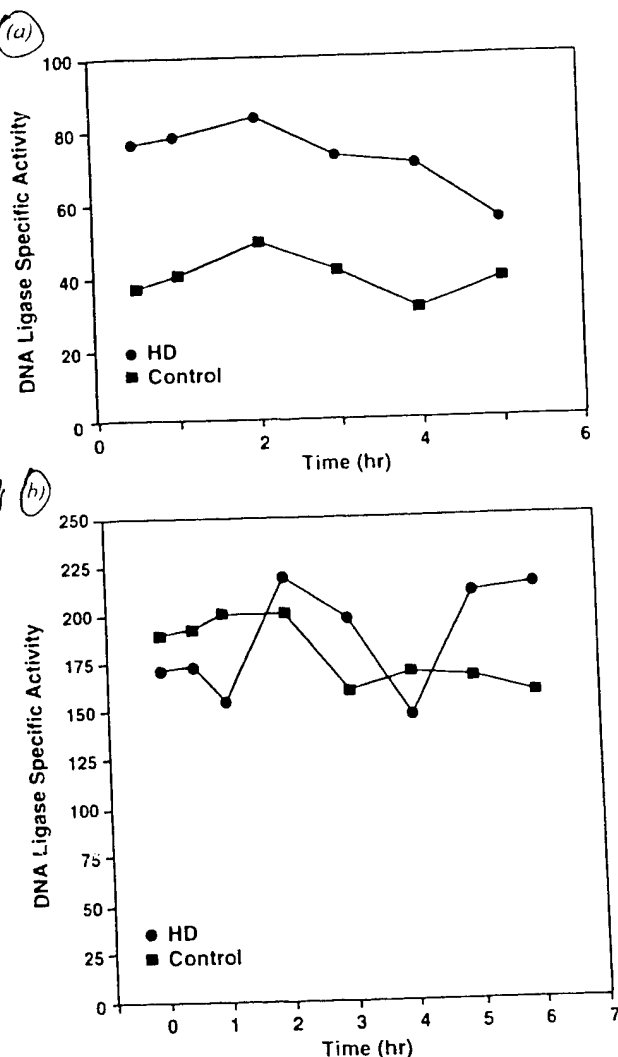


Figure 1. Activation of DNA ligase following HD exposure. Variation of DNA-ligase-specific activity with time (HD-exposed and control): (A) NHEK; (B) PARP(-) HEK. One unit of DNA ligase is the amount of enzyme required to ligate 10 pmol of substrate in 30 min at 37°C. Specific activity is in U mg^{-1} protein. Values are the mean $\pm$ SEM from three separate experiments. The SEM bars are too small to be visible outside the symbols.

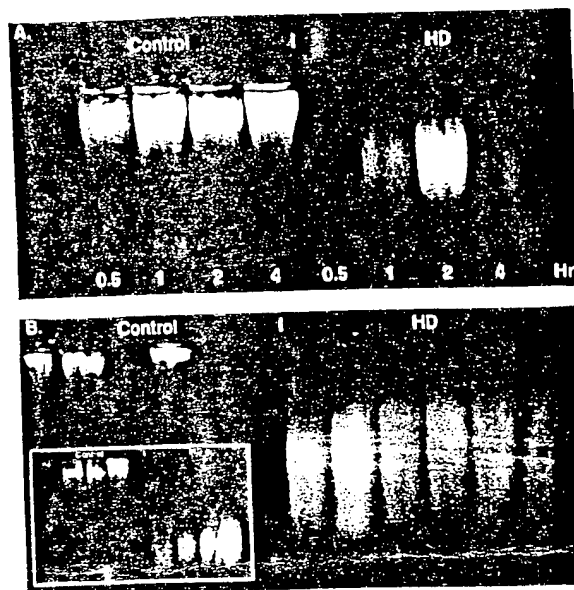


Figure 2. Alkaline agarose gel analysis of 300 μM HD-exposed cellular DNA: (A) NHEK DNA at time intervals of 0.5, 1, 2 and 4 h after HD and the corresponding unexposed control cellular DNA; (B) PARP(-) HEK DNA at time intervals of 0.5, 1, 2, 3, 4 and 6 h after HD (from left to right) and the corresponding unexposed control cellular DNA. Inset: PARP(-) HEK with 1000 μM HD at 0.5, 1, 2 and 4 h (left to right).

30' were obtained from Sigma Chemical Co. (St. Louis, MO) and the protein assay reagent was from Biorad (Richmond, CA). All other chemicals used were of analytical grade.

Cell culture

Stock NHEK (passage 2) were cultured up to three passages in 5% CO_2 95% air at 37°C in a humid atmosphere¹³ in KGM. Inducible PARP(-) human epidermal keratinocytes (HEK), previously described,¹⁴ were cultured in a medium containing 75% KGM, 22.5% Dulbecco's minimum essential medium (DMEM) and 2.5% fetal calf serum in the same incubating conditions used for NHEK. The PARP antisense mRNA was induced in these cells by culturing them in the presence of 1 μM dexamethasone for 72 h when PARP activity was reduced to ca. 10–15% of that in uninduced cells.

Exposure of cells to HD

The culture medium from the control and experimental cultures was aspirated and then replaced with KGM prewarmed to 37°C (control cells) or KGM containing HD (experimental cells). The cells were incubated for 30 min in the hood and then the medium from both control and experimental cells was removed. The cells were washed twice with warm 37°C phosphate-buffered saline (PBS) and set to culture in fresh prewarmed KGM. The cells then were harvested at fixed time intervals for cell-free extract preparation, DNA ligase assay and/or preparation of DNA for repair assay.

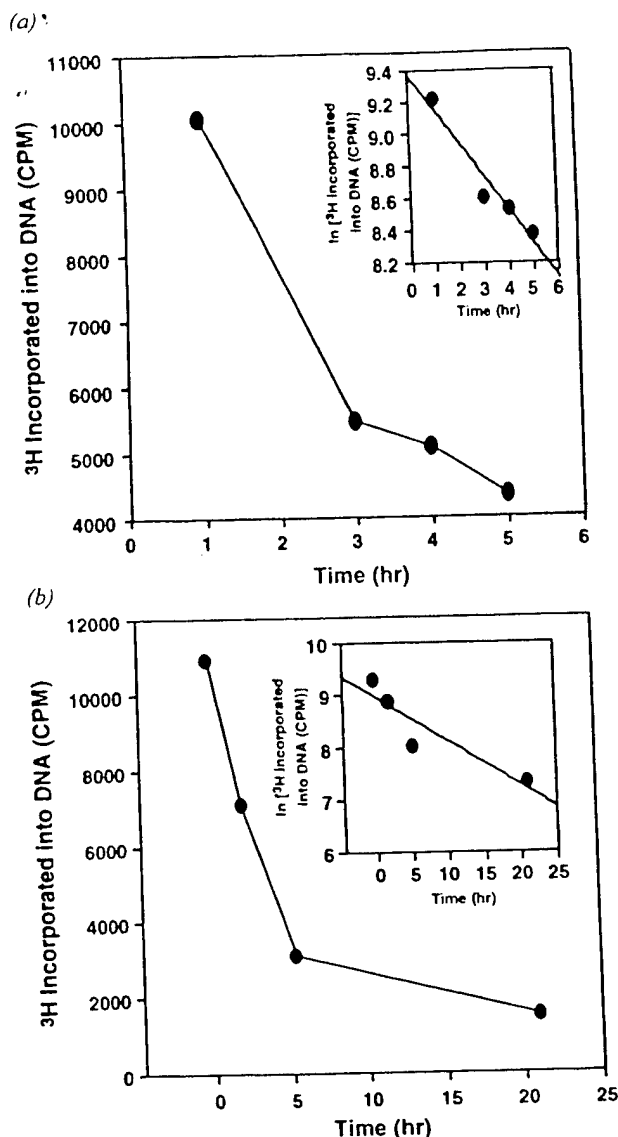


Figure 3. Kinetics of DNA repair in 300 μ M HD-exposed NHEK (A) and dexamethasone-induced PARP(-) HEK (B). Values are the mean $\pm$ SEM from three separate experiments. The SEM bars are too small to be visible outside the symbols.

Table 1. Estimated first-order rate constants for the repair of HD damage in human keratinocytes

Cell type	DNA repair rate constant (h^{-1}) ^a
NHEK	0.210
PARP(-), uninduced	0.072
PARP(-), induced	0.068

^aAverage of two separate determinations.

DNA ligase assay

The DNA ligase assays were done using the oligo-dT-poly-dA solid-state substrate¹⁵ used by Bhat and Grossman¹⁶ for the purification of DNA ligases from human placenta.

DNA repair assay

The action of HD on cellular DNA results in both single- and double-strand breaks that may be similar to apoptosis. A sensitive assay described by Basnakian and James¹⁷ to study apoptosis has been adapted to examine the repair of damaged DNA with time, when a pattern opposite to that of apoptosis is expected and was observed. The assay measures the decreased incorporation of ³H-labeled nucleotide to the available 3'-OH priming sites. With the progress of repair, the number of priming sites decreases and, as a result, the incorporation of radiolabel decreases. This gives a measure of the progress of repair. The assay mixture (total volume of 25 μ l) contained the following: 0.25 μ g of heat-denatured test DNA; 0.05 mM dATP, dGTP and dCTP; 0.0064 mM TTP and 0.5 μ Ci of [³H]TTP (activity, 2.5 mCi ml⁻¹) in a reaction buffer of 10 mM TRIS-HCl (pH 7.5), 5 mM MgCl₂ and 1 mM DTT; 0.5 U of Klenow enzyme per assay. The reaction mixture was incubated for 30 min at 22°C and then terminated by adding an equal volume of a termination buffer containing 12.5 mM EDTA and 100 mM NaCl in 10 mM TRIS-HCl (pH 7.5). The reaction mixtures then were passed through 'spin column-30', and the elutes were counted in a scintillation counter.

Agarose gel

Agarose gels were run according to the procedures described.¹⁸

Protein assay

Protein assays were done using Biorad protein assay reagent. Bovine γ -globulin was used as the standard.

RESULTS AND DISCUSSION

The DNA ligase specific activity in cell-free extracts of control and HD-exposed NHEK and PARP(-)-induced HEK as a function of time is shown in Fig. 1. The DNA ligase activation observed in the case of NHEK appears to be absent in PARP(-)-induced HEK, and a small increase is observed at much longer times compared with NHEK. The data show marked differences between the two cell systems with respect to specific activity as well as HD-induced activation. Moreover, a somewhat perplexing observation was the lack of activation in PARP(-)-uninduced HEK (data not shown). The reason is not obvious. However, it has to be recognized that NHEK are low-passage (P3) primary cells, whereas PARP(-) HEK represent a transformed cell line with higher mitotic and DNA replication rates. The DNA ligase activity is higher during DNA replication.¹⁰ The differences observed

between NHEK and PARP(-) HEK may, therefore, be reflecting the differences in their mitotic activity and in the speed of response, i.e. activation of the DNA repair pathway following HD insult.

To examine further the DNA repair process following HD exposure of these cells, DNA was prepared from these cells at different time intervals and was analyzed on alkaline agarose gels, which is shown in Fig. 2. In HD-exposed NHEK, DNA is nearly completely repaired in ca. 4 h, whereas the repair of PARP(-) HEK is comparatively much slower. This suggests that in PARP(-) HEK an essential component of the DNA repair pathway is missing, affecting the overall repair process. Because specifically PARP is inhibited, this missing component may be identified with PARP. Interestingly, it has been observed that, in a reconstituted human DNA repair system using HeLa cell extracts, ligation efficiency/DNA repair efficiency was found to be low,¹⁹ which may be attributed to the lack of added PARP in the repair system. However, it should be noted that DNA repair proceeds in the absence of PARP but at a lower rate. These results, therefore, suggest an auxiliary but essential role for PARP in the repair of DNA damage.

We examined the kinetics of the repair process by using a sensitive 'strand break' assay. In Fig. 3 the incorporation of radio nucleotide into DNA as a function of time is presented. The incorporation profile may be described by an exponential. Based on this assumption, a monomolecular rate constant for the repair can be derived (see the inset to figures). In Table 1, the estimated rate constants are given. The data for uninduced PARP(-) HEK are not shown in Fig. 3.

It is clear from the derived rate constants that in NHEK the repair of the damage is rapid, in agreement with the results of alkaline agarose gel analysis. The DNA repair in dexamethasone-induced PARP(-) HEK

is ca. 60–70% slower compared with NHEK. However, the assay shows a marginal difference between induced and uninduced PARP(-) HEK. The kinetic data are in conformity with the results of agarose gel analysis. More careful studies are needed to examine the difference between the uninduced and induced cells. In addition, similar data corresponding to the untransformed parent HEK are also needed. We determined the level of PARP in NHEK as well as in uninduced and induced PARP(-) HEK by Western blotting using PARP antibody. The PARP was reduced by ca. 90% in induced cells compared with the other two cell types, indicating that the dexamethasone treatment successfully induced PARP antisense mRNA (data not shown). Very recently, the existence of a human PARP gene family with at least three closely related members—ca. 113-kDa PARP-1 and ca. 60-kDa PARP-2 and PARP-3—was reported.^{20,21} Studies on the characterization and biological functions of these isoforms in the cell types used in the present investigation may explain the results described in Table 1. Overall, the results from this study suggest that a decreased or an absence of PARP appears to lower the rate of DNA repair. This conclusion is supported by a similar observation made by Trucco *et al.*, who reported a substantial delay in DNA strand-break repair following treatment of PARP-deficient cells with monofunctional alkylating agents.²² Previously we have suggested a mechanism¹² in which PARP stabilizes strand breaks and alters chromatin structure. The PARP and DNA ligase compete for strand breaks, and this competition is eliminated on self-ADP-ribosylation of PARP, a mechanism consistent with the observed decrease in the rate of DNA ligation in the absence of PARP.¹⁹ Our conclusion of an essential role of PARP in the DNA repair mechanism based on our observations of a delayed DNA ligase activation and DNA strand-break repair due to PARP deficiency is supported by other reports.^{22–24}

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Appended Publication F

Rosenthal, D. S., Simbulan-Rosenthal, C.M., Iyer, S., Smith, W.J., Ray, R. & **Smulson, M.E.** Calmodulin, Poly(ADP-Ribose) Polymerase and p53 are Targets for Modulating the Effects of Sulfur Mustard. *J Appl Toxic* 21, 00-00 (2001)

Calmodulin, Poly(ADP-Ribose) Polymerase and p53 are Targets for Modulating the Effects of Sulfur Mustard

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Key words: apoptosis; BAPTA; calmodulin; caspase-3; differentiation; keratinocytes; knockout mice; p53; poly(ADP-ribose) polymerase; sulfur mustard; W-7

We describe two pathways by which the vesicating agent sulfur mustard (HD) may cause basal cell death and detachment: induction of terminal differentiation and apoptosis. Following treatment of normal human epidermal keratinocytes (NHEK) with 10 or 100 μ M HD, the differentiation-specific keratin pair K1/K10 was induced and the cornified envelope precursor protein, involucrin, was cross-linked by epidermal transglutaminase. Fibronectin levels were reduced in a time- and dose-dependent manner. The rapid increase in p53 and decrease in Bcl-2 levels was consistent not only with epidermal differentiation but with apoptosis as well. Further examination of biochemical markers of apoptosis following treatment of either NHEK or human papillomavirus (HPV)-immortalized keratinocytes revealed a burst of poly(ADP-ribose) synthesis, specific cleavage of poly(ADP-ribose)polymerase (PARP) *in vivo* and *in vitro* into characteristic 89 and 24 kDa fragments, processing of caspase-3 into its active form and the formation of DNA ladders. The intracellular calcium chelator BAPTA suppressed the differentiation markers, whereas antisense oligonucleotides and chemical inhibitors specific for calmodulin blocked both markers of differentiation and apoptosis. Modulation of p53 levels utilizing retroviral constructs expressing the E6, E7 or E6 + E7 genes of HPV-16 revealed that HD-induced apoptosis was partially p53-dependent. Finally, immortalized fibroblasts derived from PARP $-/-$ 'knockout mice' were exquisitely sensitive to HD-induced apoptosis. These cells became HD resistant when wild-type PARP was stably expressed in these cells. These results indicate that HD exerts its effects via calmodulin, p53 and PARP-sensitive pathways. Copyright © 2001 John Wiley & Sons, Ltd.

INTRODUCTION

Sulfur mustard (bis-(2-chloroethyl) sulfide; HD) is a strong vesicating agent. The ability of HD to cause DNA breaks is a potential mechanism for its cytotoxic effects.¹ The DNA strand breaks bind to and catalytically activate poly (ADP-ribose) polymerase (PARP), which utilizes NAD as a substrate to poly (ADP-ribosyl)ate key nuclear proteins. Activation of PARP can therefore cause a drastic reduction in the levels of cellular NAD and ATP.^{2,3} We have recently examined this process using a human skin graft derived from human keratinocytes stably transfected with a PARP antisense inducible vector.⁴ Recent studies by our laboratory and others have further implicated PARP as an important player in apoptosis. Proteolytic cleavage of PARP was first demonstrated in chemotherapy-induced apoptosis⁵ and the specific proteolysis of PARP is now closely associated with apoptosis in different systems.⁶⁻⁸ We recently showed that the reversible stage of apoptosis is characterized by the

transient activation of PARP and poly(ADP-ribosyl)ation of nuclear proteins followed by the breakdown of poly(ADP-ribose) (PAR) and PARP,⁹ and that this 'burst' of PAR synthesis is a requirement for Fas receptor-mediated apoptosis.¹⁰

Sulfur mustard has also been shown to alter calcium homeostasis in keratinocytes¹¹⁻¹³ and calcium-buffering experiments have supported the role of calcium in the etiology of HD-induced cytotoxicity.¹⁴ The cytotoxic effect of increased levels of intracellular calcium in keratinocytes may stem from its role in keratinocyte differentiation and apoptosis. Elevated levels of intracellular calcium induce terminal differentiation in murine and human keratinocytes.¹⁵⁻¹⁸ The role of calcium in apoptosis was first established by Kaiser and Edelman¹⁹ using glucocorticoid-stimulated thymocytes, and has since been confirmed by numerous other studies. In addition to indirect effects, calcium plays a direct role in apoptosis by inducing the endonuclease responsible for the internucleosomal DNA cleavage, yielding the characteristic apoptotic DNA ladders.²⁰ The observed rise in intracellular calcium during apoptosis can occur by two mechanisms. The first involves stimulation of protein tyrosine kinases, leading to the activation of phospholipase C, the formation of IP3 and calcium mobilization.^{21,22} The second mech-

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anism involves oxygen radicals, which damage calcium transport systems localized in the endoplasmic reticulum (ER), mitochondria and plasma membrane, leading to a disruption in calcium homeostasis and a sustained increase in intracellular calcium levels.²³

Experiments utilizing specific inhibitors have also demonstrated the importance of calmodulin in programmed cell death. The sensitivity of apoptosis to cyclosporin A also suggests a role for calcium-calmodulin 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 calcium-calmodulin complexes in programmed cell death.²⁴

The targets of these apoptotic pathways are a family of cysteine proteases known as 'caspases', named for their preference for aspartate at their substrate cleavage site.²⁵ In collaboration with others, we have characterized caspase-3, which appears to be a converging point for different apoptotic pathways.⁷ 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.^{6,7,26,27}

We presently demonstrate that HD induces both terminal differentiation and apoptosis in human keratinocytes. Further, we demonstrate that the apoptotic process is dependent upon calmodulin, p53 and PARP. The induction of terminal differentiation and apoptosis may in part explain the death and detachment of basal cells of the epidermis that occurs following exposure to HD.

MATERIALS AND METHODS

Cells

Normal human epidermal keratinocytes (NHEK) were obtained as primary cultures from Clonetics (San Diego, CA) and maintained in serum-free keratinocyte growth medium (KGM). The NHEK were grown in 75-cm² tissue culture flasks to 60–80% confluency and then exposed to HD diluted in KGM to final concentrations of 10, 100 or 300 μ M.

Chemicals

Bis-(2-chloroethyl) sulfide (HD, >98% purity) was obtained from the US Army Edgewood Research, Development and Engineering Center. Glycine, *N,N'*-[1,2-ethanediylbis(oxy-2,1-phenylene)]bis[*N*-[2-[(acetyloxy)methoxy]-2-oxoethyl]]-bis[(acetyloxy)methyl]ester (BAPTA-AM) was purchased from Molecular Probes (Eugene, OR). *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was obtained from Sigma (St Louis, MO).

Antibodies

For immunoblotting. The following antibodies were obtained from Sigma: mouse monoclonal antibody (clone 8.60) that recognizes both K1 and K10 keratins; monoclonal antibody (clone SY5) against the 68-kDa cornified envelope precursor, involucrin; and affinity

purified rabbit antiserum against the attachment protein fibronectin, recognizing both a 220-kDa and a 94-kDa form of the protein.

For FACS. Phycoerythrin-conjugated antibodies specific for p53 nuclear antigen (G59-12 and PAB 1801) were obtained from Pharmingen (San Diego, CA). The FITC-conjugated anti-human Bcl-2 antibody (clone 124) was from DAKO (Capintaria, CA).

Immunoblot analysis

For immunoblot analysis, proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to nitrocellulose filters. Proteins were measured and normalized prior to gel loading. Immune complexes were visualized by electrochemiluminescence (Amersham).

FACS analysis

Medium was decanted, trypsin-EDTA was added for 5 min and the cells were removed from the flasks by scraping. The cell suspension was mixed with trypsin neutralizing solution, washed in KGM and fixed with 1% formaldehyde for 15 min followed by 70% ethanol. Fixed cells were stored at -200°C until stained for cytometry. Flow cytometric analyses were conducted on a Becton-Dickinson FACStar Plus cytometer using a 100-mW air-cooled argon laser at 488 nm.

Poly(ADP-ribose)polymerase cleavage assay

In vitro transcription and translation of full-length PARP cDNA, purification and *in vitro* cleavage by cell extracts have been described in detail. The PARP cleavage products were visualized either by fluorography or else the 89-kDa cleavage product of [³⁵S]PARP was quantified relative to the full-length PARP using a Storm 840 PhosphorImage analyzer (Molecular Dynamics). Quantification included a correction for background, as well as for the difference in methionine residues present in the 89-kDa fragment (18 Met residues) vs full-length PARP (25 Met residues).

RESULTS AND DISCUSSION

Sulfur mustard induces markers of terminal differentiation in both primary and immortalized keratinocytes

Immunoblot analysis revealed that the suprabasal-specific keratin pair K1/K10 was induced after NHEK were exposed to 10 μ M HD (Fig. 1A). Stronger induction was observed following treatment with 100 μ M HD (not shown). In extracts derived from untreated cells, the cornified envelope precursor protein involucrin migrates as a 68-kDa monomer form. The staining pattern shifted to higher molecular weight forms following 24 h exposure to 10 μ M (Fig. 1B) or 100 μ M HD, suggesting that the protein is cross-linked in response to HD. Fibronectin is produced in keratinocytes (as well as fibroblasts) in 220-kDa

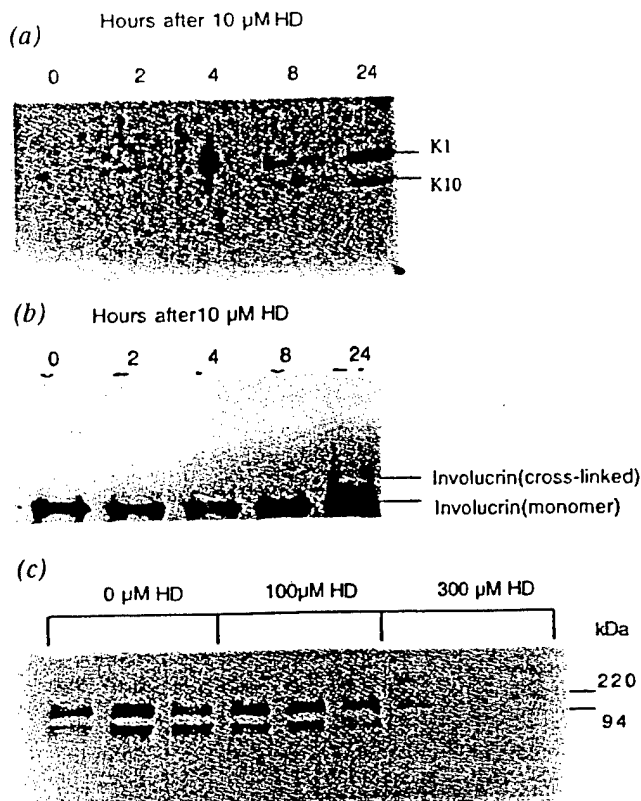


Figure 1. Modulation of differentiation markers and attachment proteins by HD. The NHEK were treated with 10 μ M HD (A, B) and 100 or 300 μ M HD (C) for the indicated times, harvested and total cell extracts were immunoblotted using antibodies specific for K1 + K10 (A), involucrin (B) or fibronectin (C).

and 94-kDa isoforms. The levels of both isoforms are reduced in a dose-dependent fashion after HD exposure (Fig. 1C).

Sulfur mustard can induce an increase in intracellular free calcium,¹² which is associated with terminal differentiation.^{17,28,29} Pre-incubation with BAPTA-AM for 30 min prior to HD treatment suppressed keratin K1 expression in response to HD (Fig. 2A). Although BAPTA treatment suppressed total protein synthesis by 50% after 24 h (not shown), this effect was not enough to account for the complete suppression of K1. Calcium may induce differentiation via its role in the activation of protein kinase C,³⁰ and calcium-calmodulin complexes modulate this response.³¹ Figure 2B shows that a 30-min pretreatment with the calmodulin inhibitor W-7 prior to exposure to 100 or 300 μ M HD inhibited K1 expression. Interestingly, calmodulin itself was down-regulated by HD, and reverse-transcription polymerase chain reaction revealed this down-regulation to be at the mRNA level (not shown).

Sulfur mustard suppresses Bcl-2 and induces p53

To examine possible mechanisms by which HD altered the differentiation response, we initially examined the expression of the *bcl-2* gene product, which inhibits both keratinocyte differentiation and apoptosis. The Bcl-2 levels are high in basal keratinocytes and are reduced in the differentiating layers of the epidermis.³² Furthermore, expression of *bcl-2* antisense RNA can

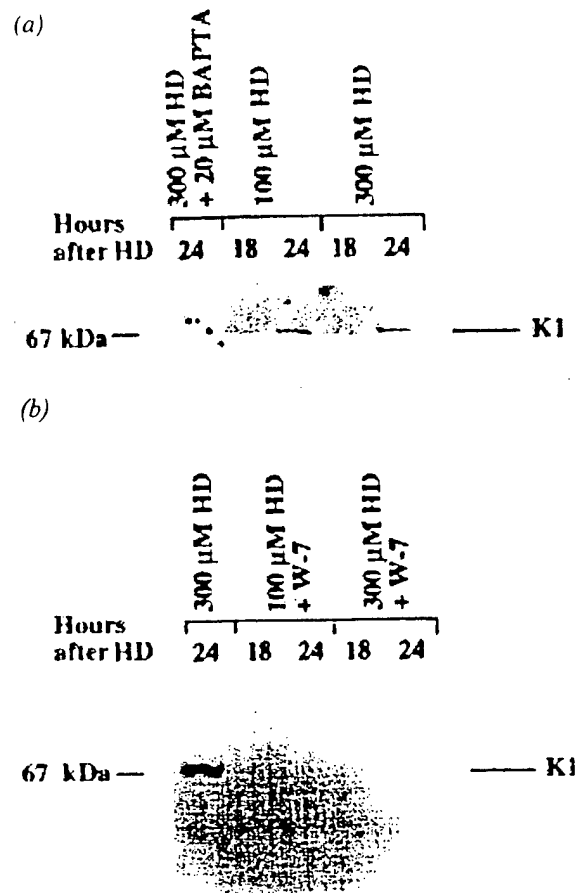


Figure 2. Calcium and calmodulin are required for the induction of markers of terminal differentiation by HD. The NHEK were treated with HD as above, either with or without pretreatment with 20 μ M BAPTA-AM (A) or W-7 (B). Cell extracts were immunoblotted using antibodies specific for K1.

lower endogenous levels of Bcl-2 and induce markers of terminal differentiation in mouse keratinocytes.³³ Following HD treatment, there is a significant decrease in Bcl-2 protein levels in NHEK, as determined by FACS analysis (Fig. 3A).

p53 has also been postulated to play important roles in both the differentiation and apoptotic responses. The FACS analysis showed a significant increase in the protein levels of p53 24 h after exposure to 200 μ M HD (Fig. 3B), whereas immunoblot analysis (not shown) shows that this increase in p53 levels occurs within 2 h.

Sulfur mustard induces apoptosis via caspase-3

The striking decrease in Bcl-2 levels and increase in p53 levels suggested that, in addition to modulating differentiation, HD may induce apoptosis as well. Thus, we assayed for markers of apoptosis following HD treatment. A sensitive technique to verify that HD induces apoptosis is to determine the activation of caspase-3 from its precursor (pro-caspase-3; CPP32) via the use of *in vitro* translated PARP. We therefore used a combination transcription/translation system to radiolabel full-length which was subsequently incubated with extracts derived from primary or immortalized keratinocytes treated with HD. Figure 4 shows that PARP cleavage activity is clearly seen in NHEK in

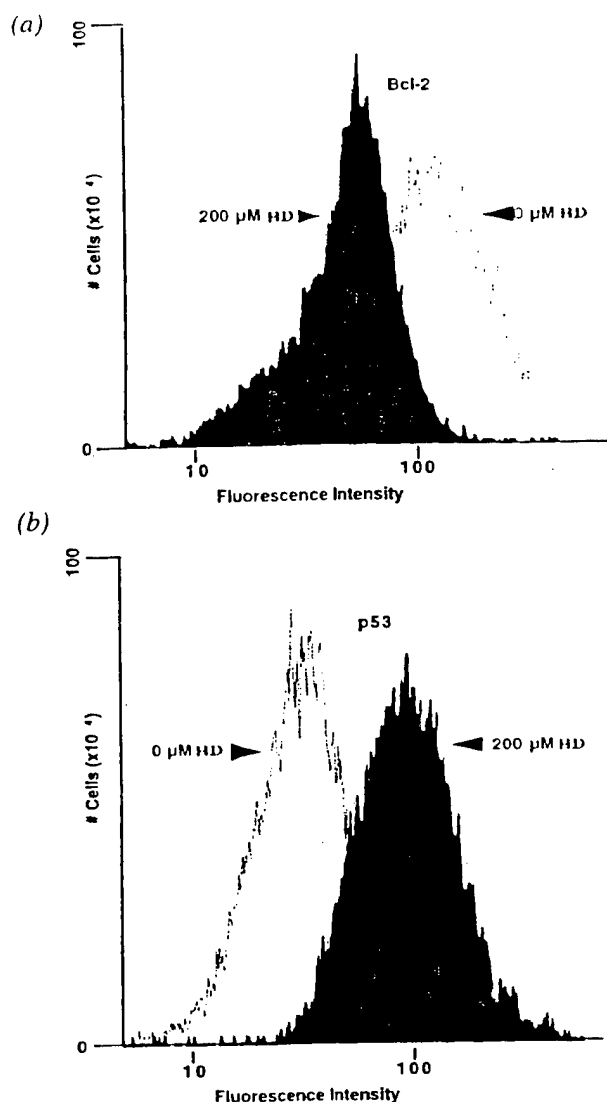


Figure 3. The Bcl-2 levels are lowered and p53 levels are elevated in NHEK treated with HD. The NHEK were treated with 200 μ M HD. At 24 h, cells were harvested, fixed and analyzed by flow cytometry using antibodies specific for Bcl-2 (A) or p53 (B).

300 μ M HD after 24 h, as evidenced by the strong appearance of the 24-kDa and 89-kDa cleavage products. At 4 h PARP cleavage activity is barely detectable in NHEK, although in immortalized keratinocytes it is seen more clearly. Figure 5 shows, by quantitative phosphor image analysis, the relative PARP cleavage activities that result from the treatment of keratinocytes with HD for 24 h. We further verified that HD induces apoptosis by determining that the caspase-3 activity *in vitro* could be associated with the processing of pro-caspase-3/CPP32 into its active protease form. Following treatment with 300 μ M HD, NHEK showed complete processing of a portion of caspase-3 into the active p17 form (not shown). The high level of caspase-3 activity observed is indicative that this vesicant is also a strong inducer of apoptosis in both primary and immortalized keratinocytes, and that apoptosis is occurring via a caspase-3-like pathway.

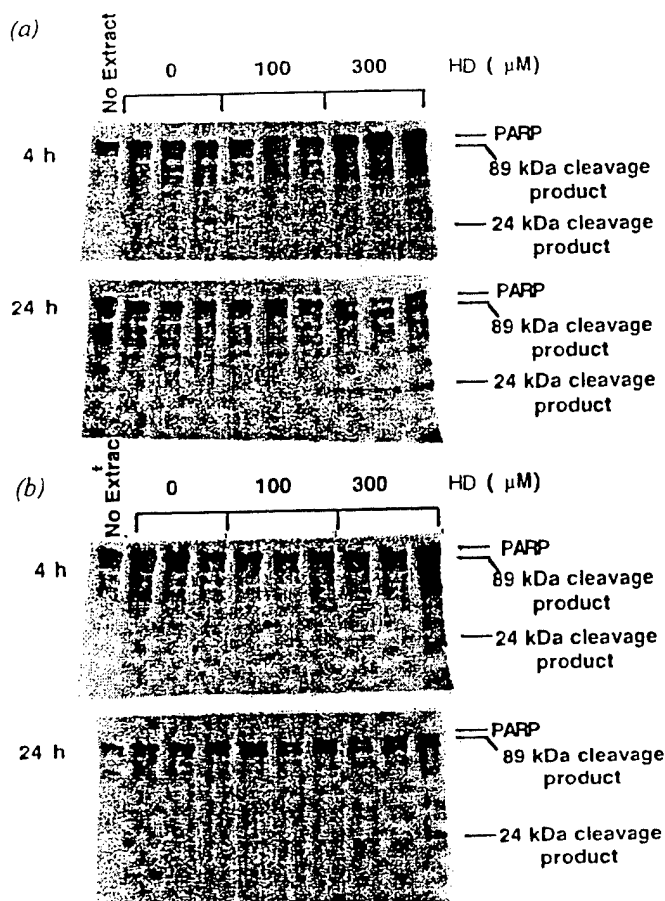


Figure 4. Extracts of keratinocytes treated with HD show *in vitro* PARP-cleavage activity. Triplicate cultures of NHEK (A) or immortalized keratinocytes (B) were treated with 100 or 300 μ M HD. Cytoplasmic extracts were then derived after 4 and 24 h and assayed for caspase-3 activity using [³⁵S]PARP as a substrate.

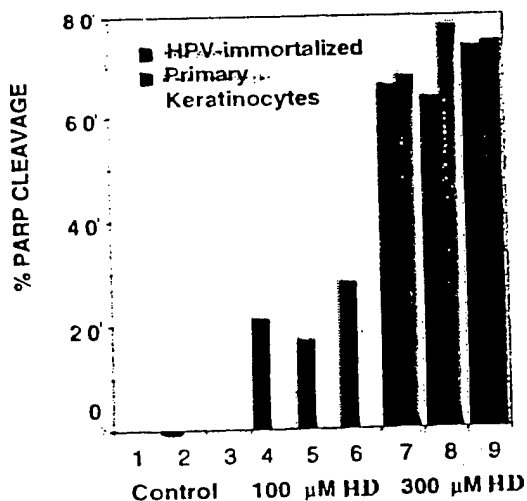


Figure 5. Quantification of PARP-cleavage activity shown in Fig. 4 by phosphor image analysis.

concentration of calmodulin in keratinocytes. Furthermore, treatment of keratinocytes with antisense oligonucleotides, but not with nonsense calmodulin nucleotides, attenuated or eliminated the apoptotic response of keratinocytes in response to HD, as measured by either *in vitro* PARP cleavage or by immunoblot analysis of caspase-3 activation (not shown).

A recent advance in molecular biological technology has been in the establishment of improved retroviral vectors that are capable of delivering a cloned gene or cDNA into cells. These retroviral vectors, originally established from mouse murine leukemia virus (MMLV), have been engineered to infect any type of cell, including human keratinocytes. We have cloned all of the human calmodulin cDNAs into a particularly useful retroviral vector, LXS.N. Calmodulin cDNA was obtained from Dr Emmanuel Strehler (Children's Hospital Center of Cincinnati). We took advantage of the unique site for Eco RI within the multicloning site of LXS.N. All calmodulin cDNAs were cloned using the strategy shown for calmodulin 2 and calmodulin 3 (Fig. 6). Digestion of the recombinant clones with Eco RI revealed that the cDNA was inserted, while restriction digestion using wither Xho I or Hpa I revealed the orientation of these clones with respect to the MMLV LTR promoter. The constructs are currently being used to transfect a packaging cell line in order to produce infectious retroviral particles. These recombinant retroviruses then can be used to infect immortalized cells in order to generate stable lines of keratinocytes that express antisense to calmodulin. In addition, these retroviral recombinants can be used also to infect primary keratinocytes at high titer. These primary keratinocytes then can be tested directly and immediately for their resistance to HD, or grafted onto nude mice. In addition to these clones, we have also cloned the calmodulin and CLP cDNA inserts into pMAMneo and K1 vectors.

CONCLUSION

Sulfur mustard induces terminal differentiation in keratinocytes: K1/K10 is induced, involucrin is cross-linked, fibronectin levels are reduced, p53 levels are elevated and Bcl-2 levels are lowered in response to 10 or 100 μ M HD. Further examination of biochemical markers of apoptosis following treatment of either NHEK or HPV-immortalized keratinocytes revealed a burst of PAR synthesis, specific cleavage of PARP *in vivo* and *in vitro* into characteristic 89-kDa and 24-kDa fragments, processing of caspase-3 into its active form and the formation of DNA ladders. BAPTA suppressed differentiation, whereas antisense oligonucleotides and chemical inhibitors specific for calmodulin blocked both markers of differentiation and apoptosis. Modulation of p53 levels utilizing retroviral constructs expressing the E6, E7 or E6 + E7 genes of HPV-16 revealed that HD-induced apoptosis was partially p53 dependent. Finally, immortalized fibroblasts derived from PARP $-/-$ 'knockout mice' were exquisitely sensitive to HD-induced apoptosis. These cells became HD-resistant when wild-type PARP was stably expressed in these cells. These results indicate that HD exerts its effects via calmodulin, p53 and PARP-sensitive pathways. An understanding of the mechanisms for HD vesication will hopefully lead to strategies for prevention of treatment of HD toxicity.

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