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Postexposure Application of Fas Receptor Small-Interfering RNA to Suppress Sulfur Mustard–Induced Apoptosis in Human Airway Epithelial Cells: Implication for a Therapeutic Approach

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ABSTRACT

Sulfur mustard (SM) is a vesicant chemical warfare and terrorism agent. Besides skin and eye injury, respiratory damage has been mainly responsible for morbidity and mortality after SM exposure. Previously, it was shown that suppressing the death receptor (DR) response by the dominant-negative Fas-associated death domain protein prior to SM exposure blocked apoptosis and microvesication in skin. Here, we studied whether antagonizing the Fas receptor (FasR) pathway by small-interfering RNA (siRNA) applied after SM exposure would prevent apoptosis and, thus, airway injury. Normal human bronchial/tracheal epithelial (NHBE) cells were used as an in vitro model with FasR siRNA, FasR agonistic antibody CH11, and FasR antagonistic antibody ZB4 as investigative tools. In NHBE cells, both SM (300 μM) and CH11 (100 ng/ml) induced caspase-3 activation, which was inhibited by FasR siRNA and ZB4, indicating that SM-induced apoptosis was via the Fas response. FasR siRNA inhibited SM-induced caspase-3 activation when added to NHBE cultures up to 8 hours after SM. Results using annexin V/propidium iodide-stained cells showed that both apoptosis and necrosis were involved in cell death due to SM; FasR siRNA decreased both apoptotic and necrotic cell populations. Bronchoalveolar lavage fluid (BALF) of rats exposed to SM (1 mg/kg, 50 minutes) revealed a significant ($P < 0.05$) increase in soluble Fas ligand and active caspase-3 in BALF cells. These findings suggest an intervention of Fas-mediated apoptosis as a postexposure therapeutic strategy with a therapeutic window for SM inhalation injury and possibly other respiratory diseases involving the Fas response.

Introduction

Sulfur mustard [SM; bis-(2-chloroethyl) sulfide] is a vesicating compound and has been used as a chemical warfare agent beginning in World War I and most recently in the Iraq/Iran conflict. Its use is now re-emerging as a major threat not only to the military but also to civilians (Saladi et al., 2006). However, in spite of intense research on its mechanisms of action and intervention of toxicity, there is no available therapy against SM-exposure effects. The target organs for SM injury are skin, lung, and the eye. SM exposure can result in severe skin blisters, pulmonary damage, and eye damage causing irritation/inflammation, photophobia, and neovascularization (Papirmeister et al., 1985; Smith et al., 1990; Smith and Dunn, 1991; Petrali et al., 1993). Although SM can cause these different tissue effects, most mortality following SM exposure has been attributed to pulmonary damage and respiratory tract lesions. Respiratory complications of SM poisoning in Iranian veterans have included laryngitis, tracheobronchitis, bronchiolitis, bronchopneumonia, chronic obstructive pulmonary disease, bronchiectasis, asthma, large airway narrowing, and pulmonary fibrosis (Bijani and Moghadamnia, 2002; Balali-Mood and Hefazi, 2005; Emad and Emad, 2007; Ghanei and Harandi, 2007). Since SM is a highly reactive chemical, its pathogenic mechanisms, e.g., DNA damage, are initiated without much delay but the tissue injury is not seen until after a lag period of 12 to 24 hours (Papirmeister et al., 1985; Rosenthal et al., 2003). This provides a therapeutic window for medical management of SM injury.

It has been previously indicated that epithelial damage via apoptosis is a major mechanism of SM injury (Rosenthal et al., 1998, 2003). In SM injury, both the intrinsic mitochondrial pathway and the extrinsic death receptor (DR)-mediated

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ABBREVIATIONS: ALI, acute lung injury; BALF, bronchoalveolar lavage fluid; BEBM, basal cell growth medium; DISC, death-inducing signaling complex; DR, death receptor; ELISA, enzyme-linked immunosorbent assay; FasL, Fas ligand; FasR, Fas receptor; NHBE, normal human bronchial/tracheal epithelial; sFasL, soluble Fas ligand; siRNA, small-interfering RNA; SM, sulfur mustard.

pathway are involved in the apoptotic response. The intrinsic pathway utilizes the initiator caspase-9, and the extrinsic pathway utilizes the initiator caspase-8, which interacts with caspase-9 via truncated Bid (Fig. 1). It has been reported previously that the DR-mediated pathway plays an important role in SM toxicity in normal human epidermal keratinocytes (Rosenthal et al., 1998, 2003). However, what type(s) of DR(s) might be involved in apoptosis due to SM was not established. The DR pathway is initiated by the clustering and activation of the membrane receptors (e.g., Fas), leading to the formation of a death-inducing signaling complex (DISC). For the Fas-mediated mechanism, the DISC contains the Fas receptor (FasR), an adapter protein consisting of the Fas-associated death domain protein, which contains a death domain and a death effector domain and procaspase-8; this complex leads to the autocatalytic activation of procaspase-8 (Xu and Shi, 2007). Active caspase-8 then cleaves and activates the effectors, caspases-3/-7, which in turn cleave protein substrates within the cell, resulting in apoptosis.

In the present study, we investigated the effects of selective FasR antagonists, ZB4 (monoclonal antagonistic antibody), and small-interfering RNA (siRNA), directed toward the FasR, on SM-induced apoptosis in normal human bronchial/tracheal epithelial (NHBE) cells as measured by caspase-3 activation. Our goal was to elucidate the potential role and therapeutic applications of the FasR, a member of the tumor necrosis factor-related superfamily of death receptors, in SM-induced apoptosis. The results from this study support previous findings that the Fas-mediated mechanisms are responsible for apoptosis due to SM in NHBE cells (Ray et al., 2008, 2010). Previous reports (Ray et al., 2008, 2010) have shown that suppressing the Fas response by using a peptide inhibitor of caspase-8 prior to SM exposure attenuates SM-induced apoptosis in NHBE cells. In this study, our new findings are the following: 1) antagonizing the Fas response at an earlier stage, i.e., by blocking the FasR, markedly reduces SM-induced apoptosis; 2) the FasR can be effectively blocked by siRNA, which has been proposed as a viable novel respiratory therapeutic approach; and, moreover, 3) inhibiting the Fas response by FasR siRNA as late as 8 hours after SM exposure attenuates SM-induced apoptosis in NHBE cells. These findings lead us to propose that Fas suppression via blocking the FasR by siRNA is a prospective experimental therapeutic approach for SM inhalation injury.

Materials and Methods

Cell Culture and Chemicals. SM [bis-(2-chloroethyl) sulfide; >98% pure] was obtained from the U.S. Army Edgewood Chemical Biologic Center (Aberdeen Proving Ground, MD). Frozen-stock NHBE cells and their growth media (basal cell growth medium [BEBM]) were obtained from Lonza (Walkersville, MD). NHBE cells were maintained in serum-free growth media supplemented with bovine pituitary extract, human epidermal growth factor, hydrocortisone, epinephrine, transferrin, insulin, and retinoic acid. Cells were subcultured or used before they reached 80% confluence and up to the third passage. All other chemicals were from Sigma-Aldrich (St. Louis, MO) and were of highest purity available.

SM Exposure of Cells. Cells were exposed to SM as described previously (Ray et al., 1995). Cells were grown to ~80% confluence (visual assessment) and then exposed to SM diluted in BEBM to a final concentration of 300 μM. A concentration of 100–300 μM is considered to be the in vitro equivalent of a vesicating SM concentration in vivo in an animal model. SM undergoes rapid hydrolysis in aqueous solution and was, therefore, diluted in growth media immediately before use. Briefly, a formulation consisting of 5 μl of a neat SM oily globule frozen in 10 ml of cell-growth medium was thawed by warming to room temperature and vortexed at top speed for 1 minute to solubilize the SM in the medium and produce a 4-mM stock solution. The stock solution was then added to the cell-culture

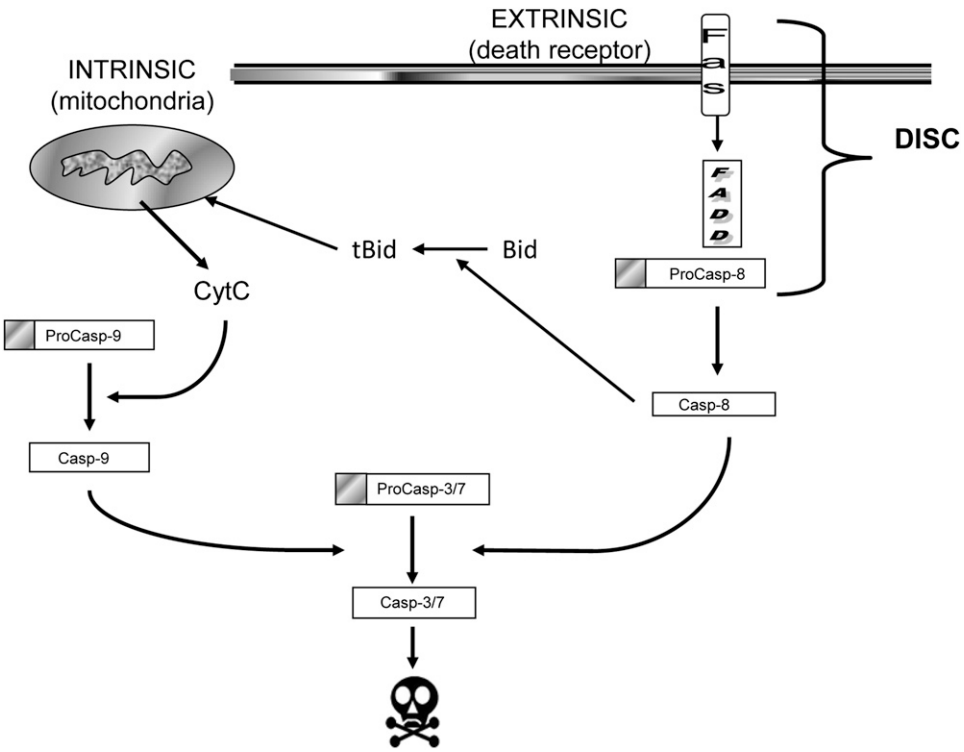

Fig. 1. Extrinsic and intrinsic apoptosis cascade.

medium in flasks or multiwell plates for the indicated SM concentration, and media were not changed until cells were harvested after indicated times for further experimental steps and analyses.

siRNA Constructs and Transfection. siRNA was obtained from Ambion (Austin, TX). The sequences were sense 5′-GCGUAUGACACAUUGAUUAtt-3′ and antisense 5′-UAACAAUGUGUCAUACGCtt-3′. All siRNA constructs were dissolved in nuclease-free water that was supplied by the manufacturer. The day of the transfection, cells were approximately at 50% confluence. Cell-culture medium was aspirated and replaced with fresh BEBM. Next, siRNA was suspended in 1.5 ml of BEBM without growth supplements for 5 minutes, then FuGENE HD (Roche, Indianapolis, IN) was added in a 6:2 ratio (microliters of FuGENE HD:micrograms of siRNA) for 15 minutes. The siRNA:FuGENE HD complex was then added to NHBE cells within 15 minutes. Cells were transfected and 48 hours later, the media were exchanged for fresh BEBM prior to SM or the FasR CH11 exposure.

Caspase-3 Activation. Caspase-3 activation and sequential activity was measured by a Caspase-Glo 3/7 assay from Promega (Madison, WI). Caspase-Glo 3/7 assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activity by measuring the luminescence generated due to the cleavage of a luminogenic substrate containing a DEVD sequence; following this cleavage, a substrate for luciferase (amino-luciferin) is released. Caspase-Glo 3/7 assay was performed according to the manufacturer's instructions. Briefly, Caspase-Glo reagent mixture was added in a 1:1 ratio in BEBM. Approximately 1 hour following Caspase-Glo reagent addition to the cells, caspase activity was measured with a SpectraMax Gemini EM (MDS Analytical Technologies, Toronto, ON, Canada).

Western Blot. The level of FasR in NHBE cells was assessed by SDS-PAGE and Western blotting. Briefly, cells harvested in ice-cold physiologic saline were lysed by boiling in 40% (w/v) urea, 1% (w/v) SDS, and 3% (v/v) β-mercaptoethanol. Several protease inhibitors present in a protease inhibitor cocktail (Sigma-Aldrich) were included throughout the wash and solubilization steps to prevent FasR degradation during the assay. The lysates were cleared by centrifugation at 16,000*g* for 10 minutes and immediately analyzed by SDS-PAGE. Each gel (8–16.5% acrylamide) lane was loaded with approximately 50 μg of protein. Semidry transfer to nitrocellulose membranes was performed at 250 mA for 60 minutes, followed by 1 hour of blocking in 2% (w/v) bovine serum albumin (this and all subsequent incubations were performed in physiologic saline, pH 7.5, room temperature). Rabbit monoclonal antibody to FasR protein was used at a 1:1000 dilution and incubated overnight at 4°C. This was followed by 1-hour incubation with a 1:1000 dilution of polyclonal peroxidase-conjugated anti-rabbit IgG. Detection was performed by fluorescent detection of enzymatic chemiluminescence substrate (Amersham, Piscataway, NJ).

Enzyme-Linked Immunosorbent Assay. High-binding enzyme-linked immunosorbent assay (ELISA) Strip Plates (Santa Cruz Biotechnology, Santa Cruz, CA) were coated with biotinylated anti-rat Fas ligand (FasL) antibody (R&D Systems, Minneapolis, MN) in 50 mM bicarbonate buffer and blocked with BSA in Tris-buffered saline. Samples and FasL protein standard were added and incubated overnight at 4°C. The wells were then incubated at room temperature for 1 hour with FasL (N-20) antibody (Santa Cruz Biotechnology) and probed with a horseradish peroxidase-conjugated secondary antibody (Invitrogen, Carlsbad, CA). The peroxidase activity was detected using 3,3′,5,5′−tetramethylbenzidine as a substrate and stopped with a 3,3′,5,5′−tetramethylbenzidine stop reagent (Sigma-Aldrich). The absorbance was measured at 450 nm using a ThermoMax microplate reader (MDS Analytical Technologies, Sunnyvale, CA). The recombinant human FasL/TNFSF6 produced in CHO cells (Sigma-Aldrich) was used as a standard.

Flow Cytometry. Cells were stained with annexin V Alexa Fluor 488 and PI utilizing the dead cell apoptosis kit from Invitrogen (Carlsbad, CA). After the final incubation step, cells were analyzed by flow cytometry. A minimum of 10,000 cells were analyzed using a FACSAria II flow cytometer (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo (Tree Star, Inc., Ashland, OR) flow cytometry analysis software.

Animals and Treatments. Male Sprague-Dawley rats (250–300 g) were purchased from Charles River Laboratories (Wilmington, MA) and maintained in an Association for Assessment and Accreditation of Laboratory Animal Care–approved animal care facility at the U.S. Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, MD). SM (>98% pure) was diluted in absolute ethanol (EtOH). Animals were exposed to EtOH alone or SM solutions by intratracheal inhalation to achieve a final dose of 1.0 mg/kg as previously described (Anderson et al., 1996).

At 6 and 24 hours after SM exposure, the rats were euthanized, and blood and bronchoalveolar lavage fluid (BALF) were collected for biochemical analyses. BALF was collected by lavaging the whole lung three times with 3 ml of aliquots of sterile saline at room temperature and washing three times before removal. The BALF was pooled and centrifuged, and the supernatant and cells were collected and stored at −80°C until analyses.

Statistical Analysis. Assays were performed in replicates of three. The results were averaged and expressed as the mean ± S.E.M. The differences between groups at each time point were determined using a one-way analysis of variance followed by post hoc comparison of multiple treatment group means using the Tukey's test. Statistical significance was defined as $P < 0.05$.

Results

ZB4 Blockade of CH11-Induced Apoptosis in NHBE Cells. The first experiments conducted were concentration-response (100–500 ng/ml) and time-course studies for CH11-induced caspase-3 activation in NHBE cells; the experiments found that 100 ng/ml CH11 added to the culture medium activated caspase-3 maximally at 24 hours (Fig. 2A). The concentration response (100–600 ng/ml) for ZB4 to block CH11-induced caspase-3 was determined: 100 ng/ml ZB4 was the minimum concentration needed to cause 80–85% inhibition of caspase-3 activation (Fig. 2B). The maximum tested concentration of 500 ng/ml ZB4 did not cause a significant caspase-3 activation, indicating that ZB4 itself does not activate caspase-3 in NHBE cells (Fig. 2B). These results clearly show that Fas-mediated caspase-3 activation is blocked by ZB4.

ZB4 Antagonism of SM-Induced Apoptosis in NHBE Cells. The effect of ZB4 (100–500 ng/ml) on caspase-3 activation at 24 hours after SM (300 μM) exposure in NHBE cells was then investigated. A concentration of 100 ng/ml ZB4 was able to reduce the SM-induced caspase-3 activation by 23 ± 10%; this decrease was small but significant ($P < 0.05$) (Fig. 2C). A higher concentration of ZB4, approximately 500 ng/ml, was needed to attenuate the SM-induced caspase-3 activation by 55 ± 3% (Fig. 2C). Since CH11 and ZB4 act on FasRs as a specific agonist and a blocker, respectively, the results presented here and above indicate that SM-induced caspase-3 activation, i.e., apoptosis, is partially Fas-mediated.

FasR siRNA Treatment Attenuates SM-Induced Caspase-3 Activation. The effect of siRNA directed toward the FasR was evaluated by measuring caspase-3 activation due to both CH11 (100 ng/ml) (Fig. 3) and SM (300 μM) (Fig. 4). NHBE cells were grown to ~50% confluence before transfection with 1–20 nM siRNA, scrambled siRNA (20 nM), or transfection agent only (control). Caspase-3 activation due to CH11 was measured at 48 hours after siRNA transfection. A dose-dependent reduction in CH11-induced caspase-3 activation was seen with a maximum reduction of ~80% occurring with 10 nM siRNA (Fig. 3A). Next, the effect

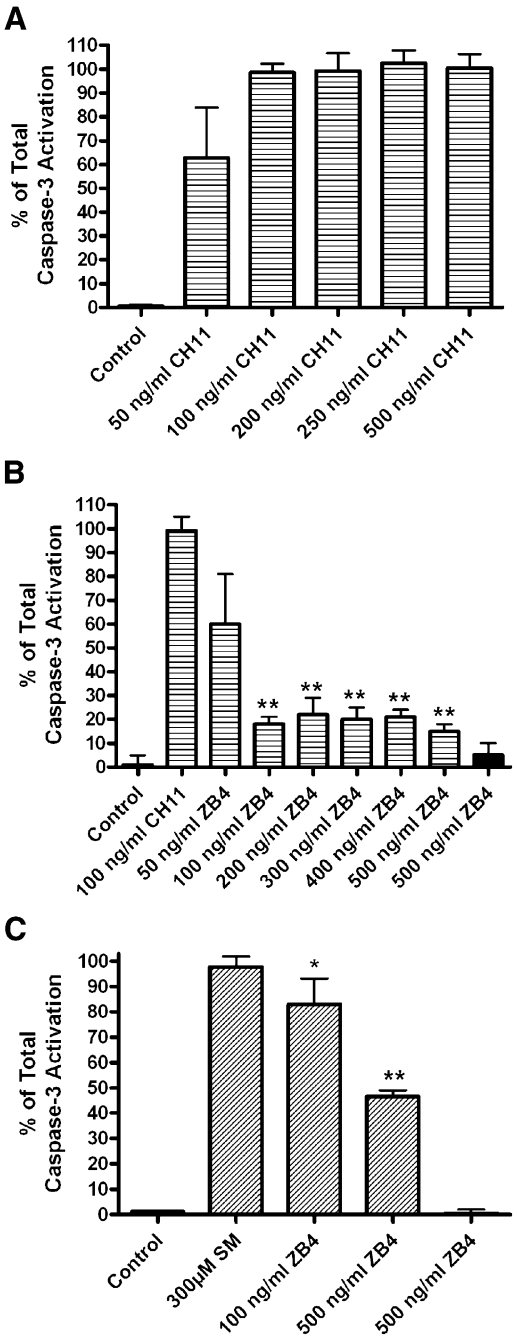

Fig. 2. Concentration response of caspase-3 activation in NHBE cells due to CH11 and ZB4. (A) NHBE cells were incubated with varying concentrations of CH11; 24 hours later, caspase-3 activity was examined. Data are shown as the percentage of total caspase-3 activation. Experiments were done in triplicate with five wells per experiment, ± SEM. (B) ZB4 at various concentrations and 100 ng/ml CH11 were presented concurrently to NHBE cells; 24 hours later, caspase-3 activation was measured. Lined bars indicate addition of 100 ng/ml CH11; solid black, ZB4 only. Data are shown as the percentage of total caspase-3 activation compared with 100 ng/ml CH11 alone. (C) NHBE cells were exposed to ZB4 at various concentrations and 300 μM SM concurrently; 24 hours later, caspase-3 activation was measured. Hatched bars indicate addition of 300 μM SM; rightmost, 500 ng/ml ZB4 is ZB4 alone. Data are shown as the percentage of total caspase-3 activation compared with 300 μM SM alone. Experiments were done in triplicate with a five wells per experiment, ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with 100 ng/ml CH11 (B) or 300 μM SM alone (C).

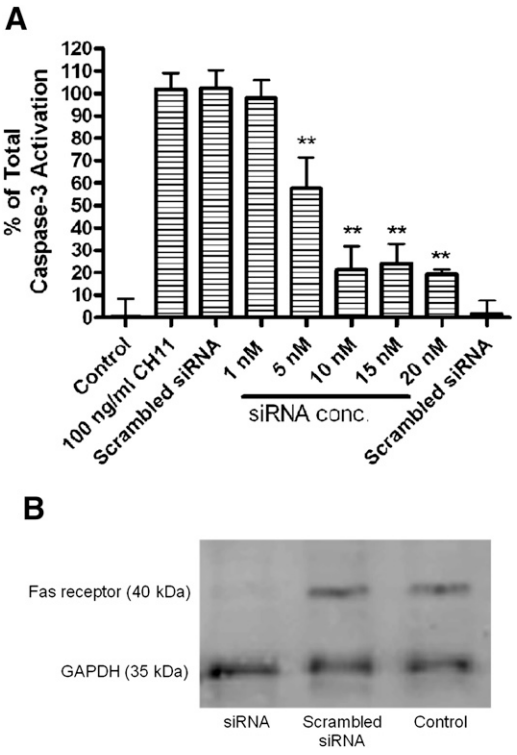

Fig. 3. (A) siRNA concentration response of caspase-3 activity after CH11 addition in NHBE cells. Various siRNA concentrations were given to NHBE cells 48 hours prior to 100 ng/ml CH11 addition. Twenty-four hours after CH11 addition, caspase-3 activation was measured. Data are shown as the percentage of total caspase-3 activation compared with 100 ng/ml CH11. Experiments were done in triplicate with five wells per experiment, ± SEM. $^{**}P < 0.01$ compared with 100 ng/ml CH11 alone. Lined bars indicate addition of 100 ng/ml CH11. (B) NHBE cells were harvested 48 hours after transfection with 10 nM siRNA. Cells were lysed and 50 μg of protein was loaded per well. FasR monoclonal antibody (1:1000) and GAPDH Ab (1:1000) were used and detected by ECF substrate.

of 10 nM siRNA on FasR protein expression by Western blotting was examined. siRNA treatment almost completely abolished the FasR band (Fig. 3B). These results showed that the siRNA construct was highly effective in suppressing FasR expression as well as Fas-mediated caspase-3 activation in NHBE cells. In other words, Fas siRNA effectively suppressed Fas-mediated apoptosis.

Whether the observed SM-induced Fas suppression by ZB4 could be reproduced by a molecular approach using FasR siRNA was then tested. NHBE cells were grown to ~50% before transfection with 1–20 nM siRNA, scrambled siRNA (20 nM), or control. Forty-eight hours post-transfection, a concentration of 300 μM SM was given to NHBE cells, and approximately 24 hours later caspase-3 activity was assayed using the Promega Caspase-Glo caspase-3 kit. As can be seen in Fig. 4, siRNA targeting toward the FasR was able to decrease the caspase-3–induced activation via SM ~65%. Using each of these antagonists, ZB4 and siRNA, a significant reduction of caspase-3–induced activation by SM was seen. These results show that the major pathway in caspase-3–induced activation of SM is the Fas pathway.

Postexposure Application of FasR siRNA. Based on the observation in NHBE cells that pretreatment with Fas siRNA was effective in decreasing (~65%) caspase-3 activation due to SM, we became interested in seeing whether

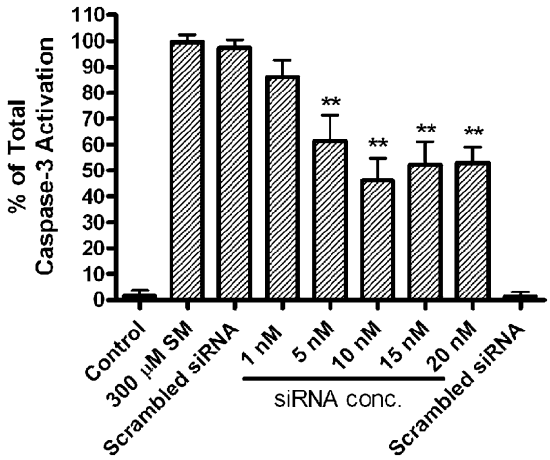

Fig. 4. FasR siRNA dose response for caspase-3 activation after 300 μM SM addition to cultured NHBE cells. Cells were treated with various siRNA concentrations 48 hours prior to 300 μM SM addition. Twenty-four hours after 300 μM SM addition, caspase-3 activation was measured. Data are shown as the percentage of total caspase-3 activation compared with 300 μM SM. Experiments were done in triplicate with five wells per experiment, ± SEM. $^{**}P < 0.01$ compared with 100 ng/ml 300 μM SM alone.

adding Fas siRNA to cell cultures at timed intervals post-SM exposure could also decrease SM-induced caspase-3 activation. First, NHBE cells (~80% confluent) were exposed to 300 μM SM, and then Fas siRNA was applied at 2, 4, or 8 hours after SM exposure. Caspase-3 activation was measured 24 hours after SM. Similar to pretreatment, postexposure treatment with siRNA also decreased (~40–60%) caspase-3 activation due to SM (Fig. 5). These results showed that Fas siRNA could prevent SM-induced apoptosis in NHBE cells even when added to cells up to 8 hours after SM; this indicated a therapeutic window out to 8 hours postexposure.

To correlate the siRNA prevention of SM-induced caspase-3 activation with preventing cell death, the status of surviving versus dead cells in NHBE cultures was determined by flow cytometry using annexin V (apoptosis marker) and PI (necrosis marker) staining. The results showed proportions of cells as live and dead (via both apoptosis and necrosis) (Fig. 6). Fas siRNA was added to NHBE cells 8 hours after 300 μM SM exposure followed by annexin V/propidium iodide (PI) staining and analyses approximately 24 hours after SM exposure. Cell death (apoptosis/necrosis) due to SM alone (Fig. 6A) was 47 ± 2% ($n = 3$). Treatment with 5–20 nM Fas siRNA decreased cell death to 18–32% ($n = 3$) (Fig. 6, B–E). Treatment with siRNA caused a reduction in the cells stained with either annexin V or PI and a concomitant increase in unstained cells. A careful examination of these results showed that in SM-exposed (300 μM) NHBE cultures, siRNA treatment was able to reduce the number of cells that were in the late apoptosis/early necrosis phases.

Soluble FasL in the Bronchoalveolar Lavage Fluid of Rats Exposed to SM via Inhalation. The findings described above indicated a Fas-mediated mechanism of cell death due to SM in vitro in an airway epithelial (NHBE) cell-culture model. To test a therapeutic approach based on this mechanism, i.e., prevention of airway injury due to SM via Fas intervention, the next step would be to validate these observations in vivo in a suitable animal model. To accomplish this objective, a rat SM inhalation model as described

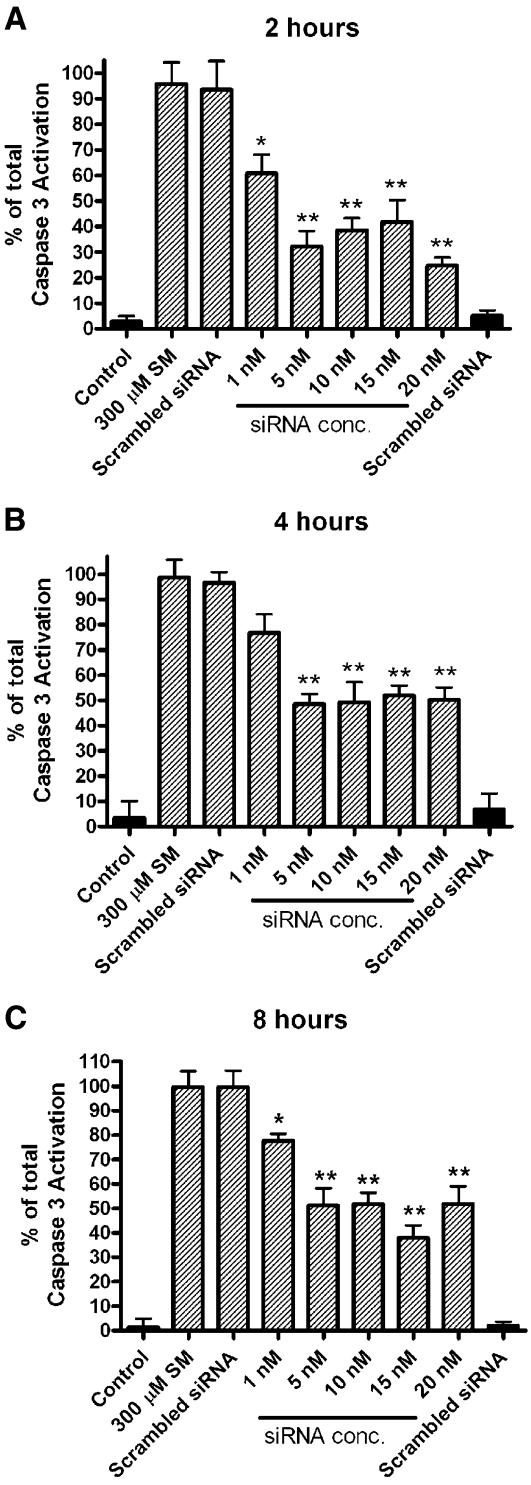

Fig. 5. FasR siRNA postexposure concentration response to 300 μM SM. FasR siRNA was added at 2, 4, and 8 hours (A–C, respectively) after 300 μM SM exposure, and caspase 3 activation was measured 24 hours after 300 μM SM exposure. Hatched bars indicate 300 μM SM exposure; black bars, vehicle control. Experiments were done in triplicate with five wells per experiment, ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with 300 μM SM alone.

previously (Anderson et al., 1996) was used. Anesthetized rats were exposed to SM (in ethanol solution) via spontaneous inhalation by intubation. Subsequently, pulmonary damage to include epithelial effects was assessed by biochemical

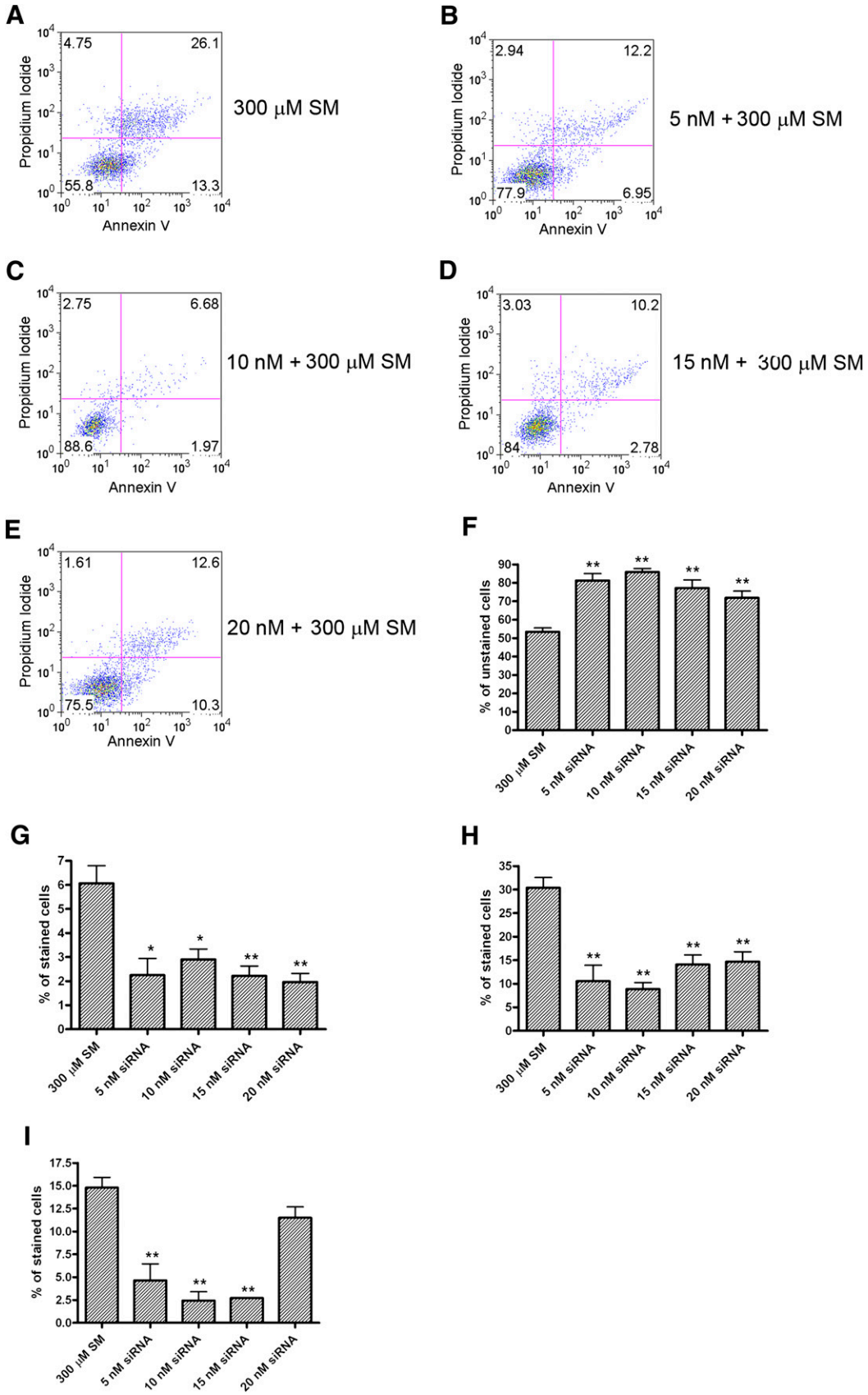

Fig. 6. Annexin V and PI staining of 300 μM SM in NHBE cells. NHBE cells were exposed to 300 μM SM, then were given 5 (B), 10 (C), 15 (D), or 20 nM (E) FasR siRNA 8 hours after SM exposure. Approximately 24 hours following SM exposure, NHBE cells were stained with Annexin V/PI and analyzed by flow cytometry. Results from a representative experiment are shown above. Averaged data for each quadrant: unstained (F), AV only (G), annexin V/PI (H), and PI only (I) is also shown. $n = 3$, ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$ compared with 300 μM SM only.

analyses of bronchoalveolar lavage fluid (BALF) constituents as well as cellular components in the BALF. In these experiments, BALF samples from sham (ethanol inhalation) control and SM-exposed (1.0 mg/kg, 50 minutes) rats were collected at either 6 or 24 hours following SM exposure. Soluble Fas ligand (sFasL) in BALF and caspase-3 activity in BALF cells were then determined using ELISA and luminometric caspase-3 assay, respectively (Fig. 7). Results showed significant ($P < 0.01$) increases in both sFasL in BALF and caspase-3 activity in BALF cells at 6 and 24 hours following both doses of SM. Although limited information has been obtained, these results suggest a Fas-mediated mechanism of apoptosis in vivo similar to the observations in vitro using NHBE cell cultures.

Discussion

The results in this study demonstrate that a highly selective and effective siRNA construct was able to down-regulate the expression and functionality of the FasR in a normal human airway epithelial cell-culture model. In cultured NHBE cells, both SM (300 μM) and the agonistic FasR antibody CH11 activated caspase-3, which was inhibited by the antagonistic FasR antibody ZB4. These results indicated that SM-induced caspase-3 activation was via the Fas response (Fig. 2). The siRNA construct at a low (10 nM) concentration markedly inhibited caspase-3 activation due to both CH11 and SM (Figs. 3 and 4). However, the extent of inhibition was more when caspase-3 was activated by CH11 than by SM, possibly because SM stimulation of caspse-3 involves mechanisms additional to those pertaining to the Fas pathway (Fig. 1). siRNA inhibited (~60%) SM-induced caspase-3 activation even when added to NHBE culture as late as 8 hours after SM exposure (Fig. 5). Moreover, flow cytometric studies revealed that the siRNA inhibition of caspase-3 activation due to SM was concomitant with reduced cell death via both apoptosis and necrosis (Fig. 6). A more detailed analysis of these results showed the following. In cells exposed to SM (300 μM), but not treated with siRNA, cell death occurred via early apoptosis, late apoptosis/early necrosis, and necrosis (Fig. 6A). It was noteworthy that the majority of the cells dying were in early and late apoptosis. However, when SM-exposed (300 μM) cells were treated with siRNA, the proportions of these types of cell deaths were decreased in a siRNA concentration-dependent manner [e.g., 5 (Fig. 6B) and 10 nM (Fig. 6C)]. These observations indicated that cell death due to SM involves early apoptosis progressing toward late apoptosis and necrosis. Moreover, it seems possible to prevent the late phases of cell death by intervening at early apoptosis.

Finally, in the in vivo studies in the rat SM inhalation model, higher levels of both sFasL in BALF (Fig. 7A) and caspase-3 activity in BALF cells (Fig. 7B) were observed. These in vivo results combined with the in vitro findings suggest a mechanism of SM inhalation injury that involves an early Fas-mediated apoptosis of airway epithelial cells, which may eventually progress to necrosis. Depending on the degree of damage, these cells may detach from the substratum and cause a sloughing off of the epithelial tissue. Respiratory distress and even death following SM inhalation are believed to be due to inflammatory responses combined with the detachment and sloughing off of the dying airway epithelial cells. These contribute to tissue aggregate, generation of pseudomembranes and mucus plug responsible for choking and death. We propose to intervene in this mechanism at one

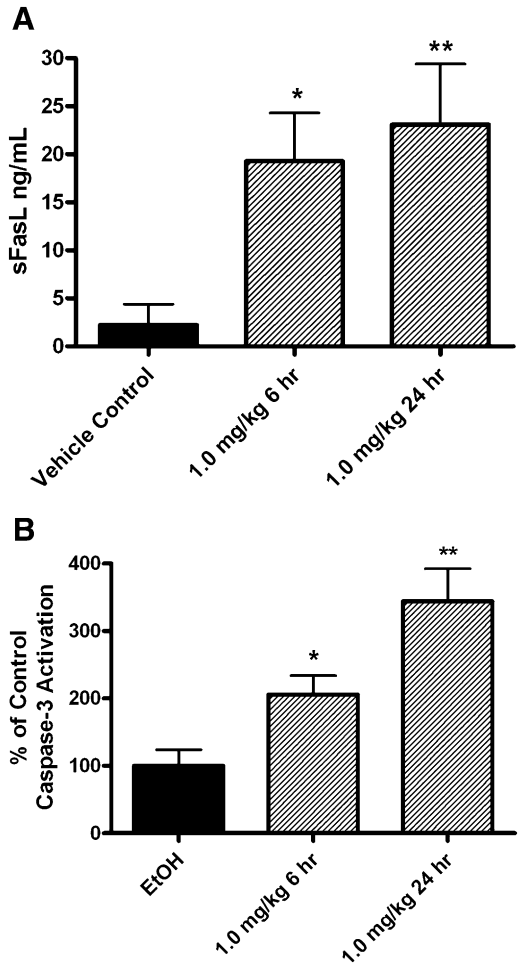

Fig. 7. Measurement of sFasL and caspase-3 in BALF in a rat SM inhalation model. sFasL (A) or caspase-3 (B) was measured in BALF or BALF cells, respectively, of rats exposed to 1.0 mg/kg SM by inhalation by intubation; BALF and BALF cells were collected at 6 or 24 hours following SM inhalation. $n = 3$ rats, ± SEM, $^*P < 0.05$, $^{**}P < 0.01$ compared with vehicle (EtOH) control.

of the component events, i.e., epithelial detachment, by inhibiting the Fas-mediated apoptosis using siRNA. If successful, the siRNA antagonism of the FasR or some other component of the DISC, e.g., the Fas-associated death domain protein, may provide a highly prospective therapeutic approach for SM inhalation injury.

In the present study, the pathway of caspase-3 activation, i.e., apoptosis due to SM, via the FasR was demonstrated. Additionally, blocking the Fas pathway by FasR siRNA-reduced cell death due to SM was shown. Most intriguing was our observation that this Fas intervention was effective even when FasR siRNA was added to cells as late as 8 hours after SM exposure. The siRNA effect is not due to its reaction with SM in the cell-culture medium. SM is sparingly soluble in water but forms a saturated solution at ~5 mM which is way above the concentration used in our experiments (i.e., 300 μM) (Papirmeister, 1991). SM is a strong electrophile due to the formation of a highly reactive sulfonium intermediate in aqueous medium. This gives rise to its bifunctional alkylating form that reacts readily with cellular functional molecules (e.g., DNA, RNA, proteins, etc.) to initiate its toxic mechanisms. At body temperature (37°C), SM hydrolyzes with a

half-life ($t_{1/2}$) of <10 minutes in water (Papirmeister, 1991), but ~15 minutes in cell-culture medium (pH 7.4) (unpublished results). Therefore, most of SM would have disappeared by about an hour, which would be long before the addition of siRNA at 2–8 hours after cell exposure. It is known that the actual manifestation of these toxic events (e.g., skin blister, corneum, and lung epithelial separation) is not observed until 12–24 hours after SM exposure. This indicates a substantial lag period between the onset of toxic cellular mechanisms and their pathologic manifestations, implying a therapeutic window. Thus, the postexposure efficacy of FasR siRNA intervention in preventing the SM effects on airway epithelial cells in vitro strongly supports the idea of its being a prospective molecular therapeutic for inhalation injury due to SM.

In addition to SM toxicity, the Fas pathway is known to be an important mediator of other disease states (e.g., idiopathic pulmonary fibrosis, acute lung injury [ALI], acute respiratory distress syndrome, etc.) (Beheshti et al., 2006; Emmler et al., 2007; Kuwano, 2008; Perl et al., 2008). In all of these cases, Fas and FasL are upregulated in the airway epithelial cells and are thought to contribute to cellular apoptosis (Matute-Bello et al., 1999; Albertine et al., 2002; Hagimoto et al., 2002; Lee et al., 2008). The severity of the infection in septic ALI was highly correlated with the Fas-FasL system (Albertine et al., 2002); furthermore, acute respiratory distress syndrome nonsurvivors were shown to have markedly higher levels of FasL than survivors (Matute-Bello et al., 1999). Active antagonism of FasR has been shown to reduce the development of endotoxin-mediated ALI, hemorrhage-induced septic ALI, and bleomycin-induced pulmonary fibrosis in animal models (Kuwano et al., 1999; Kitamura et al., 2001; Perl et al., 2007). These observations point to a critical role of Fas in each of these diseases. Therefore, the antagonism of Fas via FasR, as we have proposed, may be an effective novel treatment strategy for respiratory diseases involving the Fas-mediated mechanisms.

The idea of Fas intervention has been put forth by others as well (Perl et al., 2007; Kuwano, 2008). There are several ways to antagonize the FasR (e.g., the Fas antagonistic antibody ZB4 and Fas:Ig, a fusion protein that blocks FasL-FasR interaction); however, the pitfalls of these approaches are their possible nonselective effects. siRNA, however, does not have these pitfalls and has been shown to be highly selective. For these reasons, siRNA has emerged as a major thrust of the big pharmaceutical companies. Many have shown that siRNA is easily delivered via either intranasal or intratracheal route without any modifications to the lung (Massaro et al., 2004; Zhang et al., 2004; Perl et al., 2005; Thomas et al., 2005, 2007; Aigner, 2007; de Fougerolles et al., 2007). The delivery of siRNA via these two routes has been shown not to induce any significant activation of type I interferons via activation of TLR-3, TLR-7, TLR-8, TLR-9, or protein kinase-R pathways nor via the more classic proinflammatory responses (Alexopoulou et al., 2001; Akira, 2003; Moss and Taylor, 2003; Sledz et al., 2003; Lomas-Neira et al., 2005; Perl et al., 2005, 2008; Robbins and Rossi, 2005). This correlates with observations that intravenous delivery of siRNA does not elicit an immune response (Heidel et al., 2004). Furthermore, siRNA administrated to the lung either intranasally or intratracheally has been shown to localize only to the lung and does not become systemically available (Lomas-Neira et al., 2005; Perl et al., 2005). The above discussions clearly indicate that siRNA may be a useful tool for in vitro research as well as in vivo applications with a great potential for being on the forefront of a new class of molecular medicine.

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Authorship Contributions

Participated in research design: Keyser, Andres, Anderson, Smith, Ray.

Conducted experiments: Keyser, Andres, Nealley, Holmes, Benton, Paradiso, Appell, Anderson, Carpin.

Contributed new reagents or analytic tools: Keyser.

Performed data analysis: Keyser, Andres, Nealley, Holmes, Ray.

Wrote or contributed to the writing of the manuscript: Keyser, Andres, Nealley, Holmes, Anderson, Ray.

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