Sulfur mustard is cytotoxic to dermal fibroblasts as well as epidermal keratinocytes. We 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+/+ mice and their wildtype littermates (PARP+/−) to determine the contribution of PARP to sulfur mustard toxicity. Following sulfur mustard exposure, primary skin fibroblasts from PARP-deficient mice demonstrated increased inter-nucleosomal DNA cleavage, caspase-3 processing and activity, and annexin V positivity, compared to those derived from PARP+/+ animals. Conversely, propidium iodide staining, PARP cleavage patterns, and random DNA fragmentation revealed a dose-dependent increase in necrosis in PARP+/+ but not PARP−/− cells. Using immortalized PARP−/− fibroblasts stably transfected with the human 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 human papilloma virus. In contrast to fibroblasts, keratinocytes from both PARP+/+ and PARP+/+ mice express markers of apoptosis in response to sulfur mustard exposure. 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 design of PARP inhibitors to reduce sulfur mustard pathology.


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 (Rosenthal et al, 1998). In addition to the direct effect of 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, 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 normal human epidermal keratinocytes 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 μM, corresponding to the lowest dose we used in this study.

As SM is a strong alkylation agent, its ability to induce DNA damage via apurinic sites and endonucleolytic cleavage has been advanced as one possible pathway leading to cell death and vesication (Papirmeister et al, 1985). SM has in fact been shown to induce DNA breaks in cultured cells and activate the nuclear protein poly(ADP-ribose) polymerase (PARP) within 2 h of exposure (Rosenthal et al, 1998; Hinshaw et al, 1999). Similar to other agents that induce DNA strand breakage, SM-induced PARP activation results in the poly(ADP-ribose)ylation of a number of nuclear proteins using nictinamide adenine dinucleotide (NAD) as a substrate. We have shown that topical SM exposure induces poly(ADP-ribose)ylation of nuclear proteins in skin grafts derived from control human keratinocytes, but not those stably transfected with a PARP antisense inducible vector (Rosenthal et al, 1995). A role of PARP in SM-mediated cell death has also been investigated in peripheral blood lymphocytes, where PARP inhibitors have
been shown to extend the lifespan of cells treated with SM (Meier and Johnson, 1992; Meier, 1996).

The mechanisms by which PARP may modulate SM-induced cytotoxicity and necrosis are unknown. PARP has been implicated as an important regulator of both apoptosis and necrosis, however. Induction of PARP can drastically deplete levels of cellular NAD and adenosine triphosphate (ATP) (Wielckens et al., 1982; Alvarez et al., 1986), and the potential role for PARP in cell death via this mechanism was first proposed by Berger et al. (1983; Berger, 1985). Partial depletion of ATP by other agents has also been shown to induce apoptosis of cultured renal epithelial cells (Feldenberg et al., 1999). 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. For instance, Fas-induced apoptosis is completely blocked by reducing intracellular ATP levels (Eguchi et al., 1999). In some cells, 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). 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). Cleavage of PARP at later stages of apoptosis would prevent ATP from being lost. Consistent with this idea, it was found that PARP inhibitors prevent the SM-induced expression of necrotic markers in lymphocytes (Meier and Millard, 1998).

Thus, the cellular response to DNA damaging agents such as SM 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 versus necrosis) may depend upon a number of factors, including the time of onset of caspase activation and proteolytic cleavage of PARP.

The PARP homozygous knockout mouse (PARP−/−) model has been a powerful tool to analyze the roles of PARP in cell death induced by different agents both in cultured cells and intact animals. PARP−/− 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−/− mice neither express intact PARP nor exhibit significant poly(ADP-ribosyl)ation (Wang et al., 1995; de Murcia et al., 1997; Masutani et al., 1999). 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 a study that demonstrated that exon 2 PARP−/− animals are resistant to the neurotoxic 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). In exon 2 PARP−/− cell culture, the apoptotic response to Fas plus cycloheximide is altered, and stable transfection of PARP cDNA reverts these cells to a response similar to that of PARP−/− cells (Simbulan-Rosenthal et al., 1998). Further, 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 wildtype 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 (Herceg and Wang, 1999) 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 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 (Ha and Snyder, 1999). As yet, no studies have been published on the effects of SM on PARP−/− versus PARP+/− animals or cells derived from them.

In this study, we have utilized different cell types derived from exon 2 PARP−/− and PARP+/− animals to determine the contribution of PARP to apoptosis or necrosis following exposure to SM. We 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. These results indicate that (i) dermal fibroblasts and keratinocytes, which both contribute to SM vescation, undergo different mechanisms of cell death in response to SM, and (ii) 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, as apoptotic cell death may prevent the release of inflammatory or degradative enzymes contributing to vescation or inhibition of healing of SM-induced wounds.

MATERIALS AND METHODS

Cells Normal human epidermal keratinocytes were prepared from human foreskins as described previously (Stöppler et al., 1998) and maintained in serum-free medium (SFM; Life Technologies). Normal human epidermal keratinocytes were grown in 75 cm² tissue culture flasks to 60%-80% confluence, and then exposed to SM diluted in SFM to final concentrations of 100 μM or 300 μM. Medium was not changed for the duration of the experiments. Primary fibroblasts were derived from newborn dermus 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 human papilloma virus 16 (HPV 16) (Sherman and Schлегel, 1996) in the LXSN 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 keratin and the absence of vimentin utilizing the antipan cytokeratin (Sigma-Aldrich, St. Louis, MO) or antivimentin (Sigma-Aldrich) by both Western blot and immunofluorescent analysis.

Chemicals SM (>98% purity) was obtained from the U.S. Army Edgewood Research, Development and Engineering Center.

Antibodies Rabbit antisem against the p17 subunit of caspase-3 was obtained from Donald Nicholson (Merck Frost Center for Therapeutic Research, Montreal, Quebec, Canada). Rabbit antisem recognizing both full-length PARP and the 89 kDa apoptotic cleavage product of PARP was a kind gift from Eric Ackerman (Pacific Northwest National Laboratory, Richland, WA). Guinea pig antiserum specific for poly(ADP-ribose) have been described previously (Rosenthal et al., 1995). Antipan-keratin and antivimentin are from Sigma-Aldrich.

Immunoblot analysis For immunoblot analysis, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separated proteins were transferred to nitrocellulose filters. Proteins were measured (DCA protein assay; Bio-Rad, Hercules, CA) and normalized prior to gel loading, and all filters were stained with Ponceau-S, in order to reduce loading, and all filters were stained with Ponceau-S, in order to reduce the possibility of loading artifacts. The details for using rabbit antisem to human PARP (Ding et al., 1992) and guinea pig antisem 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).

Analysis of DNA fragmentation Cells were harvested and lysed in 0.5 ml of 7 M guanidine hydrochloride. The lysate was mixed with 1 ml of Wizard Miniprep resin (Promega, Madison, WI), incubated at room temperature for 15 min with occasional mixing, and then centrifuged at 10,000 × g for 5 min. The resulting pellet was resuspended in 2 ml of washing solution [90 mM NaCl, 9 mM Tris-HCl (pH 7.4), 0.25 mM ethylenediamine tetraacetic acid (EDTA), 55% (vol/vol) ethanol] and drawn by vacuum through a Wizard Minicolumn (Promega) mounted onto a vacuum manifold. The column was washed twice with 4 ml of washing solution and dried by centrifugation over a microfuge tube at 10,000 × g for 2 min. DNA was eluted from the column by adding 50 μl of deionized H₂O, incubating at room temperature for 15 min, and then centrifuging at 10,000 × g for 5 min. Residual RNA in the
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 (A, B) with the indicated concentrations of SM in keratinocyte growth medium, or for the indicated times with 100 μM SM (C), 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, C). All the data in (B) and (C) are presented as means ± SD of three replicates of a representative experiment; essentially the same results were obtained in three independent experiments.

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 (A, B) with the indicated concentrations of SM in keratinocyte growth medium, or for the indicated times with 100 μM SM (C), 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, C). All the data in (B) and (C) are presented as means ± SD of three replicates of a representative experiment; essentially the same results were obtained in three independent experiments.

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) and then used to drive the synthesis of PARP labeled with [35S]methionine (Dupont-NEN, Boston, MA) by coupled in vitro 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, Piscataway, NJ; 1 × 30 cm) in 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% (wt/vol) CHAPS, and 5 mM dithiothreitol (DTT). Cytosolic extracts were prepared from normal human epidermal keratinocytes by scraping PBS-washed monolayers in 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10 μg per ml pepstatin A, 20 μg per ml leupeptin, and 10 μg per ml aprotinin (at 1 × 10^6 cells per ml). The post 100,000 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 [35S]PARP (5 × 10^6 cpm), 50 mM PIPES/KOH, 2 mM EDTA, 0.1% (wt/vol) CHAPS, and 5 mM DTT 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 × SDS-PAGE sample buffer containing 4% SDS, 4% β-mercaptoethanol, 10% glycerol, 0.125 M Tris- HCl (pH 6.8), and 0.02% bromophenol blue. Samples were resolved by 10% SDS polyacrylamide gels. Either the PARP cleavage products were visualized by fluorography, or the 89 kDa cleavage product of [35S]PARP was quantified relative to the full-length PARP (25 μg per lane) using PhosphorImager 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) versus full-length PARP (25 met residues).

Assay of caspase-3 activity After harvesting the cells were resuspended in Triton X-100 lysis buffer (60 mM Tris HCl, pH 7.5, 5 mM EDTA, 10% glycerol, 2 mM sodium orthovanadate, 25 mM sodium fluoride, 10 μg per ml aprotinin, 10 μg per ml leupeptin, 1 mM AEBSF, 75 mM NaCl, 1 μg per ml pepstatin A, 1% Triton X-100) and incubated for 30 min at 4°C. The cell lysate was centrifuged at 14,000 × g for 15 min and the supernatant containing the postnuclear extract was collected. The protein concentration in the postnuclear extract was determined using the Micro BCA protein assay kit (Pierce) and then the extract was stored at −80°C until use. For the caspase-3 assay, 25 μg of extract was initially resuspended up to a volume of 50 μl with Triton X-100 lysis buffer, to which 50 μl of caspase assay buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 10 μg per ml aprotinin, 10 μg per ml leupeptin, 1 mM AEBSF, 1 μg per ml pepstatin A) was added. The aliquots were preincubated at 37°C for 30 min in a microcentrifuge tube, and then mixed with an equal amount (100 μl) of 40 μM 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-amide 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, including the early activation and late cleavage of PARP (Rosenthal et al., 1998). In addition to the direct effect of 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 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 (Fig 1A). Instead, PARP was cleaved into a 50 kDa fragment, which has been previously reported to be a major cleavage product diagnostic of necrotic cell death (Shah et al, 1996). 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-1β-converting enzyme and to CED-3, the product of a gene required for programmed cell death in Caenorhabditis elegans (Yuan et al, 1993). The identity of this protease was also demonstrated by Tewari et al (1995). Twenty-four hours after exposure, extracts were derived and analyzed for caspase-3 activity using a fluorometric assay (Materials and Methods). Whereas PARP+/+ primary fibroblasts did not exhibit SM-induced caspase-3 activity at any of the doses tested, PARP−/− fibroblasts exhibited marked caspase-3 activity in response to SM at all doses tested (Fig 1B). The greatest caspase-3 activity was observed following treatment of PARP−/− fibroblasts with 300 μM SM. Thus, PARP appears to inhibit apoptosis in primary dermal fibroblasts.

To determine if the difference in SM-induced caspase-3 activity was time dependent, we performed a time course experiment. PARP−/− or PARP+/+ primary skin fibroblasts were exposed to 300 μM SM, and then harvested at different time increments and assayed for caspase-3 activity. Figure 1(C) shows that, at all time points, PARP−/− cells displayed substantially more caspase-3 activity than PARP+/+ cells.

To confirm that the difference in the mode of cell death in SM-exposed PARP−/− and PARP+/+ skin fibroblasts was associated with a difference in processing of procaspase-3 into its catalytically active form, an immunoblot analysis was performed, using an antibody specific for the larger subunits (p17/p20) of active caspase-3. Figure 2(A) shows that SM induces the cleavage of procaspase-3 into the active p17/p20 forms in skin fibroblasts derived from PARP−/− but not PARP+/+ mice.

To further establish the relationship of cell death induced by SM in PARP−/− versus PARP+/+ skin fibroblasts, we analyzed the pattern of genomic DNA fragmentation following exposure to SM. Following SM exposure DNA was isolated from the two different cell types and analyzed by agarose gel electrophoresis, followed by ethidium bromide staining. Figure 2(B) shows that DNA isolated from SM-exposed PARP−/− primary skin fibroblasts demonstrated the characteristic apoptotic pattern of internucleosomal cleavage, whereas PARP+/+ fibroblast DNA appeared as smears or characteristic of necrosis.

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. Whereas 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 80% (Fig 3A, left).

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, whereas PARP−/− fibroblasts underwent little necrosis at all doses tested (Fig 3A, right). In a separate set of experiments, we confirmed the mode of cell death induced by SM in wildtype and PARP−/− dermal fibroblasts, using annexin V plus PI staining. Figure 3(B) clearly shows that the primary mode of cell death in PARP+/+ cells is necrotic, whereas PARP−/− cells undergo apoptosis.

In view of 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 (Fig 3C). Pretreatment with DEVD-CHO abolished the apoptotic response, however. Thus the deletion of PARP leads to an apoptotic mode of death that is 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 wildtype fibroblasts (PARP+/+), as well as PARP−/− fibroblasts stably transfected with the human PARP-1 cDNA [PARP−/− (+PARP)], but not in immortalized PARP−/− fibroblasts (Fig 4A). PARP−/− or PARP+/+ (+PARP) fibroblasts were then exposed to similar concentrations of SM and 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 μM, with > 60% cleavage observed at the highest dose of SM tested (500 μM; Fig 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 μM and 500 μM SM in the PARP ±/± cells but not in the cells stably retransfected with PARP cDNA (Fig 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 (<5%; Fig 5A). On the other hand, immortalized PARP+/+(+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+/+(+PARP) cells, indicating that the expression of PARP increases the level of necrosis in immortalized fibroblasts.

SM induces apoptosis in keratinocytes in the presence or absence of PARP We next assessed the role of PARP in the induction of apoptosis in keratinocytes. Primary mouse keratinocytes derived from PARP+/− and PARP+/+ animals following immortalization of cells with a retrovirus that expresses both the E6 and E7 genes of 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+/+(+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 the data in the left and right panels of Fig 6A are presented as means ± SD of three replicates of a representative experiment; essentially the same results were obtained in three independent experiments.

**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 keratinocyte growth medium, after which cells were prepared and assayed for annexin V binding plus PI staining at the doses indicated (B), or at 500 μM SM with or without the indicated caspase inhibitors (C) by FACS analysis. Dot plots of the results show viable (B, C, lower left quadrants), early apoptotic (annexin-V-FITC-positive; lower right quadrant), late apoptotic (upper right quadrant), and necrotic (PI-positive; upper left quadrant). (A) Percentage of cells exhibiting annexin V binding (A, left) or PI staining (A, right) as determined by FACS analysis. All the data in the left and right panels of (A) are presented as means ± SD of three replicates of a representative experiment; essentially the same results were obtained in three independent experiments.

**DISCUSSION**

SM vesication clearly involves both cytotoxicity and detachment of the epidermal basal layer in vivo. Using a cell culture model in this...
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. As 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. Consistent with this observation, in PARP−/− skin fibroblasts, SM induced proteolytic processing of procaspase-3 to its active form, and apoptotic internucleosomal cleavage of genomic DNA. In contrast, PARP+/+ cells displayed characteristic markers of necrosis upon exposure to SM; PARP was cleaved to a 50 kDa fragment, no caspase-3 processing was evident, and genomic DNA displayed random fragmentation. Furthermore, FACS analysis with annexin V revealed no binding to PARP+/+ cells compared to cells derived from PARP−/− animals. PI staining also 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 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 normal human epidermal keratinocytes (Rosenthal et al, 1998).

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 as 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), whereas keratinocyte basal cell death is primarily due to apoptosis at the doses tested (Rosenthal et al, 1998). The mode of cell death induced by SM probably also affects the levels of cytokines and other factors that determine the severity of the inflammatory response, as well as the time required for healing. For example, SM-pretreated dermal fibroblasts were shown to exert inhibitory effects on the adhesion and maturation of SM-untreated keratinocytes (Gentilhomme et al, 1998).

Although no in-depth study has been performed previously to determine the mode of cell death induced by SM in skin fibroblasts, this study indicates that these cells undergo necrosis at all concentrations of SM tested (100–500 µM). Several investigators have examined the mode of cell death induced by SM in other cell types. SM induces an apoptotic response in HeLa cells (10–100 µM; Sun et al, 1999), peripheral blood lymphocytes (6–300 µM; Meier and Millard, 1998), keratinocytes (50–300 µM; Rosenthal et al, 1998; Stöppler et al, 1998), and endothelial cells (< 250 µM; Dabrowska et al, 1996). A time-dependent shift to necrosis was observed in SM-treated lymphocytes, however (Meier and Millard, 1998), whereas markers of necrosis were observed at higher levels of SM in endothelial cells (>500 µM; Dabrowska et al, 1996) and HeLa (1 mM; Sun et al, 1999).
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 within 2 h of exposure (Rosenthal et al., 1998; Hinshaw et al., 1999). 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. The potential role of PARP in determining the role of cell death has been examined with SM-exposed peripheral blood lymphocytes, in which inhibition of necrotic but not apoptotic markers was observed when cells were treated with PARP inhibitors (Meier and Millard, 1998). These results are consistent with those of this study, our preliminary studies in which newborn wildtype and PARP-de®cient mice have been exposed to SM by vapor cup inhalation (Meier and Johnson, 1992; Meier, 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 either be directly related to the levels or activity of PARP in these cells, or result from other events that regulate cellular NAD and ATP levels.

PARP inhibitors have been shown to extend the lifespan of lymphocytes treated with SM (Meier and Johnson, 1992; Meier, 1996), and inhibitors of PARP have been reported to signi®cantly 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, such as p53 (Whitacre et al., 1995; Simbulan-Rosenthal et al., 1999) and the Ca2+/Mg2+- dependent nucleases 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, as partial depletion of ATP induces apoptosis (Feldenberg et al., 1999), whereas depletion of ATP below a threshold level blocks the later events in apoptosis (Eguchi et al., 1999), thereby shutting the mode of cell death to necrosis.

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. This 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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