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Low-dose sulfur mustard primes oxidative function and induces apoptosis in human polymorphonuclear leukocytes ☆

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Abstract

Although considerable work has focused on understanding the processes of direct tissue injury mediated by the chemical warfare vesicant, sulfur mustard (2,2′ -bis-chloroethyl sulfide; SM), relatively little is known regarding the mechanisms of secondary injury caused potentially by the acute inflammatory response that follows SM exposure. Polymorphonuclear leukocytes (PMNs) play a central role in the initiation and propagation of inflammatory responses that, in some cases, result in autoimmune tissue damage. The potential for PMN-derived tissue damage following SM exposure may, in part, account for the protracted progression of the injury before it resolves. The current study was undertaken to evaluate the priming, oxidative function, and viability of PMN following exposure to low doses of SM such as those that might remain in tissues as a result of topical exposure. Our results demonstrate that doses of SM ranging from 25 to 100 μM primed PMN for oxidative burst in response to activation by fMLP, and that doses of SM ranging from 50 to 100 μM induced PMN apoptosis. Understanding the mechanisms through which SM directly affects PMN activation and apoptosis will be of critical value in developing novel treatments for inflammatory tissue injury following SM exposure.

Keywords: Sulfur mustard; Inflammation; Apoptosis; Priming; Neutrophil

1. Introduction

The skin and other epithelial surfaces are the first targets to be injured by sulfur mustard (SM). SM exposure leads to a reproducible pattern of histological injury [1]. Considerable work has been focused on understanding mechanisms of direct cellular injury mediated by SM. We have previously demonstrated that doses of SM in the 500–1000 μM range induce necrosis in endothelial cells and keratinocytes, and that doses of SM in the 250–500 μM range induce apoptosis [2,3]. These observations have been confirmed and extended to other cell lines [4–6].

It is also becoming apparent that direct SM injury is followed by a secondary inflammatory

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response, which may cause extension and prolongation of the initial tissue injury mediated by SM [7–9]. Studies have demonstrated the presence of chemokines/cytokines in acute SM wounds, which are known to attract immune cells to sites of injury and infection [10,11]. Polymorphonuclear leukocytes (PMNs) are terminally differentiated granular phagocytes and play an essential role in innate immune responses. PMNs are the primary mediators of inflammation following tissue injury and are the initial immune cells recruited to foci of injury. Warthin and Weller [9] in 1918, using human volunteers, observed leukocyte infiltration into the site of SM exposure within 30 min of the injury and persisting for at least 6 days. In a hairless guinea pig model of SM injury, Millard et al. [12] documented recruitment of PMNs into acute SM wounds as early as 3 h postinjury. The PMN infiltration peaked at 6–12 h postinjury and preceded epidermal–dermal separation and other adverse changes in the subepidermal region [12].

Activation of PMN is generally thought to be a two-step process where an initial stimulus "primes" the cells, making them hyperresponsive to subsequent activating stimuli [13–15]. Because PMNs are recruited into areas of SM injury as early as 30 min post SM exposure, the possibility exists that the infiltrating cells may be directly exposed to low residual concentrations of SM present at the foci of injury. This is born out by several studies that examined rate of SM degradation over time in aqueous media containing normal levels of saline. The half-life ($T_{1/2}$) of SM in normal saline is 19–24 min and, in the blood, the $T_{1/2}$ of SM is 30–60 min [16–18]. Since the peak time of PMN infiltration is 6–12 h postexposure and the concentration of neat SM at the skin surface would be approximately 8 M, there would be a residual SM concentration of 300 μM after 6 h at a $T_{1/2}$ of 24 min. The effects of direct SM exposure on PMNs are unknown. We hypothesized that low levels of SM would prime PMNs, prolonging their survival and potentiating autoimmune tissue injury through the production of reactive oxygen species (ROS). In this study, we evaluate the direct effect of low-dose SM exposure (50–100 μM) on the function and viability of PMN.

2. Materials and methods

2.1. Reagents

All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted. granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-8 (IL-8) were obtained from R&D Systems (Minneapolis, MN). Dose–response curves using 10-fold serial dilutions of GM-CSF or IL-8 determined the optimal doses for priming oxidant production by PMNs in response to the bacterial chemotactic peptide, fMLP, to be 1000 U/ml GM-CSF and 100 ng/ml IL-8 (data not shown). Sulfur mustard used in these studies was synthesized at the US Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, MD) and supplied as a 9.5-mg/ml solution in pure ethanol, assayed at 98.2% purity by gas chromatography. The SM was stored at −80 °C in air-tight glass vials. Under these conditions, SM is stable for many years (USAMRICD, personal communication).

2.2. Isolation of PMN

All materials and solutions used in the preparation of PMNs were nonpyrogenic [containing undetectable (<0.1 ng/ml) endotoxin] by amoebocyte lysate assay (Sigma). Briefly, venous blood was collected from healthy volunteers into 1/6 vol acid citrate dextrose (ACD) anticoagulant solution. PMNs were then partially purified from red blood cells by dextran-70 sedimentation, and further purified by Percoll gradient separation as previously described [19]. This method of PMN isolation yielded a PMN population of ≥98% purity.

2.3. Ultraviolet (UV) radiation accelerated PMN apoptosis

Induction of PMN apoptosis by UV irradiation was performed as previously described [20]. Briefly, 5×10^6/ml of purified PMNs were seeded in 24-well polystyrene tissue culture plate (Costar) and allowed to settle into a monolayer. After settling, the cells were exposed to 50 or 100 μM SM for 60 min. PMNs were then exposed from below to 312-nm UV irradiation for 15 min at room temperature at a distance of 2.5 cm

from the transilluminator surface (model 3-3000; Fotodyne). The UV intensity at 2.5 cm (measured through a standard tissue culture plate with a UVX Digital Radiometer; UVP, San Gabriel, CA) was 3.2 mW/cm^2. After UV irradiation, cells were incubated for 5 h at 37 °C/5% CO_2 prior to apoptosis determination.

Apoptosis of PMNs was induced by SM exposure as follows: 5×10^6/ml purified PMNs were seeded in 24-well polystyrene tissue culture plate and allowed to settle into a monolayer. After settling, 50 or 100 μM SM was added to the culture medium and the cells were incubated at 37 °C/5% CO_2 for 5 h prior to apoptosis determination [21].

2.4. Analysis of CD16

The analysis of CD16 levels was undertaken as previously described [22,23]. Briefly, PMNs (1×10^6/ml) were washed in phosphate-buffered saline (PBS) containing 0.2% BSA and 0.1% sodium azide and resuspended in 100 μl of PBS, to which was added 20 μl of phycoerythrin (PE)-labeled anti-CD16 (Becton Dickinson) for 30 min at 4 °C. Cells were then washed twice in PBS plus 0.2% BSA and 0.1% sodium azide and fixed in 2% paraformaldehyde. PMNs were then analyzed using a FACS scan flow cytometer (Becton Dickinson).

2.5. Analysis of caspase-3 activity

Caspase-3 activity was analyzed using a colorimetric assay kit (BF3100; R&D Systems). PMNs (1×10^6/ml) were assayed in flat-bottom 96-well microtiter plates as per the manufacturer's protocol after incubation with or without SM or 15 min of UV irradiation as in Section 2.3 above. Following UV irradiation, cells were incubated for 2 h at 37 °C before analysis. Caspase-3 activity is reported as optical density at 405 nm.

2.6. Production of reactive oxygen species

Extracellular production of ROS in response to 100 nM fMLP was quantified in primed PMNs by oxidation of the chromophore, *p*-hydroxy-phenylacetic acid (PHPA), to its fluorescent, 2,2′-dihydroxy-biphenyl-5,5′-diacetate [$(PHPA)_2$] [24]. PMNS were incubated with GM-CSF (positive control for PMN priming) or various concentrations of SM, or for 60 min prior to the addition of PHPA followed by 100 nM fMLP. Cumulative fluorescence was measured over a 240 s time course. Intracellular production of ROS was quantified by treating PMNs with 30 μM dihydrorhodamine 123 (H_2Rh123; Molecular Probes, Eugene, OR) prior to incubation with GM-CSF or SM, or for 60 min [25]. Dihydrorhodamine 123 is oxidized by ROS to its fluorescent product, Rh123, simultaneously trapping the fluor within the cell due to its charged state. Primed rhodamine-labeled cells were harvested and washed once with PBS. Production of ROS was quantitated as the mean fluorescent intensity of rhodamine 123 using a FACS scan flow cytometer (Becton Dickinson).

2.7. Statistics

At least three experiments were performed in each treatment condition. Parametric data are presented as the mean ± standard error of the mean (S.E.M.) for the indicated number of experiments. A repeated-measures analysis of variance (ANOVA) with a Tukey–Kramer multiple comparison test was used to compare treatment groups to control in Fig. 4B. Analyses where $p \leq 0.05$ were considered significant.

3. Results

3.1. Sulfur mustard primes PMN oxidant production

Early infiltration of PMNs into foci of SM injury suggests the possibility that they may be directly exposed to low levels of SM. To determine if SM could prime PMN production of ROS, purified PMNs were incubated for 60 min with medium alone, 1000 U/ml granulocyte–macrophage colony-stimulating factor, or SM at the indicated concentrations. The chromophore PHPA was added to the primed cells followed by 100 nM fMLP as the activating stimulus. Extracellular production of ROS by PMNs primed with GM-CSF showed a >12-fold increase in cumulative fluorescence at 240 s over than cells incubated with medium alone (Fig. 1). Cells treated with SM showed a dose-dependent increase in ROS production. This was manifested by a 10-fold increase in

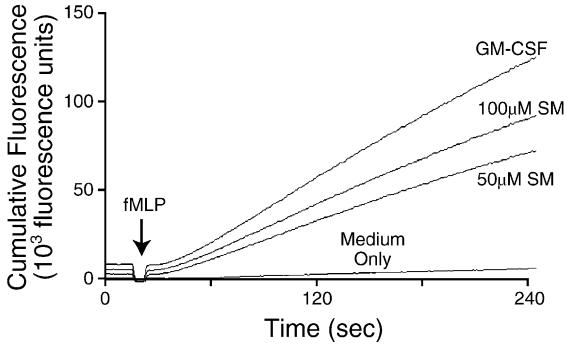

Fig. 1. SM primes extracellular PMN oxidant production. PMNs were preincubated with 50 μM SM, 100 μM SM, or 1000 U/ml GM-CSF for 60 min. PMNs incubated in medium alone served as control. Oxidant production in response to activation with 100 nM fMLP was measured over a 4-min time course as described in Materials and Methods. The *y*-axis represents fluorescence units. These results are representative of three separate experiments.

cumulative fluorescence over incubation with medium alone for doses of 50 and 100 μM SM, respectively. These data demonstrate that doses of SM at least as low as 50 μM can prime the release of ROS by PMNs.

Intracellular oxidant production by PMNs following SM priming was also determined. PMNs were loaded with 30 μM dihydrorhodamine 123 and then treated with medium alone (Fig. 2A), 0.2% EtOH (solvent only; Fig. 2B) or the following doses of SM (μM): 10 (Fig. 2C), 25 (Fig. 2D), 50 (Fig. 2E), or 100 (Fig. 2F), for 60 min. Generation of fluorescent rhodamine (mean channel fluorescence) is proportional to the ROS production within each cell and was then quantitated by flow cytometry in PMNs either without postpriming stimulation (filled histogram) or following stimulation with 100 nM fMLP (open histogram). There was no detectable difference in mean channel fluorescence between non-fMLP-stimulated PMNs (filled histograms, Fig. 2A–F), the media controls, or SM treatment groups. This demonstrates that at the concentrations tested, SM does not directly stimulate PMN oxidant production. PMNs primed with SM exhibited a dose-dependent increase in cytosolic ROS production following stimulation with 100 nM fMLP. A concentration of 50 μM SM appeared to be the optimal priming dose for intracellular ROS.

The ability of 50 μM SM to prime intracellular PMN oxidant production was compared to that of interleukin-8 and GM-CSF—cytokines that are known to prime ROS production by PMNs [26]. Also, both IL-8 and GM-CSF have been shown to be present in SM wounds [10,11]. Cells were labeled with rhodamine, primed, and stimulated as described above. While SM alone does not directly stimulate ROS production (compare Fig. 3A and B) in the absence of stimulation with fMLP (filled histograms), incubation with either IL-8 (Fig. 3C) or GM-CSF (Fig. 3D) alone resulted in a twofold and threefold increase in ROS, respectively, compared to incubation with medium alone (Fig. 3A). When primed PMNs were stimulated with 100 nM fMLP (open histograms), SM (Fig. 3B), IL-8 (Fig. 3C), or GM-CSF (Fig. 3D) treatment resulted in approximately a 10-fold increase in intracellular ROS when compared to their primed but unstimulated production. Although IL-8 and GM-CSF primed greater absolute levels of ROS production than SM, these experiments confirm that low doses of SM prime PMN oxidant production in response to a second stimulus.

3.2. SM induces PMN apoptosis

Many agents that are known to prime PMN functional activities have also been shown to prolong PMN survival by inhibiting PMN apoptosis [21]. Because the above experiments demonstrated that low doses of SM can prime PMN oxidant production in response to fMLP, we hypothesized that SM might also have a similar effect in delaying apoptosis. We therefore evaluated the effect of low-dose SM treatment on PMN apoptosis using an in vitro culture, UV-accelerated model system. We have previously demonstrated that PMN priming agents including GM-CSF and LPS protect PMN from UV-induced apoptosis [20]. To evaluate the ability of SM to protect PMNs from apoptosis in this model, PMNs were pretreated with 50 and 100 μM SM for 60 min and then exposed to UV irradiation for 15 min as described in the Materials and Methods. After UV irradiation, cells were incubated at 37 °C/5% CO_2 for 5 h, harvested, and assessed for apoptosis by loss of surface FcγIII receptor, CD16 [22,23]. PMNs were stained with phycoerythrin-labeled anti-CD16 and analyzed using flow cytometry. PMNs expressing low membrane levels of CD16 were considered apoptotic, while high expressers of CD16 were considered viable. Nonirradiated PMNs were

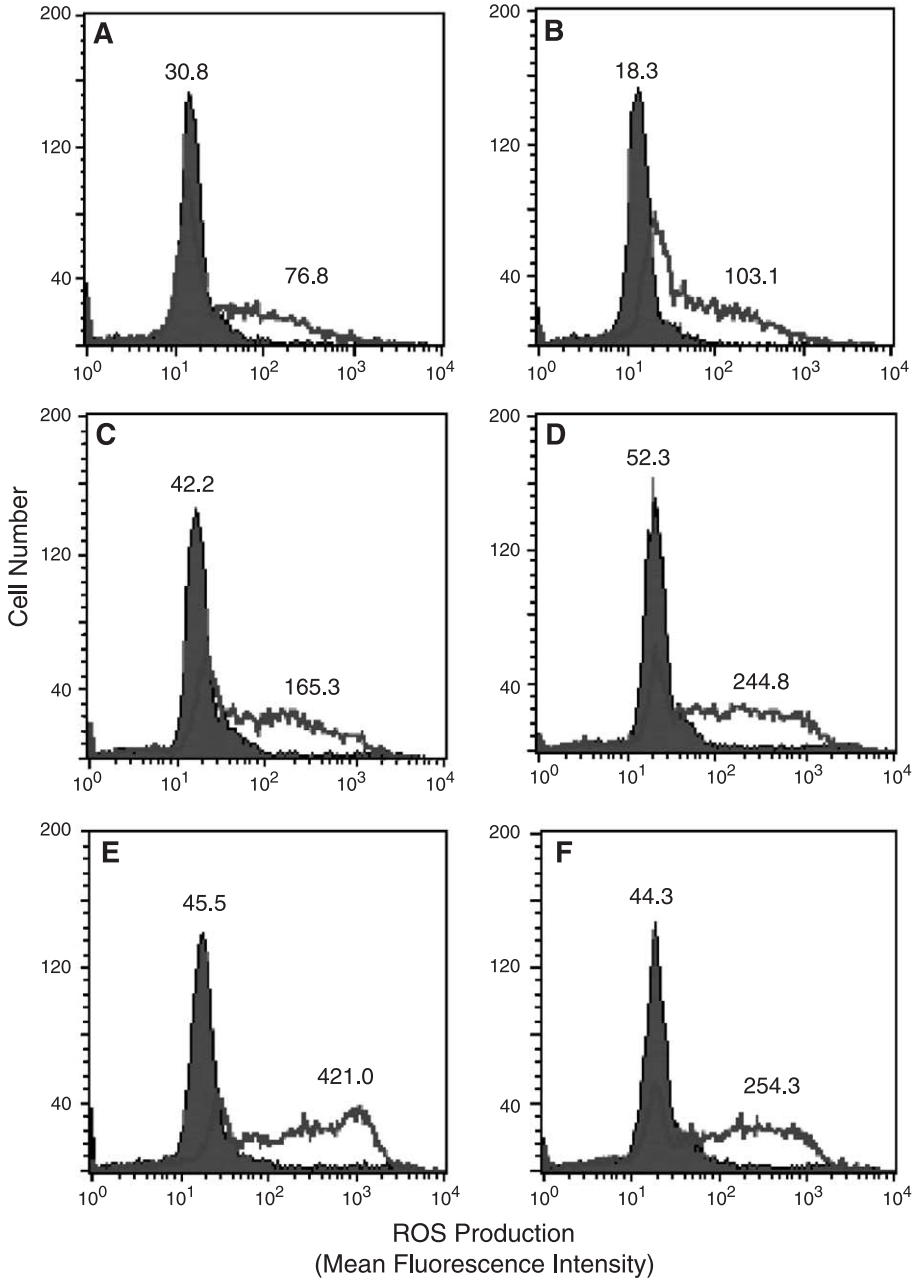

Fig. 2. SM primes intracellular PMN oxidant production. PMNs were loaded with 30 μM dihydrorhodamine 123 and then incubated with medium alone (A), 0.2% EtOH (B), 10 μM SM (C), 25 μM SM (D), 50 μM SM (E), or 100 μM SM (F) for 60 min. The EtOH treatment corresponds to the diluent concentration to which PMNs were exposed in the 100-μM SM dose. PMNs were activated with 100 nM fMLP (open histogram) or not exposed to fMLP (filled histogram), and ROS was determined by flow cytometry. The numbers in each panel correspond to the mean channel fluorescence for PMNs treated with or without 100 nM fMLP. These results are representative of four separate experiments.

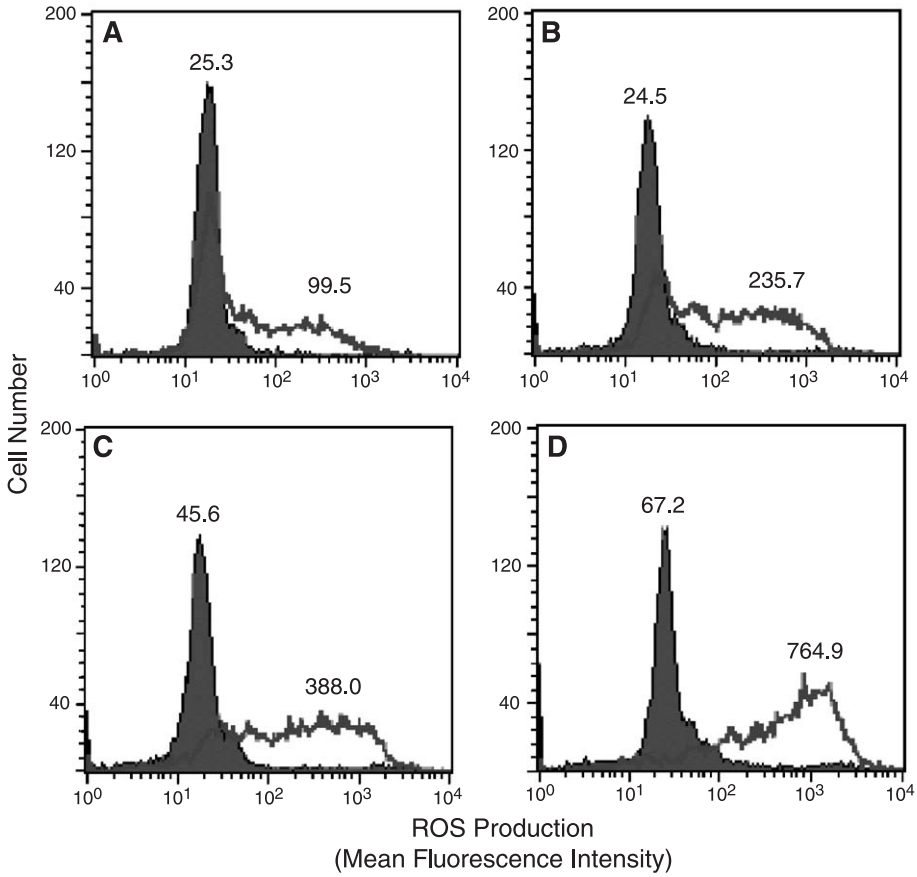

Fig. 3. Priming of intracellular PMN oxidant production by SM is proportionally similar to PMN priming by IL-8 or GM-CSF. PMNs were loaded with 30 μM dihydrorhodamine 123 and then incubated with medium alone (A), 50 μM SM (B) or 100 μM SM, 100 ng/ml IL-8 (C) or 1000 U/ml GM-CSF (D) for 60 min. PMNs were activated with 100 nM fMLP (open histogram) or not exposed to fMLP (filled histogram), and ROS was determined by flow cytometry. The numbers in each panel correspond to the mean channel fluorescence for PMNs treated with or without 100 nM fMLP. These results are representative of four separate experiments.

largely $CD16^{Hi}$ with only a small $CD16^{Lo}$ population after culture in medium alone, where UV irradiation alone increased the proportion of $CD16^{Lo}$ PMN by approximately threefold over unirradiated cells (Fig. 4A, upper row). Treatment of PMN with 50 or 100 μM SM alone increased the apoptotic population by approximately twofold over untreated and unirradiated cells. SM pretreatment not only failed to protect PMN from UV-induced apoptosis, but also appeared to enhance the proportion of $CD16^{Lo}$ cells. Fig. 4B represents the combined results from three experiments.

To confirm the proapoptotic effects of SM on PMN, cells were incubated in the absence or in the presence of SM at a concentration between 5 and 100 μM for 60 min. The PMNs were then exposed to 15 min of UV irradiation, as above, or not exposed to UV before being incubated at 37 °C/5% CO_2 for 2 h. After treatment, cells were lysed and analyzed for caspase-3 activity. Caspase-3 exists as a proenzyme that is cleaved and activated during the induction of apoptosis [27]. Caspase-3 activation in PMNs increased with increasing doses of SM in the absence of UV irradiation (Fig. 4C). Exposure to UV irradiation caused a higher level of caspase-3 activation than incubation with 100 μM SM alone, but there was no detectable enhancement of UV-induced apoptosis in SM-treated cells. These data suggest that SM does

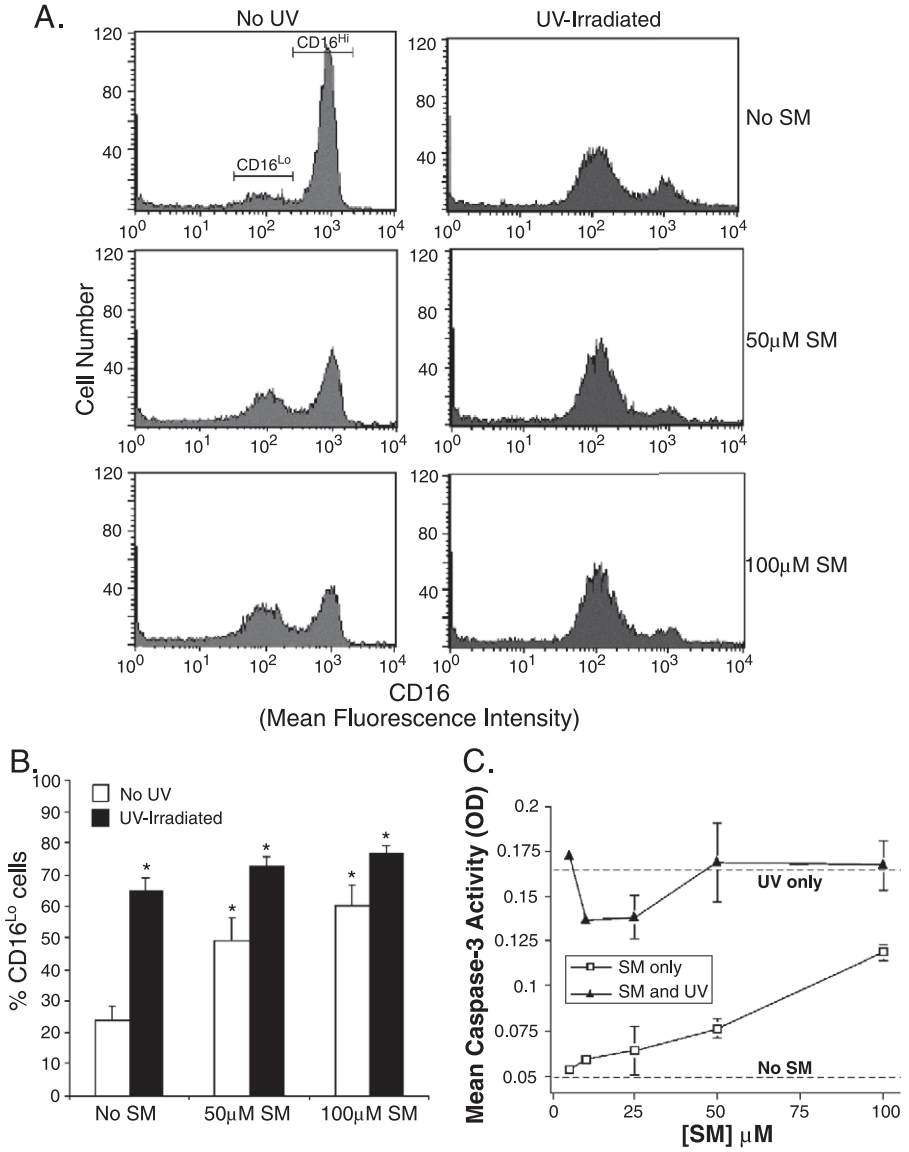

Fig. 4. SM priming does not rescue PMNs from UV-induced apoptosis. (A) PMNs were incubated with 50 or 100 μM SM for 60 min, exposed to UV irradiation, and then cultured for 5 h at 37 °C. PMNs were stained with phycoerythrin-labeled anti-CD16 and analyzed by flow cytometry. As PMNs become apoptotic, they lose CD16 and move from the $CD16^{Hi}$ to the $CD16^{Lo}$ gate. PMNs in the $CD16^{Lo}$ gate were considered apoptotic, while cells in the $CD16^{Hi}$ gate were considered viable. (B) The cumulative percentages of $CD16^{Lo}$ PMNs (apoptotic) from the unirradiated (open bars) or UV-irradiated (filled bars) condition, from the experiment described in (A), are shown. Bars represent the mean ± S.E.M. of three separate experiments. *$p < 0.05$, repeated-measures ANOVA with Tukey–Kramer multiple comparisons test. (C) PMNs were incubated with either 5, 10, 25, 50, or 100 μM SM for 60 min; exposed to UV irradiation; and then cultured for 2 h at 37 °C. Cells were lysed and caspase-3 activity was measured using a colorimetric assay. Filled triangles are cells exposed to SM and UV. Open squares represent PMN incubated with SM but not subsequently exposed to UV. Error bars represent the standard deviation of three experiments. The upper dashed line represents caspase-3 activity for cells exposed to UV only in the absence of SM incubation; the lower dashed line represents the background caspase-3 activity for cells not exposed to either SM or UV.

not prolong survival of PMN following UV irradiation and, in addition, appears to promote apoptotic progression.

4. Discussion

Although considerable work has focused on understanding the mechanisms of direct cellular injury mediated by sulfur mustard exposure, relatively little is known regarding the phenomena surrounding the acute inflammatory response that follows SM exposure. The ability to control secondary tissue damage mediated by inflammation may prove useful in treating SM lesions and reducing the time required for resolution of the injury. PMNs play a crucial role in the initiation and propagation of inflammatory responses in humans. These terminally differentiated effector cells are the first line of defense in host responses to injury and infection. PMN influx into acute SM wounds has been documented as early as 30 min post-SM exposure [9]. This raises the possibility that early infiltrating PMNs in acute SM wounds may be exposed to low doses of SM. The current study was therefore undertaken to evaluate the effect of low-dose (sub-100 μM) SM on PMN oxidative function and PMN survival.

Preparing PMNs for an enhanced response to a second stimulus is referred to as priming [13,14] and the present study demonstrates that low doses of SM can prime PMN oxidant production in response to fMLP stimulation. The priming of PMN oxidant production was dose-dependent, with 50 μM SM appearing to be the optimal dose for intracellular ROS generation in our experiments. The priming effect of 50 μM SM on ROS production by PMN was proportionally equivalent to that of GM-CSF or IL-8, although the total ROS production by SM primed cells was twofold to threefold lower. This discrepancy results from the ability of both GM-CSF and IL-8 to directly induce some degree of ROS production in the absence of stimulation by fMLP. At the concentrations tested in our experiments, no direct stimulation of PMN oxidant production was observed with SM treatment. These data demonstrate that in addition to the direct tissue injury mediated by SM, SM may also play a role in amplifying secondary inflammation-mediated injury by priming PMN oxidative function.

Recent studies have shown that several proinflammatory factors, which are known to prime PMN antimicrobial functions, also prolong PMN survival by inhibiting apoptosis. Bacterial lipopolysaccharide (LPS), tumor necrosis factor (TNF), interleukin-1 (IL-1), granulocyte–macrophage colony-stimulating factor, and granulocyte colony-stimulating factor (G-CSF) have all been shown to delay PMN apoptosis in vitro [21,28–31]. Our examination of apoptosis, following PMN exposure to 50 or 100 μM SM, indicates a different trend than that of exposure to inflammatory cytokines. Sulfur mustard exposure not only failed to rescue PMNs from UV-induced apoptosis, but also appeared to induce or accelerate the apoptotic process. Although the ability of SM to prime PMN oxidative function while simultaneously inducing apoptosis appears contradictory, we have found similar results with another PMN priming agent, tumor necrosis factor-alpha (TNF-α; J. Sweeney, unpublished observations). The mechanism by which SM mediates oxidant production and apoptosis in PMN is unknown, but it is possible that SM directly alters the redox potential within the cell, thus priming ROS generation and stimulating mitochondrially driven apoptosis. Further investigation in these areas is currently underway in our laboratory. Understanding the mechanisms through which SM directly affects PMN priming and apoptosis will be of critical value in developing effective therapies to treat secondary SM-induced tissue injury mediated by acute inflammatory responses.

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