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Emerging targets for treating sulfur mustard-induced injuries

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Abstract

Sulfur mustard (SM, bis- (2-chlororethyl) sulfide) is a highly reactive, potent warfare agent that has currently reemerged as a major threat to military and civilians. Exposure to SM is often fatal primarily due to pulmonary injuries and complications caused by its inhalation. Profound inflammation, hypercoagulation and oxidative stress are the hallmarks that define SM-induced pulmonary toxicities. Despite advances, effective therapies are still limited. This current review focuses on inflammatory and coagulation pathways that influence the airway pathophysiology of SM poisoning and highlights the complexity of developing an effective therapeutic target.

Keywords

sulfur mustard; pulmonary; coagulation; inflammation; extracellular RNA

Introduction

Sulfur mustard (SM, bis- (2-chlororethyl) sulfide) is a highly reactive, potent warfare agent that has currently reemerged as a major threat to military and civilians. Initial exposures of SM are not obvious due to lack of particular odor, however atmospheric accumulations of higher concentrations smell like mustard. Victims of SM exposure have a diverse variety of symptoms depending on the dose, duration and environment of SM exposure. SM-induced injuries are difficult to treat and cause many long-term complications. Skin, eye and lung are the immediate targets of SM where it causes quick irreversible reactions within the tissues. Profound injury to upper and lower conducting airways causes significant mortality at higher doses, and survivors of high dose exposure and those exposed to lower doses exhibit a host of clinical manifestations that include acute respiratory distress syndrome (ARDS). This review focuses on inflammatory and coagulation pathways that influence the airway pathophysiology of SM poisoning.

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Sulfur mustard toxicity

Biochemical consequences

The chemical reactions caused by sulfur mustard and its analogs are fairly well characterized. Some of the mechanisms by which it causes injury are known. Despite this, effective therapies are still lacking and there is a need to better understand its pathogenesis. The actions of SM are mainly due to its two functional chlorine groups that can form highly reactive sulfonium and carbenium ions [1] (Figure 1). These ions are potent alkylating molecules that react with nucleotides, proteins and lipids. Due to its strong alkylating properties, primarily due to activity at guanine residues, SM forms DNA adducts and crosslinks [2, 3]. SM is partially absorbed from the skin into the dermis where it can form DNA adducts or enter systemically and react with other organs including the lung [2, 4]. SM-mediated DNA alkylation and crosslinking may cause DNA damage and activation of DNA reparative enzymes [5, 6], and may ultimately result in extensive cell death and lead to malignant transformations [7]. The maximum toxicity of SM is primarily due to its modulation of DNA that specifically kills proliferative cells even at low doses. Further, SM is also highly reactive with sulfhydryl-containing compounds. Therefore, glutathione is depleted after SM exposure leading to intracellular accumulation of reactive oxygen species (ROS) [8]. Antioxidant enzymes such as superoxide dismutase are also decreased after SM inhalation leading to increased reactive oxygen species levels [9]. Reactive oxygen species cause apoptotic and necrotic cell death thereby contributing a secondary role in SM-induced toxicity [10]. SM also reacts with lipids to cause lipid peroxidation. Oxidative stress and depletion of anti-oxidant are important players in SM-induced airway injury [11]. Activation of poly (ADP-ribose) polymerase, MAPK and activating protein, AP-1 signaling pathways has been demonstrated after SM inhalation in the lung [12–14]. Additionally, proteolytic enzymes and cytokines produced by cells and secondary inflammatory responses amplify SM toxicity. More recently involvement of chemosensory channels such as transient receptor potential (TRP) family has been identified as specific sensor of the vesicant SM in the lung [15]. These effects of sulfur mustard are to a large extent mimicked by its analog, CEES (aka: half mustard; 2 chloroethyl ethyl sulfide). Analogs are frequently used as safer alternative to study vesicants in laboratories.

Pathophysiological consequences

Sulfur mustard causes a host of pathophysiological consequences. These findings are based on actual human exposures and exposure of animals to SM or CEES. The extent of injury depends on whether SM or its analogs are used. For instance, an LD50 of CEES is about 100 times less potent that SM. The extent of injury also depends on the animal model used, route, dose and duration of exposure [16]. In general, larger animals like pigs and non-human primates mimic more closely human exposures. The most common organ systems affected by SM exposures are eyes, skin and lungs [17, 18]. However sufficient evidence indicates dysfunction in brain, kidney, heart and bone marrow as well [17]. High doses are fatal and may cause convulsions, coma and death during the exposure. With lower doses the effects are delayed and symptoms may appear slowly over time. Within 6h of exposure symptoms such as nausea, fatigue, headache, eye irritation, soreness of throat and difficulty in breathing occur. Conjunctivitis, edema and inflammation of the eyelids often lead to temporary

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blindness. Other ocular symptoms include lacrimation, photophobia, blepharospasm and corneal ulceration [16]. In the next 24 h skin inflammation and blistering become apparent. Depending on exposure doses, SM also causes rhinorrhea, tracheobronchitis, airway hyperreactivity, vascular injuries, fibrin deposition followed by airway obstruction and fatalities from multi organ failure [16–19]. In the lung most acute effects of SM in the lung occur in the upper respiratory tract, however, injury to the lower airways is not uncommon [20]. Epithelial sloughing, pseudomembrane formation and airway occlusion has also been documented [16]. Studies on cadavers of SM-related fatalities have revealed maximum chronic bronchitis, pulmonary fibrosis, pulmonary infections, cellular infiltration and aspergillioma [21]. In individuals who survived there was a higher incidence of bronchiolitis obliterans and tissue biopsies revealed necrotic airways, dense cast formation, constrictive bronchiolitis, and respiratory and chronic cellular bronchiolitis [22]. Increased airway remodeling due to transforming growth factor beta 1 (TGF-β1) has also been observed upon SM inhalation [23]. Therefore, survivors of SM exposure present with a variety of respiratory anomalies including pulmonary fibrosis, bronchiectasis, acute respiratory distress syndrome (ARDS) and vascular injury and remodeling [24, 25], and are highly prone to chronic obstructive pulmonary disease (COPD) and cancers [26].

Role of coagulation in sulfur mustard poisoning

In acute lung injury and ARDS the coagulation cascade is often activated with an associated decrease in the fibrinolytic activities [27, 28]. Activation of coagulation in these situations causes extravascular fibrin deposition that can promote pulmonary dysfunction and inflammation [28]. In this context, SM exposure has great parallels with ARDS as both the coagulation and inflammatory pathways are activated [29–31]. The earliest reported indications of the role of coagulation in SM-induced injuries were from individuals exposed during the Iran-Iraq conflict in 1980–88. Autopsy of one patient that died of airway obstruction indicated formation of bronchial casts [32], in conjunction with formation of fibrin rich pseudomembranes and inflammation of the trachea and bronchial tubes [32]. These findings have also been reproduced in animal experiments with CEES or sulfur mustard [30, 31, 33, 34].

Exposure to SM/CEES causes hypercoagulation through both an increase in procoagulation factors and a decrease in the fibrinolytic factors. Veress et al. have demonstrated the formation of casts in the conducting airways of rats exposed to CEES and SM [31, 35]. These fibrin rich casts cause obstruction and impaired gas exchange that is one of the principal causes of mortality following SM exposure [31, 32]. Initiation of the procoagulatory pathways can occur through a number of different mechanisms. SM inhalation causes profound inflammation of the airways, apoptotic and necrotic cell death and vascular injuries, which can initiate coagulation via both intrinsic and extrinsic coagulation cascades. In the extrinsic pathway, tissue factor (TF) activation by SM/CEES appears to be the primary initiator. In addition to TF activation, SM/CEES exposures causes increases in fibrinogen, prothrombin and thrombin-antithrombin (TAT) complexes in the bronchoalveolar lavage fluid (BALF) of CEES-exposed rats [34]. These exposures cause an increase in TF activity and an increase in Factor X (FX) expression [33]. SM/CEES exposure also causes a parallel inhibition of the fibrinolytic pathway by increasing plasminogen activator inhibitor-1 (PAI-1), thrombin-activatable fibrinolysis inhibitor (TAFI) and α2-antiplasmin [33]. In rats, treatment with tissue

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plasminogen activator (TPA) after SM inhalation prevented cast formation and mortality [35]. Similarly, treatment with heparin and tissue factor pathway inhibitor (TFPI) also prevented mortality [34, 36]. These studies underscore the importance of coagulation in SM/CEES-induced injuries. Our studies suggest a significant contribution of airway epithelium to TF and other components of the clotting cascade [37]. Similarly, other studies have also shown that alveolar epithelium contributes significantly to coagulation [38]. However, the mechanisms and factors responsible for SM-induced increased airway coagulation are yet to be addressed.

Several additional factors can also activate the clotting cascade following SM exposure. SM exposed individuals have increased serum levels of the proinflammatory IL-6, IL-8 and MMP9 [5, 20]. They also have increased levels of oxidants that include cholesterol, triglycerides and gamma-glutamyl transferase activity [39]. Activation of the clotting cascade can also occur as a result of increased extracellular nucleic acids such as DNA and RNA that are actively released from the cells as a result of injury or necrosis. Extracellular DNA plays a dual role in both promoting and inhibiting fibrinolysis [40]. Moreover, presence of nucleic acids and histones has been shown to increase the stability of fibrin clots [41]. Growing evidence suggests that extracellular RNA (exRNA), which has been found in thrombi of mice subjected to carotid artery injury, can also provoke a significant procoagulant response [42, 43]. While in vitro studies have shown that scavengers of exRNA can prolong clotting times, *in vivo* studies have demonstrated that these scavengers can prevent thrombus formation [42]. Importantly, unlike most anticoagulants, these scavengers do not increase bleeding. Our studies have shown that there is increased exRNA in the plasma samples from rats exposed to CEES [44]. Extracellular RNA can bind to PAI-1 and stabilize it [45]. PAI-1 inhibits tissue plasminogen activator (TPA), which cleaves the proenzyme plasminogen to the active fibrinolytic plasmin (Figure 2). Interestingly PAI-1 secretion is also stimulated by pro-inflammatory cytokines. Given that PAI-1 is already increased in SM/CEES-exposed animals, further stabilization can result in prolonged inhibition of the fibrinolytic pathway. Additionally, pro-inflammatory cytokines and vascular injury expose TF and activate the extrinsic clotting pathway. Extracellular RNA from the blood can also initiate procoagulation events by activating Factor VII (FVII) [46]. The activated FVII then binds to TF forming a complex that catalyzes the conversion of Factor X to Xa. FXa then converts prothrombin to thrombin. Thrombin cleaves fibrinogen to fibrin and can also activate platelets and endothelial cells. Fibrin in turn can enhance lung inflammation by increasing the expression of IL-1β, a specific marker of inflammation [47]. Fibrin and its degradation products can also increase vascular permeability [48], enhance recruitment of neutrophils to the lung [49] and influence inflammatory cell proliferation and migration [50]. These studies underscore the importance of extracellular nucleic acids in inflammation and coagulation and could have parallels with the SM-induced injuries. Therefore potential therapies against these targets are worth exploring.

Activation of the coagulation cascade can also confer protection in a number of situations. In this context TF can play multiple roles. While the role of TF in activation of the coagulation cascade is well recognized, its role in repair is less well appreciated. Our previous studies have demonstrated that TF is critical to the survival of epithelial basal progenitor cell and required for repair of the damaged epithelium [37]. We have also shown that these TF-

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mediated effects are modulated through PARs (protease activated receptors PAR-1 and PAR-2) [37]. Therefore, therapies targeting TF can potentially impair the repair process and lead to comorbidities (Figure 3). A recent study in ARDS patients linked immune activation and expression of procoagulant proteins to survivability, suggesting that they provide compensatory survival responses [51]. In several models of ARDS it has been demonstrated that prevention of fibrin formation protects against injury with improvements in oxygenation, lung compliance and inflammation [52]. In spite of these promising studies there is little evidence that human patients benefit from such treatments, and in some cases there was even increase in mortality [53]. This is conceivable since anticoagulation therapies in a number of cases did not correct inflammatory responses and were more prone to bleeding disorders [54]. Apparently, blocking the procoagulation pathway alone may not be sufficient to offer long-term benefits. While other effective strategies are needed, the role of coagulation cannot be ignored.

Role of inflammation in sulfur mustard poisoning

Pathways of inflammation and coagulation are interdependent and intrinsically linked. Significant inflammation is often associated with increased coagulation. Inflammation can influence coagulation by increasing cytokine levels of IL-6, IL-1 and IL-12, diminishing activated protein C (APC), decreasing fibrinolysis and increasing platelet activation [54]. The role of inflammation in mediating TF activation and subsequent coagulation is increasingly being recognized [55]. Bacterial lipopolysaccharide (LPS), a known inflammatory molecule, can promote coagulation by inducing TF activation and increasing thrombin generation [20, 55]. Thrombin, in addition to its effect on coagulation, can promotes endothelial barrier dysfunction and further potentiate inflammation by its effects on cytokine production and activation of its receptor, protease activated receptors (PAR-1). In this context one would expect that by blocking thrombin generation or inhibition of PAR-1, inflammation could be suppressed. However, this may not always hold true. While the role of PAR-1 in promoting coagulation is unequivocal, its role as an anti-inflammatory molecule is equally important. Binding of APC to PAR-1 results in an anti-inflammatory phenotype [54]. Additionally, PARs (PAR-1 and PAR-2) can confer pro-survival and pro-proliferative effects through a TF dependent mechanism [37]. Taken together these studies point to the complexity of inflammatory and coagulation pathways involved in the pathogenesis of SM poisoning.

SM exposure causes severe blistering and inflammation of skin [56]. Dermal exposure to SM caused immune activation and infiltration of CD4 and CD8 positive T cells along with a delayed type hypersensitivity response and inflammation in distal organs such as lungs [57]. Dose dependent activation of mast cells and dermal neutrophil accumulation have been observed in SM-induced skin lesions [58]. Dose and time dependent increases in expression of specific mRNA of inflammatory mediators such as IL-1β, IL-8 and IL-6, tumor necrosis factor alpha (TNFα), cyclooxygenase-2 (COX 2), macrophage inflammatory proteins and keratinocyte chemoattractants have also been observed [59, 60]. Increased expression of adhesion molecules such as L-selectin and VCAM along with growth factors, granulocyte colony-stimulating factor and matrix metalloproteinases were also observed upon SM

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exposure of skin [61, 62]. Some of these effects could be reversed using anti-inflammatory therapies [63].

Inflammatory pathways are also activated by ROS, generated as a result of exposure to SM or CEES. SM also causes a marked increase in proapoptotic proteins [64] and markers of oxidative stress, along with a decrease in antioxidants [20]. Therefore it is conceivable that therapies like N-acetyl cysteine, that targets the antioxidant pathway, have shown greater promise in preventing mortality for up to 12 hours [65]. Importantly, there was decreased neutrophil infiltration in these animals, indicative of reduced inflammation. It would also be interesting to know the effects of this therapy in prolonging survival and long-term morbidity.

Pulmonary airway mucosa is first to encounter inhaled SM. Epithelial cell death and release of cellular debris causes increased activation of the immune system. The markers of inflammation may persist in circulation of victims for a very long time after SM inhalation [66, 67]. Serum levels of IL-6 and IL-8 were elevated in Iran war veterans [20]. Similar increases in inflammatory markers like IL-6, TNF-α and myeloperoxidase activity have also been observed in animal models after CEES inhalation [20]. Therapies targeting the inflammatory pathway have also shown efficacy in a number of studies. For instance, anti-TNF-α antibodies are protective against nitrogen mustard-induced injuries [68]. Similarly, pentoxifylline (TNF-α inhibitor) can also mitigate effects on acute lung injury [69]. Interestingly, TNF-α can also stimulate TF expression [70], suggesting that targeting pro-inflammatory molecules could also diminish coagulation.

SM exposure is accompanied by infiltration of inflammatory cell, mainly neutrophils, in to the airways [71]. Similar increases in blood neutrophil counts were observed in Iranian SM victims several years post exposure. Analysis of blood samples of these victims demonstrated increased CD56/CD25 positive natural killer cells in patients with increased disease severity [20]. Similarly, the BALF of these patients had increased proinflammatory cytokines and cytotoxic T cells [20]. Activation of T cell mediated responses has also been reported in animal models of SM inhalation [20]. However in one model, SM caused decreased T cell proliferation [72]. The importance of inflammatory cells in CEES induced injuries is further supported by a study that showed protection in rats following neutrophil depletion [73]. While these studies underscore the critical role of neutrophils in contributing towards the inflammatory phenotype, severe exposures lead to neutropenia, resulting in increased susceptibility to infections and increased mortality. In experimental settings, SM as well as nitrogen mustard exposures in non-human primates have been shown to cause neutropenia. In these animals administration of granulocyte colony stimulating factor (G-CSF) restored neutrophil counts much faster when compared to the untreated ones [74]. Taken together, these studies underscore the importance of the inflammatory cells in CEES and SM-induced lung injuries and highlight the complexity of associated pathways. Therefore, molecules that interfere with the activation of these pathways can potentially alleviate SM-induced lung injury.

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Challenges and future directions

SM exposures cause a host of clinical manifestations that involve multiple pathways, principal among them being the antioxidant, inflammatory and coagulation pathways. Although our current understanding of events leading to the pathogenesis of SM injuries has advanced significantly, effective therapies are still lacking. Therapies that prevent mortality against acute lethal exposures should perhaps be employed as the first line of defense. The choice of such therapies is still debatable and long-term effects should be carefully considered. Apparently in acute lethal exposures coagulation pathways are important as anticoagulant therapies in animal models can be used to prevent mortality. However, prolonged use can also lead to bleeding disorders and other comorbidities. Further, therapies that prevent hypercoagulation can potentially cause inhibition of repair pathways and lead to chronic effects. These confounding effects limit effective therapies. Evidently there is a need to develop drugs that target multiple pathways. Unfortunately, this may not come from a single wonder drug and may require combination therapies. Finally, safety and toxicity profiles of such therapies have to be looked into more carefully given the recent tragic incident with an experimental drug trial in France [75].

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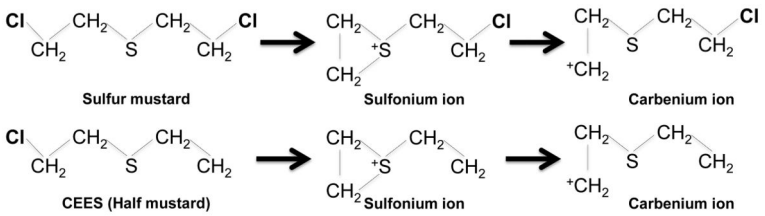

Figure 1.

Reactions of sulfur mustard and its analog CEES. Sulfur mustard and its analog 2-chloroethyl ethyl sulfide (CEES) can undergo internal cyclization to form the reactive sulfonium and carbenium ion intermediates. These intermediates react with functional groups in protein, carbohydrates and nucleic acids.

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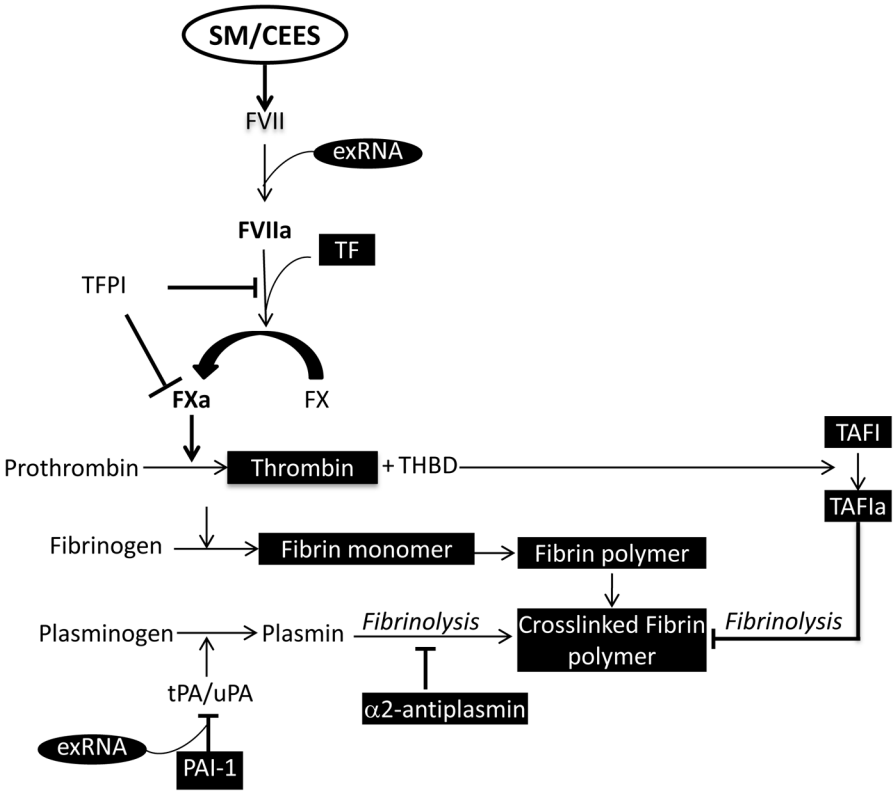

Figure 2.

Schematic representation of the coagulation pathway in SM-induced injuries. Factors in black boxes are increased in SM/CEES-induced injuries. FVII: Factor VII, FVIIa: Activated Factor VII; exRNA: extracellular RNA; TF: Tissue factor; TFPI: Tissue factor pathway inhibitor; FX: Factor X; FXa: Activated Factor X; THBD: Thrombomodulin; TAFI: Thrombin activatable fibrinolysis Inhibitor; TAFIa: Activated Thrombin activatable fibrinolysis Inhibitor; PAI-1: Protease activated Inhibitor 1.

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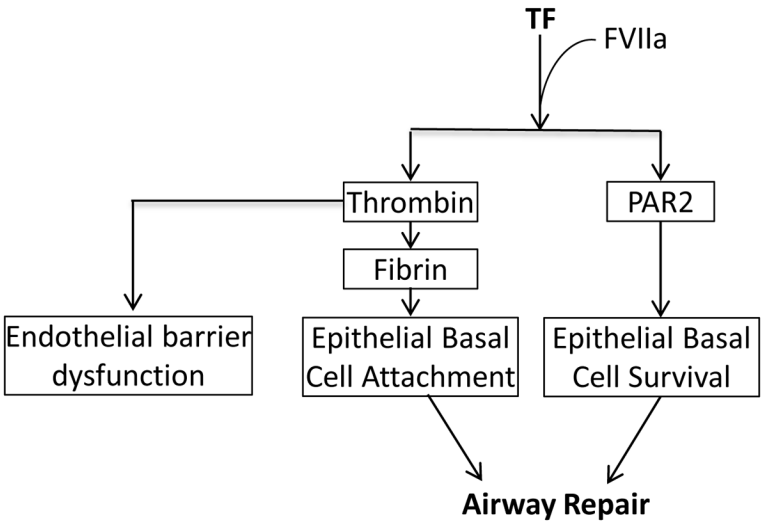

Figure 3.
Schematic illustration of tissue factor-dependent pathways in repair an injury.